



Report Information from ProQuest

10 November 2023 06:37

TABLE OF CONTENTS

Search Strategy.....	v
1. Spectroscopic Determination of Fluoride Using Eriochrome Black T (EBT) as a Spectrophotometric Reagent from Groundwater.....	1
2. Study of the Catalytic Activity of the Compounds Hydrotalcite Type Treated by Microwave in the Self-Condensation of Acetone.....	12
3. Microdroplet Actuation via Light Line Optoelectrowetting (LL-OEW).....	19
4. Rapid Simultaneous Determination of 43 Pesticide Residues in <i>Schizonepeta tenuifolia</i> by Gas Chromatography Mass Spectrometry.....	28
5. Synthesis, Optimization, and Characterization of Ecofriendly Production of Gold Nanoparticles Using Lemon Peel Extract.....	44
6. Standardization and Quality Control of the Herbal Medicine Mist Nibima, Employed to Treat Malaria and COVID-19, Using Physicochemical and Organoleptic Parameters and Quantification of Chemical Markers via UHPLC-MS/MS.....	55
7. Antioxidant Activity In Vitro Guided Screening and Identification of Flavonoids Antioxidants in the Extract from <i>Tetrastigma hemsleyanum</i> Diels et Gilg.....	65
8. Simultaneous Determination of Amphenicols and Metabolites in Animal-Derived Foods Using Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry.....	76
9. Optimization of Residual Hexane in Edible Oils Analysis Using Static Headspace Gas Chromatography..	92
10. Electrospun Membrane for the Extraction of Acrylamide in Pet Food Samples.....	98
11. Formulation and development of Serratiopeptidase enteric coated tablets and analytical method validation by UV Spectroscopy.....	105
12. Determination of Dabigatran Concentration in Human Plasma and Breast Milk.....	117
13. Chromatographic Fingerprinting Based on Column Switching Technology for Quality Evaluation of Tianmeng Oral Liquid.....	124
14. Proximate Analysis of Poultry-Mix Formed Feed Using Maize Bran as a Base.....	133
15. Determination of Carrageenan in Livestock and Poultry Meat by Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry.....	139
16. Simultaneous and Rapid Determination of Six Tyrosine Kinase Inhibitors in Patients with Non-Small Cell Lung Cancer Using HPLC-MS/MS.....	148
17. Identification and Simultaneous Determination of the Main Toxic Pyrrolizidine Alkaloids in a Compound Prescription of Traditional Chinese Medicine: Qianbai Biyan Tablet.....	161
18. Novel Trends in Electrochemical Biosensors for Early Diagnosis of Alzheimer's Disease.....	175
19. Chemical and Antimicrobial Analyses of <i>Juniperus chinensis</i> and <i>Juniperus seravschanica</i> Essential Oils and Comparison with Their Methanolic Crude Extracts.....	192
20. A Rapid Screening Method for Sibutramine Hydrochloride in Natural Herbal Medicines and Dietary Supplements.....	207
21. Evaluation of Neuraminidase Inhibitory Activity of Compounds and Extracts from Traditional Medicines by HPLC-FLD.....	221
22. Synthesis of Zearalenone Immunogen and Comparative Analysis of Antibody Characteristics.....	231

TABLE OF CONTENTS

23. Comparison of Essential Oils of <i>Houttuynia cordata</i> Thunb. from Different Processing Methods and Harvest Seasons Based on GC-MS and Chemometric Analysis.....	245
24. Simultaneous Determination of 25 Ginsenosides by UPLC-HRMS via Quantitative Analysis of Multicomponents by Single Marker.....	264
25. Determination of Pendimethalin Dynamic Residual Distribution in Crucian Carp Tissues and Associated Risk Assessment.....	282
26. Phytochemical Composition, Antioxidant, Antiacetylcholinesterase, and Cytotoxic Activities of <i>Rumex crispus</i> L.....	284
27. The Use of Sugarcane Bagasse to Remove the Organic Dyes from Wastewater.....	301
28. A Fast and Validated HPLC Method for the Simultaneous Analysis of Five 5-HT 3 Receptor Antagonists via the Quantitative Analysis of Multicomponents by a Single Marker.....	313
29. Ultraviolet-Visible Spectroscopy and Chemometric Strategy Enable the Classification and Detection of Expired Antimalarial Herbal Medicinal Product in Ghana.....	323
30. Development of a Gas Chromatography-Time-of-Flight Method for Detecting Glucosinolate Metabolites and Volatile Organic Compounds in Kimchi.....	331
31. Synthesis and Characterization of Chitosan Particles Loaded with Antioxidants Extracted from Chia (<i>Salvia hispanica</i> L .) Seeds.....	339
32. Fluorescence Spectroscopy and ¹³ C NMR Spectroscopy Characteristics of HA in Black Soil at Different Corn Straw Returning Modes.....	341
33. A Brief Review of Analytical Methods for the Estimation of Allopurinol in Pharmaceutical Formulation and Biological Matrices.....	353
34. Extraction of Pregabalin in Urine Samples Using a Sulfonated Poly(ether ether ketone) Membrane.....	369
35. An Effective and Efficient Sample Preparation Method for 2-Methyl-Isoborneol and Geosmin in Fish and Their Analysis by Gas Chromatography-Mass Spectrometry.....	379
36. A RP-HPLC Method for the Analysis of Neostigmine Methylsulfate and Process-Related Impurities, Forced Degradation Studies, in the Injection Formulation.....	387
37. Techniques for Detection of Clinical Used Heparins.....	403
38. Bismuth-Remaining Cupellation Fire Assay Preconcentration Combined with Inductively Coupled Plasma Mass Spectrometry for the Simultaneous Determination of Ultratrace Au, Pt, Pd, Ru, Rh, and Ir in Geologic Samples.....	417
39. Remarkable Phytochemical Characteristics of Chi-Nan Agarwood Induced from New-Found Chi-Nan Germplasm of <i>Aquilaria sinensis</i> Compared with Ordinary Agarwood.....	428
40. Simultaneous Quantification of Aspirin, Its Metabolite Salicylic Acid, and Salvianolic Acid B in Human Plasma Using UPLC-MS/MS.....	441
41. Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Vardenafil and Its Application of Bioequivalence.....	443
42. Identification of 24 Unknown Substances (NIAS/IAS) from Food Contact Polycarbonate by LC-Orbitrap Tribrid HRMS-DDMS 3 : Safety Assessment.....	456
43. Study on the Spectrum-Effect Relationship of the Traditional Effect of Saponins in <i>Glycyrrhiza uralensis</i> Fisch.....	473

TABLE OF CONTENTS

44. A Novel Nonenzymatic Hydrogen Peroxide Sensor Based on Magnetic Core-Shell Fe ₃ O ₄ @C/Au Nanoparticle Nanocomposite.....	484
45. Rapid Quantification of Chlorpromazine Residues in Pork Using Nanosphere-Based Time-Resolved Fluorescence Immunoassay Analyzer.....	494
46. Characterization and Quantification of Luteolin-Metal Complexes in Aqueous Extract of <i>Lonicerae Japonicae</i> Flos and Huangshan Wild Chrysanthemum.....	503
47. Development and Validation of a LC-MS/MS Method for the Determination of Nitrofurantoin Metabolites in Soft-Shell Turtle Powder Health Food Supplement.....	514
48. Rapid Determination of Pemetrexed Concentration and Distribution in Human Breast Cancer Cells (McF-7) Based on UHPLC-MS/MS.....	527
49. Study of Zuojin Pill in Treating Chronic Atrophic Gastritis by UPLC-Q-TOF/MS Based on Serum and Urine Metabolomics Combined with Network Pharmacology.....	537
50. HPLC-MS/MS-Mediated Analysis of the Pharmacokinetics, Bioavailability, and Tissue Distribution of Schisandrol B in Rats.....	539
51. Assessment of Some Physicochemical Parameters and Heavy Metals in Hand-Dug Well Water Samples of Kafta Humera Woreda, Tigray, Ethiopia.....	553
52. Total Oxidation of Isopropanol in Its Liquid Phase, at a Low Temperature in the Presence of Prepared and Characterized Zinc Oxide.....	565
53. Analytical Quality by Design Approach of Reverse-Phase High-Performance Liquid Chromatography of Atorvastatin: Method Development, Optimization, Validation, and the Stability-Indicated Method.....	572
54. Rapid Identification and Systematic Mechanism of Flavonoids from <i>Potentilla freyniana</i> Bornm. Based on UHPLC-Q-Exactive Orbitrap Mass Spectrometry and Network Pharmacology.....	588
55. Optimization of 25% Sulfosalicylic Acid Protein-to-Creatinine Ratio for Screening of Low-Grade Proteinuria.....	599
56. Determination and Pharmacokinetics of Omeprazole Enantiomers in Human Plasma and Oral Fluid Utilizing Microextraction by Packed Sorbent and Liquid Chromatography-Tandem Mass Spectrometry.....	606
57. Point-of-Care Diagnoses and Assays Based on Lateral Flow Test.....	617
58. Computer-Aided Rapid Establishment of Fingerprint of Xiaojin Capsule by HPLC.....	630
59. Corrigendum #2 to "Quantitative Detection of miRNA-21 Expression in Tumor Cells and Tissues Based on Molecular Beacon".....	641
Bibliography.....	643

SEARCH STRATEGY

Set No.	Searched for	Databases	Results
S1	Journal of Analytical Methods in Chemistry	Ebook Central, Public Health Database, Publicly Available Content Database	139992*

* Duplicates are removed from your search, but included in your result count.

Spectroscopic Determination of Fluoride Using Eriochrome Black T (EBT) as a Spectrophotometric Reagent from Groundwater

Atinafu G/Mariam

; Abebe Diro; Tsegaye Girma Asere

; Jado, Demelash

; Melak, Fekadu

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Fluoride health problem is a great concern worldwide, most often as a result of groundwater intake. Thus, determination of fluoride is vital to take appropriate measures upon fluoride contamination of water. Potentiometric method of analysis is reliable for the determination of fluoride in various samples. In addition, spectroscopic methods are found important to quantify fluoride levels from water; however, several factors hinder its easier determination. Among the bottlenecks, the use of toxic chemicals and tedious steps in preparing chemicals (e.g., SPADNS method) are to mention a few. In this study, a spectrophotometric method was developed for the determination of fluoride from groundwater using Eriochrome Black T (EBT) as a spectroscopic reagent. Experimental parameters that influence the determination of fluoride including ligand type, kinetics, pH, and ligand-to-metal ratio were assayed. Evaluation of fluoride levels showed that Beer–Lambert’s law is obeyed in the range of 0.3–5.0 mg/L at 544 nm. The calibration curve, resulting in good linearity ($R^2=0.9997$), was considered during quantitative analysis of the samples and in the spiking analysis. The limits of detection (LOD) and quantification (LOQ) of the method were found to be 0.19 and 0.64 mg/L, respectively. The precision studied in terms of intraday and interday at three concentration levels showed less than 5.4% RSD. Applicability of the method was investigated by analyzing groundwater samples spiked with fluoride standards, and satisfactory recoveries in the range of 98.18–111.4 were demonstrated. The developed spectrophotometric method has been successfully applied for fluoride determinations in groundwater samples. Thus, it could be used as an attractive alternative for the determination of fluoride from groundwater.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Fluoride is a common groundwater pollutant, mostly from geogenic sources. It is widely distributed in the Earth’s crust existing in several mineral forms such as fluorite (CaF_2), cryolite (Na_3AlF_6), monofluorophosphate (PO_3F^{2-}), and fluoroapatite ($\text{Ca}_5(\text{PO}_4)_3\text{F}$) [1]. Fluoride is used in a number of applications including dental care [2], agrochemical use [3], pharmaceuticals [4], water fluoridation [5, 6], and even in chemical industries including nuclear reactors [7, 8]. Fluoride leaching in water bodies is dependent on mineralogical chemistry, well depth, hydrologic conditions, and geologic structures, being calcium-poor aquifers expected to possess an enhanced level of fluoride [9].

Human exposure to fluoride occurs from various sources including food, drug, cosmetics, and drinking water being the major source of daily intake [10]. It has been shown to cause a significant impact on human health when excessive fluoride via drinking water occurs, including dental fluorosis and skeletal fluorosis [11, 12]. The World

Health Organization (WHO) has set a maximum contamination limit value of 1.5 mg/L for fluoride in drinking water to prevent health risks [13]. In fact, a low level (0.5–1.5 mg/L) of fluoride is assumed to be an essential component of human health in preventing dental caries [14, 15]. As a result, when fluoride sources are negligible, pharmaceutical preparations including prescription drops, lozenges, or tablets and fluoridated toothpastes are recommended [16]. Several techniques have been applied in the determination of fluoride from various matrices. Fluoride analysis techniques including electrochemical, chromatographic, titrimetric, capillary zone electrophoresis, fluorescence sensing, and spectroscopic methods have been developed [16–20]. Ion chromatography is expensive; ion-selective method is economical and sensitive in relation to UV-Vis spectroscopy. Ion-selective electrode method is widely used and suitable for industrial and field monitoring [21]; however, there are several disadvantages associated with it. It requires advanced cautions and buffer systems that require extra care in steps of chemical preparation. Buffer system during fluoride analysis using the ion-selective electrode involves citrate, cyclohexanediaminetetraacetic acid (CDTA), total ionic strength adjustment buffer (TISAB), diethylenetriamine pentaacetate (DTPA), and ethylenediaminetetraacetic acid (EDTA) [16, 22, 23]. The effect of coexisting ions in water that form complexes with fluoride has been another challenge in potentiometric/ion-selective determination of fluoride [24].

Spectroscopic determination of fluoride using chromophore-based reagents has been extensively studied. For instance, the use of complexes of flavonoid chrysin [25], aluminum complexes of triphenylmethane dyes [18, 26], interaction of fluoride with Al(III)-salicylic aldehyde acylhydrazones [18], reaction with zirconium ions and SPADNS [27], coordination of Al(III) with Alizarin Red-S [28], Al-xylene orange complex [29], and aluminum quinalizarin complex [30] are to mention a few fluoride extraction ways. In fact, the use of the aforementioned ligands would help in enhancing sensitivity and selectivity of fluoride extraction for the spectrophotometric determination. Similar phenomena have been enacted in the spectrophotometric determination of silver(I), copper(II), and cobalt(II) [31–33]. However, methods such as SPADNS possess lengthy solution preparations and use toxic chemicals. Therefore, a relatively simple, easier method of fluoride determination using a common indicator (EBT) has been developed in this research. Eriochrome Black T is among essential azo dyes used as a common indicator in complexometric titrations for the estimation of various metal cations such as Ca^{2+} , Mg^{2+} , and Zn^{2+} ions [34, 35]. A reactive azo dye such as EBT contains one or more azo bonds ($-\text{N}=\text{N}-$) that act as a chromophore. The visible spectra of azo dyes (e.g., EBT) exhibit intense bands at 510–560 nm. The spectra of azo dye to M(III) complexes are very similar to those of free dyes except changes of about 10–30 nm bathochromic shift in its absorption maxima [36].

Accordingly, this study was carried out to develop a simple spectrophotometric method for the determination of fluoride from groundwater using EBT as spectrophotometric reagent. Thus, parameters including metal-to-ligand ratio, pH effect, interference study, stoichiometry of the Al-EBT complex with fluoride, and real sample analysis have been explored.

2. Materials and Methods

2.1. Reagents

Chemicals used in the experiment include 99.0% NaF (Finkem, India), Eriochrome Black T (Merck, Germany), glacial acetic acid (Sigma-Aldrich, Germany), sodium acetate buffer, 37% HCl (Fisher Chemicals, UK), and ammonia solution (Merck, Germany). Sodium chloride (Fisher Chemicals, UK), 69% HNO_3 (Fine Chemical Industries, India), hydrated aluminum nitrate $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (Nice Chemicals Ltd., India), sodium sulphate (Blulux Laboratory reagent, India), and hydrated aluminum chloride ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) were used during the study. Eriochrome Black T (EBT) was also used in total hardness determination besides its use as a ligand in fluoride determination. A 1,10-phenanthroline method was used to determine iron in well water that is used to test the method in real groundwater sample analysis. In this method, concentrated HCl, hydroxylamine, acetate buffer, and 1,10-phenanthroline were employed. Nitrate was measured using a UV-Vis spectrophotometer (Analytik Jena Specord 200, Germany). H_2SO_4 (titrant), phenolphthalein and bromocresol green indicators, and calcon indicator were used to determine both calcium hardness and alkalinity.

2.2. Instruments and Apparatuses

A double-beam spectrometer (Analytik Jena, Germany) was used to measure the absorbance of the sample. pH meter (Bante Instruments, pH 902, USA) equipped with the electrode was used to measure the pH values of sample solutions. Quartz cuvette (1 cm), micropipettes (10–100 μL and 100–1000 μL) (Dragonmed, Shanghai, China), and filter paper (Whatman No. 542, England) were utilized during the experiment.

2.3. Solution Preparation

1000 mg/L fluoride stock solution was prepared by dissolving 2.21 g of dried sodium fluoride in 1 L of deionized water. The stock solution was kept in a refrigerator using plastic bottles until it was used. A range of calibration standards were prepared by serial dilution to determine fluoride levels at each parameter optimization. $1.0 \times 10^{-3} \text{ M}$ Al^{+3} and $1.0 \times 10^{-3} \text{ M}$ of EBT were prepared from 0.1 g of $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and 0.12 g EBT, respectively.

2.3.1. Preparation of the Al-EBT Complex

The Al-EBT complex was prepared by mixing 1:1 ratio of aluminum to EBT, $1.0 \times 10^{-3} \text{ M}$ of aluminum and $1.0 \times 10^{-3} \text{ M}$ of EBT in 250 mL deionized water, and diluted to $5.0 \times 10^{-5} \text{ M}$ for the spectrophotometric measurements.

2.4. Reaction of Fluoride with the Prepared Complex

To optimize the stoichiometry of the reaction mixture, EBT-Al with fluoride, variable concentration of the prepared Al-EBT complex and fluoride was considered, making the concentration of fluoride constant on the one hand. Also, experiments were performed by varying the concentration of fluoride with constant concentration of the complex, Al-EBT, on the other hand. The formation of the product, EBT-Al-F, was confirmed by wine red color observation at the acidic medium.

2.5. Dye Selection

The dyes including methylene orange, brilliant green, bromocresol green, methyl violet, malachite green, and Eriochrome Black T were studied to select appropriate ligand for the fluoride determination. This is to find stable complexes of aluminum to ligand that obey the Beer–Lambert law when the reaction is complete with fluoride. Trial tests for each of the dyes have been made (figure not shown).

2.6. Reaction Time

Reaction time is among the important parameters that affects the reaction and can influence quantitative evaluation of reaction products [37]. Equilibrium time is essential to quantify the analyte and hence examined during the reaction progress. The stability of the complex in water was examined by measuring the absorbance of the prepared admixture (fluoride with the Al-EBT complex) solution at different time intervals, starting from initial 15 min to 48 hr.

2.7. Effect of pH

The influence of pH on the formation of the complex was studied by carrying out the reaction in sodium acetate/acetic acid buffer solution of pH ranging from 4.0 to 5.4.

2.8. Stoichiometry of the Al-EBT Complex with Fluoride

The stoichiometric ratio between aluminum and EBT was evaluated by Job's method of continuous variation [38]. In this method, solutions of aluminum and EBT with identical molar concentrations ($5.0 \times 10^{-5} \text{ M}$) were mixed in varying volume ratios (1+9 to 9+1 mL) in such a way that the total volume of each mixture was the same which is 10 mL. The absorbance of the solutions was measured at a wavelength of 544 nm, and mole fraction was calculated by using the following formula. For the reaction, $M + n\text{LML}_n$, the composition of the complex was given by the volume fraction ratio as follows:

$n = x/1-x$, where n is the subscript of the ligand and

$X = [1-X]$, where $X = V_m/V_m + V_L$ and V_m = volume of metal and V_L = volume of the ligand

2.9. Method Validation

Method validation is the process of proving (through scientific studies) that an analytical method is acceptable for its intended use. It is used to ensure confidence in the analytical data throughout product development. The validation was carried out in terms of linearity, the limit of detection, the limit of quantification, precision, and accuracy studies [39, 40].

2.9.1. Limit of Detection (LOD)

LOD is the lowest concentration or mass of species under investigation in a sample that can be detected with a

specified level of confidence but not necessarily quantified, under stated condition of the test. It was evaluated from $LOD=3.3\sigma/S$, where σ =the standard deviation of y-intercepts of regression lines and S =the slope of the calibration curve.

2.9.2. Limit of Quantification (LOQ)

LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with a suitable precision and accuracy. $LOQ=10\sigma/S$, where σ =the standard deviation of y-intercepts of regression lines and S =the slope of the calibration curve.

2.9.3. Precision

Precision can be determined by repeatability (intraday), intermediate precision (interday), and reproducibility. Repeatability is a measure to express precision obtained with the same method, on the same test material, in the same laboratory, by the same operator, and using the same equipment within short intervals of time. Thus, in this research, repeatability was evaluated assaying three level determinations with the same concentration, during the same day, under the same experimental conditions in the morning session and afternoon session. Intermediate precision was analyzed by comparing the assays in three determinations at the same concentration within five different days and expressed as relative standard deviation (RSD).

2.9.4. Accuracy

Accuracy is an agreement between measured and real values. The accuracy of the proposed method was evaluated using recovery studies after spiking standard fluoride on a known amount of analyte of interest. The spiking has been made on a real groundwater sample, initially well characterized for its composition. Three different solutions of fluoride were prepared for each concentration level (0.5, 1, and 1.5 mg/L), and the accuracy was calculated on the basis of percentage recovery. The percentage recovery values were calculated by comparing the amount obtained from the spiked samples with the amount of fluoride in which the concentration was actually added, as follows: (1) % Recovery = $\frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100$, where C is the amount of the sample.

2.10. Interference Study

The interference studies were evaluated by measuring the influence of anions such as chloride, nitrate, and sulphate on the determination of 1.5 mg/L fluoride. Therefore, the expected interfering anions were added in such concentrations of 2–300, 25–500, and 40–700 mg/L for nitrate, sulphate, and chloride, respectively.

2.11. Determination of Fluoride in a Real Groundwater Sample

The method under investigation was tested using a real groundwater sample by appropriate dilution. The water sample was diluted, and then spike analysis was performed upon it. Fluoride was analyzed using a double-beam UV-Vis spectrophotometer (Analytik Jena Specord 200, Germany). All experiments, unless mentioned, were performed in triplicate, and the average results were reported together with error bars based on standard deviation of the triplicate measurements.

3. Results and Discussion

3.1. Optimization of Parameters

3.1.1. Dye Selection

In previous works, several dyes have been tested for the determination of fluoride including zirconium-xylene orange, m-cresol red, crystal violet, leucomalachite green, bromocresol purple, brilliant blue R, Patent blue VF sodium salt, acid violet, Alphazurine A, pararosaniline, Victoria blue R, ethyl violet, light green, bromocresol green, malachite green oxalate, lissamine green B, pyrocatechol violet, aluminon, chromeazurol B, and malachite green carbinol base [26, 41, 42]. However, only few work well in the determination of fluoride by complexing with M(III), most often aluminum ion. In this work as well, dyes as a ligand for the determination of fluoride including methylene orange, brilliant green, bromocresol green, methyl violet, and Eriochrome Black T have been tried. However, most of them fail to be sensitive and stable for analysis. Due to the sensitive absorption maxima of the dye and its aluminum complex to conform Beer–Lambert's law for certain concentration ranges, EBT was considered for further analysis. The ionic nature of the dye could be one possible factor to influence the complex formation. EBT is an anionic dye and can react with cation aluminum to form Al-EBT complex. Azo dyes such as EBT are reactive with one or more

azo bonds ($-N=N-$) that act as a chromophore in the molecule [43].

3.1.2. Selection of Maximum Wavelength

The spectra of EBT alone showed a maximum absorbance at 535nm, whereas the ternary mixtures of Al-EBT-F, as shown in Figure 1, were observed at 544nm, which is a working wavelength in the determination of fluoride. Bathochromic shift has been observed because of the displacement of the naphthalic hydrogens during the chelation process. It is known that deprotonation of phenols is accompanied by a bathochromic shift of the absorption band [44, 45].

[figure omitted; refer to PDF]

The data showed that chloride and nitrate did not interfere with the determination of fluoride, whereas sulphate showed to interfere as it is commonly reported to inhibit fluoride detection as in most visual and photometric methods [49, 50]. This might be due to the competition of SO_4^{2-} with fluoride to form a complex with the metal. In the present work, when the amount of sulphate is higher than 200mg/L, it showed to interfere with the determination of fluoride. However, sulphate usually presents at lower concentration levels than 200mg/L in Ethiopian groundwaters, which makes the method to work in real groundwaters with less interference from sulphate.

3.2. Determination of Fluoride

3.2.1. Calibration Curve

The calibration curve was drawn using series of concentrations of fluoride in the range of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0mg/L. This was obtained at optimum conditions of pH 5.0 and reaction duration of 24h. Each concentration level was read in triplicate, and average values were taken. Then, standard calibration curve was plotted from the absorbance values obtained for the five standards of fluoride solutions by taking concentration (mg/L) on the x-axis and absorbance values on the y-axis. The linear regression equation was found to be $y=0.2339x+0.2264$ and $R^2=0.9991$, which is satisfactory for quantitative analysis of the analyte in a proposed groundwater sample as shown in Figure 8.

[figure omitted; refer to PDF]

3.2.2. Precision Study

The precision of the proposed spectrophotometric method was investigated in terms of intraday precision (repeatability) and interday precision (within-lab reproducibility). Intraday and interday precisions were studied by preparing three different concentration levels (0.5, 1.5, and 2.5mg/L) of fluoride. For the case of intraday precision study, each concentration level was analyzed in triplicate on the same day (in morning and afternoon sessions). Under the same experimental conditions, interday precision of the method was evaluated at three concentration levels earlier used for intraday precision studies, for five consecutive days with the same time interval. The relative standard deviation (RSD) of the intraday (repeatability) and interday (within-lab reproducibility) precisions of the proposed method is presented in Table 1. The %RSD of both intra- and interday precisions of the three concentration levels was found below 9.00%, which indicated that the developed method showed reasonable acceptable range for the analysis of the target analyte.

Table 1

Reproducibility and intraday and interday precisions during fluoride determination.

Levels of F^- in mg/L	Precision	
Intraday (%RSD)	Interday (%RSD)	
		0.5
4.91	2.51	1.5
6.94	7.30	2.5

3.3. Analysis of the Real Sample and Recovery Studies

The applicability of the developed method was evaluated by performing relative recovery studies utilizing three level standards spiked upon real groundwater samples. In order to demonstrate the applicability and reliability of the method for real samples, three samples of groundwater were prepared and analyzed by the method. For each level, blank samples (unspiked) were also analyzed by the same method. The %RR of the target analyte for each level is demonstrated in Table 2. The relative recovery (%RR) was calculated as in the following equation: $\%RR = \frac{C_{spiked} - C_{unspiked}}{C_{added}} \times 100$, where C is the concentration of a sample.

Table 2

Analytical results of groundwater sample analysis via spiking ($n=3$).

Sample type	Fluoride standard spiked (mg/L)	This method, F^- found (mg/L) in sample waters after dilution ^a	Fluoride found (spiked + sample water) ^b	% Recovery	RS D, %
GW-1	0.25	2.50	2.70	98.18	3.20
GW-1	0.5	2.50	3.06	111.40	6.20
GW-1	1.5	2.50	2.86	110.60	4.50

^aFluoride found in groundwater after 7-fold dilution. ^bTotal fluoride in 7-fold-diluted groundwater plus spiked standard.

The observed percent recovery of the target analyte in groundwater samples ranged from 98.18 to 111.40, which briefly indicated that the proposed method had acceptable relative recoveries for the determination of fluoride from groundwater samples. The physicochemical parameters of groundwater samples (well water #1) employed in the experiment are analyzed and presented in Table 3.

Table 3

Physicochemical parameters of the well water #1 used in the experiment.

Parameters	Concentration
pH	6.77
Turbidity, NTU	7.45
TDS, mg/L	215
Chloride, mg/L	4.6
Nitrate, mg/L	0.70
Total hardness, mg/L	36
Iron, mg/L	0.012
Magnesium, mg/L	16.90

Calcium, mg/L	21.50
Conductivity, $\mu\text{S}/\text{cm}$	95.55
Alkalinity (by bicarbonate), mg/L	17
Fluoride, mg/L	17.51

3.4. Comparison of Methods in Fluoride Detection

The developed method for the determination of fluoride has been compared with related analytical techniques reported in the literature. The comparison is made in terms of linear range, limit of detection (LOD), and limit of quantification (LOQ) and given in Table 4.

Table 4

Comparison method validation parameters with previously reported methods.

Ligands used in spectroscopic analysis of fluoride	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)	Reference
Al to quinalizarin	0.5–2.0	0.1	0.3	[67]
Al to resorcin blue	0.2–1.0	0.2	0.5	[72]
Al to flavonoid chrysin	0.5–3.0	0.1	0.3	[68]
Al to malachite green	0.5–4.0	0.1	0.3	[71]
Al to chromeazurol B	0.5–4.0	0.2	0.5	[71]
Zr to resorcin blue	0.1–4.0	0.07	0.2	[87]
Al to EBT	0.3–5.0	0.19	0.64	This work

As it can be seen from Table 4, it was noted that the method developed has provided better linearity and similar or better LOD and LOQ compared with reported literature studies. Thus, the method can be considered as an attractive alternative and less toxic for determining fluoride in groundwater samples.

4. Conclusion

The analytical method for the measurement of fluoride with appropriate ligand (EBT) is presented here. EBT was well suited for the spectrophotometric reagent of fluoride analysis. Promising results were obtained with 1:1 ratio of Al and EBT complex. This complex was used as a spectrophotometric reagent for fluoride determination in the range of 0.3–5.0 mg/L. The obtained results demonstrated that the developed method was fully functional with 0.19 mg/L detection limit and 0.64 mg/L quantification limit. The method showed promising applications in real groundwater samples, providing 98.18 to 111.40% recoveries.

References

- [1] V. Bharti, "Fluoride sources, toxicity and its amelioration: a review," *Peertechz Journal of Environmental Science and Toxicology*, pp. 021-032, DOI: 10.17352/pjest.000009, 2017.
- [2] H. Sun, F. Luo, Q. Wan, "The application of fluoride in dental caries," *Dental Caries*, DOI:

10.5772/intechopen.91810, 2020.

- [3] T. Fujiwara, D. O'Hagan, "Successful fluorine-containing herbicide agrochemicals," *Journal of Fluorine Chemistry*, vol. 167, pp. 16-29, DOI: 10.1016/j.jfluchem.2014.06.014, 2014.
- [4] R. S. Bhat, A. El Gezeery, A. Ben Bachan, M. A. Alonazi, L. S. Alsuhaibani, A. El-Ansary, "Prenatal exposure to the fluoride containing psychiatric drug fluoxetine and anti-oxidative alterations in the neonatal rat brain," *Biocell*, DOI: 10.32604/biocell.2019.07012, 2019.
- [5] Z. Iheozor-Ejiofor, H. V. Worthington, T. Walsh, L. O'Malley, J. E. Clarkson, R. Macey, R. Alam, P. Tugwell, V. Welch, A.-M. Glenny, "Water fluoridation for the prevention of dental caries," *Cochrane Database of Systematic Reviews*, DOI: 10.1002/14651858.CD010856.pub2, 2015.
- [6] J. A. Cury, A. P. Ricomini-Filho, F. L. P. Berti, C. P. Tabchoury, "Systemic effects (Risks) of water fluoridation," *Brazilian Dental Journal*, vol. 30 no. 5, pp. 421-428, DOI: 10.1590/0103-6440201903124, 2019.
- [7] T. B. P. Luiz, J. L. S. D. Silva, L. L. V. Descovi Filho, "Hydrogeochemical modeling of fluoride contents in groundwater in outcrop area of Guarani Aquifer System, southern Brazil," *Geologia USP: Série Científica*, vol. 19 no. 1, pp. 69-82, DOI: 10.11606/issn.2316-9095.v19-141639, 2019.
- [8] P. N. Rao, A. D. Rao, J. S. Bhargav, K. Siva Sankar, G. Sudarshan, "Regional appraisal of the fluoride occurrence in groundwaters of Andhra Pradesh," *Journal of the Geological Society of India*, vol. 84 no. 4, pp. 483-493, DOI: 10.1007/s12594-014-0154-7, 2014.
- [9] K. Kim, G. Y. Jeong, "Factors influencing natural occurrence of fluoride-rich groundwaters: a case study in the southeastern part of the Korean Peninsula," *Chemosphere*, vol. 58 no. 10, pp. 1399-1408, DOI: 10.1016/j.chemosphere.2004.10.002, 2005.
- [10] S. K. Jha, R. K. Singh, T. Damodaran, V. K. Mishra, D. K. Sharma, D. Rai, "Fluoride in groundwater: toxicological exposure and remedies," *Journal of Toxicology and Environmental Health, Part B*, vol. 16 no. 1, pp. 52-66, DOI: 10.1080/10937404.2013.769420, 2013.
- [11] H. Zuo, L. Chen, M. Kong, L. Qiu, P. Lü, P. Wu, Y. Yang, K. Chen, "Toxic effects of fluoride on organisms," *Life Sciences*, vol. 198, pp. 18-24, DOI: 10.1016/j.lfs.2018.02.001, 2018.
- [12] R. Ullah, M. S. Zafar, N. Shahani, "Potential fluoride toxicity from oral medicaments: a review, Iran," *J. Basic Med. Sci.*, DOI: 10.22038/ijbms.2017.9104, 2017.
- [13] Who, WHO | Guidelines for Drinking-Water Quality, 2011.
- [14] Y. Dimitropoulos, A. Blinkhorn, M. Irving, J. Skinner, S. Naoum, A. Holden, A. Masoe, B. Rambaldini, V. Christie, H. Spallek, K. Gwynne, "Enabling Aboriginal dental assistants to apply fluoride varnish for school children in communities with a high Aboriginal population in New South Wales, Australia: a study protocol for a feasibility study," *Pilot and Feasibility Studies*, vol. 5 no. 1, DOI: 10.1186/s40814-019-0399-4, 2019.
- [15] S. Dey, B. Giri, "Fluoride fact on human health and health problems: a review," *Medical & Clinical Reviews*, vol. 2 no. 1, DOI: 10.21767/2471-299x.1000011, 2016.
- [16] H. Yahyavi, M. Kaykhani, M. Mirmoghaddam, "Recent developments in methods of analysis for fluoride determination," *Critical Reviews in Analytical Chemistry*, vol. 46 no. 2, pp. 106-121, DOI: 10.1080/10408347.2014.985814, 2016.
- [17] S. Soares, F. Rocha, "Fast spectrophotometric determination of iodine value in biodiesel and vegetable oils," *Journal of the Brazilian Chemical Society*, DOI: 10.21577/0103-5053.20180044, 2018.
- [18] O. Sukhareva, R. Mariychuk, S. Sukharev, S. Delegan-Kokaiko, S. Kushtan, "Application of microextraction techniques for indirect spectrophotometric determination of fluorides in river waters," *Journal of Environmental Management*, vol. 280, DOI: 10.1016/j.jenvman.2020.111702, 2021.
- [19] P. Martins, A. C. Lopes, S. Lanceros-Mendez, "Electroactive phases of poly(vinylidene fluoride): determination, processing and applications," *Progress in Polymer Science*, vol. 39 no. 4, pp. 683-706, DOI: 10.1016/j.progpolymsci.2013.07.006, 2014.
- [20] H. Altundag, S. Agar, E. Altıntig, A. Ates, S. Sivrikaya, "Use of ion chromatography method on the determination of some anions in the water collected from Sakarya, Turkey," *Journal of Chemical Metrology*, vol. 13 no. 1, pp. 14-

20, DOI: 10.25135/jcm.26.19.03.1221, 2019.

[21] A. Bratovčić, A. Odošević, S. Čatić, "The advantages of the use of ion-selective potentiometry in relation to UV/VIS spectroscopy," *Agric. Conspec. Sci.*, 2009.

[22] M. S. Hosseini, H. Rahiminejad, "Potentiometric determination of ultratrace amounts of fluoride enriched by zirconia in a flow system," *Journal of Analytical Chemistry*, vol. 61 no. 2, pp. 166-171, DOI: 10.1134/S1061934806020134, 2006.

[23] S. Borjigin, Y. Ashimura, T. Yoshioka, T. Mizoguchi, "Determination of fluoride using ion-selective electrodes in the presence of aluminum," *Analytical Sciences*, vol. 25 no. 12, pp. 1437-1443, DOI: 10.2116/analsci.25.1437, 2009.

[24] A. Wilken, V. Kraft, S. Girod, M. Winter, S. Nowak, "A fluoride-selective electrode (Fse) for the quantification of fluoride in lithium-ion battery (Lib) electrolytes," *Analytical Methods*, vol. 8 no. 38, pp. 6932-6940, DOI: 10.1039/c6ay02264b, 2016.

[25] Z. Barghouthi, S. Amereih, S. Khayat, *Spectrophotometric Determination of Fluoride in Groundwater Using Complexes of Flavonoid Chrysin*, 2013.

[26] Z. Barghouthi, S. Amereih, "Spectrophotometric determination of fluoride in drinking water using aluminium complexes of triphenylmethane dyes," *WaterSA*, vol. 38 no. 4, pp. 543-548, DOI: 10.4314/wsa.v38i4.9, 2012.

[27] T. L. Marques, N. M. M. Coelho, "Proposed flow system for spectrophotometric determination of fluoride in natural waters," *Talanta*, vol. 105, pp. 69-74, DOI: 10.1016/j.talanta.2012.11.071, 2013.

[28] P. K. Tarafder, R. K. Mondal, *Spectrophotometric Determination of Fluoride in Water*, 2007.

[29] J. Zolgharnein, A. Shahrjerdi, G. Azimi, J. Ghasemi, "Spectrophotometric determination of trace amounts of fluoride using an Al-xylene orange complex as a colored reagent," *Analytical Sciences*, vol. 25 no. 10, pp. 1249-1253, DOI: 10.2116/analsci.25.1249, 2009.

[30] Z. Barghouthi, S. Amereih, "Field method for estimation of fluoride in drinking groundwater by photometric measurement of spot on aluminium quinalizarin reagent paper," *Arabian Journal of Chemistry*, vol. 10, pp. S2919-S2925, DOI: 10.1016/j.arabjc.2013.11.024, 2017.

[31] G. S. Kamble, A. P. Gaikwad, B. N. Kokare, S. S. Kolekar, S. H. Han, M. A. Anuse, "Rapid and sensitive synergistic extraction and spectrophotometric determination of silver(I) using 1-(2',4'-dinitro aminophenyl)-4,4,6-trimethyl-1,4-dihydropyrimidine-2-thiol: analysis of real samples," *Industrial & Engineering Chemistry Research*, vol. 50 no. 19, pp. 11270-11279, DOI: 10.1021/ie200812w, 2011.

[32] G. S. Kamble, S. S. Kolekar, M. A. Anuse, "Synergistic extraction and spectrophotometric determination of copper(II) using 1-(2',4'-dinitro aminophenyl)-4,4,6-trimethyl-1,4-dihydropyrimidine-2-thiol: analysis of alloys, pharmaceuticals and biological samples," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 78 no. 5, pp. 1455-1466, DOI: 10.1016/j.saa.2011.01.027, 2011.

[33] G. S. Kamble, A. A. Ghare, S. S. Kolekar, S. H. Han, M. A. Anuse, "Development of an reliable analytical method for synergistic extractive spectrophotometric determination of cobalt(II) from alloys and nano composite samples by using chromogenic chelating ligand," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 84 no. 1, pp. 117-124, DOI: 10.1016/j.saa.2011.09.015, 2011.

[34] P. Jarujamrus, N. Malahom, S. Puchum, R. Meelapsom, M. Amatongchai, A. Siripinyanond, S. Chairam, C. Kulsing, "Complexometric and argentometric titrations using thread-based analytical devices," *Talanta*, vol. 183, pp. 228-236, DOI: 10.1016/j.talanta.2018.02.058, 2018.

[35] S. S. Nielsen, "Complexometric determination of calcium," *Food Analysis Laboratory Manual*, pp. 61-67, DOI: 10.1007/978-1-4419-1463-7_8, 2010.

[36] S. Pysarevska, L. Dubenska, I. Spanik, J. Kovalyshyn, S. Tvorynska, "Reactions of o,o'-dihydroxy azo dyes with the third group M(III) ions: a spectroscopic and electrochemical study," *Journal of Chemistry*, vol. 2013, DOI: 10.1155/2013/853763, 2013.

[37] H. Bisswanger, "Enzyme assays," *Perspectives in Science*, vol. 1 no. 1-6, pp. 41-55, DOI: 10.1016/j.pisc.2014.02.005, 2014.

- [38] K. C. Ingham, "On the application of Job's method of continuous variation to the stoichiometry of protein-ligand complexes," *Analytical Biochemistry*, DOI: 10.1016/0003-2697(75)90666-1, 1975.
- [39] P. Ravisankar, C. Naga Navya, D. Pravallika, D. N. Sri, "A review on step-by-step analytical method validation," *IOSR Journal of Pharmacy*, 2015.
- [40] A. Gustavo González, M. Ángeles Herrador, "A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles," *TRAC Trends in Analytical Chemistry*, vol. 26 no. 3, pp. 227-238, DOI: 10.1016/j.trac.2007.01.009, 2007.
- [41] Ł. Lenarczyk, Z. Marczenko, "Spectrophotometric determination of fluoride using ternary complex of zirconium with Eriochrome Cyanine R and cetylpyridinium ions," *Microchemical Journal*, vol. 34 no. 1, pp. 115-120, DOI: 10.1016/0026-265X(86)90109-8, 1986.
- [42] A. G. Macejunas, "Spectrophotometric determination of fluoride using zirconium-xylene orange," *Journal of the American Water Works Association*, vol. 61 no. 6, pp. 311-313, DOI: 10.1002/j.1551-8833.1969.tb03762.x, 1969.
- [43] M. D. G. de Luna, E. D. Flores, D. A. D. Genuino, C. M. Fultalan, M.-W. Wan, "Adsorption of Eriochrome Black T (EBT) dye using activated carbon prepared from waste rice hulls-Optimization, isotherm and kinetic studies," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 44 no. 4, pp. 646-653, DOI: 10.1016/j.jtice.2013.01.010, 2013.
- [44] H. Y. Kim, H. G. Im, S.-K. Chang, "Colorimetric and fluorogenic signaling of fluoride ions by thiophosphinated dichlorofluorescein," *Dyes and Pigments*, vol. 112, pp. 170-175, DOI: 10.1016/j.dyepig.2014.06.030, 2015.
- [45] M. Demnitz, S. Hilpmann, H. Lösch, F. Bok, R. Steudtner, M. Patzschke, T. Stumpf, N. Huittinen, "Temperature-dependent luminescence spectroscopic investigations of uranyl(vi) complexation with the halides F⁻ and Cl⁻," *Dalton Transactions*, vol. 49 no. 21, pp. 7109-7122, DOI: 10.1039/d0dt00646g, 2020.
- [46] G. F. Brunzie, R. T. Pflaum, G. F. BRUNZrn, *The Direct Spectrophotometric Determination of Fluoride Ion*, 1962.
- [47] M. Okumura, T. Yoshimura, K. Fujinaga, Y. Seike, "Direct spectrophotometric determination of traces of fluoride in rain water," *Bunseki Kagaku*, vol. 52 no. 6, pp. 485-488, DOI: 10.2116/bunsekikagaku.52.485, 2003.
- [48] C.-Q. Zhu, J.-L. Chen, H. Zheng, Y.-Q. Wu, J.-G. Xu, "A colorimetric method for fluoride determination in aqueous samples based on the hydroxyl deprotection reaction of a cyanine dye," *Analytica Chimica Acta*, vol. 539 no. 1-2, pp. 311-316, DOI: 10.1016/j.aca.2005.03.002, 2005.
- [49] H. E. Bumsted, J. C. Wells, "Spectrophotometric method for determination of fluoride ion," *Analytical Chemistry*, vol. 24 no. 10, pp. 1595-1597, DOI: 10.1021/ac60070a019, 1952.
- [50] J. A. Arancibia, A. Rullo, A. C. Olivieri, S. D. Nezio, M. Pistonesi, A. Lista, B. S. F. Band, "Fast spectrophotometric determination of fluoride in ground waters by flow injection using partial least-squares calibration," *Analytica Chimica Acta*, vol. 512 no. 1, pp. 157-163, DOI: 10.1016/j.aca.2004.02.026, 2004.

DETAILS

Subject:	Dyes; Reagents; Acids; Electrodes; Groundwater; Spectrophotometry; Sodium; Ligands; Water sampling; Spectroscopy; Aluminum; Drinking water; Chemicals; Fluorides; Product development
Business indexing term:	Subject: Product development
Location:	India; Germany; United Kingdom--UK
Company / organization:	Name: Analytik Jena; NAICS: 334513
Editor:	Spas D Kolev

Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-08-21 (Received); 2021-11-29 (Revised); 2021-12-10 (Accepted); 2021-12-28 (Pub)
DOI:	https://doi.org/10.1155/2021/2045491
ProQuest document ID:	2618118656
Document URL:	https://www.proquest.com/scholarly-journals/spectroscopic-determination-fluoride-using/docview/2618118656/se-2?accountid=211160
Copyright:	Copyright © 2021 Atinafu G/Mariam et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2022-01-09
Database:	Publicly Available Content Database

Study of the Catalytic Activity of the Compounds Hydrotalcite Type Treated by Microwave in the Self-Condensation of Acetone

Houssaini, Jamal

; Mohammed Naciri Bennani

; Ziyat, Hamid

; Arhzaf, Said; Qabaqous, O; Amhoud, Abdellatid

[ProQuest document link](#)

ABSTRACT (ENGLISH)

The self-condensation reaction of acetone, producing diacetone alcohol (DAA), is of great industrial importance. It was used to study the catalytic activity of Mg-Al catalysts synthesized by the coprecipitation method. For this purpose, we synthesized Mg-Al based hydrotalcite with a molar ratio of 3, obtained either after conventional heating or after microwave irradiation with of 100W for three minutes. Structural and chemical properties of the obtained catalysts were characterized, using different techniques: X-ray diffraction (XRD), Fourier transform infrared spectra (FTIR), scanning electron microscope (SEM), equipped with energy dispersive X-ray (EDX), and specific surface area of the catalysts were determined by the methylene blue (MB) adsorption method. Also, these catalysts were tested in the self-condensation reaction of acetone at 273K in the liquid phase without solvent, a reaction which requires very high catalytic activity. The microwave treatment improves the catalyst activity, and the conversion of acetone to diacetone alcohol increases from 13.2 to 18.3% after 8 h of reaction. Moreover, the microwave-treated hydrotalcite catalyst, calcined at 723K and rehydrated under a flow of N_2 , is the most active and gives conversion of acetone of 52% under the same reaction conditions.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Several important industrial reactions such as condensation, hydrogenation, dehydrogenating reactions, oxidation, alkylation, and addition are carried out in the homogeneous phase by the liquid bases, for instance, KOH, NaOH, and Na_2CO_3 . Heterogeneous catalysis, unlike to this point mostly used homogeneous catalysis, brings advantages, like higher selectivity, easier catalyst recovery, elimination of wastewater streams, and by-products that might contribute to pollution of the environment [1]. Hydrotalcites (HT), which are layered double hydroxides (LDHs) family, are well-known as heterogeneous base catalysts for various reactions. They need layered structures containing anions and H_2O molecules between charged metal hydroxides with the chemical formula: $(1)M1-x2+Mx3+OH2X+Ax/nn-mH2Ox-$, where M^{2+} (divalent cations=Mg, Zn, Ni, Cu, and Mn) and M^{3+} (trivalent cations=Al, Cr, Co, Mn, and Fe); A is the exchangeable anions like OH^- , Cl^- , NO_3^- , CO_3^{2-} , and SO_4^{2-} . Particularly, Mg-Al hydrotalcites, where M^{2+} and M^{3+} sites comprise Mg and Al, are representative hydrotalcites [2, 3]. The structure of hydrotalcite $Mg_6Al_2(OH)_{16}CO_3 \cdot 4H_2O$ resembles that of brucite $Mg(OH)_2$. Within the latter structure, the magnesium cations are octahedrally coordinated by hydroxyl ions, leading to the stacks of edge-shared layers of the octahedral. For the HT structure, a part of the Mg^{2+} ions is replaced by Al^{3+} ions leading to positive charge layers, whose charge is compensated by interlayer anions, essentially the carbonate ions. Furthermore, water molecules are present within

the interlayer [4]. To create catalytic activity in aldol condensation reactions, HT should undergo a selected treatment. For gas-phase condensation reactions at a hot temperature, calcination at 723K is sufficient [5]. The resulting $\text{Mg-Al}(\text{O})_x$ mixed oxide is capable of catalyzing various reactions just like the self-condensation of acetone exhibiting strong Lewis basicity [5] and therefore the condensation of formaldehyde and acetone [6]. To boost the catalytic activity at lower temperatures, the calcined samples must be rehydrated under inert atmosphere without carbonate of air. This treatment provides the restoration of the layered structure and formation of the meixnerite with OH^- ions within the interlayer domain. A useful and comparatively well-described condensation reaction within the liquid-phase using rehydrated calcined HT is the self-condensation of acetone yielding diacetone alcohol (Scheme 1) [7]. It may be concluded that a reaction temperature of 273K is advantageous to prevent the dehydration of DAA to mesityl oxide and thus to avoid the liberation of water which could influence the accessibility and the reactivity of the catalyst.

[figure omitted; refer to PDF]

The catalysts show crystalline hydrotalcite patterns. All the diffraction peaks are in good agreement with the characteristics of the hexagonal phase as the (003), (006), and (012) peaks and indicate the formation of the compounds with a good crystallinity and no impurities (Figure 1) [9]. The building block parameters were calculated assuming a 3R stacking sequence, that is, $a=2d_{110}$ and $c=3d_{003}$. Accordingly, the (c) parameter is the interlayer thickness and is regulated by water content together with the amount, size, orientation, and charge of the anions located between the brucite-like layers. The cell parameter (a) corresponds to the everyday cation–cation distance inside the brucite-like layers [10].

These parameters of the synthesized hydrotalcite are calculated by knowing the reticular planes (*hkl*) diffraction peaks from the Bragg reflection, and thus, the interreticular distance $d(hkl)$ is given by the following relation [11], and these parameters are given in Table 1. $(3)1dhkl^2=4h^2+hk+k^23a^2+l^2c^2$.

Table 1

Main physicochemical characteristics of the catalysts.

Catalysts	$\theta_{(003)}$ (°)	$\theta_{(110)}$ (°)	$d_{(003)}$ (Å)	$d_{(110)}$ (Å)	$a(\text{Å})$	$c(\text{Å})$
HT3	5.62	30.24	7.87	1.530	3.061	23.61
HT3-MW	5.56	30.16	7.95	1.534	3.068	23.86
HT3-E	5.66	30.28	7.81	1.528	3.057	23.44
HT3-MW-E	5.61	30.25	7.88	1.530	3.060	23.65

The values of parameters (a) and (c) obtained (Table 1) are closer to those reported in the literature [12]. For all solids, parameter (a) remains virtually unchanged. However, (c) parameter for HT3-E and HT3-MW-E is, respectively, smaller than that of HT3 and HT3-MW. This can be explained by the load effect after anion exchange Cl^- by CO_3^{2-} , which generated an excess of negative load in the interfoliar space favoring an electrostatic attraction between carbonate ions and loaded sheets positively, and as a result, there is a decrease in interleaf distance after exchange [12]. The Debye–Scherrer method is a crystallographic analysis technique based on X-ray diffraction and used to determine the average size of crystalline materials, according to the following formula [13]: $(4)L=K\cdot\lambda\beta\times 1\cos\theta$, where K is a constant related to crystallite shape, normally taken as 0.89, λ is the X-ray wavelength in (Å), and β is the width of the diffraction peak at half maximum height in radians. The theta can be in degrees or radians. Mean crystallite size estimation obtained for the different hydrotalcite synthesis is given in Table 2.

Table 2

Values of β and L for different hydrotalcites synthesized.

Catalysts	$\theta_{(003)} (^{\circ})$	$\beta_{(003)} (^{\circ})$	$L_{(003)} (\text{nm})$
HT3	5.62	0.5130	15.4
HT3-MW	5.56	0.6158	12.8
HT3-E	5.66	0.4194	18.8
HT3-MW-E	5.61	0.4716	16.7

According to the values given in Table 2, it can be seen that for all the samples synthesized, the average particle size is in the nonmetric range. This size increases after the exchange of chlorides Cl^- by carbonates CO_3^{2-} for catalysts treated or not treated by microwave. However, samples treated by microwaves have a lower average particle size than those of solids that have undergone conventional heating at 338K. This suggests an increase in the specific surface area and may be an increase in catalytic activity, which will be checked in the study part of the condensation reaction. The synthesized samples were also analyzed by infrared spectroscopy to verify the main existing molecular groupings and vibration types between the atoms. FT-IR spectra of the as-prepared samples are shown in Figure 2. The FT-IR spectrum of the various hydrotalcite is similar to those published elsewhere [14].
[figure omitted; refer to PDF]

Peak in the range of $3450\text{--}3550\text{cm}^{-1}$ was attributed to the stretching vibrations of physisorbed water, structural hydroxyl groups in the brucite-like layer [14], and/or the valence vibration mode of the $\text{OH}-(\text{M}-\text{OH})$ of divalent and trivalent cations (Mg^{2+} and Al^{3+}) [15]. A shoulder at 3010cm^{-1} was assigned to hydrogen bonding between water molecules and the interlayer carbonate anions. The bands at 1635 and 1374cm^{-1} showed the presence of interlayer water molecules and carbonate anions, respectively [16]. The low-frequency region showed a band at about 870cm^{-1} which is characteristic for the out-of-plane deformation of carbonate, whereas the in-plane bending is located at 680cm^{-1} [16]. The band at 564cm^{-1} corresponding to the translation modes of hydroxyl groups is influenced by Al^{3+} cations [17].

From the spectra obtained (Figure 2), it is found that the characteristic bands of the carbonate species located at 1374cm^{-1} are more intense for the samples exchanged (HT3-E and HT3-MW-E) compared to those observed for the samples not exchanged (HT3 and HT3-MW), indicating the insertion of CO_3^{2-} ions into the interfoliar space, and the replacement of chloride ions from the starting salts and not removed after washing. The hydroxylation treatment was carried out on the calcined sample at 723K. The X-ray diffraction diffractograms of the two catalysts HT3-MW-Ec and HT3-MW-EcR are shown in Figure 3.

[figure omitted; refer to PDF]

The diffractogram of the calcined sample (HT3-MW-Ec) at 723K (Figure 3) shows the disappearance of peaks (003), (006), and (012) of the lamellar structure, and therefore, the appearance of recent peaks at $2\theta=44^{\circ}$ and $2\theta=63^{\circ}$ characteristics the formation of the mixed oxide $\text{Mg-Al}(\text{O})_x$. However, the X-ray diffraction spectrum of the rehydroxylated sample (HT3-MW-EcR) shows the reappearance of all peaks (003), (006), and (012) characteristics of the lamellar structure (Figure 3). Therefore, the rehydroxylation treatment transforms the mixed oxide into a lamellar compound having a well-organized structure. This treatment reveals the reconstitution of the lamellar structure of hydrotalcite compounds type and also the formation of the meixnerite phase $\text{Mg}_6\text{Al}_2(\text{OH})_{18}\cdot 4\text{H}_2\text{O}$ [18]. SEM images were recorded to observe the effect of the synthesis method on the morphology of the catalysts. For this purpose, we compared the morphology of the two catalysts prepared by two methods, either by the conventional heating method or by the microwave irradiation method. SEM images of the two catalysts calcined at 723K (HT3-Ec and HT3-MW-Ec) are shown in Figure 4.

[figure omitted; refer to PDF]

The micrographs of the two solids show well-developed layers with a platelet structure (Figure 4). The morphology of

the two mixed oxides obtained by calcination at 723K is similar to that of the calcined double lamellar hydroxides [19, 20]. However, the microwave-treated catalyst indicates a homogeneous structure with much smaller platelet sizes than those obtained by the conventional heating method, with a larger pore surface. These results are in good agreement with crystallite size calculated by the Debye–Scherrer method (Table 2). The results obtained by energy dispersive X-ray for the two calcined catalysts are shown in Figure 5.

[figures omitted; refer to PDF]

The EDX results of the two analyzed catalysts show that the elements (oxygen, magnesium, and aluminum) characteristics of the mixed oxides are almost identical (Figure 5), with a small difference in the percentage of these elements of about 1–5%. The results of the elemental analysis are given in Table 3.

Table 3

Elements analysis results from EDX data for the two catalysts.

Catalysts	Mass (%) of chemical elements		
O	Mg	Al	HT3-Ec
16.32	54.24	29.44	HT3-MW-Ec

The specific surface of the solids was calculated by the methylene blue (MB) adsorption method [21]. This technique gives good results compared to the BET method [22]. For this purpose, we use this method to measure the specific surface of the catalysts.

The experimental protocol used is the following: a methylene blue solution was prepared by mixing 1 g of dry MB powder with 200 mL of deionized water. Then, 5 g of calcined catalyst was mixed with 15 mL of deionized water. Then, the MB solution was added to this solution by addition of 1 mL. After each addition of 1 mL of MB, the suspension was mixed by a magnetic stirrer for two minutes. A drop was taken from the solution and placed on Fisher brand filter paper. If the unadsorbed MB formed a permanent blue halo around the aggregate spot of the solid, the end point was reached. Therefore, the specific surface area of the particles can be determined from the amount of MB adsorbed, when all sites of the solid are covered by MB molecules. At this optimal point, the SSA is computed by the following equation [23]:

$$(5) \text{SSA} = \frac{m_{\text{MB}}}{m_s} \times \frac{N}{A_{\text{MB}}} \times \frac{1}{N_A} \times \frac{1}{200} \times \frac{1}{A_{\text{MB}}}$$

In equation (5), m_{MB} is the mass of the MB, N is the number of MB increments added to the suspension solution, m_s is the mass of the solid, A_N is Avogadro's number ($6.02 \times 10^{23} \text{ mol}^{-1}$), and A_{MB} is the surface area covered by one molecule of methylene blue which is generally considered to be $130 \text{ \AA}^2$. The chemical formula of MB is $\text{C}_{16}\text{H}_{18}\text{ClN}_3\text{S}$, with a corresponding molecular weight of $319.86 \text{ g} \cdot \text{mol}^{-1}$. The obtained SSA results for the two solids are given in Table 4.

Table 4

Specific surface area of the solids by the MB spot-test method.

Catalysts	Specific surface area ($\text{m}^2 \cdot \text{g}^{-1}$)
HT3-Ec	207 ± 5
HT3-MW-Ec	232 ± 5

These results show that the specific surface area value of the catalyst obtained by the microwave irradiation method (HT3-MW-Ec) is higher than that prepared by the conventional heating method (HT3-Ec). Therefore, the HT3-MW-Ec catalyst has a more ordered structure than HT3-Ec. This is confirmed by the analytical results obtained by scanning electron microscopy (Figure 4).

3.2. Catalytic Activity

The catalytic activity of the catalysts was investigated for the formation of the diacetone alcohol by the aldol condensation of acetone. Initially, the reaction was disbursed with the unmodified (HT3 and HT3-MW) catalysts, and these solids showed no activity. It is generally known that chlorides poison the reaction and reduce catalytic activity [18], which is why, within the course of the work; the reactions are conducted with the catalysts that have undergone the exchange by the carbonates. After 8h of reaction time, a small quantity of DAA is obtained giving a conversion rate of about 0.2%, indicating that these modified catalysts had low catalytic activity. This will be explained by their low basicity, which led us to check the calcined catalysts, and the results obtained with calcined catalysts are given in Table 5.

Table 5

Conversion in (%) of acetone with the catalysts: HT3-Ec and HT3-MW-Ec, as a function of time.

Time (h)	Conversion (%) with HT3-Ec	Conversion (%) with HT3-MW-Ec
2	4.7	5.6
4	7.1	9.5
6	11.0	15.4
8	13.2	18.3

It was found that calcination improves the catalytic activity of the catalysts, with an increase in the conversion for the microwave-treated calcined catalyst (HT3-MW-Ec), which shows a high conversion of 18.3% after 8h of reaction, compared to calcined and not irradiated (HT3-Ec), which gives a conversion of 13.2% (Table 5). The best conversion was obtained using the microwave-treated calcined catalyst at 723K for the self-condensation of acetone.

3.3. Influence of Rehydration

To examine the effect of the rehydroxylation treatment on the conversion of acetone at 273K, we tested the catalyst HT3-MW-Ec, which produced the highest conversion (18.3%). The conversion rates deducted after each 8h of reaction are shown in Figure 6.

[figure omitted; refer to PDF]

There is a significant increase in conversion rates with the catalyst treated by microwaves calcined and rehydroxylated, with a maximum conversion of about 52% after 8h of reaction (Figure 6). This can be explained by the formation of the news sites as Bronsted type following the reconstruction of the sheet structure of the mixed oxide and the formation of the meixnerite phase. Therefore, the presence of new basic Bronsted sites of OH^- type is favorable to the self-condensation of acetone under the reaction conditions used [18].

3.4. Initial Rate of Formation of DAA

The evolution of the catalytic activity of the catalysts treated by the microwave (HT3-MW-Ec and HT3-MW-EcR) was also followed by the determination of the initial speeds of the reaction. The latter was determined from the slope of the tangent at the origin of the curves representing the evolution of the conversion rate (%) as a function of time (Figure 5) by the following formula: $(6)V_0 = C_0 \times \frac{d\alpha}{dt}$.

The values of the initial rate for the two catalysts are given in Table 6.

Table 6

Initial reaction rate for the conversion of acetone with the catalysts HT3-MW-Ec and HT3-MW-EcR.

Catalysts	V_0 (mol·l ⁻¹ ·h ⁻¹)
HT3-MW-Ec	2.57×10^{-2}
HT3-MW-EcR	17.1×10^{-2}

All the activated hydrotalcites (thermally treated and rehydrated) were active, and they present much higher catalytic activity for the conversion of acetone to diacetone alcohol. The best catalytic performance was found for HT3-MW-EcR activated and rehydrated catalyst, with an initial rate of 17.1×10^{-2} (mol·l⁻¹·h⁻¹). On the other, activated catalyst HT3-MW-Ec showed a noteworthy lesser activity with an initial rate of 2.57×10^{-2} (mol·l⁻¹·h⁻¹). The great increase of initial rate may be due to the great hydrophilic character of the hydrotalcite surface in the rehydrated form.

4. Conclusion

The Mg-Al-based hydrotalcites with an Mg/Al ratio of 3 were synthesized by the coprecipitation method. The microwave treatments and rehydroxylation of the mixed oxides obtained after calcination were performed. These catalysts were characterized by various physicochemical techniques, which confirmed their crystallinity and purity. The obtained catalysts were studied for the acetone self-condensation reaction in order to evaluate the basicity of these catalysts. It can therefore be concluded that the hydrotalcite compounds were successfully synthesized, and it was shown that the unactivated catalysts give a lower catalytic activity than that found in the case of the calcined catalysts. Since, such self-condensation reactions require a high basicity. In addition, microwave treatment increases the conversion of acetone to diacetone alcohol, and the basicity of the catalysts can also be adjusted by rehydration. Thus, the rehydration of microwave-treated catalyst calcined at 723 K (HT3-MW-EcR) was one of the most active and selective for this reaction and significantly increases the conversion of acetone.

References

- [1] K. K. Rao, M. Gravelle, J. S. Valente, F. Figueras, "Activation of Mg-Al hydrotalcite catalysts for aldol condensation reactions," *Journal of Catalysis*, vol. 173 no. 1, pp. 115-121, DOI: 10.1006/jcat.1997.1878, 1998.
- [2] Q. Wang, D. O'Hare, "Recent advances in the synthesis and application of layered double hydroxide (LDH) nanosheets," *Chemical Reviews*, vol. 112 no. 7, pp. 4124-4155, DOI: 10.1021/cr200434v, 2012.
- [3] S. Abelló, F. Medina, D. Tichit, J. Pérez-Ramírez, J. C. Groen, J. E. Sueiras, P. Salagre, Y. Cesteros, "Aldol condensations over reconstructed Mg-Al hydrotalcites: structure-activity relationships related to the rehydration method," *Chemistry-A European Journal*, vol. 11 no. 2, pp. 728-739, DOI: 10.1002/chem.200400409, 2005.
- [4] J. C. A. A. Roelofs, A. J. Van Dillen, K. P. De Jong, "Base-catalyzed condensation of citral and acetone at low temperature using modified hydrotalcite catalysts," *Catalysis Today*, vol. 60 no. 3-4, pp. 297-303, DOI: 10.1016/S0920-5861(00)00346-1, 2000.
- [5] M. Naciri Bennani, D. Tichit, F. Figueras, S. Abouarnadasse, "Synthèse et caractérisation d'hydrotalcites Mg-Al. Application à l'aldolisation de l'acétone," *Journal de Chimie Physique et de Physico-Chimie Biologique*, vol. 96 no. 3, pp. 498-509, DOI: 10.1051/jcp:1999156, 1999.
- [6] S. Arhazaf, M. N. Bennani, S. Abouarnadasse, A. Amhoud, "Study of the basicity of hydrotalcite MgAl-CO₃ and its mixed oxide by adsorption of phenol and measurement of the catalytic activity in the condensation of furfural with acetone," *Journal of Materials and Environmental Science*, vol. 7 no. 11, pp. 4226-4236, 2016.
- [7] S. Lippert, W. Baumann, K. Thomke, "Secondary reactions of the base-catalyzed aldol condensation of acetone," *Journal of Molecular Catalysis*, vol. 69 no. 2, pp. 199-214, DOI: 10.1016/0304-5102(91)80145-s, 1991.
- [8] R. Zhao, C. Yin, H. Zhao, C. Liu, "Synthesis, characterization, and application of hydrotalcites in hydrodesulfurization of FCC gasoline," *Fuel Processing Technology*, vol. 81 no. 3, pp. 201-209, DOI: 10.1016/S0378-3820(03)00012-2, 2003.
- [9] W. Hofmeister, H. V. Platen, "Crystal chemistry and atomic order in brucite-related double-layer structures," *Crystallography Reviews*, vol. 3 no. 1, DOI: 10.1080/08893119208032964, 1992.
- [10] I. N. Dialysed, "Preparation and characterization of compounds formed in dialysed systems," *Clay Minerals*, vol.

7, 1967.

- [11] Y.-J. Lin, D.-Q. Li, D. G. Evans, X. Duan, "Modulating effect of Mg-Al-CO₃ layered double hydroxides on the thermal stability of PVC resin," *Polymer Degradation and Stability*, vol. 88 no. 2, pp. 286-293, DOI: 10.1016/j.polymdegradstab.2004.11.007, 2005.
- [12] E. S. Publishers, Coldysis T&y, pp. 173-301, 1991.
- [13] A. Monshi, M. R. Foroughi, M. R. Monshi, "Modified scherrer equation to estimate more accurately nano-crystallite size using XRD," *World Journal of Nano Science and Engineering*, vol. 02 no. 03, pp. 154-160, DOI: 10.4236/wjnse.2012.23020, 2012.
- [14] M. Xu, E. Iglesia, C. R. Apestegu, D. I. Cosimo, E. T. Al, "Structure and surface and catalytic properties of Mg-Al basic oxides," *Journal of Catalysis*, vol. 510, pp. 499-510, 1998.
- [15] T. T. X. Hang, T. A. Truc, N. T. Duong, P. G. Vu, T. Hoang, T. Hoang, "Preparation and characterization of nanocontainers of corrosion inhibitor based on layered double hydroxides," *Applied Clay Science*, vol. 67-68, pp. 18-25, DOI: 10.1016/j.clay.2012.07.004, 2012.
- [16] H. Ziyat, M. Naciri Bennani, H. Hajjaj, S. Mekdad, O. Qabaqous, "Synthesis and characterization of crude hydrotalcite Mg-Al-CO₃ : study of thymol adsorption," *Research on Chemical Intermediates*, vol. 44 no. 7, pp. 4163-4177, DOI: 10.1007/s11164-018-3361-9, 2018.
- [17] S. K. Sharma, P. A. Parikh, R. V. Jasra, "Reconstructed Mg/Al hydrotalcite as a solid base catalyst for synthesis of jasminaldehyde," *Applied Catalysis A: General*, vol. 386 no. 1-2, pp. 34-42, DOI: 10.1016/j.apcata.2010.07.021, 2010.
- [18] D. Tichit, M. Naciri Bennani, F. Figueras, R. Tessier, J. Kervennal, "Aldol condensation of acetone over layered double hydroxides of the meixnerite type," *Applied Clay Science*, vol. 13 no. 5-6, pp. 401-415, DOI: 10.1016/s0169-1317(98)00035-0, 1998.
- [19] J. M. Oh, S. H. Hwang, J. H. Choy, "The effect of synthetic conditions on tailoring the size of hydrotalcite particles," *Solid State Ionics*, vol. 151 no. 1-4, pp. 285-291, DOI: 10.1016/s0167-2738(02)00725-7, 2002.
- [20] F. Kovanda, D. Koloušek, Z. Cílová, V. Hulínský, "Crystallization of synthetic hydrotalcite under hydrothermal conditions," *Applied Clay Science*, vol. 28 no. 1-4, pp. 101-109, DOI: 10.1016/j.clay.2004.01.009, 2005.
- [21] J. C. Santamarina, K. A. Klein, Y. H. Wang, E. Prencke, "Specific surface: determination and relevance," *Canadian Geotechnical Journal*, vol. 39 no. 1, pp. 233-241, DOI: 10.1139/t01-077, 2002.
- [22] Y. Yukselen, A. Kaya, "Comparison of methods for determining specific surface area of soils," *Journal of Geotechnical and Geoenvironmental Engineering*, vol. 132 no. 7, pp. 931-936, DOI: 10.1061/(asce)1090-0241(2006)132:7(931), 2006.
- [23] Y. Yukselen, A. Kaya, "Suitability of the methylene blue test for surface area, cation exchange capacity and swell potential determination of clayey soils," *Engineering Geology*, vol. 102 no. 1-2, pp. 38-45, DOI: 10.1016/j.enggeo.2008.07.002, 2008.

DETAILS

Subject: Liquid phases; Magnesium; Conversion; Catalytic activity; Spectrum analysis; Chemical synthesis; Methylene blue; Chemical properties; Acetone; Particle size; Catalysis; Fourier transforms; Catalytic converters; Morphology; Infrared spectra; Aluminum; Catalysts

Editor: David Touboul

Publication title: International Journal of Analytical Chemistry; New York

Volume: 2021

Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-04-26 (Received); 2021-10-17 (Revised); 2021-11-19 (Accepted); 2021-12-23 (Pub)
DOI:	https://doi.org/10.1155/2021/1551586
ProQuest document ID:	2615859627
Document URL:	https://www.proquest.com/scholarly-journals/study-catalytic-activity-compounds-hydrotalcite/docview/2615859627/se-2?accountid=211160
Copyright:	Copyright © 2021 Jamal Houssaini et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2022-01-02
Database:	Publicly Available Content Database

Document 3 of 59

Microdroplet Actuation via Light Line Optoelectrowetting (LL-OEW)

ABSTRACT (ENGLISH)

Meanwhile, electrowetting-on-dielectric (EWOD) is a well-known phenomenon, even often exploited in active micro-optics to change the curvature of microdroplet lenses or in analytical chemistry with digital microfluidics (DMF, lab on a chip 2.0) to move/actuate microdroplets. Optoelectrowetting (OEW) can bring more flexibility to DMF because in OEW, the operating point of the lab chip is locally controlled by a beam of light, usually impinging onto the chip perpendicularly. As opposed to pure EWOD, for OEW, none of the electrodes has to be structured, which makes the chip design and production technology simpler; the path of any actuated droplet is determined by the movement of the light spot. However, for applications in analytical chemistry, it would be helpful if the space both below as well as that above the lab chip were not obstructed by any optical components and light sources. Here, we report on the possibility to actuate droplets by laser light beams, which traverse the setup parallel to the chip surface and inside the OEW layer sequence. Since microdroplets are grabbed by this surface-parallel, nondiverging, and nonexpanded light beam, we call this principle “light line OEW” (LL-OEW).

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Electrowetting-on-dielectric (EWOD) is a phenomenon [1–13], which has been widely used to change microdroplet curvature. E.g., this way, microfluidic lenses with variable focal lengths can be realized [14–18]. For EWOD, the droplets are usually made of ionized water or an aqueous solution and sandwiched between a top electrode and a dielectric layer above a bottom electrode. (Often, for both the substrate as well as for the superstrate, glass wafers with indium tin oxide (ITO) coatings as electrode layers are used, with the ITO films pointing inwards.) Attached to a voltage source, this setup constitutes a loaded capacitor (with the droplet as the “upper capacitor plate” and the top electrode layer just to contact the droplet). The stored electrostatic energy modifies the surface energy of the droplet on the dielectric layer such that the droplet’s contact angle and curvature are reduced (the radius of curvature is increased).

If the bottom electrode is structured and initially only part of the droplet is above the electrode structure, the change of the contact angle will be local. This way, a net force arises parallel to the substrate surface, which drags/pulls the droplet onto the electrode [19–22]. Thus, EWOD can be used to move/actuate droplets, also allowing for splitting and merging/fusion of droplets. This is beneficial for digital microfluidics (DMF) in analytical chemistry, i.e., in lab-on-a-chip applications with droplets (lab on a chip 2.0) [23–33].

The necessity of a structured electrode for EWOD-based droplet actuation has the disadvantage that all desired positions and movements of the droplets have to be known in advance because the bottom electrode has to be prepared lithographically accordingly. If another actuation path becomes necessary, another chip will have to be designed and produced.

Optoelectrowetting (OEW) solves this problem [34–44]. Neither of the electrodes has to be structured. However, another layer, i.e., a photoconductive film, has to be incorporated into the layer sequence between the dielectric layer and the bottom electrode. The capacitance of the layer sequence changes locally, wherever light is impinging and entering the photoconductive layer. With illumination, the photoconductive layer is locally relatively well

electrically conductive and can be considered part of the bottom electrode, thus leaving the dielectric layer with its small thickness as the only insulator between the “plates” of the capacitor. Where and when no light impinges, the photoconductive layer is not conductive and has to be considered part of a dielectric two-layer sequence. This way, the operating point of the module changes locally. It is determined by the EWOD voltage and its modulation frequency as well as by the wavelength and intensity of the impinging light. If parameters are set right, the droplet will move wherever the light spot is moved to, which, e.g., can be manipulated and computer-controlled by a set of external mirrors.

Side note 1: In [45], the authors use OEW in combination with a structured bottom electrode. The structure is a metallic regular close-mesh grid. The droplets can only move along the lines of the grid. However, since each stitch is smaller than the droplets’ projected areas, they can be moved to any point on the lab chip. The mesh grid stabilizes the droplets’ movements. The OEW light is impinging perpendicularly from below the chip.

OEW comes with two disadvantages. First, it is virtually impossible to optimize the setup (layer materials, layer thicknesses, EWOD voltage and frequency, OEW light intensity and wavelength, and electrical droplet conductivity) in order to guarantee droplet actuation for a wide set of parameters; for example, a slightly different OEW light intensity or wavelength might change the whole situation. Secondly, the light usually impinges onto the lab chip perpendicularly, and thus the light source and optical elements will block the space above or below the lab chip. One example for an application, where this is a restriction, is formed by SERS (surface-enhanced Raman scattering) [46], i.e., molecule detection by Raman scattering spectroscopy with signal enlargement due to local field enhancement, especially in DMF [47–49] (the enhancement is achieved by the attachment of the droplets of diluted molecules, e.g., to metallic surfaces or clusters).

Side note 2: In [50], the absorption of the photoconductive layer, in that case, made from titanium oxide phthalocyanine (TiOPc), is enhanced and controlled by plasmonic nanoparticle incorporation into the TiOPc layer. Thus, the metallic particles are not intended to enhance scattering, but performance of the photoconductive layer. An improvement might come from light, which is entering the chip from the side. A principle possibility might be to couple the OEW light into a film waveguide, which has to be incorporated into the layer sequence of the lab chip. The guided film wave, divergent in the other lateral dimension, might provide for a funnel-like path of any droplet between two regions on the chip with different light intensities.

However, as we came to know in the work leading to [51], it is also virtually impossible to fulfill the requirements of OEW and film wave guiding simultaneously. To give an example, on one hand, the light has to be absorbed to some extent in the photoconductive layer for the latter’s very purpose. Yet, on the other hand, not only the film wave divergence but also the absorption will result in different light intensities in different parts of the lab chip. Thus, OEW will not work equally well across the chip.

In this contribution, we report on a successful *variation* of the last concept, which we call “*light line OEW*” (LL-OEW) because a nondivergent, nonexpanded laser beam is passing the lab chip from side to side. And the droplet is not moved along this light beam, but rather perpendicular to it whenever the light beam/line as a whole is moved into that direction; see Figure 1.

[figure omitted; refer to PDF]

Moreover, Moench and others even proved that by the right choice of modulation frequency and voltage amplitude, the droplet cannot only be pulled/dragged by the light spot, but even pushed [44].

The modified Young–Lippmann equation to be applied is
$$(3)\cos\theta=\cos\theta_0\frac{1-2\gamma/3\gamma_0+C_{\text{dpc}}/2\gamma_0A-V_{\text{dpc}}/21+\gamma/3\gamma_0\cos\theta_0}{V_{\text{dpc}}}$$
, with C_{dpc} as the capacitance of the double-layer (dielectric plus photoconductive layer) and V_{dpc} as the voltage drop across the double-layer. Both the denominator as well as the parameter γ in the above equation are related to the Laplace pressure and its case-dependent significance, respectively [52, 53], which get strong for microdroplets with volumes well below $1\mu\text{l}$; the Laplace pressure (related to the strong surface curvature of small droplets) works against the reduction of surface tension.

The abovementioned interplay of the complex-valued impedances Z is incorporated in the voltage drop V_{dpc} according to
$$V_{\text{dpc}}=Z_{\text{dpc}}Z_{\text{dpc}}+R_{\text{path}}V$$
, with the supply voltage V , where R_{path} is the overall ohmic path resistance

of the droplet itself and the supply lines (electrode layers).

Mathematical notes: Since the impedance Z_{dpc} is a complex-valued quantity, in principle, the voltage V_{dpc} , too, has to be considered a complex-valued quantity according to equation (4). Thus, we have taken the absolute value squared of this voltage and not just the square in equation (3). Moreover, if the definition of an effective voltage V_{eff} is applied in its version with a complex-valued voltage and current, one will automatically get the absolute value (squared) of the voltage in equation (3).

Equation (3) has to be applied in the cases with and without illumination to calculate the change of the contact angle through illumination as compared to the case without illumination (which might be called the case of pure EWOD):
 $\Delta\theta = \theta_{illumination} - \theta_{no-illumination}$.

Coarsely speaking, negative contact angle changes $\Delta\theta > 0$ to push actuation. Which situation applies strongly depends on the applied voltage and its modulation frequency (and all other parameters).

The OEW force can be estimated to be $F = C_{dpc,illumination} 2A \cdot V_{dpc,illumination}^2 \cdot L$, with L as the length of the illuminated portion of the droplet's contact line. Light line OEW (LL-OEW) has the advantage over OEW that this portion is longer, such that the overall actuation force is larger than for usual OEW with perpendicular light incidence. Nevertheless, the forces are small; in our LL-OEW case, the maximum force amounts to just $0.75 \mu\text{N}$. As mentioned above, we follow the notion that a negative contact angle change is related to pulling, while a positive change stands for pushing. This is not strictly true, because the pure EWOD case (i.e., the case without light) is already related to a pulling force. This force has to be overcompensated to initialize pushing. But, the applied notion eases explanation.

3. Lab Chip Layout, Electrical and Optical Setup

The OEW chip consists of a substrate and a superstrate, each with an area of $25\text{mm} \times 25\text{mm}$. A cross-sectional view of the chip is given in Figure 3 (with typical parameter values in the caption).

[figure omitted; refer to PDF]

The substrate part is formed of several layers deposited onto an ITO-coated glass slide from Merck KGaA, Darmstadt, Germany. The glass slide has a thickness of $1.1 \mu\text{m}$, the ITO layer is $(140 \pm 20) \text{ nm}$ thick, and the optical transmission of the ITO glass slide is about 87% in the visible spectral range. A hydrogenized amorphous silicon (a-Si:H) layer is deposited onto the ITO-coated bottom glass slide by plasma-enhanced chemical vapor deposition (PECVD) with a PlasmaLab 80 Plus machine from Oxford Instruments Plasma Technology, Bristol, UK. This photoconductive a-Si:H layer has a thickness of $(790 \pm 10) \text{ nm}$. It is covered with a $(360 \pm 10) \text{ nm}$ thick dielectric layer consisting of Parylene C and a hydrophobic top layer of approx. 10 nm thin PTFE AF 1600 [54]. The Parylene C raw material is from Specialty Coating System Inc., Indianapolis, IN, USA and is deposited by chemical vapor deposition using a PDS 2010 also from Specialty Coating System Inc. PTFE AF 1600 is from Sigma Aldrich, Taufkirchen, Germany, and is spin-coated using a Delta BM Gyrset from SÜSS MicroTech, Garching, Germany.

The superstrate part consists of an ITO-coated glass slide of the same type, and a 100 to $250 \mu\text{m}$ thick (depending on designated droplet size) Ordyl® SY 355 film is used as the spacer. Ordyl® SY 355 is a dry film resist from ElgaEurope s.r.l, Milano, Italy. The thickness of one single fabricated Ordyl® SY 355 layer is $50 \mu\text{m}$. The dry film resist is applied to the substrate by hot-roll lamination of two to five layers (giving the total thickness of 100 to $250 \mu\text{m}$) and structured photolithographically. Like the substrate part, the superstrate part is hydrophobized with an approx. 10 nm thin, porous PTFE AF 1600 layer. In the superstrate case, this layer is deposited directly onto the ITO layer.

The 10 nm thin PTFE AF 1600 layers are porous, such that the electrical contact of the droplets to the dielectric layer and to the top electrode layer does not deteriorate.

To some extent, the optimization of the layer thicknesses has been a matter of trial and error, in combination with calculations in advance incorporating the dielectric numbers ϵ_{diel} and ϵ_{pc} of the materials for the dielectric and the photoconductive layer (at small frequencies). The aim has been to achieve capacitances and impedances of the layer sequence, which allow for a strong interplay, and to find a situation, where a small change in voltage or frequency has a strong impact, thus maximizing forces on the droplets.

The determination of the layer thicknesses achieved in a chip production run is performed by interpolation of data from calibration runs, in which the material deposition time is varied, and the resulting layer thicknesses are measured, e.g., with a surface profiler comparing a part of the wafer with deposited layer to another part, which has been obstructed during deposition, by measuring the step from one to the other.

The droplets of 2% NaCl aqueous solution with volume in a range between 0.2 and 0.8 μl are placed within an area of 10mm $\times$ 10mm within the spacer pattern. Then, the substrate and the superstrate part of the module are joined by spring-loaded pins 0906-1-15-20-75-14-11-0 from Mill-Max Manufacturing Corp., Oyster Bay, NY, USA, and clamps. Figure 4 contains an exploded assembly view of the mechanical bond.

[figure omitted; refer to PDF]

A rectangular alternating voltage in the range of $\pm(30\dots70)$ V at a frequency of 50Hz is applied for OEW and LL-OEW.

In both cases, the illumination is done with a laser diode L520P50 from ThorLabs GmbH, Bergkirchen, Germany. The laser diode has an emission wavelength of $\lambda=520\text{nm}$, and the light intensity is reduced by a neutral density filter to a continuous wave power of $P=150\text{nW}$ on the chip. The laser beam (collimated with a lens right behind the laser diode) is neither focused nor expanded further down along its path. The light beam has an elliptical cross section with semiaxes of 0.75 and 0.27 mm lengths, giving an intensity of 0.74W/m^2 . Higher intensities do not necessarily mean better OEW actuation.

4. Droplet Movement with Usual OEW

To demonstrate the usual OEW for comparison, Figure 5 shows different frames from a video on droplets' movements and merging. The applied voltage is $V_{ac} = \pm 40\text{V}$ at 50Hz. A laser spot is directed *perpendicularly* onto the respective droplet from below by the movement of a manually controlled external mirror.

[figure omitted; refer to PDF]

Both for Figure 5 as well as for Figure 6, the videos are provided as supplementary materials to this contribution (see below).

In the videos, the movements of the droplets are rather slow due to the slow shift of the light line, which is actuated manually. However, the EWOD response time of the droplets with a similar layer sequence is

Videos and frames from them for other voltages and frequencies would look very similar. Thus, to shed some more light on the complex situation, we give the results of calculations regarding the setup from Figure 6. The additional parameters are given in the figure caption of Figure 7; the figure itself shows the calculated results. The contact angle change is plotted as a function of supply voltage V and modulation frequency ν .

[figure omitted; refer to PDF]

For small frequencies and larger voltages, there is an area of undefined contact angle changes, in which no values are plotted. This is the area, where the right side of equation (3) is larger than 1 and, thus, out of bounds for any cosine function. In physical reality, this range is related to a saturation of the contact angle. For voltages below about $\pm 39\text{V}$, there is droplet pulling ($\Delta\theta > 0$) for frequencies still well below 1 kHz (on the contrary to the findings in [44]). The maximum values of the contact angle change for pulling and pushing are similar for this set of parameters with amounts of $\approx 40^\circ$.

The calculated results reveal that the ranges of voltage and frequency, where the contact angle changes are not negligible, are very narrow. A change of the voltage by $\pm 2.5\text{V}$ or a change of the frequency by $\pm 25\text{Hz}$ might result in just minor contact angle changes (or mathematically even undefined values). This is another disadvantage of OEW (and LL-OEW) as compared to EWOD. The freedom of droplet actuation paths is paid for by the narrow window of optimized parameters.

Another problem is that not only the supply voltage and its modulation frequency have an impact, but also all other parameters, even the droplet's electrical conductance (leading to a specific ohmic resistance), but also the droplet's size. The smaller a droplet, the stronger is the Laplace pressure. Moreover, the droplet size also determines the projected area A of the droplet and the length L of the illuminated portion of the contact line. Thus, any optimization of the chip can only be done considering a certain range of droplet sizes (volumes and projected areas). For another

range, another chip has to be prepared after appropriate calculations. – In practice, the smallest allowable droplet diameter is given by the distance between the substrate with its layers and the superstrate with its layers (in our case, 100 to 250 μm), because the droplet has to touch both the dielectric layer (with PTFE on top) and the upper electrode layer (with PTFE below).

Despite their problems, OEW and LL-OEW add some flexibility to DMF module use.

6. Conclusions

We have presented a new concept, called light line optoelectrowetting (LL-OEW), to use OEW for droplet actuation, possibly in digital microfluidics (DMF) for any application in analytical chemistry on labs on a chip (lab on a chip 2.0). The OEW light is traversing the lab chip sideways, thus keeping the space above and below the chip free of optical components and the (laser) light source. This is a necessity for applications, where the droplets also have to be monitored/manipulated/analyzed otherwise.

Light line optoelectrowetting (LL-OEW) adds another opportunity to the EWOD/OEW toolbox.

Acknowledgments

This research was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) under contract FO 157/60. The authors are thankful for the financial support. Moreover, general technological assistance by the Nano Structuring Center (NSC) of the Technische Universität Kaiserslautern (TUK), Germany, is gratefully acknowledged. The authors would also like to thank Egbert Oesterschulze and his group at TUK for the opportunity to use the CVD and PECVD machines to prepare the dielectric and the photoconductive layer, respectively. This research has been funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) under contract FO 157/60.

- [1] B. Berge, "Électrocapillarité et mouillage de films isolants par l'eau," *Comptes Rendus de l'Academie des Sciences Serie II B Mecanique-Physique-Chimie-Astronomie*, vol. 317, pp. 157-163, 1993.
- [2] M. Vallet, M. Vallade, B. Berge, "Limiting phenomena for the spreading of water on polymer films by electrowetting," *The European Physical Journal B*, vol. 11 no. 4, pp. 583-591, DOI: 10.1007/s100510051186, 1999.
- [3] H. J. J. Verheijen, M. W. J. Prins, "Reversible electrowetting and trapping of charge: model and experiments," *Langmuir*, vol. 15 no. 20, pp. 6616-6620, DOI: 10.1021/la990548n, 1999.
- [4] J. Buehrle, S. Herminghaus, F. Mugele, "Interface profiles near three-phase contact lines in electric fields," *Physical Review Letters*, vol. 91, DOI: 10.1103/PhysRevLett.91.086101, 2003.
- [5] B. Shapiro, H. Moon, R. L. Garrell, C.-J. C. Kim, "Equilibrium behavior of sessile drops under surface tension, applied external fields, and material variations," *Journal of Applied Physics*, vol. 93 no. 9, pp. 5794-5811, DOI: 10.1063/1.1563828, 2003.
- [6] K. H. Kang, "How electrostatic fields change contact angle in electrowetting," *Langmuir*, vol. 18 no. 26, pp. 10318-10322, DOI: 10.1021/la0263615, 2002.
- [7] T. B. Jones, "On the relationship of dielectrophoresis and electrowetting," *Langmuir*, vol. 18 no. 11, pp. 4437-4443, DOI: 10.1021/la025616b, 2002.
- [8] H. Moon, S. K. Cho, R. L. Garrell, C.-J. C. Kim, "Low voltage electrowetting-on-dielectric," *Journal of Applied Physics*, vol. 92 no. 7, pp. 4080-4087, DOI: 10.1063/1.1504171, 2002.
- [9] F. Mugele, J.-C. Baret, "Electrowetting: from basics to applications," *Journal of Physics: Condensed Matter*, vol. 17 no. 28, pp. R705-R774, DOI: 10.1088/0953-8984/17/28/r01, 2005.
- [10] T. B. Jones, "An electromechanical interpretation of electrowetting," *Journal of Micromechanics and Microengineering*, vol. 15 no. 6, pp. 1184-1187, DOI: 10.1088/0960-1317/15/6/008, 2005.
- [11] F. Mugele, J. Buehrle, "Equilibrium drop surface profiles in electric fields," *Journal of Physics: Condensed Matter*, vol. 19 no. 37, DOI: 10.1088/0953-8984/19/37/375112, 2007.
- [12] J. Berthier, P. Dubois, P. Clementz, P. Claustre, C. Peponnet, Y. Fouillet, "Actuation potentials and capillary forces in electrowetting based microsystems," *Sensors and Actuators A: Physical*, vol. 134 no. 2, pp. 471-479, DOI: 10.1016/j.sna.2006.04.050, 2007.
- [13] K.-L. Wang, T. B. Jones, "Saturation effects in dynamic electrowetting," *Applied Physics Letters*, vol. 86 no.

5, DOI: 10.1063/1.1861501, 2005.

[14] B. Berge, J. Peseux, "Variable focal lens controlled by an external voltage: an application of electrowetting," *The European Physical Journal E*, vol. 3 no. 2, pp. 159-163, DOI: 10.1007/s101890070029, 2000.

[15] C. Gabay, B. Berge, G. Dovillaire, S. Bucourt, "Dynamic study of a varioptic variable focal lens," *Proceedings of SPIE*, vol. 4767, pp. 159-165, DOI: 10.1117/12.468224, 2002.

[16] S. Kuiper, B. H. W. Hendriks, "Variable-focus liquid lens for miniature cameras," *Applied Physics Letters*, vol. 85 no. 7, pp. 1128-1130, DOI: 10.1063/1.1779954, 2004.

[17] F. Krogmann, W. Monch, H. Zappe, "Electrowetting for tunable microoptics," *Journal of Microelectromechanical Systems*, vol. 17 no. 6, pp. 1501-1512, DOI: 10.1109/jmems.2008.2007256, 2008.

[18] H. Zappe, C. Duppé, *Tunable Micro-optics*, 2015.

[19] M. G. Pollack, R. B. Fair, A. D. Shenderov, "Electrowetting-based actuation of liquid droplets for microfluidic applications," *Applied Physics Letters*, vol. 77 no. 11, pp. 1725-1726, DOI: 10.1063/1.1308534, 2000.

[20] M. G. Pollack, A. D. Shenderov, R. B. Fair, "Electrowetting-based actuation of droplets for integrated microfluidics Electronic supplementary information (ESI) available: six videos showing droplet flow, droplet dispensing and electrowetting," *Lab on a Chip*, vol. 2 no. 2, pp. 96-101, DOI: 10.1039/b110474h, 2002.

[21] S. Kwon Cho, H. Moon, C. Kim, "Creating, transporting, cutting, and merging liquid droplets by electrowetting-based actuation for digital microfluidic circuits," *Journal of Microelectromechanical Systems*, vol. 12 no. 1, pp. 70-80, DOI: 10.1109/jmems.2002.807467, 2003.

[22] S. N. Pei, M. C. Wu, "On-chip blade for accurate splitting of droplets in light-actuated digital microfluidics," *Proceedings of the 16th International Conference on Miniaturized Systems for Chemistry and Life Sciences MicroTAS 2012*, .

[23] J. Cao, S. Schneider, R. Schultheiß, A. Schober, J. M. Köhler, G. A. Groß, "From microtiter plates to droplets" tools for micro-fluidic droplet processing," *Microsystem Technologies*, vol. 21 no. 3, pp. 539-548, DOI: 10.1007/s00542-013-1981-y, 2015.

[24] P. C. H. Li, *Microfluidic Lab-On-A-Chip for Chemical and Biological Analysis and Discovery*, 2005.

[25] Y. Fouillet, D. Jary, C. Chabrol, P. Claustre, C. Peponnet, "Digital microfluidic design and optimization of classic and new fluidic functions for lab on a chip systems," *Microfluidics and Nanofluidics*, vol. 4 no. 3, pp. 159-165, DOI: 10.1007/s10404-007-0164-5, 2007.

[26] R. B. Fair, "Digital microfluidics: is a true lab-on-a-chip possible?," *Microfluidics and Nanofluidics*, vol. 3 no. 3, pp. 245-281, DOI: 10.1007/s10404-007-0161-8, 2007.

[27] P. Day, A. Manz, Y. Zhang, *Microdroplet Technology: Principles and Emerging Applications in Biology and Chemistry*, 2012.

[28] A. Banerjee, E. Kreit, Y. Liu, J. Heikenfeld, I. Papautsky, "Reconfigurable virtual electrowetting channels," *Lab on a Chip*, vol. 12 no. 4, pp. 758-764, DOI: 10.1039/c2lc20842c, 2012.

[29] A. Banerjee, J. Noh, Y. Liu, P. Rack, I. Papautsky, "Programmable electrowetting with channels and droplets," *Micromachines*, vol. 6 no. 2, pp. 172-185, DOI: 10.3390/mi6020172, 2015.

[30] K. Choi, A. H. C. Ng, R. Fobel, A. R. Wheeler, "Digital microfluidics," *Annual Review of Analytical Chemistry*, vol. 5 no. 1, pp. 413-440, DOI: 10.1146/annurev-anchem-062011-143028, 2012.

[31] M. J. Jebrail, M. S. Bartsch, K. D. Patel, "Digital microfluidics: a versatile tool for applications in chemistry, biology and medicine," *Lab on a Chip*, vol. 12 no. 14, pp. 2452-2463, DOI: 10.1039/c2lc40318h, 2012.

[32] E. Samiei, M. Tabrizian, M. Hoorfar, "A review of digital microfluidics as portable platforms for lab-on a-chip applications," *Lab on a Chip*, vol. 16 no. 13, pp. 2376-2396, DOI: 10.1039/c6lc00387g, 2016.

[33] S. L. S. Freire, "Perspectives on digital microfluidics," *Sensors and Actuators A: Physical*, vol. 250, pp. 15-28, DOI: 10.1016/j.sna.2016.08.007, 2016.

[34] P. Y. Chiou, H. Moon, H. Toshiyoshi, C.-J. Kim, M. C. Wu, "Light actuation of liquid by optoelectrowetting," *Sensors and Actuators A: Physical*, vol. 104 no. 3, pp. 222-228, DOI: 10.1016/s0924-4247(03)00024-4, 2003.

[35] P.-Y. Chiou, Z. Chang, M. C. Wu, "Droplet manipulation with light on optoelectrowetting device," *Journal of*

- Microelectromechanical Systems, vol. 17 no. 1, pp. 133-138, DOI: 10.1109/jmems.2007.904336, 2008.
- [36] P. Y. Chiou, S.-Y. Park, M. C. Wu, "Continuous optoelectrowetting for picoliter droplet manipulation," *Applied Physics Letters*, vol. 93 no. 22, DOI: 10.1063/1.3039070, 2008.
- [37] S.-Y. Park, P.-Y. Chiou, "Light-driven droplet manipulation technologies for lab-on-a-chip applications," *Advances in OptoElectronics*, vol. 2011, DOI: 10.1155/2011/909174, 2011.
- [38] J. K. Valley, S. NingPei, A. Jamshidi, H.-Y. Hsu, M. C. Wu, "A unified platform for optoelectrowetting and optoelectronic tweezers," *Lab on a Chip*, vol. 11 no. 7, pp. 1292-1297, DOI: 10.1039/c0lc00568a, 2011.
- [39] P.-Y. Chiou, S.-Y. Park, "Single-sided continuous optoelectrowetting (SCEOW) device for droplet manipulation with light patterns," 2012. *Lab on Chip*, US-Patent US20120024708A1
- [40] T.-M. Yu, S.-M. Yang, C.-Y. Fu, M.-H. Liu, L. Hsu, H.-Y. Chang, C.-H. Liu, "Integration of organic optoelectrowetting and poly(ethylene) glycol diacrylate (PEGDA) microfluidics for droplets manipulation," *Sensors and Actuators B: Chemical*, vol. 180, pp. 35-42, DOI: 10.1016/j.snb.2011.12.059, 2013.
- [41] V. Shekar, M. Campbell, S. Akella, "Towards automated optoelectrowetting on dielectric devices for multi-axis droplet manipulation," *Proceedings of the IEEE International Conference on Robotics and Automation (ICRA)* Karlsruhe, pp. 1431-1437, DOI: 10.1109/icra.2013.6630760, .
- [42] C. M. Collier, K. A. Hill, M. A. DeWachter, A. M. Huizing, J. F. Holzman, "Optoelectrowetting for continuous microdroplet actuation," *Biophotonics: Photonic Solutions for Better Health Care IV*, vol. IV, DOI: 10.1117/12.2052040, 2014.
- [43] C. M. Collier, K. A. Hill, M. A. DeWachter, A. M. Huizing, J. F. Holzman, "Nanophotonic implementation of optoelectrowetting for microdroplet actuation," *Journal of Biomedical Optics*, vol. 20 no. 2, DOI: 10.1117/1.JBO.20.2.025004, 2015.
- [44] F. Krogmann, H. Qu, W. Mönch, H. Zappe, "Push/pull actuation using opto-electrowetting," *Sensors and Actuators A: Physical*, vol. 141 no. 2, pp. 499-505, DOI: 10.1016/j.sna.2007.08.017, 2008.
- [45] J. Loo, S. N. Pei, M. C. Wu, "Co-planar light-actuated optoelectrowetting microfluidic device for droplet manipulation," *Journal of Optical Microsystems*, vol. 1 no. 3, DOI: 10.1117/1.JOM.1.3.034001, 2021.
- [46] R. Pilot, R. Signorini, C. Durante, L. Durante, M. Orian, L. Bhamidipati, A. Fabris, "Review on Surface-Enhanced Raman Scattering," *Biosensors*, vol. 9 no. 2, DOI: 10.3390/bios9020057, 2019.
- [47] C. Benz, H. Retzbach, S. Nagl, D. Belder, "Protein-protein interaction analysis in single microfluidic droplets using FRET and fluorescence lifetime detection," *Lab on a Chip*, vol. 13 no. 14, pp. 2808-2814, DOI: 10.1039/c3lc00057e, 2013.
- [48] T.-A. Meier, E. Poehler, F. Kemper, O. Pabst, H.-G. Jahnke, E. Beckert, A. Robitzki, D. Belder, "Fast electrically assisted regeneration of on-chip SERS substrates," *Lab on a Chip*, vol. 15 no. 14, pp. 2923-2927, DOI: 10.1039/c5lc00397k, 2015.
- [49] T.-A. Meier, R. J. Beulig, E. Klinge, M. Fuss, S. Ohla, D. Belder, "On-chip monitoring of chemical syntheses in microdroplets via surface-enhanced Raman spectroscopy," *Chemical Communications*, vol. 51 no. 41, pp. 8588-8591, DOI: 10.1039/c4cc09595b, 2015.
- [50] S. K. Thio, S. Bae, S.-Y. Park, "Plasmonic nanoparticle-enhanced optoelectrowetting (OEWE) for effective light-driven droplet manipulation," *Sensors and Actuators B: Chemical*, vol. 308, DOI: 10.1016/j.snb.2020.127704, 2020.
- [51] C. Heisel, "Micro-fluidic droplet array actuated via electrowetting," 2016. Ph.D. thesis
- [52] W. J. Jasper, S. Rasipuram, "Relationship between contact angle and contact line radius for micro to atto [10⁻⁶ to 10⁻¹⁸] liter size oil droplets," *Journal of Molecular Liquids*, vol. 248, pp. 920-926, DOI: 10.1016/j.molliq.2017.10.134, 2017.
- [53] W. J. Jasper, N. Anand, "A generalized variational approach for predicting contact angles of sessile nano-droplets on both flat and curved surfaces," *Journal of Molecular Liquids*, vol. 281, pp. 196-203, DOI: 10.1016/j.molliq.2019.02.039, 2019.
- [54] H. Zhang, S. G. Weber, "Teflon AF materials," *Fluorous Chemistry*, vol. 308, pp. 307-337, DOI: 10.1007/128_2011_249, 2011.

[55] J. Strassner, C. Heisel, D. Palm, H. Fouckhardt, "Microfluidic droplet array as optical irises actuated via electrowetting," Advances in OptoElectronics, vol. 2018,DOI: 10.1155/2018/1262947, 2018.

DETAILS

Subject:	Optical components; Actuation; Microfluidics; Electrodes; Analytical chemistry; Light; Light beams; Contact angle; Droplets; Laser beams; Light sources; Micro-optics; Light spots
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-08-11 (Received); 2021-10-15 (Revised); 2021-12-10 (Accepted); 2021-12-23 (Pub)
DOI:	https://doi.org/10.1155/2021/3402411
ProQuest document ID:	2615859558
Document URL:	https://www.proquest.com/scholarly-journals/microdroplet-actuation-via-light-line/docview/2615859558/se-2?accountid=211160

Copyright: Copyright © 2021 Christoph Doering et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2022-01-02

Database: Publicly Available Content Database

Document 4 of 59

Rapid Simultaneous Determination of 43 Pesticide Residues in *Schizonepeta tenuifolia* by Gas Chromatography Mass Spectrometry

Guo-wei, Zhou ¹
; Yong-mei, Li ¹
; Chun-ni, Liu ¹
; Hong-min, Ren ²
; Hai-ying, Li ³

¹ Chengyang District People's Hospital of Qingdao, Qingdao, Shandong 266109, China ² Baoding Customs Office, Baoding 071000, China ³ College of Pharmacy, Hebei University, Key Laboratory of Pharmaceutical Quality Control of Hebei Province, Baoding 071002, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

A simple, fast, and reliable method was established for simultaneous determination of 43 pesticides in *Schizonepeta tenuifolia*. The samples were prepared using solid-phase extraction (SPE) method. Pesticides were extracted from *Schizonepeta tenuifolia* using acetonitrile, cleaned with Pesticarb/NH₂, and eluted by mixed solvents of acetonitrile and toluene (3:1, v/v). Selected pesticides were identified using DB-35MS capillary column and detected by gas chromatography mass spectrometry. Samples were quantified by external standard method. Recoveries for the majority of pesticides at spike levels of 0.2, 0.5, and 1 mg kg⁻¹ ranged between 70 and 120% (except for Chlorothalonil, Thiamethoxam, and Dicofol), and the relative standard deviations (RSDs *n*=6) were 1.32%–13.91%. Limits of detection (LODs) were 0.0011–0.0135 mg kg⁻¹, whereas limits of quantification (LOQs) were 0.0038–0.0451 mg kg⁻¹. The satisfactory accuracy and precision, in combination with a good separation and few interferences, have demonstrated the strong potential of this technique for its application in *Schizonepeta tenuifolia* analysis.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Chinese herbal medicines (CHMs) have been widely used as a means of medication for their mild pharmaceutical effects and minimum side effects [1]. What is more, they have been considered to be gentle, nontoxic, and even harmless mainly because of their natural origin [2]. On account of the increasing requirements, a large amount of pesticides are applied for reducing loss from insects and diseases, which could lead to a high risk of contamination from agricultural chemicals [3]. What is worse, the bioaccumulation and persistent character of pesticides also pose a threat to human health as well as the environment [4–6]. Therefore, pesticides residue determination in CHMs plays an important role in market monitoring, environmental research, risk assessment of dietary intakes, and especially in the exportation of CHMs worldwide.

Schizonepeta tenuifolia is one of the most frequently used crude drugs for oriental medicine in China, Korea, Japan, and other Asian areas. It plays an important role in agricultural production and people's living. Pharmacological studies and clinical practice have proven that it is used as an antipyretic, analgesic, antipathogenic microorganisms, anti-inflammatory, antioxidation, and hemostasis drug for the treatment of colds, headaches, measles, rubella, sores, etc. [7]. Like other crops, *Schizonepeta tenuifolia* is susceptible to insect and disease attacks both in field and storage, so pesticides are widely used for their protection. Hence, it is significative to monitor pesticide residues in *Schizonepeta tenuifolia*.

Pesticides can be mainly categorized into five classes, namely, organochlorine pesticides (OCPs), organophosphorus pesticides (OPPs), pyrethroid pesticides (PYRs), carbamate pesticides (CBs), and other type of pesticides. Only a few analytical methods for the determination of pesticide residues in *Schizonepeta tenuifolia* have been described in recent literature [3, 8, 9]. Yi and Lu [10] reported a multiresidue method for the determination of 17 pesticides with only OCPs and OPPs in *Schizonepeta tenuifolia*. Yet these methods are showing their limits about pesticide varieties. In the case of treatment with sulfuric acid, it cannot be applied to the analysis of the pesticides that decompose easily under strong acidic conditions, such as PYRs.

Mass spectrometry (MS) is a good multiresidue analysis technique for its universal property and higher sensitivity. It not only can effectively discriminate the signals between analyte and impurities, but also can be identified and quantify the results via the selected ion monitoring (SIM) spectra. Therefore, it can be used to detect different types of pesticides in a variety of different substances, such as fruits [11], vegetables [12], spices [13], grape wine [14], or Chinese teas [15]. To eliminate the interferences of matrixes and keep the chromatographic system in good working order, an effective sample preparation process for different detected objects is necessary. There are many pretreatment techniques for the extraction and clean-up of pesticides in CHMs. Fu et al. [16] reported a multiresidue method using gas chromatography mass spectrometry for the determination of 201 pesticides in different CHMs. Yet, these methods are not capable of removing the complex coextractives that may interfere with the detection of the analytes of *Schizonepeta tenuifolia*. Besides, some of these technologies are relatively time-consuming and require large volumes of organic solvents. This is hazardous to human health and causes serious pollution problems. Therefore, the achievement of a good separation and few interferences from *Schizonepeta tenuifolia* samples is considered to be a difficult, in some respect even more challenging task.

To the best of our knowledge, this is the first report on the multiresidue analysis by gas chromatography mass spectrometry in *Schizonepeta tenuifolia*. A multiresidue extraction method using the orthogonal test coupled with dispersive SPE technique was firstly proposed to determine 43 pesticide residues in *Schizonepeta tenuifolia*, including seventeen OCPs, nine OPPs, five PYRs, two CBs, and ten other pesticides. An additional purpose was to find the best performance of the different pretreatments. In this study, the extraction method was optimized using an orthogonal test of $L_9(3^4)$, according to different extraction solvent, extraction solvent volume, and the extraction time. Meanwhile, the type of sorbents and eluents which affected the efficiency of SPE was investigated. A simple, fast, and reliable method was finally established for simultaneous determination of 43 pesticides in *Schizonepeta tenuifolia*. Moreover, this might be helpful for detecting pesticide residues in other CHMs in addition.

2. Materials and Methods

2.1. Chemicals and Materials

Pesticide standards including dichlorvos, carbofuran, methamidophos, ethoprophos, omethoate, phorate, hexachlorobenzene, α -BHC, monocrotophos, quitozene, γ -BHC, dimethoate, acetochlor, β -BHC, prometryn, δ -BHC, metalaxyl, chlorothalonil, aldrin, chlorpyrifos, parathion, dicofol, pendimethalin, *cis*-heptachlor epoxide, *trans*-heptachlor epoxide, *cis*-chlordane, *trans*-chlordane, thiamethoxam, *pp'*-DDE, dieldrin, fludioxonil, *op'*-DDT, nitrofen, *pp'*-DDD, β -endosulfan, propiconazole, *pp'*-DDT, bifenthrin, fenpropathrin, cyhalothrin, cypermethrin, fenvalerate, and difenoconazole were purchased from Agro-Environment Protection Institute (Tianjin, China). The individual stock standard solution of each pesticide is 100mgL⁻¹. This solution was further diluted with n-hexane to give working solution concentrations of 50, 100, 200, 500, and 1000 μ gL⁻¹, respectively, and all solutions were stored at 4°C. Acetonitrile, petroleum ether, methylbenzene, and acetone (HPLC grade) were supplied by Tianjin Chemical Reagents Co. (Tianjin, China). Florisil (250mgmL⁻¹), TPT (2000mg/12mL), and Pesticarb/NH₂ (500mg/500mg/6mL) were purchased from Tianjin Agela Technologies Co. (Tianjin, China).

2.2. GC-MS Conditions

The chromatographic system was a QP-2010Plus gas chromatograph coupled with a mass spectrometer and a AOC-20i autosampler (Shimadzu instrument Co., Japan). All 43 pesticides were separated on DB-35ms capillary column (30m×0.25mm, 0.25 μ m film thickness; Agilent Co., USA). The oven temperature was programmed as follows: the initial column temperature is 50°C, increased at 45°Cmin⁻¹ to 130°C, then at 10°Cmin⁻¹ to 190°C, then at 4°Cmin⁻¹ to 240°C, hold for 1 min, finally at 15°Cmin⁻¹ to 305°C, hold for 10 min. The inlet temperature was set at 290°C. The injection volume was 1 μ L with a splitless mode. Helium gas (99.999% purity) was chosen as the carrier gas with a constant flow of 1.1 mLmin⁻¹. The mass spectrometer was operated at 70 eV in electron impact (EI) mode with an ion source temperature of 230°C. The transmission line temperature and solvent delay time were 280°C and 5 min, respectively. Under the same chromatographic conditions, target and qualifier abundances were determined by injection of individual pesticide standards in full-scan mode with the mass/charge ratio ranging from *m/z* 50 to 500. Analysis was performed in the selected ion monitoring mode (SIM) and the mass fragment with the highest intensity was used for quantification, while two or three are selected as the qualifier ions from the remaining fragments.

Table 1 summarizes the retention times and characteristic mass fragments of the pesticides. The mixture of 43 active ingredients at 0.5mgL⁻¹ was made by each pesticide standard and the total ion chromatogram of the 43 pesticides in standard mixture solution is shown in Figure 1.

Table 1

Retention times, target ions, and qualifier ions of the selected pesticides.

Pesticide	Retention time	Target ion	Qualifier ion 1	Qualifier ion 2	Qualifier ion 3
Dichlorvos	5.53	109	185	220	
Carbofuran	6.03	164	122	123	
Methamidophos	6.18	94	95	126	
Ethoprophos	10.60	158	200	242	
Omethoate	11.58	156	110	126	
Phorate	11.63	121	231	260	
Hexachlorobenzene	12.07	284	286	282	

α -BHC	12.40	219	183	221	254
Monocrotophos	12.96	127	109	192	
Quintozene	13.50	237	249	295	
γ -BHC	13.87	183	219	221	254
Dimethoate	14.03	87	93	125	
Acetochlor	15.07	146	162	223	
β -BHC	15.12	219	217	181	254
Prometryn	16.14	241	184	226	
δ -BHC	16.22	219	181	217	254
Metalaxyl	16.42	206	234	249	
Chlorothalonil	16.52	266	231	194	
Aldrin	16.52	263	265	329	293
Chlorpyrifos	17.31	314	258	286	
Parathion	17.98	291	186	235	
Dicofol	18.60	139	141	252	250
Pendimethalin	18.81	252	220	162	
<i>cis</i> -Heptachlor epoxide	19.00	353	351	355	
<i>trans</i> -Heptachlor epoxide	19.34	353	351	355	
<i>cis</i> -Chlordane	20.12	373	375	377	
<i>trans</i> -Chlordane	20.53	373	375	377	
Thiamethoxam	20.88	182	212	247	
<i>pp'</i> -DDE	21.73	318	316	246	248
Dieldrin	22.10	263	277	345	380

Fludioxonil	23.39	248	127	154	
<i>op'</i> -DDT	23.50	235	237	165	199
Nitrofen	23.60	283	139	202	
<i>pp'</i> -DDD	23.89	235	237	165	199
β -Endosulfan	24.21	241	265	339	
Propiconazole	24.62	259	173	261	
<i>pp'</i> -DDT	24.65	235	237	165	246
Bifenthrin	24.92	181	165	166	
Fenpropathrin	25.75	181	265	349	
Cyhalothrin	26.40	181	197	141	
Cypermethrin	29.88	181	152	180	
Fenvalerate	32.06	167	181	225	419
Difenoconazole	34.88	323	325	265	

[figure omitted; refer to PDF]

2.3. Sample Preparation and Measurement Procedure

The sample preparation procedure is shown in Figure 2. *Schizonepeta tenuifolia* plant samples were ground mechanically to a homogeneous powder and sieved through a no. 50 mesh sieve ($355\mu\text{m} \pm 13\mu\text{m}$ aperture). 1.0g powder samples were accurately weighed and placed into a 50mL polystyrene centrifuge tube, then 8mL acetonitrile was added. Subsequently, the extraction was centrifuged for 10min at 4000rpm with a 3-18K centrifuge (Sigma Co. Germany) after ultrasonic extraction for 20min. The upper organic solution was collected and the remaining drugs were repeatedly extracted according to the above operation. The mixed organic solution was filtered and concentrated with a vacuum rotary evaporator (40°C).

[figure omitted; refer to PDF]

The Pesticarb/ NH_2 column (500mg/500mg/6mL) was fixed to a support and filled with 1cm anhydrous sodium sulfate. After being activated with 4mL acetonitrile/toluene (3:1, v/v), the extraction sample was added and eluted with 25mL acetonitrile/toluene (3:1, v/v). The eluant was collected into a heart-shaped flask and concentrated to dryness using a rotary vacuum evaporator (40°C). Finally, the residue was dissolved in 1mL n-hexane and filtered through a $0.45\mu\text{m}$ disposable syringe filter before injecting into an autosampler vial for GC-MS analysis.

3. Results and Discussion

3.1. Optimization of Extraction Conditions

Due to the complex nature of the matrices in which the target compounds are present and the low detection levels required by regulatory bodies, efficient sample preparation is an important aspect of analytical methods [2].

Extraction and purification are important steps in the sample preparation process. The ultrasonic extractor which is

the most commonly used laboratory equipment has good extraction efficiency [17–19]. In this study, the extraction method was optimized using an orthogonal test of $L_9(3^4)$, according to different extraction solvent, extraction solvent volume, and the extraction time. A portion (1 mL) of the 1.0 mg L^{-1} standard mixture solutions was added to 1.0000 g of the *Schizonepeta tenuifolia*. For each experimental trial, three replicate experiments were performed. The selected conditions and the results of the orthogonal test are shown in Table 2. Table 2 shows which were found with higher recovery and which is a satisfying recovery under conditions of 16 mL acetonitrile and extraction for 20 min—considered to save time and reduce the solvent. The order of the effect on the recovery was extraction solvent > extraction time > solvent volume. As a result, we applied the best optimized conditions at $A_1 B_2 C_2$ (i.e., acetonitrile as extraction solvent, extraction solvent volume of 16 mL, extraction time of 20 min) in the following experiments.

Table 2

Design and results of orthogonal test.

Trial no.	Factor			Average recovery (%)
A^a	B^b	C^c	1	1
1	1	79.6	2	1
2	2	84.6	3	1
3	3	83.2	4	2
1	2	78.4	5	2
2	3	78.3	6	2
3	1	70.6	7	3
1	3	69.4	8	3
2	1	67.3	9	3
3	2	70.5	K_1	82.5
75.8	72.5		K_2	75.8
76.8	77.8		K_3	69.1
74.8	77.0		Range	13.4

K_i , mean effect of each factor at level i ($i=1, 2, 3$). ^a Factor A, extraction solvent; level 1, acetonitrile; level 2, petroleum ether; level 3, acetone. ^b Factor B, volume; level 1, 10 mL; level 2, 16 mL; level 3, 20 mL. ^c Factor C, extraction time; level 1, 15 min; level 2, 20 min; level 3, 25 min.

3.2. Optimization of Clean-Up Conditions

During the extraction of *Schizonepeta tenuifolia* samples, not only the analyses are isolated. There are different

types of interfering compounds, mainly volatile oil, monoterpenoids and pigments and others [20], which get coextracted. The use of coextraction leads to an unsatisfactory peak shape and an increased or inhibited response, which adversely affects the quantification [21]. Therefore, another analytical step is needed, i.e., extract purification. SPE sorbents, used in dispersive form or packed in a cartridge, are demonstrated suitable to a wide variety of food and agricultural products when appropriate adsorbing/sorbent materials are selected [22–24]. The current study compared three different SPEs, namely, Florisil, TPT, and Pesticarb/ NH_2 , on their adsorption capacity for 43 pesticide, the selected conditions are shown in Table 3. The comparison results of various clean-up conditions are shown in Figure 3. Florisil and Pesticarb/ NH_2 do a better job of removing additional matrix components from the extracts than TPT, but Pesticarb/ NH_2 adsorbs more pigments and gets a higher recovery. Therefore, Pesticarb/ NH_2 was chosen as the adsorbent in the extract purification process. Meanwhile, the proportion of acetonitrile/toluene was optimized. The results showed that the recoveries decreased when the proportion of acetonitrile increased. When the samples were eluted with acetonitrile/toluene (3:1, v/v), the interference coextractives can be efficiently removed and the recoveries for most pesticides are in the range of 70%–120%.

Table 3

Experimental program of clean-up.

No.	Type of SPEs	Eluant
1	Florisil	Hexane:ethyl acetate (85:15,v/v)
2	TPT	Acetonitrile:methylbenzene (3:1,v/v)
3	Pesticarb/ NH_2	Acetonitrile:methylbenzene (3:1,v/v)
4	Pesticarb/ NH_2	Acetonitrile:methylbenzene (85:15,v/v)

[figures omitted; refer to PDF]

3.3. Validation of the Method

To validate the developed analytical method for 43 pesticides in *Schizonepeta tenuifolia*, linearity of calibration curve, LOD, LOQ, accuracy (recovery %), and precision (relative standard deviation or RSD %) experiments were performed.

3.3.1. Linearity of Calibration Curve, LOD, and LOQ

Linearity was evaluated by matrix-matched calibration curves prepared by spiking 43 pesticides standards (50, 100, 200, 500, 1000 $\mu\text{g L}^{-1}$) in *Schizonepeta tenuifolia*. Based on the result, all the 43 pesticides showed a good linearity with the coefficients of determination (R^2) over than 0.995 (Table 4), which was conducive to accurate quantity. The LOD and LOQ of the method were determined as the minimum detectable concentrations of analyte with signal-to-noise (S/N) ratio exceeding 3 and 10 by sequential injection of decreasing levels of spiked samples. With this present method, pesticide analyses presented LOD and LOQ in the range of 0.0011–0.0135 mg kg^{-1} and 0.0038–0.0451 mg kg^{-1} , respectively. The LOD and LOQ for the test pesticides are presented in Table 5. Compared with the maximum residue limits (MRLs, 0.01–5.0 mg kg^{-1}) set by the European Union (EU), United States Environmental Protection Agency (EPA), and Japan [25], the LODs of all pesticides using this method are lower and the determination of pesticide residues would be achieved.

Table 4

Regression equations, determination coefficients (R^2), LODs, and LOQs of pesticides.

Pesticide	Regression equation	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})

Dichlorvos	$Y=6.88 \times 10^4 X - 4\,313.8$	0.996 3	0.001 8	0.005 8
Carbofuran	$Y=3.18 \times 10^4 X - 765$	0.999 4	0.003 9	0.012 9
Methamidophos	$Y=4.90 \times 10^3 X - 664$	0.995 9	0.012 2	0.040 7
Ethoprophos	$Y=3.79 \times 10^4 X - 1\,022.7$	0.999 4	0.002 1	0.007 0
Omethoate	$Y=1.73 \times 10^3 X - 553.6$	0.998 4	0.013 5	0.045 1
Phorate	$Y=3.49 \times 10^4 X - 1\,189.7$	0.997 5	0.002 0	0.006 7
Hexachlorobenzene	$Y=3.87 \times 10^4 X - 765.3$	0.997 8	0.001 1	0.003 8
α -BHC	$Y=1.78 \times 10^4 X - 605.8$	0.997 1	0.002 4	0.008 0
Monocrotophos	$Y=1.35 \times 10^4 X - 2\,173.5$	0.999 3	0.011 1	0.037 1
Quintozene	$Y=1.03 \times 10^4 X - 514.6$	0.998 4	0.001 2	0.003 9
γ -BHC	$Y=2.26 \times 10^4 X - 969.5$	0.996 7	0.002 3	0.007 7
Dimethoate	$Y=2.93 \times 10^4 X - 2\,078.2$	0.995 3	0.003 4	0.011 3
Acetochlor	$Y=3.75 \times 10^4 X - 135.5$	0.998 6	0.003 9	0.012 9
β -BHC	$Y=1.04 \times 10^4 X - 386.7$	0.997 9	0.001 6	0.005 5
Prometryn	$Y=4.61 \times 10^4 X - 1\,485.4$	0.998 2	0.004 8	0.015 8
δ -BHC	$Y=1.68 \times 10^4 X - 596.9$	0.998 2	0.001 7	0.005 7
Metalaxyl	$Y=2.65 \times 10^4 X - 1\,004.2$	0.998 8	0.002 6	0.008 7
Chlorothalonil	$Y=6.88 \times 10^4 X - 11\,033.2$	0.996 4	0.038 0	0.012 7
Aldrin	$Y=1.83 \times 10^4 X - 666.9$	0.999 4	0.003 8	0.012 5
Chlorpyrifos	$Y=1.94 \times 10^4 X - 976.2$	0.995 2	0.002 2	0.007 3
Parathion	$Y=1.47 \times 10^4 X - 859.9$	0.995 6	0.002 8	0.009 2
Dicofol	$Y=1.00 \times 10^6 X - 2\,599.8$	0.997 6	0.003 0	0.009 9
Pendimethalin	$Y=3.45 \times 10^4 X - 2\,108.9$	0.997 1	0.007 3	0.024 4

<i>cis</i> -Heptachlor epoxide	$Y = 1.94 \times 10^4 X - 690.5$	0.997 5	0.001 9	0.006 3
<i>trans</i> -Heptachlor epoxide	$Y = 2.86 \times 10^3 X - 64.6$	0.997 6	0.001 2	0.004 1
<i>cis</i> -Chlordane	$Y = 3.27 \times 10^4 X - 1\,264.7$	0.995 6	0.001 9	0.006 2
<i>trans</i> -Chlordane	$Y = 2.49 \times 10^4 X - 807.7$	0.997 5	0.001 2	0.004 0
Thiamethoxam	$Y = 1.00 \times 10^4 X - 461$	0.998 6	0.002 7	0.009 2
<i>pp'</i> -DDE	$Y = 4.88 \times 10^4 X - 1\,159.8$	0.996 9	0.006 2	0.020 7
Dieldrin	$Y = 8.88 \times 10^3 X - 347.5$	0.997 1	0.003 0	0.010 1
Fludioxonil	$Y = 2.25 \times 10^4 X - 936.1$	0.998 4	0.010 0	0.033 3
<i>op'</i> -DDT	$Y = 6.47 \times 10^4 X - 3\,256.6$	0.999 4	0.005 1	0.016 9
Nitrofen	$Y = 1.95 \times 10^4 X - 1\,081.4$	0.995 3	0.001 4	0.004 7
<i>pp'</i> -DDD	$Y = 9.49 \times 10^4 X - 2\,277.2$	0.997 9	0.003 6	0.011 9
β -Endosulfan	$Y = 3.04 \times 10^3 X - 17.7$	0.998 5	0.001 3	0.004 4
Propiconazole	$Y = 2.19 \times 10^4 X - 882.9$	0.998 3	0.001 1	0.037 0
<i>pp'</i> -DDT	$Y = 4.84 \times 10^4 X - 1\,800.2$	0.999 0	0.002 6	0.008 5
Bifenthrin	$Y = 1.69 \times 10^4 X - 2\,085.6$	0.998 1	0.007 8	0.026 0
Fenpropathrin	$Y = 3.28 \times 10^4 X - 1\,073.1$	0.996 4	0.005 8	0.019 3
Cyhalothrin	$Y = 4.81 \times 10^4 X - 2\,338.9$	0.995 8	0.001 5	0.004 9
Cypermethrin	$Y = 1.78 \times 10^4 X - 963.2$	0.997 7	0.001 9	0.006 3
Fenvalerate	$Y = 2.24 \times 10^4 X - 1\,585.6$	0.995 3	0.001 3	0.004 3
Difenoconazole	$Y = 5.74 \times 10^4 X - 2\,995.7$	0.997 6	0.005 3	0.017 7

Table 5
Results of recovery test ($n=6$).

Pesticide	0.2mgkg ⁻¹	0.5mgkg ⁻¹	1 mgkg ⁻¹

Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Dichlorvos
80.97	7.50	75.92	8.61	88.10	7.36	Carbofuran
90.64	4.20	89.77	2.96	96.88	1.58	Methamidophos
76.33	3.48	70.33	5.79	72.94	7.75	Ethoprophos
72.60	8.01	75.68	9.03	70.42	8.77	Omethoate
75.32	6.86	84.91	7.90	86.87	8.45	Phorate
92.80	4.17	87.69	3.33	85.60	7.15	Hexachlorobenzene
77.82	6.30	76.81	8.17	71.93	8.33	α -BHC
117.29	8.33	105.85	6.39	109.63	8.35	Monocrotophos
92.94	3.78	97.58	7.31	92.91	5.78	Quintozene
72.21	9.29	79.66	5.04	75.42	6.11	γ -BHC
79.29	7.74	81.26	10.38	70.11	8.32	Dimethoate
100.02	4.39	82.25	5.51	84.30	3.79	Acetochlor
70.43	6.79	70.88	12.86	72.94	7.76	β -BHC
89.08	11.60	77.79	9.23	73.87	10.61	Prometryn
70.43	8.07	80.51	8.20	83.98	8.22	δ -BHC

79.12	10.61	75.13	7.76	74.02	9.75	Metala xyl
71.11	5.99	84.38	7.59	74.13	4.53	Chlorot halonil
48.43	11.77	49.33	10.43	54.93	10.32	Aldrin
71.75	8.96	82.31	10.68	79.65	10.48	Chlorp yrifos
83.33	8.92	74.89	6.45	71.43	2.45	Parathi on
79.68	2.36	77.32	8.93	75.94	4.86	Dicofol
45.61	8.75	57.42	8.29	61.89	11.38	Pendi methali n
81.18	8.07	73.90	2.76	76.90	3.30	<i>cis</i> - Heptac hlor epoxid e
78.91	12.87	73.92	9.41	75.92	10.17	<i>trans</i> - Heptac hlor epoxid e
78.42	8.11	76.51	9.56	75.81	6.63	<i>cis</i> - Chlord ane
75.11	4.01	85.17	2.14	79.27	11.71	<i>trans</i> - Chlord ane
74.01	7.76	70.45	5.25	80.58	8.41	Thiame thoxam
57.89	4.23	58.91	13.61	65.53	10.06	<i>pp'</i> - DDE

70.37	6.80	81.90	10.55	74.21	9.95	Dieldrin
75.80	12.55	74.28	4.81	72.88	5.70	Fludioxonil
83.25	8.56	78.20	8.89	77.78	7.94	<i>op'</i> -DDT
80.61	8.63	72.20	6.28	85.19	5.60	Nitrofen
92.22	2.36	92.81	1.38	91.32	1.32	<i>pp'</i> -DDD
80.77	12.46	72.65	8.28	83.78	10.14	β -Endosulfan
83.12	13.86	81.89	7.39	87.13	9.91	Propiconazole
94.19	4.84	87.21	8.73	95.53	3.25	<i>pp'</i> -DDT
73.68	6.82	74.75	5.90	75.45	8.22	Bifenthrin
70.34	11.64	83.63	11.55	74.27	10.37	Fenpropathrin
84.31	9.15	80.61	12.81	82.45	8.93	Cyhalothrin
86.32	7.07	88.28	8.23	99.06	9.48	Cypermethrin
99.53	10.22	91.62	13.91	87.47	9.06	Fenvalerate
108.70	9.83	101.28	12.93	103.91	8.35	Difenoconazole

3.3.2. Accuracy and Precision

It is generally known that recovery (%) and relative standard deviations (RSDs) represented the accuracy and

precision of the quantitative analysis methods. The recovery experiments were performed to estimate the accuracy of the method with three spiked levels (0.2mgkg^{-1} , 0.5mgkg^{-1} , and 1mgkg^{-1}) in six replicates. As shown in Table 5, the recovery of most pesticide residues was in the range of 70%–120% with peak area RSDs of 1.32%–13.91%, except Chlorothalonil, Thiamethoxam, and Dicofol. The results demonstrate that the method has a good accuracy and meets the EU's criteria of method validation procedures (SANTE, 2017) [26]. The optimized method is reliable for the simultaneous detection of 43 pesticide residues in *Schizonepeta tenuifolia*. On the other hand, regarding the problem of the low recovery rate of Chlorothalonil, Thiamethoxam, and Dicofol, with no obvious experimental evidences obtained to explain the phenomenon, some instrumental condition variations, analytical error, decomposition of target in blank extract, and any other mechanisms were believed to be linked [27, 28]. The other ways should be investigated to improve the recovery of them in our future work.

3.3.3. Evaluation Method

The comparative study was accomplished between the present technique and some reported methods that the pesticides were detected in *Schizonepeta tenuifolia* or other CHMs. A analytical method was reported by Wu et al. [29] in which just 9 OCPs and PYRs were determined in *Schizonepeta tenuifolia* samples, and the method has the disadvantages of low applicability and the types of pesticide limited. In the present method, the 47 pesticides which most likely to be detected in *Schizonepeta tenuifolia* were selected from the research literature, and they include the four classes mentioned above. Furthermore, the determination of samples is prone to false positive by GC-ECD or GC-FPD.

Besides, Liang et al. [30] developed a method in 2017 in which 33 pesticides in Ginseng samples were detected by gas chromatography mass spectrometry (GC-MS) detector. This method LODs of 33 pesticides ranged from 5 to 25mgkg^{-1} , and RSD is higher than 15%. Compared with the reported method, the LODs of the present method (ranging from 1.1 to 13.5mgkg^{-1}) are comparable to them; however, RSD are lower than 15% which demonstrates that the present method has better sensitivity.

3.4. Sample Analysis

Thirty samples were collected from Hebei province, where it is the main producing area of *Schizonepeta tenuifolia* in China. These samples were determined by the established method, and the results are shown in Table 6. From the analytical results, omethoate, monocrotophos, and dimethoate were detected in one sample, while cyhalothrin and parathion were found in 5 and 2 samples, respectively. The contents of detected pesticides in *Schizonepeta tenuifolia* samples were lower than the MRL of European food standard. The pesticide residues in *Schizonepeta tenuifolia* samples may originate from the environmental soil or the administration for controlling pests.

Table 6

Pesticide residues (mg kg^{-1}) determined in *Schizonepeta tenuifolia* samples.

Pesticide	No. of positive samples	Min.	Max.	MRL
Cyhalothrin	5	0.056	0.680	1
Omethoate	1	ND ^a	0.065	0.1
Monocrotophos	1	ND	0.068	0.1
Dimethoate	1	ND	0.099	0.1
Parathion	2	0.081	0.088	0.5

^a Not detectable or lower than limit of detection.

4. Conclusions

In this study, a rapid sample preparation for multiresidues detection in *Schizonepeta tenuifolia* was developed by dispersive solid-phase extraction technology. Optimum extraction methods were identified for *Schizonepeta tenuifolia* sample (acetonitrile as extraction solvent, extraction solvent volume of 16mL, and extraction time of 20 min) and purification (Pesticarb/NH₂ as SPE sorbents, eluted with acetonitrile/toluene (3:1, v/v) 25mL) steps. A validation procedure was performed, which showed good results for suitability, recovery, and repeatability. The developed method was applied to the determination of 30 *Schizonepeta tenuifolia* samples, and some pesticides were detected, which demonstrated that it is essential to constantly monitor pesticide residues in *Schizonepeta tenuifolia*. To our knowledge, this approach was first applied to the multiresidue analysis in *Schizonepeta tenuifolia*. The good performance of this method confirmed that it has a strong potential for its application in the monitoring of pesticide residues in *Schizonepeta tenuifolia*.

Acknowledgments

The authors gratefully acknowledge the financial support to this study by the Natural Science Foundation Project of Hebei Province (H2018201045 and C2021201026), the Higher Education Science and Technology Research Project of Hebei Province (ZD2018054), and the Science and Technology Planning Project of Inspection and Quarantine Bureau in Hebei Province (HE2017K011).

References

- [1] X. Mao, Y. Wan, A. Yan, M. Shen, Y. Wei, "Simultaneous determination of organophosphorus, organochlorine, pyrethroid and carbamate pesticides in Radix astragali by microwave-assisted extraction/dispersive-solid phase extraction coupled with GC-MS," *Talanta*, vol. 97, pp. 131-141, DOI: 10.1016/j.talanta.2012.04.007, 2012.
- [2] X. Huang, J. Xue, Y. Wang, X. Wu, H. Tong, "Rapid simultaneous determination of organochlorine and pyrethroid pesticide residues in Lycium barbarum L. using gas chromatography with electron-capture detector," *Analytical Methods*, vol. 4 no. 4, pp. 1132-1141, DOI: 10.1039/c2ay05625a, 2012.
- [3] J. Y. Xue, Y. J. Xu, F. M. Liu, J. Xue, H. Li, W. Peng, "Comparison of different sample pre-treatments for multi-residue analysis of organochlorine and pyrethroid pesticides in chrysanthemum by gas chromatography with electron capture detection," *Journal of Separation Science*, vol. 36, pp. 1311-1316, DOI: 10.1002/jssc.201201067, 2013.
- [4] B. Lozowicka, E. Rutkowska, M. Jankowska, I. Hrynko, P. Kaczynski, "Toxicological evaluation of multi-class pesticide residues in vegetables and associated human health risk study for adults and children," *Human and Ecological Risk Assessment: An International Journal*, vol. 22 no. 7, pp. 1480-1505, DOI: 10.1080/10807039.2016.1185690, 2016.
- [5] S. Elgueta, S. Moyano, P. Sepúlveda, C. Quiroz, A. Correa, "Pesticide residues in leafy vegetables and human health risk assessment in North Central agricultural areas of Chile," *Food Additives and Contaminants: Part B*, vol. 10 no. 2, pp. 105-112, DOI: 10.1080/19393210.2017.1280540, 2017.
- [6] W. Tang, D. Wang, J. Wang, Z. Wu, L. Li, M. Huang, S. Xu, D. Yan, "Pyrethroid pesticide residues in the global environment: an overview," *Chemosphere*, vol. 191, pp. 990-1007, 2018.
- [7] X. D. Liu, Y. Zhang, M. H. Wu, Z.-G. Ma, H. Cao, "Textual research of schizonepetae herba and schizonepetae spica," *China Journal of Chinese Materia Medica*, vol. 46 no. 9, pp. 5145-5151, 2021.
- [8] J. Li, Y. Gu, J. Xue, H. Y. Jin, S. Ma, "Analysis and risk assessment of pesticide residues in a Chinese herbal medicine, *Lonicera japonica* thunb," *Chromatographia*, vol. 80 no. 3, pp. 503-512, DOI: 10.1007/s10337-017-3243-0, 2017.
- [9] V. Drumea, C. Saviuc, L. Olariu, R. Nita, "Application of the GC-MS-SIM method for rapid determination of pesticide residues in medicinal herb samples used in pharmaceutical and cosmetic industries," *Toxicology Letters*, vol. 229, DOI: 10.1016/j.toxlet.2014.06.593, 2014.
- [10] X. H. Yi, Y. T. Lu, "Study on residues of pesticides and heavy metals in Ligusticum wallichii franch. and other seven kinds of traditional Chinese medicine," *Research and Practice of Chinese Medicines*, vol. 18, 2004.
- [11] U. Koesukiwat, S. J. Lehotay, N. Leepitapiboon, "Fast, low-pressure gas chromatography triple quadrupole tandem mass spectrometry for analysis of 150 pesticide residues in fruits and vegetables," *Journal of*

Chromatography A, vol. 1218 no. 39, pp. 7039-7050, DOI: 10.1016/j.chroma.2011.07.094, 2011.

[12] H. R. Norli, A. Christiansen, B. Holen, "Independent evaluation of a commercial deconvolution reporting software for gas chromatography mass spectrometry analysis of pesticide residues in fruits and vegetables," *Journal of Chromatography A*, vol. 1217 no. 13, pp. 2056-2064, DOI: 10.1016/j.chroma.2010.01.022, 2010.

[13] E. Hakme, A. Lozano, S. Uclés, M. M. Gómez-Ramos, A. R. Fernández-Alba, "High-throughput gas chromatography-mass spectrometry analysis of pesticide residues in spices by using the enhanced matrix removal-lipid and the sample dilution approach," *Journal of Chromatography A*, vol. 1573, pp. 28-41, DOI: 10.1016/j.chroma.2018.08.046, 2018.

[14] J. J. Xie, L. I. Ju, J. Chen, J. Xi, W.-R. Chen, M. Liu, "Determination of 43 pesticide residues in grape wine by solid-phase dispersion and gas chromatography-mass spectrometry," *Journal of Food Safety and Quality*, vol. 5 no. 8, pp. 2488-2495, 2014.

[15] Z. Huang, Y. Li, B. Chen, S. Yao, "Simultaneous determination of 102 pesticide residues in Chinese teas by gas chromatography-mass spectrometry," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 853 no. 1-2, pp. 154-162, DOI: 10.1016/j.jchromb.2007.03.013, 2007.

[16] Y. Fu, X. Dou, L. Zhang, J. Qin, M. Yang, J. Luo, "A comprehensive analysis of 201 pesticides for different herbal species-ready application using gas chromatography-tandem mass spectrometry coupled with QuEChERS," *Journal of Chromatography B*, vol. 1125, DOI: 10.1016/j.jchromb.2019.121730, 2019.

[17] Y. Tong, J. Xue, X. Wu, "Multi-residue pesticide determination in flos chrysanthemi by mixed mode SPE purification with GC-MS/MS analysis," *Analytical Letters*, vol. 46 no. 4, pp. 615-629, DOI: 10.1080/00032719.2012.726680, 2013.

[18] H. Yan, J. Du, X. Zhang, G. Yang, K. H. Row, Y. Lv, "Ultrasound-assisted dispersive liquid-liquid microextraction coupled with capillary gas chromatography for simultaneous analysis of nine pyrethroids in domestic wastewaters," *Journal of Separation Science*, vol. 33 no. 12, pp. 1829-1835, DOI: 10.1002/jssc.200900716, 2010.

[19] A. Bidari, M. R. Ganjali, P. Norouzi, M. R. M. Hosseini, Y. Assadi, "Sample preparation method for the analysis of some organophosphorus pesticides residues in tomato by ultrasound-assisted solvent extraction followed by dispersive liquid-liquid microextraction," *Food Chemistry*, vol. 126 no. 4, pp. 1840-1844, DOI: 10.1016/j.foodchem.2010.11.142, 2011.

[20] F. Dennis, B. Clara, "Schizonepeta tenuifolia: chemistry, pharmacology, and clinical applications," *The Journal of Clinical Pharmacology*, vol. 42, 2002.

[21] S. Li, P. Yu, C. Zhou, L. Tong, D. Li, Z. Yu, Y. Zhao, "Analysis of pesticide residues in commercially available chenpi using a modified QuEChERS method and GC-MS/MS determination," *Journal of Pharmaceutical Analysis*, vol. 10 no. 1, pp. 60-69, 2020.

[22] E. Hakme, M. E. Poulsen, "Evaluation of the automated micro-solid phase extraction clean-up system for the analysis of pesticide residues in cereals by gas chromatography-Orbitrap mass spectrometry," *Journal of Chromatography A*, vol. 1652, DOI: 10.1016/j.chroma.2021.462384, 2021.

[23] S. S. Herrmann, M. E. Poulsen, "Clean-up of cereal extracts for gas chromatography-tandem quadrupole mass spectrometry pesticide residues analysis using primary secondary amine and C18," *Journal of Chromatography A*, vol. 1423, pp. 47-53, DOI: 10.1016/j.chroma.2015.10.086, 2015.

[24] E. Boes, R. T. Rosmalina, Y. S. Ridwan, W. C. Nugraha, R. Yusiasih, "Development of validated method using QuEChERS technique for organochlorine pesticide residues in vegetable," *Procedia Chemistry*, vol. 16, pp. 229-236, DOI: 10.1016/j.proche.2015.12.052, 2015.

[25] C.-y. Shen, X.-w. Cao, W.-j. Shen, Y. Jiang, Z.-y. Zhao, B. Wu, K.-y. Yu, H. Liu, H.-z. Lian, "Determination of 17 pyrethroid residues in troublesome matrices by gas chromatography/mass spectrometry with negative chemical ionization," *Talanta*, vol. 84 no. 1, pp. 141-147, DOI: 10.1016/j.talanta.2010.12.041, 2011.

[26] European Commission, Document No. SANTE/11813/2017, Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues and Analysis in Food and Feed, 2017.

[27] L. Song, Y. Han, J. Yang, Y. Qin, W. Zeng, S. Xu, C. Pan, "Rapid single-step cleanup method for analyzing 47

pesticide residues in pepper, chili peppers and its sauce product by high performance liquid and gas chromatography-tandem mass spectrometry," Food Chemistry, vol. 279 no. 5, pp. 237-245, DOI: 10.1016/j.foodchem.2018.12.017, 2019.

[28] T. Fujiyoshi, T. Ikami, T. Sato, K. Kikukawa, M. Kobayashi, H. Ito, A. Yamamoto, "Evaluation of the matrix effect on gas chromatography - mass spectrometry with carrier gas containing ethylene glycol as an analyte protectant," Journal of Chromatography A, vol. 1434, pp. 136-141, DOI: 10.1016/j.chroma.2015.12.085, 2016.

[29] T. Wu, A. W. Ding, L. Zhang, "Study on organochlorine and pyrethroid pesticide residues in traditional Chinese medicine," Journal of Nanjing University of Technology (Natural Science Edition), vol. 24, pp. 48-51, 2008.

[30] L. P. Liang, Q. H. Lai, H. Ding, H. S. Yu, L. J. Zhang, X. B. Song, "Fast pesticide multiresidue analysis in Ginseng (Panax ginseng C.A. Mey) by gas chromatography with QuEChERS," Liaoning University of Traditional Chinese Medicine, vol. 19, pp. 63-66, 2017.

DETAILS

Subject:	Toluene; Mass spectrometry; Acetonitrile; Agricultural production; Gas chromatography; Solvents; Pesticides; Spectroscopy; Retention; Scientific imaging; Methods; Chromatography; Solid phases
Location:	China; Japan
Editor:	Neil D Danielson
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	

Milestone dates: 2021-08-16 (Received); 2021-10-25 (Revised); 2021-11-09 (Accepted); 2021-12-13 (Pub)

DOI: <https://doi.org/10.1155/2021/8934998>

ProQuest document ID: 2613972047

Document URL: <https://www.proquest.com/scholarly-journals/rapid-simultaneous-determination-43-pesticide/docview/2613972047/se-2?accountid=211160>

Copyright: Copyright © 2021 Guo-wei Zhou et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-12-26

Database: Publicly Available Content Database

Document 5 of 59

Synthesis, Optimization, and Characterization of Ecofriendly Production of Gold Nanoparticles Using Lemon Peel Extract

Alzahrani, Eman
; Alkhudidy, Ashwaq T

[ProQuest document link](#)

ABSTRACT (ENGLISH)

This study examines the importance of utilizing green synthesis using lemon peel for gold nanoparticles over other chemicals since it is environmentally friendly, available, and cheap. Several parameters were optimized to ensure the extraction of the GNPs concentration of lemon peels using HAuCl_4 and lemon peel extract having a ratio of 2:1. For the optimum result, the ratio used was 2:1. The gold nanoparticles fabrication happened in 10 minutes. The initial observation was the color change of the solution. The UV-visibility spectroscopic studies are performed to confirm the result. The experiments are done concurrently to ensure the solution is mixed on the proper ratio. The GNP is also characterized by the different techniques in their sizes and electronic transmission microscopy, essential in extracting gold nanoparticles. Other elements of the composition are removed by the EDAX methods, the FTIR method, and the TEM methods, all of which reveal the real reason behind the required extraction capacity. Most gold nanoparticles show a maximum absorption rate at the peak of 535 to 579 nm. The result obtained from the TEM and the SEM analysis revealed that the grain size is analogized to the average size of 6.67 nm. With a simple synthesis of the price, some processes show that the medically available nanoparticles are necessary. The used method in this paper to fabricate GNPs is cheap, easy, fast, and sustainable and it can be done with ease in any

laboratory.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

"Nano" comes from the Greek word "Nanos," which depicts a minute subject. This is also used to the billionth prefix to show the magnified size of the particle. According to ASTM International 2006, nanoparticles have two dimensions [1]. The particles have unique chemical and physical characteristics that show the materials' bulkiness to form total products properly. The exposure to the quantum effect is the disruption of the particles, which shows the appropriate shape of the particles [2–11]. Nanoparticles are also different in size since they represent the nature of small materials, which often depict the characteristics of most chemicals [12–16]. Generally, NPs is found in quantum dots, carbon nanotubes, nanocapsules, fullerenes, ceramic NPs, and nanoemulsions [17].

The gold nanoparticles are known as noble metals since they are resistant to rust and oxidation. They also attract attention due to their shiny physical appearance, stability, smallness, and significant biocompatibility [5]. Due to their high surface area to volume ratio, they are easily converted to other forms of affinity to show their commitment to new physical use, especially with their complex matrices, which often play a crucial role [3–5]. The nanoparticles have an attractive appearance with high electrical conductivity and a diverse microorganism. Other characteristics also possess close relations to the nanosized particles, which are turning in shape and can include the morphology of the surface [3].

Different methods to fabricate the gold nanoparticles have been devised to ensure following the rules and preparation methods of the particles [6]. A general system for connecting GNPs joins made physical and average points of view [7]. The standard compound structure for mixing gold nanoparticles wires the usage of shocking facilitated substances, for instance, sodium borohydride [18]. Such strategies are rich, and the hazardous composed materials foster that going with nanoparticles is noncompatible for human use. More lately, nanoparticles have been made using microorganisms [19–21], compounds [22], creatures [23], and plants or plant extracts [24–28] which have been proposed as possible ecofriendly choices as opposed to substance and authentic frameworks. Green science has been applied considering the exact outcomes that the world is trying to achieve along with the limited open opportunity to find convincing courses concerning progress [8–10]. Fittingly, different improvements have been relied on to add to this field utilizing green cycles; for instance, the diagram of nanoparticles from plants is taken out [5, 11].

Extraction methodology is the principal headway for the store of plant metabolites from unrefined materials. To do an extraction working with exertion, some focal cutoff networks ought to be considered since these affect the chance of a concentrate [29]. The extraction of the interest packs is all through ward upon the picked part from the plant material, other than as the solvents, which need to show straightforwardness of scattering, and the delicacy to wrongly change the solutes. Since the outcome will follow from the used dissolvable, the last ought to have a low hurting penchant. Additionally, an extraction framework is picked paying exceptional brain to the length of extraction, temperature, pH of solvent, dissolvable to-test degree, and particle size of the callous materials. These parts can incite plans in the metabolite relationship of the concentrates [30]. The ideal extraction method should be the short head, less drawn-out, and finished sufficiently in any laboratory.

As proposed before, green science likes benefit over the compound, and standard method since it is unassuming, environment particularly coordinated [27], and does not need to use high beating piece, energy, temperature or ruinous made materials. Furthermore, plants have been used to produce nanoparticles since they are immediately open, found the opportunity to direct, and have an expansive drive of metabolites that can help in reduction [31]. Therefore, we expect to use innocuous to the environment and greener plans to mix gold nanoparticles. In this work, lemon peel extract was used for a mix of metal nanoparticles, and distinctive cutoff common environmental elements for saving GNPs were examined for most principal GNP creation. After that, the materials were depicted using

Visual information, UV-Visible spectroscopy evaluation, TEM assessment, FT-IR examination, and SEM-EDAX appraisal. In this evaluation, lemon peel extract was used as a reductant for reducing of HAuCl_4 solution [32]. This potential aftereffect of lemons is a rich wellspring of citrus discard and ascorbic acid [33, 34]. In like manner, the other reason for choosing lemon is a commonly available fruit.

2. Experiment

2.1. Chemicals and Materials

Chemicals and materials are chloral auric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.9%) and the filter paper by Whatman with a pore size of $25\mu\text{m}$ and a diameter of 15 cm. The chemicals are procured from Sigma Aldrich (Poole, UK), and the lemon is collected from the local market (Taif, KSA). Distilled water is best used for this purpose.

2.2. Instrumentation

Instruments are a hot plate from VWR International LLC (West Chester, PA, US), transmission electron microscopy from the JOEL Ltd. (Welwyn Garden City, UK), a UV spectrophotometer from the GENESYS (Toronto, Canada), and an oven from the GALLI company (Milano, Italy). The FT-IR spectra from the PerkinElmer with the diamond ATR and energy dispersive analysis performed X-ray for the INCA 350 EDX systems from the Oxford Industry (Abingdon, UK). A sonicator from the Ultra wave Sonicator 300HD (Cardiff, UK) was used. A scanning electron microscope from Cambridge S360 from Cambridge Instrument Company (Cambridge, UK) and the Perkin Elmer 785A UV/Visible Detector from the Perkin Elmer (California, USA) were utilized.

2.3. Preparation of Gold Nanoparticles

Green synthesis is used with the lemon peel extract of 20g with distilled water for 100 ml. The mixture then boiled at 80°C for 5 minutes. The solution is then filtered with the Whatman paper, and an additional 2 mM of HAuCl_4 solution is added for a ratio of 1:1 at room temperature ($25 \pm 3^\circ\text{C}$), with stirring and kept at a dark place for 24 hours to ensure the extraction occurs. The reduction of gold ions into gold nanoparticles proceeded by a color change and the formation of the particles as observed [35, 36]. The color change is monitored in real-time to track the evolution and construction of the gold nanoparticles.

Some of the effects of the reaction, like the concentration of the lemon peel extract and the HAuCl_4 solution, and the reactants ratio are studied to maximize the yield of the GNPs. The experiment was also performed in triplicate for reproductivity. The resulting solutions are also monitored using the UV-V spectrophotometer.

2.3.1. Effect of Lemon Peel Concentration

The procedure described above is performed at different concentrations of the lemon peel extract to increase the amount of the formed GNPs, and their concentrations include 20, 40, 60, 80, and 100%.

2.3.2. Effect of HAuCl_4 Solution Concentration

The proceeding factors investigated are performed through different concentrations of HAuCl_4 solutions of (0.5, 1.0, 1.5, 2.0 & 2.5 mM). These were investigated in order to optimize the concentration of HAuCl_4 solution.

2.3.3. Effect of Lemon Peel Extract and HAuCl_4 Solution Ratio

Different ratios of HAuCl_4 solution and lemon peel extract (1:1, 1:2, and 2:1) were investigated in order to find the maximum production of GNPs.

2.4. Characterization of the Formed GNPs

2.4.1. Visual Observation and UV-Visible Spectra Analysis

The naked eye can observe the color of the solution under the formation of the nanoparticles. The reduction of the mixture of HAuCl_4 solution during the exercise to extract the lemon was quickly made by the exposure to the correct formulation of the solution. The distilled water is also observed under the distilled water, and the UV-V spectrum is taken from a 1 ml sample for the distilled water. The result of the absorption is monitored spectrophotometrically. The GNPs plasmon resonance is also found in 529 nm to 500 nm in the electromagnetic spectrum [37, 38].

2.4.2. TEM Analysis

The formation of gold nanoparticles was confirmed by using the electron microscope transmission. $5\mu\text{l}$ sample of the solution was put on the lacy carbon coated with 3 mm diameter copper grids. The TEM images were acquired with the Gatan UltraScan 4000 digital cameras attached to the JEOL 2010 transmission electron microscope running at

20kV.

2.4.3. SEM- EDAX Analysis

The scanning Electron Microscope of the model JSM-5800 was used to determine the shape of the developed nanoparticle. Samples were prepared by depositing drops of colloidal soliton on an aluminum grid sample holder and drying the room temperature. The elemental composition of the sample was also analyzed using the X-ray spectroscopy (EDAX) coupled to the SEM instrument.

2.4.4. FT-IR Analysis

The Fourier transform infrared spectrum of the sample was recorded by an FTIR spectrophotometer. They ranged from 4000 to 500 cm^{-1} through the making of a KBr pellet with GNPs at a resolution of 4 cm^{-1} .

3. Results and Discussion

3.1. Formation of GNPs

The formation of GNPs by the citrate reduction method can be summarized in three steps: reduction of Au^{3+} in solution, disproportionation of Au^+ to gold atoms and their nucleation, growth by disproportionation on particle surface, and coagulation. Oxidation of citrate results in the formation of dicarboxy acetone, aiding nucleation [39]. The HAuCl_4 salt disproportionates when added to water followed by ionization of the citric acid. Then, citrate will be oxidized to form dicarboxy acetone. (1) $\text{HAuCl}_4 + \text{H}_2\text{O} \rightarrow \text{AuCl}_3 + \text{H}^+ + \text{Cl}^-$ (2) $\text{C}_3\text{H}_5\text{OCO}_2\text{H}_3 + \text{H}_2\text{O} \rightarrow \text{C}_3\text{H}_5\text{OCOO}_3^- + \text{C}_3\text{H}_5\text{OCOO}_3^- + 3\text{H}^+$ (3) $\text{C}_3\text{H}_5\text{OCOO}_3^- + 3\text{C}_3\text{H}_4\text{OCOO}_2^- + 2\text{CO}_2 + \text{H}^+ + 2\text{e}^-$

This is followed by the reduction of auric salt to aurous salt, and then the aurous species are disproportionate to gold atoms. In the last step, the Au atoms adsorb Au^+ complexes and form large aggregates, which leads to the formation of aggregates, by complexation with dicarboxy acetone [40, 41]. (4) $\text{AuCl}_3 + 2\text{e}^- \rightarrow \text{AuCl}_2^- + \text{Cl}^-$ (5) $3\text{AuCl}_2^- + 2\text{Au}^+ + \text{AuCl}_3$ The overall stoichiometry of the reduction reaction can then be represented: (6) $2\text{AuCl}_3 + 3\text{C}_6\text{H}_5\text{O}_7^- + 2\text{Au}^0 + 3\text{C}_5\text{H}_4\text{O}_5 + 6\text{Cl}^- + \text{H}^+ + 3\text{CO}_2$

GNPs are usually produced by the addition of a reducing agent to a solution of chloroaurate ions (AuCl_4^-), causing reduction of the gold ions and aggregation of the Au atoms into GNPs (Scheme 1).

[figure omitted; refer to PDF]

While utilizing 20% concentration of lemon peel extract, the nanoparticles formation and size reduction were begun rapidly because of the more availability of functional groups in the lemon peel extract. Table 1 sums up the impact of various concentration of lemon peel extract in the nanoparticle's preparation. After 10min, the color of the mixture changes from yellow to ruby red, grey or blue color for the formed GNPs utilizing different concentration of lemon peel extract at room temperature. The higher speed of reduction happened at lower concentration of the reducing reagent (20% of lemon peel extract). The broadening peak was gotten at 60% of lemon peel extract showing the sized nanoparticles. The narrowest peak was obtained at 20%, which shows the laid-out nanoparticles are small in size and the higher speed of decreasing of gold particles happened in the 20%. At last, it was considered that lower concentration was optimum for fabrication of GNPs.

Table 1

Synthesis of GNPs using different concentrations of lemon peel extract.

Concentration of HAuCl_4 solution (mM)	Concentration of lemon peel extract (%)	Ratio	Color of solution	Absorbance (nm)	SPR band (nm)
2.0	20	1:1	Ruby red	0.941	566
2.0	40	1:1	Ruby red	—	—
2.0	60	1:1	Grey	—	—

2.0	80	1:1	Grey	—	—
2.0	100	1:1	Blue	—	—

The size distribution of nanoparticles, all things considered, is a fundamental issue as nanoparticles show specific physical and substance properties relying upon their shape and size. TEM is in this strategy for the most adapted techniques to study the size and shape of the nanoparticles and give their distribution [42]. In this evaluation, the prepared GNPs were characterized utilizing TEM analysis. Figure 3 shows the size distribution histogram and TEM micrographs showing the formation of nanoparticles. The TEM micrographs showed that the amount of the prepared GNPs utilizing the ideal concentration of lemon peel extract at various magnification was spherical in shape, and they were dispersed in a watery medium. It was tracked down that the size of the prepared GNPs relies upon the concentration of the lemon peel extract. It was found that the size of GNPs was 40.5nm.

[figure omitted; refer to PDF]

Table 2 summaries the color, absorbance, and wavelength of the prepared GNPs using different concentration of HAuCl_4 solution. After addition of the reducing reagent to the HAuCl_4 solution, the color changes from yellow to grey or ruby red color for the arranged GNPs in a brief timeframe at room temperature. The absorption was extended while increasing the concentration of the gold ions from 0.5mM to 2mM, and by using concentration of 2.5mM of HAuCl_4 solution, the absorbance of the prepared GNPs was reduced. Fittingly, the optimization study showed a significant effect of the HAuCl_4 solution on the synthesis of GNPs. This investigation sees that the best HAuCl_4 solution was 2mM for nanoparticles preparation. The prepared GNPs using ideal concentration of the HAuCl_4 solution (2mM) were depicted using TEM analysis. Figure 6 shows the size distribution histogram and TEM electron microscopy micrographs showing the prepared gold nanoparticles. The TEM micrographs showed that the extent of the made GNPs using the optimum concentration of HAuCl_4 solution at different magnifications was spherical and dispersed in a watery medium. It was found that the size of the prepared GNPs depends on the concentration of the HAuCl_4 solution. The size of the prepared GNPs was 38.42nm when the concentration of the HAuCl_4 solution was 2 mM.

Table 2

Synthesis of GNPs using different concentrations of HAuCl_4 solution.

Concentration of HAuCl_4 solution (mM)	Concentration of lemon peel extract (%)	Ratio	Color of solution	Absorbance (a.u)	SPR band (nm)
0.5	20	1:1	Grey	—	—
1.0	20	1:1	Grey	—	—
1.5	20	1:1	Ruby red	1.082	548
2.0	20	1:1	Ruby red	1.279	542
2.5	20	1:1	Ruby red	1.223	539

[figure omitted; refer to PDF]

3.2.3. Effect of Ratio of HAuCl_4 Solution and Reducing Reagent

The concentration of lemon peel extract (20%) and HAuCl_4 solution (2mM) in the ranges of 1:1, 1:2, and 2:1 was utilized to find the optimum composition of the preparation of GNPs since reactants ratio is an important factor affecting the formation of GNPs and even nanoparticles sorting out [44]. GNPs were prepared using different ratio of

HAuCl₄ solution to lemon peel extract. On mixing the reducing reagent with the aqueous solution of HAuCl₄, the color of the mixture was changed from yellow to colored solution, as can be seen in Figure 7. The color change indicated the reduction of the chloroauric acid ions using lemon peel extract, forming GNPs. From Figure 8, it was found that the best ratio to get a high absorbance of GNPs was when the ratio of HAuCl₄ solution and lemon peel extract in the reaction mixture was 2:1 based on the number of trials and the best yield.

[figure omitted; refer to PDF]

Table 3 presents the effect of ratio of HAuCl₄ solution and lemon peel extract. The color changes from yellow to ruby red for 1:1, 1:2, and 2:1. The UV spectra show the SPR band in the space of 523nm, and the absorbance was 2.023 a.u. (Figure 8). Contemplating everything, the best condition for preparation of GNPs when using 20% of lemon peel extract, 2mM of HAuCl₄ solution, and ratio of HAuCl₄ solution to lemon peel extract was 2:1.

Table 3

Synthesis of GNPs using different ratios of HAuCl₄ solution to lemon peel extract.

Concentration of HAuCl ₄ solution (mM)	Concentration of lemon peel extract (%)	Ratio	Color of solution	Absorbance (a.u)	SPR band (nm)
2.0	20	1:1	Ruby red	—	—
2.0	20	1:2	Ruby red	—	—
2.0	20	2:1	Ruby red	2.023	523

The prepared GNPs using optimum ratio (2:1) of HAuCl₄ solution (2mM) to lemon peel extract (20%) were depicted using TEM analysis, since it can give information about the morphology and size of the prepared nanoparticles.

Figure 9 shows the size distribution histogram and TEM micrographs showing the formation of nanoparticles. The TEM micrographs showed that the prepared GNPs were spherical. The size of GNPs was measured by measuring the diameter of whole particles on TEM images. The average diameter of prepared GNPs was in the range of 25.36 nm with very few particles of higher and lower size distribution. The TEM micrographs show that most of the gold nanospheres are round or spherical in shape with a degree of faceting due to their crystalline nature.

[figure omitted; refer to PDF]

The green-fabricated GNPs were assessed further utilizing EDAX spectrometry, which upheld gold without any contaminants. The optical adsorption peak was typically seen at 2.30keV (Figure 11), which is the ordinary adsorption of gold nanocrystallites because of surface plasmon resonance. The EDAX profile for the prepared GNPs showed solid gold molecule signals at around 2.30keV. EDAX for the GNPs offered substantial clues for gold particles and weaker signals for carbon, oxygen, and chloride, prevenient from the lemon peel's biomolecules. The strong signals signs are gotten at around 2.30keV [46, 47]. Table 4 presents the atomic percentages of the nanoparticle, which were gold, carbon, oxygen, potassium, and chloride. It was tracked down that the Au atomic was on a fundamental level of 6.17%.

[figure omitted; refer to PDF]

Table 4

EDAX elemental microanalysis of the synthesized GNPs.

Element	Weight%	Atomic%
C K	39.44	80.83

O K	6.32	9.73
Cl K	3.22	2.24
K K	1.64	1.03
Au M	49.38	6.17
Totals	100.00	100.00

FT-IR spectrum identified the functional groups which are involved in the gold nanoparticles synthesis. Figure 12 shows FT-IR spectra of GNPs utilizing lemon peel extract as a reducing reagent while Table 5 presents the experimental wavenumber, their assignments, and the wavenumber limits for the integration. It can be seen peaks at 3266cm^{-1} , 2935cm^{-1} , 1614cm^{-1} , 1406cm^{-1} , 1258cm^{-1} , and 1023cm^{-1} . From the outcomes, it could be inferred that the C. limon pectin shows sharp and strong peaks at 3266.25cm^{-1} as O-H stretch, C-H stretch in the frequency 2935cm^{-1} displayed as carbohydrate ring [48, 49]. The absorption peaks 1023cm^{-1} were from the ether (C-O-C) [50], C-H scissoring and bowing conduct is displayed by a top between 1406cm^{-1} [51], peak for group C-O stretching vibrations is coordinated in 1258cm^{-1} , and at last peak credited to group C=C stretching was located at 1614cm^{-1} [52].

[figure omitted; refer to PDF]

Table 5

Assignment of bands observed in the FT-IR spectrum of the synthesized GNPs.

Experimental wavenumber (cm^{-1})	Assignment	Range (cm^{-1})
2935	C-H stretch	2950-2800
1406	C-H in-plane bend C=C	1430-1290
1614	Stretch (conjugated)	1640-1610
1023	C-O stretch	1260-1000
3266	O-H stretch	3550 - 3200

4. Conclusion

Green synthesis is an alternative way for preparation of gold nanoparticles since it is environmentally friendly, safe, and available. Several parameters are used for the optimum conditions to prepare gold nanoparticles that were concentration of lemon peel extract, concentration of HAuCl_4 solution, and ratio of HAuCl_4 solution to lemon peel extract. A surface Plasmon resonance typically has a peak from 535 to 579nm confirming the gold nanoparticles synthesis. Additionally, different characterization techniques were used to study the formed metal nanoparticles such as SEM, EDAX analysis, and FT-IR spectroscopy. The spherical sized nanoparticles were confirmed; moreover, size distribution and morphology of the GNPs were characterized in detail. The results of the investigation showed that the optimum condition for preparation of GNPs using lemon peel extract was 20% of lemon peel extract, and 2mM of HAuCl_4 solution and ratio of HAuCl_4 solution to lemon peel extract was 2: 1. The method was simple to perform and yielded stable GNPs. The synthesized GNPs using lemon extract can be exploited towards the development of

potential antibacterial agents.

Additional Points

No animals/humans were used for studies that are the basis of this research.

Acknowledgments

The authors acknowledge the support of Taif University Researchers Supporting Project no. TURSP-2020/136, Taif University, Taif, Saudi Arabia.

References

- [1] F. K. Alanazi, A. A. Radwan, I. A. Alsarra, "Biopharmaceutical applications of nanogold," *Saudi Pharmaceutical Journal*, vol. 18 no. 4, pp. 179-193, DOI: 10.1016/j.jsps.2010.07.002, 2010.
- [2] C. Di Guglielmo, D. R. López, J. De Lapuente, J. M. L. Mallafre, M. B. Suárez, "Embryotoxicity of cobalt ferrite and gold nanoparticles: a first in vitro approach," *Reproductive Toxicology*, vol. 30 no. 2, pp. 271-276, DOI: 10.1016/j.reprotox.2010.05.001, 2010.
- [3] P. Dauthal, M. Mukhopadhyay, "Noble metal nanoparticles: plant-mediated synthesis, mechanistic aspects of synthesis, and applications," *Industrial & Engineering Chemistry Research*, vol. 55 no. 36, pp. 9557-9577, DOI: 10.1021/acs.iecr.6b00861, 2016.
- [4] R. Vajtai, *Springer Handbook of Nanomaterials*, 2013.
- [5] S. Gamboa, E. Rojas, V. Martínez, "Synthesis and characterization of silver nanoparticles and their application as an antibacterial agent," *International Journal of Biosensors & Bioelectronics*, vol. 5 no. 5, pp. 166-173, 2019.
- [6] M. R. Papasani, G. Wang, R. A. Hill, "Gold nanoparticles: the importance of physiological principles to devise strategies for targeted drug delivery," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 8 no. 6, pp. 804-814, DOI: 10.1016/j.nano.2012.01.008, 2012.
- [7] A. Khan, R. Rashid, G. Murtaza, A. Zahra, "Gold nanoparticles: synthesis and applications in drug delivery," *Tropical Journal of Pharmaceutical Research*, vol. 13 no. 7, pp. 1169-1177, DOI: 10.4314/tjpr.v13i7.23, 2014.
- [8] B. A. de Marco, B. S. Rechelo, E. G. Tótolí, A. C. Kogawa, H. R. N. Salgado, "Evolution of green chemistry and its multidimensional impacts: a review," *Saudi Pharmaceutical Journal*, vol. 27 no. 1, DOI: 10.1016/j.jsps.2018.07.011, 2019.
- [9] G. A. Hurst, "Systems thinking approaches for international green chemistry education," *Current Opinion in Green and Sustainable Chemistry*, vol. 21, pp. 93-97, DOI: 10.1016/j.cogsc.2020.02.004, 2020.
- [10] P. M. Falcone, M. Hiete, "Exploring green and sustainable chemistry in the context of sustainability transition: the role of visions and policy," *Current Opinion in Green and Sustainable Chemistry*, vol. 19, pp. 66-75, DOI: 10.1016/j.cogsc.2019.08.002, 2019.
- [11] P. Vaid, P. Raizada, A. K. Saini, R. V. Saini, "Biogenic silver, gold and copper nanoparticles—a sustainable green chemistry approach for cancer therapy," *Sustainable Chemistry and Pharmacy*, vol. 16, DOI: 10.1016/j.scp.2020.100247, 2020.
- [12] P. N. Sudha, K. Sangeetha, K. Vijayalakshmi, A. Barhoum, "Nanomaterials history, classification, unique properties, production and market," *Emerging Applications of Nanoparticles and Architecture Nanostructures*, pp. 341-384, DOI: 10.1016/b978-0-323-51254-1.00012-9, 2018.
- [13] A. Guleria, S. Neogy, B. S. Raorane, S. Adhikari, "Room temperature ionic liquid assisted rapid synthesis of amorphous Se nanoparticles: their prolonged stabilization and antioxidant studies," *Materials Chemistry and Physics*, vol. 253, DOI: 10.1016/j.matchemphys.2020.123369, 2020.
- [14] L. Castillo-Henríquez, K. Alfaro-Aguilar, J. Ugalde-Álvarez, L. Vega-Fernández, G. M. de Oca-Vásquez, J. R. Vega-Baudrit, "Green synthesis of gold and silver nanoparticles from plant extracts and their possible applications as antimicrobial agents in the agricultural area," *Nanomaterials*, vol. 10 no. 9, 2020.
- [15] I. Khan, K. Saeed, I. Khan, "Nanoparticles: properties, applications and toxicities," *Arabian journal of chemistry*, vol. 12 no. 7, pp. 908-931, DOI: 10.1016/j.arabjc.2017.05.011, 2019.
- [16] P. Mondal, A. Anweshan, M. K. Purkait, "Green synthesis and environmental application of iron-based nanomaterials and nanocomposite: a review," *Chemosphere*, vol. 259, DOI: 10.1016/j.chemosphere.2020.127509,

2020.

- [17] P. I. Dolez, "Nanomaterials definitions, classifications, and applications," *Nanoengineering*, DOI: 10.1016/b978-0-444-62747-6.00001-4, 2015.
- [18] I. Hussain, M. Brust, A. J. Papworth, A. I. Cooper, "Preparation of acrylate-stabilized gold and silver hydrosols and Gold-Polymer composite films," *Langmuir*, vol. 19 no. 11, pp. 4831-4835, DOI: 10.1021/la020710d, 2003.
- [19] T. Klaus, R. Joerger, E. Olsson, C.-G. Granqvist, "Silver-based crystalline nanoparticles, microbially fabricated," *Proceedings of the National Academy of Sciences*, vol. 96 no. 24, pp. 13611-13614, DOI: 10.1073/pnas.96.24.13611, 1999.
- [20] B. Nair, T. Pradeep, "Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains," *Crystal Growth & Design*, vol. 2 no. 4, pp. 293-298, DOI: 10.1021/cg0255164, 2002.
- [21] Y. Konishi, K. Ohno, N. Saitoh, T. Nomura, S. Nagamine, H. Hishida, Y. Takahashi, T. Uruga, "Bioreductive deposition of platinum nanoparticles on the bacterium *Shewanella* algae," *Journal of Biotechnology*, vol. 128 no. 3, pp. 648-653, DOI: 10.1016/j.jbiotec.2006.11.014, 2007.
- [22] I. Willner, R. Baron, B. Willner, "Growing metal nanoparticles by enzymes," *Advanced Materials*, vol. 18 no. 9, pp. 1109-1120, DOI: 10.1002/adma.200501865, 2006.
- [23] N. Vigneshwaran, N. M. Ashtaputre, P. V. Varadarajan, R. P. Nachane, K. M. Paralikar, R. H. Balasubramanya, "Biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus*," *Materials Letters*, vol. 61 no. 6, pp. 1413-1418, DOI: 10.1016/j.matlet.2006.07.042, 2007.
- [24] S. S. Shankar, A. Rai, B. Ankamwar, A. Singh, A. Ahmad, M. Sastry, "Biological synthesis of triangular gold nanoprisms," *Nature Materials*, vol. 3 no. 7, pp. 482-488, DOI: 10.1038/nmat1152, 2004.
- [25] S. P. Chandran, M. Chaudhary, R. Pasricha, A. Ahmad, M. Sastry, "Synthesis of gold nanotriangles and silver nanoparticles using aloe vera plant extract," *Biotechnology Progress*, vol. 22 no. 2, pp. 577-583, DOI: 10.1021/bp0501423, 2006.
- [26] J. Y. Song, B. S. Kim, "Rapid biological synthesis of silver nanoparticles using plant leaf extracts," *Bioprocess and Biosystems Engineering*, vol. 32 no. 1, pp. 79-84, DOI: 10.1007/s00449-008-0224-6, 2009.
- [27] S. Ponarulselvam, C. Panneerselvam, K. Murugan, N. Aarthi, K. Kalimuthu, S. Thangamani, "Synthesis of silver nanoparticles using leaves of *Catharanthus roseus* Linn. G. Don and their antiplasmodial activities," *Asian Pacific Journal of Tropical Biomedicine*, vol. 2 no. 7, pp. 574-580, DOI: 10.1016/s2221-1691(12)60100-2, 2012.
- [28] M. Dubey, S. Bhadauria, B. Kushwah, "Green synthesis of nanosilver particles from extract of *Eucalyptus hybrida* (safeda) leaf," *Digest Journal of Nanomaterials and Biostructures*, vol. 4 no. 3, pp. 537-543, 2009.
- [29] A. Pandey, S. Tripathi, "Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug," *Journal of Pharmacognosy and Phytochemistry*, vol. 2 no. 5, 2014.
- [30] S. S. Belokurov, I. A. Narkevich, E. V. Flisyuk, I. E. Kaukhova, M. V. Aroyan, "Modern extraction methods for medicinal plant raw material (review)," *Pharmaceutical Chemistry Journal*, vol. 53 no. 6, pp. 559-563, DOI: 10.1007/s11094-019-02037-5, 2019.
- [31] T. C. Prathna, L. Mathew, N. Chandrasekaran, M. Ashok, A. Mukherjee, "Biomimetic synthesis of nanoparticles: science, technology & applicability," *Biomimetics Learning from Nature*, DOI: 10.5772/8776, 2010.
- [32] T. Prathna, A. Raichur, N. Chandrasekaran, A. Mukherjee, "Heat mediated synthesis of silver nanoparticles using citrus limon (lemon) extract," *Asian Journal of Chemistry*, vol. 25, 2013.
- [33] O. Benavente-García, J. Castillo, F. R. Marin, A. Ortuño, J. A. Del Río, "Uses and properties of citrus flavonoids," *Journal of Agricultural and Food Chemistry*, vol. 45 no. 12, pp. 4505-4515, 1997.
- [34] J. A. Vinson, X. Su, L. Zubik, P. Bose, "Phenol antioxidant quantity and quality in foods: fruits," *Journal of Agricultural and Food Chemistry*, vol. 49 no. 11, pp. 5315-5321, DOI: 10.1021/jf0009293, 2001.
- [35] O. Aysha, S. Najimu Nisha, "Biosynthesis of silver nanoparticles, using Citrus limon (LINN.) Burm. F. Peel extract and its antibacterial property against selected urinary tract pathogens," *Journal of Biological and Scientific Opinion*, vol. 2 no. 3, pp. 248-252, 2014.
- [36] E. Alzahrani, K. Welham, "Optimization preparation of the biosynthesis of silver nanoparticles using watermelon

- and study of its antibacterial activity," *International Journal of Basic and Applied Sciences*, vol. 3 no. 4, DOI: 10.14419/ijbas.v3i4.3358, 2014.
- [37] S. Kapoor, "Preparation, characterization, and surface modification of silver particles," *Langmuir*, vol. 14 no. 5, pp. 1021-1025, DOI: 10.1021/la9705827, 1998.
- [38] G. Cao, *Nanostructures & Nanomaterials: Synthesis, Properties & Applications*, 2004.
- [39] S. Kumar, K. S. Gandhi, R. Kumar, "Modeling of formation of gold nanoparticles by citrate method," *Industrial & Engineering Chemistry Research*, vol. 46 no. 10, pp. 3128-3136, DOI: 10.1021/ie060672j, 2007.
- [40] C. Humbert, B. Busson, J.-P. Abid, C. Six, H. H. Girault, A. Tadjeddine, "Self-assembled organic monolayers on gold nanoparticles: a study by sum-frequency generation combined with UV-vis spectroscopy," *Electrochimica Acta*, vol. 50 no. 15, pp. 3101-3110, DOI: 10.1016/j.electacta.2004.10.084, 2005.
- [41] C. H. Gammons, Y. Yu, A. E. Williams-Jones, "The disproportionation of gold(I) chloride complexes at 25 to 200°C," *Geochimica et Cosmochimica Acta*, vol. 61 no. 10, pp. 1971-1983, DOI: 10.1016/s0016-7037(97)00060-4, 1997.
- [42] P. Rauwel, S. Küünal, S. Ferdov, E. Rauwel, "A review on the green synthesis of silver nanoparticles and their morphologies studied via TEM," *Advances in Materials Science and Engineering*, vol. 2015, DOI: 10.1155/2015/682749, 2015.
- [43] H. Bar, D. K. Bhui, G. P. Sahoo, P. Sarkar, S. P. De, A. Misra, "Green synthesis of silver nanoparticles using latex of *Jatropha curcas*," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 339 no. 1, pp. 134-139, DOI: 10.1016/j.colsurfa.2009.02.008, 2009.
- [44] A. Amany, S. F. G. El-Rab, F. Gad, "Effect of reducing and protecting agents on size of silver nanoparticles and their anti-bacterial activity," *Der Pharma Chemica*, vol. 4 no. 1, pp. 53-65, 2012.
- [45] E. Polievková, "Synthesis of spherical gold nanoparticles for biomedical applications," 2014.
- [46] K. Arunachalam, S. Annamalai, S. Hari, "One-step green synthesis and characterization of leaf extract-mediated biocompatible silver and gold nanoparticles from *Memecylon umbellatum*," *International Journal of Nanomedicine*, vol. 8, pp. 1307-1315, DOI: 10.2147/ijn.s36670, 2013.
- [47] T. Elavazhagan, T. Elavazhagan, "Memecylon edule leaf extract mediated green synthesis of silver and gold nanoparticles," *International Journal of Nanomedicine*, vol. 6, pp. 1265-1278, DOI: 10.2147/ijn.s18347, 2011.
- [48] S. Silva, S. Martins, A. Karmali, E. Rosa, "Production, purification and characterisation of polysaccharides from *Pleurotus ostreatus* with antitumour activity," *Journal of the Science of Food and Agriculture*, vol. 92 no. 9, pp. 1826-1832, DOI: 10.1002/jsfa.5560, 2012.
- [49] P. Kanmani, "Extraction and analysis of pectin from citrus peels: augmenting the yield from citrus limon using statistical experimental design," *Iranica Journal of Energy and Environment*, vol. 5, pp. 303-312, DOI: 10.5829/idosi.ijee.2014.05.03.10, 2014.
- [50] N. S. M. Ismail, N. Ramli, N. M. Hani, "Extraction and characterization of pectin from dragon fruit (*Hylocereus polyrhizus*) using various extraction conditions," *Sains Malaysiana*, vol. 41 no. 1, pp. 41-45, 2012.
- [51] M. R. Johan, L. C. Chong, N. A. Hamizi, "Preparation and stabilization of monodisperse colloidal gold by reduction with monosodium glutamate and poly (methyl methacrylate)," *International Journal of Electrochemical Science*, vol. 7, pp. 4567-4573, 2012.
- [52] K. H. Hassan, A. A. Khadom, N. H. Kurshed, "Citrus aurantium leaves extracts as a sustainable corrosion inhibitor of mild steel in sulfuric acid," *South African Journal of Chemical Engineering*, vol. 22, DOI: 10.1016/j.sajce.2016.07.002, 2016.

DETAILS

Subject:	Gold; Spectrum analysis; Fourier transforms; Nanoparticles; Grain size; Optimization; Visibility; Lemons; Quantum dots; Synthesis; Metabolites

Location:	United States--US; United Kingdom--UK
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-07-18 (Received); 2021-11-18 (Accepted); 2021-12-13 (Pub)
DOI:	https://doi.org/10.1155/2021/7192868
ProQuest document ID:	2613971661
Document URL:	https://www.proquest.com/scholarly-journals/synthesis-optimization-characterization/docview/2613971661/se-2?accountid=211160
Copyright:	Copyright © 2021 Eman Alzahrani and Ashwaq T. Alkhudidy. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-12-26
Database:	Publicly Available Content Database

Standardization and Quality Control of the Herbal Medicine Mist Nibima, Employed to Treat Malaria and COVID-19, Using Physicochemical and Organoleptic Parameters and Quantification of Chemical Markers via UHPLC-MS/MS

Kumatia, Emmanuel Kofi ¹

; Ofosu-Koranteng, Francis ² ; Appiah, Alfred Ampomah ¹ ; Barimah, Kofi Bobi ^{3 1} Department of Phytochemistry, Centre for Plant Medicine Research, Mampong-Akuapim, Ghana ² Department of Food and Agriculture, Ghana Standard Authority, Accra, Ghana ³ Department of Clinical Research, Centre for Plant Medicine Research, Mampong-Akuapim, Ghana

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Mist Nibima is an essential herbal medicine used to treat malaria, bacterial, yeast, and COVID-19 infections. However, the drug has not been standardized and its active chemical ingredients are also not known. This study employed physicochemical, organoleptic, qualitative, and quantitative phytochemical analysis to established standards for Mist Nibima. Additionally, UHPLC was used to quantify the alkaloid cryptolepine in the drug using calibration curve. The chemical ingredients in Mist Nibima were thereafter characterized using UHPLC-MS. Organoleptic evaluation shows that Mist Nibima is a very bitter, cloudy, broom yellow decoction with the following physicochemical parameters: pH=6.10±0.08 (at 28.3°C), total solid residue=5.34±0.27%w/v, and specific gravity=1.0099±0.0000. The total alkaloid (23.71±1.311%) content of the drug is 3 times its total saponins (7.993±0.067%) content. Methyl cryptolepinoate (37.10%), cryptolepine (33.56%), quindoline (20.78%), 11-isopropylcryptolepine (5.16%), and hydroxycryptolepine (3.14%) were the active chemical ingredients in the drug with the concentrations of 18.64±0.255, 16.85±0.231, 10.42±0.143, 2.56±0.034, and 1.70±0.023 µg/mL, respectively. Administration of a single oral therapeutic dose (30mL) of Mist Nibima corresponds to ingestion of 559.2±7.662, 505.5±6.930, 312.6±4.285, 76.8±1.028, and 51.0±0.699 µg of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine, respectively. This translates into a corresponding daily dose of 1677.6±22.986, 1516.5±20.790, 937.8±12.855, 230.4±3.084, and 153.0±2.097 µg of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine. These results could now serve as tools for authentication, standardization, and quality control of Mist Nibima to ensure its chemical and pharmacological consistency and safety.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Mist Nibima is a decoction of the root of *Cryptolepis sanguinolenta* produced by the Center for Plant Medicine Research (CPMR), Mampong-Akuapim, Ghana. *C. sanguinolenta* (Lindl.) Schlechter is a climbing, twining, perennial shrub which is a member of the Apocynaceae family. The plant is locally known in Ghana as Nibima or Kadze in the Twi and Ewe dialects, respectively [1]. Mist Nibima is being sold and dispensed at the Center's Clinic as a key

antimalaria herbal medicine in Ghana for over four decades. It is also used to treat other diseases such as candidiasis, upper respiratory tract infections, viral infections, urinary tract infections, diabetes, and lung diseases. Recently, there were reports of COVID-19 patients being cured after taking Mist Nibima. Furthermore, the Food and Drugs Authority (FDA), Ghana, and the National Medicine Regulatory Agency (NMRA), Ghana, approved the clinical trial of Mist Nibima (which is being sourced from CPMR) against COVID-19 to be undertaken by some scientist of the School of Public Health at the Kwame Nkrumah University of Science and Technology [2].

However, the chemical composition of Mist Nibima and a greater majority of herbal medicines on the global market are not known due to lack of standardization and chemical finger printing. This leads to variations in the chemical components, effectiveness, and/or periodic cases of toxicity due to toxic compounds which may be produced or absorbed by the plant since plants are variable raw materials [3, 4]. Additionally, medicinal plants and herbal medicinal products are also contaminated by assimilation of environmental contaminants such as toxic metals, pesticides, microbiological contaminants, foreign matter, and by-products of microorganisms as a result of improper postharvest handlings or during production and/or storage which makes the products deteriorate and unwholesome for consumption. These problems could be resolved by standardization, chemical finger print analysis, and quality control to help safeguard the uniformity, safety, and effectiveness of various batches of a particular herbal medicinal product [3, 4]. During standardization, various scientific assays and observations are employed to establish a set of standards or constant parameters or absolute qualitative and quantitative values for a set of inherent properties of the medicine which guarantees the assurance of quality, efficacy, safety, and reproducibility [5].

It had been reported that the most active antimalaria constituent of *C. sanguinolenta* root was the indoloquinoline alkaloid cryptolepine [6]. Although constituents in *C. sanguinolenta* root have been extensively revealed, the exact chemical ingredients and their quantities in Mist Nibima are not known. The aim of this study, therefore, is to employ UHPLC-MS to characterize and quantify the chemical ingredients in Mist Nibima and to also standardize the decoction with the alkaloid cryptolepine isolated from *C. sanguinolenta* root using the calibration curve method in addition to other phytochemical and physicochemical studies.

2. Materials and Methods

2.1. Chemicals and Reagents

Ethyl acetate was obtained from Merck KgaA, Darmstadt, Germany. Chloroform and petroleum ether (40–60°C) were procured from Fisher Scientific, Loughborough, and Park Scientific Northampton, UK, respectively. Ethanol (99%) was also purchased from Midland International, Ghana. Silica gel (normal phase for column chromatograph) and aluminum precoated normal phase TLC plates (6 F₂₅₄) were also purchased from Merck KgaA, Darmstadt, Germany.

2.2. Isolation of Cryptolepine from the Root of *C. sanguinolenta*

The dried root of *C. sanguinolenta* was obtained from the stores of the Production Department of CPMR. It was pulverized into coarse powder and 2.0 kg was cold macerated with 5.0 L of 70% ethanol for 3 days. The extract was filtered and concentrated under low pressure in rotary evaporator to 0.5 L. Total alkaloid extract (13 g) was obtained after the crude extract (0.5 L) was basified with ammonia and exhaustively extracted with chloroform (0.5 L x 6) and dried into a solid in the rotary evaporator. The crude alkaloid extract (13 g) was chromatographed over a normal phase silica gel using petroleum ether-chloroform-ethanol in a gradient eluting manner to obtain dark purple needle crystals.

2.3. Mist Nibima

Three batches (NB. 20010; NB. 20011; NB. 20012) of Mist Nibima were obtained from the dispensary of the Cenetr for Plant Medicine Research, Mampong-Akuapim, for the study. The decoction was produced from the pulverized roots of *C. sanguinolenta* and packaged in 330 mL amber-colored plastic bottles. The drug was registered with the Food and Drugs Authority of Ghana with the number FDA/HD₁.20-02086.

2.4. Evaluation of Physicochemical Properties of Mist Nibima

2.4.1. Total Solid Residue (TSR)

Mist Nibima (10 mL) was measured into a ceramic evaporating dish with a known weight (W_o) and dried over a

water bath in the hood. The dish was removed and dried in an oven to a constant weight at 70°C. It was then removed and allowed to cool to room temperature after which its weight (W_1) was determined. TSR of the drug was calculated by relating the weight of the drug to its volume using the following formula: (1) $TSR = \frac{W_1 - W_0}{\text{Volume of sample evaporated}} \times 100\% \text{ wv}$.

2.4.2. Specific Gravity (SG)

Briefly, a dried empty gravity bottle with a known weight (W_0) was fully filled with Mist Nibima and corked with the gravity bottle's stopper and weighed (W_1) after being wiped dried. The drug in the gravity bottle was then discarded. The bottle was rinsed with distilled water. It was thereafter fully filled with distilled water, wiped dried, and weighed (W_2). The SG of the sample was then calculated as the weight of the sample divided by the weight of water using the following formula: (2) $SG = \frac{W_1 - W_0}{W_2 - W_0}$.

2.4.3. pH

The pH of the drug was determined using a digital pH meter (PL-700 PV, Taiwan) with pH and temperature probes. The pH meter was first calibrated with standard pH buffers 4, 7, and 10 prior to being dipped into the drug in a 50 mL glass beaker.

2.4.4. Color

The color of the drug was determined by observation with the eye and then matching it with colors on RAL Classic color chart (Ral color chart.pdf-e-paint.co.uk).

2.5. Identification of Phytochemical Constituents in Mist Nibima

Previously described methods [7] were employed to identify the classes of phytochemical constituents in Mist Nibima.

2.6. Quantification of Total Alkaloids in Mist Nibima

A revised method described by Kumatia et al. [8] which was earlier reported [9] was adopted to quantify the amount of alkaloid in Mist Nibima. Mist Nibima (100 mL) was evaporated to dryness on water bath, and 1.0 g of the dried sample was used for the test. The percentage of alkaloids in the drug was calculated with the following formula: (3) $\text{Alkaloid} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample}} \times 100\%$.

2.7. Identification of Cryptolepine Using UHPLC-MS

In order to establish the chemical identity of cryptolepine, 2.5 mg of the isolated compound was first taken through a similar process as described above in the UHPLC finger print evaluation of Mist Nibima. Additionally, the UHPLC was coupled with a Mass Spectrometer (Agilent Tripple Quadrupole MS) to determine the mass and fragmentation patterns of cryptolepine. The electron source ionization (ESI) (positive mode) was employed for fragmentation. The experimental conditions and the MS m/z scan range were as earlier reported [8]. The mobile phase employed in the UHPLC analysis (Sections 2.7–2.9) was 70% water and 30% methanol under isocratic conditions.

2.8. Preparation of a Standard Calibration Curve Using Cryptolepine

The quantity of cryptolepine in Nibima was determined by means of a calibration curve method using UHPLC. A standard calibration curve was generated using the pure cryptolepine isolated from *C. sanguinolenta* root. Cryptolepine (2.5 mg) was dissolved in 25 mL of HPLC grade methanol. Various concentrations of the solution (0.25, 0.5, 1.0, 2.0, 5.0, and 10.0 $\mu\text{g/mL}$) were prepared from the original solution using serial dilution. The solutions were run on a UHPLC to obtain the response, retention time (RT), peak area, and peak height. The data acquired was processed using the MassHunter Quantitative Analysis software version B.07.00 (Agilent Technologies, USA) to produce a standard calibration curve by plotting the response against the concentration of cryptolepine. The calibration curve produced a linear regression equation in the form of (4) $y = ax + b$, where y is the peak area of cryptolepine in the crude medicine/sample, a and b are constants given by the calibration curve, and x is the concentration of the cryptolepine in the crude sample/medicine.

2.9. Determination of the Quantity of Cryptolepine in Mist Nibima

In order to determine the quantity of cryptolepine in Nibima, 100 mL of the drug was evaporated to dryness on water bath. The dried sample (2.0 g) was dissolved in 4 mL of methanol in a 50 mL centrifuge tube and thoroughly mixed with an electronic mixer (Vortex mixer) for 5 minutes and then centrifuged at 3500 rpm. The top layer of the solution

(2 mL) was pipetted into a pear-shaped flask and evaporated to dryness using a rotary evaporator. The residue was redissolved in 1 mL HPLC grade methanol and filtered with filter paper (mesh size=0.45 µm) into a GC vial and used for UHPLC analysis. The analysis was performed in duplicates using three batches of Nibima. Methanol was used as a blank. The data was processed using the Mass Hunter Quantitative Analysis software version B.07.00 (Agilent Technologies, USA). The quantity of cryptolepine in Mist Nibima was calculated using the linear regression equation [10] produced above from the calibration curve of the pure cryptolepine.

2.10. Chemical Fingerprinting and Identification of Compounds in Mist Nibima by UHPLC-MS

Chemical finger print analysis and identity of the compounds in Mist Nibima were evaluated according to a previously described method [8]. The dry sample of Mist Nibima (25 mg) was used.

2.11. Determination of the Percentage Compositions

The percentage composition of a given compound in the drug was calculated as follows: (5) % Composition of a compound = $\frac{\text{Area of the given compound in the drug}}{\text{Sum of the area of all the compounds in the drug}} \times 100\%$.

2.12. Determination of the Quantities of Other Compounds in Mist Nibima

The quantity of a given chemical ingredient/compound (QGCC), in µg or mg, in Mist Nibima was calculated by relating the quantity of the cryptolepine (QCT), in µg or mg, estimated in Mist Nibima and the percentage composition of cryptolepine (%CTP) in the drug and that of the given compound (%GCC) in the UHPLC chromatogram using the following formula: (6) $QGCC \mu\text{g or mg} = \%GCC \times QCT \%CTP$.

3. Presentation of Data

Data generated from this study was analyzed using GraphPad Prism statistical software and presented as mean ± SEM.

4. Results and Discussion

4.1. Physicochemical and Organoleptic Properties of Mist Nibima

The results from the physicochemical and organoleptic tests are tabulated in Table 1.

Table 1

Physicochemical and organoleptic properties of Mist Nibima.

Physicochemical parameters		Organoleptic properties	
Parameter	Mean ± SEM	Property	Nature
pH @ 28.3°C	6.10 ± 0.08	Color	Broom yellow (RAL 1032)
Total solid residue	5.34 ± 0.27% w/v	Taste	Very bitter
Specific gravity	1.0099 ± 0.0000	Clarity	Cloudy

Physicochemical properties of a drug are properties that relate to both physical and chemical characteristics of the drug/substance.

For decoctions, the physicochemical properties that are mostly evaluated are pH, specific gravity, total solid residue, and volume. Additionally, organoleptic parameters of a drug are those properties which are evaluated using the sense organs, e.g., taste, color, odor, texture, and dosage form. In this study, the organoleptic parameters evaluated for Mist Nibima include color, taste, and clarity. Individual drugs have unique physicochemical and organoleptic parameters which serve as fingerprints for standardization and quality control of future batches. Thus, this study has established standard physicochemical and organoleptic parameters for Mist Nibima (Table 1) which will be used as reference in the quality control of the subsequent batches of the drug.

4.2. Qualitative and Quantitative Phytochemical Constituents

The results of the qualitative and quantitative phytochemical analysis of Mist Nibima are tabulated in Tables 2 and 3.

Table 2

Results of the qualitative phytochemical analysis of Mist Nibima.

Test	Result	Test	Result
Alkaloids	Present	Flavonoids	Absent
Saponins	Present	Antracenosides	Absent
Polyuronoids	Present	Phenolic compounds	Absent
Free reducing sugars	Present	Triterpenes	Absent
Phytosterols	Absent	Cyanogenic glycosides	Absent

Table 3

Percentage compositions of saponins and alkaloids in Mist Nibima.

Phytochemical constituent	Quantity (%) in Nibima
Alkaloids	23.71 ± 1.311
Saponins	7.993 ± 0.067

The qualitative analysis indicates that the drug contains alkaloids saponins polyuronoids and free reducing sugars in all the three batches. The other six phytoconstituents were absent in the drug. Furthermore, the quantification tests also indicate that the alkaloids ($23.71 \pm 1.311\%$) content of the drug is 3 times the saponins ($7.993 \pm 0.067\%$) content (Table 3).

Besides, the physicochemical and organoleptic characteristics and qualitative and quantitative phytochemical constituent data of a herbal drug are the easiest and less expensive parameters used as standardization and quality control tools especially in a resource limited environments where modern instruments are too expensive to afford. Hence, these results also constitute a unique fingerprint for Mist Nibima which shall be used as a reference standard for quality control, standardization, and authentication of future batches of the drug.

4.3. Identification of Cryptolepine Using Chromatographic Profiles of Cryptolepine

Cryptolepine was obtained as a purple needle crystal. The TLC and UHPLC chromatograms of cryptolepine are shown in Figures 1(a) and 1(b). The TLC chromatogram of the chloroform solution of cryptolepine produced a single pink spot when developed in 100% chloroform or chloroform/petroleum ether after the plate was dried in air for 10 mins and then immersed in 10% H_2SO_4 and heated at $100^\circ C$ for 3mins (Figure 1(a)). $R_f = 0.5897$ (chloroform) and 0.6111 (chloroform/petroleum ether 9:1). The purple color of cryptolepine and its former R_f value compare favorably with the color and $R_f = 0.51$ (chloroform/methanol/ammonia 90:10:1) reported for cryptolepine on a reverse phase TLC plate [11].

[figures omitted; refer to PDF]

The UHPLC chromatogram of cryptolepine also produced a single peak with a RT of 9.936mins. The compound produced a single spot in two-solvent system in the TLC analysis and a single peak in the UHPLC chromatogram indicates that the isolated compound was pure. The mass spectrum and structure of the isolated compound from *C. sanguinolenta* root are shown in in Figure 2, which consists of a total of ten (10) peaks of ion fragments with mass to charge ratios ranging 103.30–363.20 (Figure 2). The ion peak at 233.20 revealed the $[M+H]^+$ of $C_{16}H_{12}N_2$ which is

indicative of cryptolepine with a molecular mass (Mm of 232.28g/mol) [12, 13]. The isolate was hence identified as cryptolepine based on its color, R_f , and Mm.

[figure omitted; refer to PDF]

In order to develop a chemical fingerprint for the quality control of Mist Nibima and to also be sure that the alkaloid cryptolepine is part of the constituents extract into Mist Nibima by boiling *C. sanguinolenta* roots, UHPLC analysis of the drug was performed. The UHPLC chromatogram/chemical finger print of Mist Nibima shows the presence of five (5) distinct compounds in the drug with their respective RT. Cryptolepine is represented by the fourth peak with RT of 9.836min which is similar to the RT (9.926min) of pure cryptolepine (Figure 1). The RT of cryptolepine reduced by 0.021min in the crude drug because the other constituents in the crude drug interfered with/blocked the movement of the cryptolepine through the UHPLC column.

Chemical fingerprinting is a critical tool used in quality control and standardization of herbal medicines to protect the homogeneity, safety, and efficacy of different batches of a herbal product/medicine [4]. A chemical fingerprint is a specific pattern which specifies the existence of various chemical markers in a sample. Chemical markers are defined as constituents or classes of constituents in a herbal medicinal product which are relevant for quality control purposes irrespective of their effectiveness as therapeutic principles [14]. The characteristic of the UHPLC chemical fingerprint described for Mist Nibima above (Figure 4) is therefore essential to safeguard the quality, authenticity, homogeneity, safety, and effectiveness of the future batches of the drug.

4.7. Identification of Compounds in Mist Nibima Using the MS Data

4.7.1. Identification of Compound 1 (Peak 1) as Quindoline

The structure and mass spectrum of compound 1 are shown in Figure 5. Compound 1 produced an adduct ion with 100% molecular ion peak m/z at 259.10 which is indicative of $[M+H+K]^+$.

[figure omitted; refer to PDF]

This represents the molecular mass of $C_{15}H_{10}N_2$ which is indicative of quindoline with a molecular mass of 218.25 g/mol. Quindoline was previously isolated from (*C*) *sanguinolenta* root with M^+ at m/z 218 (100%) by [13].

4.7.2. Identification of Compound 2 (Peak 2) as Methyl Cryptolepine

The mass spectrum of the second compound in Mist Nibima is shown in Figure 6.

[figure omitted; refer to PDF]

Compound 2 produced a fragment ion at m/z of 291.10 (0.6%) as the last peak in the spectrum which is indicative of $[M+H]^+$. This represents the molecular formula of $C_{18}H_{14}N_2O_2$ with the molecular mass of 290.0g/mol. This data agreed with the molecular weight of 290 and $[M+H]^+$ of 291 reported for methyl cryptolepine [11]. Compound 2 was, therefore, identified as methyl cryptolepine (Figure 6).

4.7.3. Identification of Compound 3 (Peak 3) as Hydroxycryptolepine

The mass spectrum of the second compound in Mist Nibima is shown in Figure 7.

[figure omitted; refer to PDF]

Compound 3 produced at least 10 distinct peaks in its mass spectrum. The peak with m/z at 275 represents $[M+2H+Na]^+$ which is indicative of a sodiated adduct of $C_{16}H_{12}N_2O$. This is indicative of hydroxycryptolepine which has a molecular mass (Mm) of 248g/mol. The increase in Mm to 250g/mol is due to the presence of the ^{18}O isotope in hydroxyl group instead of ^{16}O . Furthermore, the m/z observed at 259.2 in the spectrum is indicative of $[M+2H+Na]^+ - CH_3$. These MS features observed for compound 3 confirmed that it is hydroxycryptolepine. Hydroxycryptolepine was isolated from *C. sanguinolenta* with $[M+H]^+$ of 249 which led to Mm of 248g/mol [11].

4.7.4. Identification of Compound 4 (Peak 4) as Cryptolepine

The mass spectrum of compound 4 is shown in Figure 8. Compound 4 showed similar mass spectral features which corresponds to those described for cryptolepine in Figure 2. Hence, compound 4 was identified as cryptolepine.

[figure omitted; refer to PDF]

4.7.5. Identification of Compound 5 (Peak 5) as 11-Isopropylcryptolepine

The mass spectrum of the 5th compound in Nibima is shown in Figure 9. Compound 5 produced a molecular ion with m/z at 277. This represents the $[M+H]^+$ of 11-isopropylcryptolepine which has a molar mass of 276g/mol.

Furthermore, the m/z at 259 and 245 represents $[M - H - NH_3]^+$ and $[M - 2H - 2CH_3]^+$, respectively, for 11-isopropylcryptolepine. Therefore, compound 5 was confirmed to be 11-isopropylcryptolepine [15].

[figure omitted; refer to PDF]

4.8. Concentrations of Active Chemical Ingredients in Mist Nibima

The names, RT, and concentrations of the five active chemical ingredients/compounds identified in the herbal drug using the UHPLC-MS characterization are presented in Table 5.

Table 5

Names of compounds/active ingredients identified in Mist Nibima and their RTs, peak areas, percentage compositions, and concentrations in Mist Nibima.

Peak	Proposed compound	RT (min)	Area	Composition in Mist Nibima (%) ($\mu\text{g/mL}$) (mg/330mL bottle)		
1	Quindoline	1.352	2070555 301.3	20.78	10.42 $\pm$ 0.143	3.44 $\pm$ 0.047
2	Methyl cryptolepinoate	1.577	3697465 123.3	37.10	18.64 $\pm$ 0.255	6.15 $\pm$ 0.084
3	Hydroxycryptolepine	6.321	3398117 26.26	3.41	1.70 $\pm$ 0.023	0.56 $\pm$ 0.011
4	Cryptolepine	9.835	3344237 823.9	33.56	16.85 $\pm$ 0.231	5.56 $\pm$ 0.076
5	11-Isopropylcryptolepine	10.96 2	5141029 21.06	5.16	2.56 $\pm$ 0.034	0.85 $\pm$ 0.007

The result indicates that methyl cryptolepinoate with 37.10% composition was the major component of Mist Nibima. This was followed by cryptolepine with 33.56%. The remaining three components followed in the decreasing order of quindoline (20.78%), 11-isopropylcryptolepine (5.16%), and hydroxycryptolepine (3.14%). Mist Nibima is a decoction (boiling water extract) of *C. sanguinolenta* root. Water is a very polar solvent due to its strong hydrogen bonds. Hence, the most polar compounds were to be extracted in larger quantities into the drug compared to the less polar ones. However, this principle is true for methyl cryptolepinoate, an ester which dissociate into a cation and anion when in water, but not for hydroxycryptolepine. This indicates that the amount of compound extracted by boiling water depended not only on the polarity of the compound, but also on the quantity of the compound present in the plant material. Hence, hydroxycryptolepine might be the smallest constituent produced by the batch of the plant used to produce the drug. The dose of Mist Nibima as indicated on the label is 30mL to be taken orally, 3 times daily after meals. Based on the results in Table 5 above, a single administration of 30mL of the drug is equivalent to ingestion of 559.2 ± 7.662 , 505.5 ± 6.930 , 312.6 ± 4.285 , 76.8 ± 1.028 , and $51.0 \pm 0.699 \mu\text{g}$ of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine, respectively. This translates into a corresponding daily dose (30mL three (3) times) of 1677.6 ± 22.986 , 1516.5 ± 20.790 , 937.8 ± 12.855 , 230.4 ± 3.084 , and $153.0 \pm 2.097 \mu\text{g}$ of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine.

C. sanguinolenta has been widely studied for its biological activities and chemical constituents. Phytochemical investigations of organic extracts and fractions of the plant led to isolation of about eighteen indolo quinoline

alkaloids in addition to three other derivatives of cryptolepine. They are cryptolepine, isocryptolepine, cryptosanguinolentine, neocryptolepine, cryptotackieine, quindoline, cryptospirolepine, cryptolepicarboline, cryptomisine, 11-isopropylcryptolepine, cryptolepinone, biscryptolepine, hydroxycryptolepine, cryptoheptine, cryptoquindoline, cryptolepinoic acid, methyl cryptolepinoate, and ethyl cryptolepinoate [6, 11, 13, 15–20]. However, the exact chemical constituents and their concentration in the herbal drugs/preparations of *C. sanguinolenta* including Mist Nibima were not known. This study has chromatographically and spectroscopically characterized the individual chemical compounds in Mist Nibima and also determined their concentration for the first time.

The UHPLC chemical finger printing analysis has shown that Mist Nibima contains five compounds out of about eighteen alkaloids previously isolated from the plant, since the chemical fingerprint has shown that the drug contains five compounds (five peaks) with specific RTs. It is now possible to discriminate contaminants in any future batch of the drug or be able to tell if an additional peak detected in any future batch of Mist Nibima is being produced by the plant or is an adulterant by using this chemical fingerprint as a reference standard. The chemical finger print chromatogram obtained for Mist Nibima could, therefore, serve as reference standard in determination of adulteration and substandard batches of the drug in future productions.

Furthermore, knowing the standard concentration of a drug makes it possible to distinguish between a substandard batch with significantly lower concentration of the active ingredients or a batch with significantly high concentrations of the active ingredients which may be toxic. These situations may arise as a result of lapses in the raw material sourcing and the manufacturing process. Since the respective compounds and their standard concentrations in Mist Nibima are now known, the necessary adjustments can be made to any batch which does not meet these standard concentrations of the active ingredients in order to bring it to the level of the reference concentrations of its active ingredients. For instance, the concentration of active ingredients in a lower concentrated batch of a herbal medicine can be increased to the standard levels by evaporations under controlled heat to a required volume or addition of a calculated amount of a high concentrated sample, whereas a batch with higher concentration of active ingredients of the drug can be diluted with calculated amount of water or low concentrated sample to reduce it to the standard concentration.

5. Conclusions

The present study has established physicochemical, organoleptic, qualitative, and quantitative phytochemical standardization and quality control parameters for Mist Nibima, a herbal decoction used for the treatment of malaria, COVID-19, and many other diseases. Five indole alkaloids, namely, quindoline, methyl cryptolepinoate, hydroxycryptolepine, cryptolepine, and 11-isopropylcryptolepine, and their corresponding concentrations have also been identified as the active chemical constituents in Mist Nibima for the first time. These results will be used as tools for authentication, standardization, and quality control of this essential herbal medicine to ensure its consistent chemical and pharmacological characteristics and safety.

Acknowledgments

The authors are very grateful to the staff of the Phytochemistry Department of the Center for Plant Medicine Research for their technical support.

References

- [1] Y. Ameyaw, "Morpho-histological characters for the identification of *cryptolepis sanguinolenta* (lindl.) schtr, inter," *Journal of Applied and Natural Science*, vol. 3 no. 2, pp. 331-339, 2012.
- [2] Food and Drug Authority (FDA), Ghana, FDA/CS/CPE/PR1/21/0003. Press Release-FDA Approves First Herbal Medicine for Clinical Trial on COVID-19 Treatment, 2021. <https://fdaghana.gov.gh/press-release.php>
- [3] E. K. Kumatia, F. Ayertey, R. Appiah-Opong, P. Bolah, E. Ehun, J. Dabo, "Antrocaryon micraster (A. Chev. And Guillaumin) stem bark extract demonstrated anti-malaria action and normalized hematological indices in *Plasmodium berghei* infested mice in the Rane's test," *Journal of Ethnopharmacology*, vol. 266, DOI: 10.1016/j.jep.2020.113427, 2021.
- [4] S. Li, Q. Han, C. Qiao, J. Song, C. Lung Cheng, H. Xu, "Chemical markers for the quality control of herbal medicines: an overview," *Chinese Medicine*, vol. 3, DOI: 10.1186/1749-8546-3-7, 2008.

- [5] O. F. Kunle, H. O. Egharevba, P. O. Ahmadu, "Standardization of herbal medicines-a review. nt," *Journal of the Biodiversity Conservation*, vol. 4, pp. 101-112, DOI: 10.5897/ijbc11.163, 2012.
- [6] K. Cimanga, T. De Bruyne, L. Pieters, A. J. Vlietinck, C. A. Turger, "In vitro and in vivo antiplasmodial activity of cryptolepine and related alkaloids from *Cryptolepis sanguinolenta*," *Journal of Natural Products*, vol. 60 no. 7, pp. 688-691, DOI: 10.1021/np9605246, 1997.
- [7] G. E. Trease, W. C. Evans, *Pharmacognosy*, 1989.
- [8] E. K. Kumatia, F. Ayertey, R. Appiah-Opong, G. K. Bagyour, K. O. Asare, V. C. Mbatcho, J. Dabo, "Intervention of standardized ethanol leaf extract of *Annickia polycarpa*, (DC.) Setten and Maas ex I.M. Turner. (Annonaceae), in *Plasmodium berghei* infested mice produced anti-malaria action and normalized gross hematological indices," *Journal of Ethnopharmacology*, vol. 267, DOI: 10.1016/j.jep.2020.113449, 2021.
- [9] J. B. Harborne, *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 1973.
- [10] A. S. Sonmezdag, H. Kelebek, S. Selli, "Characterization of aroma-active and phenolic profiles of wild thyme (*Thymus serpyllum*) by GC-MS-Olfactometry and LC-ESI-MS/MS," *Journal of Food Science & Technology*, vol. 53 no. 4, pp. 1957-1965, DOI: 10.1007/s13197-015-2144-1, 2016.
- [11] A. Paulo, E. T. Gomes, J. Steele, D. C. Warhurst, P. J. Houghton, "Antiplasmodial activity of cryptolepis sanguinolenta alkaloids from leaves and roots," *Planta Medica*, vol. 66 no. 1, pp. 30-34, DOI: 10.1055/s-2000-11106, 2000.
- [12] E. Gellért, Raymond-Hamet, E. Schlittler, "Die Konstitution des Alkaloids Cryptolepin," *Helvetica Chimica Acta*, vol. 34 no. 2, pp. 642-651, DOI: 10.1002/hlca.19510340228, 1951.
- [13] D. Dwuma-Badu, J. S. K. Ayim, N. I. Y. Fiagbe, J. E. Knapp, P. L. Schiff, D. J. Slatkin, "Constituents of west african medicinal plants XX: quindoline from cryptolepis sanguinolenta," *Journal of Pharmaceutical Sciences*, vol. 67 no. 3, pp. 433-434, DOI: 10.1002/jps.2600670350, 1978.
- [14] European Medicines Agency (EMA), New EMA Paper on Markers for Herbal Medicinal Products: Reflection Paper on Markers Used for Quantitative and Qualitative Analysis of Herbal Medicinal Products and Traditional Herbal Medicinal Products, 2008. <http://www.ema.europa.eu/pdfs/human/hmpc/25362907en.pdf>
- [15] A. Paulo, E. T. Gomes, P. J. Houghton, "New alkaloids from *Cryptolepis sanguinolenta*," *Journal of Natural Products*, vol. 58 no. 10, pp. 1485-1491, DOI: 10.1021/np50124a002, 1995.
- [16] J.-L. Poussel, M.-T. Martin, A. Jossang, B. Bodo, "Isocryptolepine from cryptolepis sanguinolenta," *Phytochemistry*, vol. 39 no. 3, pp. 735-736, DOI: 10.1016/0031-9422(94)00925-j, 1995.
- [17] M. H. M. Sharaf, P. L. Schiff, A. N. Tackie, C. H. Phoebe, G. E. Martin, "Two new indoloquinoline alkaloids from cryptolepis sanguinolenta: cryptosanguinolentine and cryptotackieine," *Journal of Heterocyclic Chemistry*, vol. 33 no. 2, pp. 239-243, DOI: 10.1002/jhet.5570330204, 1996.
- [18] M. H. M. Sharaf, P. L. Schiff, A. N. Tackie, "The isolation and structure determination of cryptomisine, a novel indolo[3,2-b]quinoline dimeric alkaloid from *Cryptolepis sanguinolenta*," *Journal of Heterocyclic Chemistry*, vol. 33 no. 3, pp. 789-797, DOI: 10.1002/jhet.5570330343, 1996.
- [19] T. Parvatkar, P. S. Parameswaran, S. G. Tilve, "Isolation, biological activities and synthesis of indoloquinoline alkaloids: cryptolepine, isocryptolepine and neocryptolepine," *Current Organic Chemistry*, vol. 15 no. 7, pp. 1036-1057, DOI: 10.2174/138527211794785118, 2011.
- [20] C. Crouch, A. O. Davis, T. D. Spitzer, "Elucidation of the structure of quindolinone, a minor alkaloid of cryptolepis sanguinolenta: submilligram ¹H-¹³C and ¹H-¹⁵N heteronuclear shift correlation experiments using micro inverse-detection," *Journal of Heterocyclic Chemistry*, vol. 32 no. 3, pp. 1077-1080, DOI: 10.1002/jhet.5570320369, 1995.

DETAILS

Subject:	Infections; Software; Malaria; Quality control; Specific gravity; Phytochemicals; Calibration; Ingredients; Saponins; Ethanol; Alkaloids; COVID-19; Qualitative analysis; Quality standards; Medicine; Ingestion; Standardization; Herbal medicine; Quantitative analysis; Coronaviruses; Parameters
Business indexing term:	Subject: Quality control Quality standards
Location:	Ghana; United States--US; Germany
Company / organization:	Name: Agilent Technologies Inc; NAICS: 334515, 334516, 334519, 339112, 513210; Name: Food & Drug Administration--FDA; NAICS: 922190
Editor:	David Touboul
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chem istry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-09-01 (Received); 2021-11-12 (Accepted); 2021-12-01 (Pub)
DOI:	https://doi.org/10.1155/2021/6390481
ProQuest document ID:	2609151162
Document URL:	https://www.proquest.com/scholarly-journals/standardization-quality-control-herbal-medicine/docview/2609151162/se-2?accountid=211160

Copyright:

Copyright © 2021 Emmanuel Kofi Kumatia et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2022-07-14

Database:

Publicly Available Content Database

Document 7 of 59

Antioxidant Activity In Vitro Guided Screening and Identification of Flavonoids Antioxidants in the Extract from *Tetrastigma hemsleyanum* Diels et Gilg

Ding, Li

; Zhang, Xiaomin; Zhang, Jiajia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

This study aimed to investigate the extract with high antioxidant activity of *Tetrastigma hemsleyanum* Diels et Gilg and identify the antioxidant components in vitro. α , α -Diphenyl- β -picrylhydrazyl (DPPH) radical assay, Trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing antioxidant power (FRAP), and hydroxyl radical scavenging method were used to screen the extract with high antioxidant activity. The antioxidant capacity of the extracts was evaluated by the free radical scavenging ability of DPPH. The ability of extracts to scavenge 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical was evaluated by TEAC assay. The FRAP method was used to evaluate the ability of extracts to reduce Fe^{3+} . The ability to scavenge hydroxyl radicals produced by the interaction of hydrogen peroxide and Fe^{2+} was measured by monitoring the change in the absorbance of the reaction mixture at 536 nm. Then, high-performance liquid chromatography-DPPH (HPLC-DPPH) and HPLC-hydroxyl radical scavenging methods were used to screen the antioxidant components in the extract. The molecular weight of the above antioxidant components was investigated using the qualitative analytical method of high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-Q-TOF LC/MS). Based on the concentrations of the samples (0.2–4 mg/mL), the DPPH free radical scavenging ability, ABTS+ free radical scavenging ability, hydroxyl free radical scavenging ability, and Fe^{3+} reducing ability of the ethyl acetate extract (EAE) were stronger than that of the crude extract (CE), petroleum ether extract (PEE), and n-butanol extract (BE). The EAE has higher antioxidant activity than CE, PEE, and BE. Six antioxidant components, rutin, quercetin, isoquercetin, astragalin, kaempferol, and kaempferol-3-o-rutside, were identified in the EAE.

FULL TEXT

1. Introduction

The rhizome of *Tetragium hemsleyanum* Diels et Gilg, a Chinese folk herbal medicine, is used for the treatment of children with high fever, pneumonia, hepatitis, viral meningitis, scrofula, carbuncle, and furuncle [1]. Modern pharmacological studies have shown that *Tetragium hemsleyanum* Diels et Gilg has anti-inflammatory, analgesic [2], antiviral [3], antiliver injury [4, 5], antitumor [6, 7], and other pharmacological effects [8, 9].

A large number of studies are focused on the chemical constituents of *Tetragium hemsleyanum* Diels et Gilg. The aboveground part of *Tetragium hemsleyanum* Diels et Gilg contains β -sitosterol, palmitic acid, pentacosane, gallic acid, ethyl gallate, catechin, 7-O-galloylcatechin, and 3,3'-dimethoxy ellagic acid-4-O- β -D-glucopyranoside [10]. Taraxerone, ergosterol, oleic acid, palmitic acid, myristic acid, hexadecylic acid, heptadecanoic acid, 9,12-octadecadienoic acid, α -linolenic acid, kaempferol, quercetin, and kaempferol-3-o-neohesperidin were found in liposolubility extract of *Tetragium hemsleyanum* Diels et Gilg [11–13]. In addition, Xu analyzed 80% methanol ultrasonic extract of *Tetragium hemsleyanum* by HPLC-Q-TOF-MS and found that it contained kaempferol-7-o-rhamnose-3-o-glucoside, rutin, isoquercetin, kaempferol rutoside, astragaloside, and quercetin [12]. Fan identified 8 flavonoids (isorientin, orientin, orientin-2"-O-rhamnoside, isoorientin-2"-O-rhamnoside, vitexin, vitexin-2"-O-rhamnoside, isovitexin, and isovitexin-2"-O-rhamnoside) in *Tetragium hemsleyanum* leaves by rapid resolution liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (RRLC-Q-TOF-MS) [14]. These results showed that oleic acid and flavonoids constituted the largest proportion of the plant [15]. Flavonoids have various biological activities, such as the cardiovascular system [16, 17], antioxidant [18–20], anti-tumor [21–23], anti-inflammatory, and analgesic [24], and are one of the most and diverse phenolic groups of natural origin [25]; their antioxidation capability is a significant property.

Free radicals that are produced in the process of life metabolism regulate the signal transmission between cells and cell growth and inhibit the viruses and bacteria [26]. Excessive free radicals attack the cell membrane and leak from the intracellular region, which in turn leads to the denaturation of protein and nucleic acid, thereby, damaging the cells, tissues, and organs, inducing various diseases, and accelerating the aging of the body. The mechanism of inflammation, tumor, aging, and cardiovascular diseases is closely related to the excessive production of free radicals or a decrease in the ability to scavenge free radicals [27]. Antioxidants quench free radicals and form nontoxic ions or molecules. The free radicals were scavenged to achieve antioxidation, and antioxidants can stop or reverse some damage caused by excessive free radicals [28]. Synthetic antioxidants used to prolong the shelf life of food, such as butyl hydroxyanisole (BHA), dibutyl hydroxytoluene (BHT), and propyl gallate (PG) [29–31], have been found to be potential hepatotoxicity and carcinogenicity [32, 33]. Due to restricted use of synthetic antioxidants [34], natural antioxidants with high safety and fewer side effects are favored. Natural antioxidants and active substances mainly originate from plants [31], including tea extract [35, 36], fruit and vegetable extracts [37], balsam pear extracts [38], Chinese herbal extracts [39], raw *Rehmanniae* extract [40], and spices extracts [41]. Natural antioxidants can eliminate free radicals [42, 43], improve blood circulation, delay cell aging, and prevent cancer [44, 45].

While screening the antitumor extract and active components of *Tetragium hemsleyanum* Diels et Gilg, we found that some of the isolated parts were oxidized [46, 47]. This phenomenon indicated that there might be antioxidant components in *Tetragium hemsleyanum* Diels et Gilg. However, active components responsible for antioxidants in *Tetragium hemsleyanum* Diels et Gilg are yet to be identified. Therefore, by utilizing the antioxidant test, we screened out the extract that harbors high antioxidant activity, and the molecular weight of the potential active components was determined by HPLC-Q-TOF LC/MS.

2. Materials and Methods

2.1. Chemicals and Plant Material

DPPH, ABTS, TPTZ (2,4,6-tri-2-pyridinyl-1,3,5-triazine), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA), and methanol, ethanol, petroleum ether, ethyl

acetate, n-butanol, and other reagents for the antioxidant test were analytically pure and purchased from China National Pharmaceutical Group Co. (Beijing, China). Rutin (lot no. 100080–201805, HPLC $\geq 98\%$), astragaloside (lot no. 110781–201908, HPLC $\geq 98\%$), isoquercetin (lot no. 111809–201804, HPLC $\geq 98\%$), quercetin (lot no. 100081–201610, HPLC $\geq 98\%$), kaempferol (lot no. 110861–201703, HPLC $\geq 98\%$), and kaempferol-3-o-rutside (lot no. 112007–201903, HPLC $\geq 98\%$) standard reference materials were obtained from National Institutes for Food and Drug Control (Beijing, China). Chromatography grade and LC-MS grade acetonitrile were obtained from Tedia (Fairfield, OH, USA) and Fisher (Waltham, MA, USA), respectively.

The rhizome of *Tetrastigma hemsleyanum* Diels et Gilg from Guangxi Province, China, was purchased from Guangxi, China (lot no. 180615), and identified by Professor Xia Miao-fen at Zhejiang Pharmaceutical College.

2.2. Extraction, Fractionation, and Antioxidant Evaluation

The extracts were prepared according to the method of Bao et al. [48] and Yong [49] with minor modifications. The dried powder (5kg) of the rhizomes of *Tetrastigma hemsleyanum* Diels et Gilg was soaked in three volumes of 60% methanol at room temperature. Subsequently, the soaking solution was filtered, combined, and concentrated under reduced pressure; the crude extract (CE) (364 g) was prepared by vacuum drying. Then, it was suspended in water and extracted successively with petroleum ether, ethyl acetate, and n-butanol to obtain petroleum ether extract (PEE) (28 g), ethyl acetate extract (EAE) (25 g), and n-butanol extract (BE) (68 g), respectively. The antioxidant activities of each extract were measured using the DPPH assay, TEAC assay, ferric reducing antioxidant power (FRAP), and hydroxyl radical scavenging method to screen the extract with high antioxidant activity. The antioxidant components in this extract were detected by HPLC-DPPH and HPLC-hydroxyl radical scavenging methods. The molecular weight of the antioxidant components was deduced by HPLC-Q-TOF LC/MS. The extracts were solubilized in ethanol solution (80% v/v), except for PEE, which was dissolved in dimethyl sulfoxide (DMSO).

2.3. DPPH Assay

The DPPH free radical scavenging activity was assessed according to Leong et al. [50] with a slight improvement. Briefly, 3.0 mL of DPPH 80% ethanol solution (10^{-4} M) was mixed with 0.1 mL of the sample solution. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 517 nm using a UV-vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The radical scavenging activity (%) was calculated as follows: (1) Scavenging activity % = $1 - A_1/A_0 \times 100\%$, where A_1 is the absorbance of the sample, and A_0 is the absorbance of the blank group.

2.4. TEAC Assay

The antioxidant activity against ABTS⁺ was measured as described by Strail et al. [51] with some modifications. ABTS⁺ was formed by reacting 7.0 mM ABTS with 4.95 mM potassium persulfate solution at room temperature in the dark for 12 h. Then, the solution was diluted with 80% ethanol before measuring the absorbance (0.70 ± 0.02) at 734 nm. After incubation of 20 μ L sample solution (0.2–4 mg/mL) with 3.0 mL ABTS⁺ at 30°C for 10 min, the absorbance was measured at 734 nm by the UV-vis spectrophotometer. The antioxidant capacity was expressed as mg of Trolox/g of samples (TEAC), and the TEAC was calculated as follows: (2) $TEAC = (A_0 - A_1) / (b \times VW)$, where A_1 is the absorbance of the sample, A_0 is the absorbance of the blank group, b is the slope of the standard curve, a is the intercept of the standard curve, V is the volume of Trolox (μ L), and W is the weight of extracts (g).

2.5. FRAP

The antioxidant activity against Fe^{3+} -TPTZ was performed according to the method established by Benzie et al. [52] with some modifications. Fresh FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, and 20 mM $FeCl_3 \cdot 6H_2O$ solution at the ratio of 10:1:1. A volume of 3.0 mL FRAP reagent was added to 20 L sample solution (0.2–4 mg/mL) and incubated at 37°C for 30 min. The absorbance of the reaction mixture was measured at 593 nm. FRAP was expressed as the number of moles of $FeSO_4$ required to achieve the same absorbance per g of samples and calculated as follows: (3) $FRAP = (A_1 - A_0) / (b \times 1W)$, where A_1 is the absorbance of the sample, A_0 is the absorbance of the blank group, b is the slope of the standard curve, a is the intercept of the standard curve, and W is the weight of extracts (g).

2.6. Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging capacity was evaluated according to the method described by Jin et al. [53] with some modifications. Briefly, 1.0 mL phosphate-buffered saline (PBS, pH 7.4) was mixed with 0.5 mL (0.148 mg/mL) o-phenanthrene solution, 0.5 mL (0.208 mg/mL) ferrous sulfate solution, and 1.0 mL sample solution (0.2–4 mg/mL). After the mixture was vortexed, 1.0 mL (0.01%) hydrogen peroxide (H_2O_2) solution was added and incubated at 37°C for 60 min. The absorbance was measured at 536 nm. The hydroxyl radical scavenging capacity was calculated as follows: (4) Scavenging activity% = $\frac{A_1 - A_0}{A_2 - A_0} \times 100\%$, where A_1 is the absorbance of the sample, A_0 is the absorbance of the blank control group, and A_2 is the absorbance of the sample and reagent blank group.

2.7. HPLC Conditions

Herein, we used traditional methods to select the extract with high antioxidant activity, and the antioxidant components were screened using the HPLC-DPPH scavenging method and HPLC-hydroxyl radical scavenging method [54]. The chromatography selected optimal chromatographic conditions to separate the components in the extract. Then, the content of the sample components before and after the addition of the free radicals was detected using a UV detector. Next, the components with antioxidant capacity were selected by comparing the changes in the peak area or peak height of the corresponding components in the two chromatograms. Antioxidant components in the extract with high oxidation activity were screened by using an Agilent HPLC system with a photodiode-array detector (1260 series, Santa Clara, CA, USA). The chromatographic separation was carried out using a Shimadzu VP-ODS C18 (150 × 4.6 mm, 5 μm) with solvent A (0.1% formic acid) and solvent B (acetonitrile). The gradient condition was as follows: 0 min, 18% B; 0–5 min, 25% B; 5–10 min, 35% B; 10–15 min, 60% B; 15–20 min, 90% B; and 20–30 min, 18% B. The flow rate was 1.0 mL/min, the injection volume was 20 μL , the column temperature was set at 30°C, and the detection wavelength was 254 nm.

2.8. HPLC-DPPH Scavenging Method

The majority of the phenolic antioxidants can quench DPPH radicals, and hence, DPPH radicals are often used to screen the antioxidant activities of phenolic compounds [55]. In this study, we used the HPLC-DPPH scavenging method to determine the peak area of each component in the sample before and after adding DPPH. The components of the reduced peak area harbored antioxidant activity. Briefly, 250 μL sample solution (4 mg/L) and 250 μL DPPH solution (500 μM) were mixed and incubated at 37°C in the dark for 30 min. The mixture was then filtered through a 0.45 μm membrane and determined according to the chromatographic conditions in 2.2.5.

2.9. HPLC-Hydroxyl Radical Scavenging Method

Briefly, 200 μL H_2O_2 solution (25 mM) and 200 μL of FeSO_4 solution (250 μM) were added to 250 μL sample solution (4 mg/L). The mixture was incubated at room temperature in the dark for 7 min, filtered with 0.45 μm membrane, and measured according to the chromatographic conditions in 2.2.5.

2.10. Identification of Active Antioxidant Ingredients

The identification experiment was carried out using an Agilent HPLC-Q-TOF LC/MS (1260 series, 6520 series) and an Agilent HPLC system with a photodiode array detector (1260 series). The chromatographic separation was conducted using a Extend Agilent C18 (50 × 2.1 mm, 1.8 μm) with solvent A (0.1% formic acid) and solvent B (acetonitrile). The gradient condition was as follows: 0 min, 18% B; 0–5 min, 25% B; 5–10 min, 35% B; 10–15 min, 60% B; and 15–20 min, 90% B. The flow rate was 0.2 mL/min, injection volume was 1 μL , the column temperature was room temperature, the scanning range was 50–1500, and the capillary voltage was 4000 V. Nitrogen was used as dry gas at a flow rate of 10 mL/min, the temperature was 350°C, and the atomizer pressure was 40 psi. The mass spectrogram and chromatogram were recorded under the negative ion source. After acquiring the molecular weight, the antioxidant components in the sample were further identified based on their standard products by HPLC, according to the chromatographic conditions in 2.2.5.

2.11. Statistical Analysis

All experimental data were expressed as mean ± standard deviation (SD) of three independent experiments in triplicate and analyzed by SPSS (version 17.0). One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test any significant differences between the means. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Screening of Antioxidant Extracts

3.1.1. Comparison of DPPH Free Radical Scavenging Capacity of Extracts

DPPH has been widely used in the determination of antioxidant free radical scavenging capacity [56]. The scavenging rate of each extract is shown in Figure 1. Subsequently, the DPPH scavenging activity of EAE and CE was similar to the sample concentrations of 0.2 mg/mL ($P>0.05$) and 4 mg/mL ($P>0.05$), respectively, while DPPH scavenging activity of EAE and all the other extracts is the largest difference at the sample concentration of 1 mg/mL ($P<0.05$). The DPPH scavenging activity of EAE was dose-dependent in the concentration range of 0.2–4 mg/mL and occurred in the following order: EAE>CE>BE>PEE (Figure 2).

[figure omitted; refer to PDF]

3.1.2. Comparison of ABTS+ Free Radical Scavenging Capacity of Extracts

The TEAC values of different concentrations of each extract are shown in Figure 2. In addition to PPE, the other extracts showed a robust ability to scavenge ABTS+ radicals. However, no significant difference was detected in the TEAC values between EAE and CE at the sample concentrations of 0.2, 0.5, and 1 mg/mL ($P>0.05$). On the other hand, in the sample concentration range of 0.2–4 mg/mL, the TEAC values of EAE were 150.78 ± 20.94 , 241.72 ± 30.34 , 281.36 ± 3.17 , 265.54 ± 7.79 , and 207.59 ± 14.45 mg Trolox/g extract, respectively. The TEAC value was not dose-dependent because the weight of the extract (W) varied in the five different concentrations of each extract. If calculated according to formula (1), the antioxidant capacity was dose-dependent and expressed in terms of scavenging activity.

3.1.3. Comparison of the Reducing Power of Extracts

In this experiment, the reduction ability of CE and EAE was dissimilar ($P<0.05$). At different concentrations, the reduced ability of EAE was significantly higher than that of other extracts, indicating a high reduction ability of Fe^{3+} . In the concentration range of 0.2–4 mg/mL, the FRAP values of EAE were 0.74 ± 0.06 , 1.12 ± 0.04 , 1.02 ± 0.04 , 0.71 ± 0.01 , and 0.68 ± 0.00 mM FeSO_4 /g extract, respectively (Figure 3).

[figure omitted; refer to PDF]

3.1.4. Comparison of Hydroxyl Radicals Scavenging Capacity of Extracts

Figure 4 shows the highest clearance rate of EAE, although the clearance rates of CE of 1 and 2 mg/mL concentrations are close to that of EAE ($P>0.05$). At 0.2, 0.5, and 0.4 mg/mL, the clearance rate of EAE was significantly different from that of other extracts ($P<0.05$). In the concentration range of 0.2–4 mg/mL, the scavenging activity of hydroxyl radicals in EAE was $32.69\pm3.66\%$, $59.73\pm2.76\%$, $56.80\pm1.69\%$, $51.28\pm3.47\%$, and $47.60\pm1.63\%$, respectively.

[figure omitted; refer to PDF]

3.2. Screening Antioxidant Components in the Extract with High Antioxidant Activity by HPLC

The present study demonstrated that the EAE had the strongest antioxidant activity among the four extracts. Then, we used HPLC to analyze and screen the antioxidant components by HPLC-DPPH and HPLC-hydroxyl radical scavenging methods.

When no reagent was added to the sample solution, nine chromatographic peaks were obtained (Figure 5(a)). After the DPPH solution (Figure 5(b)) or hydroxyl radical reagent (Figure 5(c)) was added to the sample solution, distinct changes were detected in the area and height of the nine peaks. Compared to the chromatograms of the sample before and after the reaction, the amount of free radicals and the antioxidant content decreased with the progress in the antioxidant reaction (Figure 5). A total of nine components were identified as peaks.

[figures omitted; refer to PDF]

3.3. Identifying Antioxidant Components in the Extract with High Antioxidant Activity

The response of each component in the EAE extract to the negative ion source is better than that of the positive ion source, which is the same as reported previously [57, 58]. Based on the total ion chromatogram (Figure 6) and six-component mass spectrogram (Figure 7) under the negative ion detection mode and the molecular weight and molecular structure (Table 1) analyzed by the data of the workstation, we speculated the potential compounds and

compared them with the corresponding standards by HPLC. Finally, we identified six compounds, namely, rutin, isoquercetin, astragaloside, quercetin, kaempferol, and kaempferol-3-o-rutside (Figure 8).

[figure omitted; refer to PDF]

[figures omitted; refer to PDF]

Table 1

Molecular weight and formula of six chemical components in EAE (4 mg/mL).

No.	tR/min	Molecular formula	Measured value (<i>m/z</i>)	Theoretical value (<i>m/z</i>)	Relative error (ppm)
1	14.393	$C_{27}H_{30}O_{16}$	610.1494	610.1473	2.31
2	14.682	$C_{21}H_{19}O_{12}$	464.0955	464.0944	2.24
3	15.181	$C_{27}H_{29}O_{15}$	594.1585	594.1576	1.41
4	15.422	$C_{21}H_{19}O_{11}$	448.1006	448.0996	2.19
5	17.160	$C_{15}H_9O_7$	302.0427	302.0421	1.82
6	18.077	$C_{15}H_9O_6$	286.0477	286.0477	0.09

[figures omitted; refer to PDF]

4. Discussion

The current results are different from those of Sun et al. that 80% methanol extract of *Tetragium hemsleyanum* had better scavenging ability to DPPH and ABTS+ free radicals than ethyl acetate extract and hexane extract [49], which could be attributed to different extraction methods, origins of the herb, and concentrations of the samples. In this study, the total antioxidant capacity of CE was not equivalent to that of EAE, although the trend of CE in scavenging DPPH radicals, ABTS+ radicals, hydroxyl radicals, and reducing Fe^{3+} was consistent with that of EAE (Figures 1–4). This might be ascribed to the lower content of flavonoids in CE than that in EAE.

Figure 5 shows that the content of peaks 1, 2, and 3 also decreased; however, the molecular weight was not determined by HPLC-Q-TOF LC/MS. Furthermore, phenolic acids, such as catechin, epicatechin, gallic acid, procyanidin B₁, and procyanidin B₂ [59, 60], with strong antioxidant activity, have not been identified in EAE, CE, PEE, and BE, which might be due to the extraction process or the low content of phenolic acids in the extracts. The antioxidative activity of EAE in vivo and the distribution and metabolism of each antioxidant component in the organisms are not clear, and the mechanism of EAE underlying the antioxidative effect remains to be elucidated. The six identified compounds were flavonoids and polyhydroxy compounds, and their antioxidant activity has been reported. Among these, rutin is a strong antioxidant [57], and its antioxidant activity is based on three mechanisms: the o-dihydroxy structure in B ring, the 2,3 double bond of the 4-oxo-functional group coupling in C ring, and the 5-OH and 7-OH groups of the 4-oxo-functional group in A and C rings. Rutin alleviates early brain injury of rats with subarachnoid hemorrhage by antioxidation and antiapoptosis [58]. Quercetin inhibits the activities of acetylcholinesterase and butyryl cholinesterase and Fe^{2+} induces lipid peroxidation in the brain homogenates of the rat, which are the putative mechanisms that underlie the quercetin-mediated management of oxidative stress-induced neurodegeneration [61]. Isoquercetin, a potent antioxidative compound, has neuroprotective capacities that are beneficial for the treatment of ischemic stroke and related diseases [62]. Astragalin, a major flavonoid, is a potential beneficial agent that protects the diabetic-induced spermatogenic dysfunction in male mice by increasing the antioxidant enzyme activities and inhibiting inflammation [63, 64]. Kaempferol and kaempferol-3-o-rutside increases the DPPH and ABTS free radical scavenging activity and inhibits the T cell proliferation induced by

concanavalin A and nitric oxide (NO) or reactive oxygen species (ROS) production in LPS-induced 264.7 macrophages cells [65].

5. Conclusions

In this study, for the first time, we used in vitro traditional antioxidant tests (DPPH assay, TEAC assay, FRAP, and hydroxyl radical scavenging assay) combined with HPLC-DPPH and HPLC-hydroxyl radical scavenging methods to screen out EAE with high antioxidant activity from *Tetrastigma hemsleyanum* and identified six antioxidant components by Q-TOF LC/MS and HPLC. The EAE may be used in food, medicine, and other fields to prevent the disease and assist the treatment of some oxidative stress-related diseases as these six compounds have significant biological activities. However, further studies are needed on the correlation between the content of each antioxidant component in EAE and the antioxidant capacity of EAE and the antioxidant mechanism.

Acknowledgments

This work was supported by the Ningbo Natural Science Foundation (2018A610244) and the science and technology benefiting people technology R &D project of Ningbo Science and Technology Bureau (2015C50063).

Glossary

Abbreviations

DPPH:1,1-Diphenyl-2-picrylhydrazyl radical, 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl

TEAC:Trolox equivalent antioxidant capacity

FRAP:Ferric reducing antioxidant power

ABTS:2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

HPLC:High-performance liquid chromatography

HPLC-Q-TOF LC/MS:High-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry

EAE:The ethyl acetate extract

CE:The crude extract

PEE:Petroleum ether extract

BE:n-Butanol extract

BHT:Dibutyl hydroxytoluene

BHA:Butyl hydroxyanisole

PG:Propyl gallate

TPTZ:2,4,6-Tri-2-pyridinyl-1,3,5-triazine

Trolox:6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

References

- [1] S. B. Liao, W. W. Cai, D. Chen, P. Xie, J. Huang, Z. X. Mu, "Anti-inflammatory and analgesic effects of the extracts of *Tetrastigma hemsleyanum* 's aerial parts from Fujian in vivo," Chinese Journal of Modern Applied Pharmacy, vol. 34 no. 2, pp. 319-324, 2017.
- [2] W. Dan-Dan, G. Rong, Y. Bin, "Experimental study on the effects of Radix *Tetrastigmae* on respiratory syncytial virus," Natural Product Research and Development, vol. 31, pp. 1070-1074, 2019.
- [3] X. M. Zhong, Q. Q. Mao, Z. Huang, "Experimental study on the protective effect and acute toxicity of *Tetrastigma hemsleyanum* extract on CCl₄ induced acute liver injury in mice," Chinese Traditional Patent Medicine, vol. 28 no. 3, pp. 422-424, 2006.
- [4] L. I. Ping, J. I. Weiwei, P. Xin, D. O. Pathology, "Study on the trifolium extract in anit-induced liver injury in mice," China Modern Doctor, vol. 56 no. 30, pp. 32-35, 2018.
- [5] H. Y. Wu, Y. T. Wu, Y. Y. Chen, H. L. Luo, H. F. Jiang, "Effect of Radix *Tetrastigma hemsleyani* flavones on apoptosis and MAPK signaling pathway in myeloid leukemia NB-4 cells," Chinese Journal of Pathophysiology, vol. 35 no. 8, pp. 1451-1456, 2019.
- [6] T. Hu, Z. Q. Feng, L. R. Zhong, "Effect of flavonoids from *Tetrastigma hemsleyani* Diels et Gilg on myeloid derived suppressive cells in mice bearing Lewis lung cancer," Chinese Journal of Integrated Traditional and Western

Medicine, vol. 38 no. 10, pp. 1229-1233, 2018.

[7] J. N. M. College, Dictionary of Traditional Chinese Medicine, 1977.

[8] X. L. Wang, "Influence of the water extract of Radix Tetrastigmae on IL-23 and IL-17 in rats of rats of chronic obstructive pulmonary disease," Journal of Practical Traditional Chinese Medicine, vol. 32 no. 9, pp. 848-850, 2016.

[9] D. D. Liu, G. Cao, Q. Zhang, Y. L. Ye, L. K. Han, W. H. Ge, "Inhibition effect of flavonoids from Radix tetrastigmae on acute lung injury of aged mice through p38MAPK and NF- κ B pathway," Chinese Pharmacological Bulletin, vol. 12, pp. 1725-1729, 2015.

[10] D. Liu, J. H. Ju, J. S. Yang, "Studies on chemical constituents form Tetrastigma hypoglaucum," Chinese Journal of traditional Chinese Medicine, vol. 34 no. 1, 2004.

[11] D. Liu, J. S. Yang, "A Study on chemical components of Tetrastigma hypoglaucum Diels et Gilg. native to China," Chinese Traditional and Herbal Drugs, vol. 24 no. 10, pp. 611-612, 1999.

[12] Y. J. Hu, L. Cheng, J. B. Pu, "GC-MS analysis of petroleum ether extract from Tetrastigma hemsleyanum," Chinese Traditional Medicine Science and Technology, vol. 20 no. 1, pp. 46-47, 2013.

[13] Y. Q. Li, W. C. Lu, Y. Z. Guo, "Studies on the chemical constituents of Tetrastigma hemsleyanum Diels et Gilg," Chinese Traditional and Herbal Drugs, vol. 34 no. 11, pp. 982-983, 2003.

[14] F. Shi-Ming, X. Xin-Yue, Z. Fan-Tian, Z. Xiao-Hong, C. Bi-Ya, X. U. Wen, "Identification of chemical components and determination of flavonoids in Tetrastigma hemsleyanum leaves," Chinese Journal of Pharmaceutical Analysis, vol. 37 no. 8, pp. 1481-1486, 2017.

[15] M. L. Zeng, N. T. Shen, S. W. Wu, Q. Li, "Analysis on chemical constituents in Tetrastigma hemsleyanum by UPLC-Triple-T O F/MS," Chinese Traditional and Herbal Drugs, vol. 4 no. 5, pp. 874-883, 2017.

[16] Y. Jiang, Q. Zheng, A. Zhang, L. Cui, L. Xia, M. Luo, "Flavone from Zhongjiefeng (Herba Sarcandrae Glabrae) inhibits platelet apoptosis in immune-induced bone marrow failure through mitochondrial pathway," Journal of traditional Chinese medicine=Chung i tsa chih ying wen pan, vol. 37 no. 5, pp. 643-649, 2017.

[17] C. L. Peng, S. M. Zhang, Y. Zhu, "Differential inhibition effects of ginkgo flavones and ginkgolide on the proliferation of vascular smooth muscle cell and the mechanism," Central South Pharmacy, vol. 15 no. 10, pp. 1351-1356, 2017.

[18] L. Lu, J. Mi, Q. Luo, "Optimization of extraction process of flavonoids from lycium barbarum L. var. Auranticarpum K. F. Ching and its antioxidant activities in vitro," Science and Technology of Food Industry, vol. 40 no. 24, pp. 165-171, 2019.

[19] W. Yan-Bo, S. Yan, Y. Yi-Jun, "Ultrasonic assisted extraction of polyphenols and flavonoids from Maijishan wild acanthopanax and their antioxidant activities in vitro," Natural Product Research and Development, vol. 31 no. 12, pp. 2153-2162, 2019.

[20] J. J. Diao, R. A. Cao, C. Y. Li, "Antioxidant activity and structure of flavonoid compounds extracted from raspberry root," Chinese Journal of Biologicals, vol. 32 no. 12, pp. 1350-1357, 2019.

[21] H. Jin, J. Yu, Z. Y. Wang, "Mechanism of flavonoids of sophorae fructus in inhibiting proliferation, migration and invasion of hepatocellular carcinoma cells by regulating LncRNA FBXL 19-AS1/miR-342-3p pathway," China Journal of Chinese Materia Medica, vol. 10, 2020.

[22] Y. S. Qi, X. Li, X. M. Qin, "Anti- cancer effect of the flavonoids of Astragalus combined with cisplatin on lewis lung carcinoma-bearing mice," Acta Pharmaceutica Sinica, vol. 8, 2020.

[23] L. H. Zhang, T. T. Wu, L. G. Zhao, X. Wei, S. H. Zhang, C. X. Wang, "Advances in studies anticancer activity of flavonoids from Ginkgo biloba extract," Chinese Pharmaceutical Journal, vol. 54 no. 6, pp. 444-449, 2019.

[24] G. G. Lv, Z. K. Qiu, S. Z. Chang, H. Lin, J. Lu, D. D. Ye, "Anti-inflammatory, analgesic and analgesic effects of total flavone from Stigma maydis and its effect on acute gouty arthritis," Drug Evaluation Research, vol. 41 no. 2, pp. 206-209, 2018.

[25] A. S. Mecnas, C. R. Adão Malafaia, L. S. Sangerito, D. L. R. Simas, T. B. Machado, A. C. F. Amaral, A. L. S. Dos Santos, D. M. G. Freire, I. C. R. Leal, "Rutin derivatives obtained by transesterification reactions catalyzed by Novozym 435: antioxidant properties and absence of toxicity in mammalian cells," PLoS One, vol. 13 no. 9, DOI:

10.1371/journal.pone.0203159, 2018.

- [26] L. Živković, V. Bajić, M. Bruić, S. Borozan, K. Popić, D Topalović, J Santibanez, B Spremo-Potporević, "Antigenotoxic and antioxidant potential of medicinal mushrooms (Immune Assist) against DNA damage induced by free radicals-an in vitro study," *Mutation Research*, vol. 845, DOI: 10.1016/j.mrgentox.2019.06.008, 2019.
- [27] R. Rohman, R. Kar, "How does the presence of an oxyradical influence the behavior of polyphenolic antioxidant? A case study on gallic acid," *Journal of Molecular Modeling*, vol. 24 no. 7, DOI: 10.1007/s00894-018-3701-0, 2018.
- [28] Y. Sun, K. Yang, Q. Cao, J. Sun, Y. Xia, Y. Wang, W. Li, C. Ma, S. Liu, "Homogenate-assisted vacuum-powered bubble extraction of moso bamboo flavonoids for on-line scavenging free radical capacity analysis," *Molecules*, vol. 22 no. 7, DOI: 10.3390/molecules22071156, 2017.
- [29] P. Xiao, H. Huang, X. Li, J. Chen, J.-a. Duan, "Characterization, evaluation of nutritional parameters of *Radix isatidis* protein and its antioxidant activity in D-galactose induced ageing mice," *BMC Complementary and Alternative Medicine*, vol. 19 no. 1, DOI: 10.1186/s12906-019-2726-y, 2019.
- [30] M. D. Mira-Sánchez, J. Castillo-Sánchez, J. M. Morillas-Ruiz, "Comparative study of rosemary extracts and several synthetic and natural food antioxidants. Relevance of carnosic acid/carnosol ratio," *Food Chemistry*, vol. 309, 2020.
- [31] A. M. Mahmoud, F. L. Wilkinson, M. A. Sandhu, J. M. Dos Santos, M. Y. Alexander, "Modulating oxidative stress in drug-induced injury and metabolic disorders: the role of natural and synthetic antioxidants," *Oxidative Medicine and Cellular Longevity*, vol. 2019, DOI: 10.1155/2019/3206401, 2019.
- [32] I.-C. Sheih, T.-K. Wu, T. J. Fang, "Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems," *Bioresource Technology*, vol. 100 no. 13, pp. 3419-3425, DOI: 10.1016/j.biortech.2009.02.014, 2009.
- [33] N. Chen, H. Yang, Y. Sun, J. Niu, S. Liu, "Purification and identification of antioxidant peptides from walnut (*Juglans regia* L.) protein hydrolysates," *Peptides*, vol. 38 no. 2, pp. 344-349, DOI: 10.1016/j.peptides.2012.09.017, 2012.
- [34] B. L. Zhao, *Oxygen Free Radicals and Natural Antioxidants*, 1999.
- [35] Z. Li, H. Caiyun, R. Fazheng, "Anti-obesity efficacy of extracts from several kinds of tea in high-fat diet-induced obese mice," *Food Science*, vol. 39 no. 17, pp. 192-198, 2018.
- [36] L. I. Yan-Hui, L. Miao-Ling, L. Si-Hui, S. U. Han-Tao, S. Hai-Yan, "Effects of tea extracts of different fermentations on proliferation of colorectal cancer cell line HCT116," *Guangdong Agricultural Sciences*, vol. 45 no. 9, pp. 103-108, 2018.
- [37] L. Liu, L. Yu, M. Zhou, "Intervention study on that effect of grape seed extract proanthocyanidins on blood glucose and blood lipid in the patients with type 2 diabetes mellitus," *International Journal of Laboratory Medicine*, vol. 40 no. 15, pp. 1844-1849, 2019.
- [38] L. Zhang, S. Lei, "Protective effects and mechanism of *Momordica Charantia* extract on RGCS of type 2 diabetic rats," *Chinese Journal of Coal Industry Medicine*, vol. 23 no. 1, pp. 11-15, 2020.
- [39] H. Wang, Q. Chi, S. L. Xiong, C. F. Zeng, L. Wan, "Effects of *Lonicera japonica* extract on lung inflammation in LPS-induced ARDS rats," *Journal of Guangdong Pharmaceutical University*, vol. 33 no. 3, pp. 379-382, 2017.
- [40] X. Zhang, M. Y. Shi, M. Sun, Y. B. Zhang, Y. L. Zhang, L. J. Liu, "Influences of *Shengdi Huang* (*Rehmannia recens* Radix) extract on pulmonary index and monocyte chemoattractant protein-1 mRNA expression of pulmonary interstitial fibrosis rats," *LiShiZhen Medicine and Materia Medica Research*, vol. 29 no. 12, pp. 2884-2886, 2018.
- [41] X. Y. Tang, Y. L. Zhao, "Study on the effect of garlic extracts on preservation and antioxidant activity of chilled meat," *Meat Industry*, vol. 37 no. 7, pp. 435-438, 2017.
- [42] N. Huda-Faujan, A. Noriham, A. S. Norrakiah, A. S. Babji, "Antioxidant activity of plants methanolic extracts containing phenolic compounds," *African Journal of Biotechnology*, vol. 8, 2009.
- [43] W. Khan, S. Subhan, D. F. Shams, S. G. Afridi, R. Ullah, A. A. Shahat, A. S. Alqahtani, "Antioxidant potential, phytochemicals composition, and metal contents of *Datura alba*," *BioMed Research International*, vol. 2019, DOI:

10.1155/2019/2403718, 2019.

[44] L. I. Meng-Ru, Z. Yu-Zhi, D. U. Guan-Hua, Q. Xue-Mei, "Research progress about the anti-aging effect and mechanism of flavonoids from traditional Chinese medicine," *Acta Pharmaceutica Sinica*, vol. 54 no. 8, pp. 1382-1391, 2019.

[45] G. L. Wang, J. K. Li, X. Wu, K. Wang, "Extraction and purification of *Debregeasia orientalis* fruit polyphenols and its antioxidant, antitumor activity," *Natural Product Research and Development*, vol. 31 no. 1, 2019.

[46] L. Ding, J. Q. Xiong, "Study preliminary on the anti-tumor effect of the extract of *Tetrastigma hemsleyani*," *Strait Pharmaceutical Journal*, vol. 23 no. 12, pp. 46-48, 2011.

[47] L. Ding, L. X. Zhang, Y. Qiu, Y. H. Wang, "Chemical constituents in chloroform extraction of *Tetrastigma hemsleyani* Diels et. Gilg and their antitumor activities," *Chinese Pharmaceutical Journal*, vol. 50 no. 21, pp. 1857-1860, 2016.

[48] L. Bao, X. F. Ma, X. H. Song, "Two new resveratrol tetramers isolated from *Cayratia japonica* (thunb.) gagn. With strong inhibitory activity on fatty acid synthase and antioxidant activity," *Chemistry and Biodiversity*, vol. 7 no. 12, pp. 2931-2940, 2011.

[49] S. Yong, Chemical composition, activities of anti-oxidation and anticancer of *Terastigma hemsleyanum*, 2018.

[50] G. Shui, "An investigation of antioxidant capacity of fruits in Singapore markets," *Food Chemistry*, vol. 76 no. 60-75, 2002.

[51] P. Stratil, B. Klejdus, V. Kubáň, "Determination of total content of phenolic compounds and their antioxidant activity in vegetables evaluation of spectrophotometric methods," *Journal of Agricultural and Food Chemistry*, vol. 54 no. 3, pp. 607-616, DOI: 10.1021/jf052334j, 2006.

[52] I. F. F. Benzie, J. J. Strain, "The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay," *Analytical Biochemistry*, vol. 239 no. 1, pp. 70-76, DOI: 10.1006/abio.1996.0292, 1996.

[53] M. Jin, Y. X. Cai, J. R. Li, H. Zhao, "1, 10-Phenanthroline-Fe²⁺ oxidative assay of hydroxyl radical produced by H₂O₂/Fe²⁺," *Progress in Biochemistry and Biophysics*, vol. 23 no. 6, pp. 553-555, 1996.

[54] J. D. Fu, New Method for Rapid Screening of Antioxidants from Nature Products, pp. 14-54, 2010.

[55] W. M. Brand-Williams, M. E. Cuvelier, C. L. W. T. Berset, "Use of a free radical method to evaluate antioxidant activity," *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, vol. 28 no. 1, DOI: 10.1016/s0023-6438(95)80008-5, 1995.

[56] X. Duan, Y. Jiang, X. Su, Z. Zhang, J. Shi, "Antioxidant properties of anthocyanins extracted from litchi (*Litchi chinensis* Sonn.) fruit pericarp tissues in relation to their role in the pericarp browning," *Food Chemistry*, vol. 101 no. 4, pp. 1365-1371, 2007.

[57] H. T. H. Vu, S. M. Hook, S. D. Siqueira, M. Anette, R. Thomas, M. D. Arlene, "Are phytosomes a superior nanodelivery system for the antioxidant rutin?," *International Journal of Pharmaceutics*, vol. 548, DOI: 10.1016/j.ijpharm.2018.06.042, 2018.

[58] Y. W. Han, C. C. Wang, L. X. Ming, "Protective effect of rutin on early brain injury in mice with subarachnoid hemorrhage by anti-oxidation and anti-apoptosis," *Drugs & Clinic*, vol. 35 no. 3, pp. 421-425, 2020.

[59] W. Xu, Z. Q. Fu, J. Lin, X. C. Huang, S. M. Fan, "Qualitative and quantitative analysis of major constituents in *Tetrastigma hemsleyanum* by HPLC-Q-T O F-MS and UPLC-QqQ-MS," *Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica*, vol. 39 no. 22, pp. 4365-4372, 2014.

[60] X. U. Wen, F. U. Zhi-Qin, L. Jing, H. Xue-Cheng, Y. U. Hong-Min, H. Ze-Hao, "Rapid simultaneous determination of ten major flavonoids in *Tetrastigma hemsleyanum* by UPLC-MS/MS," *Yao xue xue bao = Acta pharmaceutica Sinica*, vol. 49 no. 12, pp. 1711-1717, 2014.

[61] A. O. Ademosun, G. Oboh, F. Bello, P. O. Ayeni, "Antioxidative properties and effect of quercetin and its glycosylated form (rutin) on acetylcholinesterase and butyrylcholinesterase activities," *Journal of Evidence-Based Complementary and Alternative Medicine*, vol. 21, DOI: 10.1177/2156587215610032, 2016.

[62] C.-P. Wang, Y.-W. Shi, M. Tang, X.-C. Zhang, Y. Gu, X.-M. Liang, Z.-W. Wang, F. Ding, "Isoquercetin ameliorates cerebral impairment in focal ischemia through anti-oxidative, anti-inflammatory, and anti-apoptotic

effects in primary culture of rat hippocampal neurons and hippocampal CA1 region of rats," *Molecular Neurobiology*, vol. 54 no. 3, pp. 2126-2142, DOI: 10.1007/s12035-016-9806-5, 2017.

[63] B. H. Xia, Y. M. Zhou, S. L. Pi, et al, "Determination of antioxidant activity of isoquercetin, rutin and astragaloside in mulberry leaves by UPLC," *Journal of Chinese Medicinal Materials*, vol. 39 no. 3, pp. 586-589, 2016.

[64] S. O. Abarikwu, A. B. Pant, E. O. Farombi, "Dietary antioxidant, quercetin, protects sertoli-germ cell coculture from atrazine-induced oxidative damage," *Journal of Biochemical and Molecular Toxicology*, vol. 26 no. 11, pp. 477-485, DOI: 10.1002/jbt.21449, 2012.

[65] W. Jingqiu, F. Xianying, G. Lin, C. Fuliang, Z. Linguo, W. Zhenzhong, "Antitumor, antioxidant and anti-inflammatory activities of kaempferol and its corresponding glycosides and the enzymatic preparation of kaempferol," *PLoS One*, vol. 13 no. 5, 2018.

DETAILS

Subject:	Mass spectrometry; Butanol; Acids; Reagents; Hydrogen peroxide; Free radicals; Crude oil; Antioxidants; Chromatography; Ethyl acetate; Ethanol; Analgesics; Sulfonic acid; Scavenging; Qualitative analysis; Hydroxyl radicals; Quadrupoles; Assaying; Aging; Flavonoids; Molecular weight; High performance liquid chromatography
Location:	Beijing China; United States--US; China
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	

Milestone dates: 2021-07-17 (Received); 2021-11-02 (Accepted); 2021-11-23 (Pub)

DOI: <https://doi.org/10.1155/2021/7195125>

ProQuest document ID: 2606666610

Document URL: <https://www.proquest.com/scholarly-journals/antioxidant-activity-vitro-guided-screening/docview/2606666610/se-2?accountid=211160>

Copyright: Copyright © 2021 Li Ding et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-12-05

Database: Publicly Available Content Database

Document 8 of 59

Simultaneous Determination of Amphenicols and Metabolites in Animal-Derived Foods Using Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry

Wu, Xinyi; Shen, Xixi; Cao, Xiangyue; Nie, Rongrong; Zhang, Haonan; Tang, Changbo; Wang, Wei

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Amphenicols are widely used to prevent and treat animal diseases. However, amphenicol residues accumulate in livestock and poultry and harm consumers. We hypothesized that one can combine solid-phase extraction (SPE) with ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to simultaneously determine amphenicols and metabolites in pork, beef, lamb, chicken, and their products and meet government regulations for maximum residue limits. We extracted crude samples with ethyl acetate and ammonia water (98:2, v/v), purified the samples with a CNW Si SPE column, defatted the samples with acetonitrile-saturated *n*-hexane, and then determined the resulting analytes by UHPLC-MS/MS. The limit of detection of the analytes in livestock and poultry meat was 0.03–1.50 µg/kg, and the limit of quantification was 0.05–5.00 µg/kg. Measured chloramphenicol, thiamphenicol, and florfenicol concentrations were linear over the range 0.50–50 µg/kg; and the florfenicol amine concentration was linear over the range 5.00–200 µg/kg (all with correlation coefficients >0.9990). The recovery of the spiked samples was between 72% and 120%. The intraday relative standard deviation (RSD) ranged from 1% to 9%, and the interday RSD ranged from 1% to 12%. Based on the above results, the current method is sensitive,

accurate, and reproducible with the detection limits being well below the maximum residue limits as per Chinese standard GB 31650-2019, and thus, our research hypothesis could be confirmed.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

China produces and consumes a large quantity of livestock and poultry meat; corresponding quality and safety problems harm consumer health and create barriers to the import and export trade [1]. Among these quality and safety problems, veterinary drug residues are especially pertinent. Chloramphenicol (CAP), thiamphenicol (TAP), and florfenicol (FF) are amphenicols, substances that are widely used in livestock and poultry breeding and production. These substances have broad-spectrum antibacterial properties, are low cost, and have high potency [2–4]. However, CAP is highly toxic to the human hematopoietic system and can cause side effects such as aplastic anemia [5, 6]. As a derivative of CAP, TAP replaces the *para*-nitro group of CAP with a methanesulfonyl group and is less toxic than CAP but can inhibit the production of red blood cells, white blood cells, and platelets [7, 8]. FF is synthesised from TAP by replacing the 3-hydroxy group with fluorine; it is an animal-specific amphenicol and has higher antibacterial activity than CAP and TAP but has embryo toxicity [9, 10]. FF is metabolized to florfenicol amine (FFA) via bioconversion pathways in treated animals (Figure 1) [11]. Therefore, to regulate the use of toxic amphenicols and reduce their prevalence in livestock and poultry products, the use of amphenicols is strictly restricted by laws and regulations. Currently, China, the United States, the European Union, Japan, and other countries have banned the use of CAP in animal feeds. As shown in Table 1, regulations in China, the United States (US), and the European Union (EU) have stipulated that the maximum residue limits (MRLs) of TAP in animal-derived foods such as the muscle, fat, liver, kidney, and milk are 50 µg/kg; and the MRLs of FF (calculated as the sum of both FF and FFA) in the muscle, liver, kidney, skin, and fat are 100–3700 µg/kg [12–17]. TAP is not recommended for animals from which eggs are produced for human consumption, and FF is not recommended for animals from which milk and eggs are produced for human consumption in China and the EU

[figure omitted; refer to PDF]

Table 1

Comparison of the MRLs for amphenicols in China, the US, and the EU.

Analyte	Species	Target tissue	MRL (µg/kg)		
China	US	EU	TAP	All species	All tissues
50		50	-		
FF (sum of FF and FFA)	Porcine	Muscle	300	200	300
Skin and fat	500		500	Liver	2000
2500	2000	Kidney	500		500
Bovine	Muscle	200	300	200	Liver
3000	3700	3000	Kidney	300	

300	Ovine caprine	Muscle	200		200
Liver	3000		3000	Kidney	300
	300	Poultry	Muscle	100	
100	Skin and fat	200		200	Liver
2500		2500	Kidney	750	

Various approaches for the determination of amphenicols in animal-derived foods are available in the literature and apply detection systems such as biosensors [18–21], enzyme-linked immunosorbent assays [22, 23], high-performance liquid chromatography (HPLC) [24–26], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [27–30], gas chromatography (GC) [31], and GC-MS/MS [32, 33]. Among them, LC-MS/MS is the main detection and positive confirmation method for amphenicols. Ultrahigh-performance liquid chromatography (UHPLC)-MS/MS is an LC-MS/MS technology that is more suitable than conventional LC-MS/MS for the analysis and detection of amphenicols because of a short analysis time, higher separation efficiency, and higher sensitivity. Currently, several analytical methods have been reported for the analysis of some of amphenicols and metabolites [28, 30, 34, 35]. In addition, these methods are usually developed for one or two matrices [7, 36, 37]. To our knowledge, there are few reports on simultaneously detecting the entire family of amphenicols and metabolites in animal-derived foods. In this study, we combined solid-phase extraction (SPE) with UHPLC-MS/MS in both negative and positive electrospray ionization (ESI) mode for simultaneous determination of CAP, TAP, FF, and FFA in pork, beef, lamb, chicken, and their products under the optimal experimental conditions. Our goal is to promote the development of international trade in animal-derived foods, provide references for quality and safety monitoring, and standardize supervision and testing methods of animal-derived foods.

2. Materials and Methods

2.1. Chemicals and Reagents

CAP, TAP, FF, FFA, and CAP-D5 standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). FFA-D3 standard was obtained from Toronto Research Chemicals (Toronto, ON, Canada). HPLC-grade acetonitrile (ACN), methanol, ethyl acetate, and *n*-hexane were supplied by Merck (Darmstadt, Germany). Reagent grade ammonium hydroxide, HPLC-grade acetone, and formic acid were supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). Water was purified with an Arium Pro ultrapure water purification system (Sartorius, Göttingen, Germany).

2.2. Preparation of Standard Stock and Working Solutions

Standard stock solutions of CAP, TAP, FF, FFA, CAP-D5, and FFA-D3 were prepared at a concentration of 1000 μ g/mL by dissolving each analyte in methanol. All the aforementioned standard stock solutions were stored at -20°C for up to 6 mo.

Mixed standard working solutions of CAP, TAP, FF, and FFA at a concentration of 10 μ g/mL were prepared by diluting the standard stock solutions with methanol. An internal standard working solution at a concentration of 1.0 μ g/mL was prepared by diluting the internal standard stock solutions with methanol. The aforementioned standard working solutions were stored at -20°C for up to 3 mo.

2.3. UHPLC-MS/MS Instrumentation and Operating Conditions

A Thermo Scientific Vanquish ultrahigh-performance liquid chromatograph coupled to a Thermo Scientific TSQ Quantis mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used. Separation was performed with a Waters Acquity UPLC high-strength silica (HSS) C18 column (50 mm $\times$ 2.1 mm, 1.8 μ m; Waters, Milford, MA, USA), with the front end connected to a Waters Acquity UPLC HSS C18 VanGuard precolumn (5 mm $\times$ 2.1 mm, 1.8 μ m; Waters, Milford, MA, USA). The column temperature was 40°C , and the sample injection volume was 2.0 μ L.

Gradient elution was performed with water and ACN as the mobile phases, at a flow rate of 0.3 mL/min: 0–4 min, 4% (v/v) ACN increased to 96% (v/v); 4–6 min, 96% (v/v) ACN; and 6–10 min, 96% (v/v) ACN decreased to 4% (v/v). The MS/MS was equipped with an ESI source operating in the positive ionization (PI) mode, negative ionization (NI) mode, and selected reaction monitoring mode. CAP, TAP, FF, and CAP-D5 were analyzed in the NI mode, whereas FFA and FFA-D3 were analyzed in the PI mode. The ESI source was operated with the following capillary voltage: 3.5 kV in PI mode and 2.5 kV in NI mode; sheath gas: 50 Arb; auxiliary gas: 10 Arb; ion transfer tube temperature: 325°C; and vaporizer temperature: 350°C.

2.4. Sample Preparation

Blank samples used in the validation procedure were obtained from the Supervision, Inspection, and Testing Center for Quality of Meat Products (Nanjing, China). Pork, beef, lamb, and chicken muscle were chopped and homogenized by an HM6300 intelligent homogenizer (Lab Precision Beijing Technology Co., LTD, Beijing, China) at 10,000 r/min, and aliquots of 5.0 g were weighed in a 50-mL polypropylene centrifuge tube. Samples were spiked with 10 μ L of the internal standard working solution, corresponding to a concentration of 2 μ g/kg of CAP-D5 and FFA-D3 in tissue. Then 15 mL of ethyl acetate with 2% (v/v) ammonia were added. The mixture was thoroughly homogenized using an IKA Vortex 3 (IKA, Staufen, Germany). After mixing, the homogenate was centrifuged at 10,621 g for 5 min at 4°C, and the supernatant was transferred into a 50-mL polypropylene tube. The extraction step was repeated by the addition of 15 mL of ethyl acetate with 2% (v/v) ammonia, shaken, and centrifuged again under the same conditions. The resulted supernatant was merged with the previous extract in the 50 mL polypropylene tube, and the extracts were evaporated to dryness at 40°C for 2.5 h under a nitrogen stream with an N-EVAP-11 nitrogen evaporator (Organomation, Berlin, MA, USA).

The residual solution was dissolved in acetone/*n*-hexane (1:9, v/v; 5 mL) and loaded onto a CNW Si SPE (200 mg) cartridge, which was preconditioned with water (5 mL) and acetone/*n*-hexane (1:9, v/v; 5 mL). The cartridge was eluted with acetone/*n*-hexane (6:4, v/v; 5 mL), and then the eluate was evaporated to dryness at 40°C for 10 min under a nitrogen stream. The residual solution was reconstituted by adding 1.0 mL of ACN-water (50:50; v/v) and then vortexed for 1 min. Next, 5 mL of hexane saturated with pure ACN was added to the tube, and the mixture was vortexed for 30 s. After centrifugation for 1 min at 10,621 g, the hexane layer was discarded. This defatting step was repeated twice. Finally, an aliquot of 1 mL of the bottom layer was poured through a 0.22 μ m filter into an autosampler vial, and then 2 μ L of the liquid in the aforementioned vial was analyzed using UHPLC-MS/MS.

3. Method Validation

3.1. Determination of Linearity, Limit of Detection, and Limit of Quantification of Amphenicols

A series concentration of matrix-matched standard solution was prepared by diluting the mixed standard working solutions with the blank matrix extract. Among them, CAP, TAP, and FF were set at 0.5, 1.0, 5.0, 10.0, and 50.0 μ g/kg within the range 0.5–50.0 μ g/kg, and FFA was set at 5.0, 10.0, 50.0, 100.0, and 200.0 μ g/kg within the range 5.0–200.0 μ g/kg (the concentrations of CAP-D5 and FFA-D3 were 10 μ g/L). The standard curves were constructed using the mass concentration of amphenicols (μ g/kg) as the abscissa (*x*) and the analyte/internal standard peak area ratio versus as the ordinate (*y*), and the squares of the correlation coefficients (R^2) were calculated.

Negative livestock and poultry meat were added to the internal standard working solution and the mixed standard working solution and then detected with reference to the method described above. The limit of detection (LOD) was the minimum added concentration when the signal-to-noise ratio (S/N) ≥ 3 , and the limit of quantification (LOQ) was the minimum added concentration when S/N ≥ 10 [38].

3.2. Recovery and Precision Test

Solutions were spiked into livestock and poultry meat as follows: standard working solutions of CAP, TAP, and FF at three concentration levels (1, 5, and 10 μ g/kg) and the standard working solution of FFA at three concentration levels (5, 10, and 50 μ g/kg). Pretreatment was performed in accordance with the method described above, and each concentration was set in six parallels. The recovery was calculated as follows: (1) recovery% = $C_E / C_M \times 100\%$, where C_E is the experimental concentration determined from the calibration curve and C_M is the spiked concentration. The precision was determined by calculating the relative standard deviation (RSD). Samples with three levels of spiked

concentrations were measured in the same day and in six consecutive days, and then the intraday RSD and the interday RSD were calculated. The RSD was calculated as follows: $(2)RSD\% = \frac{SD}{C_A} \times 100\%$, where SD is the standard deviation and C_A is the average of the experimental concentration determined from the calibration curve.

3.3. Application to Livestock Meat, Poultry Meat, and their Products

To validate our strategy, 28 real samples of commercial livestock meat, poultry meat, and their products from various locations in China were used to detect amphenicols. Amphenicols were extracted from the samples as previously described, and the extracts were analyzed with UHPLC-MS/MS.

3.4. Data Analysis

TraceFinder 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition and processing, and Origin 8.0 software (Microcal Software Inc., Northampton, MA, USA) was used for plotting.

4. Results and Discussion

4.1. Optimization of UHPLC-MS/MS Conditions

The use of formic acid improved the response and peak shape corresponding to FFA but generated ion suppression of CAP, TAP, and FF—which we monitored in the NI mode [28]. Thus, to better separate amphenicols and metabolite residues, we used ACN-0.1 v/v% formic acid, ACN-0.2 v/v% formic acid, and ACN-0.3 v/v% formic acid as the mobile phase for UHPLC-MS/MS analyses in this experiment. The separation effect of the four target compounds was excellent when ACN-water was a mobile phase (Figure 2). The FFA peak became bimodal after adding formic acid to the mobile phase. Therefore, we selected ACN-water as a mobile phase to separate the four analytes.

[figure omitted; refer to PDF]

We directly injected $1 \mu\text{g/mL}$ of CAP, TAP, FF, FFA, CAP-D5, and FFA-D into the mass spectrometer at a flow rate of $20 \mu\text{L/min}$, and the instrument scanned in Full Scan Q1 simultaneously in ESI (+) and ESI (–) to determine the precursor ion of each compound. Then the instrument scanned in SIM Q3. For each compound, we optimized two pairs of characteristic ion pairs with the highest response as qualitative and quantitative ion pairs. We also optimized the declustering voltage and collision energy of each ion pair. In doing so, we obtained corresponding mass spectrometry parameters (Table 2).

Table 2

Mass spectrometry conditions and range of relative intensities for each compound.

Analyte	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering voltage (V)	Collision voltage (V)
CAP	–	321	257	113	–10
152*	113	–12	–		
CAP-D5	–	326	157	113	–16
–					
TAP	–	354	290*	290	–12
185	164	–10	–		
FF	–	356	185*	141	–20
119	141	–38	–		

FFA	+	248	230*	100	10
130	100	27	-		
FFA-D3	+	251	233	85	10

*Quantification ion. CAP, chloramphenicol; FF, florfenicol; FFA, florfenicol amine; TAP, thiamphenicol.

4.2. Optimization of Sample Preparation

The extraction solvent was ACN, which showed satisfactory recoveries for CAP. However, when the other analytes were introduced in the method, it was necessary to choose the extraction solvent to provide better recoveries rates for all analytes, not just for CAP. The solvent of election was ethyl acetate, which can promote the extraction of all analytes. Moreover, the use of ammonium hydroxide as an additive can promote an alkaline environment, which is favorable for the extraction of FFA. Taking into account the extraction efficiency of all the amphenicols and metabolites, it was necessary to choose an extraction solvent that was able to provide better recoveries for all the analytes. Therefore, we chose the following extraction methods for comparison:

- (i) Method A: ethyl acetate: ammonium hydroxide (98:2, v/v), extract twice
- (ii) Method B: ACN: ammonium hydroxide (98:2, v/v), extract twice
- (iii) Method C: ACN, extract twice
- (iv) Method D: ACN: ethyl acetate: ammonium hydroxide (49:49:2, v/v/v), extract twice
- (v) Method E: ethyl acetate: ammonium hydroxide (98:2, v/v), extracted once; and ACN: ammonium hydroxide (98:2, v/v), extracted once

After the extraction by method A, the recovery of amphenicols and metabolites in the four livestock and poultry meat products was all in the range 60% to 120% (Table 3). Therefore, we chose method A as the optimal extraction method.

Table 3

Recoveries of amphenicols and metabolites under various extraction methods.

Extraction method	Matrix	Number of amphenicols and metabolites		
Recovery <60%	Recovery 60% to 120%	Recovery >120%	A	Pork
0	4	0	Beef	0
4	0	Lamb	0	4
0	Chicken	0	4	0
-				
B	Pork	1	1	2
Beef	1	3	0	Lamb
1	1	2	Chicken	3

1	0	-		
C	Pork	1	1	2
Beef	1	2	1	Lamb
1	1	2	Chicken	1
3	0	-		
D	Pork	1	1	2
Beef	1	2	1	Lamb
0	2	2	Chicken	0
4	0	-		
E	Pork	1	2	1
Beef	1	1	2	Lamb
0	2	2	Chicken	2

Because livestock and poultry meat contain a large quantity of fat and protein and are complex matrices, the samples require further purification after extraction. Therefore, we compared the purification effects of the following columns: HyperSep Retain PEP (60mg/3mL; Thermo Fisher Scientific, Waltham, MA, USA), Oasis HLB (200mg/6 mL; Waters, Milford, MA, USA), and CNW Si SPE (60mg/3mL; ANPEL Experimental Technology Co., Shanghai, China). After purification with the CNW Si SPE column, the recovery of amphenicols and metabolites in the four livestock and poultry meat products was in the range from 60% to 120% (Table 4). Therefore, we chose the CNW Si SPE column as the optimal purification method.

Table 4

Recoveries of amphenicols and metabolites under various purification methods.

Purification method	Matrix	Number of amphenicols and metabolites		
Recovery <60%	Recovery 60% to 120%	Recovery >120%	HyperSep Retain PEP	Pork
1	1	2	Beef	0

4	0	Lamb	1	2
1	Chicken	0	2	2
-				
Oasis HLB SPE	Pork	2	0	2
Beef	0	3	1	Lamb
1	2	1	Chicken	1
1	2	-		
CNW Si SPE	Pork	0	4	0
Beef	0	4	0	Lamb
0	4	0	Chicken	0

4.3. Linearity of Standard Curves, LOD, and LOQ for Amphenicols and Metabolites

We used CAP-D5 as the internal standard for CAP, TAP; and FF; and FFA-D3 as the internal standard for FFA. In four types of livestock and poultry meat, the method was linear over the range of 0.5–50.0 $\mu\text{g/kg}$ for CAP, TAP, and FF; and of 5.0–200.0 $\mu\text{g/kg}$ for FFA. The R^2 was greater than >0.999. The LOD and LOQ of the four tested compounds ranged from 0.03–1.50 $\mu\text{g/kg}$ and 0.11–5.0 $\mu\text{g/kg}$ in four types of livestock and poultry meat (Table 5). Compared with other literature reports [27,28,38], this method is fast and has high sensitivity and meets the requirements of the National Food Safety Standard on Maximum Residue Limits for Veterinary Drugs in Foods (GB 31650–2019) in China.

Table 5

Linearity of standard curve, R^2 , LOD, and LOQ of amphenicols and metabolites in livestock and poultry meat.

Matrix	Analyte	Linearity ($\mu\text{g/kg}$)	Linear equation	R^2	LOD ($\mu\text{g/kg}$)	LOQ ($\mu\text{g/kg}$)
Pork	CAP	0.50–50.0	$y=0.05933x+0.02204$	0.9996	0.11	0.36
TAP	0.50–50.0	$y=0.01864x+0.02831$	0.9992	0.12	0.37	FF
0.50–50.0	$y=0.1219x+0.05289$	0.9998	0.03	0.11	FFA	5.00–200.0
$y=0.07707x-0.00162$	0.9991	0.60	2.00	-		

Beef	CAP	0.5–50.0	$y=0.06262x-0.01198$	0.9995	0.05	0.18
TAP	0.5–50.0	$y=0.01741x+0.006421$	0.9991	0.06	0.20	FF
0.5–50.0	$y=0.1233x-0.09559$	0.9992	0.04	0.13	FFA	5.0–200.0
$y=0.06948x-0.9006$	0.9998	1.50	5.00	-		
Lamb	CAP	0.5–50.0	$y=0.05427x+0.07979$	0.9995	0.06	0.21
TAP	0.5–50.0	$y=0.02154x+0.02831$	0.9992	0.06	0.21	FF
0.5–50.0	$y=0.1553x+0.2424$	0.9998	0.04	0.12	FFA	5.0–200.0
$y=0.05435x-0.1845$	0.9992	0.50	5.00	-		
Chicken	CAP	0.5–50.0	$y=0.08144x-0.1681$	0.9991	0.03	0.05
TAP	0.5–50.0	$y=0.03328x-0.04784$	0.9992	0.03	0.11	FF
0.5–50.0	$y=0.1599x-0.2144$	0.9991	0.03	0.11	FFA	5.0–200.0

CAP, chloramphenicol; FF, florfenicol; FFA, florfenicol amine; LOD, limit of detection; LOQ, limit of quantification; TAP, thiamphenicol.

4.4. Recovery and Precision of Amphenicols and Metabolites

To verify the recovery and precision of this method, we spiked blank samples at three concentrations within the ranges 1.0–10.0 µg/kg for CAP, TAP, and FF and 5.0–50.0 µg/kg for FFA ($n=6$ at each level). Table 6 summarizes the results of the recovery and precision experiments. The average recovery of the four analytes in livestock and poultry meat samples was 71.77% to 120.30%, with an intraday RSD <9.22% and interday RSD <12.30%. Thus, our method has high recovery and good precision and can achieve simultaneous detection of amphenicols and metabolites in livestock and poultry meat.

Table 6

Recovery and precision of amphenicols and metabolites ($n=6$).

Matrix	Analyte	Added level (µg/kg)	Recovery (%)	Intraday RSD (%)	Interday RSD (%)
Pork	CAP	1	98.93	4.90	6.38

5	115.81	2.16	3.71	10	113.16
5.00	5.29	TAP	1	95.80	8.30
9.11	5	112.92	7.44	7.82	10
106.31	6.93	7.92	FF	1	83.81
5.62	5.68	5	93.02	2.82	4.59
10	95.63	1.83	4.12	FFA	5
97.07	2.51	6.24	10	89.22	6.23
10.42	50	89.61	7.93	11.94	.
Beef	CAP	1	113.00	7.44	7.31
5	99.73	4.41	8.24	10	93.30
2.50	10.35	TAP	1	87.91	5.53
9.47	5	93.99	5.10	11.90	10
80.10	3.02	7.10	FF	1	80.13
6.08	8.22	5	89.50	4.90	4.93
10	96.81	2.12	2.41	FFA	5
86.43	9.22	9.60	10	80.29	7.18
7.22	50	100.22	3.90	10.8	.
Lamb	CAP	1	97.20	1.83	2.30
5	110.91	2.61	6.00	10	120.30
3.88	7.43	TAP	1	71.77	6.15
6.20	5	105.44	4.50	4.86	10
99.02	1.93	2.83	FF	1	81.21
6.27	12.30	5	109.00	1.21	1.37

10	99.03	1.90	4.19	FFA	5
101.14	4.58	11.00	10	103.91	5.24
8.25	50	92.02	3.72	8.33	.
Chicken	CAP	1	101.27	4.71	8.34
5	101.05	1.63	3.60	10	97.03
5.74	5.42	TAP	1	84.00	6.88
11.70	5	105.23	6.30	8.70	10
103.54	6.91	11.02	FF	1	101.10
3.90	5.22	5	85.44	7.16	9.70
10	82.82	4.28	4.21	FFA	5
108.71	6.30	5.54	10	101.63	6.62

CAP, chloramphenicol; FF, florfenicol; FFA, florfenicol amine; RSD, relative standard deviation; TAP, thiamphenicol.

4.5. Real Sample Analyses

We selected 28 commercial livestock meat, poultry meat, and their products to verify practical applications of our method in real food matrices with possible interferences. We used one positive meat sample as the quality control sample. We detected 1.38 $\mu\text{g}/\text{kg}$ of the banned veterinary drug CAP in fresh chicken, 0.35 $\mu\text{g}/\text{kg}$ of FF in pork sausage, and 0.54 $\mu\text{g}/\text{kg}$ of FF in fried pork jerky (Qingdao, Shandong), yet none of the samples exceeded the MRL of 300 $\mu\text{g}/\text{kg}$ for porcine muscle (GB 31650-2019; Table 7).

Table 7

Test results of commercial livestock meat, poultry meat, and their products.

Sample name	Place of origin (China)	CAP	TAP	FF	FFA
Fresh pork (positive control)	Nanjing, Jiangsu	+	+	+	+
Fresh pork	Nanjing, Jiangsu				
Pork sausage	Linyi, Shandong			+	
Pork ham	Nanjing, Jiangsu				
Fried pork jerky	Zibo, Shandong				
Fried pork jerky	Qingdao, Shandong			+	

Roasted pork jerky	Zhangzhou, Fujian				
Roasted pork sausage	Yantai, Shandong				
Fresh beef	Nanjing, Jiangsu				
Spiced beef	Pingxiang, Jiangxi				
Beef sausage	Linyi, Shandong				
Fried beef jerky	Bazhong, Sichuan				
Fried beef jerky	Zibo, Shandong				
Charcoal grilled beef jerky	Tongliao, Nei Mongol				
Charcoal grilled beef jerky	Chifeng, Nei Mongol				
Fresh lamb	Nanjing, Jiangsu				
Boiled lamb	Suzhou, Jiangsu				
Spiced lamb	Hulunbair, Nei Mongol				
Fried lamb jerky	Tongliao, Nei Mongol				
Fried lamb jerky	Bayannaoer, Nei Mongol				
Roast leg of lamb	Hulunbair, Nei Mongol				
Roast leg of lamb	Xilin Gol League, Nei Mongol				
Fresh chicken	Nanjing, Jiangsu	+			
Instant chicken breast	Pingxiang, Jiangxi				
Chicken sausage	Linyi, Shandong				
Crispy chicken	Nanjing, Jiangsu				
Fried chicken	Nanjing, Jiangsu				
Roast chicken	Yantai, Shandong				
Beggar's chicken	Hangzhou, Zhejiang				

+, detected; no entry indicates not detected. CAP, chloramphenicol; FF, florfenicol; FFA, florfenicol amine; TAP, thiamphenicol.

5. Conclusions

Amphenicols are widely used in livestock and poultry breeding and production, but illegal or noncompliant use may occur. This causes amphenicol residues to accumulate in the body of livestock and poultry animals. Such accumulation is a threat to livestock and poultry food safety, as well as human health. Therefore, we developed a detection method for amphenicols and metabolites in livestock meat, poultry meat, and their products. By optimizing MS, LC, and sample pretreatment conditions, we established a UHPLC-MS/MS detection method that can simultaneously detect amphenicols and metabolites in livestock meat, poultry meat, and their products. The peak time of the analytes was within 5min; the LOD was 0.03–1.50 $\mu\text{g/kg}$; the LOQ was 0.05–5.00 $\mu\text{g/kg}$; the recovery of spiked samples at low, medium, and high concentrations was between 71.77% and 120.30%; the intraday RSD was <9.22%, and the interday RSD was <12.30%. Thus, this method has a short analysis time, a high sensitivity, and a good repeatability. We have thus provided scientific support and technical assurance for reliable detection of amphenicols and metabolites in animal-derived foods for food safety monitoring.

Acknowledgments

The authors thank Qingqin Lü for her valuable advice on UHPLC-MS/MS analysis. This research was financially supported by grants from the National Key R &D Program of China (2018YFC1602804) and a special intergovernmental project for International Cooperation in Science, Technology and Innovation (2019YFE0103800).

References

- [1] G. Zhou, W. Zhang, X. Xu, "China's meat industry revolution: challenges and opportunities for the future," *Meat Science*, vol. 92 no. 3, pp. 188-196, DOI: 10.1016/j.meatsci.2012.04.016, 2012.
- [2] M. Pastor-Belda, N. Campillo, N. Arroyo-Manzanares, M. Hernández-Córdoba, P. Viñas, "Determination of amphenicol antibiotics and their glucuronide metabolites in urine samples using liquid chromatography with quadrupole time-of-flight mass spectrometry," *Journal of Chromatography B*, vol. 1146, DOI: 10.1016/j.jchromb.2020.122122, 2020.
- [3] N. R. Barveen, T.-J. Wang, Y.-H. Chang, "Photochemical decoration of silver nanoparticles on silver vanadate nanorods as an efficient SERS probe for ultrasensitive detection of chloramphenicol residue in real samples," *Chemosphere*, vol. 275, DOI: 10.1016/j.chemosphere.2021.130115, 2021.
- [4] L. R. Guidi, F. A. Santos, A. C. S. R. Ribeiro, C. Fernandes, L. H. M. Silva, M. B. A. Gloria, "A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish," *Talanta*, vol. 163, pp. 85-93, DOI: 10.1016/j.talanta.2016.10.089, 2017.
- [5] J. P. Cerutti, V. Aiassa, M. A. Fernández, M. R. Longhi, M. A. Quevedo, A. Zoppi, "Structural, physicochemical and biological characterization of chloramphenicol multicomponent complexes," *Journal of Molecular Liquids*, vol. 331, DOI: 10.1016/j.molliq.2021.115761, 2021.
- [6] P. Ma, H. Guo, N. Duan, X. Ma, L. Yue, Q. Gu, Z. Wang, "Label free structure-switching fluorescence polarization detection of chloramphenicol with truncated aptamer," *Talanta*, vol. 230, DOI: 10.1016/j.talanta.2021.122349, 2021.
- [7] L. R. Guidi, P. A. S. Tette, M. B. A. Gloria, C. Fernandes, "A simple and rapid LC-MS/MS method for the determination of amphenicols in Nile tilapia," *Food Chemistry*, vol. 262, pp. 235-241, DOI: 10.1016/j.foodchem.2018.04.087, 2018.
- [8] F. Wang, W. Wang, S. Yuan, W. Wang, Z.-H. Hu, "Comparison of UV/H₂O₂ and UV/PS processes for the degradation of thiamphenicol in aqueous solution," *Journal of Photochemistry and Photobiology A: Chemistry*, vol. 348, pp. 79-88, DOI: 10.1016/j.jphotochem.2017.08.023, 2017.
- [9] R. Li, Z.-J. Lin, J.-Y. Yang, Z.-L. Xu, H. Wang, H.-T. Lei, Y.-M. Sun, Y.-D. Shen, "An indirect competitive enzyme-linked immunosorbent assay for simultaneous determination of florfenicol and thiamphenicol in animal meat and urine," *Chinese Journal of Analytical Chemistry*, vol. 46 no. 8, pp. 1321-1328, DOI: 10.1016/s1872-2040(18)61104-1, 2018.
- [10] G. Wang, B. Wang, X. Zhao, X. Xie, K. Xie, X. Wang, G. Zhang, T. Zhang, X. Liu, G. Dai, "Determination of

- thiamphenicol, florfenicol and florfenicol amine residues in poultry meat and pork via ASE-UPLC-FLD," *Journal of Food Composition and Analysis*, vol. 81, pp. 19-27, DOI: 10.1016/j.jfca.2019.05.004, 2019.
- [11] A. Anadón, M. A. Martínez, M. Martínez, A. Ríos, V. Caballero, I. Ares, M. R. Martínez-Larrañaga, "Plasma and tissue depletion of florfenicol and florfenicol-amine in chickens," *Journal of Agricultural and Food Chemistry*, vol. 56 no. 22, pp. 11049-11056, DOI: 10.1021/jf802138y, 2008.
- [12] L. Campone, R. Celano, A. L. Piccinelli, I. Pagano, N. Cicero, R. D. Sanzo, S. Carabetta, M. Russo, L. Rastrelli, "Ultrasound assisted dispersive liquid-liquid microextraction for fast and accurate analysis of chloramphenicol in honey," *Food Research International*, vol. 115, pp. 572-579, DOI: 10.1016/j.foodres.2018.09.006, 2019.
- [13] L. R. Guidi, P. A. S. Tette, C. Fernandes, L. H. M. Silva, M. B. A. Gloria, "Advances on the chromatographic determination of amphenicols in food," *Talanta*, vol. 162, pp. 324-338, DOI: 10.1016/j.talanta.2016.09.068, 2017.
- [14] X. Ma, H. Li, S. Qiao, C. Huang, Q. Liu, X. Shen, Y. Geng, W. Xu, C. Sun, "A simple and rapid sensing strategy based on structure-switching signaling aptamers for the sensitive detection of chloramphenicol," *Food Chemistry*, vol. 302, DOI: 10.1016/j.foodchem.2019.125359, 2020.
- [15] J. Ding, Q. Li, X. Xu, X. Zhang, Y. Su, Q. Yue, B. Gao, "A wheat straw cellulose-based hydrogel for Cu (II) removal and preparation copper nanocomposite for reductive degradation of chloramphenicol," *Carbohydrate Polymers*, vol. 190, pp. 12-22, DOI: 10.1016/j.carbpol.2018.02.032, 2018.
- [16] Ministry of Agriculture and Rural Affairs of the People's Republic of China, National Health Commission of the People's Republic of China, State Administration for Market Regulation, National Standard of the People's Republic of China, National Food Safety Standard on Maximum Residue Limits for Veterinary Drugs in Foods, GB31650-2019, 2019. <https://www.aqsc.agri.cn/tzgg/201910/P020191012570175865095.pdf>
- [17] Commission European, Commission Regulation (EU) No 37/2010 of 22 December 2009 on Pharmacologically Active Substances and their Classification Regarding Maximum Residue Limits in Foodstuffs of Animal Origin (Text with EEA Relevance), 2009. https://ec.europa.eu/health/sites/default/files/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf
- [18] A. S. Sadeghi, N. Ansari, M. Ramezani, K. Abnous, M. Mohsenzadeh, S. M. Taghdisi, M. Alibolandi, "Optical and electrochemical aptasensors for the detection of amphenicols," *Biosensors and Bioelectronics*, vol. 118, pp. 137-152, DOI: 10.1016/j.bios.2018.07.045, 2018.
- [19] H. Li, W. Geng, M. M. Hassan, M. Zuo, W. Wei, X. Wu, Q. Ouyang, Q. Chen, "Rapid detection of chloramphenicol in food using SERS flexible sensor coupled artificial intelligent tools," *Food Control*, vol. 128, DOI: 10.1016/j.foodcont.2021.108186, 2021.
- [20] L. V. De Faria, T. P. Lisboa, N. D. S. Campos, G. F. Alves, M. A. C. Matos, R. C. Matos, R. A. A. Munoz, "Electrochemical methods for the determination of antibiotic residues in milk: a critical review," *Analytica Chimica Acta*, vol. 1173, DOI: 10.1016/j.aca.2021.338569, 2021.
- [21] A. S. Sadeghi, M. Mohsenzadeh, K. Abnous, S. M. Taghdisi, M. Ramezani, "Development and characterization of DNA aptamers against florfenicol: fabrication of a sensitive fluorescent aptasensor for specific detection of florfenicol in milk," *Talanta*, vol. 182, pp. 193-201, DOI: 10.1016/j.talanta.2018.01.083, 2018.
- [22] N. Sai, Y. Chen, N. Liu, G. Yu, P. Su, Y. Feng, Z. Zhou, X. Liu, H. Zhou, Z. Gao, "A sensitive immunoassay based on direct hapten coated format and biotin-streptavidin system for the detection of chloramphenicol," *Talanta*, vol. 82 no. 4, pp. 1113-1121, DOI: 10.1016/j.talanta.2010.06.018, 2010.
- [23] A. Yibar, F. Cetinkaya, G. E. Soyutemiz, "ELISA screening and liquid chromatography-tandem mass spectrometry confirmation of chloramphenicol residues in chicken muscle, and the validation of a confirmatory method by liquid chromatography-tandem mass spectrometry," *Poultry Science*, vol. 90 no. 11, pp. 2619-2626, DOI: 10.3382/ps.2011-01564, 2011.
- [24] A. Posyniak, J. Zmudzki, J. Niedzielska, "Evaluation of sample preparation for control of chloramphenicol residues in porcine tissues by enzyme-linked immunosorbent assay and liquid chromatography," *Analytica Chimica Acta*, vol. 483 no. 1-2, pp. 307-311, DOI: 10.1016/s0003-2670(02)01487-3, 2003.
- [25] V. Samanidou, L.-D. Galanopoulos, A. Kabir, K. G. Furton, "Fast extraction of amphenicols residues from raw

- milk using novel fabric phase sorptive extraction followed by high-performance liquid chromatography-diode array detection," *Analytica Chimica Acta*, vol. 855, pp. 41-50, DOI: 10.1016/j.aca.2014.11.036, 2015.
- [26] F. Moragues, C. Igualada, N. León, "Validation of the determination of chloramphenicol residues in animal feed by liquid chromatography with an ion trap detector based on European decision 2002/657/EC," *Food Analytical Methods*, vol. 5 no. 3, pp. 416-421, DOI: 10.1007/s12161-011-9261-x, 2011.
- [27] F. Moragues, C. Igualada, N. León, "Confirmatory method for the determination of amphenicols in muscle and kidney of several animal species," *Food Analytical Methods*, vol. 10 no. 3, pp. 610-617, DOI: 10.1007/s12161-016-0623-2, 2016.
- [28] H.-Y. Liu, S.-L. Lin, M.-R. Fuh, "Determination of chloramphenicol, thiamphenicol and florfenicol in milk and honey using modified QuEChERS extraction coupled with polymeric monolith-based capillary liquid chromatography tandem mass spectrometry," *Talanta*, vol. 150, pp. 233-239, DOI: 10.1016/j.talanta.2015.12.045, 2016.
- [29] R. W. Fedeniuk, D. McKenzie, M. Mizuno, C. Neiser, C. O'Byrne, B. Shurmer, "Development and validation of determinative and confirmatory LC-MS/MS methodologies for total florfenicol and tulathromycin residues in bovine, equine and porcine kidney, liver and muscle tissues," *Journal of Chromatography B*, vol. 983-984, DOI: 10.1016/j.jchromb.2014.12.035, 2015.
- [30] X.-D. Pan, P.-G. Wu, W. Jiang, B.-J. Ma, "Determination of chloramphenicol, thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass spectrometry," *Food Control*, vol. 52, pp. 34-38, DOI: 10.1016/j.foodcont.2014.12.019, 2015.
- [31] S. Zhang, F. Sun, J. Li, L. Cheng, J. Shen, "Simultaneous determination of florfenicol and florfenicol amine in fish, shrimp, and swine muscle by gas chromatography with a microcell electron capture detector," *Journal of AOAC International*, vol. 89 no. 5, pp. 1437-1442, DOI: 10.1093/jaoac/89.5.1437, 2006.
- [32] Y. Yikilmaz, A. Filazi, "Detection of florfenicol residues in salmon trout via GC-MS," *Food Analytical Methods*, vol. 8 no. 4, pp. 1027-1033, DOI: 10.1007/s12161-014-9982-8, 2014.
- [33] W.-L. Liu, R.-J. Lee, M.-R. Lee, "Supercritical fluid extraction in situ derivatization for simultaneous determination of chloramphenicol, florfenicol and thiamphenicol in shrimp," *Food Chemistry*, vol. 121 no. 3, pp. 797-802, DOI: 10.1016/j.foodchem.2009.12.087, 2010.
- [34] H. Kikuchi, T. Sakai, R. Teshima, S. Nemoto, H. Akiyama, "Total determination of chloramphenicol residues in foods by liquid chromatography-tandem mass spectrometry," *Food Chemistry*, vol. 230, pp. 589-593, DOI: 10.1016/j.foodchem.2017.03.071, 2017.
- [35] S. Saito-Shida, K. Shiono, J. Narushima, S. Nemoto, H. Akiyama, "Determination of total florfenicol residues as florfenicol amine in bovine tissues and eel by liquid chromatography-tandem mass spectrometry using external calibration," *Journal of Chromatography B*, vol. 1109, pp. 37-44, DOI: 10.1016/j.jchromb.2019.01.018, 2019.
- [36] X. Xie, B. Wang, M. Pang, X. Zhao, K. Xie, Y. Zhang, Y. j. Wang, Y. w. Guo, C. j. Liu, X. n. Bu, R. Wang, H. Shi, G. Zhang, T. Zhang, G. Dai, J. Wang, "Quantitative analysis of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in eggs via liquid chromatography-electrospray ionization tandem mass spectrometry," *Food Chemistry*, vol. 269, pp. 542-548, DOI: 10.1016/j.foodchem.2018.07.045, 2018.
- [37] Z. Xiao, R. Song, Z. Rao, S. Wei, Z. Jia, D. Suo, X. Fan, "Development of a subcritical water extraction approach for trace analysis of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in poultry tissues," *Journal of Chromatography A*, vol. 1418, pp. 29-35, DOI: 10.1016/j.chroma.2015.09.047, 2015.
- [38] U.S. Department of Health and Human Services, U.S. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Bioanalytical Method Validation: Guidance for Industry (2018), 2001. <https://www.fda.gov/media/70858/download>

DETAILS

Subject:	Mass spectrometry; Government regulations; Livestock; Food; Analytical chemistry; Animals; Animal health; Liquid chromatography; Scientific imaging; Metabolites; Chromatography; Water purification; Ethyl acetate; Meat; Chloromycetin; Correlation coefficients; Solid phases; Nitrogen; Acetonitrile; Liver; Poultry; Ammonia; Residues; Spectroscopy; Pork; Meat quality; Hexanes
Location:	Beijing China; United States--US; Germany; China
Company / organization:	Name: European Union; NAICS: 926110, 928120
Editor:	Barbara Bojko
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-08-09 (Received); 2021-09-15 (Revised); 2021-10-22 (Accepted); 2021-11-03 (Pub)
DOI:	https://doi.org/10.1155/2021/3613670
ProQuest document ID:	2597343209
Document URL:	https://www.proquest.com/scholarly-journals/simultaneous-determination-amphenicols/docview/2597343209/se-2?accountid=211160

Copyright: Copyright © 2021 Xinyi Wu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-11-14

Database: Publicly Available Content Database

Document 9 of 59

Optimization of Residual Hexane in Edible Oils Analysis Using Static Headspace Gas Chromatography

Azrul Hisyam Samsuri¹; May Yen Ang¹; Ng, Shean Yeaw²

¹ Shimadzu Malaysia Sdn. Bhd, No. 6, Lorong Teknologi 3/4A, Nouvelle Industrial Park 2, Taman Sains Selangor 1, Kota Damansara, 47810 Petaling Jaya, Selangor, Malaysia ² Shimadzu Malaysia Sdn. Bhd, No. 6, Lorong Teknologi 3/4A, Nouvelle Industrial Park 2, Taman Sains Selangor 1, Kota Damansara, 47810 Petaling Jaya, Selangor, Malaysia; Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

This study aims to determine the residual hexane in four edible oils in Malaysia using a simple, rapid, and automated method in order to improve the efficiency and productivity of the analysis. Gas chromatography (GC/FID) equipped with a headspace autosampler (HS-20) was used to perform the analysis. Incubation time for each injection was successfully optimized from one hour to 30 minutes (50% reduction) compared to the official AOCS method Ca 3b-87. Out of the four tested edible oils, only the hexane residues detected in sunflower oil exceeded the maximum residue limit (MRL) set by the European Union regulation. Significant difference of the results obtained between large calibration range (0–938 mg kg⁻¹) and small calibration range (0–68 mg kg⁻¹) suggests that there is a need to use a lower standard calibration concentration to avoid misinterpretation of analysis results. Method validation applies to the technical hexane; 2-methylpentane, 3-methylpentane, cyclohexane, and methylcyclopentane, the signal-to-noise (S/N), as well as the limit of quantification (LoQ) values was found to be 218.20, 221.45, 746.37, 97.37 and 0.85, 0.84, 0.25, 1.93 mg kg⁻¹, respectively. Good linearity, repeatability, and low carryover of this method have provided an alternative way to analyze the content of the residual hexane in edible oils in a more efficient manner. Current study might provide a fundamental reference for the improvement of the AOCS official Ca 3b-87 method for determination of hexane residues in fats and oils analysis in the future.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Oil palm, rubber, cocoa, and rice represent the major crops in Malaysia, which have substantial contribution to our economy growth. It is noteworthy that the role of oil palm is particularly important as it accounting 28% of world palm oil production and 33% of world exports. Edible vegetable oils can be derived from the fruits of oil palms. In edible oil production, mechanical pressing and solvent extraction are the two common methods used in the industry, whereby the former method is the most traditional method of oil extraction [1]. About 60–80% of the oil can be obtained by mechanical pressing, and the pressed-cake will be sent to a solvent extraction plant to optimize the oil recovery efficiency ranging from 5–20% [2]. These oils yielded from solvent extraction could be extremely valuable, especially when the crude palm oil prices surge. One of the most effective solvents used for oil extraction is hexane due to its nonpolar nature. However, the commercial hexane used in this production typically consists of some isomers (2-methylpentane, 3-methylpentane, methylcyclopentane, and n-hexane), which are known to have a detrimental health effect [3]. Main toxic effects reported on human studies are muscular weakness, headache, dizziness, and slight nausea [4]. Due to that, according to European Union regulations, maximum residue limit (MRL) of hexane has been set at 1 mg kg^{-1} when it is used in the production or fractionation of fats and oils as well as production of cocoa butter [5]. This regulation has led to various methods being developed for the determination of hexane residue in fats and oils.

Several studies have been carried out on the analysis of residual hexane in food products. Mirghani and Che Man [6] developed a method for the determination of hexane residues in palm and peanut oils based on FTIR spectroscopy with attenuated total reflectance (ATR). Despite of its speed, convenience, and environment friendly, difficulty in interpreting the results have urged most of the industries are still prone to follow the gas chromatography method. The headspace-solid phase microextraction (SPME) method was developed to analyze the hexane residues in olive oil [7]. Similarly, the advantages of short analysis time, without the need of using solvent, are still not able to attract the interest of most industries to adopt this method due to the challenge in terms of collecting the highly concentrated solvent [8]. On the other hand, the AOCS official method Ca 3b-87 derived from International Union of Pure and Applied Chemistry Standard Method (IUPAC) has also evaluated the presence of hexane using HS-GC-FID [9]. This method has been successfully applied and accepted worldwide in the routine analysis of residual solvents in fats and edible oils.

However, preanalysis and postanalysis procedures highlighted in the AOCS official method are manual, labor intensive, time consuming, and hence, susceptible to errors. Several industrial managers have giving out their opinion regarding this matter in a Palm Oil Workshop organized by Shimadzu Sdn Bhd in 2019 (unpublished data). There are increasing numbers of industries looking forward to use an automated system that is not only able to increase productivity but also shorten the analysis time. To the best of our knowledge, there is no study to optimize the official method procedures of hexane residues in edible oil in Malaysia. Hence, current study aims to evaluate the hexane residues of four edible oil samples from the market of Malaysia using a rapid, simple, and automated method.

2. Materials and Methods

General: sunflower oil, corn oil, sesame oil, and palm oil were purchased from hypermarket in Kota Damansara, Malaysia, on March 2019. Headspace crimp vials (20 ml) were purchased from Shimadzu Corporation (Shimadzu Asia Pacific, Singapore). Eppendorf pipettes were purchased from Sigma Aldrich (St. Louis, MO, USA).

Reagents: HPLC-grade analytical reagents including hexane, hexane isomers, and heptane were obtained from Merck (Germany).

Instrumentation: GC/FID (GC-2030; Shimadzu, Japan) and the headspace sampler (HS-20; Shimadzu) were used in this study. Helium was used as the carrier gas for the analysis using GC/FID. The column flow was set at 0.8 ml min^{-1} with a flow control mode using constant linear velocity. The detector and injection temperature were set at 120°C with a 1:100 split mode.

Headspace sampler: headspace sampler (HS-20; Shimadzu, Japan) was used in this study. The incubation oven temperature of 80°C, sample line temperature of 90°C, transfer line temperature of 100, agitating level 3, equilibrating time of 30 min, pressurizing time of 1 min, load time of 0.5 min, and injection time of 1 min were configured. For method optimization, incubation time was set ranging from 10 to 60 min at a 10-minute interval.

Column: SOLGEL-1MS (30.0m; 0.25mm ID; 0.25 μ m df) was used in this study. The column was maintained at 40°C for 10 min, and then, it was heated up to 100°C at a rate of 30°C min⁻¹ and maintained at 100°C for 3 min.

Standard preparation: standards were prepared using a matrix-match method. 5g ($\pm$ 0.01g) of solvent-free edible oils was weighed in a headspace vial. 5 μ l of n-heptane (ISTD) was added into the oil. Next, technical hexane was added into the vial, leaving one vial with no added solvent as blank as given in Table 1. Each vial was covered with the headspace cap and tightly crimped. After that, all standards were vortexed for five minutes before being transferred to the headspace sampler for analysis.

Sample preparation: edible oils available in Malaysia's market were analyzed. 5g ($\pm$ 0.01g) of the oils was weighed in the headspace vial. 5 μ l of n-heptane (ISTD) was added into oil. The oil was then vortexed for five minutes before being transferred to the headspace sampler for analysis.

Statistical analyses: all analyses were carried out in triplicates, and the data were statistically analyzed by one-way ANOVA. The level of statistical significance was set at $p < 0.05$ using SPSS software version 23.0.

3. Results and Discussion

The effect of incubation time was tested to optimize the method. Incubation time ranged from 10 to 60 minutes at a 10-minute interval was studied. Based on the result, 30 minutes of the incubation time was the best for the analysis because there is no significant increase of the total peak area of hexane thereafter as shown in Figure 1. Within this range, headspace extraction has already reached its equilibrium. The result has shortened 50% of the incubation time for each injection as compared to the official AOCS method.

[figure omitted; refer to PDF]

Table 1

Amount of technical hexane added in five gram of oil.

Concentration	Vial number					
1	2	3	4	5	6	μ l/5g
0	0.5	1	2	4	7	mg kg ⁻¹

Quantification of residual hexane was based on matrix-match calibration and internal standard. The same amount of internal standard was spiked into oil matrix prior to addition of different amount of technical hexane to plot the calibration curve. Chromatogram and the group-calibration curve of the standards are shown in Figure 2. These four peaks are belonging to hydrocarbons that make up the technical hexane. Hydrocarbons that usually make up the technical hexane include 2-methylpentane, 3-methylpentane, cyclohexane, and methylcyclopentane. These four peaks were grouped together to plot a group-calibration curve. The group-calibration curve with good coefficient of determination (R^2) value of more than 0.999 was obtained for these analyzed standards (0–938 mg kg⁻¹). Using this calibration curve, residual hexane in four different types of edible oils available in Malaysia's market was quantified.

[figure omitted; refer to PDF]

According to AOCS [9], this official method is only suitable for the determination of quantities of hexane between 10 and 1500 mg kg⁻¹. Hence, slight modification of the official AOCS method was made to improve the evaluation.

Since the amount of residual hexane in edible oil often present in trace amount, which is outside the working range of the calibration curve (lowest standard is 67 mg kg⁻¹), a new calibration curve with lower standard concentration (0–68 mg kg⁻¹) was prepared as given in Table 2, in order to better match the low concentration of residual hexane in sample. Coefficient of determination (R^2) value of more than 0.998 for the new calibration curve was obtained

(Figure 3). Comparison of results between the official AOCS method with higher standard calibration concentration and the instrumental automated method with lower standard calibration concentrations was tabulated and is given in Table 3. Of all the four tested edible oils, only sunflower oil was found to contain the residual hexane that exceeded the maximum residue limit (MRL). Besides, the residual hexane detected in the large calibration range (0–938 mg kg⁻¹) is significantly different as compared to the results from the small calibration range (0–68 mg kg⁻¹). These results suggest that a large difference of calibration range might induce misinterpretation of analysis results while extrapolating from the calibration curve.

Table 2

Amount of technical hexane added in 20g of oil.

Concentration	Vial number				
1	2	3	4	5	μl/5g
0	0.5	1	1.5	2	mg kg ⁻¹

[figure omitted; refer to PDF]

Table 3

Comparison of results of residual hexane in different edible oils.

Edible oils	Residual hexane concentration (mg kg ⁻¹)	
AOCS official method with higher standard calibration concentration (0–938 mg kg ⁻¹)	Modified AOCS official method with lower standard calibration concentration (0–68 mg kg ⁻¹)	Sunflower oil
3.074	2.688 ^a	Corn oil
0.414	0.358 ^a	Sesame oil
0.711	0.602 ^a	Palm oil

^aSignificant difference at p<0.05.

For method validation, performance checks have been performed. Theoretical quantitation limit (LOQ) based on the signal-to-noise (S/N) value was calculated using the Shimadzu LabSolutions software (Table 4). The repeatability of the system was tested using five different vials containing 5g of oil spiked with 134 mg kg⁻¹ of hexane and internal standard (ISTD). The average result obtained was 137 mg kg⁻¹ (n=5) with a low level of relative standard deviation (RSD) of 1.69% and 102% recovery (Figure 4). A measurement using blank was also performed to check for carryover. The result showed an extremely low carryover indicating the good system performance of Shimadzu HS-20 as well as the reliability of this method (Figure 5).

Table 4

Theoretical quantitation limit (LOQ) and signal-to-noise (S/N) based on 17 mg kg⁻¹ standard.

Compound	S/N	LOQ (mg kg ⁻¹)
2-Methylpentane	218.20	0.85
3-Methylpentane	221.45	0.84
n-Hexane	746.37	0.25
Methylcyclopentane	97.37	1.93

[figure omitted; refer to PDF][figure omitted; refer to PDF]

4. Conclusion

The sunflower oil was found to contain the residual hexane that exceeded the maximum residue limit (1 mg kg⁻¹) among the four tested edible oils in Malaysia. In this analysis, optimization of the incubation time has drastically reduced half of the analysis time following the official AOCS Ca 3b-87 method. Besides, modification of the large calibration range to a lower calibration range (0–68 mg kg⁻¹) in the calibration curve showed a significant difference compared to the results obtained following the official AOCS Ca 3b-87 method (0–938 mg kg⁻¹). The static autoheadspace analysis method could be a recommended tool for the quantitative analysis method of residual hexane in vegetable liquid oil samples. With various automated features, this system has the capability to produce highly reliable quantification analysis. In conclusion, these results provide a fundamental reference for the improvement of the official AOCS Ca 3b-87 method in the future.

Acknowledgments

The authors would like to thank Mr. Stanley Tan (General Manager, Shimadzu, Malaysia) and Mr. Hazni (Assistant Manager, Customer Support Centre, Shimadzu Malaysia) for giving the advice and guidance while performing the analysis as well as completion of the manuscript. This work was funded by Shimadzu Malaysia Sdn Bhd.

References

- [1] L. M. Khan, M. A. Hanna, "Expression of oil from oilseeds-a review," *Journal of Agricultural Engineering Research*, vol. 28 no. 6, pp. 495-503, DOI: 10.1016/0021-8634(83)90113-0, 1983.
- [2] W. M. J. Achten, L. Verchot, Y. J. Franken, E. Mathijs, V. P. Singh, R. Aerts, B. Muys, "Jatropha bio-diesel production and use," *Biomass and Bioenergy*, vol. 32 no. 12, pp. 1063-1084, DOI: 10.1016/j.biombioe.2008.03.003, 2008.
- [3] P. J. Wan, R. J. Horn, "Extraction solvents for oilseeds," *Proceedings of the Oilseed Processing and Utilization World Conference*, vol. 9, pp. 707-709, .
- [4] F. Peña, S. Cárdenas, M. Gallego, M. Valcárcel, "Direct sampling of orujo oil for determining residual hexane by using a chemsensor," *Journal of the American Oil Chemists' Society*, vol. 80 no. 6, pp. 613-618, DOI: 10.1007/s11746-003-0747-4, 2003.
- [5] European Parliament and of The Council, "On the approximation of the laws of the member states on extraction solvents used in the production of foodstuffs and food ingredients," *Official Journal of the European Union*, vol. 52, 2009.
- [6] M. E. S. Mirghani, Y. B. Che Man, "Determination of hexane residues in vegetable oils with FTIR spectroscopy," *Journal of the American Oil Chemists' Society*, vol. 80 no. 7, pp. 619-623, DOI: 10.1007/s11746-003-0748-3, 2003.
- [7] N. S. Kim, J. H. Lee, O. S. Heo, K. T. Lee, "Study of the presence of residual hexane on olive oils," *Journal of the Korean Society of Food Science and Nutrition*, vol. 35, pp. 1405-1411, 2006.
- [8] E.-J. Jeong, S. H. Lee, B.-T. Kim, G. Lee, S. S. Yun, H. S. Lim, Y.-S. Kim, "An analysis method for determining residual hexane in health functional food products using static headspace gas chromatography," *Food Science and*

DETAILS

Subject:	Oil recovery; Hydrocarbons; Solvent extraction processes; Sunflowers; Gas chromatography; Calibration; Cyclohexane; Optimization; Residues; Edible oils; Vegetable oils; Chromatography; Automation; Sunflower oil; Hexanes; Oils & fats
Business indexing term:	Subject: Automation
Location:	Malaysia; Japan
Editor:	Spas D Kolev
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-07-27 (Received); 2021-10-12 (Accepted); 2021-10-29 (Pub)
DOI:	https://doi.org/10.1155/2021/1941336
ProQuest document ID:	2594361456
Document URL:	https://www.proquest.com/scholarly-journals/optimization-residual-hexane-edible-oils-analysis/docview/2594361456/se-2?accountid=211160

Copyright:

Copyright © 2021 Azrul Hisyam Samsuri et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2021-11-07

Database:

Publicly Available Content Database

Document 10 of 59

Electrospun Membrane for the Extraction of Acrylamide in Pet Food Samples

Basheer, Chanbasha ¹

¹ Department of Chemistry, King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia; Interdisciplinary Research Centre for Membrane and Water Security, King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

A simple microextraction procedure was developed using an electrospun nanostructured membrane to determine acrylamide in pet food samples. Polyvinyl chloride, polyvinyl alcohol, and polyvinyl alcohol/hydroxyethyl cellulose electrospun membranes were prepared and investigated as a sorbent to extract acrylamide. The characterization of the synthesized electrospun membrane was accomplished using field-emission scanning electron microscopy (FESEM). FESEM images showed uniform morphology and beadless nanofibers. Quantification was done by high-performance liquid chromatography with ultraviolet detection. A series of microextraction parameters were optimized before quantitative analysis of dry pet food samples. The calibration curve exhibited good linearity with a correlation coefficient of 0.996 across a 1–100 µg/kg concentration range. The recovery of acrylamide for pet food samples spiked with 5 and 10 µg/kg was in the range of 79.6–113.9 ($n=3$). The intraday precision of the method was less than 12% for three replicated real spiked samples at the 5 µg/kg level. The results demonstrated that the electrospun nanostructured membrane has good extraction selectivity and minimal matrix effect with an enrichment factor of 180-fold.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

The Swedish National Food administration listed acrylamide as a probable human carcinogen [1]. A large amount of acrylamide is detected in common food products such as French fries, potato crisp, breakfast cereal, and many other baked products. It was first discovered by a research group [2] that the concentration of acrylamide present in a range of fried and oven-cooked foods could go up to 5 mg/kg. It had become one of the most significant

controversies in food science ever since. Acrylamide was proven to have mutagenicity in rats, and it has been classified as a probable human carcinogen and a known human neurotoxin [3–5]. The Maillard reaction was believed to be the primary source of acrylamide found in food [6]. It involves the reaction between asparagine and a carbonyl source that will undergo a series of reactions to give acrylamide as a byproduct [7]. Acrylamide is usually found in high-temperature processed carbohydrate-rich and roasted foods [4]. In order to supply an energy source to pets, most dry pet foods are made of high-carbohydrate ingredients such as rice bran, oatmeal, millet, barley, and potatoes, and these foods are often prepared by baking. Previous studies suggested that dry pet foods may likely contain acrylamide [8].

A sample preparation process is necessary before the analysis of dry pet food samples. The concentration of acrylamide in pet food is expected to be low; it is necessary to have a preconcentration step before analysis. A commonly used technique is solid-phase extraction (SPE), which involves a large sample size volume [7]. Various extraction techniques have been reported for the determination of acrylamide in food samples: solid-phase microextraction (SPME) [9] and matrix solid-phase dispersion [10]. The SPME technique has limitations; the fibers are easily fragile and costly [11]. The SPE requires a large amount of sample and solvent, and the cartridges are expensive [8, 12]. Solvent-minimized extraction methods, dispersive liquid-liquid microextraction (DLLME) and liquid-phase microextraction (LPME), are other methods reported for acrylamide in food samples [13, 14]. A major limitation for the solvent-minimized approach is selecting suitable solvents for the polar target analytes [15]. Recently, electrospun polymer nanofiber-assisted extractions are reported in the literature for a wide range of target analytes [16–22]. All these reports showed considerable improvement in the extraction efficiency and recovery with a small sample size.

The main objective of this work is to demonstrate the applicability of the electrospun nanostructure membrane used in the microextraction method. After extraction, the quantification of acrylamide was performed by high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Chemicals and Reagents

The following polymers were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA): polyvinyl alcohol (PVA) (Mw 85,000–140,000), hydroxyethyl cellulose (HEC) (Mw 250,000), and polyvinyl chloride (Mw 120,000). Acrylamide was purchased from Bio-Rad Laboratories (Richmond, CA, USA). HPLC-grade acetonitrile was purchased from M. Tedia Company Inc. (Fairfield, IL, USA) and sulfuric acid from E. Merck (Germany). Ultrapure water was prepared on a Milli-Q (Milford, MA, USA) system. A standard stock solution of 100 mg/L of acrylamide was prepared in water. A working standard solution at 1 mg/L was used for low-concentration spiking. All solutions were stored at 4°C.

2.2. Electrospinning Setup

The electrospinning setup is self-assembled and consists of an electrospun control system, a DW-P403-1AC voltage supply unit, and a TS 2–60 multisyringe pump, all supplied by OptroBio Technologies Pte. Ltd. (Singapore). The collector was a conducting plate spread with aluminum foil for the collection of nanofibers formed.

2.3. Fabrication of the Nanostructured Membrane

2.3.1. Polystyrene (Mw 45,000)

Polystyrene solution for electrospinning was prepared by dissolving 50 wt. % PS in tetrahydrofuran (THF), and electrospinning was performed by applying a high voltage of 18 kV between the syringe needle containing the PS solution and the collector. The working distance between the tip of the needle and the collector was 10 cm. The flow rate of the polymer solution was maintained at 50 μ L/min.

2.3.2. Polyvinyl Alcohol

Polyvinyl alcohol solution for electrospinning was prepared by dissolving 15 wt. % PVA in water, and electrospinning was performed by applying a high voltage of 20 kV between the needle of the syringe containing the PVA solution and the collector. The working distance between the tip of the needle and the collector was 10 cm. The flow rate of the polymer solution was maintained at 10 μ L/min.

In addition to pure PVA nanofibers, it is also interesting to investigate different types of nanofibers formed by combining another compatible polymer with PVA. In this case, hydroxyethyl cellulose (HEC) was chosen; it was prepared by dissolving 5 wt. % HEC in water and mixed with 15 wt. % PVA in a 1:1 ratio with continuous stirring for at least 12 hrs, followed by electrospinning using the same condition as above to give PVA/HEC nanofibers. As PVA is highly soluble in water, an additional step was needed to make the PVA-based nanofibers insoluble in water used for microextraction with water as the medium. The additional step was the cross-linking of PVA nanofibers using glutaraldehyde in the presence of acid for 6 hours. In this additional step, the hydroxyl groups present in PVA and HEC cross-linked to form acetal ring groups or ether linkages, as a result, rendering them insoluble in water.

2.3.3. Polyvinyl Chloride

Polyvinyl chloride solution for electrospinning was prepared by dissolving 20 wt. % PVC in tetrahydrofuran (THF), and electrospinning was performed by applying a high voltage of 20kV between the needle of the syringe containing the PVC solution and the collector. The working distance between the tip of the needle and the collector was 10 cm. The flow rate of the polymer solution was maintained at 50 μ L/min.

2.4. Sample Preparation

All dry pet foods were purchased from local pet shops or supermarkets. Five grams of each sample were grounded in a commercial blender and 20 mL of water to obtain a homogenized suspension for 5 min. Another 10 mL of water was added, and the homogenates were left to stand in a water bath under agitation. After 30 min, the homogenates were centrifuged (12,000 rpm, 20 min) to sediment solid particles and filtered through Whatman No. 2 filter paper. The filtrates were transferred to a 50 mL volumetric flask and topped up to the mark with water. Any unused portions were stored at 4°C.

2.5. Microextraction Procedure

The initial extraction procedure was carried out on 10 mL of the 50 μ g/kg spike sample (acrylamide-free food sample) prepared in a 20 mL sample vial. A piece of nanostructured membrane weighing approximately 5 mg ($\approx 1 \times 1$ cm) was added to the sample solution together with a magnetic stirring bar. The sample was stirred for 30 min at 300 rpm for extraction to proceed. During extraction, the nanostructured membrane tumbled freely in the sample solution. After extraction, the nanostructured membrane was taken out from the sample solution with a pair of forceps and placed in a 500 μ L microvial together with 100 μ L of 0.01 M sulfuric acid desorbed via ultrasonication for 30 min. After desorption was done, the nanostructured membrane was removed. The extract was filtered through a 0.45 μ m (13 mm dia.) syringe filter unit before 10 μ L was injected into the HPLC system for analysis. All extractions were performed in triplicate ($n=3$) to calculate the repeatability.

2.6. HPLC Analysis

Analysis was carried out on a Shimadzu (Kyoto, Japan) HPLC system comprising an LC6AD binary pump, a DGU-14A degasser, an SPD-20A UV/vis spectrophotometric detector set at 200 nm, and a CBM-20A communication bus module. Data were collected and processed using LC Solution (Shimadzu) data analysis software. A PAH C₁₈ column (250 mm $\times$ 4.6 mm; 5 μ m) from Waters (Milford, MA, USA) was used with a mobile-phase flow rate of 0.6 mL/min. The mobile phase used was a 20–80% acetonitrile–0.01 M sulfuric acid mixture (pH of the mobile phase mixture was adjusted to 2). A column temperature of 25°C was maintained. 10 μ L of the sample was injected each time through a manual injector. No derivatization was needed before injection.

3. Results and Discussion

3.1. Morphology of the Nanostructured Membrane

The nanostructured membranes have a porous network with lightweight, making them suitable as a sorbent material for microextraction. Morphologies were observed under a JEOL JSM-6701F field-emission scanning electron microscope (FESEM). Morphologies that can be observed under the FESEM include the size of the individual fiber diameter by using the scale bar and also the uniformity in the size of the fibers formed. The surface of the fibers can also be examined under high magnification for the porosity. It can be told from the micrographs that PS nanofibers are not long and continuous (Figure 1(a)), which explains why the nanofibers obtained from PS are fragile and

brittle. Beaded structures were also observed (Figure 1(b)). As such, PS nanofibers were not suitable for use in microextraction (i.e., they disintegrated upon agitation under stirring).

[figures omitted; refer to PDF]

For PVP nanofibers, the fibers dissolved too quickly before any SEM images can be taken. Scanning electron micrographs of PAA nanofibers produced from 6 wt. % in DMF polymer solution were taken (Figure 2) before they began to dissolve. The nanofibers formed were very fine and sticky and were unsuitable for use in microextraction.

[figures omitted; refer to PDF]

The electrospinning conditions stated earlier result from their higher elasticity and ability to form membranes that remain. The duration for electrospinning of all the nanostructured membranes was maintained at 60 min. Even though the polymer solution's flow rate for PVA and PVA/HEC ($10\mu\text{L}/\text{min}$) was lower than PVC, the thickness of the membranes obtained was relatively constant. Figure 3 shows the scanning electron micrographs of the nanostructured membranes used in this project under various magnifications.

[figures omitted; refer to PDF]

Looking at the scanning electron micrograph of PVC nanofibers (Figure 4), it can be told from the higher-magnification ($\times 10,000$) micrograph that PVC nanofibers are highly porous which gives them a higher surface area for interaction with acrylamide.

[figures omitted; refer to PDF]

3.2. Optimization of Microextraction Conditions

Solid pet food samples were homogenized with ultrapure water and filtered and the filtered samples were used for optimization. The extraction conditions that affect the extraction efficiency were optimized one after another.

3.2.1. Selection of the Nanostructured Membrane as Sorbent Material

Extraction was first performed according to the conditions stated in Section 2.5. The first parameter to optimize was the choice of a suitable nanostructured membrane as a sorbent material. Three different nanostructured membranes from different polymer solutions were investigated. To maintain consistency in the extraction process, the weight of the membrane was kept constant. The extraction efficiency of the five different nanostructured membranes is summarized and shown in Figure 5.

[figure omitted; refer to PDF]

The PVC nanostructured membrane showed the highest extraction efficiency compared to the other membranes. The possible extraction mechanism might be the electrostatic interactions between chlorine in PVC and the nitrogen moiety in acrylamide. In the case of PVA and PVA/HEC membranes, hydrogen bonding might play a role in the extraction of acrylamide. Given the higher amount of water molecules present in the sample solution, it is inevitable that water molecules compete with acrylamide molecules for hydrogen-bonding sites with the polymer. Hence, the extraction efficiency of acrylamide was lowered for the PVA and PVA/HEC nanostructured membranes.

3.2.2. Extraction Time

As microextraction is equilibrium-based, the amount of time needed for equilibrium to establish has to be studied. The extraction time effect on the extraction efficiency was investigated by observing the peak area with an extraction time of 10, 20, 30, and 40 min. The analyte partition coefficient between the aqueous sample and the nanostructured membrane is an essential factor in determining the amount of analyte extracted by the nanostructured membrane. The extraction time profile (Figure 6) shows that the extraction efficiency directly proportional to peak area is optimum at an extraction time of 20 min and decreases subsequently with increased extraction time. One proposed explanation could be that, after the membrane has reached its maximum capacity at a longer extraction time, analytes might be desorbed from the nanostructured membrane, causing a decreased extraction efficiency. Similar behavior has been reported in many microextraction procedures. Another possible explanation can be attributed to the nanostructured membrane physical property: the nanofibers within the membrane are not firmly held together. Hence, the nanostructured membrane cannot withstand a more extended period of agitation, as a result, causing the extraction efficiency to be significantly reduced.

[figure omitted; refer to PDF]

It was observed that, at low pH (i.e., acidic medium), extraction efficiency is lowered, while there is no significant change at high pH (i.e., basic medium). Being an amide, acrylamide protonated at low pH, and thus, it prefers to stay in water as an ionic compound rather than interacting with PVC. There was no effect on extraction efficiency at higher pH, and hence, for the subsequent experiments, no adjustment of pH was needed.

The choice of desorption solvent to use was investigated by changing the desorption solvent used. Higher acrylamide was desorbed in 0.01 M sulfuric acid than 0.01 M hydrochloric acid (Figure 10). This could be due to the higher affinity of acrylamide for sulfuric acid than hydrochloric acid.

[figure omitted; refer to PDF]

Acrylamide is formed naturally at high temperatures in food/feedstuff rich in starch. Conventional liquid-liquid extraction followed by sample cleanup is the traditional method adopted in the industry [23]. The LLE method generates a large amount of toxic and expensive solvents. Thus, the cost of analyzing pet food samples is high, and search for cheaper methods for pet food samples is in demand. Recently, a simple electrochemical square-wave voltammetry method has been reported [24]. High detection limits are registered for the electrochemical process. The nanofiber extraction method is simple, cost-effective, and reusable for multiple extractions, and the small sample volume is sufficient to obtain low detection limits.

4. Conclusion

Three different nanostructured membranes were successfully prepared by the electrospinning procedure and utilized as a sorbent. A microextraction procedure was developed to quantitate acrylamide in pet food samples. The method showed recoveries (>79.7%) with low detection limits of parts per billion concentration range for complex pet samples. The polyvinyl chloride electrospun nanostructure membrane showed better performance over other membranes. The study strengthens the use of membranes for multiple applications. The PVC electrospun membranes were stable and reusable; no loss and no carryover were observed up to 20 times. This method can be further developed by producing different nanostructured membranes from different polymer solutions to extract other analytes present in food samples or our environment.

Ethical Approval

This article does not contain any studies with human participants or animals performed by the author.

Acknowledgments

The author gratefully acknowledges the funding support of the Deanship of Scientific Research at King Fahd University of Petroleum and Minerals (KFUPM) through the KFUPM-National University of Singapore collaboration project grant (Project no. KFUPM-NUS-06).

References

- [1] Swedish Nation Food Administration, Acrylamide, 2021. <https://www.livsmedelsverket.se/en/food-and-content/oonskade-amnen/akrylamid>
- [2] E. Tareke, P. Rydberg, P. Karlsson, S. Eriksson, M. Törnqvist, "Analysis of acrylamide, a carcinogen formed in heated foodstuffs," *Journal of Agricultural and Food Chemistry*, vol. 50 no. 17, pp. 4998-5006, DOI: 10.1021/jf020302f, 2002.
- [3] I. M. Andačić, A. Tot, M. Ivešić, A. Krivohlavek, R. Thirumdas, F. J. Barba, M. B. Sabolović, J. G. Kljusurić, S. R. Brnčić, "Exposure of the croatian adult population to acrylamide through bread and bakery products," *Food Chemistry*, vol. 322 no. 12677, 2020.
- [4] EC (European Commission), Commission Regulation of 20 November 2017 on Establishing Mitigation Measures and Benchmark Levels for the Reduction of the Presence of Acrylamide in Food, 2018.
- [5] J. Tabeshpour, S. Mehri, K. Abnous, H. Hosseinzadeh, "Neuroprotective effects of thymoquinone in acrylamide-induced peripheral nervous system toxicity through MAPKinase and apoptosis pathways in rat," *Neurochemical Research*, vol. 44 no. 5, pp. 1101-1112, DOI: 10.1007/s11064-019-02741-4, 2019.
- [6] D. V. Zyzak, R. A. Sanders, M. Stojanovic, D. H. Tallmadge, B. L. Eberhart, D. K. Ewald, D. C. Gruber, T. R. Morsch, M. A. Strothers, G. P. Rizzi, M. D. Villagran, "Acrylamide formation mechanism in heated foods," *Journal of Agricultural and Food Chemistry*, vol. 51 no. 64, pp. 4782-4787, DOI: 10.1021/jf034180i, 2003.

- [7] R. Bortolomeazzi, M. Munari, M. Anese, G. Verardo, "Rapid mixed mode solid phase extraction method for the determination of acrylamide in roasted coffee by HPLC-MS/MS," *Food Chemistry*, vol. 135 no. 4, pp. 2687-2693, DOI: 10.1016/j.foodchem.2012.07.057, 2012.
- [8] H. S. Canbay, M. Doğantürk, "Analysis of acrylamide in drinking water by SPE and GC-MS," *Applied Water Science*, vol. 9 no. 42, DOI: 10.1007/s13201-019-0918-8, 2019.
- [9] A. Nematollahi, M. Kamankesh, H. Hosseini, J. Ghasemi, F. Hosseini-Esfahani, A. Mohammadi, "Investigation and determination of acrylamide in the main group of cereal products using advanced microextraction method coupled with gas chromatography-mass spectrometry," *Journal of Cereal Science*, vol. 87, pp. 157-164, DOI: 10.1016/j.jcs.2019.03.019, 2019.
- [10] C. M. Soares, R. C. Alves, S. Casal, M. B. Oliveira, J. O. Fernandes, "Development and validation of a matrix solid-phase dispersion method to determine acrylamide in coffee and coffee substitutes," *Journal of Food Science*, vol. 75, pp. T57-T63, DOI: 10.1111/j.1750-3841.2010.01545.x, 2010.
- [11] C. Cagliero, T. D. Ho, C. Zhang, C. Bicchi, J. L. Anderson, "Determination of acrylamide in brewed coffee and coffee powder using polymeric ionic liquid-based sorbent coatings in solid-phase microextraction coupled to gas chromatography-mass spectrometry," *Journal of Chromatography A*, vol. 1449, DOI: 10.1016/j.chroma.2016.04.034, 2016.
- [12] F. Ning, T. Qiu, Q. Wang, H. Peng, Y. Li, X. Wu, Z. Zhang, L. Chen, H. Xiong, "Dummy-surface molecularly imprinted polymers on magnetic graphene oxide for rapid and selective quantification of acrylamide in heat-processed (including fried) foods," *Food Chemistry*, vol. 221, pp. 1797-1804, DOI: 10.1016/j.foodchem.2016.10.101, 2017.
- [13] M. Saraji, S. Javadian, "Single-drop microextraction combined with gas chromatography-electron capture detection for the determination of acrylamide in food samples," *Food Chemistry*, vol. 274, pp. 55-60, DOI: 10.1016/j.foodchem.2018.08.108, 2019.
- [14] N. Khorshidian, M. Yousefi, M. Shadnough, S. D. Siadat, M. Mohammadi, A. M. Mortazavian, "Using probiotics for mitigation of acrylamide in food products: a mini review," *Current Opinion in Food Science*, vol. 32, pp. 67-75, DOI: 10.1016/j.cofs.2020.01.011, 2020.
- [15] M. Pan, K. Liu, J. Yang, L. Hong, X. Xie, S. Wang, "Review of research into the determination of acrylamide in foods," *Foods*, vol. 9 no. 4, DOI: 10.3390/foods9040524, 2020.
- [16] Y. Xie, T. Zhang, Y. Chen, Y. Wang, L. Wang, "Fabrication of core-shell magnetic covalent organic frameworks composites and their application for highly sensitive detection of luteolin," *Talanta*, vol. 213, DOI: 10.1016/j.talanta.2020.120843, 2020.
- [17] B. Habib, P.-M. Hamed, R. Soroush, "Magnetic and electric field-assisted electrospun polyamide nanofibers for on-line μ -solid-phase extraction and HPLC," *RSC Advances*, vol. 4, pp. 52590-52597, 2014.
- [18] N. Eskandarpour, H. Sereshti, "Electrospun polycaprolactam-manganese oxide fiber for headspace-solid phase microextraction of phthalate esters in water samples," *Chemosphere*, vol. 191, pp. 36-43, DOI: 10.1016/j.chemosphere.2017.10.029, 2018.
- [19] J.-J. Xu, M. An, R. Yang, Z. Tan, J. Hao, J. Cao, L.-Q. Peng, W. Cao, "Determination of tetracycline antibiotic residues in honey and milk by miniaturized solid phase extraction using chitosan-modified graphitized multiwalled carbon nanotubes," *Journal of Agricultural and Food Chemistry*, vol. 64 no. 12, pp. 2647-2654, DOI: 10.1021/acs.jafc.6b00748, 2016.
- [20] N. Hajebi, S. Seidi, M. Ramezani, M. Manouchehri, "Electrospun polyamide/graphene oxide/polypyrrole composite nanofibers: an efficient sorbent for headspace solid phase microextraction of methamphetamine in urine samples followed by GC-MS analysis," *New Journal of Chemistry*, vol. 44 no. 34, pp. 14429-14437, DOI: 10.1039/d0nj03240a, 2020.
- [21] S. Seidi, M. Doroudian, "Electrospun NiFe layered double hydroxide/nylon 6 composite nanofibers as a sorbent for micro solid phase extraction by packed sorbent of non-steroidal anti-inflammatory drugs in human blood," *Journal of Chromatography A*, vol. 1614, DOI: 10.1016/j.chroma.2019.460718, 2020.

[22] S. Seidi, E. Sadat Karimi, A. Rouhollahi, M. Baharfar, M. Shanehsaz, M. Tajik, "Synthesis and characterization of polyamide-graphene oxide-polypyrrole electrospun nanofibers for spin-column micro solid phase extraction of parabens in milk samples," Journal of Chromatography A, vol. 1599, pp. 25-34, DOI: 10.1016/j.chroma.2019.04.014, 2019.

[23] H. H. Lim, H. S. Shin, "A new derivatization approach with d-cysteine for the sensitive and simple analysis of acrylamide in foods by liquid chromatography-tandem mass spectrometry," Journal of Chromatography A, vol. 1361, pp. 117-124, 2014.

[24] Y. Zhang, G. Y. Zhang, Y. Zhang, "Occurrence and analytical methods of acrylamide in heat-treated foods, review and recent developments," Journal of Chromatography A, vol. 1075, 2005.

DETAILS

Subject:	Polymers; Sample size; Polyvinyl chloride; Pet food; Nanostructure; Morphology; Acrylamide; Cellulose; Nanofibers; Solvents; Petfoods; Polyvinyl alcohol; Electrospinning; High performance liquid chromatography; Membranes; Hydroxyethyl celluloses; Correlation coefficients; Sorbents; Selectivity
Location:	United States--US
Editor:	Maria Jose Ruiz Angel
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-07-12 (Received); 2021-10-13 (Accepted); 2021-10-22 (Pub)

DOI: <https://doi.org/10.1155/2021/1285501>

ProQuest document ID: 2589571545

Document URL: <https://www.proquest.com/scholarly-journals/electrospun-membrane-extraction-acrylamide-pet/docview/2589571545/se-2?accountid=211160>

Copyright: Copyright © 2021 Chanbasha Basheer. This is an open access article distributed under the Creative Commons Attribution License (the “License”), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-11-01

Database: Publicly Available Content Database

Document 11 of 59

Formulation and development of Serratiopeptidase enteric coated tablets and analytical method validation by UV Spectroscopy

Panthi, Vijay Kumar ¹

; Jha, Saurav Kumar ² ; Chaubey, Raghvendra ³ ; Pangen, Rudra ⁴

¹ Department of Pharmacy, Tribhuvan University, Sunsari Technical College, Dharan, Sunsari, Nepal; Research & Development Department, Asian Pharmaceuticals, Rupandehi, Nepal; Research & Development Department, Corel Pharmaceuticals, Rupandehi, Nepal ² Himalayan Parenteral and Formulations, Janakpur, Nepal ³ Quality Assurance Department, Asian Pharmaceuticals, Rupandehi, Nepal ⁴ Department of Pharmaceutical Sciences, School of Health and Allied Sciences, Pokhara University, Pokhara, Nepal

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Serratiopeptidase (SRP) is a proteolytic enzyme that emerged as one of the most potent anti-inflammatory and analgesic drugs. The purpose of the present study was to formulate and evaluate enteric-coated tablets for SRP and investigate their stability using a simple and validated analytical method by ultraviolet (UV) spectroscopy. The colloidal silicon dioxide (2.50%), sodium starch glycolate (3.44%), and croscopolone (2.50%) were used as appropriate excipients for the development of core part of tablets. To protect the prepared tablets from acidic environment in the stomach, white shellac, castor oil, HPMC phthalate 40, and ethyl cellulose were used. The seal coating and enteric coating attained were 2.75% and 6.74%, respectively. SRP was found to be linear at 265 nm in the concentration range of 25–150 µg/mL. The results revealed that our developed method was linear ($R^2=0.999$), precise (RSD % = 0.133), and accurate (% recovery = 99.96–103.34). The formulated SRP tablets were found to be

stable under accelerated conditions as well as under room temperature for 6 months (assay %: >97.5%). The *in vitro* drug release study demonstrated that enteric-coated tablets were able to restrict SRP release in both acidic environments: 0.1 N HCl and simulated gastric fluid (pH 1.2). Moreover, at 60 minutes, the formulated SRP tablets revealed 13.0% and 8.98% higher drug release in phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8), respectively, compared to the marketed tablet formulation. This study concludes that enteric-coated tablets of SRP with higher drug release in the intestine can be prepared and examined for their stability using validated analytical technique of UV spectroscopy.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

A wide range of proteolytic enzymes mixtures have been used as supportive therapy in clinical condition such as trauma and orthopedic. There are several reports supporting the use of these proteolytic enzymes for the treatment of inflammatory disorders [1]. Moreover, these enzymes linked to metalloprotease family have been profitably assessed for their anti-inflammatory properties, which include trypsin, chymotrypsin, and serratiopeptidase (SRP) [2]. Chemically, SRP is an extracellular metalloprotease derived from the nonpathogenic enterobacteria *Serratia* E15 [3]. It consists of a polypeptide chain of 470 residues and a catalytic zinc ion per molecule with molecular weight of 45–60 kDa. SRP is normally recommended for oral administration at a dose of 5–10 mg three times a day [1]. This enzyme is rapidly and readily absorbed through the intestine and transported directly into the blood circulation [4]. SRP has been extensively used in Europe and Asia for more than 30 years but is relatively new in the United States and Canada. In addition, its anti-inflammatory property was first studied in Japan in 1967. Later, during the 1970s, these parenteral enzyme preparations were replaced by oral enteric-coated tablet form. During the 1980s and 1990s, this research was proposed to separately be conducted in Europe and Japan and demonstrated that SRP is the most effective agent in diminishing inflammation among all available enzyme formulations [3, 5]. Currently, SRP has been broadly used in Japan and Europe as the anti-inflammatory and analgesic agent of choice [6].

The most common problem associated with SRP oral delivery is high risk of enzymatic degradation in the gastrointestinal tract due to its proteinaceous nature. In addition, hydrophilic nature of SRP leads to low intestinal membrane permeability, due to which a very high dosage of SRP needs to be orally administered to elicit significant anti-inflammatory responses [7]. In order to increase the stability of SRP (reduced acid hydrolysis) and improve the oral bioavailability simultaneously, several techniques like delivering the SRP entrapped in Eudragit S100 microspheres, liposomal formulations, alginate gel encapsulated with SRP, chitosan-coated ceramic nanocores containing SRP, in situ cubic phase transforming system of glyceryl monooleate containing SRP, tetracycline-SRP-containing periodontal gel, and polar lipid-based lipospheres were approached previously [1]. Oral tablet dosage form with enteric coating and combination with controlled and sustained release property could be an effective way to decrease the frequency of dosing and increase the bioavailability of SRP [8–10]. Therefore, *in vitro* release profiles and *in vivo* efficacy are considered as important parameters to be studied for developing suitable delivery system. Another major challenge in developing a formulation of SRP is to retain its structural backbone, inhibiting the folding tendency during manufacturing, storage, digestion, and absorption in the stomach and intestines [11]. In order to determine the qualitative and quantitative evaluation of SRP, different analytical techniques have been advised including thin layer chromatography (TLC), X-ray powder diffractometry (XRPD), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), and ultraviolet (UV) spectroscopy. Meanwhile, some drawbacks still exist behind the successful application of these techniques. Although TLC technique is easy and fast for the qualitative measurement and identification of SRP, it has demerits like long plate running time and laboriousness. Furthermore, XRPD is very expensive, and the cost involved in purchase of the instrument and its upkeep may prevent its use for estimation of SRP as a simple routine tool. Moreover, although ELISA appears to be simple, it is very difficult to receive the antibodies against SRP. The cost involved to carry out a

single analysis is a factor for the technique being widely used. In addition, requirement of specific column and solvents may limit the adequate utilization of HPLC for SRP determination. On the contrary, many studies revealed that UV spectrophotometric method was found to be precise, accurate, robust, simple, and economical for the determination of SRP alone and in combination with other drugs including aceclofenac, diclofenac, and nimesulide [3]. In addition, one of the advantages of the application of UV method over other analytical techniques is that the UV method does not require time consuming treatments and procedures usually associated with chromatographic methods as well as rapid analytical techniques in the early phase of product development [12, 13]. The main objective of this research was to formulate stable enteric-coated tablets of SRP to prevent the degradation in gastric environment and develop and validate simple and cost-effective UV spectroscopy method for the determination of SRP.

2. Materials and Methods

2.1. Materials

Serratiopeptidase (SRP) powder was purchased from Biocon Limited, Bangalore, India. Isopropyl alcohol and methylene chloride were procured from Thermo Fisher GmbH, Karlsruhe, Germany. Microcrystalline cellulose powder (MCCP) pH 101 and 102, colloidal silicon dioxide, crospovidone, magnesium stearate, and stearic acid were purchased from Thermax Pvt. Ltd., India. Hydroxypropyl methyl cellulose (HPMC) phthalate 40 was obtained from Shandong Head Co., Ltd., China. The other coating materials were obtained as a gift sample from Colorcon Asia Pvt. Ltd, Industrial Estate, Goa, India. Marketed enteric-coated tablet formulation of SRP was procured from local medical store and used as a reference sample. All the reagents and solvents used in the analysis were of analytical grade.

2.2. Instrumentation

The following instruments were used in the formulation and analysis of prepared enteric-coated tablets: dissolution study was performed using dissolution tester (Electrolab, model: EDT-14XL), UV/visible spectrophotometer (Agilent Technologies, model: Cary-60 UV-Vis), tray dryer (Techmac Engineering Works, Azadpur, Delhi-33, India), compression machine (Fluidpack, Machine No. 145, Ahmedabad, India), analytical balance (Ohaus Corporation, model: PA423C, USA), thickness and diameter (Electronic Express, model: 0604CAL6++), hardness tester (Electrolab, EH-01P), and coating machine (Captech Systems, model: CCP-12).

2.3. Analytical Method Development

2.3.1. Determination of Wavelength of Maximum Absorption of SRP

The 25–150 $\mu\text{g/mL}$ of concentration range of SRP was dissolved in deionized (DI) water and the UV-visible spectrum of SRP was examined using UV spectrophotometer between 200 and 400 nm. Next, the derivative spectra of SRP at various concentrations were recorded. The interfering effect of all excipients on the maximum absorption was assessed by scanning the spectrum of each excipient alone and in combination with SRP. The maximum absorbance was observed at 265 nm.

2.3.2. Preparation of Standard Stock Solution of SRP

Standard solution was prepared by weighing 50 mg of SRP accurately and then was transferred to a 100 mL volumetric flask and dissolved by adding 25 mL of DI water. The flask was allowed to sonicate for 15 minutes and volume was made up to the mark with DI water to give a solution containing 500 $\mu\text{g/mL}$ of SRP.

2.3.3. Determination of Calibration Curve of SRP

The required volume from the standard stock solution of SRP was withdrawn and transferred to a 10 mL volumetric flask. The final volume was adjusted up to the mark with DI water to obtain different concentration of SRP including 25, 50, 75, 100, 125, and 150 $\mu\text{g/mL}$. The calibration curve of concentration versus absorbance was plotted and then straight-line equation was determined.

2.3.4. Analysis of Formulated and Commercial Tablet Formulation

The developed method was also applied to determine SRP in formulated and marketed tablets. An average weight of SRP tablet equivalent to 50 mg was taken in 50 mL volumetric flask and volume was made up to the mark with diluents. From this, 5 mL was taken and transferred to a 50 mL volumetric flask and the final volume was made up to

the mark with diluent to give a final concentration of 100 µg/mL. The absorption was recorded at 265nm and the concentrations of the drug were calculated from the linear regression equation. The percentage assay and relative standard deviation (RSD %) of the SRP in tablets were calculated in triplicates.

2.4. Analytical Method Validation

2.4.1. Linearity

The linearity and range of the developed method were analyzed by measuring the absorption of a series of SRP standard solutions (25–150 µg/mL) at λ_{max} of 265nm. The absorption of these standard solutions was plotted against different concentration. The regression line equation and the square of the correlation coefficient (R^2) were calculated.

2.4.2. Accuracy

In this research, the accuracy of the method was determined by addition of standard drug to the sample at three different concentration levels (50, 100, and 150%) taking into consideration percentage purity of added bulk drug samples. It was determined by calculating the recovery of SRP by standard addition method. In addition, accuracy of the developed method in SRP enteric-coated tablet formulation was studied by the analysis of spiked samples at different concentrations as mentioned above.

2.4.3. Precision

Precision studies were performed by analyzing standard solutions containing 50, 100, and 150 µg/mL on three different days by two different analysts as an interday precision. The absorbance of the solutions was examined and then standard deviation (SD) and RSD % were calculated. Moreover, to determine intraday precision, standard solutions containing 50, 100, and 150 µg/mL of SRP were analyzed three times on the same day. Next, absorbance of the solutions was examined and standard deviation (SD) and RSD % were calculated.

2.4.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The calibration curve was repeated for 6 times and SD of the intercepts was calculated using the following formula:

(1) $\text{LOD} = 3.3 \times \text{SDSlope}$, (2) $\text{LOQ} = 10 \times \text{SDSlope}$,

where SD is standard deviation of Y- intercept of 6 calibration curves.

Furthermore, $\text{SD intercept} = \text{SE intercept} \times \sqrt{N}$.

N is number of test samples.

Slope is the mean slope of the 6 calibration curves [14, 15].

2.4.5. Robustness

The robustness of the method was evaluated by examining the different mixture of same concentration of samples to find out the effect of slight changes on absorption at wavelengths of 265 ± 1.0 , and then average mean, SD, and RSD % of absorbance were determined.

2.5. Formulation and Development of SRP Tablets

The core part of SRP tablets was prepared by dry mixing process followed by slugging using roller compactor prior to improving the powder flow properties of granules. This process was performed without applying heat to avoid thermal denaturation. Slugging is a dry granulation that uses mechanical compression (slugs) or compaction (roller compaction) to facilitate the agglomeration of dry powder particles. The conformational changes in proteins that occur when they are heated are referred to as denaturation [16, 17]. Before commencement of manufacturing process, MCCP pH 101 and 102 and crospovidone were allowed to dry at 100°C for 30 minutes. After drying, MCCP pH 101 and 102 and crospovidone were sieved through 30 meshes and half quantities of three excipients (colloidal silicon dioxide, purified talc, and stearic acid) were sieved through 60 meshes and mixed properly for 10 minutes. Subsequently, mixed powder was sieved through roller compactor as a slugging process and materials were passed through 20 meshes. Finally, remaining amounts of colloidal silicon dioxide, purified talc, and stearic acid were sifted through 60 meshes for lubrication. Consequently, lubricated granules were compressed in 8-station rotary compression machine using dies and punch having 7.4 mm diameter without break line.

To seal-coat the tablet, white shellac was dissolved in isopropyl alcohol (IPA) with continuous stirring for about 30 minutes and then castor oil was added in it and it was allowed to stir for 10 minutes. For preparation of enteric

coating solution, HPMC phthalate 40 was dispersed in IPA with continuous stirring for 10 minutes and then methylene chloride was added in it followed by stirring continuously for 30 minutes prior to dissolving. Subsequently, ethyl cellulose (EC), castor oil, titanium dioxide, and Lake Erythrosine were added in this solution with regular stirring approximately for 30 minutes. In this study, coating of tablets was done using a side-vented, perforated pan coating apparatus machine. Firstly, weighed quantity (about 1.0kg) of tablets was kept in the coating pan which was preadjusted at 40°C for 5–10 minutes and actual weight of tablet was evaluated. Secondly, tube was put in the coating solution, and then the various parameters including spray rate (8–20 mL/min), inlet air temperature (20–50°C), atomizing air pressure (1–3 bar), and the rotating speed of coating pan (8–15 rpm) were adjusted and optimized. Furthermore, after completion of coating, tablets were kept in the pan at 37°C and 3 rpm for curing. Finally, coated tablets were taken from the pan and the various parameters were assessed. Although EC is not an enteric coating material, this excipient was used in this study owing to its binding properties to enhance the hardness of tablet as well as to avoid drug release in gastric region. EC is water-insoluble cellulose having various appealing characteristics including biocompatibility, gastroresistance, and degradation to nontoxic and readily excreted components. This material is widely applied in pharmaceutical technology because it is convenient for processing alone or in combination with other excipients such as plasticizers or other polymers [18]. Furthermore, due to the potentiality of EC to modify release rate of drug, it is extensively utilized as a coating agent. A study done by Shah et al. developed a colon targeted multiunit formulation of metronidazole using EC and Eudragit® S 100 as coating polymers to prevent initial drug release in the gastric region. The drug release was remarkably hindered by the outer EC-based coating layer [19]. Moreover, EC, on the other hand, has ethoxy functional groups with a very low propensity to react chemically. This polymer, then, could provide the formulator good binding characteristics with low chemical reactivity [20]. The detailed composition of the tablet formulation is mentioned in Table 1 and the overall procedure was illustrated in flowchart shown in Figure 1.

Table 1

Composition of formulated tablets.

S. No.	Materials (mg/tablet)	F1	F2	F3	F4	F5
1.	Serratiopeptidase	10.50	10.50	10.50	10.50	10.50
2.	MCCP pH 101	141.00	—	—	—	—
3.	MCCP pH 102	—	139.50	132.50	132.50	132.50
4.	Colloidal silicon dioxide	2.00	2.50	4.00	4.00	4.00
5.	Sodium starch glycolate	3.50	4.50	5.50	5.50	5.50
6.	Crospovidone	—	—	4.00	4.00	4.00
7.	Magnesium stearate	1.50	1.50	2.00	2.00	2.00
8.	Stearic acid	1.50	1.50	1.50	1.50	1.50
Total weight (uncoated)		160.00	160.00	160.00	160.00	160.00
—						

Seal coating materials						
1.	White shellac	—	—	5.10	5.70	6.37
2.	Castor oil	—	—	0.062	0.062	0.062
3.	Isopropyl alcohol	—	—	105.00	105.00	105.00
—						
Enteric coating materials						
1.	HPMC phthalate 40	—	—	8.00	8.00	13.44
2.	Ethyl cellulose	—	—	2.74	2.74	3.820
3.	Castor oil	—	—	0.47	0.47	0.97
4.	Titanium dioxide	—	—	0.55	0.55	0.55
5.	Lake Erythrosine	—	—	0.11	0.11	0.11
6.	Isopropyl alcohol	—	—	85.00	85.00	85.00
7.	Methylene chloride	—	—	150.00	150.00	150.00
Targeted weight in mg (coated)		—	—	170.00	170.00	170.00

[figure omitted; refer to PDF]

2.6. Loss on Drying (LOD) of Granules

After successful drying, approximately 5 gm sample was taken from tray dryer and poured in moisture analyzer (Sartorius MA37) containing weighing pan and distributed over its surface area and finally allowed to heat at 80°C. Then LOD value of dried granules was reported from moisture analyzer.

2.7. Carr's Index and Hausner Ratio of Lubricated Granules

After complete drying of moistened granules, lubricants were added and mixed properly for 5 minutes in order to enhance the flow properties of lubricated granules. The 10gm sample of lubricated granules was poured in 100mL measuring cylinder and tapped 100 times. Next, Carr's index and Hausner ratio of lubricated granules were determined. The ratio of tapped density to bulk density of the powders is called the Hausner ratio [21].

2.8. Weight Variation and Physical Specification

Weight variation of the formulated tablets was evaluated according to USP method recommended for uncoated tablets. This parameter is performed by weighing 20 tablets separately and if it satisfies the criteria approved by official pharmacopoeia, test will be considered successful [15]. Tablet physical specifications such as hardness were evaluated by digital portable hardness tester (Electrolab, Model EH-01P), while thickness and diameter were examined by Vernier caliper (Electronic Express, model: 0604CAL6++). The test was performed on ten tablets; the average reading was assigned as the tablet hardness, thickness, and diameter specifications of the formulated tablets. In addition, for appearance examination, twenty tablets of each formulation were randomly selected to check any discoloration or degradation of drug in the tablets by visual method. If any discoloration or black spots appear, it

shows the degradation or decomposition of drug in the tablet formulation [22].

2.9. Determination of Content Uniformity

According to European Pharmacopeia (Ph. Eur.), for the assessment of content uniformity, 10 tablets were subdivided and ten different subdivided parts were randomly selected. The active content in each part was determined using a suitable analytical method. Ten tablets were finely powdered; amount of the powder equivalent to 10.0mg of SRP was accurately weighed and transferred to a 100mL of volumetric flask. Subsequently, the flask was filled with phosphate buffer pH 6.8 and mixed thoroughly. The volume make-up was done up to the mark with the diluent to give a final concentration of 100 µg/mL and filtered. Moreover, the absorbance of the resulting solution was measured at 265.0nm using UV spectroscopy. Finally, the linearity equation obtained from calibration curve as mentioned previously was used for the estimation of SRP in the tablet formulations. The preparation complies with the test if each individual content is between 85.0% and 115.0% of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75.0–125.0% of the average content. If one individual content is outside the limits of 85.0–115.0% but within the limits of 75.0–125.0%, the individual contents of 20 other units (subdivided tablet parts) taken at random are determined. The preparation complies with the test if not more than one of the individual contents of the 30 units is outside 85.0–115.0% of the average content and none are outside the limits of 75.0–125.0% of the average content [23].

2.10. *In Vitro* Dissolution Studies

2.10.1. Drug Release Profile in 0.1 N HCl (pH 1.2) and Phosphate Buffer (pH 6.8)

The dissolution of formulated SRP enteric-coated tablets was examined as per the USP and International Conference on Harmonization (ICH) guidelines. This study was performed using USP apparatus 2 (paddle). The drug release study was carried out in two different dissolution media, 0.1 N HCl (pH 1.2) and phosphate buffer (pH 6.8), according to USP. After temperature reached $37 \pm 0.5^\circ\text{C}$, single tablet was added individually to separate dissolution dishes containing 900mL of dissolution medium and run for 60 minutes at 50rpm. Subsequently, 5mL of the solution was withdrawn using syringes from each dissolution container at predetermined time intervals of 15, 30, 45, and 60 minutes. After each withdrawal, 5mL of fresh dissolution media was added to maintain sink condition. The mean value obtained from triplicate measurements was taken to assess the percentage of dissolved SRP using the following formula [24]: $(\%) \text{ of dissolved SRP} = \frac{\text{actual amount of released SRP}}{\text{theoretical amount of SRP}} \times 100$. The comparative evaluation of dissolution was established on the readings of the evaluated similarity factor (f_2) and dissimilarity factor (f_1) of the dissolution profile for the formulated and marketed SRP tablets. Moreover, the values for f_1 and f_2 were examined using (4) and (5), respectively. The f_2 factor evaluates the vicinity between two release profiles and similarly f_1 also calculates the difference between two profiles. R_t and T_t in the equations represent the percentages of drug dissolved at each time point for the reference (market tablet) and test (formulated tablet), respectively. The f_1 value higher than 15 demonstrates significant dissimilarity, while the f_2 value more than 50 reveals significant similarity [15, 24–26]. $(4) f_1 = \sum_{t=1}^n |R_t - T_t| \times 100$, $(5) f_2 = 50 \cdot \log \frac{1 + \sum_{t=1}^n |R_t - T_t|^2}{\sum_{t=1}^n |R_t - T_t|^2} \times 100$.

2.10.2. Drug Release Profile in Simulated Gastric Fluid (pH 1.2) and Simulated Intestinal Fluid (pH 6.8)

The release assessment of SRP enteric-coated tablets applied a calibrated dissolution apparatus with paddles at 50 rpm, and the bath temperature was maintained at $37 \pm 0.5^\circ\text{C}$. It is suggested that, for all the enteric-coated tablets, they must tolerate simulated gastric fluid (pH 1.2) in the initial 2h followed by 1h in simulated intestinal fluid (pH 6.8) according to the standard method, since SRP will hydrolyze in aqueous solution. Therefore, in this study, we employed simulated intestinal fluid (pH 6.8) that consisted of 0.25% sodium dodecyl sulphate (SDS) as release medium. For preparation of simulated gastric fluid, NaCl (3g) was dissolved in about 1450 mL of DI water with continuous stirring and then pH was adjusted to 1.2 ± 0.1 with diluted HCl. Furthermore, the simulated intestinal fluid was also composed by dissolving potassium phosphate monobasic (10.2g) and SDS (3.75g) in a same 1000mL of DI water and then pH adjusted to 6.8 ± 0.1 with 1 N NaOH. Finally, the volume make-up of each prepared fluid was done up to 1500mL with DI water [27].

(a) Tolerance test in simulated gastric fluid

(i) Tolerance test of SRP enteric-coated tablets was performed in 250 mL simulated gastric fluid (pH 1.2) with a paddle rotation of 50 rpm and with the bath temperature of 37°C. After 2 h, 5 mL sample was taken by syringes from each vessel and filtered through a nylon filter (0.45 µm, 25 mm). After each withdrawal, same volume (5 mL) of fresh dissolution media was added to maintain sink condition. The mean value obtained from triplicate measurements was taken to assess the percentage of dissolved SRP using the formula mentioned in equation (1).

(b) Release profile in simulated intestinal fluid

The release test of the enteric-coated tablets required enduring for 2 h in 250 mL of simulated gastric fluid, and the release amount was determined in 250 mL of the simulated intestinal fluid. Sample was withdrawn from each dissolution apparatus at 15, 30, 45, and 60 min during dissolution in simulated intestinal fluid, and the same amount of release medium was added promptly. Moreover, the withdrawn sample solutions were filtered through a nylon filter (0.45 µm, 25 mm), and then the release percentage of SRP enteric-coated tablets in simulated intestinal fluid (pH 6.8) was determined and calculated according to the formula mentioned in equation (1).

2.11. Accelerated Stability Studies

Stability studies were carried out on optimized formulation according to ICH guidelines. The formulated tablet packed in blister strips was subjected to accelerated stability testing for 6 months as per ICH norms at a temperature of $40 \pm 2^\circ\text{C}$ and relative humidity of $75 \pm 5\%$. Samples were taken at regular time intervals of 1 month for over a period of 6 months and analyzed for the change in color, hardness, drug content, and *in vitro* drug release in acid media (0.1 N HCl) by procedure stated earlier. Any changes in evaluation parameters, if observed, were noted [28]. Tests were carried out in triplicates and mean value of the observed values was noted along with standard deviation.

3. Statistical Analyses

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The data were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to indicate statistical significance. All results are expressed as mean $\pm$ standard deviation.

4. Results and Discussion

4.1. Analytical Method Validation

4.1.1. Linearity

The SRP was found to be linear at 265 nm in the concentration range of 25–150 µg/mL in water, which was demonstrated in calibration curve. The obtained correlation coefficient (R^2) and regression equation were 0.999 and $Y = 0.001x + 0.005$, respectively. The linearity range of SRP is shown in Figure 2. Furthermore, Nirale and Menon in 2010 studied the stability of SRP over a period of 12 h in DIW and Tris and phosphate buffer at room temperature and 37°C. The study that measured enzyme activity using proteolytic activity measurement method demonstrated retention of SRP enzyme activity of higher than 90% in Tris and phosphate buffer at both temperatures for at least 8 h. However, the stability of SRP was significantly decreased in DIW [2].

[figure omitted; refer to PDF]

In addition, *in vitro* release profile of SRP from enteric-coated tablets was carried out in simulated gastric fluid (pH 1.2) for 2 h and findings are revealed in Table 11. The percentage of the residual content of SRP in enteric-coated tablets was determined in accordance with its initial amount. The results demonstrated that little amount of SRP was released from formulated enteric-coated tablets within 2 h of agitation. However, percentage of SRP released in simulated gastric fluid (pH 1.2) was 1.023-fold lower than market sample. Furthermore, no evidence of disaggregation, cracking, or melting was observed at the surface of enteric-coated tablets in simulated gastric fluid solution. Moreover, release profile of SRP from enteric-coated formulation and market sample over the period of 60 minutes in simulated intestinal fluid (pH 6.8) is revealed in Figure 8. Both samples showed significant release profile until 60 minutes. At 60 min, the formulated SRP tablets and market sample demonstrated 98.22% and 89.24% of drug release, respectively. In this medium, SRP tablets showed 8.98% higher dissolution as compared to market sample at 60 minutes. Interestingly, there was no significant variation in release profile of both formulated SRP and market sample in both phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8). Thus, the dissolution data showed that there was no statistical significance of drug release ($P > 0.05$) from 15 to 60 minutes between the

formulated tablets and the marketed one in simulated intestinal fluid (pH 6.8) medium. Moreover, in this study, similarity factor (f_2) and dissimilarity factor (f_1) of optimized SRP enteric-coated tablets and market sample were determined in both phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8). The results of similarity factor (f_2) applied in phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8) were 51.14 and 54.22, respectively. Therefore, it was concluded that optimized formulation of SRP enteric-coated tablets has shown good similarity (more than 50) with market sample. Furthermore, findings of dissimilarity factor (f_1) in phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8) were 16.61 and 16.67, respectively. Thus, it was also further justified that an f_1 value greater than 15 indicates significant dissimilarity between optimized SRP formulation and reference sample. In addition, SRP is soluble in water; therefore, in order to prevent it from hydrolysis during the preparation of enteric-coated tablets, it is suggested to adopt dry granulation or ethanol granulation method.

Table 11

The percentage of the residual content of SRP enteric-coated tablets after agitation in simulated gastric fluid (pH 1.2) for 2h.

Samples tested	Times (%)						Average (%)
1	2	3	4	5	6	SRP formulated tablets	98.40
99.14	98.35	99.24	98.12	97.69	98.49	Market sample	98.22

[figure omitted; refer to PDF]

4.2.7. Determination of Assay

The assay percentage of SRP was analyzed as per the procedure mentioned in Section 2.3.4. During day 1, the calculated assay for room temperature and accelerated analysis were 101.82 and 101.64%, respectively. Until 6 months of stability, both room temperature and accelerated stability analysis exhibited assay % within the limit. There was no significant degradation in assay throughout the stability period. The corresponding figures for room temperature and accelerated stability were 99.32 and 97.91%, respectively, in 6th month's analysis. The overall mean RSD % was 1.51%. The detailed observations of assay are demonstrated in Figure 9.

[figure omitted; refer to PDF]

5. Conclusions

In this study, enteric-coated tablet formulation of SRP was prepared and a simple validated UV method was developed for analytical determination and quantification of SRP in formulated tablets. The drug release profile of our formulated tablet at phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8) was relatively higher than the marketed sample of SRP tablet. The results of initial and accelerated stability until 6 months revealed that the formulated tablets were both physically and chemically stable. However, still adequate studies based on biological stability and hydrolysis are suggested to verify the stability and efficacy. From this study, it was concluded that, by employing commonly available pharmaceutical excipients such as diluents, superdisintegrants, and hydrophilic and hydrophobic polymers, a commercial enteric-coated tablet of SRP could be developed successfully. This study can contribute as a guide to the pharmaceutical companies to manufacture SRP containing dosage forms and apply validated analytical methods to assess its product quality.

Acknowledgments

The authors are thankful to Asian Pharmaceuticals, Rupandehi, Nepal, for providing opportunity to conduct this

research.

References

- [1] U. A. Shinde, S. S. Kanojiya, "Serratiopeptidase niosomal gel with potential in topical delivery," *Journal of Pharmaceutics*, vol. 2014, DOI: 10.1155/2014/382959, 2014.
- [2] N. M. Nirale, M. D. Menon, "Topical formulations of serratiopeptidase: development and pharmacodynamic evaluation," *Indian Journal of Pharmaceutical Sciences*, vol. 72 no. 1, pp. 65-71, DOI: 10.4103/0250-474X.62246, 2010.
- [3] V. Gupte, U. Luthra, "Analytical techniques for serratiopeptidase: a review," *Journal of Pharmaceutical Analysis*, vol. 7 no. 4, pp. 203-207, DOI: 10.1016/j.jpha.2017.03.005, 2017.
- [4] P. Rajaram, A. Bhattacharjee, S. Ticku, "Serratiopeptidase-a cause for spread of infection," *Journal of Clinical and Diagnostic Research: Journal of Clinical and Diagnostic Research*, vol. 10 no. 8, DOI: 10.7860/JCDR/2016/21388.8302, 2016.
- [5] S. Bhagat, M. Agarwal, V. Roy, "Serratiopeptidase: a systematic review of the existing evidence," *International Journal of Surgery*, vol. 11 no. 3, pp. 209-217, DOI: 10.1016/j.ijssu.2013.01.010, 2013.
- [6] M. Tiwari, "The role of serratiopeptidase in the resolution of inflammation," *Asian Journal of Pharmaceutical Sciences*, vol. 12 no. 3, pp. 209-215, DOI: 10.1016/j.ajps.2017.01.003, 2017.
- [7] M. Rawat, S. Saraf, S. Saraf, "Influence of selected formulation variables on the preparation of enzyme-entrapped Eudragit S100 microspheres," *AAPS PharmSciTech*, vol. 8 no. 4, DOI: 10.1208/pt0804116, 2007.
- [8] M. Rawat, D. Singh, S. Saraf, S. Saraf, "Development and in vitro evaluation of alginate gel-encapsulated, chitosan-coated ceramic nanocores for oral delivery of enzyme," *Drug Development and Industrial Pharmacy*, vol. 34 no. 2, pp. 181-188, DOI: 10.1080/03639040701539479, 2008.
- [9] M. H. Shah, A. Paradkar, "Cubic liquid crystalline glyceryl monooleate matrices for oral delivery of enzyme," *International Journal of Pharmaceutics*, vol. 294 no. 1-2, pp. 161-171, DOI: 10.1016/j.ijpharm.2005.01.019, 2005.
- [10] M. Maheshwari, G. Miglani, A. Mali, A. Paradkar, S. Yamamura, S. Kadam, "Development of tetracycline-serratiopeptidase-containing periodontal gel: formulation and preliminary clinical study," *AAPS PharmSciTech*, vol. 7 no. 3, DOI: 10.1208/pt070376, 2006.
- [11] S. B. Jadhav, N. Shah, A. Rathi, V. Rathi, A. Rathi, "Serratiopeptidase: insights into the therapeutic applications," *Biotechnology Reports*, vol. 28, DOI: 10.1016/j.btre.2020.e00544, 2020.
- [12] S. M. Dhole, N. D. Amnerkar, P. B. Khedekar, "Comparison of UV spectrophotometry and high performance liquid chromatography methods for the determination of repaglinide in tablets," *Pharmaceutical Methods*, vol. 3 no. 2, pp. 68-72, DOI: 10.4103/2229-4708.103875, 2012.
- [13] J. Almeida, M. Bezerra, D. Markl, A. Berghaus, P. Borman, W. Schlindwein, "Development and validation of an in-line API quantification method using AQbD principles based on UV-vis spectroscopy to monitor and optimise continuous hot melt extrusion process," *Pharmaceutics*, vol. 12 no. 2, DOI: 10.3390/pharmaceutics12020150, 2020.
- [14] Ü. Şengül, "Comparing determination methods of detection and quantification limits for aflatoxin analysis in hazelnut," *Journal of Food and Drug Analysis*, vol. 24 no. 1, pp. 56-62, DOI: 10.1016/j.jfda.2015.04.009, 2016.
- [15] M. N. Abualhasan, J. Mansour, N. Jaradat, A. N. Zaid, I. Khadra, "Formulation and development of a validated UV-spectrophotometric analytical method of rutin tablet," *International Scholarly Research Notices*, vol. 2017, DOI: 10.1155/2017/2624947, 2017.
- [16] S. Shanmugam, "Granulation techniques and technologies: recent progresses," *BiolImpacts: BI*, vol. 5 no. 1, pp. 55-63, DOI: 10.15171/bi.2015.04, 2015.
- [17] A. Strzelczak, J. Balejko, M. Szymczak, A. Witczak, "Effect of protein denaturation temperature on rheological properties of baltic herring (*Clupea harengus membras*) muscle tissue," *Foods*, vol. 10 no. 4, DOI: 10.3390/foods10040829, 2021.
- [18] K. Wasilewska, K. Winnicka, "Ethylcellulose-A pharmaceutical excipient with multidirectional application in drug dosage forms development," *Materials*, vol. 12 no. 20, DOI: 10.3390/ma12203386, 2019.
- [19] N. Shah, O. P. Sharma, T. Mehta, A. Amin, "Design of experiment approach for formulating multi-unit colon-

- targeted drug delivery system: in vitro and in vivo studies," *Drug Development and Industrial Pharmacy*, vol. 42 no. 5, pp. 825-835, DOI: 10.3109/03639045.2015.1082581, 2016.
- [20] R. P. Desai, S. H. Neau, S. I. Pather, T. P. Johnston, "Fine-particle ethylcellulose as a tablet binder in direct compression, immediate-release tablets," *Drug Development and Industrial Pharmacy*, vol. 27 no. 7, pp. 633-641, DOI: 10.1081/ddc-100107319, 2001.
- [21] R. B. Shah, M. A. Tawakkul, M. A. Khan, "Comparative evaluation of flow for pharmaceutical powders and granules," *AAPS PharmSciTech*, vol. 9 no. 1, pp. 250-258, DOI: 10.1208/s12249-008-9046-8, 2008.
- [22] J. A. Seitz, G. M. Flessland, "Evaluation of the physical properties of compressed tablets I," *Journal of Pharmaceutical Sciences*, vol. 54 no. 9, pp. 1353-1357, DOI: 10.1002/jps.2600540926, 1965.
- [23] M. T. am Ende, S. K. Moses, A. J. Carella, R. A. Gadkari, T. W. Graul, A. L. Otano, R. J. Timpano, "Improving the content uniformity of a low-dose tablet formulation through roller compaction optimization," *Pharmaceutical Development and Technology*, vol. 12 no. 4, pp. 391-404, DOI: 10.1080/10837450701369253, 2007.
- [24] J. Rivera-Leyva, M. García-Flores, A. Valladares-Méndez, L. Orozco-Castellanos, M. Martínez-Alfaro, "Comparative studies on the dissolution profiles of oral ibuprofen suspension and commercial tablets using biopharmaceutical classification system criteria," *Indian Journal of Pharmaceutical Sciences*, vol. 74 no. 4, pp. 312-318, DOI: 10.4103/0250-474x.107062, 2012.
- [25] J. Emami, "In vitro-in vivo correlation: from theory to applications," *Journal of Pharmacy & Pharmaceutical Sciences: a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques*, vol. 9 no. 2, pp. 169-189, 2006.
- [26] V. Raju, K. V. Murthy, "Development and validation of new discriminative dissolution method for carvedilol tablets," *Indian Journal of Pharmaceutical Sciences*, vol. 73 no. 5, pp. 527-536, DOI: 10.4103/0250-474X.99000, 2011.
- [27] X.-M. Pan, J. Li, R. Gan, X.-N. Hu, "Preparation and in vitro evaluation of enteric-coated tablets of rosiglitazone sodium," *Saudi Pharmaceutical Journal*, vol. 23 no. 5, pp. 581-586, DOI: 10.1016/j.jsps.2015.02.018, 2015.
- [28] J. R. Dixon, "The international conference on harmonization good clinical practice guideline," *Quality Assurance*, vol. 6 no. 2, pp. 65-74, DOI: 10.1080/105294199277860, 1998.
- [29] M. Maleque, M. R. Hasan, F. Hossen, S. Safi, "Development and validation of a simple UV spectrophotometric method for the determination of levofloxacin both in bulk and marketed dosage formulations," *Journal of Pharmaceutical Analysis*, vol. 2 no. 6, pp. 454-457, DOI: 10.1016/j.jpha.2012.06.004, 2012.
- [30] A. B. Nair, R. Gupta, R. Kumria, S. Jacob, M. Attimarad, "Formulation and evaluation of enteric coated tablets of proton pump inhibitor," *Journal of Basic and Clinical Pharmacy*, vol. 1 no. 4, pp. 215-221, 2010.
- [31] G. Crotts, A. Sheth, J. Twist, I. Ghebre-Sellassie, "Development of an enteric coating formulation and process for tablets primarily composed of a highly water-soluble, organic acid," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 51 no. 1, pp. 71-76, DOI: 10.1016/s0939-6411(00)00129-6, 2001.
- [32] S. Roy, "A systemic approach on understanding the role of moisture in pharmaceutical product degradation and its prevention: challenges and perspectives," *Biomedical Research-tokyo*, vol. 29, pp. 3336-3343, DOI: 10.4066/biomedicalresearch.29-18-978, 2018.
- [33] A. N. Zaid, R. J. Al-Ramahi, A. A. Ghoush, A. Qaddumi, Y. A. Zaaror, "Weight and content uniformity of lorazepam half-tablets: a study of correlation of a low drug content product," *Saudi Pharmaceutical Journal*, vol. 21 no. 1, pp. 71-75, DOI: 10.1016/j.jsps.2011.12.009, 2013.
- [34] M. Saravanan, K. S. Nataraj, K. S. Ganesh, "The effect of tablet formulation and hardness on in vitro release of cephalexin from Eudragit L100 based extended release tablets," *Biological and Pharmaceutical Bulletin*, vol. 25 no. 4, pp. 541-545, DOI: 10.1248/bpb.25.541, 2002.

DETAILS

Subject:	Standard deviation; Accuracy; Mathematical analysis; pH; Ethyl cellulose; Room temperature; Protective coatings; Chromatography; Shellac; Castor oil; Tablets; Drug dosages; Bioavailability; Spectrum analysis; Ultraviolet spectroscopy; Cellulose; Intestine; Silicon dioxide; Stability analysis; Enzymes; Product development; Spectroscopic analysis
Business indexing term:	Subject: Product development
Location:	Asia; Europe; United States--US; India; Japan
Editor:	Adil Denizli
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-05-25 (Received); 2021-10-04 (Revised); 2021-10-05 (Accepted); 2021-10-19 (Pub)
DOI:	https://doi.org/10.1155/2021/9749474
ProQuest document ID:	2589571505
Document URL:	https://www.proquest.com/sc-holarly-journals/formulation-development-serratiopeptidase-enteric/docview/2589571505/se-2?accountid=211160

Copyright: Copyright © 2021 Vijay Kumar Panthi et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-11-01

Database: Publicly Available Content Database

Document 12 of 59

Determination of Dabigatran Concentration in Human Plasma and Breast Milk

Sidgwick, F ¹ ; Porter, A ¹
; Ayuk, P ² ; Kamali, F ³
; Truemann, A ¹

¹ Newcastle University Protein and Proteome Analysis (NUPPA), Newcastle Upon Tyne, UK ² Newcastle Upon Tyne Hospitals Foundation Trust, Newcastle Upon Tyne, UK ³ Translational and Clinical Research Institute, Newcastle University and Newcastle Upon Tyne Hospitals Foundation Trust, Newcastle Upon Tyne, UK

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Venous thromboembolism (VTE) is an important cause of death following childbirth. Dabigatran etexilate can be a useful prophylaxis in susceptible women during the postpartum period. However, it is not clear whether dabigatran is excreted into breast milk in amounts which can be harmful to the suckling baby. We have developed an accurate, sensitive, and specific assay for the quantitation of dabigatran in both human plasma and breast milk. This is particularly useful for the determination of the extent by which dabigatran is secreted into breast milk in relation to its systemic availability. Dabigatran was enriched from both matrices using solid-phase extraction prior to separation on a C8-RPLC column and detection using SRM on a QqTrap mass spectrometer. The assay was validated for specificity, sensitivity, linearity, precision, accuracy, and stability of the analyte in human plasma and breast milk. The lower limit of detection for dabigatran was 20 pg/ml in plasma and 75 pg/ml in breast milk. This assay will aid future studies for the measurement of dabigatran concentrations in human breast milk to help determine if dabigatran etexilate can safely be administered to breast-feeding women.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Venous thromboembolism (VTE) is an important cause of death following childbirth [1]. Dabigatran etexilate, a direct thrombin inhibitor, is a member of the non-vitamin K antagonist oral anticoagulants (NOACs) used for the treatment

and prophylaxis of thromboembolic disorders [2–4]. Dabigatran etexilate is rapidly absorbed following oral ingestion with mean peak plasma concentrations ($C_{\max}$ = 160 ng/ml) of the active agent, dabigatran, reaching within 2 h of administration [5]. Dabigatran etexilate is currently not recommended for use as a prophylaxis in women susceptible to thromboembolism during the postpartum period. This is because it is not known at this stage whether dabigatran is excreted into breast milk in amounts which can be harmful to the suckling baby. Investigations exploring dabigatran secretion into breast milk will require the use of a sensitive and robust assay. To date, several analytical methods for determining dabigatran concentrations in human plasma samples have been reported, most of which utilise liquid chromatography-mass spectrometry (LC-MS) because of its superior accuracy, sensitivity, and specificity [6–12]. These assays have a LC turnaround time of 4.5–7 minutes and a lower limit of quantification (LOQ) between 25 pg/ml and 0.3 ng/ml [6–12]. However, there is no assay reported which measures dabigatran concentration in human breast milk. Here, we report a flexible, robust, rapid, and sensitive assay that can be utilised to determine dabigatran concentration in both plasma and breast milk over a concentration range of three orders of magnitude. This assay is particularly useful for future studies aimed at determining the extent by which dabigatran is secreted into breast milk in relation to its systemic availability in postpartum women.

2. Materials and Methods

Expressed human breast milk that was surplus to requirement was donated for use in this study. Dabigatran and $^{13}\text{C}_6$ -dabigatran were obtained from Alsachim (France). Agilent Bond Elut C18 SPE cartridges (1 mL) were obtained from Crawford Scientific (UK). Formic acid and HPLC-grade acetonitrile were obtained from Fisher Scientific (UK). An Ace 5 C8-300 column (250 × 1 mm) was supplied by Advanced Chromatography Technologies, UK. All other solvents used were of analytical grade and were obtained from VWR (UK). All the stock standard solutions, calibration standards, and quality control samples of dabigatran were prepared using a calibrated accurate weighing balance. Dilutions were prepared from stock solutions on the day of analysis. The dabigatran concentration range chosen for the standard curve allowed for the variance in plasma dabigatran concentrations observed in both healthy volunteers [5] and patient populations [13] following the administration of a therapeutic dose of dabigatran etexilate.

$^{13}\text{C}_6$ -dabigatran (2.5 ng in 5 μL of water) was added to 500 μL of breast milk or plasma. Samples were centrifuged in an Eppendorf benchtop centrifuge at 13,000 rpm for 5 min at room temperature. 200 μL of the aqueous layer or 200 μL of plasma were applied to a Bond Elute C18 1 mL SPE cartridge that had been conditioned sequentially with 500 μL of methanol and 500 μL of HPLC grade water. Cartridges were washed with 500 μL of HPLC grade water and eluted into glass tubes first with 500 μL of methanol and then with 500 μL of propan-2-ol. The eluent was dried in a nitrogen stream at room temperature prior to redissolving in 200 μL of 0.1% formic acid in HPLC-grade water. The resulting solution was centrifuged at 13,000 rpm for 2 min at room temperature in an Eppendorf bench centrifuge prior to transfer into a glass HPLC vial.

2.1. LCMSMS Analysis

The resultant solution (1 μL) from each vial was injected onto a Dionex Ultimate 3000 HPLC system (Thermo Fisher, UK), equipped with an Ace 5 C8-300 column (250 mm × 1 mm, Advanced Chromatography Technologies, UK) equilibrated with 4% B (HPLC solvents: A: 0.1% formic acid in HPLC-grade water; B: acetonitrile, and C: 0.1% formic acid in methanol) at a flow rate of 50 $\mu\text{L}/\text{min}$ through the loading pump of the HPLC system. The column was eluted using the gradient shown in Table 1, and the eluent was monitored online using a QTrap 4000 mass spectrometer (Sciex, UK).

Table 1

Gradient programme on the Dionex Ultimate HPLC system using an Ace 5 C8-300 column (250 mm × 1 mm).

Minutes	Flow ($\mu\text{L}/\text{min}$)	% B	% C
0.0	50	4	0

1.5	50	4	0
2.5	50	15	0
10.0	50	40	0
12.5	50	65	0
14.0	50	95	0
14.1	50	95	0
14.2	50	4	0
15.0	50	4	96
15.8	50	4	96
17.0	50	4	0
25.0	50	4	0

Source parameters of the QTrap mass spectrometer were as follows: entrance potential 12, curtain gas 25, CAD 4, ion source voltage 5kV, temperature 200°C, GS1 20, GS2 20, and interface heater ON. Six SRM transitions were monitored as described in Section 3. N₂ was used as the curtain gas, nebuliser, and collision gas.

3. Results

3.1. LCMSMS Optimisation

Dabigatran and the ¹³C-labelled dabigatran standard were infused into a QTrap4000 mass spectrometer using positive mode electrospray ionisation. After optimisation of source parameters, product ion spectra were recorded (Figure 1) and three transitions were chosen for both the internal standard and the unlabelled analyte (Tables 2 and 3), and collision energy and source parameters were optimised separately for each transition. Following MS optimisation, samples were separated using reversed-phase HPLC (RP-HPLC) on an Ace C8 column and a 15 min gradient from 4% acetonitrile to 95% acetonitrile. Figure 2 shows the chromatogram in plasma and breast milk obtained from a postpartum woman following the oral administration of 220 mg of dabigatran etexilate.

[figure omitted; refer to PDF]

Table 2

MRM transitions monitored for dabigatran.

Q1 mass	Q3 mass	Time (msec)	DP	CE
472.2	289.2	50	96	41
472.2	306.3	50	96	29
472.2	324.0	50	101	29

Table 3

MRM transitions monitored for ^{13}C dabigatran.

Q1 mass	Q3 mass	Time (msec)	DP	CE
478.2	295.2	50	96	41
478.2	312.4	50	96	29
478.2	330.3	50	101	29

[figures omitted; refer to PDF]

3.2. Solid-Phase Extraction

To extract dabigatran from either milk or plasma, ten different solid-phase extraction columns were evaluated for recovery and reproducibility (supplementary figure S1). The best performance (76% recovery of dabigatran) was obtained using Agilent 30 mg 1 ml BondElute C18 SPE cartridges.

3.3. Specificity and Sensitivity

Specificity and sensitivity were tested using chromatograms of blank breast milk and blank plasma samples and samples of both matrices spiked with 0.01 ng dabigatran. Neither matrix showed interference at the retention time of dabigatran (Figure 3).

[figures omitted; refer to PDF]

3.4. Linearity and Dynamic Range

The assay was linear ($R^2 > 0.99$, $n=9$ data points) for dabigatran in breast milk and plasma over a range of 5 ng/ml to 2 mg/ml. Figure 4 shows typical standard curves for dabigatran constructed in plasma and breast milk. During the acquisition of the measured values for dabigatran in breast milk, a blank was run as every third sample while data for a standard curve and data for the breast milk samples were acquired. The mean $\pm$ SE dabigatran area in all blanks ($n=24$) was 3210 ± 435 , and the mean $\pm$ SE ^{13}C -labelled dabigatran area in all breast milk standard samples (0.5 nmol of dabigatran on column; $n=33$) was 720773 ± 27011 . This implies that the noise level (including potential carryover) is 2.2 pmol on column, corresponding to a breast milk dabigatran concentration of 20 pg/ml. Defining the lower limit of detection (LLOD) as three times the noise level yielded an LLOD of 75 pg/ml. Defining the lower limit of quantitation (LLOQ) as ten times the noise level yielded an LLOD of 2200 pg/ml.

[figure omitted; refer to PDF]

3.5. Precision and Accuracy

Using the optimised assay, plasma and breast milk samples were spiked with 0.5 ng of dabigatran and analysed in triplicate on the same day and on separate days in order to determine intraday and interday reproducibility (Table 4).

Table 4

Intraday and interday reproducibility.

Dabigatran concentration (ng/ml)	Intraday CV (%)	Interday CV (%)
<i>Milk</i>		
10	6.22	18.68
30	8.40	18.98

100	4.37	6.97
500	2.42	9.05
1000	2.67	2.41
2000	0.39	3.43
–		
<i>Plasma</i>		
3	19.43	38.75
10	13.89	35.76
30	4.83	7.00
100	2.84	7.27
500	2.76	2.86
1000	3.06	4.21
2000	2.84	6.35

3.6. Stability of Dabigatran Samples

To determine if dabigatran is undergoing biological or chemical modification during storage in breast milk, triplicate aliquots of dabigatran were subjected to a variety of different treatments, including a single and a double freeze-thaw cycle (–80°C), storage for 20h or 44h at 4°C, and storage at room temperature for 1h, 4h, or 20h. The results as shown in Table 5 indicate that none of these treatments was associated with a change in dabigatran concentration.

Table 5

Stability testing of triplicates of a 300 µl aliquot of breast milk containing 48pg of dabigatran. Three technical replicates were treated as indicated, and dabigatran concentration was determined. ANOVA indicated that none of the treatments showed a significant difference in dabigatran concentration. Frozen samples were stored at –20°C for one week. RT=room temperature.

Treatment	Dabigatran (pg)	<i>n</i>
None	52.7	3
Freeze, thaw, freeze	44.5	2
4°C, 44h	46.0	3

4°C, 20h	47.0	3
RT, 20h	47.3	3
RT, 4h	42.0	3
RT, 1h	41.3	3
-20°C, 1 week	46.7	3

4. Discussion

We have developed a highly sensitive and reproducible assay to determine dabigatran concentration in human plasma and breast milk. The assay is linear over more than three orders of magnitude. It takes four hours to prepare a batch of 24 samples plus 30 min per sample for the LCMSMS analysis on a Sciex Qtrap 4000 mass spectrometer, an instrument that is frequently available in many clinical laboratories. To our knowledge, this is the first published procedure to determine dabigatran concentration in human breast milk samples.

Dabigatran etexilate is currently licensed for the prevention of venous thromboembolism (VTE) following hip and knee surgery. VTE is an important cause of death following childbirth [1], and current preventive strategies include the administration of injectable heparin [1]. Dabigatran etexilate is administered orally and would, therefore, have advantages over injectable heparin. This assay will aid future studies to determine if dabigatran is detectable in breast milk in clinically significant concentrations following oral administration of dabigatran etexilate to breast-feeding women. Such studies are essential if dabigatran etexilate is to be considered as an alternative to injectable heparin. Our assay permits the detection of very low concentrations of dabigatran in breast milk and ensures that clinical implications can be fully evaluated before dabigatran etexilate is considered for use in breast-feeding women.

Disclosure

Boehringer-Ingelheim did not have any role in the interpretation of the data or in the writing of the manuscript.

Authors' Contributions

P.A., and F.K., and A. T. designed the study. A.T., F.S., and A. P. performed MS-HPLC analyses. All authors contributed to the manuscript.

References

- [1] Royal College of Obstetricians and Gynaecologists, Reducing the Risk of Venous Thromboembolism during Pregnancy and the Puerperium, 2015.
- [2] L. Wallentin, S. Yusuf, M. D. Ezekowitz, M. Alings, M. Flather, M. G. Franzosi, P. Pais, A. Dans, J. Eikelboom, J. Oldgren, J. Pogue, P. A. Reilly, S. Yang, S. J. Connolly, "Efficacy and safety of dabigatran compared with warfarin at different levels of international normalised ratio control for stroke prevention in atrial fibrillation: an analysis of the RE-LY trial," *The Lancet*, vol. 376 no. 9745, pp. 975-983, DOI: 10.1016/s0140-6736(10)61194-4, 2010.
- [3] A. Gomez-Outes, A. I. Terleira-Fernandez, M. L. Suarez-Gea, E. Vargas-Castrillon, "Dabigatran, rivaroxaban, or apixaban versus enoxaparin for thromboprophylaxis after total hip or knee replacement: systematic review, meta-analysis, and indirect treatment comparisons," *BMJ*, vol. 344, DOI: 10.1136/bmj.e3675, 2012.
- [4] K. Suen, R. N. Westh, L. Churilov, A. J. Hardidge, "Low-molecular-weight heparin and the relative risk of surgical site bleeding complications: results of a systematic review and meta-analysis of randomized controlled trials of venous thromboprophylaxis in patients after total joint arthroplasty," *The Journal of Arthroplasty*, vol. 32 no. 9, pp. 2911-2919, DOI: 10.1016/j.arth.2017.04.010, 2017.
- [5] J. Stangier, K. Rathgen, H. Stähle, D. Gansser, W. Roth, "The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects," *British Journal of Clinical Pharmacology*, vol. 64, pp. 292-303, DOI: 10.1111/j.1365-2125.2007.02899.x, 2007.

- [6] S. Blech, T. Ebner, E. Ludwig-Schwellinger, J. Stangier, W. Roth, "The metabolism and disposition of the oral direct thrombin inhibitor, dabigatran, in humans," *Drug Metabolism and Disposition*, vol. 36 no. 2, pp. 386-399, DOI: 10.1124/dmd.107.019083, 2008.
- [7] X. Delavenne, J. Moracchini, S. Laporte, P. Mismetti, T. Basset, "UPLC MS/MS assay for routine quantification of dabigatran—a direct thrombin inhibitor—in human plasma," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 25 no. 58, pp. 152-156, DOI: 10.1016/j.jpba.2011.09.018, 2012.
- [8] Z. Y. Hu, R. B. Parker, V. L. Herring, S. C. Laizure, "Conventional liquid chromatography/triple quadrupole mass spectrometry based metabolite identification and semi-quantitative estimation approach in the investigation of in vitro dabigatran etexilate metabolism," *Analytical and Bioanalytical Chemistry*, vol. 405 no. 5, pp. 1695-704, DOI: 10.1007/s00216-012-6576-4, 2012.
- [9] M. Korostelev, K. Bihan, L. Ferreol, N. Tissot, J. S. Hulot, C. Funck-Brentano, N. Zahr, "Simultaneous determination of rivaroxaban and dabigatran levels in human plasma by high-performance liquid chromatography-tandem mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 100, pp. 230-235, DOI: 10.1016/j.jpba.2014.08.011, 2014.
- [10] J. Li, J. Fang, F. Zhong, W. Li, Y. Tang, Y. Xu, S. Mao, G. Fan, "Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous determination of dabigatran etexilate, intermediate metabolite and dabigatran in 50 μ L rat plasma and its application to pharmacokinetic study," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 973, pp. 110-119, DOI: 10.1016/j.jchromb.2014.09.025, 2014.
- [11] E. M. Schmitz, K. Boonen, D. J. van den Heuvel, J. L. van Dongen, M. W. Schellings, J. M. Emmen, F. van der Graaf, L. Brunsveld, D. van de Kerkhof, "Determination of dabigatran, rivaroxaban and apixaban by ultra-performance liquid chromatography—tandem mass spectrometry (UPLC-MS/MS) and coagulation assays for therapy monitoring of novel direct oral anticoagulants," *Journal of Thrombosis and Haemostasis*, vol. 12 no. 10, pp. 1636-1646, DOI: 10.1111/jth.12702, 2014.
- [12] E. G. Nouman, M. A. Al-Ghobashy, H. M. Lotfy, "Development and validation of LC-MSMS assay for the determination of the prodrug dabigatran etexilate and its active metabolites in human plasma", " *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 989, pp. 37-45, DOI: 10.1016/j.jchromb.2015.02.042, 2015.
- [13] Boehringer Ingelheim Limited, Summary of Product Characteristics, Pradaxa 150mg Hard Capsules, 2019.

DETAILS

Subject:	Plasma; Acids; Childbirth & labor; Assaying; Thromboembolism; Blood plasma; Stability analysis; Noise; Disease prevention; Chromatography; Breastfeeding & lactation; Reproducibility; Solid phases; Prophylaxis
Location:	United Kingdom--UK
Editor:	Victoria F Samanidou
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021

Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-06-21 (Received); 2021-09-01 (Revised); 2021-09-21 (Accepted); 2021-10-22 (Pub)
DOI:	https://doi.org/10.1155/2021/5949385
ProQuest document ID:	2589570801
Document URL:	https://www.proquest.com/scholarly-journals/determination-dabigatran-concentration-human/docview/2589570801/se-2?accountid=211160
Copyright:	Copyright © 2021 F. Sidgwick et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-11-01
Database:	Publicly Available Content Database

Document 13 of 59

Chromatographic Fingerprinting Based on Column Switching Technology for Quality Evaluation of Tianmeng Oral Liquid

Meng Yuan¹ ; Liu, Xiaoyan¹ ; Sun, Yue¹ ; Wang, Linlin¹ ; Pian Jin¹ ; Zhuang, Xiaoqi¹ ; Zang, Hengchang² ; Zhang, Zhonghu³ ; Nie, Lei⁴

¹ School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan 250012, China ² School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan 250012, China; National Glycoengineering Research Center, Jinan 250012, China; National Medical Products Administration Key Laboratory for Technology Research and Evaluation of Drug Products, Shandong University, Jinan 250012, China ³ Shandong Institute for Food and Drug Control, Jinan 250100, China ⁴ School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan 250012, China; National Medical Products Administration Key Laboratory for Technology Research and Evaluation of Drug Products, Shandong University, Jinan 250012, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Separation power was limited when the conventional high-performance liquid chromatography (HPLC) fingerprinting method based on a single column was used to analyze very complex traditional Chinese medicine (TCM) preparations. In this research, a novel HPLC fingerprinting method based on column switching technology by using a single pump was established for evaluating the quality of Tianmeng oral liquid (TMOL). Twelve batches of TMOL samples were used for constructing HPLC fingerprints. Compared with the 16 common peaks in fingerprinting with a single column, 25 common peaks were achieved with two columns connected through a six-way valve. The similarity analysis combined with bootstrap method was applied to determine the similarity threshold, which was 0.992 to distinguish expired samples and unexpired samples. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were also applied to classify the TMOL samples, and results revealed that expired and unexpired samples are classified into two categories. The HPLC fingerprinting based on column switching technology with better separation power and higher peak capacity could characterize chemical composition information more comprehensively, providing an effective and alternative method to control and evaluate the quality of TMOL, which would offer a valuable reference for other TCM preparations.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Tianmeng oral liquid (TMOL) is a traditional Chinese medicine (TCM) formula preparation composed of seventeen Chinese herbs following the principles of “Jun (emperor)-Chen (minister)-Zuo (adjuvant)-Shi (courier),” which has the effect of replenishing Qi and tonifying kidney, nourishing heart, and calming mind, and commonly used in the treatment of insomnia caused by deficiency of spleen and kidney [1, 2]. Jun (emperor) includes *Acanthopanax senticosus* and *Polygonatum sibiricum*. Chen (minister) includes *Rehmannia glutinosa*, *Lycium chinense*, *Fructus Mori*, *Epimedium brevicornu*, *Silkworm moth*, *Astragalus membranaceus*, and *Codonopsis pilosula*. Zuo (adjuvant) includes *Wolfiporia cocos*, *Pericarpium Citri Reticulatae*, *Rhizoma Pinelliae*, and *Crataegus pinnatifida*. Shi (courier) is *Strychnos nux-vomica*. For the complex system of TCM, the establishment of its fingerprint is an effective method to evaluate the interbatch consistency and stability of quality and has been internationally recognized [3–5]. TMOL is a compound preparation of TCM with the characteristics of multicomponents and multitargets, and its fingerprint has been established on a single column by high-performance liquid chromatography (HPLC) [6]. In addition, other relevant analytical methods available in the literature for quality evaluation of Tianmeng capsules are primarily based on quantification of a single bioactive component, which is difficult to reflect the quality comprehensively [7–9]. The

more chromatographic peaks obtained in HPLC fingerprinting could reflect the complete information about chemical components, which is more conducive to reflect the inner quality of TCM comprehensively [10–12]. However, most researches on fingerprinting are based on a single column and the corresponding fingerprint has limited selectivity and separation power to analyze complex TCM preparations [13–15].

The column switching technique is used to connect chromatographic columns with different stationary phases, and the selectivity and resolution of chromatography could be improved to obtain more chromatographic peaks characterizing more chemical information, which is already widely applied to analyze complex systems [16–18]. Usually, the mode of column switching can be divided into two-column double-pump system, three-column three-pump system, and complex coupling system [19], while more pumps require more hardware costs. To carry out automatic column switching, the instrument should be equipped with a double-pump infusion system and a high-pressure flow path selection valve at least [20, 21]. There are few studies on HPLC fingerprinting methods based on column switching of a single pump to obtain more chromatographic peaks for TCM. Given the existing shortcomings, we proposed to establish a single-pump double-column HPLC column switching system by connecting different polarity columns with a six-way valve, which fully combined the advantages of separation selectivity of different polarity chromatographic columns to effectively increase peak capacity, improve the separation effect of characteristic components, and consequently reflect more comprehensive information in fingerprint.

In this study, the column switching technology based on a single pump was employed for the development of the HPLC fingerprinting method to carry out the quality evaluation of TMOL. The present method was compared with a single-column method in different aspects. Various chemometric methods such as similarity analysis, principal component analysis (PCA), and hierarchical clustering analysis (HCA) were all applied to distinguish different batches of TMOL samples for quality evaluation.

2. Materials and Methods

2.1. Materials and Reagents

Syringin (purity: >98%) and calycosin 7-O-glucoside (purity: >98%) were purchased from Ruifensi Biotechnology Co., Ltd. (Chengdu, China). Icarin (purity: >98%), hesperidin (purity: >98%), and chlorogenic acid (purity: >98%) were purchased from Mansite Biotechnology Co., Ltd. (Chengdu, China). HPLC grade acetonitrile and methanol were obtained from Tedia (Fairfield, USA) and all other solvents used, including acetic acid, phosphoric acid, and formic acid were analytical grade, were purchased from Sinopharm Chemical Reagent (Beijing, China). Deionized water was purified with a Milli-Q water purification system (Molsheim, France). TMOL samples were produced by Rongchang Pharmaceutical Co., Ltd. (Yantai, China) and were purchased from retail pharmacies. The batch numbers (S1–S12) were 170202, 170204, 170706, 171112, 171121013, 171121021, 171218, 180101, 180103, 180104, 180117, and 180203, respectively.

2.2. Column Switching Technology

The HPLC column switching system with a single-pump double-column system was successfully established by adding a six-way injection valve. The two chromatographic columns were connected through a six-way valve. The first kind of connection method is shown in Figures 1(a) and 1(b). The position of the six-way valve in the first 35 min is 1 2 and the two-column analysis was performed. After 35 min, the six-way valve was switched to 1 6 and the single-column analysis was performed. The second kind of connection method is shown in Figures 1(c) and 1(d). The position of the six-way valve in the first 35 min is 1 2 for single-column analysis. The six-way valve was switched to 1 6 after 35 min, and two-column analysis was performed. Samples were injected twice for one sample analysis according to two connection methods, and we focused on the comparison of the single-column method and the two-column method based on column switching technology.

[figures omitted; refer to PDF]

2.3. Apparatus and Chromatographic Conditions

Chromatographic separations were carried out by an Agilent 1260 HPLC instrument (Agilent Technologies, USA), including a quaternary pump, an autosampler, a column temperature controller, and a diode array detector, connected to Agilent ChemStation software. The two chromatographic columns employed were an ACE EXCEL C₁₈

column (250×4.6mm, 5 μ m) and a JADE-PAK C₈ column (50×4.6mm, 3.5 μ m). The mobile phase was comprised of 0.1% aqueous phosphoric acid (A) and acetonitrile (B). The elution condition was set as follows: 5%–5% B for 0–5 min, 5%–14% B for 5–15 min, 14%–18% B for 15–21 min, 18%–21% B for 21–30 min, 21%–24% B for 30–35 min, 24%–30% B for 35–40 min, 30%–36% B for 40–50 min, 36%–95% B for 50–60 min, 95%–95% B for 60–65 min, and 95%–5% B for 65–75 min. A low rate of 0.6 mL/min and a temperature of 25°C were maintained. A wavelength used to detect signal was selected as 230 nm and the injection volume was set as 10 μ L.

2.4. Preparation of Reference Standard Solution and Sample Solution

Five kinds of reference substances were dissolved in methanol to prepare mixed reference standard solution, and the final concentration was as follows: calycosin 7-O-glucoside (0.00150 g/mL), syringin (0.00135 g/mL), icariin (0.00178 g/mL), chlorogenic acid (0.00294 g/mL), and hesperidin (0.00225 g/mL). One TMOL sample (10 mL) was taken from each batch for analysis. Methanol was added to the samples for ultrasonic extraction for 5 minutes; then the volume of the extract was adjusted to 25 mL. Furthermore, to remove the insoluble components, the centrifugation was performed for 10 min under the condition of 9668.16×g to obtain supernatant, which was filtered with 0.22 μ m membrane prior to injecting.

2.5. Data Analysis

“Similarity Evaluation System for Chromatographic Fingerprint of TCM” (Chinese Pharmacopoeia Commission, 2012A) was applied to analyze fingerprints. The similarity was calculated by the cosine of the angle between the sample fingerprinting and the reference fingerprinting with MATLAB (MathWorks, R2019a). The variability of HPLC fingerprint analysis of 12 batches of samples was evaluated by principal component analysis (PCA) with MATLAB (MathWorks, R2019a). Hierarchical clustering analysis was performed using Origin software (OriginLab, 2021b).

3. Results and Discussion

3.1. Optimization of Coupled Columns for Fingerprinting

Choices of chromatographic columns are based on key parameters such as stationary phase and lengths, which have a significant influence on selectivity and resolution. In this research, considering the relatively strong polarity of components of TMOL, the column with a shorter chain length has relatively greater polarity, and so reversed-phase chromatographic columns with different stationary phases and different chain lengths are combined for analysis. The optimal result of two connected columns was determined by trial and error according to the columns available in our research laboratory.

With the purpose of obtaining more chromatographic peaks in the HPLC fingerprinting of TMOL, two columns with different stationary phases, such as Phenomenex Synergi Hydro-RP C₁₈ (4 μ m, 250×4.6 mm) and JADE-PAK C₈ (3.5 μ m, 50×4.6 mm), ACE EXCEL C₁₈ (5 μ m, 250×4.6 mm) and JADE-PAK C₈ (3.5 μ m, 50×4.6 mm), JADE-PAK C₈ (5 μ m, 150×4.6 mm) and JADE-PAK ODS-AQ (5 μ m, 150×4.6 mm), and JADE-PAK C₈ (5 μ m, 150×4.6 mm) and Sharsil-U C₄ (5 μ m, 150×4.6 mm), were connected through a six-way valve. In addition, the combination of ACE EXCEL C₁₈ (5 μ m, 250×4.6 mm) and JADE-PAK C₈ (5 μ m, 150×4.6 mm) was also tried; that is, the total column length is 400 mm, but the results showed the column pressure increased and the separation effect was not improved significantly. Therefore, we did not try further and the total length of the two columns was limited to 300 mm to prevent excessive column pressure. As shown in Figure 2, it is found that ACE EXCEL C₁₈ (250×4.6 mm, 5 μ m) and JADE-PAK C₈ (50×4.6 mm, 3.5 μ m) (combination b) could get the better peak capacity and resolution. A total of 56 peaks with peak heights greater than 10 mAU were separated by the two columns based on ACE EXCEL C₁₈ (5 μ m, 250×4.6 mm) and JADE-PAK C₈ (3.5 μ m, 50×4.6 mm), which was the best among the different column-connection modes, and the resolution of 34 peaks was more than 1.5. Through the first kind of connection method (shown in Figures 1(a) and 1(b)), more polar components can be separated by double-column analysis before 35 min. Through the second kind of connection method (shown in Figures 1(c) and 1(d)), more components with less polarity can be obtained by double-column analysis after 35 min.

[figure omitted; refer to PDF]

3.2. Optimization of HPLC Chromatographic Conditions

The HPLC conditions including mobile phase with different acids, gradient elution, column temperature, flow rate,

and detection wavelength were optimized for obtaining fingerprints with satisfactory separation. Acetonitrile was selected as a solvent eluent to obtain good chromatographic behavior and low background noise. Since the addition of acid additives in the mobile phase can reduce the tailing degree of the chromatographic peak and improve the peak shape, the effect of adding different acids (0.1% formic acid, 0.1% acetic acid, and 0.1% phosphoric acid) into mobile phase A was investigated, and the results showed that the effect of adding phosphoric acid was the best (Figure S1). By adjusting the proportion of the mobile phase, the baseline was stable, the retention time of each chromatographic peak was moderate, more peaks were obtained, and better separation was achieved. Different column temperatures (25°C, 30°C, and 35°C) were compared and the most appropriate column temperature was 25°C (Figure S2). According to rate theory, the flow rate can affect the plate height, and thus separation effect. Compared with different flow rates (0.5 mL/min, 0.6 mL/min, and 0.7 mL/min), the best condition was 0.6 mL/min with more peaks and better resolution (Figure S3). After testing different wavelengths (210 nm, 230 nm, 254 nm, 260 nm, and 270 nm), 230 nm was selected as the most appropriate detection wavelength because most compounds had satisfactory absorption (Figure S4).

3.3. Validation of the HPLC Fingerprint Method

The characteristic peak of chlorogenic acid (peak 14 shown in Figure 3) was selected as a reference peak, and the relative standard deviations (RSDs, %) of relative retention time (RRT) and relative peak area (RPA) of the common peaks were used for validation of the HPLC fingerprint method, including the stability, repeatability, and intraday and interday precision. The specific results are displayed in Table S1. The stability test was performed by analysis of the same sample solution with different time intervals (0, 2, 4, 8, 12, and 24 h), and the RSDs values of RRT and RPA were less than 0.75% and 4.8%, respectively. The repeatability test was performed by analysis of six independent TMOL samples of the same batch, and the RSDs values of RRT and RPA were less than 0.72% and 4.8%, respectively. The intraday precision test was performed by analysis of the same solution six consecutive times within one day, and the RSDs values of RRT and RPA were less than 0.81% and 4.9%, respectively. The interday precision test was performed by analysis of the same solution three consecutive times over three consecutive days, and the RSDs values of RRT and RPA were less than 0.71% and 5.0%, respectively. The above results showed that the established method was feasible for the chromatographic fingerprint of TMOL.

[figure omitted; refer to PDF]

3.4. Chromatographic Fingerprinting of TMOL

Under the above optimal chromatographic conditions, the fingerprints of 12 batches samples were obtained. According to their retention times (Figure 3) and ultraviolet (UV) spectra, five peaks are identified as syringin, chlorogenic acid, calycosin 7-O-glucoside, hesperidin, and icariin, respectively. The contrast of UV spectra for the above five components is shown in Figure S5.

The fingerprints of 12 batches of samples by column switching technology were input to professional software named Similarity Evaluation System for Chromatographic Fingerprint of TCM. A reference fingerprint obtained by the average method and the sample fingerprints are both shown in Figure 3. Because the column switching technology based on a single pump was used to establish the fingerprint method, the fingerprint measurement required two injections. The reference fingerprints and TMOL fingerprints of 12 batches of the first injection (before 35 min) and the second injection (after 35 min) are shown in Figures S6 and S7, respectively.

3.5. Similarity Analysis

Based on obtained common peaks, similarity analysis was performed between the reference fingerprint and the fingerprints of TMOL samples using MATLAB R2019a. The similarities were evaluated with the cosine method. The similarity of 10 unexpired samples were 0.999, 0.999, 0.999, 0.999, 0.998, 0.999, 0.997, 0.996, 0.996, and 0.998, and they shared similar chromatographic patterns with similarity values higher than 0.995. The two expired samples had lower similarity values, which were 0.978 and 0.984, suggesting the differences existed in quality between the expired and the unexpired TMOL.

The similarity threshold is the lowest limit of fingerprint similarity for a class of samples. Interval estimation is often used to determine the threshold in statistics, which requires that the overall distribution of the data is known or

approximately empirical. The bootstrap method proposed by Professor Efron in 1979 is based on the existing small amount of sample data and extends to a large amount of sample data by replacement sampling [22]. Therefore, according to the results of similarity evaluation of 12 batches of samples, the bootstrap method was used to determine the identification threshold of the unexpired TMOL samples (the number of bootstrap samples to draw is 10000, $\alpha=0.05$). After calculation, the similarity threshold is 0.992. The results showed that the similarity of unexpired samples (S3-S12) was higher than 0.992, which could be considered similar and belong to the same category, while the similarity of expired samples (S1-S2) was lower than 0.992, which could be considered to be different from the quality of unexpired samples. The HPLC fingerprinting combined with similarity analysis and threshold determination could judge the quality discrepancy of TMOL samples.

3.6. Principal Component Analysis

PCA is a multivariate analysis technique widely used for feature extraction and dimensionality reduction to investigate the correlation between multiple variables [23]. The areas of 25 common peaks in chromatographic fingerprints as variables and 12 samples as objects were employed to form the data matrix, which was analyzed directly with PCA using MATLAB R2019a. Based on the eigenvalues and contribution rate of principal components, two principal components (PC1, PC2) were extracted to account for 87.1% and 10.9% of the 25 data variance in 12 batches of samples, respectively, and could well represent most information in the fingerprints of samples. The PCA score plot is shown in Figure S8. Twelve batches of TMOL samples distribute in two domains, which are labeled as group 1 (S1, S2) and group 2 (S3-S12), respectively. The boundary between the two categories is apparent. S1 and S2 are in positive part on PC1, while the rest of the samples (S3-S12) form an independent cluster with scores close to zero or negative values on PC1. On PC2, the scores of samples (S1, S2, S4, S7, and S11) are negative values, while other samples have positive scores.

Furthermore, to identify the variables causing the significant category separation, the loading plot for PC1 and PC2 is also shown in Figure S9. From the loading plot, peak 20 and peak 6 significantly contribute to the two categories. Peak 18 and peak 19 moderately lead to separation, while other peaks have little contribution to classification.

3.7. HCA with Heatmaps

In order to further clarify the influence of common peaks on classifiers, HCA with heatmaps was performed to group TMOL samples into different clusters. The peak areas of 12 batches of TMOL samples were normalized, and the cluster dendrograms were generated based on Euclidean distance with group average method for measuring the dissimilarity. Visibly, all TMOL samples were gathered into two clusters (Figure 4). Specifically, the expired samples 1 and 2 are located near to each other in one cluster, and the unexpired samples 3-10 are involved in another cluster. In addition, some peaks like 6, 20, 18, and 19 seem to have an obvious difference in peak area between the two clusters, which corresponds to the result obtained by the loading plot (Figure S9) of PCA. The results indicated that the fingerprints combined with HCA realized the identification of the expired and the unexpired TMOL samples. [figure omitted; refer to PDF]

3.8. Comparison with Fingerprinting Based on a Single Column

Three batches of TMOL samples (180101, 180104, and 180203) and the solution of the mixed standards were analyzed under the chromatographic conditions of the fingerprinting method based on a single column and column switching technology with one pump, respectively. HPLC fingerprints of 3 batches of TMOL samples and the chromatogram of mixed reference standard solution with the single-column method are shown in Figure S10. The peak capacities of the three batches of samples analyzed by the single-column method were 45, 46, and 46, while the corresponding peak capacities increased to 56, 63, and 62 by the column switching method, respectively. The column switching method could obtain a higher peak capacity than the single-column method (at least 11 chromatographic peaks) under the similar conditions. In addition, compared with the single-column method, the column switching method could increase the number of theoretical plates of the same component (Table S2) and achieve a better resolution of the five components (Table S3). Resolution (R) can be expressed as $R=N/4\alpha-1/k_2/k_2+1$, and theoretical plate number (N), separation factor (α), and retention factor of the latter peak (k_2) are three main parameters affecting resolution. Compared with the single column, the length of the column is increased by the

column switching technology, which can acquire a higher theoretical plate number. Under the condition of the same mobile phase, the change of column stationary phase affects α and k_2 . Therefore, the improvement of resolution by column switching is attributed to the integrated effect of N , α , and k_2 .

As a comparison, there were 25 common peaks (Figure 3) in the fingerprints of different batches, including 16 peaks (Figure S6) in the first injection analysis and 9 peaks (Figure S7) in the second injection analysis, while 16 common peaks (Figure S11) were only obtained if we used a single C18 column for analysis [6] under similar measurement time. More common peaks occurred in the fingerprints, indicating more separation power with column switching technology than a single column, and could reflect chemical composition more comprehensively, which is highly meaningful for the quality evaluation of TMOL.

Traditional Chinese medicine (TCM) is different from chemically synthesized drugs in that its chemical composition is complex and the sources are extensive, making it difficult to control and evaluate its quality. HPLC fingerprinting technology is to separate the complicated chemical composition of TCM with chromatographic column under the optimal chromatographic conditions to acquire the corresponding chromatogram as fingerprint. The characteristic peaks of the fingerprint are the manifestation of the chemical composition of TCM, which can indicate its quality. Therefore, the fingerprint plays a key role in quality control and evaluation of TCM. The more peaks in fingerprint, the stronger characterization of TCM's quality. Although the column switch using a single pump due to laboratory conditions has the limited improvement on the HPLC fingerprinting separation, it would be still an alternative method to develop the fingerprint, which could have a certain positive point in quality evaluation of TCM.

4. Conclusions

A reliable fingerprinting method based on column switching technology by using a single pump was developed and applied successfully to analyze 12 batches of very complex preparation of TMOL in the present work. Through chemometric methods including similarity analysis, PCA, and HCA, the discrimination of expired and unexpired TMOL samples was successfully achieved. Column switching technology with a pump would make fingerprints have better separation power and higher peak capacity and could provide an effective method to establish fingerprints for quality control and evaluation of complex TCM preparations like TMOL.

Authors' Contributions

Meng Yuan contributed to software, data curation, and manuscript review and editing. Xiaoyan Liu carried out methodology and writing of original draft. Yue Sun contributed to validation. Linlin Wang performed formal analysis. Pian Jin carried out investigation. Xiaoqi Zhuang provided resources. Hengchang Zang was responsible for supervision. Zhonghu Zhang performed visualization. Lei Nie was responsible for conceptualization, project administration, and funding acquisition. Meng Yuan and Xiaoyan Liu contributed equally to this work.

References

- [1] D. Xiao, "Clinical observation of Tianmeng Oral Liquid combined with eszopiclone in treatment of post-stroke insomnia," *Drugs & Clinic*, vol. 31 no. 10, pp. 1612-1615, 2016.
- [2] J. Zhao, M. Su, Q. Fang, "Clinical observation of Tianmeng Oral Liquid in treatment of chronic obstructive pulmonary disease and insomnia," *Chinese Traditional and Herbal Drugs*, vol. 48 no. 17, pp. 3587-3589, 2017.
- [3] Y. Liang, P. Xie, F. Chau, "Chromatographic fingerprinting and related chemometric techniques for quality control of traditional Chinese medicines," *Journal of Separation Science*, vol. 33 no. 3, pp. 410-421, DOI: 10.1002/jssc.200900653, 2010.
- [4] Z. Shu, X. Li, K. Rahman, L. Qin, C. Zheng, "Chemical fingerprint and quantitative analysis for the quality evaluation of Vitex negundo seeds by reversed-phase high-performance liquid chromatography coupled with hierarchical clustering analysis," *Journal of Separation Science*, vol. 39 no. 2, pp. 279-286, DOI: 10.1002/jssc.201500796, 2016.
- [5] H. Wang, M. Chen, J. Li, Y. Chang, Z. Dou, Y. Zhang, "Quality consistency evaluation of Kudiezi Injection based on multivariate statistical analysis of the multidimensional chromatographic fingerprint," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 177, 2020.
- [6] X. Liu, H. Zhang, M. Su, "Comprehensive quality evaluation strategy based on non-targeted, targeted and

- bioactive analyses for traditional Chinese medicine: Tianmeng oral liquid as a case study," *Journal of Chromatography A*, vol. 1620, 2020.
- [7] D. Ge, Z. Deng, "Determination of astragaloside in Tianmeng capsules by HPLC-ELSD," *China Pharmaceuticals*, vol. 24 no. 5, pp. 31-32, DOI: 10.1097/01.bmsas.0000461705.82350.f2, 2015.
- [8] X. Ma, W. Qu, "HPLC determination of the contents of strychnine in Tianmeng capsules," *Herald of medicine*, vol. 27 no. 4, pp. 467-468, DOI: 10.1111/j.1469-8749.1996.tb15106.x, 2008.
- [9] X. Gao, H. Cao, P. Wang, "HPLC determination of the contents of syringin in Tianmeng capsules," *Chin J Pharm Anal*, vol. 27 no. 12, pp. 1953-1955, 2007.
- [10] F. Gao, Z. Xu, W. Wang, G. Lu, Y. Vander Heyden, T. Zhou, G. Fan, "A comprehensive strategy using chromatographic profiles combined with chemometric methods: application to quality control of *Polygonum cuspidatum* Sieb. et Zucc," *Journal of Chromatography A*, vol. 1466, pp. 67-75, DOI: 10.1016/j.chroma.2016.08.050, 2016.
- [11] X. Lu, W. Tong, S. Wang, J. Li, J. Zheng, X. Fan, L. Liu, "Comparison of the chemical constituents and immunomodulatory activity of *ophiopogonis radix* from two different producing areas," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 134, pp. 60-70, DOI: 10.1016/j.jpba.2016.11.025, 2017.
- [12] W. Jiang, X. Liu, M. Su, H. Zang, Y. Zeng, L. Nie, "Chromatographic fingerprint based on serially coupled columns combined with multiple-component quantitation with a single reference standard for quality evaluation of Shen-Zhi-Ling oral liquid," *Analytical Methods*, vol. 11 no. 3, pp. 367-374, DOI: 10.1039/c8ay01744a, 2019.
- [13] F. P. Prencipe, V. Brighenti, M. Rodolfi, A. Mongelli, C. dall'Asta, T. Ganino, R. Bruni, F. Pellati, "Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in *Humulus lupulus* L," *Journal of Chromatography A*, vol. 1349, pp. 50-59, DOI: 10.1016/j.chroma.2014.04.097, 2014.
- [14] Y. Liu, Z. Liu, G. Sun, Y. Wang, J. Ling, J. Huang, "Monitoring and evaluating the quality consistency of compound bismuth aluminate tablets by a simple quantified ratio fingerprint method combined with simultaneous determination of five compounds and correlated with antioxidant activities," *PloS One*, vol. 10 no. 3, DOI: 10.1371/journal.pone.0118223, 2015.
- [15] S. Liu, G. Zhang, Y. Qiu, X. Wang, L. Guo, Y. Zhao, M. Tong, L. Wei, L. Sun, "Quality evaluation of Shenmaidihuang Pills based on the chromatographic fingerprints and simultaneous determination of seven bioactive constituents," *Journal of Separation Science*, vol. 39 no. 23, pp. 4557-4567, DOI: 10.1002/jssc.201600718, 2016.
- [16] D. R. Stoll, X. Li, X. Wang, P. W Carr, S. E Porter, S. C Rutan, "Fast, comprehensive two-dimensional liquid chromatography," *Journal of chromatography. A*, vol. 1168 no. 1-2, DOI: 10.1016/j.chroma.2007.08.054, 2007.
- [17] M. Kula, D. Głód, M. Krauze-Baranowska, "Two-dimensional liquid chromatography (LC) of phenolic compounds from the shoots of *Rubus idaeus* 'Glen Ample' cultivar variety," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 121, pp. 99-106, DOI: 10.1016/j.jpba.2015.12.047, 2016.
- [18] M. Iguiniz, S. Heinisch, "Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 145, pp. 482-503, DOI: 10.1016/j.jpba.2017.07.009, 2017.
- [19] K. Zhao, D. Yang, Z. Wang, "Column-switching high performance liquid chromatography and its application to food inspection," *National Institute for Nutrition and Food Safety*, vol. 21 no. 2, pp. 156-161, 2009.
- [20] M. Hädener, P. S. Bruni, W. Weinmann, M. Frübis, S. König, "Accelerated quantification of amphetamine enantiomers in human urine using chiral liquid chromatography and on-line column-switching coupled with tandem mass spectrometry," *Analytical and Bioanalytical Chemistry*, vol. 409 no. 5, pp. 1291-1300, DOI: 10.1007/s00216-016-0056-1, 2017.
- [21] C.-L. Hsieh, P.-Y. Lin, F. Akita, M. Mita, T. Ide, J.-A. Lee, K. Hamase, "Development of a three-dimensional HPLC system for the simultaneous determination of lactate and 3-hydroxybutyrate enantiomers in mammalian urine," *Chromatography*, vol. 40 no. 1, pp. 25-32, DOI: 10.15583/jpchrom.2018.021, 2019.
- [22] B. Efron, "Rietz lecture-bootstrap methods-another look at the jackknife," *Annals of Statistics*, vol. 7 no. 1, 1977.

[23] W. Zhao, F. Sun, J. Cui, Y. Ren, M. Liu, F. Zhao, "Quantitative analysis and chromatographic fingerprinting of WeiChangShu tablet using high-performance liquid chromatography combined with chemometric methods," Chromatographia, vol. 81 no. 9, pp. 1293-1304, DOI: 10.1007/s10337-018-3553-x, 2018.

DETAILS

Subject:	Similarity; Quality assessment; Principal components analysis; Acids; Cluster analysis; Technology assessment; Fingerprinting; Clustering; Switching; Separation; Traditional Chinese medicine; Statistical methods; Methods; Chromatography; High performance liquid chromatography; Chemical composition; Chinese medicine; Control methods
Location:	United States--US; China
Editor:	Neil D Danielson
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-07-19 (Received); 2021-08-17 (Revised); 2021-09-13 (Accepted); 2021-09-29 (Pub)
DOI:	https://doi.org/10.1155/2021/2514762

ProQuest document ID: 2580585663

Document URL: <https://www.proquest.com/scholarly-journals/chromatographic-fingerprinting-based-on-column/docview/2580585663/se-2?accountid=211160>

Copyright: Copyright © 2021 Meng Yuan et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-10-10

Database: Publicly Available Content Database

Document 14 of 59

Proximate Analysis of Poultry-Mix Formed Feed Using Maize Bran as a Base

Solagbade Saheed Afolabi ¹

; Oyeyode, John Oluwafemi ²

; Wasswa Shafik ³

; Sunusi, Zubair A ⁴; Adegoke Abdullahi Adeyemi ⁵ ¹ Department of Chemistry, Yazd University, P.O. Box 89175-741, Yazd, Iran ² Polymer Department, Hussaini Adamu Federal Polytechnic, Kazaure, Jigawa, Nigeria ³ Department of Computing & Mathematics, King Fahd University of Petroleum & Minerals, Dhahran 31261, Saudi Arabia ⁴ Federal Government Girls' College, Kazaure, Jigawa, Nigeria ⁵ Biological Sciences Department, Federal University of Agriculture Abeokuta, Abeokuta, Nigeria

[ProQuest document link](#)

ABSTRACT (ENGLISH)

The purpose of this research was to demonstrate the proximate analysis of poultry-mix made using maize bran as a basis. Red beans, soya beans, and benny beans were the three samples utilised in this study. This work investigates the appropriate poultry mix for birds breed for meat and egg. Thirty grammes of proteinous feedstock were weighed and homogeneously combined with 70 grammes of maize bran. The following was revealed in a proximate analysis of the feeds: moisture ranged from 1.18% to 1.54%, unrefined lipids 0.99–3.08%, total carbohydrate 57% to 72%, ash content 38.48% to 38.92%, unrefined protein 18.38% to 22.53% and unrefined fiber 2.0% to 4.65% respectively for broilers and layers. In terms of nutritional concentrations, all feed samples showed a substantial variation. Based on the findings of the study, it can be stated that Soya bean-maize bran is an excellent poultry-mix formulation that has deep well-disposed benefits and meets nearly all nutritional needs for meat and egg-producing birds.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

For many years, the continual rise in the human population has had a significant impact on the demand for animal-based foods. In third-world nations like Nigeria, population growth has not kept pace with agricultural expansion, particularly in livestock output. Feedstocks like maize bran and soya bean are generally of high quality, providing all of the necessary nutrients in the right quantities for the poultry's healthy growth and development. Rice milling waste in broiler chicken diets significantly increased nutrient utilization and economic value, and may thus be utilised to replace maize in broiler chicken diets between 10% and 40% as demonstrated [1]. The world's livestock producers are having difficulties in meeting the demand for animal production, and this disparity has led to severe malnutrition [2].

It was reported years after that the number of undernourished people is still increasing world-wide by about five million each year with overwhelming majority in the developing world [3]. Unfortunately, the egg, which is a compact source of vital nutrients and should have been a more natural way of delivering the needed nutrients, is prohibitively expensive for the typical man. In the retail market, eggs had varying quality and nutritional content, which may impact human RDA fulfilment and perhaps enhance egg quality and nutritional value [4]. Numerous studies have been carried out for the proximate analysis of poultry-mix formed. To show this, the authors of [5] studied and evaluated the intake of low-graded-cassava meal, finding that the newly created drink "dusa" contained more protein than maize grains. Furthermore, an experiment was conducted to compare the energy sources of Cashew nut meal and conventional-plant-proteins meal. Based on the results of this investigation, Cashew nut in diets can be an excellent alternative to conventional-plant-proteins. [6].

To attain the exact quantities of nutrients, it is vital to balance the ratio of diets. The research's findings revealed substantial differences in the quality of chicken diets made from soya beans, red beans, and benny beans. It can be stated that Soya bean-maize bran proven to be an appropriate poultry-mix formulation with minimal side effects that meets nearly all nutritional needs for poultry birds. It has a high percentage of carbohydrates, which are an essential source of energy for broilers and layers. It also has a high proportion of natural protein, which is the fundamental ingredient of structural and protective tissues for poultry birds.

The main contribution of this paper is to derive a balanced diet that resolve to provide appropriate quantities of biologically available nutrients required by birds breed for meat and egg. Moisture content, ash content, unrefined protein, unrefined fiber content, and unrefined lipid content are all subjected to an experimental analysis. The total carbohydrate content of maize bran, red and soya beans, and the Benny, on the other hand, was determined in order to provide farmers with useful information on the nutritional composition of poultry feeds.

2. Materials and Methods

In this section, the used methodology and content samples are briefly laid that includes Sample Assortment and Preparation.

2.1. Sample Assortment and Preparation

The maize bran, red beans (*Phaseolus vulgaris*), soya beans (*Glycine max*), and Benny seed (*Sesame indicum*) were bought from the local market in Masaka, Nassarawa state Nigeria.

2.2. Preparation of Poultry-Mix

A 30g each of the proteinous feedstock was weighed and mixed homogeneously with 70g of the maize bran, each detailed below:

2.3. Analysis for Moisture Content

A 5g sample of poultry-mix was put into a crucible and placed in an oven at 105°C for 12 hours. After cooling in desiccator, the sample was re-weighed using the technique described by [7]. The percentage of moisture content (%) was calculated using, $(1)100\beta - \alpha - \gamma - \alpha\beta - \alpha$, where α is the weight of clean, dry crucible (g), β is the weight of crucible + wet sample (g), γ is the weight of crucible + dry sample (g).

2.4. Analysis for Ash Content

A 5g of the previously calcined sample obtained by heating at high temperature in the absent of air was weighed into a dry crucible and brought to constant weight. The crucible was placed in a furnace and heated for 12 hours at 550°C and was left to cool in a desiccator, after which the crucible with the ash was carefully weighed. The percentage of ash content was calculated using, (2) $\text{Ash content \%} = 100\alpha - \beta\gamma$, where α is the weight of crucible with sample (g), β is the weight of crucible with ash (g), γ is the weight of sample (g).

2.5. Analysis for Lipid Content

Different types of lipids in nature differ structurally from one another. The qualitative analysis of the lipid was carried out as follow; The extraction flasks were removed from the kiln, it was exposed to cool by the use of a dryer, and lastly, it was weighed to milligrams. A 5g of the dry sample was weighed into insertion thimble, with focal handling of the dried sample through tongs, and then the sample was positioned within the extraction unit. The flask that contained the hexane at 75% of totality volumes to an extractor was connected the solution brought to boiling and the heat was adjusted to attain almost ten refluxes within sixty minutes based on the lipids within the sample. When all the above steps were done to that point, the hexane was meant to be evaporated in rotary-evaporator. The used flasks within the experiment were cooled in a desiccator, and the weighed respectively to within milligrams. The defatted samples were used in determining unrefined fiber. The Unrefined lipid was calculated using, (3) $\text{Unrefined lipid content \%} = 100\beta - \alpha\gamma$.

Given that, α depict the weight of clean, dry flask (g), β is the weight of flask with fat (g), γ is the weight of the sample (g).

2.6. Analysis for Fiber Content

Fiber content is the most important factor in determining how fiber composites behave. Acid hydrolysis was used to determine the crude fiber content. A 3g defatted sample was carefully weighed and was placed in the 200ml flask, then boiling sulphuric acid solution was added. The boiling point of the attached condenser was maintained, the sample was boiled for an accurately half an hour, the quantity of distilled water was kept steady and the flask was swirling from often in order to eliminate particles that may then attached to the sides. The filtration funnel was lined with the filter paper and pre-heated with boiling water. Finally, the flask was detached and allowed to rest for 60 seconds, then contents were filtered with suction.

Residual product from the treated filter paper was transferred into sodium hydroxide solution boiled for half hour. The hydrolyzed mixture from the crucible was carefully separated after standing for 1 min. The residue was washed with boiling water, with the HCl solution and then again with boiling water, finishing with three washes with hexane. The crucible was placed in a kiln set at 105°C for 12 hours then cooled in desiccator. The crucible was weighed quickly with the residue inside and was placed in the crucible furnace at 550°C for 3 hours and was left to cool in a desiccator and weighed again. Unrefined fiber was calculated using, (4) $\text{Unrefined fiber content \%} = 100\alpha - \beta\gamma$ where, α is weight of crucible with dry residue (g), β is the weight of crucible with ash (g), γ is the weight of sample (g).

2.7. Analysis for Protein Content

A 1g of sample was weighed and transferred to a Kjeldahl flask; 10g Na_2SO_4 , 0.7g HgO , 25ml sulphuric acid and a few grains of pumice stone was added after which the flask was heated at 320°C, shaking irregularly until the material was carbonized and the bubbles disappeared, the temperature was raised to 360°C, then brought to a moderate bubble. The flask walls were not allowed to burn in order to prevent organic particles from adhering to them.

As a clear solution was observed with no color possessions, it was then boiled for 2 hours longer and cool. The distilled water of 350ml was carefully added onto the flask, where the contents were stirred, given that some zinc pellets were added and left to cool. Considerably, the sulphuric acid with 0.5N of the capacity of 25ml was collected into a single flask of the distilling apparatus, anticipated nitrogen, with noticeable droppings of methyl red indicator added concurrently.

The experiment added both a 100ml of the sodium hydroxide and 10ml sodium sulphate to the sample in order to maintain ammonia where the solution is well mixed connected to the distilling apparatus. The flask was heated in

order to distill approximately 150ml of the liquids in half an hour. The use of litmus paper depicted alkaline, although the distillation process continues. The content in the flask was stirred irregularly. In the collecting flask, the excess sulphuric acid was titrated with sodium hydroxide 0.25N, the methyl-red-indicator. (5) Nitrogen in sample % = $100\alpha\beta\gamma$ 0.014, where, α is the chlorohydric acid used in titration (mL), β is the normality standard, γ is the weight of the sample.

2.8. Analysis for Carbohydrate Content (Clegg-Anthrone Method)

The 2.5 grams of the wet samples were ranged from 0.001 g to 300mg of sum carbohydrates, transferred to a stopped of 100ml graduated cylinder, with water of 10mL capacity was added and stirred in combination glass rod to diffuse the samples. The solution was transferred to 1 mL spectrophotometer cells. The green color was found stable for a period of only 2 hours. The absorption-rates were read at 630nm adjacent to the blank solution calculated [8]. (6) Total available carbohydrates % of $C_6H_{12}O_6 = 25.\beta\alpha.W$, Given that, W is the weight of the sample (g), α is the absorption of diluted standard, β is the concentration of the sample.

3. Results and Discussion

The experimental results and discussion of the nutritional value of a poultry feed that are considered when formulating diets are unrefined protein, moisture, unrefined lipid, unrefined fiber, ash and carbohydrate content.

3.1. Unrefined Lipid Content of Poultry-Mix

The result in Figure 1 indicates that Soya beans-maize bran mix has the lowest unrefined lipid content of 0.99% while the Benny seed-maize bran and red beans-maize bran has an unrefined lipid content of 3.08% and 2.12% respectively. Poultry-mix with high unrefined lipid content will be ideal for poultry birds raised for meat; hence Benny seed and red bean-maize bran will be a good choice for such poultry farmer/farm. In poultry farms where fat is not a superior demand, soya bean-maize bran will be appropriate as it will provide the required energy needed by the birds [9].

[figure omitted; refer to PDF]

Nevertheless, the feeds with stumpy ash-content are co-operative in monitoring the urinary-tract complications, literature have not recorded that feeds that are high in ash-content are beneficial to poultry birds that are being raised for meat and egg [12]. Therefore, all three samples are good choices for poultry-mix formulation considering their ash content but different mineral compositions.

3.5. Unrefined Protein Content of Poultry-Mix

Soya bean-maize bran had a slightly high amount of unrefined protein to be 22.53% compared to Red bean-maize bran and Benny seed-maize bran with 20.13% and 18.38% respectively as shown in Figure 5. A high amount of unrefined protein contains the main crucial components of essential, and protecting soft tissue, like the skin feather, the bone matrix, and the ligaments and the soft tissues comprising structures and muscle for both broilers and layers, where animal additives were put under consideration [13].

[figure omitted; refer to PDF]

Higher altitudes of unrefined protein advance effectiveness by accumulative broiler enactment, and the dispensation yield. Nonetheless, when protein is inadequate in poultry feed as seen in the case of using Benny seed-maize bran, there is a decrease of growth or even efficiency and an insertion of protein from a smaller amount of vigorous body tissues to preserve the utilities of additional vigorous tissues. In chicken feed, crude protein is an important nutrient. Lowering the usage of crude protein not only lowers feed costs, but it also reduces pollution in the chicken industry as illustrated [14]. Given its protein content (22.53%), soya bean-maize bran is an excellent source of protein in poultry diets.

3.6. Unrefined Fiber Content of Poultry-Mix

Benny seed-maize bran contained the highest amount of unrefined fiber to be 4.65%, Soya beans-maize bran, and red bean-maize bran contained a low amount of unrefined fiber to be 2.0% and 2.43% respectively as shown in Figure 6. Poultry feeds high in unrefined fiber should be discouraged due to the adverse effects on nutrient, utilization, and performance such as a decrease in body weight gain and feed conversion [14]. Therefore, any

poultry farmer that is breeding poultry birds raised for meat and egg should ensure that the poultry-mix should be low in unrefined fiber and these shows why Soya bean-maize bran is an ideal poultry feed considering its low unrefined fiber content (2.0%) as demonstration [15].

[figure omitted; refer to PDF]

4. Conclusion

The relatively low percentage moisture content indicates low susceptibility of micro-organisms and with a low probability of affecting the physical and chemical aspects of the feed, which relates its freshness and stability for the storage of the feed over a long period of time. Furthermore, it has a low percentage of unrefined lipids, which signifies that where fat is not a significant demand, it would be appropriate to provide the required energy needed by the birds. The diets for broilers and layers at the same, but the layers diet typically has slightly more calcium and is fortified with extra vitamins for proper embryo development and egg-shell formation. In contrast, broilers require high protein, calcium, metabolizable energy and less fiber in their feed. Therefore, it can be concluded that soya bean-maize bran, which has all these requirements for layers and broiler feed, can be used. As our future research, this experiment is to be subjected to other chemical content analysis of poultry feeds mix in aspect to increase poultry productivity.

Disclosure

The paper was providing a detailed mix of poultry and was from the combined efforts of authors involve in experimentation; apparatus set ups, results structuring and figure plotting among others.

Acknowledgments

The authors acknowledge the Department of Chemistry, HussainiAdamu Federal Polytechnic Kazaure, for giving us their skills in mainly apparatus measurements and doing experiments. This study has been supported and funded by Hussaini Adamu Federal Polytechnic Kazaure of Nigeria.

References

- [1] R. S. Onabanjo, G. S. Ojewola, D. N. Onunkwo, O. O. Adedokun, E. U. Ewa, A. O. Nzotta, K. K. Safiyu, "Performance of broiler chickens fed rice milling waste based diets," *Nigerian Journal of Animal Production*, vol. 48 no. 2, pp. 148-161, DOI: 10.51791/njap.v48i2.2943, 2021.
- [2] K. Lokaewmanee, K. Seedarak, "The effects of dietary maoberry (*Antidesma* sp.) pomace supplementation on broiler growth performance, blood parameters, carcass quality and intestinal histology," *Indian Journal of Animal Research*, vol. 1 no. 7, DOI: 10.18805/ijar.b-1362, 2021.
- [3] F. J. Elgar, A. Sen, G. Gariépy, W. Pickett, C. Davison, K. Georgiades, N. Hammami, M. A. Da Silva, D. Gordon, H. R. Melgar-Quinonez, "Food insecurity, state fragility and youth mental health: a global perspective," *SSM-Population Health*, vol. 14 no. 14, DOI: 10.1016/j.ssmph.2021.100764, 2021.
- [4] M. Hossain, W. Rahman, M. Ali, T. Sultana, K. Hossain, "Identification and antibiogram assay of *Escherichia coli* isolated from chicken eggs," *Journal of Bio-Science*, vol. 11, pp. 123-133, DOI: 10.3329/jbs.v29i0.54828, 2021.
- [5] A. A. Kirrella, S. E. Abdo, K. El-Naggar, M. M. Soliman, S. M. Aboelenin, M. A. O. Dawood, A. A. Saleh, "Use of corn silk meal in broiler diet: effect on growth performance, blood biochemistry, immunological responses, and growth-related gene expression," *Animals*, vol. 11 no. 4, DOI: 10.3390/ani11041170, 2021.
- [6] J. O. Ocheja, A. Halilu, B. A. Shittu, S. E. Eniolorunda, A. D. Ajagbe, "Haematology and serum biochemistry of yearling west african dwarf goats fed cashew nut shell based diets," *Veterinary Medicine and Public Health Journal*, vol. 2 no. 1, pp. 17-22, 2021.
- [7] M. Rózewicz, "Production, use and efficiency of utilising grains of various cereal species as feed resources for poultry production," *Polish Journal of Agronomy*, vol. 38, pp. 66-74, 2019.
- [8] V. Krishnan, M. Awana, A. Singh, S. Goswami, T. Vinutha, R. R. Kumar, S. P. Singh, T. Sathyavathi, A. Sachdev, S. Praveen, "Starch molecular configuration and starch-sugar homeostasis: key determinants of sweet sensory perception and starch hydrolysis in pearl millet (*Pennisetum glaucum*)," *International Journal of Biological Macromolecules*, vol. 183 no. 183, pp. 1087-1095, DOI: 10.1016/j.ijbiomac.2021.05.004, 2021.
- [9] A. Čirović, I. Ivanovski, A. Čirović, D. Nikolić, A. Ivanovski, "The adjuvant aluminum fate-metabolic tale based on

- the basics of Chemistry and biochemistry," Journal of Trace Elements in Medicine & Biology, vol. 16, 2021.
- [10] M. Kačániová, L. Hleba, M. Džugan, A. Pasternakiewicz, V. Kňazovická, "Microbiological properties and antimicrobial effect of Slovakian and Polish honey having regard to the water activity and water content," Journal of Microbiology, Biotechnology and Food Sciences, vol. 6, pp. 272-281, 2021.
- [11] H. M. Hafez, Y. A. Attia, "Challenges to the poultry industry: current perspectives and strategic future after the COVID-19 outbreak," Frontiers in veterinary science, vol. 7, DOI: 10.3389/fvets.2020.00516, 2020.
- [12] G. Tobin, A. Schuhmacher, "Laboratory animal nutrition in routine husbandry and experimental and regulatory studies," Handbook of Laboratory Animal Science, DOI: 10.1201/9780429439964-13, 2021.
- [13] K. Y. Man, K. L. Chow, Y. B. Man, W. Y. Mo, M. H. Wong, "Use of biochar as feed supplements for animal farming," Critical Reviews in Environmental Science and Technology, vol. 51 no. 2, pp. 187-217, DOI: 10.1080/10643389.2020.1721980, 2021.
- [14] Y. A. Attia, F. Bovera, J. Wang, M. A. Al-Harthi, W. K. Kim, "Multiple amino acid supplementations to low-protein diets: effect on performance, carcass yield, meat quality and nitrogen excretion of finishing broilers under hot climate conditions," Animals, vol. 10 no. 6, DOI: 10.3390/ani10060973, 2020.
- [15] A. Giri, P. Ranjan, V. K. Bharti, "Selenium toxicity in domestic animals: sources, toxicopathology, and control measure," Selenium Contamination in Water, DOI: 10.1002/9781119693567.ch4, 2021.

DETAILS

Subject:	Carbohydrates; Acids; Experiments; Sodium; Poultry; Eggs; Soybeans; Lipids; Nutrients; Caustic soda; Meat; Moisture content; Beans; Proteins
Location:	Nigeria
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English

Document type: Journal Article

Publication history :

Milestone dates: 2020-08-08 (Received); 2021-08-31 (Revised); 2021-09-03 (Accepted); 2021-09-21 (Pub)

DOI: <https://doi.org/10.1155/2021/8894567>

ProQuest document ID: 2578641868

Document URL: <https://www.proquest.com/scholarly-journals/proximate-analysis-poultry-mix-formed-feed-using/docview/2578641868/se-2?accountid=211160>

Copyright: Copyright © 2021 Solagbade Saheed Afolabi et al. This is an open access article distributed under the Creative Commons Attribution License (the “License”), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. <https://creativecommons.org/licenses/by/4.0/>

Last updated: 2023-08-31

Database: Publicly Available Content Database

Document 15 of 59

Determination of Carrageenan in Livestock and Poultry Meat by Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry

Sun, Yanqin ¹ ; Zhu, Xudong ² ; Shen, Xixi ¹ ; Wang, Wei ¹

¹ Key Laboratory of Meat Processing and Quality Control, MOE, Key Laboratory of Meat Processing, MARA, Jiangsu Synergetic Innovation Center of Meat Processing and Quality Control, Nanjing Agricultural University, Nanjing 210095, China ² Key Laboratory of Meat Processing and Quality Control, MOE, Key Laboratory of Meat Processing, MARA, Jiangsu Synergetic Innovation Center of Meat Processing and Quality Control, Nanjing Agricultural University, Nanjing 210095, China; College of Sciences, Nanjing Agricultural University, Nanjing 210095, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has become the main method for the detection and analysis of food additives because of its good separation, high selectivity, and high sensitivity. The aim of this study was to establish an UHPLC-MS/MS method that can quickly and accurately measure the content of carrageenan in livestock and poultry meat. Chromatographic separation was performed on

an ACQUITY UPLC BEH HILIC C18 column (2.1 mm×50 mm, 1.7 μ m) using a gradient elution with methanol and 0.1% (v/v) formic acid in water as a mobile phase. The quantitative analysis was executed using a triple quadrupole mass spectrometer in which electrospray ionization, multiple reaction monitoring, and negative mode were operated. The retention time was about 1.3 min for carrageenan. The carrageenan content showed a good linear relationship from 0.05 to 1.00 g/kg. The limit of detection (LOD) was 0.06 g/kg, and the limit of quantification (LOQ) was 0.18 g/kg. The standards were spiked at three levels (low, medium, and high) and were analyzed in six replicates. The recovery values of carrageenan in pork, beef, lamb, chicken, and duck meat were 82.06–111.55%, 85.43–112.50%, 89.55–116.00%, 83.80–102.15%, and 82.41–110.90%, respectively. The relative standard deviations (RSDs) were all lower than 7.51%. The developed method shows a high recovery rate and good precision and can be used for the rapid detection of carrageenan in livestock and poultry meat.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Carrageenan is a polysaccharide extracted from marine red algae and widely used in the production and processing of meat and meat-based products to improve meat texture [1–3] because of its good water retention capacity and stability [4–6]. Carrageenan is a safe and nontoxic natural food additive. The carrageenan added to meat and meat-based products is nondegradable. Even this nondegradable type, however, can be degraded under high temperature and an acidic environment during meat processing [7–9]. According to the references and results of animal experiments, excessive intake of degraded carrageenan damages the gastrointestinal tract, induces inflammation, and leads to the development of tumors and other diseases [10–14]. In this regard, the safety of carrageenan in meat and meat-based products should be carefully regulated.

The European Union currently prohibits the addition of carrageenan to infant food formulas but allows its use in meat and meat-based products. The *National Food Safety Standard for the Use of Food Additives* (GB2760-2014) issued by China clearly indicates that carrageenan cannot be used as a thickening agent in fresh meat, but it can be used in the production of premade meat products and cooked meat products with clear marking on the product packaging. Carrageenan is not strictly regulated in the current marketplace, but driven by profits, some unethical manufacturers use carrageenan to increase the weight of meat and meat-based products. The unregulated use of carrageenan not only changes the composition of meat but also increases the risk of contamination from foreign impurities and pathogenic microorganisms. This seriously affects meat quality and safety [14]. In addition, carrageenan is often used as an adhesive to reconstitute meat to maintain its sensory state. To attract consumers, manufacturers do not always correctly or accurately label the food packaging as required by government standards. Thus, it is important to detect the presence of carrageenan in livestock and poultry meat and related products.

Current carrageenan detection methods in complex food products mainly include fluorescence spectroscopy [15, 16], infrared spectroscopy [17–21], Raman spectroscopy [22], and nuclear magnetic resonance technology [23–26]. These methods all require models established in conjunction with chemometrics, and data processing is cumbersome. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) combined with chromatographic separation and mass spectrometry identification can help determine a variety of compounds—this technique has been widely used in the analysis of food quality and safety including for aflatoxin [27], free fatty acids [28], and phenols [29]. However, there are few reports on the detection of carrageenan in livestock and poultry meat via this method. Ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has even higher separation speed, better peak identification, and higher sensitivity than HPLC-MS/MS. Therefore, in view of the fact that carrageenan is mainly artificially added and does not exist in fresh meat, we established a UHPLC-MS/MS method to quickly measure the content of carrageenan in livestock and poultry meat. This method can realize sensitive and quantitative identification of the carrageenan present in livestock and poultry meat and detect carrageenan-adulterated meat and poultry.

2. Materials and Methods

2.1. Materials and Reagents

Food-grade carrageenans were purchased from ShiShi Globe Agar Industries Co., Ltd. (Fujian, China). Hydrochloric acid (density approximately 1.19g/mL, guaranteed reagent grade) was obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Chromatographically pure acetonitrile was purchased from Merck Co., Ltd. (Branchburg, NJ, USA). All solutions were prepared with ultrapure water (18.2MΩ cm, an Arium® Pro ultrapure water purification system; Sartorius, Göttingen, Germany). We obtained positive samples of pork, beef, lamb, chicken, and duck containing 0.20g/kg carrageenan labeled as A, B, C, D, and E, respectively. Samples were from the Supervision, Inspection and Testing Center for Quality of Meat Products (Nanjing, China). In addition, we acquired commercial food products including various kinds of livestock and poultry meat and meat products from a local supermarket in Nanjing, China. Other analytically pure reagents were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Sample Pretreatment

Food-grade carrageenans have an average molecular weight of 100,000 Da or higher [24], which makes it difficult to detect them directly using UHPLC-MS/MS. Therefore, in this experiment, we pretreated the meat samples according to the following steps: (1) about 10.00g of meat sample was placed in a 50 mL centrifuge tube followed by the addition of 23 mL of water; (2) the mixture was homogenized at 10,000 rpm for 1 min, and then 2 mL of 12 mol/L hydrochloric acid was added and the mixture was vortexed for 1 min; (3) the sample was placed on a shaker at 200 rpm for 30 min; (4) the mixture was then put in a water bath at 80°C for 20 min and agitated every 10 min; (5) the sample was taken out from the water bath and cooled to room temperature (22°C–28°C) before centrifugation at 10,000 rpm for 10 min; and (6) the supernatant was diluted with acetonitrile at a ratio of 1:1 (v/v) and passed through a 0.22-μm filter into a UHPLC-MS/MS autosampler vial.

2.3. Preparation of Standard Solutions

In a 10 mL volumetric flask, 0.10g of carrageenan was dissolved in 8 mL of water at 60°C. The flask was cooled to room temperature (22°C–28°C) before further dilution with water to prepare a stock solution with a mass concentration of 10 mg/mL. We weighed 10.00g of five types of livestock and poultry meat without carrageenan into a 50 mL centrifuge tube. We then added 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, or 1.00 mL of carrageenan reference stock solution to the tubes along with water to reach a final volume of 23 mL. The mixture was homogenized for 1 min at 10,000 rpm. The subsequent pretreatment was performed in accordance with the method described above, and the extracts were analyzed through UHPLC-MS/MS.

2.4. Chromatographic and Mass Spectrometric Conditions

We used an ACQUITY I-Class UHPLC system equipped with a XEVO TQ-S micro triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) for separation and detection. MassLynx 4.1 software (Waters Corp., Milford, MA, USA) was used to acquire data and control the system. The analytes were separated on a Waters ACQUITY UPLC BEH HILIC C18 column (2.1 mm × 50 mm, 1.7 μm). The column temperature was 35°C, and the sample injection volume was 5.0 μL. We performed a gradient elution with solutions A (methanol) and B (0.1% (v/v) formic acid in water) as the mobile phases at a flow rate of 0.4 mL/min: 0–1 min, 80% A and 20% B; 1–4 min, 40% A and 60% B; and 4–5 min, 80% A and 20% B.

Mass spectrometry equipped with electrospray ionization (ESI) source in negative mode was used for multiple reaction monitoring (MRM). We performed mass spectroscopic analysis under the following conditions: quantitative ion pair (m/z): 402.97–96.96; qualitative ion pair (m/z): 402.97–96.96, 402.97–241.07; desolvation gas flow: 700 L/h; cone gas flow: 150 L/h; collision energy: 30–34 eV; capillary voltage: 2800 V; nebulizer pressure: 6.5 bar; and desolvation gas temperature: 550°C.

2.5. Determination of the Linear Range, Detection Limit, and Quantification Limit

We measured the prepared matrix standard solutions using UHPLC-MS/MS via an established UHPLC-MS/MS method. The concentration gradient had seven concentration points including 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 g/kg. A standard curve was plotted using the mass concentration of carrageenan (g/kg) as the abscissa (X) and

the peak area ($\mu\text{S} \times \text{min}$) as the ordinate (Y). The plot was then used to determine the optimal linear range and correlation coefficient. We used three times the signal-to-noise ratio to calculate the limit of detection (LOD), and 10-fold the signal-to-noise ratio to calculate the limit of quantitation (LOQ) [30].

2.6. Determination of Accuracy and Precision of UHPLC-MS/MS

The carrageenan standard stock solutions at three concentrations (0.20 g/kg, 0.40 g/kg, and 0.80 g/kg) were spiked into pork, beef, lamb, chicken, and duck. We performed the pretreatment according to the method described above and measured the recovery of the spiked standard. We measured six replicates of standard samples of the same concentration to calculate the relative standard deviation (RSD) and determine the method's precision.

2.7. Application in Real Meat Samples

To validate our strategy, we selected 12 types of commercially available livestock and poultry meat and meat-based products in addition to the five positive samples. We used the methods described above to measure the content of carrageenan in various meat products. The HPLC-MS/MS technique in the Food Supplementary Inspection Method of State Administration for Market Regulation (BJS 201804) was used for verification and to evaluate the practical value of the proposed method [31].

3. Results and Discussion

3.1. Standard Curve, Linearity, LOD, and LOQ of UHPLC-MS/MS

We used the UHPLC-MS/MS method to detect the standard working solutions of carrageenan in five types of poultry and meat. The standard curve was plotted to determine the LOQ, the LOD, and the linear range of carrageenan in different matrices. Data from Figures 1 and 2 and Table 1 show that this method efficiently detected carrageenan in livestock and poultry meat and obtained good peak shapes. All samples had a good linear dynamic range of 0.05–1.00 g/kg. The correlation coefficients were greater than 0.99, and the LOD and LOQ were 0.06 g/kg and 0.18 g/kg, respectively, for all samples. Considering the functional characteristics of carrageenan, the amount added in meat products was reported to be 0.25–0.50 g/kg [32]. This study used 0.05 g/kg as the lowest concentration of the standard curve, which met the sensitivity requirements for carrageenan detection. This method offered a shorter detection time, better linear range, and higher sensitivity than previously reported methods [20, 22, 25, 33].

[figures omitted; refer to PDF]

[figures omitted; refer to PDF]

Table 1

Linear equation, correlation coefficient, detection limit, and quantification limit of carrageenan as determined by UHPLC-MS/MS ($n=6$).

Matrix	Linear equation	Correlation coefficient (R^2)	Linear range (g/kg)	LOD (g/kg)	LOQ (g/kg)
Pork	$Y=3398780X+287068.44$	0.995	0.05–1.00	0.06	0.18
Beef	$Y=2657540X+136688.22$	0.999	0.05–1.00	0.06	0.18
Lamb	$Y=2422610X+162849.48$	0.996	0.05–1.00	0.06	0.18
Chicken	$Y=1936610X+251091.83$	0.998	0.05–1.00	0.06	0.18
Duck	$Y=1408280X+93223.84$	0.997	0.05–1.00	0.06	0.18

LOD: limit of detection; LOQ: limit of quantitation; UHPLC-MS/MS: ultrahigh-performance liquid chromatography-tandem mass spectrometry.

3.2. Accuracy and Precision of UHPLC-MS/MS

To verify the accuracy of the method, different concentrations of carrageenan standard were spiked into pork, beef, lamb, chicken, and duck to determine the recovery and precision. We measured each solution concentration in six replicates (Table 2). At the spiked levels, the recovery of carrageenan was 82.06–111.55%, 85.43–112.50%, 89.55–116.00%, 83.80–102.15%, and 82.41–110.90% for pork, beef, lamb, chicken, and duck, respectively; the precision values were 3.27–5.88%, 3.20–5.68%, 4.02–6.84%, 3.61–7.51%, and 0.84–6.11%, respectively. These results indicated that the method has high recovery and good precision and can offer accurate measurements of carrageenan content in complex meat matrices.

Table 2

The recovery and precision of spiked carrageenan standard in livestock and poultry meat ($n=6$).

Matrix	Spiked standard (g/kg)	Measured value (g/kg)	Recovery (%)	RSD (%)
<i>Pork</i>	0.20	0.21±0.02	104.75	5.88
	0.40	0.45±0.03	111.55	3.27
	0.66±0.07	82.06	5.24	-
<i>Beef</i>	0.20	0.23±0.01	112.50	5.68
	0.40	0.38±0.02	95.32	3.20
	0.68±0.05	85.43	4.61	-
<i>Lamb</i>	0.20	0.23±0.01	116.00	4.02
	0.40	0.36±0.03	89.55	5.40
	0.84±0.06	105.25	6.84	-
<i>Chicken</i>	0.20	0.20±0.01	102.15	5.53
	0.40	0.39±0.04	96.95	7.51
	0.67±0.04	83.80	3.61	-
<i>Duck</i>	0.20	0.22±0.01	110.90	0.84
	0.40	0.41±0.02	101.73	6.11

RSD: relative standard deviation.

3.3. Determination of Real Meat Samples

To examine the applicability of the established UHPLC-MS/MS, we next used 12 types of commercially available

livestock and poultry meat and meat-based products in addition to the positive controls to measure the carrageenan content. The results are given in Table 3. Carrageenan was detected in only two kinds of restructured steaks; the content was 0.28g/kg and 0.41 g/kg, respectively. The thin-cut sirloin steak had a clear label of carrageenan addition, but the thick-cut sirloin steak did not. Carrageenan was not detected in the other tested products except for the positive samples. The results were compared with HPLC-MS/MS, and there was no statistical significance between the two methods ($P>0.05$). The results showed that the method was suitable for the rapid detection of carrageenan content in livestock and poultry meat products and could be used to verify the labeling of food additives and monitor the quality and safety of meat and meat-based products.

Table 3

Analysis of carrageenan in commercial livestock and poultry meat and meat-based products ($n=6$).

Sample	Place of production	Carrageenan found (g/kg)	
UHPLC-MS/MS	HPLC-MS/MS	Pork	Jiangsu, China
Nd	Nd	Beef	Jiangsu, China
Nd	Nd	Lamb	Jiangsu, China
Nd	Nd	Chicken	Jiangsu, China
Nd	Nd	Duck	Jiangsu, China
Nd	Nd	Thick-cut sirloin steak	Zhejiang, China
0.28 ± 0.04	0.29 ± 0.05	Thin-cut sirloin steak	Zhejiang, China
0.41 ± 0.07	0.42 ± 0.09	Prime rib steak	Shanghai, China
Nd	Nd	Charcoal braised pork chop	Beijing, China
Nd	Nd	Bacon	Hubei, China
Nd	Nd	Smoked chicken breast	Shandong, China
Nd	Nd	Chicken breast	Hebei, China
Nd	Nd	A	Jiangsu, China
0.21 ± 0.02	0.22 ± 0.01	B	Jiangsu, China
0.23 ± 0.01	0.22 ± 0.03	C	Jiangsu, China

0.23±0.01	0.24±0.02	D	Jiangsu, China
0.20±0.01	0.21±0.01	E	Jiangsu, China

Nd: not detected.

4. Conclusions

Carrageenan adulteration has become a problem in meat and meat-based products. The addition of carrageenan to livestock and poultry meat may cause contamination risks for heavy metals, foreign substances, and pathogenic microorganisms in meat and meat-based products. Moreover, it adversely affects the health and safety of consumers. Therefore, it is of great significance to develop a rapid detection method for carrageenan content in meat and meat-based products. Here, we developed a UHPLC-MS/MS method to measure the content of carrageenan in livestock and poultry meat. The LOD was 0.06g/kg, the LOQ was 0.18g/kg, the recovery of the spiked standard was 82.06–116.00%, and the RSD was lower than 7.51%. These results indicated that the method has a short analysis time, high recovery, good precision, and high repeatability. Furthermore, this method could rapidly measure carrageenan content in livestock and poultry meat and meat-based products.

Authors' Contributions

Yanqin Sun and Xudong Zhu contributed equally to this work.

Acknowledgments

The authors thank Dr. Yong Liu for his valuable advice on UHPLC-MS/MS analysis. This research was financially supported by grants from the National Key R & D Program of China (2018YFC1602804).

References

- [1] G. Zhou, W. Zhang, X. Xu, "China's meat industry revolution: challenges and opportunities for the future," *Meat Science*, vol. 92 no. 3, pp. 188-196, DOI: 10.1016/j.meatsci.2012.04.016, 2012.
- [2] P. Montero, J. L. Hurtado, M. Pérez-Mateos, "Microstructural behaviour and gelling characteristics of myosystem protein gels interacting with hydrocolloids," *Food Hydrocolloids*, vol. 14 no. 5, pp. 455-461, DOI: 10.1016/s0268-005x(00)00025-4, 2000.
- [3] V. D. Prajapati, P. M. Maheriya, G. K. Jani, H. K. Solanki, "Retracted: carrageenan: a natural seaweed polysaccharide and its applications," *Carbohydrate Polymers*, vol. 105, pp. 97-112, DOI: 10.1016/j.carbpol.2014.01.067, 2014.
- [4] D. Verbeken, N. Neirinck, P. Van Der Meeren, K. Dewettinck, "Influence of κ -carrageenan on the thermal gelation of salt-soluble meat proteins," *Meat Science*, vol. 70 no. 1, pp. 161-166, DOI: 10.1016/j.meatsci.2004.12.007, 2005.
- [5] M. Pérez-Mateos, J. L. Hurtado, P. Montero, F. Fernández-Martín, "Interactions of κ -carrageenan plus other hydrocolloids in fish myosystem gels," *Journal of Food Science*, vol. 66 no. 6, pp. 838-843, DOI: 10.1111/j.1365-2621.2001.tb15183.x, 2001.
- [6] Y. Chen, M.-L. Liao, D. V. Boger, D. E. Dunstan, "Rheological characterisation of κ -carrageenan/locust bean gum mixtures," *Carbohydrate Polymers*, vol. 46 no. 2, pp. 117-124, DOI: 10.1016/s0144-8617(00)00293-9, 2001.
- [7] B. B. Sedayu, M. J. Cran, S. W. Bigger, "A review of property enhancement techniques for carrageenan-based films and coatings," *Carbohydrate Polymers*, vol. 216, pp. 287-302, DOI: 10.1016/j.carbpol.2019.04.021, 2019.
- [8] E. García-García, A. Totosaus, "Low-fat sodium-reduced sausages: effect of the interaction between locust bean gum, potato starch and κ -carrageenan by a mixture design approach," *Meat Science*, vol. 78 no. 4, pp. 406-413, DOI: 10.1016/j.meatsci.2007.07.003, 2008.
- [9] Z. Pietrasik, A. Jarmoluk, "Effect of sodium caseinate and κ -carrageenan on binding and textural properties of pork muscle gels enhanced by microbial transglutaminase addition," *Food Research International*, vol. 36 no. 3, pp. 285-294, DOI: 10.1016/s0963-9969(02)00170-9, 2003.
- [10] A. Borthakur, S. Bhattacharyya, A. N. Anbazhagan, A. Kumar, P. K. Dudeja, J. K. Tobacman, "Tu1884 carrageenan exposure stimulates BCL10-NF-KappaB - BCL10 loop and prolonged inflammation in colonic epithelial

cells," *Gastroenterology*, vol. 142 no. 5, 2012.

- [11] A. Borthakur, S. Bhattacharyya, P. K. Dudeja, J. K. Tobacman, "Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 292 no. 3, pp. G829-G838, DOI: 10.1152/ajpgi.00380.2006, 2007.
- [12] J. K. Tobacman, "Review of harmful gastrointestinal effects of carrageenan in animal experiments," *Environmental Health Perspectives*, vol. 109 no. 10, pp. 983-994, DOI: 10.1289/ehp.01109983, 2001.
- [13] S. N. Marcus, A. J. Marcus, R. Marcus, S. W. Ewen, J. Watt, "The pre-ulcerative phase of carrageenan-induced colonic ulceration in the Guinea-pig," *International Journal of Experimental Pathology*, vol. 73, pp. 515-526, 1992.
- [14] W. Zheng, J. Wang, S. Zhao, "Advances in research of detection techniques for carrageen illegally used in the pre-slaughtering pigs," *Chinese Journal of Animal Health Inspection*, vol. 32 no. 9, pp. 69-71, 2015.
- [15] F. B. Kong, G. Q. Chen, Q. F. Huang, C. Chen, R. Li, "Detection of carrageenan in the orange juice using fluorescence spectra methods," *Spectroscopy and Spectral Analysis*, vol. 33 no. 1, pp. 126-130, 2013.
- [16] Q. F. Huang, S. M. Gao, G. Q. Chen, C. Chen, F. B. Kong, R. Li, "Study on the fluorescence spectra of carrageenan and prediction of its concentration," *Journal Of Jiangnan University, Natural Science*, vol. 11 no. 5, pp. 592-596, 2012.
- [17] H.-J. He, Y.-D. Zhu, W. Wang, "Rapid nondestructive detection of glue-injected meat by NIR hyperspectral imaging technology," *Science and Technology of Food Industry*, vol. 41 no. 10, pp. 219-223, 2020.
- [18] J. Prado-Fernández, J. A. Rodríguez-Vázquez, E. Tojo, J. M. Andrade, "Quantitation of κ -, ι - and λ - carrageenans by mid-infrared spectroscopy and PLS regression," *Analytica Chimica Acta*, vol. 480 no. 1, pp. 23-37, DOI: 10.1016/s0003-2670(02)01592-1, 2003.
- [19] K. M. Nunes, M. V. O. Andrade, A. M. P. Santos Filho, M. C. Lasmar, M. M. Sena, "Detection and characterisation of frauds in bovine meat in natura by non-meat ingredient additions using data fusion of chemical parameters and ATR-FTIR spectroscopy," *Food Chemistry*, vol. 205, pp. 14-22, DOI: 10.1016/j.foodchem.2016.02.158, 2016.
- [20] X. Zheng, Y. Li, W. Wei, Y. Peng, "Detection of adulteration with duck meat in minced lamb meat by using visible near-infrared hyperspectral imaging," *Meat Science*, vol. 149, pp. 55-62, DOI: 10.1016/j.meatsci.2018.11.005, 2019.
- [21] Y. Meng, Y.-H. Zhang, L.-D. Xu, D.-J. Chen, Y.-L. Zhang, Y.-M. Zhang, "Rapid detection of meat injected with water or gum by near infrared spectroscopy," *Food Science*, vol. 35 no. 8, pp. 299-303, 2014.
- [22] K. M. Nunes, M. V. O. Andrade, M. R. Almeida, C. Fantini, M. M. Sena, "Raman spectroscopy and discriminant analysis applied to the detection of frauds in bovine meat by the addition of salts and carrageenan," *Microchemical Journal*, vol. 147, pp. 582-589, DOI: 10.1016/j.microc.2019.03.076, 2019.
- [23] H. C. Bertram, H. J. Andersen, A. H. Karlsson, "Comparative study of low-field NMR relaxation measurements and two traditional methods in the determination of water holding capacity of pork," *Meat Science*, vol. 57 no. 2, pp. 125-132, DOI: 10.1016/s0309-1740(00)00080-2, 2001.
- [24] M. Li, B. Li, W. Zhang, "Rapid and non-invasive detection and imaging of the hydrocolloid-injected prawns with low-field NMR and MRI," *Food Chemistry*, vol. 242, pp. 16-21, DOI: 10.1016/j.foodchem.2017.08.086, 2018.
- [25] X. Wang, Z.-Y. Wang, L.-H. Chen, B.-L. Liu, "Hydrophilic colloid injected meat LF-NMR relaxation characteristics and its comparison of NIR discrimination," *Food and Fermentation Industries*, vol. 42 no. 2, pp. 150-156, 2016.
- [26] Y.-Y. Wu, Q.-R. Zhang, J.-Z. Han, D.-F. Qu, "Rapid detection of gum-injected meat by low-field nuclear magnetic resonance," *Meat Research*, vol. 27 no. 3, pp. 26-29, 2013.
- [27] N. Abdullah AlFaris, J. Zaidan AlTamimi, Z. A. AlOthman, S. Fahad Al Qahtani, S. M. Wabaidur, A. A. Ghfar, T. Saleh Aldayel, "Analysis of aflatoxins in foods retailed in Saudi Arabia using immunoaffinity column cleanup and high-performance liquid chromatography-fluorescence detection," *Journal of King Saud University Science*, vol. 32 no. 2, pp. 1437-1443, DOI: 10.1016/j.jksus.2019.11.039, 2020.
- [28] S. M. Wabaidur, A. AlAmmari, A. Aqel, S. A. AL-Tamrah, Z. A. Alothman, A. Y. B. H. Ahmed, "Determination of

free fatty acids in olive oils by UPHLC-MS," Journal of Chromatography B, vol. 1031, pp. 109-115, DOI: 10.1016/j.jchromb.2016.07.040, 2016.

[29] A. Al-Rifai, A. Aqel, T. Al-Warhi, "Antibacterial, antioxidant activity of ethanolic plant extracts of some convolvulus species and their DART-ToF-MS profiling," Evidence-Based Complementary and Alternative Medicine, vol. 2017, DOI: 10.1155/2017/5694305, 2017.

[30] Z. A. ALOthman, S. M. Wabaidur, "Application of carbon nanotubes in extraction and chromatographic analysis: a review," Arabian Journal of Chemistry, vol. 12 no. 5, pp. 633-651, DOI: 10.1016/j.arabjc.2018.05.012, 2019.

[31] State Administration for Market Regulation of the People's Republic of China, Methods for Supplementary Inspection of Food, Determination of Carrageenan In Livestock Meat, BJS 201804, 2018.

[32] E. A. Foegeding, S. R. Ramsey, "Rheological and water-holding properties of gelled meat batters containing iota carrageenan, kappa carrageenan or xanthan gum," Journal of Food Science, vol. 52 no. 3, pp. 549-553, DOI: 10.1111/j.1365-2621.1987.tb06672.x, 1987.

[33] B. Quemener, C. Marot, L. Mouillet, V. Da Riz, J. Diris, "Quantitative analysis of hydrocolloids in food systems by methanolysis coupled to reverse HPLC. Part 1. Gelling carrageenans," Food Hydrocolloids, vol. 14 no. 1, DOI: 10.1016/s0268-005x(99)00042-9, 2000.

DETAILS

Subject:	Mass spectrometry; Formic acid; Livestock; Food products; Reagents; Food additives; Liquid chromatography; Separation; Recovery; Scientific imaging; Chromatography; Gas flow; Food quality; Meat; Selectivity; Quadrupoles; Signal to noise ratio; Hydrochloric acid; Poultry; Carrageenan; Spectroscopy; Pork; Methods; Meat quality
Location:	United States--US; China
Company / organization:	Name: Waters Corp; NAICS: 334516
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779

Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-07-09 (Received); 2021-08-11 (Revised); 2021-09-15 (Accepted); 2021-09-25 (Pub)
DOI:	https://doi.org/10.1155/2021/5277453
ProQuest document ID:	2578641865
Document URL:	https://www.proquest.com/scholarly-journals/determination-carrageenan-livestock-poultry-meat/docview/2578641865/se-2?accountid=211160
Copyright:	Copyright © 2021 Yanqin Sun et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-10-03
Database:	Publicly Available Content Database

Document 16 of 59

Simultaneous and Rapid Determination of Six Tyrosine Kinase Inhibitors in Patients with Non-Small Cell Lung Cancer Using HPLC-MS/MS

Liu, Yanping ¹ ; Liu, Hua ² ; Xia, Zhewei ³ ; Wang, Zhipeng ⁴
 ; Yun, Yunlei ⁴ ; Zhang, Guanying ⁵ ; Huang, Lifeng ⁵
 ; Gao, Shouhong ⁴
 ; Chen, Wansheng ⁴

¹ Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China; Department of Pharmacy, The Affiliated Suzhou Science and Technology Town Hospital of Nanjing Medical University, Suzhou 215153, China ² Department of Pharmacy, Taixing People's Hospital, Taizhou, Jiangsu 225400, China ³ Pharmaceutical Analysis and Testing Center, School of Pharmacy, The Second Military Medical University, Shanghai 200003, China ⁴ Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China ⁵ Department of Pharmacy, The Affiliated Suzhou Science and Technology Town Hospital of Nanjing Medical University, Suzhou 215153, China

ABSTRACT (ENGLISH)

Objective. To develop a new method for quantitatively analyzing six tyrosine kinase inhibitors (gefitinib, erlotinib, icotinib, afatinib, osimertinib, and crizotinib) used in the treatment of non-small cell lung cancer (NSCLC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Methods.** The analytes were detected in the selected reaction monitoring mode on a triple quadrupole mass spectrometer with the positive ionization mode. Carbamazepine was utilized as the internal standard. The pretreatment of the plasma sample was completed based on protein precipitation with acetonitrile, and the analytes were separated on an Agilent Zorbax SB-C18 reversed-phase column (2.1 mm × 100 mm, 3.5 μ m, Agilent, USA) using gradient elution. The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The flow rate was 0.3 mL/min, and the injection volume was 5 μ L. The column temperature was set and maintained at 35°C. **Results.** The calibration curves were linear over the range from 5.0 to 1000.0 ng/mL for gefitinib, crizotinib, and osimertinib; from 50.0 to 4000.0 ng/mL for icotinib and erlotinib; and from 5.0 to 400.0 ng/mL for afatinib. Linear correlation coefficients were >0.990 for all regression curves. The intra- and interday accuracy and precision of the method were within $\pm 15.0\%$ and not more than 15.0%, respectively. The mean recovery of all the analytes ranged from 70.18% to 110.76%, the matrix effect was from 88.85% to 127.58%, and stability was within $\pm 15.0\%$. **Conclusion.** This newly developed method was sensitive, simple, and robust and could be used in therapeutic drug monitoring of six tyrosine kinase inhibitors in NSCLC patients.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Lung cancer is one of the most common malignant tumors in the world, among which the number of patients with non-small cell lung cancer (NSCLC) is about 85–90%, and 14% of cancer deaths in the world are caused by NSCLC [1]. In the last 50 years, treatments for NSCLC mainly included chemical drugs. Chemotherapy regimens of cisplatin, vincristine combined with cisplatin, and paclitaxel have prolonged the survival time and improved the quality of life of lung cancer patients, but the 5-year overall survival rate is about 5% [2]. In recent years, tumor treatment has developed rapidly, and oral small-molecule targeting drugs have emerged successively. These oral small-molecule targeting drugs bind to certain receptors on the tumor cells, thereby inhibiting downstream tyrosine kinase signaling and tumor proliferation. The primary small-molecule target drugs mainly target epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK). EGFR and ALK mutations occur mainly in nonsmoking NSCLC patients, and nonsmoking patients also have higher survival rates than smokers [3]. In newly treated patients with EGFR-mutated NSCLC, compared with platinum chemotherapy, first-generation EGFR inhibitors (gefitinib and erlotinib) or second-generation EGFR inhibitors (afatinib and icotinib) had significant higher survival and better quality of life [4]. However, EGFR (T790M) secondary mutations occur in approximately 50% of patients with progressive lung cancer after 9–13 months of treatment. Osimertinib, the third-generation EGFR inhibitor, showed a greater survival advantage in patients with secondary mutations at EGFR (T790M) than platinum-based chemotherapy [5]. Crizotinib significantly improved the quality of life in patients of nonprogressive lung cancer or NSCLC of ALK rearrangement [6].

Molecular targeted drugs are all given orally on fixed dose, which have good efficacy, but there are also many adverse reactions. Adverse reactions are one of the factors leading to interruption of treatment. Many pharmacokinetic studies have reported the relationship between clinical efficacy or adverse reactions and plasma exposure [7–10]. However, there were significant individual differences in plasma exposure levels. The therapeutic window of these drugs was narrow, and there were significant differences in pharmacokinetics among individual

patients [11]; therefore, these drugs are candidates for therapeutic drug monitoring (TDM). TDM is a dose optimization strategy to achieve faster and more effective clinical efficacy. The use of the rapid and sensitive LC-MS/MS detection method to determine the concentration of drugs in human blood or other body fluids can effectively adjust the dosage of drugs, improve the therapeutic effect of drugs, reduce the adverse reactions, and ensure the rational use of drugs. In order to solve this problem, several LC-MS/MS methods have been developed [12–15], but these methods have high requirements for instruments, complicated operation, and long analysis time, which hinder the clinical application. Therefore, the purpose of this study was to establish a simple, rapid, and sensitive method for the simultaneous determination of six TKIs in human plasma and verify its clinical application.

2. Materials and Methods

2.1. Chemicals and Reagents

The analytes including gefitinib (lot: F1102AS), icotinib (lot: J0615A), afatinib (lot: M0320A), erlotinib (lot: J0615A), crizotinib (lot: A0320A), and carbamazepine (internal standard, IS) (lot: M1001AS) were supplied by Meilun Biotech Co., Ltd. (Dalian City, China). Osimertinib (lot: 1-NJL-79-1) was purchased from Toronto Research Chemicals (Toronto, Canada). Mass spectrometric reagents methanol and acetonitrile were obtained from Merck (Merck Company, Darmstadt, Germany).

Formic acid was purchased from Tedia Company Inc. (Tedia, Fairfield, OH, USA). Isopropanol reagent was purchased from Shanghai Titan Technology Co., Ltd. (Titan, Shanghai, China). Distilled water was purchased from Watsons Distilled Water Co., Ltd. (Watsons, Guangzhou, China). Human blank plasma was donated by healthy volunteers in our laboratory (Shanghai, China).

2.2. Mass Spectrometry

The experiment was performed on an Agilent 1200 series HPLC system, consisting of an online degasser, a binary pump, an autosampler, and a column oven and interfaced to an Agilent 6410A triple quadrupole mass spectrometer equipped, in which the ionization source is the electrospray ionization source (ESI source, Agilent Technologies, USA). The data were processed using Agilent MassHunter data processing software (version B.01.04; Agilent Technologies, USA).

2.3. Liquid Chromatographic Conditions

All analytes were performed on a Zorbax SB-C18 analytical column (2.1 mm × 100 mm, 3.5 μm, Agilent, USA). The mobile phases contained 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B), and the flow rate was set at 0.3 mL/min. The gradient program started at 30% B, increased gradually to 90% B in 3 min, and then was held at 90% B until 7 min. The pastime was 5 min. The column temperature was set at 35°C, and the autosampler was maintained at room temperature. The volume injected into the chromatographic system was 5 μL.

2.4. Mass Spectrometry Conditions

All analytes were collected under the multiple reaction monitoring (MRM) and positive ionization mode (Figure 1). The mass spectrum parameters were as follows: HPLC flow rate of 250 L/min, sheath gas flow rate of 12 L/min, and temperature of 250°C. Nozzle voltage is 500 V. The atomizer pressure is 45 psi. Capillary voltage is 4000 V. The drying gas and atomizing gas are nitrogen, the flow rate of drying gas is 5 L/min, and the temperature is 350°C. Table 1 shows the optimized MRM parameters for six analytes and IS.

[figures omitted; refer to PDF]

Table 1

Optimized mass spectrometry parameters of six analytes and IS.

Analytes	Ionization mode (±)	Precursor ions (m/z)	Fragmentor (V)	Collision energy (V)	Product ions (m/z)
Gefitinib	+	447.2	90	22	128.2

Icotinib	+	392.1	155	32	304.2
Afatinib	+	486.2	140	32	371.1
Erlotinib	+	394.2	100	30	278.1
Crizotinib	+	450.2	140	26	260.1
Osimertinib	+	499.9	105	26	72.0
Carbamazepine (IS)	+	237.1	115	19	194.1

2.5. Preparation of Standard and Quality Control Samples

The stock solutions of all analytes were prepared, respectively, in 70% methanol (methanol-water, 70:30, V/V), and 2.05, 2.02, 1.99, 2.02, 2.08, and 2.00 mg of gefitinib, icotinib, afatinib, erlotinib, crizotinib, and osimertinib were accurately weighed and dissolved to obtain 1.0 mg/mL for all of them. The stock solutions were aliquoted and stored at -80°C . The stock solution of analytes was further diluted with 10% methanol (methanol-water, 10:90, V/V) to obtain combined work solutions at the following concentrations: 100, 200, 500, 1000, 2000, 5000, and 10000 ng/mL for gefitinib, crizotinib, and osimertinib; 500, 1000, 2000, 5000, 10000, 20000, and 40000 ng/mL for icotinib and erlotinib; and 50, 100, 200, 500, 1000, 2000, and 4000 ng/mL for afatinib; they were diluted with blank human plasma 10 times, and their concentrations ranged from 10 to 1000 ng/mL for gefitinib, crizotinib, and osimertinib; from 50 to 4000 ng/mL for icotinib and erlotinib; and from 5 to 400 ng/mL for afatinib. Quality control (QC) samples were also prepared in the same way for each TK, and their concentrations were set at 20.0, 100.0, and 500.0 ng/mL for gefitinib, crizotinib, and osimertinib; at 100.0, 500.0, and 2000.0 ng/mL for icotinib and erlotinib; and at 10.0, 50.0, and 200.0 ng/mL for afatinib. The stock solutions were stored at -80°C . They were brought to room temperature (25°C) for thaw before pretreatment. For IS stock solution, 1.99 mg carbamazepine was dissolved in 70% methanol and stored at -80°C after aliquot. The IS work solution was freshly prepared with acetonitrile at a concentration of 100 ng/mL for carbamazepine and stored at -20°C .

2.6. Sample Pretreatment

The samples were prepared as follows: for all analytes, sample pretreatment was performed by protein precipitation. The blood sample ($50\ \mu\text{L}$) was transferred to a 1.5 mL Eppendorf tube prior to spiking with $100\ \mu\text{L}$ of acetonitrile (containing 100 ng/mL of IS solution). After being vortexed for 1 min, the mixture was centrifuged at $14500\times g$ for 15 mins at room temperature. Then, $100\ \mu\text{L}$ of the supernatant solution was transferred to a 1.5 mL Eppendorf tube prior to adding $200\ \mu\text{L}$ mobile phase (A:B, 70:30, V/V). The sample was centrifuged at 14500 rpm for another 15 mins after being vortexed for 1 min at room temperature. Then, $5\ \mu\text{L}$ of the supernatant solution was directly injected into the HPLC-MS/MS system for analysis.

2.7. Human Sample

This research was approved by and performed at Changzheng Hospital (Shanghai, China) from March 2019 to May 2019. Blood samples were collected in EDTA-3K tubes from NSCLC patients after treatment with TKIs, and a total of 25 patients with NSCLC were enrolled in this study. And 3 mL venous blood samples were collected after a food fasting overnight, gently mixed after the TKI in NSCLC patients had reached steady concentration (two weeks after the first dose), and then centrifuged at $3000\times g$ for 10 minutes. The plasma was harvested and measured by the above method.

2.8. Method Validation

According to the Chinese Pharmacopoeia (version 2010) and FDA guidelines [16], method validation includes specificity, lower limit of quantitation (LLOQ), linearity, inter- and intraprecision and accuracy, carryover, extraction

recovery, matrix effect, and stability.

3. Results and Discussion

3.1. Chromatography Condition Optimization

In this experiment, some universal columns containing Agilent Zorbax SB-C18 (2.1 mm × 100 mm, 3.5 μm), Waters XSELECT™ HSS PFP (2.1 mm × 100 mm, 3.5 μm), and XBridge™ BEH C18 (2.1 mm × 50 mm, 2.5 μm) were tested for their retention and separation ability. By comparing their chromatographic features (such as resolution, retention time, response value, and peak shape), the results showed a better peak shape and response on the Agilent Zorbax SB-C18 (2.1 mm × 100 mm, 3.5 μm) column and achieved complete separation of analytes in a short time from endogenous interferents. Therefore, the Agilent Zorbax SB-C18 (2.1 mm × 100 mm, 3.5 μm) column was selected for the development of the method.

Different mobile phase additives (such as formic acid and ammonium acetate) were added to improve the peak shape, response, and retention time. When ammonium acetate was added to the mobile phase, the responses of all analytes were low, and the peak shape was poor. And then, retention time and separation effect were gained after testing with different ratios of formic acid (FA) (0.05%, 0.1%, and 0.2% FA) in the mobile phase. It was found that when 0.1% FA was added to the water phase, the response of analytes could be significantly increased.

3.2. Sample Pretreatment Optimization

Protein precipitation, solid-phase extraction (SPE), and liquid-liquid extraction are the main methods used in laboratory sample pretreatment. The protein precipitation method is a simple, economical, and time-saving method, while the cost of liquid-liquid extraction and solid-phase extraction is relatively higher. Therefore, the protein precipitation method is first tested. Acetonitrile and methanol were used as precipitating agents to remove protein from plasma. By comparing the extraction recovery and matrix effect of acetonitrile and methanol in different proportions, it was found that the ratio of acetonitrile to the sample is 2:1, and the extraction recovery is higher. In order to further purify the sample and reduce impurities in the sample, the supernatant after centrifugation was further treated. An equal volume of aqueous phase, organic phase, and their mixtures in different ratios was added to the supernatant. Results showed that the ratio of the mixed mobile phase is 7:3 (V:V), and the extraction recovery and peak shape are relatively ideal. In the end, comparing the extraction recovery and matrix effect of the supernatant and mixed mobile phase in different proportions, results found that the ratio of the mixed mobile phase to the supernatant is 2:1 (V:V), and the extraction recovery is higher. The pretreatment method is simple and economical, which can basically meet the requirements of this experiment and has good practicability.

3.3. Method Validation

3.3.1. Specificity

Comparisons of specificity from blank, IS, and six analytes spiked and clinical real samples (Figure 2) indicated no significant interferences at the same retention times of the analytes and IS. The retention time of six TKIs and IS is as follows: IS: 5.2 min, iconitib: 2.7 min, erlotinib: 4.2 min, gefitinib: 1.3 min, crizotinib: 1.3 min, afatinib: 1.3 min, and osimertinib: 1.9 min.

[figures omitted; refer to PDF]

3.3.2. Linearity

Through the construction of calibration curves, the linear correlation coefficients (R) of all analytes were greater than 0.990 under the weighing coefficient of $1/x^2$. The linear correlation coefficients (R) were more than 0.990 for all analytes. Results of regression equations for the calibration curves are presented in Table 2. The LLOQ was 10 ng/mL in the human plasma matrix of gefitinib, crizotinib, and osimertinib, 50 ng/mL in iconitib and erlotinib, and 5 ng/mL in afatinib, which were also in accordance with the accuracy within ±20% and precision less than 20%. The LLOQ and accuracy assessment results are summarized in Table 3.

Table 2

Linearity regression parameters of six analytes.

Analytes	Regression type	Linear range	Weighing factor	Regression equations	R
Gefitinib	Linearity	10.0–1000.0	$1/\chi^2$	$y=0.0025 \cdot x - 0.0029$	0.9915
Icotinib	Linearity	50.0–4000.0	$1/\chi^2$	$y=0.0011 \cdot x - 0.0129$	0.9981
Erlotinib	Linearity	50.0–4000.0	$1/\chi^2$	$y=0.0024 \cdot x - 0.0275$	0.9986
Crizotinib	Linearity	10.0–1000.0	$1/\chi^2$	$y=0.0193 \cdot x - 0.4494$	0.9940
Osimertinib	Linearity	10.0–1000.0	$1/\chi^2$	$y=0.0016 \cdot x - 0.0023$	0.9911
Afatinib	Linearity	5.0–400.0	$1/\chi^2$	$y=0.0141 \cdot x - 0.4145$	0.9934

Table 3

Precision and accuracy of the LLOQ of six analytes' calibration curves.

Analytes	Nominal concentration (ng/mL)	Measured concentration (ng/mL) $\pm$ SD	Precision RSD (%)	Accuracy mean (%)
Gefitinib	10	8.82 $\pm$ 0.29	6.31	96.22
Icotinib	50	50.22 $\pm$ 0.91	1.81	100.44
Erlotinib	50	49.01 $\pm$ 0.92	1.87	98.01
Crizotinib	10	10.07 $\pm$ 0.36	7.27	101.39
Osimertinib	10	9.75 $\pm$ 0.21	4.31	95.15
Afatinib	5	5.30 $\pm$ 0.31	5.76	105.98

3.3.3. Matrix Effect and Extraction Recovery

The matrix effect and extraction recovery of samples (low, middle, and high) were investigated. The results showed that the matrix effect of the analytes was between 96.83% and 114.09%, and the recovery was between 76.66% and 97.18%. The RSD (%) of the matrix effect and extraction recovery factors was less than 15%. The results are shown in Table 4.

Table 4

Extraction recovery and matrix effect of six analytes (%) ($n=3$).

Analytes	Nominal concentration (ng/mL)	Extraction recovery		Matrix effect	
Mean (%) $\pm$ SD	RSD (%)	Mean (%) $\pm$ SD	RSD (%)	Gefitinib	20

91.18±3.49	3.08	114.09±7.4	2.56	100	80.75 ±3.34
113.83±8.88	500	97.18±1.13	108.98±11.45	.	
Icotinib	100	81.41±1.33	3.11	107.58±4.28	3.49
500	93.89±3.91	100.91±2.91	2000	96.02±1.62	101.8 5± 5.85
-					
Erlotinib	100	85.24±3.33	3.00	108.67±8.22	5.49
500	90.10±1.8	101.26±2.47	2000	85.85±1.99	97.63 ±5.90
-					
Crizotinib	20	76.66±6.33	2.71	109.91±12.21	5.31
100	77.75±5.15	100.98±7.88	500	82.10±3.87	96.83 ± 11.76
-					
Osimertinib	20	82.25±7.63	2.92	100.58±4.94	2.97
100	90.53±2.48	102.72±5.27	500	81.76±4.03	96.85 ± 10.60
-					
Afatinib	10	85.20±1.38	3.12	102.56±17.39	2.91
50	90.55±8.06	108.45±15.34	200	95.69±8.61	107.0 8± 9.03

RSD was calculated using the IS-normalized matrix and recovery factors.

3.3.4. Precision and Accuracy

Three levels of QC samples (low, middle, and high) were chosen to analyze the inter- and intra-accuracy and precision. The results of accuracy with inter- and intra-accuracy were 85.36% to 111.38% and 85.24% to 113.04%, and the inter- and intraprecision were 1.02% to 5.41% and 0.33% to 4.97%. Table 5 summarizes the inter- and intraday accuracy and precision for the analytes.

Table 5

Inter- and intra-accuracy and precision of six analytes ($n=5$).

Analytes	Nominal concentration (ng/ml)	Interday			Intraday		
		Precision (RSD %)	Measured concentration (mean $\pm$ SD)	Accuracy (RE %)	Precision (RSD %)	Gefitinib	20
17.70 $\pm$ 0.66	-11.5	3.75	17.32 $\pm$ 0.26	-13.4	1.51	100	87.81 $\pm$ 1.81
-12.19	2.06	87.77 $\pm$ 2.22	-12.23	2.53	500	552.89 $\pm$ 15.82	10.59
2.86	565.21 $\pm$ 9.26	13.04	1.63	-			
Icotinib	100	91.09 $\pm$ 0.93	-8.91	1.02	91.56 $\pm$ 0.49	-8.44	0.53
500	439.77 $\pm$ 12.18	-12.05	2.77	426.70 $\pm$ 1.82	-14.66	0.42	20.00
1919.82 $\pm$ 39.30	-0.40	2.04	1966.42 $\pm$ 27.49	-1.68	1.39	-	
Erlotinib	100	87.59 $\pm$ 1.14	-12.41	1.31	88.94 $\pm$ 0.82	-11.06	0.93
500	441.47 $\pm$ 6.58	-11.71	1.50	450.70 $\pm$ 1.51	-9.86	0.33	20.00
1887.25 $\pm$ 94.36	-5.64	2.19	1933.51 $\pm$ 37.33	-3.25	1.93	-	
Crizotinib	20	18.17 $\pm$ 0.62	-9.15	3.42	18.55 $\pm$ 0.92	-7.25	4.97
100	91.67 $\pm$ 3.11	-8.33	3.39	88.67 $\pm$ 1.71	-11.33	1.92	50.0

538.54±12.68	7.71	2.35	538.57±4.57	7.71	0.85		
Osimertinib	20	18.72±0.92	-6.40	4.92	18.52±0.46	-7.40	2.53
100	85.36±2.96	-14.64	3.47	85.24±4.95	-14.76	5.81	500
556.91±11.80	11.38	2.12	561.76±15.24	12.35	2.71		
Afatinib	10	9.92±0.53	-0.80	5.41	9.76±0.19	-2.40	1.94
50	53.78±2.28	7.56	4.24	51.54±1.74	3.08	3.37	200

3.3.5. Stability

Short-term stability (25°C in room temperature for 12h and in the autosampler for 24h), long-term stability (1 month at -80°C), and three freeze-thaw cycles' stability were determined for all analytes in triplicate at each of the low, middle, and high concentrations. Results of stability are shown in Table 6.

Table 6

Results of stability of six analytes (n=3).

Analytes	Nominal concentration (ng/mL)	Freeze-thaw stability		12h at room temperature		24h in the autosampler		Long-term stability (1 month)	
		Measured concentration (mean)	Accuracy (RE %)	Measured concentration (mean)	Accuracy (RE %)	Measured concentration (mean)	Accuracy (RE %)	Gefitinib	20
17.58	-12.10	17.46	-12.70	17.56	-12.20	17.47	-12.65	100	88.85
-11.15	90.03	-9.97	86.11	13.89	88.34	-11.66	500	543.30	8.66
553.20	10.64	556.10	11.22	541.33	8.27				

Icotinib	100	91.87	-8.13	90.71	-9.29	91.04	-8.96	89.09	-10.91
500	447.00	-10.60	446.90	-10.62	441.40	-11.72	433.56	-13.28	20.00
1920.00	-4.00	1972.00	-1.40	1969.00	-1.55	1950.43	-2.47		
Erlotinib	100	87.75	-12.25	89.76	-10.24	87.53	-12.47	86.34	-13.66
500	440.90	-11.82	450.70	-9.86	440.90	-11.82	430.55	-13.89	20.00
1887.00	-5.65	1950.01	-2.50	1938.00	-3.10	1840.11	-7.99		
Crizotinib	20	18.05	-9.75	18.92	-5.40	18.94	-5.30	17.32	-13.40
100	91.86	-8.14	93.22	-6.78	94.22	-5.78	93.21	-6.79	50.0
541.90	8.38	550.80	10.16	550.70	10.14	544.13	8.83		
Osimertinib	20	19.01	-4.95	17.80	-11.00	17.55	-12.50	19.02	-4.90
100	88.06	-11.94	87.57	-12.43	85.95	-14.05	87.23	-12.77	50.0
551.30	10.26	561.70	12.34	549.40	9.88	561.07	12.21		

Afatinib	10	10.10	9.10	10.71	7.10	10.07	0.70	10 .2 1	2. 1
50	53.57	7.14	53.56	7.12	51.78	3.56	49.03	– 1. 94	20 0

3.4. Application of Clinical Samples Treated by TKIs

To test the applicability of this method, 25 plasma samples were collected from 25 NSCLC patients who were treated with one of six drugs. Gefitinib (250mg qd), erlotinib (150mg qd), icotinib (125mg tid), afatinib (40mg qd), osimertinib (80mg qd), and crizotinib (250mg bid) at standard doses at Changzheng Hospital were prescribed according to clinical diagnosis. Nine (erlotinib), seven (osimertinib), three (gefitinib), three (crizotinib), two (afatinib), and one (erlotinib) plasma samples were collected from patients, and the time points were designed in 7–14 days after administration (trough concentration). The TKIs were quantitatively measured in the patients' plasma. Results of the drug concentration distribution of TKIs in NSCLC patients are shown in Figure 3.

[figure omitted; refer to PDF]

The drug exposure in vivo has a close relation with the treatment efficacy and/or adverse reaction, and it was still the key point for the clinical optimization of drug dose [17–21]. There were differences in drug absorption, distribution, metabolism, and excretion between patients and within patients, and several studies in recent years were also reported [22–24]. This makes monitoring the concentration of TKI drugs' exposure in vivo particularly significant, and the method is an appropriate effective detection technology to increase the treatment efficacy and/or adverse reaction in the process of individualized dosing administration. Due to the limitation of objective conditions, the number of enrolled patients was small, due to which the relationship between the exposure and treatment efficacy could not be determined.

4. Conclusion

A simple, rapid, and sensitive method for the simultaneous determination of TKIs (including gefitinib, erlotinib, crizotinib, afatinib, osimertinib, and icotinib) in human plasma from NSCLC patients by the HPLC-MS/MS method was developed and validated. The analytical time was 7 min for six analytes after optimizing detection conditions, and the sample pretreatment method was simple, rapid, and economical. This method was suitable for clinical therapeutic drug monitoring to obtain a better treatment outcome.

Authors' Contributions

Yanping Liu and Hua Liu contributed equally to this work.

References

- [1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68 no. 6, pp. 394-424, DOI: 10.3322/caac.21492, 2018.
- [2] H. Wakelee, K. Kelly, M. J. Edelman, "50 years of progress in the systemic therapy of non-small cell lung cancer," *American Society of Clinical Oncology Educational Book*, vol. 34, pp. 177-189, DOI: 10.14694/edbook_am.2014.34.177, 2014.
- [3] A. Muth, N. S. GKulkarni, V. Parvathaneni, "Tyrosine kinase inhibitor conjugated quantum dots for non-small cell lung cancer (NSCLC) treatment," *European Journal of Pharmaceutical Sciences*, vol. 133, pp. 145-159, 2019.
- [4] J. A. Barta, C. A. Powell, J. P. Wisnivesky, "Global epidemiology of lung cancer," *Annals of Global Health*, vol. 85 no. 1, DOI: 10.5334/aogh.2419, 2019.
- [5] N. Girard, "Optimizing outcomes in EGFR mutation-positive NSCLC: which tyrosine kinase inhibitor and when?," *Future Oncology*, vol. 14 no. 11, pp. 1117-1132, DOI: 10.2217/fon-2017-0636, 2018.
- [6] A. T. Shaw, D.-W. Kim, K. Nakagawa, "Crizotinib versus chemotherapy in advanced ALK-positive lung cancer,"

New England Journal of Medicine, vol. 368 no. 25, pp. 2385-2394, DOI: 10.1056/nejmoa1214886, 2013.

[7] H. Yu, N. Steeghs, C. M. Nijenhuis, J. H. M. Schellens, J. H. Beijnen, A. D. R. Huitema, "Practical guidelines for therapeutic drug monitoring of anticancer tyrosine kinase inhibitors: focus on the pharmacokinetic targets," *Clinical Pharmacokinetics*, vol. 53 no. 4, pp. 305-325, DOI: 10.1007/s40262-014-0137-2, 2014.

[8] H. Hayashi, Y. Kita, H. Iihara, "Simultaneous and rapid determination of gefitinib, erlotinib and afatinib plasma levels using liquid chromatography/tandem mass spectrometry in patients with non-small-cell lung cancer," *Biomedical Chromatography*, vol. 30 no. 7, pp. 1150-1154, DOI: 10.1002/bmc.3642, 2016.

[9] C. J. Lucas, J. H. Martin, "Pharmacokinetic-guided dosing of new oral cancer agents," *The Journal of Clinical Pharmacology*, vol. 57 no. Suppl 10, pp. S78-S98, DOI: 10.1002/jcph.937, 2017.

[10] S. Wind, D. Schnell, T. Ebner, M. Freiwald, P. Stopfer, "Clinical pharmacokinetics and pharmacodynamics of afatinib," *Clinical Pharmacokinetics*, vol. 56 no. 3, pp. 235-250, DOI: 10.1007/s40262-016-0440-1, 2017.

[11] I. Solassol, F. Pinguet, X. Quantin, "FDA-and EMA-approved tyrosine kinase inhibitors in advanced EGFR-mutated non-small cell lung cancer: safety, tolerability, plasma concentration monitoring, and management," *Biomolecules*, vol. 9 no. 11, DOI: 10.3390/biom9110668, 2019.

[12] H. H. Huynh, C. Pressiat, H. Sauvageon, "Development and validation of a simultaneous quantification method of 14 tyrosine kinase inhibitors in human plasma using LC-MS/MS," *Therapeutic Drug Monitoring*, vol. 39 no. 1, pp. 43-54, DOI: 10.1097/ftd.0000000000000357, 2017.

[13] R. Reis, L. Labat, M. Allard, "Liquid chromatography-tandem mass spectrometric assay for therapeutic drug monitoring of the EGFR inhibitors afatinib, erlotinib and osimertinib, the ALK inhibitor crizotinib and the VEGFR inhibitor nintedanib in human plasma from non-small cell lung cancer patients," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 158, pp. 174-183, DOI: 10.1016/j.jpba.2018.05.052, 2018.

[14] C. Merienne, M. Rousset, D. Ducint, "High throughput routine determination of 17 tyrosine kinase inhibitors by LC-MS/MS," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 150, pp. 112-120, DOI: 10.1016/j.jpba.2017.11.060, 2018.

[15] Y. He, L. Zhou, S. Gao, "Development and validation of a sensitive LC-MS/MS method for simultaneous determination of eight tyrosine kinase inhibitors and its application in mice pharmacokinetic studies," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 148, pp. 65-72, DOI: 10.1016/j.jpba.2017.09.013, 2018.

[16] Z. Wang, Y. Yang, F. Zhang, "A direct, sensitive and efficient method for determination of alpha-fluoro-beta-alanine in urine: evaluating the influence of magnesium isoglycyrhizinate on excretion in rat model," *Journal of Chromatography B*, vol. 1102-1103, pp. 17-22, DOI: 10.1016/j.jchromb.2018.10.016, 2018.

[17] T. Kawata, M. Higashimori, Y. Itoh, "Gefitinib exposure and occurrence of interstitial lung disease in Japanese patients with non-small-cell lung cancer," *Cancer Chemotherapy and Pharmacology*, vol. 83 no. 5, pp. 849-858, DOI: 10.1007/s00280-019-03788-4, 2019.

[18] N. Ueno, S. Banno, Y. Endo, "Treatment status and safety of crizotinib in 2028 Japanese patients with ALK-positive NSCLC in clinical settings," *Japanese Journal of Clinical Oncology*, vol. 49 no. 7, pp. 676-686, DOI: 10.1093/jjco/hyz049, 2019.

[19] K. Nakao, S. Kobuchi, S. Marutani, "Population pharmacokinetics of afatinib and exposure-safety relationships in Japanese patients with EGFR mutation-positive non-small cell lung cancer," *Scientific Reports*, vol. 9 no. 1, DOI: 10.1038/s41598-019-54804-9, 2019.

[20] A. Almomen, H. M. Maher, N. Z. Alzoman, S. M. Shehata, A. Alsubaie, "Flavoured water consumption alters pharmacokinetic parameters and increases exposure of erlotinib and gefitinib in a preclinical study using wistar rats," *PeerJ*, vol. 8, DOI: 10.7717/peerj.9881, 2020.

[21] S. L. Groenland, D. R. Geel, J. M. Janssen, "Exposure-response analyses of anaplastic lymphoma kinase inhibitors crizotinib and alectinib in non-small cell lung cancer patients," *Clinical Pharmacology & Therapeutics*, vol. 109 no. 2, pp. 394-402, 2020.

[22] W. Zhang, Y. Zhang, Q. Zhao, "Long-term safety of icotinib in patients with non-small cell lung cancer: a retrospective, real-world study," *Journal of Thoracic Disease*, vol. 12 no. 3, pp. 639-650, DOI:

10.21037/jtd.2019.12.115, 2020.

[23] H. Zhang, Q. Li, X. Zhu, "Association of variability and pharmacogenomics with bioequivalence of gefitinib in healthy male subjects," *Frontiers in Pharmacology*, vol. 9, DOI: 10.3389/fphar.2018.00849, 2018.

[24] S. Sakamoto, K. Sato, Y. Takita, "ABCG2 C421A polymorphisms affect exposure of the epidermal growth factor receptor inhibitor gefitinib," *Investigational New Drugs*, vol. 38 no. 6, pp. 1687-1695, DOI: 10.1007/s10637-020-00946-x, 2020.

DETAILS

Subject:	Mass spectrometry; Formic acid; Plasma; Analytical chemistry; Cancer therapies; Mutation; Telemedicine; Quality control; Scientific imaging; Chromatography; Monitoring; Correlation coefficients; Drug dosages; Acetonitrile; Quality of life; Kinases; Lung cancer; Quadrupoles; Tyrosine; High performance liquid chromatography; Flow velocity; Chemotherapy
Business indexing term:	Subject: Quality control
Location:	United States--US; China
Company / organization:	Name: Agilent Technologies Inc; NAICS: 334515, 334516, 334519, 339112, 513210
Editor:	Mohamed Abdel-Rehim
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	

Milestone dates: 2021-02-23 (Received); 2021-08-13 (Accepted); 2021-09-17 (Pub)

DOI: <https://doi.org/10.1155/2021/5524361>

ProQuest document ID: 2578641738

Document URL: <https://www.proquest.com/scholarly-journals/simultaneous-rapid-determination-six-tyrosine/docview/2578641738/se-2?accountid=211160>

Copyright: Copyright © 2021 Yanping Liu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2022-07-14

Database: Publicly Available Content Database

Document 17 of 59

Identification and Simultaneous Determination of the Main Toxic Pyrrolizidine Alkaloids in a Compound Prescription of Traditional Chinese Medicine: Qianbai Biyan Tablet

Yang, Mengting ¹
; Tan, Daopeng ²
; Lu, Anjing ¹
; Lin, Qin ¹
; Wang, Changhong ³
; Ling, Hua ⁴
; Lu, Yanliu ¹
; He, Yuqi ¹

¹ Key Laboratory of Basic Pharmacology of Ministry of Education and Joint International Research Laboratory of Ethnomedicine of Ministry of Education, School of Pharmacy, Zunyi Medical University, Zunyi, Guizhou 563000, China ² Key Laboratory of Basic Pharmacology of Ministry of Education and Joint International Research Laboratory of Ethnomedicine of Ministry of Education, School of Pharmacy, Zunyi Medical University, Zunyi, Guizhou 563000, China; State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University, Guiyang 550014, China ³ The Institute of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Cailun Road 1200, Shanghai, China ⁴ School of Pharmacy, Philadelphia College of Osteopathic Medicine, 625 Old Peachtree Rd NW, Suwanee, GA 30024, USA

ABSTRACT (ENGLISH)

Qianbai biyan tablet (QT) is a compound prescription of traditional Chinese medicine which is used to treat nasal congestion, rhinitis, and nasosinusitis, with *Senecio scandens* as its main plant material. Several pyrrolizidine alkaloids (PAs) were reported in *Senecio scandens* and others of *Senecio* species. Although *Senecio scandens* is assigned as the legal plant material of QT, whether replaced use of it by other *Senecio* plants can bring toxicity is unknown because of the lack of quantitative data about toxic PAs between different *Senecio* species. In the present study, adonifoline, senkirkine, and another PA presumed as emiline have been identified in QT; however, there was no senecionine detected in all tablets. PA contents in QTs varied in different companies and different batches. Adonifoline existed only in *Senecio scandens*, and senecionine was detected in all eight *Senecio* plants investigated in the present study. Data showed that replaced use of *Senecio scandens* with a low level of senecionine by other *Senecio* plants such as *Senecio vulgaris* containing a high level of senecionine is advertised to be forbidden. Data of the present study may be used as a reference to make new drug quality regularity and recommendation guideline for the safety of QT.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Qianbai biyan tablet (QT), a compound prescription of traditional Chinese medicine (TCM) used in China, South East Asia, and some European countries, was used to treat several clinical diseases such as nasal congestion, rhinitis, and nasosinusitis for several decades and had a large number of consumers. According to the China Pharmacopoeia (CP), *Senecio scandens* Buch.-Ham. ex D. Don was used as the main component of QT mixed with six other herbs, including Herba Selaginellae, Rhizoma Seu Radix Notopterygii, Semen Cassiae, Herba Ephedrae, Rhizoma Chuanxiong, and Radix Angelicae Dahuricae [1]. Some active constituents in QT, such as ephedrine from Herba Ephedrae, chrysophanol from Semen Cassiae, and flavonoids from Herba Selaginellae, were assayed to evaluate the quality of this tablet [2, 3]. To our knowledge, however, there were hardly any reports about the determination of toxic constituents in QT.

Pyrrolizidine alkaloids (PAs) were a kind of compounds that come from esterification of carboxylic acid named necic acid and pyrrole ring named necine and considered as one of the most toxic constituents in *Senecio* plants, causing hepatotoxicity [4] with the major clinical symptom of venoocclusion disease (VOD) and other toxicities including neurotoxicity and gene toxicity [5]. The mechanism of the toxicity of PAs was considered as metabolic activation [6] by liver P450s. In the world, about 3% of all flowering plants and more than 6000 plant species contain PAs [7], such as Compositae, Boraginaceae, and Leguminosae [8]. Among these plants, *Senecio* plants (Compositae) were considered the primary source of PAs, and large numbers of reports attributed the PAs toxicity against livestock, wildlife, and humans to *Senecio* plants [9].

In our previews [8], several PAs, including senecionine, senecionine N-oxide, seneciphylline, seneciphylline N-oxide, and senkirkine, existed in *Senecio scandens* that were the main components of QT as mentioned above [1]. The last one was an OTO-type PA, and others were RET-type; all of these five compounds were considered as toxicants because of their unsaturated C1,2-bond, which was considered as the key reason leading PAs induced toxicity [10]. Among these five compounds, senecionine was the common constituent existing in several other *Senecio* plants, such as *Senecio ambraceus* [11], *Senecio vulgaris* [12], and *Senecio scandens* [13]. Senkirkine was the only otonecine-type PA found in *Senecio scandens*. Another probable toxic RET compound named adonifoline, which had unsaturated C1,2-bond and existed in *Senecio scandens*, was also reported, and the data showed that plants came from the different place had various content of adonifoline [14]. However, there was hardly any report about

the toxicity of adonifoline, including acute toxicity and cumulative toxicity. PAs that existed in *Senecio scandens* may cause potent dangers for the consumption of herbs, even compound prescriptions. However, the types and quantities of PAs existing in QT and the content of PAs in QT were still unknown.

Although *Senecio scandens* is the active pharmaceutical ingredient of QT, other *Senecio* species such as *Senecio nemorensis*, *Senecio vulgaris*, and *Senecio cannabifolius* distribute extensively in Europe, China, and other Asian countries may be misused confused as a medical material. Tens of PAs, such as senecionine [12], senecicannabine [15], jacozone [16], seneciphylline [17], senecionine N-oxide [12], adonifoline [18] and senkirkine [19] have been found in *Senecio* plants. However, to our knowledge, except *Senecio scandens* there was rare information about the quantitative determination of the main PAs in these plants and whether the replacement of *Senecio scandens* by other *Senecio* plants in QT could bring toxicity or increase toxicity is unknown.

In the present study, we performed an LC/MS/MS analysis to identify the PAs in QT from different manufactories and different batches of the same manufactory, as well as related herbs of *Senecio* species described above. Several PAs such as adonifoline and senkirkine, which represented RET-type PAs and OTO-type PAs, respectively, have been detected in QT. Senecionine, which is the most common PA and had demonstrated toxicity, was assayed in all tablets and *Senecio* plants investigated in the present study. The purpose of this study was to provide the type and content of PAs in QT and give a toxicity evaluation to QT based on the chemical analysis data and other published work; on the other hand, whether replaced use of *Senecio scandens* by others could be promised was discussed here. Data of the present study can provide a reference for the quality standard of QT in later drug regulations.

2. Experimental

2.1. Chemicals

Reference substances including adonifoline, senecionine, senkirkine, monocrotaline, isoline, and clivorine were isolated by our lab from various *Compositae* plants such as *Senecio scandens* and *Ligularia duciformis*, and the purities of reference substances are more than 98%, determined by HPLC-DAD.

Senecio plants investigated were collected by our lab. All samples were authenticated by Dr. Lihong Wu, and voucher specimens were deposited in the herbarium of Shanghai R & D Center for Standardization of TCM. Samples of QT were purchased from drug stores all over China. Different batches of QT were obtained from Qixing Pharmaceutical Company Limited (Guangdong province, China). Detailed information about plants and tablets is shown in Table 1.

Table 1

Information of QTs and *Senecio* plants tested in the present study.

Company/species	Abbreviations	Batch number	Place of production
Qianbai biyan tablets	HP		
Heping Co.	GYT	50301	Guangdong province
Guoyitang Co.	HZ	20150201	Guangdong province
Huizhou Co.	BLXF	50428	Guangdong province
BoluoXianfeng Co.	BYS	50615	Guangdong province
Baiyunshan Co.	RS	L5D001	Guangdong province

Rensheng Co.	XZ	50302	Guangxi province
Xiuzheng Co.	SF	30801	Jilin province
Sifang Co.	QX	50602	Henan province
Qixing Co.	QX	5119	Guangdong province
Qixing Co.	QX	4040	Guangdong province
Qixing Co.	QX	4121	Guangdong province
Qixing Co.	QX	4126	Guangdong province
Qixing Co.	QX	5011	Guangdong province
Qixing Co.	QX	5138	Guangdong province
Qixing Co.	QX	5141	Guangdong province
Qixing Co.	QX	5159	Guangdong province
Qixing Co.	QX	6095	Guangdong province
Qixing Co.	QX	6109	Guangdong province
Qixing Co.	QX	6153	Guangdong province
Qixing Co.	QX	6168	Guangdong province
Qixing Co.	QX	6179	Guangdong province
Qixing Co.	S.sc.	6248	Guangdong province
Plant materials	S.ar.		
<i>S. scandens</i>	S.ca.v.	—	Guangdong province
<i>S. argunensis</i>	S.ca.	—	Jilin province
<i>S. cannabifolius</i> var. <i>integrifolius</i>	S.vu	—	Jilin province
<i>S. cannabifolius</i>	T.ph	—	Jilin province
<i>S. vulgaris</i>	S.la	—	Heilongjiang province

<i>T. phaeantha</i>	S.ne	—	Jilin province
<i>S. laetus</i>		—	Chongqing city
<i>S. nemorensis</i>		—	Jilin province

HPLC-grade acetonitrile was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade formic acid was purchased from Tedia Company (Fairfield, OH, USA). HPLC-grade water was produced by a Milli-Q water purifying system. All other chemicals and agents were purchased from commercial sources and were of the highest purity grade commercially available.

2.2. Instruments

The analysis was performed using a Thermo Finigan HPLC instrument equipped with a quaternary pump, a PDA detector, an autosampler, and a column compartment. The HPLC system was coupled with an ion trap mass spectrometry of LCQ XP Plus via an ElectroSpray Ionization (ESI) interface for mass analysis and detection. Data was acquired by the Xcalibur software obtained from Thermo Finigan. A Heraeus Biofuge Primo (Germany) was used for centrifugation in the present study.

2.3. Sample Preparation

Ten tablets of each sample were ground to a fine powder (40 mesh) using a pulverizer, an aliquot of 300 mg powder was accurately weighted to a 25 mL sealed conical flask, and 10 mL HCl solution (0.2%) was added to soak the powder for 30 min and then extract it by ultrasound for 40 min. Transfer the ultrasound extracted solution to a 15 mL centrifuge tube to perform centrifugation at 5000 × g for 10 min; the supernatant was filtered with a 0.3 μm filter; an aliquot of 10 μL was injected into LC/MS for identification and determination of PAs. The plant of *Senecio scandens* and other plant materials of *Senecio* species were also ground to the powder of 40 mesh, an aliquot of 1 g was accurately weighted to the 25 mL sealed conical flask, and the later operation was the same as tablets.

2.4. HPLC Analysis

Adonifoline, monocrotaline, isoline, clivorine, and senkirkine were chosen to optimize the chromatographic separation parameters. Chromatographic separation was performed on the Phenomenex Synergi MAX-RP C12 column (4 μm, 250 × 4.6 mm) together with a supported C12 guard column. The mobile phase consisted of 1% formic acid in water as solvent A and acetonitrile as solvent B. The eluting process was as follows: beginning from 5% B and with a gradient from 5% to 28% B for 25 min, held at 28% B for 30 min, then to 95% B for 10 min, held at this percentage for 10 min. The mobile phase flow rate was 1 mL/min; the column temperature was set to 25°C, there was no use of the PDA detector because the UV absorption of pyrrolizidine alkaloids was very weak.

2.5. Mass Parameters

All mass spectrometric experiments were performed on an ion trap mass spectrometer. The LC effluent was introduced into the ESI source in a postcolumn splitting ratio of 10:1. The MS detector was optimized to obtain the maximum signal of [M+H]⁺ ions of isoline, which was one of the retronecine-type pyrrolizidine alkaloids, and clivorine, which was one of the OTO pyrrolizidine alkaloids. Scan mode was positive. The optimal parameters were as follows: Capillary Temp was 300.00°C, sheath gas flow and aux/sweep gas flow were 32 and 10, respectively (arbitrary unite), source voltage was 5 kV, source current was 80 μA, and capillary voltage was 9 V.

2.6. Identification of Main Pyrrolizidine Alkaloids in QT

A full scan mode was used to obtain the total ion chromatogram (TIC) of extracted tablet samples, and then the extracted ion chromatogram (EIC) was used to improve the signal-noise ratio (S/N ratio); based on TIC and EIC, several ions were screened as a candidate of probable PAs. [M+H]⁺ ions of even m/z number were chosen to perform MS/MS fragmentation after screening. The fragmentation regularity of PAs reported before in [20] was used to judge the type of candidate PAs based on the characteristic ions such as 120 and 138, which were used as the markers of RET PAs, as well as 122, 150, and 168, which were used as the markers of otonecine-type PAs. The fingerprint of MS/MS spectrum, as well as the retention retain time (RT) of probable peaks, was compared with that

of the reference substances available.

2.7. LC/MS/MS Analysis of *Senecio* Plants and Pure PAs

The procedure described above was conducted on tablets to identify the probable PAs in QT while on eight *Senecio* plants, including *Senecio scandens*, *Senecio argunensis*, *Senecio cannabifolius* var. *integrifolius*, *Senecio cannabifolius*, *Senecio vulgaris*, *Tephrosia phaeantha*, *Senecio laetus*, and *Senecio nemorensis* to examine whether the plant source of QT contains these main PAs. Pure reference substance solutions such as adonifoline, senecionine, and senkirkine were injected to obtain their retain time, molecular weight, and MS/MS spectrum. Both the data of herbs and reference substance were used to compare with the main PAs in QT.

2.8. Validation of Analysis Method

2.8.1. Standard Curve of Adonifoline, Senecionine, and Senkirkine

Reference substance solutions with a series of concentrations of pure adonifoline, senecionine, and senkirkine were injected to LC/MS/MS to obtain the SIC peak area or height for construction of the standard curve. The peak area of adonifoline in SIC 338 was linear with concentration range from 9.80 ng/mL to 6144.00 ng/mL; senkirkine in SIC 168 was linear with a concentration range from 10.00 ng/mL to 200.00 ng/mL. The peak area and height of senecionine in SIC 308 were linear, with a concentration range from 1.00 ng/mL to 5000.00 ng/mL.

2.8.2. Precision and Accuracy

Reference substance of adonifoline was used to perform the valuation of analysis method, including precision and accuracy. The standard curve of adonifoline was analyzed for triplication in one day and in three consecutive days to calculate the interday and the intraday precision. 10 mL solution with concentrations of 1228.80, 245.80, and 49.20 ng/mL of adonifoline dissolved by 0.2% hydrochloric acid solution was added to the three aliquots of 300 mg powder of tablets (Rensheng, Guangxi Province, China), and preparations were conducted as described in Sample Preparation. The recovery of adonifoline was assayed to represent the accuracy of the analysis method; 10 mL 0.2% hydrochloric acid solution was added to another aliquot of 300 mg powder of the same manufactory as the blank contrast sample.

2.9. Assay of Four PAs in Eight *Senecio* Plants and QTs from Different Companies and Batches

QTs collected from 9 pharmaceutical companies, named Qixing (Guangdong province, China), Heping (Guangdong province, China), Guoyitang (Guangdong province, China), Huizhou (Guangdong province, China), Boluoxianfeng (Guangdong province, China), Baiyunshan (Guangzhou city, China), Rensheng (Guangxi province, China), Xiuzheng (Jilin province, China), and Sifang (Henan province, China), as well as 10 different batches from the same company (Qixing pharmaceutical company, Guangdong province, China) were assayed by LC/MS/MS. Selective ion chromatograms (SIC) of 338 which is the MS/MS fragment of the $[M+H]^+$ ion 366, 308 which is the MS/MS fragment of $[M+H]^+$ ion 336, and 168 which is the MS/MS fragment of $[M+H]^+$ ion 366 were used to determine the content of adonifoline, senecionine, and senkirkine, respectively. Eight *Senecio* plants also were assayed the same as the tablets. Peak area was used for calculation of concentrations of adonifoline, senkirkine, and PA2, but peak height was used for senecionine because good separation of senecionine was not obtained in some plant samples (Figure 1). An external reference method was used in the assay of both tablets and plants.

[figure omitted; refer to PDF]

3. Results

3.1. Chromatogram and MS/MS Fragmentation Regularity of PA Standard

Adonifoline, monocrotaline, isoline, senecionine, senkirkine, and clivorine were used to optimize chromatogram parameters. Because PAs possess the property of a weak base, a C12 column was chosen to prevent the tailing peak. 1% formic acid was used to keep the molecular state of PAs to improve the peak symmetry in the separation process and improve the ionization efficiency of PAs in the electrospray ionization (ESI) source. Because low concentrations were used in mixture standard, these compounds could not be observed in TIC but could be observed clearly in EIC of MS/MS fragment of their $[M+H]^+$ ions.

MS/MS fragmentation of PAs was studied extensively [8, 20]. In the present study, the experiment was repeated on senecionine (RET) and senkirkine (OTO), and the same results as reported data have been reached. Ions 120 and

138 were usually generated by RET-type PAs, and 122, 150, and 168 were usually generated by OTO-type PAs under the positive scan mode. All these ions could represent the core structure of PA. Detail fragmentation regularities on the basis of senecionine and senkirkine are shown in Figures 2(b) and 2(c).
[figures omitted; refer to PDF]

3.2. Identification of Pyrrolizidine Alkaloids in QT

The identification of PAs in the QT from different manufactories was performed by mass fragmentation analysis and comparing with the reference standards. Firstly, full scan mode was used to obtain the molecular weight of probable PAs. After screening, three compounds, which had the same molecular weight of 365 and RT of 5.79 min, 11.81 min, and 14.10 min, were chosen as candidates of PAs labeled as PA1, PA2, and PA3 (Figure 3). Some samples investigated in the present study had one or two constituents of PA1, PA2, and PA3. Some samples contained all of the three candidates, such as tablets of Baiyunshan Company. Next, the $[M+H]^+$ ions of candidates were used to perform the MS/MS fragmentation, and full scan mode was used to obtain the MS/MS spectrum of corresponded $[M+H]^+$ ions. In the MS/MS spectrum of PA1, 120 and 138, which represent the RET PAs, were observed, while in that of PA2 and PA3, 122, 150, and 168, which represent the OTO PAs, were observed (Figure 4). Reported PAs, which had a molecular weight of 365, include three RETs, such as adonifoline [18], grantianine [21], and seneciocannabine [15], and two OTO such as senkirkine [19] and emiline [22]. It was reported that adonifoline and senkirkine existed in *Senecio scandens* which was the legal plant source of QT according to the CP, so these two compounds could be concluded as the most probable PAs in tablets. Except adonifoline and senkirkine, the last probable PA was of OTO, so emiline was the most probable candidate. Senecionine was the common constituent in most *Senecio* plants, but we did not detect the $[M+H]^+$ ion 336 of senecionine in all tablet samples. Further, to verify the candidates of PAs in QT, their MS/MS fragmentation and retention time were compared to 6 PAs compounds, including monocrotaline, adonifoline, senecionine, senkirkine, isoline, and clivorine. Their retention time was 4.19, 5.79, 12.36, 14.10, 17.09, and 27.10 min, respectively. In the MS/MS spectrum of adonifoline, monocrotaline, senecionine, and isoline, two characteristic ions, 120 and 138, of RET PAs were observed; in that senkirkine and clivorine, 122, 150, and 168 were observed as the characteristic ions of OTO PAs. To identify the structure of PA1, PA2, and PA3, both RT and MS/MS spectrum of six references were used to compare with the candidates. As a result, PA1 was identified as adonifoline, and PA3 was identified as senkirkine. This corresponded with our conclusion about their structure. But PA2 was not the same as any one of the six PAs, and the most probable structure was presumed as emiline. Senecionine eluted at 12.36 min; although most *Senecio* plants, including *Senecio scandens*, contained this compound, there was no peak which possessed protonation molecular ion of 336 found in all QT samples. Structures and MS/MS spectrums of pure adonifoline, senecionine, and senkirkine are shown in Figures 2(a)–2(c), and probable structure and MS/MS spectrum of PA2 are shown in Figure 2(d).

[figure omitted; refer to PDF][figure omitted; refer to PDF]

3.3. Identification of Pyrrolizidine Alkaloids in *Senecio* Plants

To confirm the PAs source in QT, eight *Senecio* plants, including *Senecio scandens*, *Senecio argunensis*, *Senecio cannabifolius* var. *integrifolius*, *Senecio cannabifolius*, *Senecio vulgaris*, *Tephroseris phaeantha*, *Senecio laetus*, and *Senecio nemorensis*, which are distributed extensively in China, were analyzed by LC/MS/MS in the present study. To identify PA1, PA2, and PA3 that were found in QT, a full scan of the MS/MS fragment of ions 366 was performed in all plants samples. In the TIC of MS/MS fragment of 366 (Figure 5), all of PA1, PA2, and PA3 were detected in *Senecio scandens*. Both of PA2 and PA3, but not PA1, were detected in *Senecio argunensis* and *Senecio laetus*. PA3 but not PA1 and PA2 was detected in *Tephroseris phaeantha*, which used to be *Senecio* plants. In *Senecio cannabifolius* var. *integrifolius*, *Senecio cannabifolius*, *Senecio vulgaris*, and *Senecio nemorensis*, all of PA1, PA2, and PA3 were not detected. Peaks A, B, C, and D are the other four PAs detected in *Senecio argunensis*, *Senecio cannabifolius* var. *integrifolius*, *Senecio cannabifolius*, *Tephroseris phaeantha*, and *Senecio laetus*, but not detected in QT. Other peaks in Figure 4 are not PAs. For confirming whether senecionine, which was reported in most *Senecio* plants and considered as the most common toxic PA in *Senecio* plants, existed in plant source of QT, the full scan of MS/MS fragment of 336, which is the $[M+H]^+$ ion of senecionine, was performed to eight plant samples.

In chromatogram of total MS/MS fragment of 336 (Figure 1), PA4 was found at RT 12.24 min. The MS/MS spectrum (Figure 6) indicated that it is senecionine by comparing with the standard substance. It is demonstrated by an LC/MS/MS method, for the first time, that all plant samples tested in the present study contain senecionine. In TIC of MS/MS fragment 336, the other three RETs were also detected and labeled as E, F, and G; because of very similar MS/MS spectrum (Figure 6) and the same molecular weight with PA4, we conclude them as three isomers of senecionine. The detailed structures of E, F, and G need advanced investigation.

[figure omitted; refer to PDF][figure omitted; refer to PDF]

3.4. Validation of the Analysis Method

3.4.1. Standard Curve of Adonifoline, Senecionine, and Senkirkine

The peak area of adonifoline in SIC 338 was linear with concentration range from 9.80 ng/mL to 6144.00 ng/mL, and that of senkirkine in SIC 168 was linear with concentration range from 10.00 ng/mL to 200.00 ng/mL. The peak area and height of senecionine in SIC 308 were linear with concentration range from 1.00 ng/mL to 5000.00 ng/mL.

3.4.2. Precision and Accuracy

Average recovery of analysis method was 99.54% at high concentration, 96.31% at medium concentration, and 76.39% at low concentration, which indicated that accuracy in high and medium concentration is better than low concentration and some low content of PAs may be relatively inaccurate. Precision was 98.11% at high concentration, 97.20% at medium concentration, and 95.42% at low concentration for interday, while it was 88.29%, 85.20%, and 82.70% for intraday at high, medium, and low concentration, respectively.

3.5. Determination of Adonifoline, Senkirkine, Senecionine, and Emiline in QT and Eight *Senecio* Plants

Three compounds were identified as PAs in QT: two of them (PA1 and PA3) were sure to be adonifoline and senkirkine and PA2 was presumed as emiline. In eight *Senecio* species, PA1, PA2, and PA3 were found in different plants, and PA4 was detected and identified as senecionine in all eight *Senecio* plants. These four constituents in QT of 9 different pharmaceutical companies and in eight *Senecio* plants were assayed. The peak in SIM chromatogram was used for quantification. For adonifoline and senecionine, the selective ions were 338 and 308, which were the base peak in the MS/MS spectrum of their $[M+H]^+$ ions, respectively. Ion 168, which was the characteristic ion of OTO PAs and the base peak in MS/MS spectrums of $[M+H]^+$ ions of PA2 and senkirkine, was chosen to be the selective ion for PA2 and senkirkine. Peak area was used to determine the content of adonifoline, senkirkine, and PA2; but peak height was used to determine the content of senecionine because a good separation was not obtained for it (Figure 1). Because of no reference compound available, PA2 was semidetermined using pure senkirkine with one point external standard method. According to the determination result of four PAs in QT tablets of 9 companies and eight *Senecio* plants, senecionine was detected in all *Senecio* plants but not in any tablets. Adonifoline was detected as the main PA in most tablets but only in one *Senecio* plant (*Senecio scandens*). For understanding whether adonifoline contents in QT tablets coming from the same manufactory were the same, QT tablets of 14 batches from Qixing (Guangdong province, China) company were assayed in the present study. According to the results of PAs content in QT tablets, the content of four PAs, including adonifoline, senecionine, senkirkine, and PA2, was significantly different in QT tablets from individual companies. Adonifoline was the most common constituent in QT, although no adonifoline was detected in Heping Co. and Rensheng Co., tablet of Qixing Co. has the highest content of adonifoline. Senecioine, the most common constituent in most *Senecio* plants which had noticeable toxicity, was not detected in any tablets. Except for Heping, Xiuzheng, and Sifang Co., senkirkine, an OTO PA, was contained in tablets of all other companies. PA2, which is presumed as emiline in the present study, was detected in tablets of Qixing, Guoyitang, Baiyunshan, and Rensheng Co. However, the quantities are very low. No PAs were detected in tablets of Heping Co., and the only adonifoline existed in tablets of Xiuzheng and Sifang Co. The results of PAs determination in tablets of different companies are expressed in Table 2.

Table 2

PAs content in tablets of different companies, batches, and *Senecio* plants.

Company/species	Batch number	Adonifoline	Senecionine	Senkirkine	PA2
Heping	50301	—	—	—	—
Guoyitang	20150201	—	—	184.16	8.64
Huizhou	50428	53.04	—	65.91	—
Boluoixianfeng	50615	25.93	—	54.44	—
Baiyunshan	L5D001	799.09	—	781.40	33.44
Rensheng	50302	—	—	279.27	11.13
Xiuzheng	30801	2380.10	—	—	—
Sifang	50602	2125.64	—	—	—
Qixing	5119	10623.04	—	362.91	8.90
Qixing	4040	—	—	—	—
Qixing	4121	—	—	—	—
Qixing	4126	—	—	—	—
Qixing	5011	—	—	—	—
Qixing	5138	—	—	—	—
Qixing	5141	3088.67	—	—	—
Qixing	5159	9762.87	—	—	—
Qixing	6095	5467.22	—	—	—
Qixing	6109	15351.60	—	—	—
Qixing	6153	—	—	—	—
Qixing	6168	—	—	—	—
Qixing	6179	—	—	—	—
Qixing	6248	3449.40	—	—	—

<i>S. scandens</i>	—	7043.64	21.31	2425.01	84.85
<i>S. argunensis</i>	—	—	94.46	95.32	9.58
<i>S. cannabifolius</i> var.	—	—	17.35	—	—
<i>S. cannabifolius</i>	—	—	4125.63	—	—
<i>S. vulgaris</i>	—	—	3950.02	—	—
<i>T. phaeantha</i>	—	—	9.75	2162.57	0.00
<i>S. laetus</i>	—	—	2116.77	393.27	18.38
<i>S. nemorensis</i>	—	—	29.56	—	—

*All units in the table are in ng/g.

According to the determination of PAs in tablets of different companies, adonifoline is the main constituent in QT. For understanding whether the PAs contents in the tablets of the same company were coincident, 14 batches of QT from Qixing Co. were collected to determine the content of adonifoline. As a result, only six batches contain adonifoline and the contents were various. All determination results are listed in Table 2.

Senecio scandens is the legal plant source of QT according to the CP, but in the actual production process, some other *Senecio* species may be used to replace it. In the present study, eight *Senecio* plants, including ones extensively distributed in China, were assayed to determine the content of adonifoline, senecionine, senkirkine, and PA2. As a result, *Senecio scandens* contained all four PAs, *Senecio argunensis* and *Senecio laetus* contained senecionine, senkirkine, and PA2 but no adonifoline, and *Tephrosia phaeantha* contained two PAs of senecionine and senkirkine. It was an exciting discovery that senecionine was detected in all plants tested in the present study, and adonifoline, the main constituent in QT, was detected only in *Senecio scandens*. All determination results are listed in Table 2.

4. Discussion

Three PAs (PA1, PA2, and PA3) were detected in QT. PA1 and PA3 were identified as adonifoline and senkirkine. PA2 was presumed as emiline in the present study. According to the determination result, adonifoline was the main RET PA and senkirkine was the main OTO PA in QT tablets. All three compounds had the unsaturated bond between C1 and C2 sites. According to the data reported, although this kind of structure is the key reason causing the PAs induced toxicity, the toxicity of adonifoline has not been reported. And, experiment data of our lab showed that the LD₅₀ value of adonifoline on mice is far lower than senecionine (data not show). Other toxicities of QT induced by adonifoline should be investigated later by pharmacology method. Chemical analysis data cannot be used as the sole evidence to evaluate the toxicity of tablets but is an important part. Senkirkine was demonstrated as a toxical PA with mutagenic activity [23, 24]. The highest contents of senkirkine in QTs observed from samples investigated were several hundred nanograms per gram tablet and far lower than adonifoline. PA2 existed in tablets of Qixing, Guoyitang, Rensheng, and Baiyunshan Co., but the quantities were so small that it nearly cannot be detected.

Tablets of different companies were assayed in the present study. Data showed that PAs content in tablets of different companies was various. Fourteen batches of tablets of Qixing Co. were chosen to be assayed, and adonifoline, which was the main constituent in QT, was assigned as the index. The result showed that the contents of adonifoline in different batches of tablets were various. Contents of some batches were high, reaching more than 10µg/g, but some batches contained no adonifoline. The factors that influence PAs content in QT may be concluded

as the pharmaceutical technology, instability of PAs compounds, and the plant source of QT. In our opinion, plant source was proposed to be the main factor that makes the PAs content vary between different batches. Zhang et al. reported that adonifoline in *Senecio scandens* from a different province, even the same province of China, had various content [14]. Adonifoline content in *Senecio scandens* of Wuhu (Anhui province of China) was 9.83 $\mu\text{g/g}$, but that of Bozhou (Anhui province of China) was 32.99 $\mu\text{g/g}$. Except for the place of production, time of plants collection also influences the PAs content by comparing the result of present and reported work; adonifoline content in *Senecio scandens* collected in March from Guizhou province of China was 47 $\mu\text{g/g}$ [14], but in present experiment adonifoline content in *Senecio scandens* collected in December also from Guizhou province of China was only 7 $\mu\text{g/g}$. These data may be used to explain that why PAs contents in QT of different companies and different batches were not the same because *Senecio scandens* was the main material of QT. Another direct proof was obtained by comparing the data of the present study with reported data [14]. In *Senecio scandens* of Guangxi province of China, adonifoline was not detected, and in our experiment, the tablet of Rensheng Company also in Guangxi province contained no adonifoline.

Except for adonifoline and senkirkine identified in the present study in QT, other PAs were identified in reported data such as senecionine, seneciophylline, neoplatyphylline, and their N-oxides [8]. But these PAs were not detected in tablets. Senkirkine was another main PAs constituent in *Senecio scandens* and had a content of 2.43 $\mu\text{g/g}$; but in tablets, the highest senkirkine content was 0.7 $\mu\text{g/g}$. Our previews data showed that senkirkine under high temperature was not stable. According to CP, the production process of QT includes a step that extracts *Senecio scandens* with boiling water [1], so extraction temperature may be one of the reasons that some PAs could be detected in plants but not in tablets.

Although *Senecio scandens* is the legal plant source of QT, the other *Senecio* plants may be used to replace it because distinguishing dried plants from each other is very difficult when lacking an experienced botanist. So, eight *Senecio* plants were assayed to determine the PAs content to understand whether the probable replacement would influence the PAs content of tablets. Data showed that PAs contents of eight plants were various. For adonifoline, only *Senecio scandens* contained it; senkirkine existed in four plants, but its contents in *Senecio scandens* and *Tephrosia phaeantha* were high and in the other two plants, *Senecio argunensis* and *Senecio laetus*, they were low. As in QT, the quantity of PA2 in some *Senecio* plants was very low, even zero. Senecionine existed in all eight plants investigated and with high content in *Senecio vulgaris*, *Senecio cannabifolius*, and *Senecio laetus*, but in *Senecio scandens*, its content was shallow. There was no toxicity reported for adonifoline but senecionine was a received toxicant in reported data. So, if some *Senecio* plants which had high content of senecionine replaced *Senecio scandens*, the toxicity of QT may be induced. By comparing these data, we can conclude that PAs content and type in different *Senecio* species are so different that replacement use of *Senecio scandens* in QT by other *Senecio* plants must not be permitted. So, although the rule that *Senecio scandens* is the legal plant source of QT in CP is based only on traditional use in China, our data may provide some experimental support of its use.

In the present study, only limited PAs compounds were detected in QT and some *Senecio* species herbs using an HPLC/MS/MS approach in full scan mode, even according to the characteristic product ions at m/z 120/138 or 168/150 in the literature [25] and comparing with the reference standards. These may be due to the low PAs content in QT and its plant resource *S. scandens* and the variation in PAs content amongst *S. scandens* from different geographic locations [25]. On the other hand, PA2 needs to be further confirmed accurately in the future. And then, there are some other types of alkaloids in the plants of *S. scandens* except for PAs [26], though whether these types of alkaloids are toxic is unknown.

5. Conclusion

In conclusion, an HPLC/MS/MS method has been used in the present study to identify and determine the main PAs in QT. And these index compounds have been determined in eight *Senecio* plants. As a result, three PAs have been identified in tablets, two of them were identified as adonifoline and senkirkine, and another was presumed as emiline. Because senecionine is a common constituent in several reported *Senecio* plants and has demonstrated toxicity, senecionine content in QT and eight *Senecio* plants has also been assayed. According to our data,

adonifoline was the main constituent of QT, and senecionine was not detected in any tablets. Contents of PAs were various between different manufactories and between different batches of the same producer. In eight *Senecio* plants, adonifoline was detected only in *Senecio scandens*, but senecionine was detected in all eight plants. *Senecio vulgaris*, *Senecio cannabifolius*, and *Senecio laetus* contained high level of senecionine but only a little senecionine existed in *Senecio scandens*. So as our advice, replace the use of *Senecio scandens* with other *Senecio* plants must be forbidden because senecionine had demonstrated toxicity. Plant source was considered as the main factors that influence the PAs content in QT as well as plant collection place and collection time. So, material standards must be found to control the tablet quality. Senkirkine content in QT was less than in plants because of its instability. According to these data, PAs content in some QTs was very high, but whether it can induce corresponded toxicity would need advanced pharmacology and toxicity study. Our chemical analysis data combined with advanced pharmacology and toxicology study can provide a reference for making a guideline for the safe use of QT.

Acknowledgments

The study was supported by the Department of Science and Technology of Guizhou Province (nos. QKHZC [2019]2961, QKHPTRC [2019]5657, QKHPTRC [2018]5772-001, QKHZDZXZ [2019]3001, QKHZC [2019]2953, QKHZC [2020]4Y072, QKHZC [2021]420, QKHZC [2019]2829, and QKHZC [2021]normal476), the Ministry of Science and Technology of China (2017YFC1702005), the Program for Excellent Young Talents of Zunyi Medical University (15zy-004), the Research Center of Dendrobium Industry Development in Guizhou Province (QSKH[2019003]), the Science and Technology Innovation Action Plan of Domestic Science and Technology Cooperation Projects in Shanghai (20025800400), the Department of Education of Guizhou Province (QJHKY[2021]049), and the project of State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University (No. FAMP201909K).

References

- [1] C. P. Commission, "Pharmacopoeia of the people's Republic of China," 2020.
- [2] P. P. Duan, Y. H. Chen, J. J. Xin, "Simultaneous determination of ephedrine hydrochloride, chlorogenic acid, P - hydroxycinnamic acid, hyperoside and Rhein in Qianbai biyan tablets by HPLC-CAD," *China Pharmacist*, vol. 23 no. 7, pp. 1432-1435, 2020.
- [3] Y. Z. Xing, S. J. Song, "Simultaneous determination of seven active constituents in Qianbai biyan tablets by HPLC-CAD," *China Pharmacist*, vol. 23 no. 6, pp. 1216-1219, 2020.
- [4] A. R. Mattocks, *Chemistry and Toxicology of Pyrrolizidine Alkaloids*, 1986.
- [5] P. P. Fu, Q. Xia, G. Lin, M. W. Chou, "Pyrrolizidine alkaloids-genotoxicity, metabolism enzymes, metabolic activation, and mechanisms," *Drug Metabolism Reviews*, vol. 36 no. 1, DOI: 10.1081/dmr-120028426, 2004.
- [6] C. E. Couet, J. Hopley, A. B. Hanley, "Metabolic activation of pyrrolizidine alkaloids by human, rat and avocado microsomes," *Toxicon*, vol. 34 no. 9, pp. 1058-1061, DOI: 10.1016/0041-0101(96)00056-6, 1996.
- [7] L. W. Smith, C. C. J. Culvenor, "Plant sources of hepatotoxic pyrrolizidine alkaloids," *Journal of Natural Products*, vol. 44 no. 2, pp. 129-152, DOI: 10.1021/np50014a001, 1981.
- [8] S.-L. Li, G. Lin, P. P. Fu, C.-L. Chan, M. Li, Z.-H. Jiang, Z.-Z. Zhao, "Identification of five hepatotoxic pyrrolizidine alkaloids in a commonly used traditional Chinese medicinal herb, *Herba Senecionis scandentis* (Qianliguang)," *Rapid Communications in Mass Spectrometry*, vol. 22 no. 4, pp. 591-602, DOI: 10.1002/rcm.3398, 2008.
- [9] B. L. Stegelmeier, J. A. Edgar, S. M. Colegate, D. R. Gardner, T. K. Schoch, R. A. Coulombe, R. J. Molyneux, "Pyrrolizidine alkaloid plants, metabolism and toxicity," *Journal of Natural Toxins*, vol. 8 no. 1, pp. 95-116, 1999.
- [10] P. Fu, Q. Xia, G. Lin, M. Chou, "Genotoxic pyrrolizidine alkaloids-mechanisms leading to DNA adduct formation and tumorigenicity," *International Journal of Molecular Sciences*, vol. 3 no. 9, pp. 948-964, DOI: 10.3390/i3090948, 2002.
- [11] H. Wiedenfeld, D. Altanchimeg, A. Gantur, S. Narantuya, "Toxic pyrrolizidine alkaloids from three Mongolian plants. A possible risk for cattle poisoning," *Journal of Natural Toxins*, vol. 11 no. 3, pp. 187-192, 2002.
- [12] T. Hartmann, A. Ehmke, U. Eilert, K. von Borstel, C. Theuring, "Sites of synthesis, translocation and

- accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris* L," *Planta*, vol. 177 no. 1, pp. 98-107, DOI: 10.1007/bf00392159, 1989.
- [13] V. Batra, T. R. Rajagopalan, "Alkaloidal constituents of *Senecio scandens*," *Current Science*, vol. 49 no. 5, 1977.
- [14] F. Zhang, C.-h. Wang, W. Wang, L.-x. Chen, H.-y. Ma, C.-f. Zhang, M. Zhang, S. W. A. Bligh, Z.-t. Wang, "Quantitative analysis by HPLC-MS2 of the pyrrolizidine alkaloid adonifoline in *Senecio scandens*," *Phytochemical Analysis*, vol. 19 no. 1, pp. 25-31, DOI: 10.1002/pca.1009, 2008.
- [15] P. B. Pelser, H. de Vos, C. Theuring, T. Beuerle, K. Vrieling, T. Hartmann, "Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (*Asteraceae*)," *Phytochemistry*, vol. 66 no. 11, pp. 1285-1295, DOI: 10.1016/j.phytochem.2005.04.015, 2005.
- [16] J. Luthy, U. Zweifel, B. Karlhuber, C. Schlatter, "Pyrrolizidine alkaloids of *Senecio alpinus* L. and their detection in feedingstuffs," *Journal of Agricultural and Food Chemistry*, vol. 29 no. 2, pp. 302-305, DOI: 10.1021/jf00104a021, 1981.
- [17] M. Deinzer, P. Thomson, D. Burgett, D. Isaacson, "Pyrrolizidine alkaloids: Their occurrence in honey from Tansy Ragwort (*Senecio jacobaea* L.)," *Science*, vol. 195 no. 4277, pp. 497-499, DOI: 10.1126/science.835011, 1977.
- [18] F. Zhang, C.-h. Wang, A.-z. Xiong, W. Wang, L. Yang, C. J. Branford-White, Z.-t. Wang, S. W. A. Bligh, "Quantitative analysis of total retronecine esters-type pyrrolizidine alkaloids in plant by high performance liquid chromatography," *Analytica Chimica Acta*, vol. 605 no. 1, pp. 94-101, DOI: 10.1016/j.aca.2007.10.021, 2007.
- [19] Y. Asada, T. Furuya, "Neosenkirkine and senkirkine from *Senecio pierotii*," *Planta Medica*, vol. 44 no. 3, DOI: 10.1055/s-2007-971438, 1982.
- [20] G. Lin, K.-Y. Zhou, X.-G. Zhao, Z.-T. Wang, P. P. H. But, "Determination of hepatotoxic pyrrolizidine alkaloids by on-line high performance liquid chromatography mass spectrometry with an electrospray interface," *Rapid Communications in Mass Spectrometry*, vol. 12 no. 20, pp. 1445-1456, DOI: 10.1002/(sici)1097-0231(19981030)12:20<1445::aid-rcm356>3.0.co;2-g, 1998.
- [21] R. Adams, M. Gianturco, "The structures of grantianine and scleratine. A suggested biogenesis of the acids in the alkaloids from *Senecio* and *Crotalaria* species," *Journal of the American Chemical Society*, vol. 78 no. 17, pp. 4458-4464, DOI: 10.1021/ja01598a072, 1956.
- [22] S. Kohlmunzer, J. Grzybek, H. Tomczyk, "Investigation of the biological activity of emiline using phytobiological tests," *Polish Journal of Pharmacology and Pharmacy*, vol. 25 no. 3, pp. 293-297, DOI: 10.1111/jphp.1973.25.issue-10, 1973.
- [23] U. Candrian, J. Lüthy, U. Graf, C. Schlatter, "Mutagenic activity of the pyrrolizidine alkaloids seneciphylline and senkirkine in *Drosophila* and their transfer into rat milk," *Food and Chemical Toxicology*, vol. 22 no. 3, pp. 223-225, DOI: 10.1016/0278-6915(84)90131-5, 1984.
- [24] L. Yu, Y. Xu, H. Feng, S. F. Y. Li, "Separation and determination of toxic pyrrolizidine alkaloids in traditional Chinese herbal medicines by micellar electrokinetic chromatography with organic modifier," *Electrophoresis*, vol. 26 no. 17, pp. 3397-3404, DOI: 10.1002/elps.200500233, 2005.
- [25] Y. Zhou, N. Li, F. F. Choi, C. F. Qiao, J. Z. Song, S. L. Li, X. Liu, Z. W. Cai, P. P. Fu, G. Lin, H. X. Xu, "A new approach for simultaneous screening and quantification of toxic pyrrolizidine alkaloids in some potential pyrrolizidine alkaloid-containing plants by using ultra performance liquid chromatography-tandem quadrupole mass spectrometry," *Analytica Chimica Acta*, vol. 681 no. 1-2, pp. 33-40, DOI: 10.1016/j.aca.2010.09.011, 2010.
- [26] D. Tan, G. Chou, Z. Wang, "Three new alkaloids from *Senecio scandens*," *Chemistry of Natural Compounds*, vol. 50 no. 2, pp. 329-332, DOI: 10.1007/s10600-014-0944-6, 2014.

DETAILS

Subject:	Traditional Chinese medicine; Low level; Pharmaceutical industry; Acids; Chromatography; Toxicity; Chinese medicine; Ultrasonic imaging; Sensors; Alkaloids
Business indexing term:	Subject: Pharmaceutical industry
Location:	China; United States--US
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-06-18 (Received); 2021-08-24 (Revised); 2021-08-26 (Accepted); 2021-09-09 (Pub)
DOI:	https://doi.org/10.1155/2021/5209618
ProQuest document ID:	2574087285
Document URL:	https://www.proquest.com/scholarly-journals/identification-simultaneous-determination-main/docview/2574087285/se-2?accountid=211160

Copyright:

Copyright © 2021 Mengting Yang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2021-09-19

Database:

Publicly Available Content Database

Document 18 of 59

Novel Trends in Electrochemical Biosensors for Early Diagnosis of Alzheimer's Disease

Valkova, Pavla; Pohanka, Miroslav

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Background. Alzheimer's disease (AD) is a multifactorial progressive and irreversible neurodegenerative disorder affecting mainly the population over 65 years of age. It is becoming a global health and socioeconomic problem, and the current number of patients reaching 30–50 million people will be three times higher over the next thirty years.

Objective. Late diagnosis caused by decades of the asymptomatic phase and invasive and cost-demanding diagnosis are problems that make the whole situation worse. Electrochemical biosensors could be the right tool for less invasive and inexpensive early diagnosis helping to reduce spend sources—both money and time. **Method.**

This review is a survey of the latest advances in the design of electrochemical biosensors for the early diagnosis of Alzheimer's disease. Biosensors are divided according to target biomarkers. **Conclusion.** Standard laboratory methodology could be improved by analyzing a combination of currently estimated markers along with neurotransmitters and genetic markers from blood samples, which make the test for AD diagnosis available to the wide public.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Biosensors are simple but very precise analytical devices suitable for the detection of a broad spectrum of biological and chemical analytes. They are based on the conversion of a biological or chemical reaction into a measurable signal. Their application increases over years to medical, environmental, industrial, food, or pharmaceutical analysis mainly due to their fast response, accuracy, low cost, portability, and suitability for the point-of-care diagnosis [1, 2]. The history of biosensors dates back to the 1960s when Clark and Lyons presented the first biosensor. It was based on an oxygen electrode, so the first electrochemical biosensor was described [2, 3]. Many new biosensor designs are currently known. We can divide them either by their detection technique (called a physicochemical transducer)

into magnetic, optical, electrochemical, mass-based, and thermal or by their biorecognition element, which is a biomolecule bound to the surface of the transducer. Enzymes, antibodies, genetic material (DNA, RNA), or whole cells and tissues are usually used as a biorecognition element. The biorecognition element provides a specific catalytic or binding reaction to the analyte, which is directly converted to a measurable signal by the transducer on its surface [4].

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by an irreversible and progressive loss of selectively vulnerable populations of neurons [5, 6]. AD is an incurable devastating disorder that mainly affects the elderly population; it is currently estimated that more than 30 million people have AD, and their number is growing rapidly. Fast response to the first symptoms of AD is the key factor in the diagnosis of early stages of the disease, so treatment can be initiated and quality of life extended. Standard tests for AD, including magnetic resonance imaging, positron emission tomography (PET), near-infrared, or cerebrospinal fluid (CSF) analysis, are invasive and expensive and are usually performed on patients with the development of mild cognitive impairment. Thus, the low-cost test appropriate for the diagnosis of all stages of AD is required, and electrochemical biosensors represent a promising alternative for currently available diagnostic techniques [2, 7].

2. Electrochemical Biosensors

Interest in electrochemical biosensors has increased over the years, mainly due to their high sensitivity, simple construction, easy handling, portability, low cost, ability to measure turbid samples, and compatibility, unlike the other types of biosensors [8, 9]. Electrochemical biosensors are based on the transformation of a biochemical signal into an amperometric signal while electrons are either generated or used. The amperometric signal is created by a potentiometric, amperometric, conductometric, or impedimetric transducer [3, 10]. The type of electrochemical sensor depends on the measured parameter: the electrical current is measured by the amperometric transducer, the generated potential is measured by the potentiometric transducer, conductance of medium is measured by the conductometric transducer, and impedance of medium is measured by impedimetric transducer [8]. Measured parameters of different electrochemical transducers are shown in Figure 1.

[figure omitted; refer to PDF]

Amperometric biosensors are among the very simple detection techniques gaining interest in many scientific fields. The current resulting from the oxidation or reduction of electroactive substance is measured using a constant potential [11, 12]. Voltammetry is a method covered by amperometric techniques, but the varying potential is applied to the working electrode, and the change of current is observed [13]. Voltammetric techniques, including cyclic voltammetry, square wave voltammetry, differential pulse voltammetry, or linear sweep voltammetry, are often used in the construction of electrochemical biosensors [14]. Potentiometric biosensors are based on measuring potential/pH variation as a response to applied current, usually with low amplitude. It is often used in food, environmental, and clinical analysis to measure many organic and inorganic substances. The simplest potentiometric sensor is a pH or any other ion-selective electrode [15, 16]. Conductometric biosensors interpret a specific biological reaction on the transducer's surface as electrical conductance measured using a low-amplitude alternating electrical potential. The conductivity of sample change relies on the production or consumption of charged species [16, 17]. Impedimetric biosensors measure changes in impedance as a frequency function. Impedance is like resistance opposite of current flow, but resistance occurs in a direct current circuit, while impedance occurs in full alternating current circuits. Impedimetric biosensors usually determine affinity interactions between molecules [18, 19]. The pros and cons of different electrochemical techniques are summarized in Table 1.

Table 1

Summary of advantages and disadvantages of various electrochemical techniques.

Electrochemical technique	Advantages	Disadvantages
Amperometry	Fastness, sensitivity, precision	Poor selectivity

Potentiometry	Measuring of low concentration and turbid samples	Possibility of false-negative result by strong buffers
Conductometry	Use in living biological system	Low specificity
No reference electrode needed easy miniaturization; they measure both electroactive and inactive analytes	Impedimetry	Possibility of label-free setup; they can be applied in living biological system and they exert high sensitivity and suitability for miniaturization

The electrochemical biosensors can also be divided into two groups regarding the electrochemical recognition process: biocatalytic sensors and bioaffinity sensors. The biocatalytic sensors are characterized by a catalytic reaction taking place on the surface of sensors and enzymes while the cells and tissues are appropriate biorecognition elements enabling this kind of reaction. On the other hand, bioaffinity sensors are typical for the affinity interaction taking place on the electrode surface, such as antigen-antibody interaction, nucleic acids interaction, or aptamer interaction. The bioaffinity biosensors with electrochemical detection are evolving rapidly because of their simplicity, low time, and high sensitivity, in contrast to traditional techniques for the detection of DNA, RNA, or antibodies such as polymerase chain reaction or fluorescence in situ hybridization. Their application in the diagnosis of AD is in great demand [7]. Biomolecules appropriate as biorecognition elements in electrochemical biosensors are shown in Figure 2.

[figure omitted; refer to PDF]

The first changes in the brain begin years, even decades, before symptoms appear. This phase is called the pathophysiological or clinical stage, and it is characterized as an asymptomatic period no different from normal aging. $A\beta$ is deposited in neocortical areas of the brain without cognitive or behavioral changes. On the other hand, it is still not clear whether all $A\beta$ deposition leads to the development of AD. The diagnostic methodology for this phase has not been established. The National Institute on Aging and the Alzheimer's Association focused on the design of diagnostic criteria and biomarkers appropriate for the diagnosis of early AD stages [5, 7, 29, 30]. The prodromal stage of AD known as mild cognitive impairment (MCI) is the period between normal aging and AD, and patients are cognitively impaired, usually in memory but not demented. Cognitive functions such as orientation, language, attention, and executive function may also be affected, and patients may have mild problems to easily perform common daily tasks. Patients suffering MCI have an increased risk of developing AD, so MCI treatment aims to reduce this risk [5, 31–33]. The third phase, called AD dementia, is characterized as a slow progression of memory disturbance followed by other cognitive domains' disturbance (language, social, and occupational dysfunctions) and behavioral changes. Plaques of deposited $A\beta$ and NFT are the main pathological features of this stage [5, 31]. Three neuroimaging markers were established for the diagnosis of Alzheimer's disease: magnetic resonance imaging (MRI) of hippocampal atrophy, fluorodeoxyglucose PET (FDG-PET) of temporoparietal hypometabolism, and PET of increased brain deposition of $A\beta$ and NFT [23].

The individual phases are overviewed in Table 2.

Table 2

Overview of AD phases [5, 10, 30–32].

Phase	Symptoms	Neuroimaging	Biomarkers

Clinical	None	None	None
MCI	Change of cognitive ability	MRI, PET, FDG-PET	CSF levels of T-tau, P-tau, and $A\beta_{42}$
Impairment of at least one cognitive domain	Mild problems in performing complex tasks not demented	AD dementia	Cognitive and behavioral dysfunctions

4. Alzheimer's Disease Biomarkers

4.1. CSF Biomarkers

The analysis of CSF biomarkers, reliable indicators of cerebral neurochemistry, is an established diagnostic method achieving high specificity in the diagnosis of AD (survey in Figure 4). CSF biomarkers are perfect as diagnostic criteria for the early stages of AD. On the other hand, obtaining CSF is an invasive and painful procedure with possible side effects, especially in the elderly. CSF analysis is performed either immunohistochemically or by enzyme-linked immunosorbent assay. Both methods are relatively expensive and time consuming. To date, three biomarkers have been established for the diagnosis of AD from CSF: phosphorylated tau protein (P-tau) 181, total tau protein (T-tau), and $A\beta$ [2, 7, 10]. Decreased $A\beta_{42}$ alone or in combination with $A\beta_{40}$ and increased P-tau₁₈₁ and T-tau is a typical profile for the diagnosis of AD from CSF [34]. The analysis of these three CSF biomarkers has high diagnostic accuracy (85–90%) in the diagnosis of AD and also in the diagnosis of MCI, so they have been incorporated in the diagnostic criteria of AD [35].

[figure omitted; refer to PDF]

4.1.1. P-tau₁₈₁ and T-tau

Tau protein is a microtubule-associated protein that normally stabilizes microtubules in the cell cytoskeleton, but it is also forming intracellular aggregates in several neurodegenerative diseases including AD. Phosphorylation of tau protein is a physiological process ensuring regulation of tau protein production. Under pathological conditions, the tau protein becomes hyperphosphorylated and causes protein separation from the microtubules and destabilizes them structurally, so axonal transport is disrupted. Hyperphosphorylated tau protein forms masses of paired helical filaments inside the nerve cell bodies—the NFT, one of the clinical criteria for AD diagnosis [10, 36, 37]. Thus, tau protein has become a biomarker evaluated in the AD diagnosis as a sign of axonal degeneration and NFT formation. However, the tau protein exists in the human brain in 6 isoforms produced from a single gene that differ in posttranslational modification including phosphorylation, glycosylation, and oxidation. The tau protein isoform phosphorylated on threonine 181 (P-tau₁₈₁) is important for AD diagnosis. While the P-tau₁₈₁ may be a more specific marker of AD, general T-tau belongs to general CSF markers of all neurodegeneration, so, elevated levels of T-tau may be also detected in other tauopathies. This is due to the NFT structure, typical for AD, which consists of hyperphosphorylated tau protein [10, 34, 37, 38].

4.1.2. $A\beta_{42}$ and $A\beta_{40}$

Any imbalance in the production or clearance of amyloidogenic $A\beta$ peptides leads to extraneuronal accumulation of $A\beta$ in amyloid plaques, the second well-documented pathology of AD, and also the second clinical diagnostic criteria of AD. $A\beta$ peptides, normally soluble, exist in many conformations consisting of 36–43 amino acids, so they are labeled according to the length of their amino acid chain. In particular, $A\beta_{42}$ most easily succumb to aggregation into $A\beta$ plaques. $A\beta_{42}$ and $A\beta_{40}$ are the most reliable indicators of AD. Compared to the healthy population, the analysis of $A\beta_{42}$ from CSF in AD patients shows a substantial decrease, probably caused by its aggregation into plaques. On the other hand, $A\beta_{40}$ CSF levels do not correlate with AD status, the levels are not decreased, and it is not formed in amyloid plaques. Despite this fact, its levels are measured in the diagnosis of AD, given that the ratio $A\beta_{42}/A\beta_{40}$ shows a significant decrease. The analysis of ratio $A\beta_{42}/A\beta_{40}$ has a more corresponding value than the measurement of $A\beta_{42}$ alone, as it compensates for intraindividual changes within AD patients and has a better association with the

pathogenesis of AD [7, 30, 36].

4.1.3. Neurotransmitters

Neurotransmitters are endogenous chemical compounds that act as communication messengers between neurons. To date, more than a hundred neurotransmitters have been discovered [9]. AD is not a disease affecting only the cognitive functions of the brain, but noncognitive symptoms have been observed, including agitation, anxiety, depression, apathy, psychosis, and sleep or appetite disorders. Various studies suggested that neurotransmitters have a key role in both cognitive and behavioral dysfunctions of AD [39]. Neurotransmitters including acetylcholine, dopamine, serotonin, glutamate, and norepinephrine have been studied as factors influencing the development of neurodegeneration and as possible biomarkers of early stages of AD [7, 40].

4.2. Blood Biomarkers

So far, the analysis of CSF and neuroimaging, mainly PET scan, has the dominant role in AD diagnosis as well as in AD drug design. Although the analysis of biomarkers from CSF in the diagnosis of AD is a specific and established method with high diagnostic accuracy, it is still an invasive intervention in the body. In recent years, therefore, there has been a growing tendency to establish a methodology for the diagnosis of AD from plasma samples [2, 41]. Possible problems when using blood samples to measure AD biomarkers could be high interferences and the ability of the biomarker to penetrate from CSF to blood through the blood brain barrier [30]. The following biomarkers described below including APOE 4, protein p53, and the other proteins are very often also CSF biomarkers (8 Toyos-Rodriguez 2020). A survey of the most promising blood biomarkers is given in Figure 5.

[figure omitted; refer to PDF]

4.2.1. $A\beta$ and Tau

Initial studies did not suggest a relevant correlation between CSF and blood levels of $A\beta_{42}$ or ratio $A\beta_{42}/A\beta_{40}$, as was summarized in the meta-analysis of Olsson and coauthors (2016) [35]. On the other hand, new studies showed promising results in the analysis of $A\beta$ from blood. In the work of Janelidze and coauthors (2016), a weak positive correlation between CSF and blood levels for both $A\beta_{42}$ and $A\beta_{40}$ was observed mainly in patients with AD dementia. Prodromal stages showed just a moderate decrease in $A\beta_{42}$ levels and no decrease in $A\beta_{40}$ levels. This work reveals that plasma changes of $A\beta$ levels occur much later in comparison with the levels of $A\beta$ in CSF [42]. The possibility of $A\beta$ measurement was also studied in the work of Ovod and coauthors (2017) and Nakamura and coauthors (2018) with a proven correlation between $A\beta$ levels in the blood and CSF [41, 43, 44]. Regarding the measurement of plasma T-tau and P-tau, the work of Toombs and Zetterger (2020) mentioned a contrast result from various studies. It probably depends on the sensitivity of the chosen method. Using ultrasensitive assays, plasma tau levels were increased compared to the healthy population, but this increase is negligible in comparison with levels of tau from CSF. On the other hand, increased plasma levels of P-tau₁₈₁ correlate perfectly with increased CSF levels of P-tau₁₈₁ [41, 45].

4.2.2. Neurofilament Light Chain

The neurofilament light chain is an intraaxonal structural protein that leaks into body fluids, both CSF and blood, during axonal damage, regardless of the cause. Although it is not clear how blood levels of NFL correlate with neurodegeneration, it belongs to the most consistent plasma biomarkers of neurodegeneration [41, 45].

4.2.3. Protein Markers

Plasma levels of proteins including clusterin, fetuin B, pancreatic prohormone, and prostate-specific antigen complexed to α 1-antichymotrypsin are altered during AD and can be analyzed as good biomarkers but only together represent a reliable tool for diagnosis with strong correlation to AD [2]. Apolipoprotein E4 (APOE4) is considered a risk factor for the development of AD and has been introduced as a plasma biomarker for early diagnosis of AD. APOE is a glycoprotein that mediates the binding of lipoproteins to their low-density receptors. They are predominantly expressed in the brain in several isoforms, APOE2, APOE3, and APOE4 belong to the most common ones, but only APOE4 presents a risk factor to AD by reducing amyloid clearance and accelerating senile plaques formation. Circa 50% of all AD patients have APOE4 isoform [7, 10]. The protein p53 responds to cellular stress, and the relationship between conformationally altered p53 and AD diagnosis in blood has been studied since 2008 when

Lanni and coauthors observed unfold p53 in peripheral blood cells of AD patients. Recently, unfolded protein p53 was determined from blood in the work of Amor-Gutiérrez and coauthors (2020) using a competitive electrochemical immunosensor with promising results [46, 47].

4.2.4. Genetic Markers

The AD, not only early-onset type, can be diagnosed by the analysis of the genome of patients. Genetic mutations and polymorphisms related to the disease can be discovered. Single gene mutation of chromosomes 1, 14, and 21 causes malformations on amyloid precursor protein, presenilin 1, and presenilin 2, and it is the cause of the early onset of AD. The above-mentioned polymorphism of APOE is the probable most common genetic cause of late onset of AD and can be also revealed by the analysis of the genetic material [2].

4.2.5. Metals

Metals, including Cu^{II}, Zn^{II}, and Fe^{III}, may play an important role in AD pathology due to their high concentration in senile plaques. Mainly redox-active metals (Cu and Fe) are able to bound on A β , stabilize its oligomeric form, and accelerate the aggregation of A β [25, 26]. Recently, altered plasma metal levels were observed in conditions involving CNS-associated disorders in the work of Nahan and coauthors (2017). According to the work of Xu and coauthors (2018) and Guan and coauthors (2017), plasma metals are suggested as potential blood markers [48–50].

5. Electrochemical Biosensors in Diagnosis of Alzheimer's Disease

5.1. Detection of A β

In recent years, highly sensitive electrochemical biosensors have been designed for the detection of A β , especially A β_{42} and A β oligomers. With respect to the type of biorecognition element, three main groups of structures were used: RNA aptamer, antibody, and molecularly imprinted polymers (MIP). The developed biosensor devices are typically portable and simple tools commonly suitable for point-of-care use when a commercial product based on the research will be introduced into the market.

In the work of Negahdary and Heli (2019), RNA aptamer was immobilized onto the surface of gold disk electrode with electrodeposited fern leaves-like gold nanostructure intended to detect A β_{42} [51].

MIP has also been used in the work of Ozcan and coauthors (2020) and Pereira and coauthors (2020), both aimed to detect A β_{42} [52, 53]. The classic electrochemical biosensor construction, a glassy carbon electrode with delaminated titanium carbide MXene and multiwalled carbon nanotubes composite covered by MIP, was prepared in the work of Ozcan and coauthors [52]. On the other hand, in the work of Pereira and coauthors, an innovative paper-based platform for carrying MIP was prepared [53]. Although Ozcan and coauthors have achieved a much lower limit of detection as well as better sensitivity, the construction of Pereira and coauthor is unique in its simplicity and low cost.

The most widely used biorecognition elements were antibodies. In the work of Le and coauthors (2020), a self-assembled monolayer functionalized interdigitated chain-shaped electrode with immobilized specific anti-A β antibody was prepared for the detection of A β_{42} and used together with atypical nonfaradaic detection [54]. The self-assembled monolayer was also used in the construction of biosensors in the work of Carneiro and coauthors (2017). They modified the gold electrode by mercaptopropionic acid SAM, gold nanoparticles, and monoclonal antibody mAb DE2B4 for the analysis of A β_{42} [55]. Sethi and coauthors (2020) used a screen-printed electrode with a dual layer of graphene-reduce graphene oxide to immobilize the H31L21 antibody. The sensor was designed for use in the rapid detection of A β_{42} [56].

A unique biorecognition element-cellular prion protein was used in the work of Qin and coauthors (2020). A gold electrode with immobilized gold dendrite and electropolymerized poly(pyrrole-3-carboxylic acid) was used as a carrier for this bioreceptor for the detection of A β oligomers [57]. A comparison of the detection limits, linear concentration ranges, or detection techniques of biosensors described above is shown in Table 3.

Table 3

Overview of electrochemical biosensors for the detection of A β in AD diagnosis.

Marker	Construction	LOD/pM	Linear range/pM	Detection	References
$A\beta_{42}$	Fern leaves-like gold nanostructure with an RNA aptamer	88.6×10^{-3}	0.440–285	DPV	[51]
$A\beta O$	PPy-3-COOH electropolymerized onto gold dendrite with bounded cellular prion protein	1×10^{-6}	10^{-6} – 10×10^3	Impedimetry	[57]
$A\beta_{42}$	SAM functionalized interdigitated chain-shaped electrode with anti- $A\beta$ antibody	1.70	2.20 – 2.20×10^3	Nonfaradaic detection	[54]
$A\beta_{42}$	GCE with titanium carbide MXene and MWCN composite including MIP	6.65×10^{-5}	2.20×10^{-4} – 2.20×10^{-2}	DPV	[52]
$A\beta_{42}$	Dual layer of graphene and rGO with immobilized H31L21 antibody achieved via Pyr-NHS	2.40	11.0 – 55.0×10^3	DPV	[56]
$A\beta_{42}$	MIP in a paper-based platform on the carbon ink electrode's surface	14.8	22.0 – 22.0×10^4	SWV	[53]
$A\beta_{42}$	Gold electrode with mercaptopropionic acid SAM, gold nanoparticles, and monoclonal antibody mAb DE2B4	1.15	2.20 – 2.20×10^2	SWV	[55]

LOD: limit of detection, DPV: differential pulse voltammetry, $A\beta O$: $A\beta$ oligomers, PPy-3-COOH: poly(pyrrole-3-carboxylic acid), SAM: self-assembled monolayer, GCE: glassy carbon electrode, MWCN: multiwalled carbon nanotubes, MIP: molecularly imprinted polymers, rGO: reduced graphene oxide, Pyr-NHS: 1-pyrenebutyric acid N-hydroxysuccinimide ester, and SWV: square wave voltammetry.

5.2. Detection of tau Protein

Recent approaches in electrochemical biosensors for tau protein detection were based on antibody biorecognition elements. Different types of tau isoforms are known. Tau-441 was detected in the work of Carlin and Martic-Milne (2018), Karaboga and Sezgenturk (2020), and Wang and coauthors (2017) [58–60]. In the first-mentioned paper, a very simple construction of gold electrode covered by anti-tau antibodies was designed [58]. The paper of Karaboga and Sezgenturk introduced a more complicated construction of indium tin oxide electrode coated by polyethylene terephthalate and utilizing nanocomposite of reduced graphene oxide and gold nanoparticles for antibodies binding [59]. Wang and coauthors prepared a four-electrode system of gold microband electrodes covered with a layer of a self-assembled monolayer and protein G. Protein G is used to interact with immobilized antibodies to ensure their optimal orientation [60]. The fact is that the use of a nanocomposite structure in biosensor construction highly increases the sensitivity of the sensor, according to the available limit of detections.

The tau-381 isoform was determined in the work of Shui and coauthors (2018). In this paper, a combination of antibodies and aptamer as biorecognition elements was used in Sandwich assay construction. The gold working electrode was used as a carrier of cysteamine-stabilized AuNPs covered by biorecognition elements [61].

T-tau protein was determined by a gold electrode coated with a self-assembled monolayer of 3-mercaptopropionic acid with immobilized anti-T-tau antibodies in the work of Dai and coauthors (2017) and a screen-printed carbon electrode with gold nanoparticles-poly(amidoamine) dendrimer nanocomposite and anti-tau capture antibody for tau protein detection was designed in work of Razzino and coauthors (2020) [62, 63]. Further details on the described electrochemical biosensors for the detection of tau protein are summarized in Table 4.

Table 4

Overview of electrochemical biosensors for tau protein detection in AD diagnosis.

Marker	Construction	LOD/ pM	Linear range/pM	Detection	Reference
Tau-441	Anti-tau antibodies immobilized onto a gold electrode	10^6-10^3	–	CV, SWV	[58]
T-tau	SAM of MPA binding anti-T-tau antibody on the gold electrode	–	–	DPV	[62]
Tau-381	Cysteamine-stabilized AuNPs with anti-tau antibody and an aptamer specific to tau-381	0.420	0.500–1.00 10^2	DPV	[61]
Tau	SPCE modified with an AuNPs-PAMAM dendrimer nanocomposite and anti-tau capture antibody	0.030	0.110–91.0	Amperometry	[63]
Tau-441	ITO-coated PET electrode with rGO-AuNPs nanocomposite and anti-tau antibodies	0.002	2.20 10^{-2} –10.9	EIS, CV	[59]
Tau-441 (2N4R)	Four gold microband electrodes with a layer of a SAM, protein G, and anti-tau antibodies	0.030	–	CV, EIS	[60]

LOD: limit of detection, CV: cyclic voltammetry, SWV: square wave voltammetry, SAM: self-assembled monolayer, MPA: 3-mercaptopropionic acid, DPV: differential pulse voltammetry, AuNPs: gold nanoparticles, SPCEs: screen-printed carbon electrode, PAMAM: poly(amidoamine), rGO: reduced graphene oxide, ITO: indium tin oxide, PET: polyethylene terephthalate, and EIS: electrochemical impedance spectroscopy.

5.3. Detection of Neurotransmitters

Chemical compounds that serve as biological messengers from a nerve cell through a synapse to a target cell are called neurotransmitters. In recent years, various neurotransmitters have been measured for early diagnosis of AD. Electrochemical biosensors for acetylcholine detection were designed by da Silva and Brett (2020), Chauhan and coauthors (2017), and Moreira and coauthors (2017), and all of these sensors contained the enzyme acetylcholinesterase as biorecognition element [64–66]. The most sensitive one, glass plate modified by iron oxide nanoparticles-poly(3,4-ethylenedioxythiophene)-reduced graphene oxide nanocomposite with immobilized enzymes acetylcholinesterase and choline oxidase, was constructed by the team of Chauhan and coauthors [65]. Moreira and coauthors prepared a biosensor based on platinum wire covered by a highly porous gold film and enzyme acetylcholinesterase, and it shows the lowest limit of detection [66]. The biosensor prepared by da Silva and Brett was based on a glassy carbon electrode modified by iron oxide nanoparticles covered by a film of poly(neural red), and acetylcholinesterase was immobilized onto its surface [64].

Another neurotransmitter, dopamine, was the target of a biosensor construction designed by Shin and coauthors (2017) and Yi and coauthors (2017). Both biosensors were enzymeless, the first one based on indium tin oxide electrode covered by graphene oxide and silver nanoparticles, and the second one based on self-supporting nanoporous gold wire with palladium nanoparticles [67, 68]. Both sensors showed similar detection limits and so similar sensitivity.

The papers of Hughes and coauthors (2015) and Alves and coauthors (2016) were focused on the determination of glutamate by electrochemical biosensors [69, 70]. The enzymatic sensor was based on a screen-printed carbon

electrode covered by a layer of chitosan with multiwalled carbon nanotubes and the enzyme glutamate dehydrogenase and its cofactor nicotinamide adenine dinucleotide [69]. The nonenzymatic sensor was based on a graphite electrode with an immobilized small protein-like chain designed to mimic a peptide that recognizes glutamate [70]. Electrochemical biosensors for the detection of neurotransmitters are listed in Table 5.

Table 5

Overview of electrochemical biosensors for the detection of neurotransmitters in AD diagnosis.

Marker	Construction	LOD/ μM	Linear range/ μM	Detection	Reference
ACh	GCE modified by IONPs with poly(neutral red) film and AChE	1.00	2.50–60.0	Amperometry	[64]
ACh	Glass plate with IONPs-PEDOT-rGO nanocomposite modified by FTO and immobilized AChE and ChO	4.00×10^{-3}	4.00×10^{-3} – 8.00×10^2	CV	[65]
ACh	Pt wire covered by highly porous gold film with immobilized AChE	10.0	0.250×10^3 – 1.90×10^3 (PBS) 0.120×10^3 – 1.40×10^3 (GB)	LSV, SWV, CA	[66]
Dopamine	ITO electrode covered by GO and SNPs	0.200	0.100 – 1.00×10^2	CV, DPV, amperometry	[67]
Dopamine	Self-supporting NPG wire with PdNPs	Up to 1.00	1 – 2.20×10^2	DPV	[68]
Glutamate	SPCE with chitosan and MWCN encapsulating GLDH and NAD^+	3.00	7.50–105	Amperometry	[69]
Glutamate	Graphite electrode with mimetic peptide recognizing glutamate	0.001	1.00×10^3 – 10.0×10^3	DPV	[70]

LOD: limit of detection, ACh: acetylcholine, GCE: glassy carbon electrode, IONPs: iron oxide (Fe_2O_3) nanoparticles, AChE: acetylcholinesterase, PEDOT: poly(3,4-ethylenedioxythiophene), rGO: reduced graphene oxide, FTO: fluorine-doped tin oxide, ChO: choline oxidase, CV: cyclic voltammetry, PBS: phosphate-buffered solution, GB: glycine buffer, LSV: linear sweep voltammetry, SWV: square wave voltammetry, CA: chronoamperometry, ITO: indium tin oxide, GO: graphene oxide, SNPs: silver nanoparticles, DPV: differential pulse voltammetry, NPG: nanoporous gold, PdNPs: palladium nanoparticles, SPCE: screen-printed carbon electrode, MWCNTs: multiwalled carbon nanotubes, GLDH: glutamate dehydrogenase, and NAD^+ : nicotinamide adenine dinucleotide.

5.4. Detection of Genetic and Protein Markers

MicroRNA has become a highly determined biomarker of AD. Four works focusing on the detection of microRNA-137, microRNA-146a, and microRNA-34a are described lower. Azimzadeh and coauthors (2017) designed an electrochemical biosensor for the detection of microRNA-137 based on screen-printed carbon electrodes modified by reduced graphene oxide-gold nanowire nanocomposite, and doxorubicin was immobilized as an intercalated label [71]. A gold electrode with self-assembled capture microRNA for bioconjugation with microRNA-146a was constructed by Khalilzadeh and coauthors (2019) [72]. Detection of microRNA-34a was the aim of two studies

performed by two teams from Ege University. An older study described the preparation of pencil graphite electrodes with a DNA probe for microRNA-34a [73]. The second article described impedimetric biosensor based on screen-printed carbon electrode with immobilized 3.5 poly(amidoamine) dendrimer and DNA probe for microRNA-34a, and it showed better sensitivity than voltammetric biosensor [74].

APOE is encoded by a gene having three isoforms (E2, E3, and E4) [75]. APOE4 is considered a risk factor for the development of AD [7]. Mutations of APOE can be detected by genosensors, and the protein itself can be detected mainly by immunosensors. Jafari and coauthors (2019) designed genosensor based on a glassy carbon electrode with an immobilized reduced graphene oxide-cerium oxide nanoparticles nanocomposite modified by ssDNA probe for APOE gene, namely, for 23-base oligonucleotide sequences with a point mutation [75]. On the other hand, Liu and coauthors (2020) prepared an immunosensor based on a glassy carbon electrode with gold nanobipyramid coated platinum nanostructure coated by anti-APOE4 antibody and gold:palladium-polydopamine nanotube nanozyme [76].

An electrochemical biosensor for another protein biomarker of AD, clusterin, was constructed in the work of Islam and coauthors (2018). Label-free immunosensor was based on a screen-printed carbon electrode with anti-clusterin antibody fragments [77].

Electrochemical immunosensors for the detection of conformationally altered (unfolded) protein p53 have also been proposed for the early diagnosis of AD. A simple screen-printed carbon electrode with bound anti-p53 antibody was prepared by Tonello and coauthors (2016), but only preliminary results were described [78]. Iglesias-Mayor and coauthors (2020) designed an immunosensor based on bifunctional core-shell Au-Pt/Au and immobilized anti-p53 antibody. This proposal was based on competition between protein p53 in the sample and p53 bounded to streptavidin modified magnetic beads added in measured solution, so, the higher concentration of p53 in the sample caused the lower catalytic current response [79]. The electrochemical geno- and immunosensors described above are summarized in Table 6.

Table 6

Overview of electrochemical biosensors for the detection of genetic and protein markers in AD diagnosis.

Marker	Construction	LOD/nM	Liner range/nM	Detection	Reference
miR-137	SPCE with rGO and Au nanowires and label doxorubicin	$1.70 \cdot 10^{-6}$	$5.00 \cdot 10^{-6}$ – $0.750 \cdot 10^{-3}$	DPV	[71]
miR-146a	Au electrode modified by capture miR	$10.0 \cdot 10^{-3}$	$10.0 \cdot 10^{-3}$ – $1.00 \cdot 10^3$	SWV	[72]
miR-34a	SPCE with PAMAM dendrimer and DNA probe for miR-34a	140	0 – $10.7 \cdot 10^2$	Impedimetry	[74]
miR-34a	PGE with GO and miRNA-34a specific DNA probe	$10.7 \cdot 10^2$	$7.10 \cdot 10^2$ – $5.00 \cdot 10^3$	DPV	[73]
APOE gene	GCE electrode modified by rGO-CONPs nanocomposite with ssDNA probe for APOE gene	$1.00 \cdot 10^{-6}$	$10.0 \cdot 10^{-6}$ – 10.0	SWV	[75]

APOE 4	GCE modified by Au nanobipyramid coated Pt nanocomposite with anti-APOE4 antibody and AuPd-PDA nanozyme	$0.450 \cdot 10^{-3}$	$1.50 \cdot 10^{-3}$ –58.0	Amperometry	[76]
Clusterin	SPCE with anti-clusterin antibody fragments	Down to $3.20 \cdot 10^{-5}$	$3.20 \cdot 10^{-5}$ – $3.20 \cdot 10^{-3}$	CV, SWV	[77]
p53	SPCE with anti-p53 antibody	—	—	ASV	[78]
p53	SPCE modified by bifunctional core-shell Au–Pt/Au NPs with anti-p53 monoclonal antibody	66.0	—	CA	[79]

6. Perspectives in the Diagnosis of Alzheimer's Disease

Because the number of AD patients is growing rapidly and no therapeutic drug to cure AD was found, early diagnosis is the key factor in managing and slowing the disease. The most important step for the future diagnosis of AD lies in early diagnosis before a severe clinical symptom appears. Preventive testing should include biomarkers that precede these symptoms [10, 80]. This examination should be noninvasive, affordable, simple, and fast. Less invasive analysis of blood biomarkers is a promising possibility, but noninvasive analysis of urine, tears, sweat, or saliva would overcome the limitation of invasive sampling [80, 81]. AD as a multifactorial disease with still unclear pathology is very hard to diagnose. Biosensors for simultaneous detection of multiple biomarkers would simplify and speed up diagnostics from just one sample. So, the important strategy is to integrate more analytical technologies into one platform. The use of nanotechnologies would provide high sensitivity and specificity of biosensors providing analysis of very low concentrations of biomarkers in noninvasively taken samples [81, 82]. Molecularly imprinted polymers allow the analysis of high-affinity aptamers and antibodies specific for AD biomarkers. This is an important challenge for the development of biosensors, which should pay close attention to this problem [10, 82].

Electrochemical biosensors are technology growing rapidly in the diagnosis of AD, but the extension of tested biomarkers would facilitate their incorporation into clinical practice. Many biomarkers including metal ions or posttranslational protein modifications are still neglected. Heavy metals have been analyzed by electrochemical biosensor from water in the work of Sciuto and coauthors (2020), copper from water samples was determined in the work of Cui and coauthors (2014), and iron from the water was tested in the work of Kamal and coauthors (2014) [83–85]. Glycan electrochemical biosensor for cancer diagnosis was presented in the work of Kveton and coauthors (2019), the electrochemical behavior of phosphotyrosine was observed in the work of Popa and Ducleescu (2013), and dual-mode sensor using an electrolyte–insulator–semiconductor field-effect device coupled with nanoplasmonic effects was developed for protein phosphorylation detection in the work of Bhalla and coauthors (2015) [86–88]. So, various electrochemical biosensors exist, but their optimization and application in AD diagnosis were not tried yet.

7. Conclusion

AD is a serious health and socioeconomic problem, and its solution is mainly in early and simple but precise diagnosis, so proper diagnostic techniques are required. Simple sensors suitable for point-of-care use are especially desired. Electrochemical biosensors are the future of AD diagnostics due to their high sensitivity, simple construction, easy handling, portability, and low cost. Although many possible diagnostic methods have recently been investigated, their integration into clinical practice and diagnostic protocol is necessary, especially since early diagnosis of AD is a crucial part of good treatment and patient's state outcome. Detection of blood biomarkers instead of CSF biomarkers is one of the key parts of this diagnostic protocol which could also make the diagnosis more accessible to all patients with or without AD symptoms. Although electrochemical biosensors for the detection of A β , tau proteins, neurotransmitters, and genetic and protein markers have been discussed in this review, laboratory determination of A β 42 alone or in combination with A β 40, P-tau₁₈₁, and T-tau has been established into practice so far. Standard laboratory methodology could be improved by analyzing a combination of the currently

estimated markers along with neurotransmitters and genetic markers from blood samples, which make the test for AD diagnosis available to the wide public. This protocol could detect AD in asymptomatic patients and prolong their quality lifetime.

Acknowledgments

A long-term organization development plan for mass destruction weapons (Faculty of Military Health Sciences, University of Defense, Czech Republic) is gratefully acknowledged.

Glossary

Abbreviations

AD:Alzheimer's disease

A β :Amyloid β

CSF:Cerebrospinal fluid

FDG-PET:Fluorodeoxyglucose positron emission tomography

MRI:Magnetic resonance imaging

NFT:Neurofibrillary tangles

PET:Positron emission tomography

P-tau:Phosphorylated tau protein

T-tau:Total tau protein.

References

- [1] P. Mehrotra, "Biosensors and their applications-a review," *Journal of Oral Biology and Craniofacial Research*, vol. 6 no. 2, pp. 153-159, DOI: 10.1016/j.jobcr.2015.12.002, 2016.
- [2] L. C. Brazaca, I. Sampaio, V. Zucolotto, B. C. Janegitz, "Applications of biosensors in Alzheimer's disease diagnosis," *Talanta*, vol. 210, DOI: 10.1016/j.talanta.2019.120644, 2020.
- [3] K. Rajpoot, "Recent advances and applications of biosensors in novel technology," *Biosensors Journal*, vol. 6, 2017.
- [4] F. J. Gruhl, B. E. Rapp, K. Länge, "Biosensors for diagnostic applications," *Molecular Diagnostics*, pp. 115-148, 2013.
- [5] N. Aggarwal, R. Shah, D. Bennett, "Alzheimer's disease: unique markers for diagnosis & new treatment modalities," *Indian Journal of Medical Research*, vol. 142 no. 4, pp. 369-382, DOI: 10.4103/0971-5916.169193, 2015.
- [6] B. N. Dugger, D. W. Dickson, "Pathology of neurodegenerative diseases," *Cold Spring Harbor Perspectives in Biology*, vol. 9 no. 7, DOI: 10.1101/cshperspect.a028035, 2017.
- [7] C. Toyos-Rodríguez, F. J. García-Alonso, A. de la Escosura-Muñiz, "Electrochemical biosensors based on nanomaterials for early detection of Alzheimer's disease," *Sensors*, vol. 20 no. 17, DOI: 10.3390/s20174748, 2020.
- [8] P. Martinkova, A. Kostelník, T. Valek, M. Pohanka, "Main streams in the construction of biosensors and their applications," *International Journal of Electrochemical Science*, vol. 12, pp. 7386-7403, DOI: 10.20964/2017.08.02, 2017.
- [9] Z. Tavakolian-Ardakani, O. Hosu, C. Cristea, M. Mazloun-Ardakani, G. Marrazza, "Latest trends in electrochemical sensors for neurotransmitters: a review," *Sensors*, vol. 19 no. 9, DOI: 10.3390/s19092037, 2019.
- [10] B. Shui, D. Tao, A. Florea, J. Cheng, Q. Zhao, Y. Gu, W. Li, N. Jaffrezic-Renault, Y. Mei, Z. Guo, "Biosensors for Alzheimer's disease biomarker detection: a review," *Biochimie*, vol. 147, pp. 13-24, DOI: 10.1016/j.biochi.2017.12.015, 2018.
- [11] V. Gaudin, "Receptor-based electrochemical biosensors for the detection of contaminants in food products," *Electrochemical Biosensors*, pp. 307-365, DOI: 10.1016/b978-0-12-816491-4.00011-5, 2019.
- [12] A. González-López, M. T. Fernández Abedul, "Electrochemical detection of melatonin in a flow injection analysis system," *Laboratory Methods in Dynamic Electroanalysis*, pp. 85-97, DOI: 10.1016/b978-0-12-815932-3.00009-7, 2020.
- [13] S. Roy, S. Pandit, "Microbial electrochemical system," *Microbial Electrochemical Technology*, pp. 19-48, DOI:

10.1016/b978-0-444-64052-9.00002-9, 2019.

[14] G. Hussain, D. Silvester, "Comparison of voltammetric techniques for the electroanalysis of dissolved solids and gases in water, acetonitrile and room temperature ionic liquids (RTILs): unusual behaviour for gases," Proceedings of the PRIME 2016/230th ECS Meeting, .

[15] E. Cesewski, B. N. Johnson, "Electrochemical biosensors for pathogen detection," Biosensors and Bioelectronics, vol. 159, DOI: 10.1016/j.bios.2020.112214, 2020.

[16] A. Pisoschi, "Potentiometric biosensors: concept and analytical applications-an editorial," Biochemistry & Analytical Biochemistry, vol. 5, DOI: 10.4172/2161-1009.1000e164, 2016.

[17] C. Okafor, D. Grooms, E. Alocilja, S. Bolin, "Comparison between a conductometric biosensor and ELISA in the evaluation of Johne's disease," Sensors, vol. 14 no. 10, pp. 19128-19137, DOI: 10.3390/s141019128, 2014.

[18] J. Leva-Bueno, S. A. Peyman, P. A. Millner, "A review on impedimetric immunosensors for pathogen and biomarker detection," Medical Microbiology and Immunology, vol. 209 no. 3, pp. 343-362, DOI: 10.1007/s00430-020-00668-0, 2020.

[19] M. Hoyos-Nogués, F. J. Gil, C. Mas-Moruno, "Antimicrobial peptides: powerful biorecognition elements to detect bacteria in biosensing technologies," Molecules, vol. 23 no. 7, DOI: 10.3390/molecules23071683, 2018.

[20] WHO, "Global action plan on the public health response to dementia 2017–2025," WHO Document Production Services, 2017.

[21] M. F. Mendez, "Early-onset Alzheimer disease," Neurologic Clinics, vol. 35 no. 2, pp. 263-281, DOI: 10.1016/j.ncl.2017.01.005, 2017.

[22] R. H. Swerdlow, "Pathogenesis of Alzheimer's disease," Clinical Interventions in Aging, vol. 2 no. 3, pp. 347-359, 2007.

[23] A. Kas, R. Migliaccio, B. Tavitian, "A future for PET imaging in Alzheimer's disease," European Journal of Nuclear Medicine and Molecular Imaging, vol. 47 no. 2, pp. 231-234, DOI: 10.1007/s00259-019-04640-w, 2020.

[24] A. K. Thakur, P. Kamboj, K. Goswami, "Pathophysiology and management of Alzheimer's disease: an overview," Journal of Analytical & Pharmaceutical Research, vol. 7 no. 2, pp. 226-235, DOI: 10.15406/japlr.2018.07.00230, 2018.

[25] M. Rana, A. K. Sharma, "Cu and Zn interactions with A β peptides: consequence of coordination on aggregation and formation of neurotoxic soluble A β oligomers," Metallomics, vol. 11 no. 1, pp. 64-84, 2018.

[26] F. Tahmasebinia, S. Emadi, "Effect of metal chelators on the aggregation of beta-amyloid peptides in the presence of copper and iron," Biometals, vol. 30 no. 2, pp. 285-293, DOI: 10.1007/s10534-017-0005-2, 2017.

[27] A. Gella, N. Durany, "Oxidative stress in Alzheimer disease," Cell Adhesion & Migration, vol. 3 no. 1, pp. 88-93, DOI: 10.4161/cam.3.1.7402, 2009.

[28] M.-S. García-Ayllón, I. Riba-Llena, C. Serra-Basante, J. Alom, R. Boopathy, J. Sáez-Valero, "Altered levels of acetylcholinesterase in Alzheimer plasma," PLoS One, vol. 5 no. 1, DOI: 10.1371/journal.pone.0008701, 2010.

[29] J. C. Vickers, S. Mitew, A. Woodhouse, C. M. Fernandez-Martos, M. T. Kirkcaldie, A. J. Canty, G. H. McCormack, A. E. King, "Defining the earliest pathological changes of Alzheimer's disease," Current Alzheimer Research, vol. 13 no. 3, pp. 281-287, DOI: 10.2174/1567205013666151218150322, 2016.

[30] J. C. Lee, S. J. Kim, S. Hong, Y. Kim, "Diagnosis of Alzheimer's disease utilizing amyloid and tau as fluid biomarkers," Experimental & Molecular Medicine, vol. 51 no. 5, DOI: 10.1038/s12276-019-0250-2, 2019.

[31] B. J. Kelley, R. C. Petersen, "Alzheimer's disease and mild cognitive impairment," Neurologic Clinics, vol. 25 no. 3, pp. 577-609, DOI: 10.1016/j.ncl.2007.03.008, 2007.

[32] E. J. Mufson, L. Binder, S. E. Counts, S. T. DeKosky, L. deToledo-Morrell, S. D. Ginsberg, M. D. Ikonomic, S. E. Perez, S. W. Scheff, "Mild cognitive impairment: pathology and mechanisms," Acta Neuropathologica, vol. 123 no. 1, pp. 13-30, DOI: 10.1007/s00401-011-0884-1, 2012.

[33] T. Yamasaki, H. Muranaka, Y. Kaseda, Y. Mimori, S. Tobimatsu, "Understanding the pathophysiology of Alzheimer's disease and mild cognitive impairment: a mini review on fMRI and ERP studies," Neurology Research International, vol. 2012, DOI: 10.1155/2012/719056, 2012.

- [34] N. R. Barthélemy, R. J. Bateman, C. Hirtz, P. Marin, F. Becher, C. Sato, A. Gabelle, S. Lehmann, "Cerebrospinal fluid phospho-tau T217 outperforms T181 as a biomarker for the differential diagnosis of Alzheimer's disease and PET amyloid-positive patient identification," *Alzheimer's Research & Therapy*, vol. 12 no. 1, DOI: 10.1186/s13195-020-00596-4, 2020.
- [35] B. Olsson, R. Lautner, U. Andreasson, A. Öhrfelt, E. Portelius, M. Bjerke, M. Hölttä, C. Rosén, C. Olsson, G. Strobel, E. Wu, K. Dakin, M. Petzold, K. Blennow, H. Zetterberg, "CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis," *The Lancet Neurology*, vol. 15 no. 7, pp. 673-684, DOI: 10.1016/s1474-4422(16)00070-3, 2016.
- [36] S. D. Targum, "Biomarkers for the identification and treatment of dementia," *Psychiatry (Edgmont (Pa.: Township))*, vol. 5 no. 2, pp. 51-56, 2008.
- [37] H. Hampel, K. Blennow, L. M. Shaw, Y. C. Hoessler, H. Zetterberg, J. Q. Trojanowski, "Total and phosphorylated tau protein as biological markers of Alzheimer's disease," *Experimental Gerontology*, vol. 45 no. 1, pp. 30-40, DOI: 10.1016/j.exger.2009.10.010, 2010.
- [38] C. Wattmo, K. Blennow, O. Hansson, "Cerebro-spinal fluid biomarker levels: phosphorylated tau (T) and total tau (N) as markers for rate of progression in Alzheimer's disease," *BMC Neurology*, vol. 20 no. 1, DOI: 10.1186/s12883-019-1591-0, 2020.
- [39] D. S. Strac, D. Muck-Seler, N. Pivac, "Neurotransmitter measures in the cerebrospinal fluid of patients with Alzheimer's disease: a review," *Psychiatria Danubina*, vol. 27 no. 1, pp. 14-24, 2015.
- [40] R. Sangubotla, J. Kim, "Recent trends in analytical approaches for detecting neurotransmitters in Alzheimer's disease," *TRAC Trends in Analytical Chemistry*, vol. 105, pp. 240-250, DOI: 10.1016/j.trac.2018.05.014, 2018.
- [41] J. Toombs, H. Zetterberg, "In the blood: biomarkers for amyloid pathology and neurodegeneration in Alzheimer's disease," *Brain Communications*, vol. 2 no. 1, DOI: 10.1093/braincomms/fcaa054, 2020.
- [42] S. Janelidze, E. Stomrud, S. Palmqvist, H. Zetterberg, D. van Westen, A. Jeromin, L. Song, D. Hanlon, C. A. Tan Hehir, D. Baker, K. Blennow, O. Hansson, "Plasma β -amyloid in Alzheimer's disease and vascular disease," *Scientific Reports*, vol. 6 no. 1, DOI: 10.1038/srep26801, 2016.
- [43] A. Nakamura, N. Kaneko, V. L. Villemagne, T. Kato, J. Doecke, V. Doré, C. Fowler, Q.-X. Li, R. Martins, C. Rowe, T. Tomita, K. Matsuzaki, K. Ishii, K. Ishii, Y. Arahata, S. Iwamoto, K. Ito, K. Tanaka, C. L. Masters, K. Yanagisawa, "High performance plasma amyloid- β biomarkers for Alzheimer's disease," *Nature*, vol. 554 no. 7691, pp. 249-254, DOI: 10.1038/nature25456, 2018.
- [44] V. Ovod, K. N. Ramsey, K. G. Mawuenyega, J. G. Bollinger, T. Hicks, T. Schneider, M. Sullivan, K. Paumier, D. M. Holtzman, J. C. Morris, T. Benzinger, A. M. Fagan, B. W. Patterson, R. J. Bateman, "Amyloid β concentrations and stable isotope labeling kinetics of human plasma specific to central nervous system amyloidosis," *Alzheimer's and Dementia*, vol. 13 no. 8, pp. 841-849, DOI: 10.1016/j.jalz.2017.06.2266, 2017.
- [45] H. Zetterberg, S. C. Burnham, "Blood-based molecular biomarkers for Alzheimer's disease," *Molecular Brain*, vol. 12 no. 1, DOI: 10.1186/s13041-019-0448-1, 2019.
- [46] O. Amor-Gutiérrez, E. Costa-Rama, N. Arce-Varas, C. Martínez-Rodríguez, A. Novelli, M. T. Fernández-Sánchez, A. Costa-García, "Competitive electrochemical immunosensor for the detection of unfolded p53 protein in blood as biomarker for Alzheimer's disease," *Analytica Chimica Acta*, vol. 1093, pp. 28-34, DOI: 10.1016/j.aca.2019.09.042, 2020.
- [47] C. Lanni, M. Racchi, G. Mazzini, A. Ranzenigo, R. Polotti, E. Sinforiani, L. Olivari, M. Barcikowska, M. Styczynska, J. Kuznicki, A. Szybinska, S. Govoni, M. Memo, D. Uberti, "Conformationally altered p53: a novel Alzheimer's disease marker?," *Molecular Psychiatry*, vol. 13 no. 6, pp. 641-647, DOI: 10.1038/sj.mp.4002060, 2008.
- [48] K. S. Nahan, K. B. Walsh, O. Adeoye, J. A. Landero-Figueroa, "The metal and metalloprotein profile of human plasma as biomarkers for stroke diagnosis," *Journal of Trace Elements in Medicine & Biology*, vol. 42, pp. 81-91, DOI: 10.1016/j.jtemb.2017.04.004, 2017.
- [49] J. Xu, S. J. Church, S. Patassini, P. Begley, K. A. B. Kellett, E. R. L. C. Vardy, R. D. Unwin, N. M. Hooper, G. J.

- S. Cooper, "Plasma metals as potential biomarkers in dementia: a case-control study in patients with sporadic Alzheimer's disease," *Biomaterials*, vol. 31 no. 2, pp. 267-276, DOI: 10.1007/s10534-018-0089-3, 2018.
- [50] C. Guan, R. Dang, Y. Cui, L. Liu, X. Chen, X. Wang, J. Zhu, D. Li, J. Li, D. Wang, "Characterization of plasma metal profiles in Alzheimer's disease using multivariate statistical analysis," *PLoS One*, vol. 12 no. 7, DOI: 10.1371/journal.pone.0178271, 2017.
- [51] M. Negahdary, H. Heli, "An ultrasensitive electrochemical aptasensor for early diagnosis of Alzheimer's disease, using a fern leaves-like gold nanostructure," *Talanta*, vol. 198, pp. 510-517, DOI: 10.1016/j.talanta.2019.01.109, 2019.
- [52] N. Özcan, O. Akyıldırım, N. Atar, M. L. Yola, M. L. au, "Electrochemical detection of amyloid- β protein by delaminated titanium carbide MXene/multi-walled carbon nanotubes composite with molecularly imprinted polymer," *Materials Today Communications*, vol. 23, DOI: 10.1016/j.mtcomm.2020.101097, 2020.
- [53] M. V. Pereira, A. C. Marques, D. Oliveira, R. Martins, F. T. C. Moreira, M. G. F. Sales, E. Fortunato, "Paper-based platform with an in situ molecularly imprinted polymer for β -amyloid," *ACS Omega*, vol. 5 no. 21, pp. 12057-12066, DOI: 10.1021/acsomega.0c00062, 2020.
- [54] H. T. N. Le, J. Park, S. Cho, "A probeless capacitive biosensor for direct detection of amyloid beta 1-42 in human serum based on an interdigitated chain-shaped electrode," *Micromachines*, vol. 11 no. 9, DOI: 10.3390/mi11090791, 2020.
- [55] P. Carneiro, J. Loureiro, C. Delerue-Matos, S. Morais, M. do Carmo Pereira, "Alzheimer's disease: development of a sensitive label-free electrochemical immunosensor for detection of amyloid beta peptide," *Sensors and Actuators B: Chemical*, vol. 239, pp. 157-165, DOI: 10.1016/j.snb.2016.07.181, 2017.
- [56] J. Sethi, M. Van Bulck, A. Suhail, M. Safarzadeh, A. Perez-Castillo, G. Pan, "A label-free biosensor based on graphene and reduced graphene oxide dual-layer for electrochemical determination of beta-amyloid biomarkers," *Microchimica Acta*, vol. 187 no. 5, DOI: 10.1007/s00604-020-04267-x, 2020.
- [57] J. Qin, S. Kim, M. Cho, Y. Lee, "Hierarchical and ultra-sensitive amyloid beta oligomer sensor for practical applications," *Chemical Engineering Journal*, vol. 401, DOI: 10.1016/j.cej.2020.126055, 2020.
- [58] N. Carlin, S. Martic-Milne, "Anti-tau antibodies based electrochemical sensor for detection of tau protein biomarkers," *Journal of the Electrochemical Society*, vol. 165 no. 12, pp. G3018-G3025, DOI: 10.1149/2.0041812jes, 2018.
- [59] M. N. Sonuç Karaboga, M. K. Sezgintürk, "Analysis of tau-441 protein in clinical samples using rGO/AuNP nanocomposite-supported disposable impedimetric neuro-biosensing platform: towards Alzheimer's disease detection," *Talanta*, vol. 219, DOI: 10.1016/j.talanta.2020.121257, 2020.
- [60] S. X. Wang, D. Acha, A. J. Shah, F. Hills, I. Roitt, A. Demosthenous, R. H. Bayford, "Detection of the tau protein in human serum by a sensitive four-electrode electrochemical biosensor," *Biosensors and Bioelectronics*, vol. 92, pp. 482-488, DOI: 10.1016/j.bios.2016.10.077, 2017.
- [61] B. Shui, D. Tao, J. Cheng, Y. Mei, N. Jaffrezic-Renault, Z. Guo, "A novel electrochemical aptamer-antibody sandwich assay for the detection of tau-381 in human serum," *The Analyst*, vol. 143 no. 15, pp. 3549-3554, DOI: 10.1039/c8an00527c, 2018.
- [62] Y. Dai, A. Molazemhosseini, C. C. Liu, "A single-use, in vitro biosensor for the detection of t-tau protein, a biomarker of neuro-degenerative disorders, in PBS and human serum using differential pulse voltammetry (DPV)," *Biosensors*, vol. 7 no. 1, DOI: 10.3390/bios7010010, 2017.
- [63] C. A. Razzino, V. Serafin, M. Gamella, M. Pedrero, A. Montero-Calle, R. Barderas, M. Calero, A. O. Lobo, P. Yáñez-Sedeño, S. Campuzano, J. M. Pingarrón, "An electrochemical immunosensor using gold nanoparticles-PAMAM-nanostructured screen-printed carbon electrodes for tau protein determination in plasma and brain tissues from Alzheimer patients," *Biosensors and Bioelectronics*, vol. 163, DOI: 10.1016/j.bios.2020.112238, 2020.
- [64] W. da Silva, C. M. A. Brett, "Novel biosensor for acetylcholine based on acetylcholinesterase/poly(neutral red)-deep eutectic solvent/Fe₂O₃ nanoparticle modified electrode," *Journal of Electroanalytical Chemistry*, vol. 872, DOI: 10.1016/j.jelechem.2020.114050, 2020.

- [65] N. Chauhan, S. Chawla, C. S. Pundir, U. Jain, "An electrochemical sensor for detection of neurotransmitter-acetylcholine using metal nanoparticles, 2D material and conducting polymer modified electrode," *Biosensors and Bioelectronics*, vol. 89 no. 1, pp. 377-383, DOI: 10.1016/j.bios.2016.06.047, 2017.
- [66] F. T. C. Moreira, M. G. F. Sale, M. Di Lorenzo, "Towards timely Alzheimer diagnosis: a self-powered amperometric biosensor for the neurotransmitter acetylcholine," *Biosensors and Bioelectronics*, vol. 87, pp. 607-614, DOI: 10.1016/j.bios.2016.08.104, 2017.
- [67] J. W. Shin, K. J. Kim, J. Yoon, J. Jo, W. A. El-Said, J. W. Choi, "Silver nanoparticle modified electrode covered by graphene oxide for the enhanced electrochemical detection of dopamine," *Sensors*, vol. 17 no. 12, DOI: 10.3390/s17122771, 2017.
- [68] X. Yi, Y. Wu, G. Tan, P. Yu, L. Zhou, Z. Zhou, "Palladium nanoparticles entrapped in a self-supporting nanoporous gold wire as sensitive dopamine biosensor," *Scientific Reports*, vol. 7 no. 1, DOI: 10.1038/s41598-017-07909-y, 2017.
- [69] G. Hughes, R. M. Pemberton, P. R. Fielden, J. P. Hart, "Development of a novel reagentless, screen-printed amperometric biosensor based on glutamate dehydrogenase and NAD⁺, integrated with multi-walled carbon nanotubes for the determination of glutamate in food and clinical applications," *Sensors and Actuators B: Chemical*, vol. 216, pp. 614-621, DOI: 10.1016/j.snb.2015.04.066, 2015.
- [70] L. Alves, A. Castro, S. Oliveira, L. Júnior, L. Goulart, A. Bonetti, A. E. Da Hora Machado, C. Ueira-Vieira, J. Madurro, A. Madurro, "Development of a mimetic system for electrochemical detection of glutamate," *Journal of Solid State Electrochemistry*, vol. 20, DOI: 10.1007/s10008-016-3236-1, 2016.
- [71] M. Azimzadeh, N. Nasirizadeh, M. Rahaie, H. Naderi-Manesh, "Early detection of Alzheimer's disease using a biosensor based on electrochemically-reduced graphene oxide and gold nanowires for the quantification of serum microRNA-137," *RSC Advances*, vol. 7 no. 88, pp. 55709-55719, DOI: 10.1039/c7ra09767k, 2017.
- [72] B. Khalilzadeh, M. Rashidi, A. Soleimanian, H. Tajalli, G. S. Kanberoglu, B. Baradaran, M.-R. Rashidi, "Development of a reliable microRNA based electrochemical genosensor for monitoring of mir-146a, as key regulatory agent of neurodegenerative disease," *International Journal of Biological Macromolecules*, vol. 134, pp. 695-703, DOI: 10.1016/j.ijbiomac.2019.05.061, 2019.
- [73] D. Isin, E. Eksin, A. Erdem, "Graphene oxide modified single-use electrodes and their application for voltammetric miRNA analysis," *Materials Science and Engineering: C*, vol. 75, pp. 1242-1249, DOI: 10.1016/j.msec.2017.02.166, 2017.
- [74] G. Congur, A. Erdem, "PAMAM dendrimer modified screen printed electrodes for impedimetric detection of miRNA-34a," *Microchemical Journal*, vol. 148, pp. 748-758, DOI: 10.1016/j.microc.2019.05.040, 2019.
- [75] S. Jafari, F. Faridbod, H. Haji-Hashemi, "An electrochemical genosensor for a fragment of apolipoprotein e gene based on reduced graphene and cerium oxide nanocomposite," *Analytical & Bioanalytical Electrochemistry*, vol. 11, pp. 1625-1637, 2019.
- [76] Y. Liu, G. He, H. Liu, H. Yin, F. Gao, J. Chen, S. Zhang, B. Yang, "Electrochemical immunosensor based on AuBP@pt nanostructure and AuPD-PDA nanozyme for ultrasensitive detection of APOE4," *RSC Advances*, vol. 10 no. 13, pp. 7912-7917, DOI: 10.1039/d0ra00298d, 2020.
- [77] K. Islam, S. Damiani, J. Sethi, A. Suhail, G. Pan, "Development of a label-free immunosensor for clusterin detection as an Alzheimer's biomarker," *Sensors*, vol. 18 no. 1, DOI: 10.3390/s18010308, 2018.
- [78] S. Tonello, M. Serpelloni, N. F. Lopomo, G. Abate, D. L. Uberti, E. Sardini, "Screen-printed biosensors for the early detection of biomarkers related to Alzheimer disease: preliminary results," *Procedia Engineering*, vol. 168, pp. 147-150, DOI: 10.1016/j.proeng.2016.11.182, 2016.
- [79] A. Iglesias-Mayor, O. Amor-Gutiérrez, A. Novelli, M.-T. Fernández-Sánchez, A. Costa-García, A. de la Escosura-Muñiz, "Bifunctional Au@Pt/Au core@shell nanoparticles as novel electrocatalytic tags in immunosensing: application for Alzheimer's disease biomarker detection," *Analytical Chemistry*, vol. 92 no. 10, pp. 7209-7217, DOI: 10.1021/acs.analchem.0c00760, 2020.
- [80] H. P. Karki, Y. Jang, J. Jung, J. Oh, "Correction to: advances in the development paradigm of biosample-based

- biosensors for early ultrasensitive detection of Alzheimer's disease," *Journal of Nanobiotechnology*, vol. 19 no. 1, DOI: 10.1186/s12951-021-00850-3, 2021.
- [81] E. Mikula, "Recent advancements in electrochemical biosensors for Alzheimer's disease biomarkers detection," *Current Medicinal Chemistry*, vol. 28 no. 20, 2021.
- [82] D. Gopalan, A. Pandey, A. T. Alex, G. Kalthur, S. Pandey, N. Udupa, S. Mutalik, "Nanoconstructs as a versatile tool for detection and diagnosis of Alzheimer biomarkers," *Nanotechnology*, vol. 32 no. 14, DOI: 10.1088/1361-6528/abdcdb, 2021.
- [83] E. L. Sciuto, S. Petralia, J. R. Meer, S. Conoci, "Miniaturized electrochemical biosensor based on whole-cell for heavy metal ions detection in water," *Biotechnology and Bioengineering*, vol. 118 no. 4, pp. 1456-1465, DOI: 10.1002/bit.27646, 2021.
- [84] L. Cui, J. Wu, J. Li, Y. Ge, H. Ju, "Electrochemical detection of Cu 2+ through ag nanoparticle assembly regulated by copper-catalyzed oxidation of cysteamine," *Biosensors and Bioelectronics*, vol. 55, pp. 272-277, DOI: 10.1016/j.bios.2013.11.081, 2014.
- [85] A. Kamal, N. Sharma, V. Bhalla, M. Kumar, R. K. Mahajan, "Electrochemical sensing of iron (III) by using rhodamine dimer as an electroactive material," *Talanta*, vol. 128, pp. 422-427, DOI: 10.1016/j.talanta.2014.04.034, 2014.
- [86] F. Kveton, A. Blsakova, L. Lorencova, M. Jerigova, D. Velic, O. Blixt, B. Jansson, P. Kasak, J. Tkac, "A graphene-based glycan biosensor for electrochemical label-free detection of a tumor-associated antibody," *Sensors*, vol. 19 no. 24, DOI: 10.3390/s19245409, 2019.
- [87] O. M. Popa, V. C. Diclescu, "Electrochemical oxidation mechanism of phosphotyrosine at a glassy carbon electrode," *Journal of Electroanalytical Chemistry*, vol. 689, pp. 216-222, DOI: 10.1016/j.jelechem.2012.11.005, 2013.
- [88] N. Bhalla, M. Di Lorenzo, G. Pula, P. Estrela, "Protein phosphorylation detection using dual-mode field-effect devices and nanoplasmonic sensors," *Scientific Reports*, vol. 5 no. 1, DOI: 10.1038/srep08687, 2015.

DETAILS

Subject:	Electrodes; Aging; Public health; Neurotransmitters; Dementia; Sensors; Biomarkers; Diagnosis; Biosensors; Voltammetry; Older people; Cognitive ability; Medical imaging; Enzymes; Proteins; Electric currents; Alzheimer's disease
Editor:	Kevin Honeychurch
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry

ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-16 (Received); 2021-08-26 (Accepted); 2021-09-02 (Pub)
DOI:	https://doi.org/10.1155/2021/9984876
ProQuest document ID:	2571755871
Document URL:	https://www.proquest.com/scholarly-journals/novel-trends-electrochemical-biosensors-early/docview/2571755871/se-2?accountid=211160
Copyright:	Copyright © 2021 Pavla Valkova and Miroslav Pohanka. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2023-10-11
Database:	Publicly Available Content Database

Document 19 of 59

Chemical and Antimicrobial Analyses of *Juniperus chinensis* and *Juniperus seravschanica* Essential Oils and Comparison with Their Methanolic Crude Extracts

Khamis, Al-Dhafri S¹ ; Chai, Lay Ching²

¹ Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; Department Supervisor, Department of Biology College of Science, Sultan Qaboos University, Muscat 123, PO Box 36, Oman ² Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Juniperus chinensis and *Juniperus seravschanica* are commonly used in the traditional folk medicine to treat microbial infection. In this study, the essential oils obtained from the leaves of *J. chinensis* growing in Malaysia and *J. seravschanica* growing in Oman were analysed by head space-solid phase microextraction-gas chromatography mass spectrometry (HS-SPME-GC-MS) and screened for antimicrobial activities against *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC 10662), *Bacillus subtilis* ATCC6059, *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (NCTC 6571), and methicillin-resistant *S. aureus* (MRSA; ATCC 33591). To compare the antimicrobial activities of extracts using different extraction methods, methanol extraction was performed to obtain crude extracts from the leaves of *J. chinensis* and *J. seravschanica* for antimicrobial analysis. The HS-SPME-GS-MS analysis of the essential oils from the leaves of *J. chinensis* and *J. seravschanica* identified 37 and 36 components, respectively. Essential oils from these two species had distinctive chemical component profiles, with α -pinene (27.2%) as the major component of *J. chinensis* essential oil, while dl-limonene (45.2%) constitutes the major component of *J. seravschanica* essential oil. Essential oils of these two species shared only six similar terpenoids compounds: α -pinene, β -pinene, γ -elemene, sabinene, elemol, and 3-cyclohexen-1-ol. Overall, the essential oils showed antimicrobial activities against all the six bacterial strains tested, with the highest antagonistic activity against *M. luteus* and *B. cereus*; while, methanolic crude extracts showed the highest activities against *S. aureus* and MRSA strains. The methanolic crude extracts demonstrated significantly higher antibacterial activity against the Gram-positive bacteria ($p < 0.005$); while, the essential oils of *Juniperus* did not show significant differences between Gram-positive and Gram-negative bacteria. Future studies are needed to investigate the active compounds present in the essential oils and methanolic crude extracts that confer the selectivity in the antimicrobial activity.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Asia has a large number of medicinal plants that have been used traditionally as folk medicine. In fact, of the 70,000 species of medicinal plants used in traditional medicine globally, a huge proportion are actually originated from Asia. Of these Asian traditional plants, *Juniperus* tree is one of the most popular and extensively studied medicinal plants. *Juniperus* belongs to the Cupressaceae family consisting of approximately 70 species that are widely distributed throughout the Northern Hemisphere. Some of the most popular species includes *J. chinensis*, *J. excelsa*, and *J. communis*. Research studies of the various species of *Juniperus* tree revealed a wide range of unique medicinal properties such as antimicrobial [1], anticancer [2] antioxidant [3], antitumor [4], antidiabetic [5], antimicrobial [6], and many other therapeutic properties [7].

Juniperus seravschanica, which is also commonly known as Pashtun juniper, is widely distributed in the central Asia from the southern Kazakhstan through Kyrgyzstan, Tajikistan, eastern Uzbekistan, and Turkmenistan to northern and eastern Afghanistan, northern Pakistan, and Kashmir, as well as in the mountains of southeastern Iran and the Al Hajar Mountains of Oman [8]. The Pashtun juniper is used by the Himalayans to treat a range of diseases including abdominal cramps, diarrhoea, asthma, headache, gonorrhoeae, and leucorrhoea [9]. The Lebanese and Turkish use *J. seravschanica* to treat asthma, cough, common cold, bronchitis, pneumonia, throat inflammation and tuberculosis, urinary tract inflammations, rheumatism, and to remove renal and gall bladder stones [9].

On the other hand, *J. chinensis*, which is also known as the Chinese juniper, is commonly found in many regions in the east and southeast Asia including China, Taiwan, Myanmar, Japan, Korea, and Malaysia [10]. The stems of *J. chinensis* is used in the Chinese medicine to treat parasitic skin problems and rheumatism; the fruit is used in the treatment of convulsions, excessive sweating, and hepatitis; and the root is used to treat burns and scalds [11]. This study aims to determine and compare the chemical compositions of the essential oils obtained from the leaves of the Omani *J. seravschanica* and the Malaysian *J. chinensis*. The antimicrobial activity of the essential oils and

methanolic crude extracts from the leaves of the Omani *J. seravschanica* and the Malaysian *J. chinensis* were also determined.

2. Materials and Methods

2.1. Plant Materials

Two types of plant species were examined in this study, namely, *Juniperus seravschanica* and *Juniperus chinensis*. *J. seravschanica* was from Oman, while *J. chinensis* was from Malaysia.

The leaves of *J. seravschanica* were collected from the tree grown in the Jabel Akhdar Mountains of northern Oman). This plant was previously thought to belong to the Persian juniper population (*J. excelsa* subsp. *polycarpus*) but has been recently confirmed as the *Zeravshan juniper J. seravschanica* [8]. It is one of the dominant species native to the montane woodlands of Oman [12]. The specimen was authenticated at the Herbarium of the Plant Unit, Biology Department, Sultan Qaboos University, Oman, with voucher code Al-Dhafri, Kh. s.n.

The leaves of *Juniperus chinensis* were collected from the trees grown in the University of Malaya campus located in Kuala Lumpur, Malaysia. The leaves of *J. chinensis* are needle shaped when young and scaly when mature [13]. For this particular plant specimen used in this study, the leaves were spiny and had a maximum diameter of 7.5 cm. The identity of the plant was confirmed by a botanist, Dr. M. Sugumaran, of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. A voucher of the specimen was deposited at the University of Malaya Herbarium with the code number KLU047364.

2.2. Essential Oils Extraction

Essential oil was extracted from the leaves of *J. chinensis* and *J. seravschanica* via using the modified steam distillation method developed by Masango [14]. In brief, 500 g of leaves sample was placed in the distillation flask that was connected to the steam generator and a condenser to collect the essential oil. After that, the water was heated to 100°C to produce steam to volatilize the essential oils from the leave samples. The volatilization process was conducted for about 5 hours. Then, the mixture was collected and separated using a separatory funnel, in which the essential oils that settled at the bottom layer of the separatory funnel were collected in a clean bottle for analysis.

2.3. Preparation of Methanol Extracts

Leaves of the respective plants (*J. chinensis* and *J. seravschanica*) were washed with sterile water and then sun-dried for 48 h. The sun-dried leaves were then grinded to fine powder with a stainless-steel blender. After that, each powdered sample (30 g) was macerated in 500 mL of 80% methanol (Sigma-Aldrich, St. Louis, MO, USA) for 3 days at 4°C to extract the phytochemicals presence in the leaf samples [3]. The liquid extract was subsequently filtered and concentrated using a rotary vacuum evaporator (BUCHI Rotavapor, Switzerland), at 40°C. The resulting semisolid extracts which had a yield of about 15% (w/w) was then freeze-dried with a freeze dryer (Flexi-Dry MP, FTS Systems, China) into the dry fine-powdered sample, which was subsequently dissolved in sterile distilled water to an extract stock solution at a concentration of 32 mg/mL. The extract stock solutions were kept at -80°C until tested.

2.4. Head Space-Solid Phase Microextraction-Gas Chromatography Mass Spectrometry Analysis

The essential oils extracted from *J. seravschanica* and *J. chinensis* were analysed using the head space-solid phase microextraction-gas chromatography mass spectrometry (HS-SPME-GC-MS) method. The solid phase microextraction fibre was exposed to the headspace of the extracted essential oils, and the sample fibre was then inserted to the injection port of a Shimadzu gas chromatograph model GC-MS-QP/5050A, equipped with a quadrupole mass spectrometer and a J & W Scientific DB-5MS (5% phenyl/95% dimethylpolysiloxane) fused silica capillary column (30 m × 0.25 mm I.D. × 0.25 mm film thickness). The oven temperature was programmed to increase from 51°C to 251°C at 3°C/min. Injector and interface temperatures were kept at 275°C and 300°C, respectively. Helium was used as the carrier gas with a linear velocity of 44.6 cm/s, column flow rate of 1.5 mL/min, and total flow rate of 36 mL/min. The split ratio was 1:21. Mass spectra were continuously recorded over the mass range 35–501 amu. The MS operating parameters were as follows: ionization voltage 70 eV and scan rate 500 amu/s. The mass spectra obtained were compared to those recorded in the computer MS library (Wiley 229,000 database). The percentage composition was determined by using the single area percentage method without considering

corrections for response factors.

2.5. Antimicrobial Assay

Bacteria strains used in this study were revived from glycerol stock cultures maintained in the laboratory. The Gram-positive bacteria used were *Bacillus subtilis* (ATCC6059), *Staphylococcus aureus* (NCTC 6571), *Micrococcus luteus* (ATCC 9341), and methicillin-resistant *S. aureus* (ATCC 33591); while, the Gram-negative bacteria used were *Pseudomonas aeruginosa* (NCTC 10662) and *Escherichia coli* (NCTC 10418). The bacteria were subsequently subcultured on nutrient agar (BDH Media, London, United Kingdom), and the purity of each strain was determined prior to the antibacterial assay.

The antimicrobial activities of the methanolic extracts of *J. seravschanica* and *J. chinensis* and the essential oil of *J. seravschanica* and *J. chinensis* were evaluated against the six strains of bacteria. The bacterial inoculums were prepared from overnight broth culture in physiological saline (0.8% of NaCl) to reach the turbidity equivalent to 0.5 McFarland standard ($\sim 10^8$ cfu/mL) and further diluted to 10^6 cfu/mL. Muller–Hinton agar (MH agar; from Sigma-Aldrich, USA) were poured in Petri dishes, solidified, and surface dried before inoculation. The extracts were dissolved in deionized water to obtain a final concentration of 10 mg/mL, and the suspension was filter-sterilized using the 0.22 μ m membrane filter. Sterilized 6 mm filter paper discs were impregnated with 10 μ L of the respective extracts and were placed on inoculated MH agar plates that had been inoculated with the test bacteria. All the plates were incubated at 37°C for 24 h and were examined for zones of growth inhibition. The diameters of the zones of inhibition obtained after incubation were measured in millilitres for all the test organisms. The tests were performed in triplicates. Tetracycline 30 μ g (Oxoid Ltd, Ontario, USA) and filter paper disc soak in sterile distilled water were used as the positive and negative controls in all experiment, respectively.

2.6. Statistical Analysis

Descriptive statistical analysis of the antibacterial data and comparison of significant differences among the plant extracts and bacterial strains were conducted using IBM SPSS Statistics version 26 (IBM, New York, USA).

3. Results and Discussion

3.1. Chemical Compositions of *J. seravschanica* and *J. chinensis* Essential Oils

The HS-SPME-GCMS analysis of the essential oils extracted from the leaves of *J. seravschanica* and *J. chinensis* contained 37 and 36 volatiles components, respectively (Table 1). Essential oils of *J. seravschanica* and *J. chinensis* had very different chemical components make up. In total, 67 chemical compounds were detected in the essential oils of both *Juniperus* species. Most of these compounds have previously been reported to present in the essential oils of the leaves of the *Juniperus* plant from Iran. The chemical composition of the *Juniperus* leaf essential oils could be classified into five different classes of components: monoterpenes (MT), monoterpenoids (MTO), sesquiterpenes (ST), sesquiterpenoids (STO), and other, nonterpene components (NT) (Table 1). Diterpene was not detected in these essential oils.

Table 1

Chemical composition of the essential oils extracted from the leaves of *J. chinensis* and *J. seravschanica* analysed using the HS-SPME-GCMS approach.

No.	Components	Formula	CAS	Class of components ^a	<i>J. chinensis</i>		<i>J. seravschanica</i>	
Retention index	Contents ^b (%)	Retention index	Contents ^b (%)	1	Alpha-pinene	C ₁₀ H ₁₆	80-56-8	MT
932	27.2	939.53	33.12	2	Sabinene	C ₁₀ H ₁₆	3387-41-5	MT

948	15.2	974.9	0.05	3	Isolongifolan-8-ol	$C_{15}H_{26}O$	1139-08-8	ST O
1593	3.4	1547.49	0.15	4	Beta-pinene	$C_{10}H_{16}$	127-91-3	MT
948	1.1	979.92	0.96	5	Gamma-elemene	$C_{15}H_{24}$	29873-99-2	ST
948	0.4	1306.7	0.18	6	3-Cyclohexen-1-ol	$C_{12}H_{20}O_2$	9/4/4821	NT
1327	0.32	9.43	0.08	7	Alpha-thujene	$C_{10}H_{16}$	2/5/2867	MT
928.34	18.6	—	—	8	Beta-myrcene	$C_{10}H_{16}$	123-35-3	MT
993.82	17.82	—	—	9	Cis-caryophyllene	$C_{15}H_{24}$	6753-98-6	ST
1418.43	3.9	—	—	10	3-Carene	$C_{10}H_{16}$	554-61-0	MT
1014.74	1.8	—	—	11	Amyl ethyl ketone	$C_8H_{16}O$	106-68-3	NT
939.53	1.6	—	—	12	(-)-Beta-elemene	$C_{15}H_{24}$	33880-83-0	ST
897	0.8	—	—	13	4-Ethylbenzoic acid, cyclopentyl ester	$C_{14}H_{18}O$	7779-65-9	NT

1114.87	0.4	—	—	14	Cis-3-hexenol leaf alcohol	$C_6H_{12}O$	928-96-1	NT
1114.87	0.4	—	—	15	Cis-4-hexen-1-ol	$C_6H_{12}O$	821-41-0	NT
1573.08	0.33	—	—	16	4-Methoxy-2-methylbut-1-ene	$C_6H_{12}O$	34752-58-4	NT
1127.31	0.32	—	—	17	Amyl vinyl carbinol	$C_8H_{16}O$	106-68-3	NT
1114.87	0.32	—	—	18	Alpha-humulene	$C_{15}H_{24}$	6753-98-6	ST
1452.52	0.27	—	—	19	Guaiol	$C_{15}H_{26}O$	489-86-1	NT
1082	0.2	—	—	20	1-Octen-3-ol	$C_{10}H_{20}O$	18479-51-1	NT
1652.21	0.2	—	—	21	3-Cyclopenten-1-one	C_6H_8O	4096-34-8	NT
1652.21	0.2	—	—	22	Hemellitol	C_9H_{12}	526-73-8	NT
1650	0.18	—	—	23	Beta-eudesmol	$C_{15}H_{26}O$	473-15-4	ST O
1650	0.16	—	—	24	Cyclopropane	$C_{10}H_{16}$	68998-21-0	MT

928	0.15	—	—	25	Safrole	$C_{10}H_{16}O$	1989 4-97- 4	MT O
1191	0.13	—	—	26	Alpha- phelland rene	$C_{10}H_{16}$	99- 83-2	MT
1018.49	0.11	—	—	27	Bicyclo[3.1.0]he x-2-ene	$C_{10}H_{16}$	5/2/2 867	NT
924.09	0.1	—	—	28	2- Amino- 5- methylb enzoic acid	$H_2NC_6H_3(C_3H_3)CO_2H$	2941 -78-8	NT
1286.23	0.1	—	—	29	3- Octen- 2-ol	$C_8H_{16}O$	7664 9-14- 4	NT
1092	0.1	—	—	30	Alpha,4- Dimethy lstyrene	$C_{10}H_{12}$	1195 -32-0	MT O
1073	0.1	—	—	31	Bicyclo[2.2.1]he ptan-7- one	$C_{10}H_{16}O$	464- 48-2	MT O
1139.69	0.1	—	—	32	Bicyclo[4.1.0]he pt-2-ene	$C_{10}H_{16}$	554- 61-0	MT
948	0.1	—	—	33	Cis-3- hexen- 1-ol	$C_{12}H_{20}O_2$	9/4/4 821	NT
1127.31	0.1	—	—	34	Cyclofe nchene	$C_{10}H_{16}$	488- 97-1	MT
729	0.1	—	—	35	Cyclohe xane	$C_{12}H_{18}$	7474 2-35- 1	NT

1148	0.1	—	—	36	Methyl- d3-1- dideuteri o-2- propenyl ether	$C_4H_5D_3O$	2936 6-08- 3	NT
855.37	0.1	—	—	37	Origane ne	$C_{10}H_{16}$	2/5/2 867	MT
902	0.1	—	—	38	DL- Limonen e	$C_{10}H_{16}$		MT
—		1037.28	45.21	39	Myrcene	$C_{10}H_{16}$	123- 35-3	MT
—		993.82	8.3	40	(+)-3- Carene	$C_{10}H_{16}$	1346 6-78- 9	MT
—		1014.74	2.81	41	Cis- calamen ene	$C_{15}H_{22}$	7293 7-55- 4	ST O
—		1306.7	0.94	42	Beta- caryoph yllene	$C_{15}H_{24}$	87- 44-5	ST
—		1418.43	0.9	43	Alpha- copaene	$C_{15}H_{24}$	3856 -25-5	ST
—		993.82	0.49	44	Bornyle ne	$C_{10}H_{16}$	464- 17-5	MT
—		908.64	0.36	45	Delta- cadinen e	$C_{15}H_{24}$	483- 76-1	ST
—		1512.77	0.34	46	Caryoph yllene oxide	$C_{15}H_{24}O$	1139 -30-6	ST O
—		1579	0.33	47	Alpha- muurole ne	$C_{15}H_{24}$	1983 -22-9	ST

—		974.9	0.3	48	Beta- elemene	$C_{15}H_{24}$	515- 13-9	ST
—		1306.7	0.3	49	Alpha- terpinen e	$C_{10}H_{16}$	99- 86-5	MT
—		1018.49	0.25	50	Alpha- terpineol	$C_{10}H_{18}O$	2012 6-76- 5	MT O
—		1640.05	0.25	51	Alpha- fench e	$C_{10}H_{16}$	471- 84-1	MT
—		950.57	0.23	52	Germac rene B	$C_{15}H_{24}$	2838 7-44- 2	ST
—		1547.59	0.23	53	2,4(10)- Thujadie ne	$C_{10}H_{14}$	3626 2-09- 6	MT O
—		928.34	0.2	54	Alpha- camphol ene aldehyd e	$C_{10}H_{16}O$	515- 00-4	MT O
—		1513.45	0.2	55	Alpha- humulen e	$C_{15}H_{24}$	6753 -98-6	ST
—		948.64	0.2	56	Camphe ne	$C_{10}H_{16}$	79- 92-5	MT
—		950.57	0.2	57	Beta- bourbon ene	$C_{15}H_{24}$	5208 -59-3	ST
—		908.64	0.16	58	Carvone	$C_{10}H_{14}O$	2244 -16-8	OM
—		1244.05	0.15	59	Trans- pinocarv eol	$C_{10}H_{16}O$	547- 61-5	MT O

—		1139.69	0.15	60	Cis-(+)-carveol	$C_{10}H_{16}O$	2102-59-2	MT O
—		1219.54	0.14	61	Beta-phellandrene	$C_{15}H_{24}$	5432-4-03-7	ST
—		1547.59	0.13	62	Gamma-cadinene	$C_{15}H_{24}$	483-74-9	ST
—		1522.53	0.11	63	Terpinolene	$C_{10}H_{16}$	586-62-9	MT
—		1091.32	0.11	64	Beta-cubebene	$C_{15}H_{24}$	1769-9-14-8	ST
—		1308.8	0.1	65	Bornyl acetate	$C_{12}H_{20}O_2$	76-49-3	NT
—		1286.23	0.1	66	Germacrene-D	$C_{15}H_{24}$	3783-9-63-7	ST
—		1573.08	0.1	67	Tricyclene	$C_{10}H_{16}$	508-32-7	MT

^a Monoterpenes (MT), monoterpenoids (MTO), sesquiterpenes (ST), sesquiterpenoids (STO), diterpenes (DT) and other nonterpene components (NT). ^b The relative peak area normalization content obtained from the chromatogram. Out of the 67 components detected in the essential oils from both of the *Juniperus* species, only 6 components, all terpenoids, were found to be present in both species. These 6 components were α -pinene, β -pinene, γ -elemene, sabinene, elemol, and 3-cyclohexen-1-ol (Table 1). The findings in this study were generally in agreement with the findings reported by others that α -pinene, β -pinene, sabinene, myrcene, dl-limonene, and bornyl acetate were the major components of *Juniperus* volatile oils [15]. Sela and coworkers [16] revealed two chemotypes of essential oils extracted from the berries and leaf of wild Greek Junipers (*J. excelsa* M.Bieb. (Cupressaceae) growing in the Republic of Macedonia: the α -pinene-type (also rich in limonene, β -pinene, and β -myrcene) and the sabinene-type (also contained α -pinene, β -myrcene, limonene, cis-thujone, terpinolene, and α -thujene). The α -pinene-type essential oil showed a moderate antibacterial activity against *S. aureus*; while, the sabinene-type essential oils were inhibitive against *E. coli*, demonstrating variable antimicrobial capacity [16] Chemical analysis conducted in this study revealed that the major chemical components in the essential oil of *J. chinensis* were α -pinene (27.2%), sabinene (15.2%), α -thujene (18.6%), and β -myrcene (17.9%); while, the essential oil of *J. seravschanica* was rich in α -pinene (33.1%) and dl-limonene (45.2%) (Table 1). The essential oils extracted from the leaf of these two species were most abundant in α -pinene, but only *J. chinensis* essential oil contains also sabinene. Both essential oils demonstrated comparable antimicrobial activity as given in Table 1.

3.2. Selective Inhibition of *J. seravschanica* and *J. chinensis* Essential Oils and Methanolic Crude Extracts against

Gram-Positive and Gram-Negative Bacteria

Overall, the methanolic extracts of *J. seravschanica* showed the strongest antibacterial activity against the six bacterial strains tested (zone of inhibition = 14.3 ± 3.0 mm) compared with the methanolic extracts of *J. chinensis* (zone of inhibition = 12.6 ± 2.0 mm) and *F. bruguieri* (zone of inhibition = 12.4 ± 1.7 mm). In comparison, the essential oils of both *J. seravschanica* (zone of inhibition = 12.0 ± 2.5 mm) and *J. chinensis* (zone of inhibition = 11.9 ± 2.4 mm) demonstrated a weaker antibacterial activity based on the disc diffusion method (Table 2).

Table 2

Antibacterial activity of the *J. seravschanica* and *J. chinensis* essential oils and methanolic crude extracts (100 μ g) of *J. seravschanica* and *J. chinensis* measured using the disc diffusion method against six bacterial strains. Each cell with different alphabet subscribes across each row was significantly different ($p < 0.05$) based on Tukey HSD post hoc analysis.

Bacteria strain	Essential oil of		Methanolic crude extracts of		Positive control (tetracycline 30 μ g)
<i>J. seravschanica</i>	<i>J. seravschanica</i>	<i>J. seravschanica</i>	<i>J. chinensis</i>	<i>Escherichia coli</i> NCTC 10418	8.1 ± 0.1^a
9.8 ± 0.2^b	10.9 ± 0.1^c	12.5 ± 0.5^d	15 ± 0.3	<i>Pseudomonas aeruginosa</i> NCTC 10662	13.2 ± 0^a
10.7 ± 0.3^b	10.5 ± 0.5^b	10.7 ± 0.6^b	15 ± 0.6	<i>Bacillus subtilis</i> ATCC 6059	12.4 ± 0.2^{ab}
12.6 ± 0.1^b	15.8 ± 0.3^d	13.8 ± 0.3^c	16 ± 0.4	<i>Micrococcus luteus</i> ATCC 10240	15.4 ± 0.2^c

16.5±0.2 ^d	13.5±0.5 ^b	9.8±0.3 ^a	18±0.2	<i>Staphylococcus aureus</i> NCTC 6571	13.2±0.2 ^a
12.2±0.1 ^a	17.3±0.6 ^c	15.3±0.6 ^b	17±0.6	Methicillin-resistant <i>S. aureus</i> ATCC 33591	9.9±0.1 ^a

It was noteworthy to point out that the methanolic extracts were found to have significantly higher antibacterial activity against the Gram-positive bacteria compared with the Gram-negative bacteria ($p < 0.005$); while, the essential oils of *Juniperus* did not show significant differences between Gram-positive and Gram-negative bacteria (Figure 1). A number of studies have also reported that plant extracts from *Juniperus* leaf [17] and fruit [18] could have a higher bactericidal activity against Gram-positive bacteria [17, 18]. Although another study from Oman that assess the antimicrobial activity of leaf extracts of Omani *Juniperus* excels reported only moderate antimicrobial activity of the extracts, reexamination of the data published indicates that the extracts seem to demonstrate a better activity against Gram-positive bacteria [19]. Similar scenario was also observed in extracts from other types of plants. Based on the study conducted by Rumolo and coworkers [20], *Aleurites moluccanus* (bark), *Curcuma mangga* (rhizome), and *Woodfordia floribunda* (leaf) extracts were inhibitive only against the Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) tested.

[figure omitted; refer to PDF]

The observed lesser inhibitory effect against the Gram-negative bacteria was not surprising, as in general, these bacteria are more resistant than Gram-positive ones [21]. These differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative bacterial cell wall is a multilayered structure and quite complex [22], with an outer membrane consisting of a hydrophilic surface rich in lipopolysaccharide molecules [23]. In addition, the periplasmic space contains enzymes that could degrade any exogenous molecules and prevent the entry of inhibitors, including antibiotic molecules [24]. Gram-positive bacteria do not possess this type of outer membrane and cell wall structure. Therefore, antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and produce a leakage of the cytoplasm and its coagulation [22].

3.3. Antibacterial Activity of *Juniperus* Methanolic Crude Extracts against Methicillin-Resistant *Staphylococcus aureus*

Methanolic crude extract of *J. seravschanica* demonstrated better antibacterial activity against MRSA compared with the methanolic extracts of *J. chinensis* (Table 2). Methanolic crude extract of *J. seravschanica* showed high antibacterial activity against both methicillin-sensitive *S. aureus* (MSSA) and MRSA, with their inhibition zone more or less similar (Table 2). On the other hand, the methanolic extracts of *J. chinensis* and essential oils of *J. seravschanica* and *J. chinensis* showed a decreased activity against MRSA when compared with the MSSA strain tested (Table 1).

Staphylococcus aureus has been identified as one of the major pathogens causing community-acquired and nosocomial infections [25]. It could cause a wide range of diseases in human, including skin infections, sepsis, osteomyelitis, pneumonia, and even death. *Staphylococcus aureus* is an opportunistic pathogen that presents in the nose and skin of about 30% and 20% of healthy adults, respectively [26]. MRSA is *S. aureus* strain that has acquired multidrug resistance to beta-lactam antibiotic that is commonly used to treat bacterial infection [27].

Therefore, due to its resistance to the broad-spectrum beta-lactam antibiotics, MRSA infections are difficult to treat and pose a higher public health risk. As such, the increased effort has been focusing on searching for an alternative treatment for MRSA infection. Numerous studies have evaluated the antibacterial activity of plant compounds against MRSA. Research by Martz and coworkers [28] on the antibacterial activity of *Juniperus communis* methanolic extracts showed a satisfactory antibacterial activity against both MSSA and MRSA. Nitta and coworkers [29] evaluated the efficacy of plant extracts from 181 species of tropical and subtropical plants in inactivation of MRSA and found that the *Shorea hemsleyana* bark and *Cyphostemma bainesii* root extracts that contained stilbene derivatives demonstrated an excellent anti-MRSA activity. Other than that, essential oils of *Eucalyptus globulus*, *Thymus vulgaris*, *Clerodendrum serratum*, *Terminalia arjuna*, and *Coridothymus capitatus* showed good capability to inhibit growth of MRSA [7–11].

The better antibacterial activity of the methanolic leaf extract of *J. seravschanica* against MRSA as observed in this study requires more in-depth investigation to identify the active compounds that contribute to its inhibitory actions against MRSA.

4. Conclusions

In conclusion, *J. seravschanica* from Oman and *J. chinensis* from Malaysia, *Juniperus* trees of similar genus but different species as well as from different geographical locations yielded essential oils with variable chemical compositions but exert minimal differences in its antimicrobial activities. Overall, the *Juniperus* essential oils demonstrated antimicrobial activities against all the six Gram-positive and Gram-negative bacteria tested. On the other hand, different extraction methods yielded extracts with different antimicrobial capabilities. While the essential oils showed minimal selectivity in its antimicrobial activities against Gram-positive and Gram-negative bacteria, the methanolic crude extracts showed higher inhibitory activities against Gram-positive bacteria tested in this study. Also, the methanolic extract of *J. seravschanica* leaves showed a potential anti-MRSA capability. It is evident from this study that despite variation in the chemical compositions, both the *J. chinensis* and *J. seravschanica* essential oils demonstrated broad-spectrum antimicrobial activities against both Gram-positive and Gram-negative bacteria. Nevertheless, further studies are required to reveal the mechanisms of actions of the *Juniperus* essential oils against the bacteria.

Disclosure

The findings presented in this study are part of the PhD project of Mr. Al-Dhafri Khamis S. This study was conducted at both the University of Malaya, Malaysia, and Sultan Qaboos University, Oman.

Authors' Contributions

Dr. Chai Lay Ching helped in data analysis and the writing up of this study.

Acknowledgments

Special appreciation is dedicated to Dr. Koshy Phillips who served as the PhD supervisor of Mr. Al-Dhafri Khamis S. during the experimental design and data collection period of this study.

References

- [1] J. Asili, S. A. Emami, M. Rahimizadeh, B. S. Fazly-Bazzaz, M. K. Hassanzadeh, "Chemical and antimicrobial studies of *Juniperus excelsa* subsp. *excelsa* and *Juniperus excelsa* subsp. *polycarpus* essential Oils," *Journal of Essential Oil Bearing Plants*, vol. 11 no. 3, pp. 292-302, DOI: 10.1080/0972060x.2008.10643633, 2008.
- [2] M. Darvishi, S. Esmaeili, N. Dehghan-Nayeri, P. Mashati, A. Gharehbaghian, "Anticancer effect and enhancement of therapeutic potential of Vincristine by extract from aerial parts of *Juniperus excelsa* on pre-B acute lymphoblastic leukemia cell lines," *Journal of Applied Biomedicine*, vol. 15 no. 3, pp. 219-226, DOI: 10.1016/j.jab.2017.04.002, 2017.
- [3] M. F. Taviano, A. Marino, A. Trovato, "Antioxidant and antimicrobial activities of branches extracts of five *Juniperus* species from Turkey," *Pharmaceutical Biology*, vol. 49 no. 10, pp. 1014-1022, DOI: 10.3109/13880209.2011.560161, 2011.
- [4] A. Salaritabar, B. Darvishi, F. Hadjiakhoondi, "Therapeutic potential of flavonoids in inflammatory bowel disease: a comprehensive review," *World Journal of Gastroenterology*, vol. 23 no. 28, DOI: 10.3748/wjg.v23.i28.5097, 2017.

- [5] H. Keskes, K. Mnafigui, K. Hamden, M. Damak, A. El Feki, N. Allouche, "In vitro anti-diabetic, anti-obesity and antioxidant proprieties of *Juniperus phoenicea* L. leaves from Tunisia," *Asian Pacific journal of tropical biomedicine*, vol. 4, pp. S649-S655, DOI: 10.12980/apjtb.4.201414b114, 2014.
- [6] S. Bano, A. Intisar, M. Rauf, "Comparative analysis of oil composition and antibacterial activity of aerial parts of *Terminalia arjuna* (Roxb.)," *Natural Product Research*, vol. 34 no. 9, pp. 1311-1314, DOI: 10.1080/14786419.2018.1557656, 2020.
- [7] S. Bais, N. S. Gill, R. Nitán, S. Shandeep, "A phytopharmacological review on a medicinal plant: *Juniperus communis*," *International Scholarly Research Notices*, vol. 2014, DOI: 10.1155/2014/634723, 2014.
- [8] R. P. Adams, A. Al-Farsi, A. E. Schwarzbach, "Confirmation of the southern-most population of *Juniperus seravschanica* in Oman by DNA sequencing of nrDNA and four cpDNA regions," *Phytologia*, vol. 96 no. 3, pp. 218-224, 2014.
- [9] K. Zahara, Y. Bibi, Q. Abdul, S. Ahmad, M. Abdul, "An Insight into therapeutic Potential of *Juniperus excelsa* M. Bieb," *Zeitschrift fur Arznei- & Gewurzpflanzen*, vol. 22 no. 2, pp. 93-96, 2017.
- [10] M. Ramdani, T. Lograda, H. Silini, "Antibacterial activity of essential oils of *Juniperus phoenicea* from Eastern Algeria," *Journal of Applied Pharmaceutical Science*, vol. 3 no. 11, 2013.
- [11] J. A. Duke, E. S. Ayensu, *Medicinal Plants of China*, 1985.
- [12] C. A. MacLaren, "Climate change drives decline of *Juniperus seravschanica* in Oman," *Journal of Arid Environments*, vol. 128, pp. 91-100, DOI: 10.1016/j.jaridenv.2016.02.001, 2016.
- [13] J. H. Kim, H. Lee, A. W. Jatoti, "Juniperus chinensis extracts loaded PVA nanofiber: Enhanced antibacterial activity," *Materials Letters*, vol. 181, pp. 367-370, DOI: 10.1016/j.matlet.2016.05.164, 2016.
- [14] P. Masango, "Cleaner production of essential oils by steam distillation," *Journal of Cleaner Production*, vol. 13 no. 8, pp. 833-839, DOI: 10.1016/j.jclepro.2004.02.039, 2005.
- [15] S. Afsharypour, M. Rahiminezhad, L. Ghaemmaghani, M. S. Soleimani, M. Khanmohammadic, N. Afsharipour, "Essential oil constituents of leaves of the male and female shrubs of *Juniperus chinensis* L. from Isfahan," *Iranian Journal of Pharmaceutical Sciences*, vol. 3 no. 3, pp. 177-180, 2007.
- [16] F. Sela, M. Karapandzova, G. Stefkov, I. Cvetkovikj, S. Kulevanova, "Chemical composition and antimicrobial activity of essential oils of *Juniperus excelsa* Bieb. (Cupressaceae) grown in R. Macedonia," *Pharmacognosy Research*, vol. 7, pp. 74-80, DOI: 10.4103/0974-8490.147212, 2015.
- [17] M. Manel, H. Nouzha, M. Rim, "Antibacterial and antioxidant activity of *Juniperus thurifera* L. leaf extracts growing in East of Algeria," *Veterinary World*, vol. 11 no. 3, pp. 373-378, DOI: 10.14202/vetworld.2018.373-378, 2018.
- [18] N. Miceli, A. Marino, A. Köroğlu, "Comparative study of the phenolic profile, antioxidant and antimicrobial activities of leaf extracts of five *Juniperus* L. (Cupressaceae) taxa growing in Turkey," *Natural Product Research*, vol. 34 no. 11, pp. 1636-1641, DOI: 10.1080/14786419.2018.1523162, 2020.
- [19] A. M. Weli, J. M. A. Al-Mjrafi, J. R. S. Alnaaimi, "Effect of different polarities leaves crude extracts of Omani *Juniperus excelsa* on antioxidant, antimicrobial and cytotoxic activities and their biochemical screening," *Asian Pacific Journal of Reproduction*, vol. 3 no. 3, pp. 218-223, DOI: 10.1016/s2305-0500(14)60029-4, 2014.
- [20] A. Romulo, E. A. M. Zuhud, J. Rondevaldova, L. Kokoska, "Screening of in vitro antimicrobial activity of plants used in traditional Indonesian medicine," *Pharmaceutical Biology*, vol. 56 no. 1, pp. 287-293, DOI: 10.1080/13880209.2018.1462834, 2018.
- [21] A. C. Kudi, J. U. Umoh, L. O. Eduvie, J. Gefu, "Screening of some Nigerian medicinal plants for antibacterial activity," *Journal of Ethnopharmacology*, vol. 67 no. 2, pp. 225-228, DOI: 10.1016/s0378-8741(98)00214-1, 1999.
- [22] R. S. Gupta, "Origin of diderm (Gram-negative) bacteria: antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes," *Antonie van Leeuwenhoek*, vol. 100 no. 2, pp. 171-182, DOI: 10.1007/s10482-011-9616-8, 2011.
- [23] Z. Gao, M. Petreikov, E. Zamski, A. A. Schaffer, "Carbohydrate metabolism during early fruit development of sweet melon (*Cucumis melo*)," *Physiologia Plantarum*, vol. 106 no. 1, DOI: 10.1034/j.1399-3054.1999.106101.x,

1999.

- [24] C. F. Duffy, R. F. Power, "Antioxidant and antimicrobial properties of some Chinese plant extracts," International Journal of Antimicrobial Agents, vol. 17 no. 6, pp. 527-529, DOI: 10.1016/s0924-8579(01)00326-0, 2001.
- [25] S. S. Magill, E. O'Leary, S. M. Ray, "Antimicrobial use in US hospitals: comparison of results from emerging infections program prevalence surveys, 2015 and 2011," Clinical Infectious Diseases, vol. 72, 2020.
- [26] H. Chambers, "The changing epidemiology of Staphylococcus aureus ?," Emerging Infectious Diseases, vol. 7 no. 2, pp. 178-182, DOI: 10.3201/eid0702.010204, 2001.
- [27] K. S. Gurusamy, R. Koti, C. D. Toon, P. Wilson, B. R. Davidson, "Antibiotic therapy for the treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections in surgical wounds," Cochrane Database of Systematic Reviews, vol. 8, 2013.
- [28] F. Martz, R. Peltola, S. Fontanay, R. E. Duval, R. Julkunen-Tiitto, S. Stark, "Effect of latitude and altitude on the terpenoid and soluble phenolic composition of juniper (Juniperus communis) needles and evaluation of their antibacterial activity in the boreal zone," Journal of Agricultural and Food Chemistry, vol. 57 no. 20, pp. 9575-9584, DOI: 10.1021/jf902423k, 2009.
- [29] T. Nitta, T. Arai, H. Takamatsu, "Antibacterial activity of extracts prepared from tropical and subtropical plants on methicillin-resistant Staphylococcus aureus," Journal of Health Science, vol. 48 no. 3, pp. 273-276, DOI: 10.1248/jhs.48.273, 2002.

DETAILS

Subject:	Mass spectrometry; Rheumatism; Bacteria; Gram-positive bacteria; Scientific imaging; Pseudomonas aeruginosa; Essential oils; Chromatography; Oils & fats; Solid phases; Selectivity; Statistical analysis; Terpenes; Microorganisms; Asthma; Herbal medicine; Gas chromatography; Antimicrobial agents; E coli
Location:	Iran; Malaysia; Asia; Oman; United States--US; China
Company / organization:	Name: University of Malaya; NAICS: 611310
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760

e-ISSN: 16878779

Source type: Scholarly Journal

Language of publication: English

Document type: Journal Article

Publication history :

Milestone dates: 2021-03-29 (Received); 2021-08-10 (Revised); 2021-08-16 (Accepted); 2021-08-30 (Pub)

DOI: <https://doi.org/10.1155/2021/9937522>

ProQuest document ID: 2571755865

Document URL: <https://www.proquest.com/scholarly-journals/chemical-antimicrobial-analyses-i-juniperus/docview/2571755865/se-2?accountid=211160>

Copyright: Copyright © 2021 Al-Dhafri S. Kha mis and Lay Ching Chai. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. <https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-09-12

Database: Publicly Available Content Database

Document 20 of 59

A Rapid Screening Method for Sibutramine Hydrochloride in Natural Herbal Medicines and Dietary Supplements

Liang, Qi ¹

; Zhuang, Yue ¹ ; Ma, Jun ¹ ; Wang, Jinyan ¹ ; Feng, Rui ² ; He, Ruisi ³ ; Luo, Zhuoya ¹

; Wang, Honghao ⁴ ; Zhan, Ruoting ⁵ ¹ Guangdong Institute for Drug Control, Guangzhou, China ²

Research Center of Chinese Herbal Resource Science and Engineering, Guangzhou University of

Chinese Medicine, Guangzhou, China ³ School of Pharmaceutical Sciences, Sun Yat-Sen University,

Guangzhou, China ⁴ Market Regulation Administration of Guangdong, Guangzhou, China ⁵ School of

Chinese Materia Medica, Ministry of Education, Key Laboratory of Chinese Medicinal Resource from

Lingnan, Guangzhou University of Chinese Medicine, Guangzhou, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Herbal weight loss drugs are becoming more widely used in the fight against obesity, but ineffective regulation of these products have resulted in harmful additives. These products may contain adulterants such as sibutramine hydrochloride that may result in serious adverse health events including death. This work established a color precipitation reaction-based rapid screening method for illegal adulteration of sibutramine hydrochloride in natural herbal medicines (NHM) and dietary supplements (DS). While a variety of chromatography- and electrophoresis-based systems have been reported to measure this analyte, they generally suffer from high costs, complicated sample preparation, and a costly analytical infrastructure. In contrast, we present a simple, handheld kit to assay for sibutramine. The performance metrics of this tool include an average detection time of approximately 3 minutes, which is markedly shorter than conventional methods (HPLC or HPLC-MS, etc.), a detection limit of 0.1 mg per aliquot, and an accuracy of 99.02% ($n=820$). More strikingly, the sensitivity is 100% ($n=278$), and the specificity is 98.52% ($n=542$). The rapid test kit developed from this screening method was evaluated by FDA. In summary, this screening method is a rapid, simple, and low-cost tool for the detection of sibutramine in NHM and DS with superior selectivity and sensitivity. For these reasons, this method is especially suitable for underdeveloped settings because it can be employed onsite without any instrumentation. In addition, this approach could rapidly exclude most of the negative samples to boost efficiency in large-scale samples assay. If necessary, positive samples can undergo further alternate testing methods to confirm the positive results of sibutramine hydrochloride content.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Sibutramine hydrochloride, N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine hydrochloride monohydrate ($C_{17}H_{26}ClN \cdot HCl \cdot H_2O$) is a reuptake inhibitor of noradrenaline and 5-hydroxytryptamine and has been used as an antiobesity drug since 1997 [1]. It has a molecular weight of 334.33 g/mol.

Sibutramine hydrochloride may cause severe adverse effects including insomnia, psychosis, affective psychosis, panic attacks, delirious state, amnesia, bipolar disorder, hypomanic or manic episodes, and increases the risk of stroke and heart attack [2–15]. Consequently, FDA has withdrawn sibutramine hydrochloride from the US market in October 2010 [16].

Recently, illegal adulteration of natural herbal medicine (NHM) and dietary supplements (DS) with sibutramine hydrochloride has been reported and is dangerous for consumers.

To date, numerous detection methods for sibutramine hydrochloride adulteration in NHM and DS for weight loss have been developed including thin-layer chromatography (TLC) [17, 18], gas chromatography mass spectrometry (GC-MS) [19, 20], liquid chromatography (LC) [21, 22], liquid chromatography-mass spectrometry (LC-MS) [21, 23–27], ion exchange chromatography (IEC) [28], capillary electrophoresis (CE) [29], capillary zone electrophoresis (CZE) [30], nuclear magnetic resonance (NMR) [31], ion mobility spectrometry (IMS) [32], infrared spectroscopy (IR) [33], micellar electrokinetic capillary chromatography (MECC) [34], X-ray powder diffraction (XRPD), and colorimetry [35, 36]. However, there are several disadvantages of these methods including long assay time, expensive instrumentation, and the need for skilled operators. As a result, these methods are an obstacle to consistent and cost-effective testing of NHM and DS.

Color tests have been widely used in drug analysis historically. They are simple, inexpensive, and easy to operate. The results of these methods are easily distinguished and commonly used to assist the identification. Without the precise but unwieldy instruments, the color tests can provide a preliminary estimate of the samples at the various scenes. These results are not as accurate as the instrument analysis, but effective enough for on-site inspection [37–40].

Therefore, this work establishes a rapid, simple, colorimetric screening method for sibutramine hydrochloride in

NHM and DS. This method can be developed as a rapid screening kit for onsite analysis of sibutramine hydrochloride in NHM and DS. The method could also be an initial rapid screen prior to instrumental analysis.

2. Materials and Methods

2.1. Instrumentation

Millipore Milli-Q water purification system (Millipore, USA) and LC-20A HPLC system with UV detector (Shimadzu, Japan) were used.

2.2. Reference Standard and Reagents

Sibutramine hydrochloride monohydrate (National Institutes for Food and Drug Control, China; Lot. no. 100624–200401, desiccated for two hours at 105°C before use). Ethyl acetate (AR, Guangzhou Chemical Reagent Co.), phosphoric acid (analytical grade, Guangzhou Chemical Reagent Co.), ammonium reineckate (analytical grade, Shanghai Hengxin Chemical reagent Co., Ltd.), methanol (HPLC grade, Guangzhou Chemical Reagent Co.), triethylamine (analytical grade, Guangzhou Chemical Reagent Co.), acetonitrile (HPLC grade, Merck & Co., Inc., USA), and sodium heptanesulfonate (analytical grade, Shandong Yuwang Industrial Co., Ltd.) were used in this study.

2.3. Samples

Zhuoyue Fengzi Slimming Capsule and Fufang Shoubao Capsule were used, courtesy of Guangdong Institute for Drug Control.

2.4. Preparation of Ammonium Reineckate Detection Reagent

0.1 g of ammonium reineckate is dissolved in 4 ml of purified water and shaken for 1 min. This solution should be freshly prepared and used within 48 hours.

2.5. Rapid Screening Method Procedure

(i) Powdered sample (0.1 to 0.5 g) was placed in a 7 ml sample vial (A). We then added 3.5 ml of ethyl acetate to vial A, inverted it 5 times, and allowed it to stand for 1 min.

(ii) We used a burette to extract 2 ml of the upper-layer liquid (organic phase) of vial A and transferred it to another sample vial (B). We then added 3 ml of 3% H_3PO_4 to vial B. This was inverted 5 times to make a dispersed phase and allowed to stand for 1 min.

(iii) Next, we used a burette to extract 1 ml of the lower-layer liquid (aqueous phase) from vial B and transferred it to a test tube. We then added 1 drop of ammonium reineckate detection reagent. If an obvious light-pink insoluble substance is formed immediately, then the sample is positive for sibutramine. Otherwise, it is negative.

2.6. LC Determination of Sibutramine

An LC-20A HPLC system (Shimadzu, Japan) equipped with an automatic sampler and a UV detector was used for the chromatographic analysis. A KQ-300DE ultrasonic generator was purchased from Kunshan Ultrasonic Instruments Co., Ltd., China.

The chromatographic analysis was carried out in isocratic mode on an ODS-2 Hypersil C_{18} analytical column (250 mm $\times$ 4.6 mm; 5 μm particle size; Thermo Fisher Scientific, USA). The mobile phase in the HPLC method was methanol-acetonitrile-sodium heptanesulfonate solution (10:3:7, v/v/v). Sodium heptanesulfonate solution was prepared as follows: add 1000 ml of water in a beaker, dissolve 2.02 g of sodium heptanesulfonate, and then add 0.8 ml of triethylamine and adjust the pH to 3.3 ± 0.1 with glacial acetic acid. The flow rate was 1.0 ml/min, the detection wavelength was 223 nm, and the injection volume was 20 μl .

10 mg of sibutramine hydrochloride was weighed and dissolved in 50 ml of mobile phase. It was then gradually diluted to 0.2 mg/ml in mobile phase with volumetric pipettes and flasks. Quality control solutions were prepared from independent stock standards at 0.5, 1.0, 2.0, 25, 50, 80, and 100 $\mu\text{g/ml}$ in mobile phase. Each quality control solution was filtered through a Sartorius model 0.45 μm polytetrafluoroethylene (PTFE) filter before application in HPLC system. The calibration curve was linear from 0.536 to 107.2 $\mu\text{g/ml}$ ($Y = 40939X + 2517.6$, $R^2 = 1$).

3. Results and Discussion

3.1. Principle of the Rapid Screening Method

The outermost electron orbital of the nitrogen atom in sibutramine is a SP^3 hybrid. Thus, there are two lone-paired

electrons in the s orbital, which approach the empty orbital of the proton in inorganic acid solutions and form an ammonium cation with proton. As the nitrogen atom combines with high pKa alkyls ($\text{pK}_{\text{a}}_{\text{CH}_4} = 49$), it donates electron part of the positive charge of the ammonium cation that can be dispersed to attain stability. Thus, sibutramine tends to be protonated and well solubilized in acid solution (Figure 1(a)) [41].

[figures omitted; refer to PDF]

Due to the electronegativity of the nitrogen atom in protonated sibutramine, it combines with the nitrogen atom to form a hydrogen bond with water and become solvated (Figure 1(b)). Therefore, sibutramine hydrochloride can easily be dissolved in inorganic acid aqueous solution. On the other hand, as it has a benzene ring and an aliphatic amine structure, sibutramine can also easily dissolve in organic solvents such as methanol, ethanol, *n*-butanol, ethyl acetate, benzene, and dichloromethane.

NHM and DS usually consist of multiple compounds, most of which are colored substances such as phytochromes. Thus, it is necessary to remove these substances before initiating the color reaction for rapid screening because the colored substances may interfere with the colored reaction.

We developed a two-step extraction protocol for sibutramine hydrochloride because of its good solubility in both organic acid solvents and inorganic solution. First, an organic solvent is applied to extract sibutramine hydrochloride from samples, which separates the interfering substances that are insoluble in organic solvents. Second, a liquid-liquid extraction (LLE) is performed with an inorganic acid to further separate sibutramine from the organic solvent that contains the interfering substances.

There are three criteria in selecting the sibutramine hydrochloride extraction buffer: (1) it must dissolve sibutramine hydrochloride; (2) it may not dissolve the inorganic acid solution; and (3) it should be environmentally friendly with no human toxicity. Fortunately, there are numerous organic solvents that meet these criteria including ethyl acetate with good extraction effect and low toxicity.

Both ethyl acetate and sibutramine are electron-donating compounds. They cannot form a hydrogen bond; however, they can attract each other with Van der Waals forces to form weak interactions. Thus, ethyl acetate can easily dissolve and extract sibutramine because they have similar polarities. In addition, ethyl acetate can exclude insoluble substances that may potentially interfere with sibutramine assay.

More importantly, LLE can protonate, solvate, and extract sibutramine and also exclude most competing substances that are insoluble in acid aqueous medium and may interfere with the test results. While many acids could be used, we selected H_3PO_4 because of its noncorrodibility, nonvolatility, and convenience.

The sibutramine is protonated on contact with inorganic acid. The polarity of the protonated sibutramine increases, so the difference in polarities between the protonated sibutramine and the ethyl acetate increases, and thus their affinity decreases. Protonated sibutramine then forms hydrogen bonds with water and is solvated and then moves into the inorganic phase from the organic phase.

Sibutramine accepts a proton to form a quaternary ammonium salt (R^+), and the detection agent should precipitate with the quaternary ammonium salt. For this, we chose ammonium reineckate, which is soluble in water and produces a cation of NH_4^+ and an anion of $[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]^-$.

The cation of R^+ reacts with the anion of $[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]^-$ to form an organic salt that is insoluble in acidic aqueous medium. The organic salt is pink, and the precipitate is easily visualized. The ion reaction formula is described in the following equation: $(1) \text{R}^+ + [\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]^- \rightarrow \text{RCr}(\text{NH}_3)_2(\text{SCN})_4 \downarrow$

3.2. Optimization of the Method

The main influencing factors of the method were the property and amount of the extraction liquids. To select ethyl acetate and phosphoric acid solution as the extraction liquid has been briefly explained in the above. Based on these results, further optimization of the method was undertaken as follows.

3.2.1. Concentration of Phosphoric Acid Solution

The high efficiency of the extraction step is important because the violent shaking, repeated steps, and long layering time are not always allowed in the rapid screening methods to enhance the performance. This study used dilute phosphoric acid solution and measured the partition coefficient of sibutramine hydrochloride in ethyl acetate and

diluted phosphoric acid to optimize the condition.

The partition coefficient was estimated by the following equation: $(2)K = C_A/C_B$.

Here, both C_A and C_B are the concentrations of the test substance in two immiscible solvents while the distribution system becomes balanced.

71.025mg of sibutramine hydrochloride standard was weighed and transferred into a 200ml volumetric flask. Then, ethyl acetate was added to dissolve the sample and diluted to the volume. Next, 10.0ml of the solvent was transferred into a 60ml separation funnel. This was repeated for 11 more samples. Each held 10.0ml of diluted phosphoric acid at 0.5%, 1.0%, 3.0%, and 5.0%, respectively. The flask was sealed, shaken, and drained. This was repeated and allowed to stand for layering. We next determined the content of the sibutramine hydrochloride in this two-phase solution with HPLC.

We removed 0.5ml from the supernatant and the lower layer and placed them in two 10ml volumetric flasks, respectively. After adding mobile phase to each, we mixed the samples, filtered, and analysed them both with HPLC (Table 1). As shown in Table 1, the concentration variation of the diluted phosphate solution had little effect on the partition coefficients. The coefficients increased slightly with the concentration rising and reached the maximum value when the concentration of the diluted phosphate solution was around 3.0%. Therefore, a 3.0% diluted phosphate solution was chosen as the extraction solution.

Table 1

The results of partition coefficients.

System*	Sample	Peak area	C^A or C^B ($\mu\text{g/ml}$)	Partition coefficients K	K (avg.)	R.S.D. % ($n=3$)
1	B	13958213	340.89	36.62	36.72	1.31
A	383660	9.31	B	13936925	340.37	36.29
A	386525	9.38	B	14138345	345.29	37.24
A	382022	9.27	-			
2	B	13961488	340.97	42.00	40.37	3.51
A	334942	8.12	B	14056876	343.30	39.64
A	357049	8.66	B	14024944	342.52	39.47
A	357868	8.68	-			
3	B	14275491	348.64	45.18	46.70	3.18
A	318567	7.72	B	14247243	347.95	48.14
A	298507	7.23	B	14171506	346.10	46.77
A	305466	7.40	-			

4	B	14118285	344.80	46.67	47.04	2.78
A	305057	7.39	B	14198116	346.75	48.50
A	295231	7.15	B	14093721	344.20	45.96

*System 1: ethyl acetate/0.5% phosphoric acid; 2: ethyl acetate/1.0% phosphoric acid; 3: ethyl acetate/3.0% phosphoric acid; 4: ethyl acetate/5.0% phosphoric acid; ^AC: the organic phase; and ^BC: the aqueous phase.

3.2.2. Volume of the Extraction Liquid

An orthogonal experiment was performed to optimize the volume of extraction liquids in each step. The concentration of the sample used in this experiment was 0.86 mg/g, which was determined by HPLC at first. The experiment operated by the following steps:

(i) Powdered sample (0.15g) was placed in a 10ml sample vial (A). Then, V_1 ml ethyl acetate was added to vial A, inverted it 5 times, and allowed it to stand for 1 min. Determine the concentration of sibutramine hydrochloride in ethyl acetate ($C_1 \mu\text{g/ml}$) by HPLC.

(ii) V_2 ml of the upper-layer liquid of vial A was transferred into another sample vial (B). V_3 ml 3% H_3PO_4 was added to vial B, inverted 5 times to make a dispersed phase and allowed to stand for 1 min. By the way, the amount of V_3 was determined according to a series of fixed ratio of the two phases ($V_2:V_3$). Determine the concentration of sibutramine hydrochloride in phosphoric acid solution ($C_2 \mu\text{g/ml}$) by HPLC.

As shown in Table 2, the extraction rates were about 40% when 2ml ethyl acetate was used. And the rates could be increased to about 80% with 3.5ml ethyl acetate. Furthermore, double the volume, the rates were increased about only 6 percent, but the concentration of the solution was nearly halved. In the LLE step, the rates were more than 90% in different conditions. It suggests that almost all the sibutramine was extracted into the phosphate acid solution. Considering the final step of the test, high concentration of the solution is favorable for the formation of the precipitate. Thus, the phosphate acid solution should be used as less as possible. However, there is a practical reason, the volume of the liquid used in rapid screening is better to be more than 2ml for easy operation and observation. Finally, 3.5ml ethyl acetate and 3ml phosphate acid solution was considered as the optimal condition.

Table 2

The analysis results of the orthogonal experiment.

No.	V_1 (ml)	C_1 ($\mu\text{g/ml}$)	Extraction rate (%)	V_2 (ml)	$V_2:V_3$	V_3 (ml)	C_2 ($\mu\text{g/ml}$)	LLE rate (%)	Transfer efficiency (%)
1	7	16.59	87.74	4.0	1:3	12.0	5.36	99.03	86.89
2	2:3	6.0	10.63	94.94	83.30	3	4:3	3.0	18.88
91.2 7	80.08	-							
4	3.5	30.91	81.67	2.0	1:3	6.0	9.97	95.80	78.24
5	2:3	3.0	20.23	104.6 4	85.46	6	4:3	1.5	41.00

103.17	84.26	-							
7	2.0	29.84	43.30	1.0	1:3	3.0	17.84	179.33	77.65
8	2:3	1.5	23.85	119.90	51.92	9	4:3	0.75	48.85

C: the concentration of sibutramine hydrochloride in the detection solution. LLE rate: the extraction rate of liquid-liquid extraction step. Transfer efficiency: the efficiency of the sibutramine transfer from the sample to the test liquid.

3.2.3. Effects of Temperature

The effect of temperature on chromic reaction and LLE rate was studied by carrying out the rapid screening method under 10°C, 15°C, 20°C, and 25°C (Table 3). At 10°C, no visible pink precipitates are formed within 30s. At 15°C or higher temperature, pink precipitates are formed within 30s. The LLE rates at 10°C, 15°C, 20°C, and 25°C are 99.39%, 99.38%, 99.32%, and 99.23% respectively.

Table 3

Effect of temperature on colorimetric reaction.

Temperature (°C)	Phenomena
10	No obvious pink insoluble substance forms within 30s
15	Pink insoluble substance forms within 30s
20	Pink insoluble substance forms within 30s
25	Pink insoluble substance forms within 30s

3.3. Transfer Efficiency of the Method

By investigating the extraction rate and LLE rate, we demonstrate the transfer efficiency of sibutramine as well as overall feasibility. Extraction rate and LLE rate were determined by HPLC quantification of sibutramine in each extraction step.

The content powder removed from 20 Zhuoyue Fengzi Slimming Capsules was homogenously mixed and weighed. The mean mass was 0.285g (R.S.D.=2.76%, $n=20$). We analytically weighed 0.1g and added 50ml of the mobile phase for extraction accompanied with 10min of treatment in an ultrasonic bath. The supernatant of the extraction solution was filtered through a 0.45 μ m PTFE filter before being applied to the HPLC system. The results include that average content of sample is 32.21 mg/g and RSD is 2.58% ($n=6$); more details are shown in Table 4.

Table 4

The results of determination of the samples.

Sample weight (g)	Peak area A	C^1 (μ g/ml)	X^2 (mg/g)	Average (mg/g)	R.S.D. % ($n=6$)
0.10685	2828469	69.03	32.30	32.21	2.58
0.10835	2848987	69.53	32.09	0.10627	2737640

66.81	31.43	0.10711	2748151	67.07	31.31
0.10810	2884583	70.40	32.56	0.10192	2805760

¹C: the concentration of sibutramine hydrochloride in the detection solution, same as below. ²X: the content of sibutramine hydrochloride in the test specimen.

0.1 g of sample was transferred to a 10ml specimen bottle followed by 8.0ml of ethyl acetate and inversion 5 times. This was incubated at room temperature for 1 min. We next transferred 3.0ml of the supernatant to another bottle for the LLE step (solution A). Here, we measured another 2.0ml of the supernatant and transferred it to a 25ml volumetric flask. Next, mobile phase was added to dilute to the mark followed by shaking. This was filtered and analyzed with HPLC. The results include that average extraction rate is 78.28% and RSD is 4.33% ($n=6$); more details are shown in Table 5.

Table 5

Results of the extraction.

Sample weight (g)	Peak area A	C ($\mu\text{g/ml}$)	Extraction rate (%)	Average (%)	R.S.D. % ($n=6$)
0.10281	1045088	25.47	76.90	78.28	4.33
0.10362	1031081	25.12	75.28	0.10281	1067915
26.02	78.59	0.10288	1042179	25.40	76.64
0.10428	1169179	28.50	84.84	0.10126	1036531

Procedure. Solution A was transferred to a 10ml bottle and mixed with 3.0ml of 3% phosphoric acid. The sample was inverted 5 times. We moved 1.0ml of the lower layer into a 10ml volumetric flask. Mobile phase was added to the mark and then mixed well. We filtered and measured the filtrate with HPLC. This was repeated for five more replicates and results in that average liquid-liquid extraction rate is 59.45% and RSD is 11.01% ($n=6$); more details are shown in Table 6.

Table 6

Results of liquid-liquid extaction rate.

Content in solution A (mg)	Peak area A	C ($\mu\text{g/ml}$)	LLE rate (%)	Average (%)	R.S.D. % ($n=6$)
0.9550	622675	15.15	47.59	59.45	11.01
0.9422	799286	19.46	61.97	0.9759	768581
18.71	57.52	0.9523	779353	18.98	59.78
1.0687	968675	23.60	66.25	0.9472	824669

The average transfer efficiency calculated according to the extraction rate and the LLE rate is 46.62% and RSD is 13.63% ($n=6$); more details are demonstrated in Table 7. It was important to only gently shake the bottle to maintain easy operation, rapid results, and high reproducibility. We also used a shortened laying time. The recommended

dosage of sibutramine hydrochloride (10mg per dose), the content of the determined samples (1.90mg to 67.02mg per capsule), and the transfer efficiency (above 30%) give acceptable precision and high detection sensitivity (0.025 mg/ml, equal to 0.112mg per aliquot, see section LOD). This rapid screening method can determine whether sibutramine hydrochloride is added to NHM or DS products used for weight loss.

Table 7

Calculation of transfer efficiency. The transfer efficiency is the product of the extraction rate and the LLE rate.

No.	Extraction rate (%)	LLE rate (%)	Transfer efficiency (%)	Average (%)	R.S.D. % (n=6)
1	76.90	47.59	36.59	46.62	13.63
2	75.28	61.97	46.65	3	78.59
57.52	45.21	4	76.64	59.78	45.81
5	84.84	66.25	56.21	6	77.44

3.4. Method Validation and Application

The purpose of this rapid screening method is to screen the samples for illegal addition of target analytes within about 3 minutes at the site of inspection. At the same time, dealing with large quantities of samples at low cost was also expected, this method generally consumed about a tenth the cost of conventional analysis methods. One out of it is to quickly screen out whether the sample is a positive one, and the positive rates of such samples are usually about 0~30% according to the data known. For negative results, no further instrumental analysis is required for confirmatory testing; for positive screening results, a conventional analytical method is used to test the sample to confirm the rapid screening results and accurately detect the content of the target component. The conventional analytical methods mentioned above are high-performance liquid chromatography with reference substances. The screening method has been validated according to Chinese Pharmacopoeia (Version 2010) 9101. These metrics include transfer efficiency, limit of detection (LOD), accuracy, sensitivity, and specificity via HPLC and comparison with the official methods approved by China Food and Drug Administration (CFDA) [42, 43].

3.4.1. LOD

We dissolved sibutramine hydrochloride in ethyl acetate and diluted it with ethyl acetate to obtain a series of concentrations of sibutramine hydrochloride (Table 8). Half of one Fufang Shoubao Capsule was transferred to 3.5 ml of one of the sibutramine hydrochloride standard solutions to a bottle. The Fufang Shoubao product was also tested with an official method and found to be negative for sibutramine and thus was considered to be an interference. Testing was performed as described in the rapid screening method procedure section, and the results are presented in Table 6. When the extraction rate (Table 5) is considered, the LOD in Table 6 will be adjusted based on equation (2). The result is 0.112mg of sibutramine per aliquot of sample (Table 9): $(3)LOD_{adjusted}=LOD \times 3.578.28\%$.

Table 8

The determination of detection limit.

Sample	Concentration of sibutramin hydrochloride (mg/ml)	Precipitate formed
1	0	No
2	0.008	No

3	0.010	No
4	0.012	Not sure
5	0.025	Yes
6	0.050	Yes
7	0.100	Yes

When testing sibutramine within a dietary supplement, it was determined that 0.025 mg/ml sibutramine hydrochloride was the lowest visually detectable limit.

Table 9

The determination of adjusted detection limit.

Sample	Content of sibutramin hydrochloride (mg per aliquot*)	Precipitate formed
1	0	No
2	0.036	No
3	0.045	No
4	0.054	Not sure
5	0.112	Yes
6	0.224	Yes
7	0.447	Yes

According to the results listed in Table 9, the LOD is 0.112 mg of sibutramine hydrochloride per aliquot for this method. The method is simple and rational. Aliquot: a certain amount of sample may vary from 0.1 to 0.5 g.

3.4.2. Method Applications and Average Accuracy, Sensitivity, Specificity, and Selectivity

The rapid screening results can be true positive, false positive, true negative, or false negative versus the official methods approved by CFDA.

Accuracy, sensitivity, and specificity are the important parameters that characterized the precision of this method.

These three parameters were calculated by the following equations, respectively: (4) accuracy = $\frac{S - SFS}{S} \times 100\%$, (5) sensitivity = $\frac{P - NFP}{P} \times 100\%$, (6) specificity = $\frac{N - PFN}{N} \times 100\%$.

Here, S is the total number of test specimens, P_T is the number of the true positive specimens, N_T is the number of the true negative specimens, N_F is the number of the false negative specimens, P_F is the number of the false positive specimens, S_F is the summation of P_F and N_F , P is the summation of P_T and N_F , and N is the summation of N_T and P_F .

Based on laboratory, civic, and provincial applications by different experimental level operators, the performance of the method is valuated below.

In our laboratory, we studied 60 batches of proprietary Chinese medicines and health foods that claimed an

antiobesity function. These were purchased from three different cities. We compared the rapid screening results to the CFDA-approved method; the findings were completely consistent except for one false positive result (lab test results in Table 10).

Table 10

The primary technical parameters of this method.

	P_T	N_T	P_F	N_F	Accuracy (%)	Sensitivity (%)	Specificity (%)
Lab test (S=60)	36	23	1	0	98.33	100	95.83
Small-scale (S=47)	34	13	0	0	100	100	100
Full-scale (S=713)	208	498	7	0	99.02	100	98.61
Total (S=820)	278	534	8	0	99.02	100	98.52

With the assistance of two civic institutes of drug control, 47 batches of proprietary Chinese medicines and health food sold for weight loss were purchased and examined by technicians at the two institutes. The results via the rapid screening method concurred with the CFDA-approved methods (small-scale results in Table 10).

In August 2008, the Guangdong province FDA launched a joint operation on antiobesity health food supervision in which 238 dealers were sampled, and 713 batches of weight loss DS were tested by the local FDA inspectors in the province who have a little experiment training. Two hundred and sixty-six brands of antiobesity health foods sold. Compared with the CFDA-approved method, only 7 out of the 713 samples were false positive by the rapid method (full-scale results in Table 10).

In this method, the target compound undergoes protonation, LLE, and a precipitation reaction (Figure 2). Each step offers selectivity and excludes interference substances. Therefore, the method produces high-accuracy results as shown above.

[figure omitted; refer to PDF]

3.5. Verification and Evaluation from FDA

We requested the FDA division of pharmaceutical analysis to evaluate the rapid test kit in 2011. They prepared standards to estimate the detection limit of this test by spiking dietary supplements with sibutramine. They found that they could visually identify spiked dietary supplements with as little as 0.1 mg of sibutramine. That is significant because a typical dose of sibutramine is 5mg, so this detection limit is 50 times lower than the expected level of sibutramine in adulterated dietary supplements. They concluded that the rapid test kits are extremely useful to screen dietary supplements for the presence of sibutramine.

4. Conclusions

This study describes a new and easy on-site inspection method with high specificity, fast assay time, and low cost. This tool is likely to be used widely by local Food and Drug Administration personnel. The assay may overcome the shortcomings of current technology and solve the difficulties encountered in the supervision and administration of food and drugs to ensure public health safety.

This study uses a fast and novel colorimetric reaction. The methodology, choice of the reagent and test solution, evaluation model, LOD values, and verification steps described here are novel and may be useful to other researchers in the field.

While this rapid screening test is simple and easy to use, it does require a skilled operator and could not be used by common consumers without training. Thus, we are working on an improved version that can be employed by unskilled operators.

Disclosure

The abstract was presented in "The 8th Global Summit on Regulatory Science Conference" (GSRS2018).

Acknowledgments

This work was supported by the Bureau of Science and Information Technology of Guangzhou Municipal Government (2014J4100175), the Guangdong Medical Produces Administration (2018JDZ01), and Guangdong Quality and Efficacy Consistency Evaluation of Generic Drugs Research Platform (2017B020221002).

References

- [1] C. Gundlah, K. F. Martin, D. J. Heal, S. B. Auerbach, "In vivo criteria to differentiate monoamine reuptake inhibitors from releasing agents: sibutramine is a reuptake inhibitor," *Journal of Pharmacology and Experimental Therapeutics*, vol. 283 no. 2, pp. 581-591, 1997.
- [2] F. Benazzi, "Organic hypomania secondary to sibutramine-citalopram interaction," *Journal of Clinical Psychiatry*, vol. 63 no. 2, DOI: 10.4088/jcp.v63n0213a, 2002.
- [3] K. Binkle, S. R. Knowles, "Sibutramine and panic attacks," *American Journal of Psychiatry*, vol. 159 no. 10, pp. 1793-1794, DOI: 10.1176/appi.ajp.159.10.1793-a, 2002.
- [4] Q. Cordeiro, H. Vallada, "Sibutramine-induced mania episode in a bipolar patient," *The International Journal of Neuropsychopharmacology*, vol. 5 no. 3, pp. 283-284, DOI: 10.1017/S1461145702003000, 2002.
- [5] T. Sayin, M. Güldal, "Sibutramine: possible cause of a reversible cardiomyopathy," *International Journal of Cardiology*, vol. 99 no. 3, pp. 481-482, DOI: 10.1016/j.ijcard.2003.11.060, 2005.
- [6] P. Fernández, A. M. Peiró, "A sibutramine-induced delusional disorder relapse," *Journal of Neuropsychiatry and Clinical Neurosciences*, vol. 19 no. 1, pp. 88-89, DOI: 10.1176/jnp.2007.19.1.88, 2007.
- [7] R. S. Padwal, S. R. Majumdar, "Drug treatments for obesity: orlistat, sibutramine, and rimonabant," *Lancet*, vol. 369 no. 9555, pp. 71-77, DOI: 10.1016/s0140-6736(07)60033-6, 2007.
- [8] F. F. Da Rocha, T. O. Bamberg, F. C. C. Pinto, L. M. Guimarães e Gomes, S. Silveira, "Hypomanic episode secondary to sibutramine in a patient with type-I bipolar disorder," *Revista Brasileira De Psiquiatria*, vol. 30 no. 4, pp. 400-401, DOI: 10.1590/s1516-44462008000400018, 2009.
- [9] B. Dogangun, N. Bolat, I. Rustamov, L. Kayaalp, "Sibutramine-induced psychotic episode in an adolescent," *Journal of Psychosomatic Research*, vol. 65 no. 5, pp. 505-506, DOI: 10.1016/j.jpsychores.2008.09.013, 2008.
- [10] M. Florentin, E. N. Liberopoulos, M. S. Elisaf, "Sibutramine-associated adverse effects: a practical guide for its safe use," *Obesity Reviews*, vol. 9 no. 4, pp. 378-387, DOI: 10.1111/j.1467-789x.2007.00425.x, 2008.
- [11] J. Lee, T. Teoh, T.-S. Lee, "Catatonia and psychosis associated with sibutramine: a case report and pathophysiologic correlation," *Journal of Psychosomatic Research*, vol. 64 no. 1, pp. 107-109, DOI: 10.1016/j.jpsychores.2007.05.014, 2008.
- [12] P. K. Fu, H. Y. Hsu, P. Y. Wang, "Transient global amnesia after taking sibutramine: a case report," *The Neurologist*, vol. 16 no. 16, pp. 129-131, DOI: 10.1097/NRL.0b013e3181c6bf1b, 2010.
- [13] S. Naik, C. L. Khoo, R. Lua, S. B. Chai, A. Liew, K. Sim, "Recurrent episodes of brief affective psychosis induced by sibutramine," *Progress in Neuro Psychopharmacology & Biological Psychiatry*, vol. 34 no. 7, pp. 1359-1360, DOI: 10.1016/j.pnpbp.2010.07.009, 2010.
- [14] P. J. Nathan, B. V. O' Neill, A. Napolitano, "Neuropsychiatric adverse effects of centrally acting antiobesity drugs," *CNS Neuroscience and Therapeutics*, vol. 17 no. 5, pp. 490-505, DOI: 10.1111/j.1755-5949.2010.00172.x, 2011.
- [15] N. Waszkiewicz, B. Zalewska-Szajda, S. D. Szajda, "Sibutramine-induced mania as the first manifestation of bipolar disorder," *BMC Psychiatry*, vol. 12 no. 1, DOI: 10.1186/1471-244X-12-43, 2012.
- [16] [FDA] Food and Drug Administration, FDA Drug Safety Communication: FDA Recommends Against the Continued Use of Meridia (Sibutramine), 2010.
- [17] E. S. Simaremare, R. A. Susilowati, Y. D. Astuti, "Analysis of acetaminophen, mefenamic acid, sibutramine hydrochloride, and sildenafil citrate," *Journal of Applied Pharmaceutical Science*, vol. 8 no. 11, pp. 48-56, DOI: 10.7324/japs.2018.81107, 2018.
- [18] H. Hayun, B. P. Maggadani, N. Amalina, "Determination of sibutramine adulterated in herbal slimming products

- using TLC densitometric method," *Indonesian Journal of Pharmacy*, vol. 27 no. 1, pp. 15-21, DOI: 10.14499/indonesianjpharm27iss1pp15, 2016.
- [19] K. Marjan, H. Mehdi, K. Farzad, "Identification and determination of synthetic pharmaceuticals as adulterants in eight common herbal weight loss supplements," *Iranian Red Crescent Medical Journal*, vol. 16 no. 3, 2014.
- [20] Y. P. Lin, Y. L. Lee, C. Y. Hung, C.-F. Chang, Y. Chen, "Detection of adulterated drugs in traditional Chinese medicine and dietary supplements using hydrogen as a carrier gas," *PLoS One*, vol. 13 no. 10, DOI: 10.1371/journal.pone.0205371, 2018.
- [21] Y. Zhong, C. Sun, J. Xiong, Y. Shi, "Simultaneous determination of eight adulterants in weight management supplements and herbs by HPLC-DAD and LC-MS/MS," *Journal of Liquid Chromatography & Related Technologies*, vol. 40 no. 12, pp. 640-648, DOI: 10.1080/10826076.2017.1343730, 2017.
- [22] Z. Zaharieva, D. Tanev, D. Danalev, "Development and validation of HPLC/DAD method for simultaneously determination of six prohibited substances in model matrices," *Acta Chromatographica*, vol. 32 no. 4, pp. 276-280, DOI: 10.1556/1326.2019.00749, 2020.
- [23] R. Jin, L. Li, L. Guo, W. Li, Q. Shen, "A graphene tip coupled with liquid chromatography tandem mass spectrometry for the determination of four synthetic adulterants in slimming supplements," *Food Chemistry*, vol. 224 no. 1, pp. 329-334, DOI: 10.1016/j.foodchem.2016.12.091, 2017.
- [24] Q. Cheng, L. Shou, C. Chen, S. Shi, M. Zhou, "Application of ultra-high-performance liquid chromatography coupled with LTQ-orbitrap mass spectrometry for identification, confirmation and quantitation of illegal adulterated weight-loss drugs in plant dietary supplements," *Journal of Chromatography B*, vol. 1064, pp. 92-99, DOI: 10.1016/j.jchromb.2017.09.009, 2017.
- [25] P. Paula, M. J. E. Rodrigues, M. Correia, J. S. Amaral, M. B. P. P. Oliveira, C. Deleure-Matos, "Analysis of pharmaceutical adulterants in plant food supplements by UHPLC-MS/MS," *European Journal of Pharmaceutical Sciences*, vol. 99 no. 1, pp. 219-227, DOI: 10.1016/j.ejps.2016.12.024, 2017.
- [26] J. Hong, J. Hur, W. Lee, "Comprehensive screening of multiclass illegal adulterants in herbal supplements and spices using specific MS/MS fragmentations by UHPLC-Q/TOF-MS," *Analytical methods*, vol. 11 no. 41, pp. 5260-5273, 2019.
- [27] L. S. Müller, A. P. L. Moreira, D. T. Muratt, C. Viana, L. M. de Carvalho, "An ultra-high performance liquid chromatography–electrospray tandem mass spectrometric method for screening and simultaneous determination of anorexic, anxiolytic, antidepressant, diuretic, laxative and stimulant drugs in dietary supplements marketed for weight loss," *Journal of Chromatographic Science*, vol. 57 no. 6, pp. 528-540, DOI: 10.1093/chromsci/bmz025, 2019.
- [28] R. D. M. Thaís, D. S. Géssica, G. C. Leal, "A new approach to ion exchange chromatography with conductivity detection for adulterants investigation in dietary supplements," *Biomedical Chromatography*, vol. 33 no. 11, DOI: 10.1002/bmc.4669, 2019.
- [29] L. M. De Carvalho, P. A. Cohen, C. V. Silva, "A new approach to determining pharmacologic adulteration of herbal weight loss products," *Food Additives & Contaminants: Part A*, vol. 29 no. 11, pp. 1661-1667, DOI: 10.1080/19440049.2012.706834, 2012.
- [30] L. M. De Carvalho, M. Martini, A. P. Moreira, S. Garcia, P. Nascimento, D. Bohrer, "Determination of synthetic pharmaceuticals in phytotherapeutics by capillary zone electrophoresis with contactless conductivity detection (CZE-C4D)," *Microchemical Journal*, vol. 96 no. 1, pp. 114-119, DOI: 10.1016/j.microc.2010.02.006, 2010.
- [31] R. Hachem, G. Assemet, N. Martins, "Proton NMR for detection, identification and quantification of adulterants in 160 herbal food supplements marketed for weight loss," *Journal of Pharmaceutical & Biomedical Analysis*, vol. 124, pp. 34-47, DOI: 10.1016/j.jpba.2016.02.022, 2016.
- [32] J. D. Dunn, C. M. Gryniewicz-Ruzicka, D. J. Mans, "Qualitative screening for adulterants in weight-loss supplements by ion mobility spectrometry," *Journal of Pharmaceutical & Biomedical Analysis*, vol. 71, pp. 18-26, DOI: 10.1016/j.jpba.2012.07.020, 2012.
- [33] N. Cebi, M. T. Yilmaz, O. A. Sagdic, "A rapid ATR-FTIR spectroscopic method for detection of sibutramine

adulteration in tea and coffee based on hierarchical cluster and principal component analyses," Food Chemistry, vol. 229 no. 15, pp. 517-526, DOI: 10.1016/j.foodchem.2017.02.072, 2017.

[34] Y. J. Xu, G. P. Xu, "Determination of sibutramine, indapamide, bumetanide and chlorothiazide in mass-reducing tonic by micellar electrokinetic capillary chromatography," Chinese Journal of Pharmaceutical Analysis, vol. 25 no. 12, pp. 1456-1459, 2005.

[35] K. Stypułkowski, A. Błażewicz, J. Maurin, K. Sarna, Z. Fijałek, "X-ray powder diffractometry and liquid chromatography studies of sibutramine and its analogues content in herbal dietary supplements," Journal of Pharmaceutical & Biomedical Analysis, vol. 56 no. 5, pp. 969-975, DOI: 10.1016/j.jpba.2011.08.028, 2011.

[36] K. Chitra, K. Sujatha, R. R. Sanjivkumar, A. Ghosh, K. V. V. Kaushik, "Extractive colorimetric determination of sibutramine from its pharmaceutical preparation," Indian Drugs, vol. 41, pp. 478-481, 2004.

[37] R. A. Velapoldi, S. A. Wicks, "The use of chemical spot kits for the presumptive identification of narcotics and drugs of abuse," Journal of Forensic Sciences, vol. 19 no. 3, pp. 636-656, 1974.

[38] K. Miecznikowski, P. J. Kulesza, M. Ordak, "Color tests and electroanalytical methods for the preliminary identification of drugs," The Electrochemical Society, 2016.

[39] National Institute of Standards and Technology (NIST) United States of America, Color Test Reagents/Kits for Preliminary Identification of Drugs of Abuse, 2000.

[40] C. L. O'Neal, D. J. Crouch, A. A. Fatah, "Validation of twelve chemical spot tests for the detection of drugs of abuse," Forensic Science International, vol. 109 no. 3, pp. 189-201, DOI: 10.1016/s0379-0738(99)00235-2, 2000.

[41] D. Lucero-Borja, O. Castilla, X. Subirats, C. Ràfols, "Solubility-pH profiles of a free base and its salt: sibutramine as a case study," ADMET & DMPK, vol. 5 no. 4, pp. 253-256, DOI: 10.5599/admet.5.4.452, 2017.

[42] National Pharmacopoeia Committee of China, The Pharmacopoeia of the People's Republic of China, 2010.

[43] Z. Xie, Theories and Practices of Rapid Screening Technologies for Adulteration in Health Products, 2011.

DETAILS

Subject:	Acids; Hydrocarbons; Reagents; Hydrogen bonds; Ions; Nuclear magnetic resonance-NMR; Quality control; Additives; Scientific imaging; Dietary supplements; Adulterants; Chromatography; Selectivity; Nitrogen; Mass spectrometry; Performance measurement; Cost analysis; Chemical precipitation; Herbal medicine; Sensitivity; Sodium; Solvents; Psychosis; Electrophoresis; Methods; High performance liquid chromatography; Weight loss; Screening
Business indexing term:	Subject: Quality control
Location:	United States--US; China; Japan
Editor:	Maria Jose Ruiz Angel
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited

Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-06-03 (Received); 2021-04-25 (Revised); 2021-07-31 (Accepted); 2021-08-26 (Pub)
DOI:	https://doi.org/10.1155/2021/8889423
ProQuest document ID:	2569268680
Document URL:	https://www.proquest.com/scholarly-journals/rapid-screening-method-sibutramine-hydrochloride/docview/2569268680/se-2?accountid=211160
Copyright:	Copyright © 2021 Qi Liang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-09-05
Database:	Publicly Available Content Database

Document 21 of 59

Evaluation of Neuraminidase Inhibitory Activity of Compounds and Extracts from Traditional Medicines by HPLC-FLD

Yu, Guangying ¹

; Fang, Dan ² ¹ Department of Traditional Chinese Medicine, Tianjin Hospital, Tianjin 300211, China ² Heping Hospital of No. 983 Hospital of Joint Service Support Force, Tianjin 300020, China

ABSTRACT (ENGLISH)

A simple and effective method was established and validated to determine 4-methylumbelliferone (4-MU) for screening the natural neuraminidase inhibitors (NAIs) from traditional medicines (TMs) by high performance liquid chromatography combined with fluorescence detection (HPLC-FLD). 4-MU and TMs compounds were separated on a Heder TM ODS column (5 μ m, 4.6 $\times$ 250 mm) using an isocratic elution of 55% methanol at 35°C. The flow rate was 1 mL min⁻¹. The excitation and emission wavelength were performed at 320 nm and 480 nm. Some extracts of TMs and compounds were selected as examples to demonstrate the feasibility of the new HPLC-FLD method. It was found that the results of most compounds except for the auto fluorescence substances determined by HPLC-FLD were in good agreement with NA enzyme-based inhibitory assays. Comparing to traditional NA enzyme-based inhibitory assays, the HPLC-FLD method could prevent interference from fluorescence pigments of compounds. It was considered a simple, effective, and economical technique for the screening the natural neuraminidase inhibitors from traditional medicines.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Influenza has become a great public concern in recent years as a major health problem all over the world. The chemically synthesized neuraminidase inhibitors (NAIs) are widely used to prevent or treat influenza as antiviral drugs [1]. Zanamivir and oseltamivir are two common drugs introduced into clinical practice acting as NAIs, but clinical relevance of some mutations remains uncertain [2–4]. Fortunately, traditional medicines (TMs) and their preparations for clearing heat and detoxification have a very good therapeutic effect in the treatment of influenza in China [5, 6]. It was reported that some small molecules were identified as the potential natural neuraminidase inhibitors in *Radix Scutellariae* and Reduning injection (*Flos Lonicerae*, *Herba Artemisiae annuae*, and *Gardenia jasminoides* extract) [7, 8]. Traditional medicine resource is an important source of potential NAIs for antiviral drug screening. Because the chemical composition of TMs is complex, it is extremely necessary to establish a rapid screening method for the new, effective, and natural NAIs from TMs.

NA enzyme-based inhibitory assays are most widely used to evaluate the potential capabilities of compounds or extracts of TMs by fluorescence (FL) spectrophotometry. Recently, one-step high-performance liquid chromatography-fraction collector and time-of-flight mass spectrometry (HPLC-FC/Q-TOF-MS) or ultra performance liquid chromatography coupled with diode-array detectors and autofraction collector/time-of-flight mass spectrometry (UPLC-DAD-FC/QTOF-MS) coupled with bioactive assays were used to screen and identify natural NAIs from TM extracts [7, 8]. The affinity-based ultrafiltration UPLC-QTOF-MS analysis has been developed to identify potent bacterial NAIs from the plant extracts [9]. Among these reported methods, the compounds with autofluorescence such as flavonoids and coumarins in the screened samples may nonlinearly interfere with fluorescence measurements of 4-methylumbelliferone (4-MU) causing false positives or highly evaluated results [10]. To solve these problems, the neuraminidase-immobilized capillary microreactor has been applied to screen natural NAIs from traditional Chinese medicines. Nevertheless, this method has low sensitivity and reproducibility. These aforementioned events raise the need to explore a new method for screening natural NAIs from TMs.

The high-performance liquid chromatography combined with the fluorescence detection (HPLC-FLD) method is one of the commonly used separation methods of TMs and has been used in various operations. There are some reports about HPLC-FLD, including determination of the estrogenic hormones, amino acid, and aflatoxins [11–13]. It is sensitive and specific to avoid the false-positive results in NA enzyme-based inhibitory assays where some compounds are with autofluorescence. Hence, a new method HPLC-FLD was established to enhance advantages

and avoid disadvantages.

The aim of the work was to establish a simple and effective method to determine 4-MU for screening the NAIs from TMs by HPLC-FLD. The HPLC-FLD method was suitable to screen the compounds from complex TMs which have potential capacity to inhibit NA. The interference from the screened compounds was eliminated by separating the components in samples, substrate, and product using HPLC-FLD. The positive drug peramivir was used to validate the assay as a reference inhibitor. Some types of compounds with or without autofluorescence such as coumarins, ridoids, flavonoids, and phenolic acids and four herbal medicines were selected to verify the feasibility of screening natural NAIs from TMs by the HPLC-FLD method. The HPLC-FLD method was highly efficient, more stable, economical, and feasible to screen NAIs compared with the result of the NA enzyme-based inhibitory assays. In addition, the new method established by HPLC-FLD can effectively avoid the false-positive result caused by some compounds with autofluorescence and improve the accuracy of the screening experiments.

2. Materials and Methods

2.1. Chemicals and Reagents

The enzyme NA, the substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (4-MuNeu5Ac), the product 4-MU (Figure 1), and positive drug peramivir were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol (Dikma Technologies Inc, USA) were of HPLC grade. Deionized water was purified by using a Milli-Q academic water purification system (Millipore, Milford, MA, USA). The analytical reagents, sodium dihydrogen phosphate, and sodium hydrogen phosphate were purchased from Tianjin Kermel Science Company. The reference compounds of five ridoids (genipin, geniposide, shanzhiside methyl ester, geniposidic acid, and 8-O-acetylshanzhiside methyl ester), five phenolic acids (chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A, and isochlorogenic acid C), two flavonoids (baicalin and baicalein), and nine coumarins (coumarin, notopteron, xanthotoxin, isoimperatorin, psoralen, angelicin, xanthoxol, bergapten, and osthole) were all purchased from Chengdu Dest Biotechnology Co., Ltd. (Chengdu, China).

[figure omitted; refer to PDF]

2.2. Instrumentation

The HPLC-FLD analysis was performed on an Agilent 1100 HPLC system with a fluorescence detector (FLD). The sample analysis was carried out on a Hadera TM ODS column (4.6×250 mm, 5 μ m) at 35°C with a flow rate of 1.0 mL min⁻¹. The elution program was isocratic 16 min with 45% water and 55% methanol, and the injection volume was 10 μ L. According to the instructions of the Neuraminidase Inhibitors Screen Kit (Beyotime Biotechnology, P0309), the excitation and emission wavelengths of FLD were set at 320 nm and 480 nm, respectively (Figure 2).

[figure omitted; refer to PDF]

2.3. Preparation of Solutions

2.3.1. Preparation of Enzyme, Substrate, and Product Solutions

The buffer solution was prepared by adding a suitable amount of sodium dihydrogen phosphate to a moderate amount of sodium hydrogen phosphate until pH value reached 6.0. Stock solutions of NA and 4-MuNeu5Ac were dissolved in a phosphate buffer solution at the concentration of 25 U mL⁻¹ and 10 μ g mL⁻¹, respectively. Product 4-MU was prepared with methanol. All solutions were stored at -20°C.

2.3.2. Preparation of Reference Compounds

The reference compounds of genipin, geniposide, shanzhiside methyl ester, geniposidic acid, 8-O-acetylshanzhiside methyl ester, chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A, isochlorogenic acid C, baicalin, baicalein, coumarin, notopteron, xanthotoxin, isoimperatorin, imperatorin, psoralen, angelicin, xanthoxol, bergaptenosthole, and peramivir were each prepared by dissolving with 50% methanol giving a stock concentration of 1.0 mg mL⁻¹ and diluted into 8 different concentrations for bioassays and then stored at 4°C.

2.3.3. Preparation of the Samples

Scutellaria baicalensis, *Notopterygium incisum*, *Gardenia jasminoides*, and *Lonicera japonica* were obtained from different markets and authenticated by Guangying Yu (Tianjin hospital). All herbal medicines were grounded into a powder in order to improve the extraction efficiency. *N. incisum* and *G. jasminoides* powder (10.00 g) were added into

a 250mL volumetric flask ultrasonically for 30min and 20min, respectively. *S. abaicalensis* powder (5.00g) was dissolved in 500mL with 70% methanol, and *L. japonica* (5.00g) was prepared with 50% methanol. The two solutions were ultrasonic for 40min and 30min, respectively. Four herbal medicine extracts were combined, condensed, and dried by rotary evaporation. The extraction efficiency of *S. abaicalensis*, *N. incisun*, *G. jasminoides*, and *L. japonica* was 29.4%, 23.03%, 23.74%, and 28.2%, respectively. Then, all samples were stored at 4°C.

2.4. Method Validation

The HPLC-FLD method validation included linearity, limits of detection (LOD), limits of quantification (LOQ), and precision following the USFDA guidelines [14]. The stability and recovery tests were performed according to a previous reference [15]. Results obtained from the HPLC-FLD method were compared with those detected by NA enzyme-based inhibitory assays to demonstrate the feasibility of the novel method.

2.4.1. Linearity, LOD, and LOQ

The calibration curve of 4-MU was constructed by plotting the peak area (y) against the corresponding concentration (x , ng mL⁻¹). The limits of detection (LOD) and limits of quantification (LOQ) values were calculated at a signal-to-noise (S/N) ratio of 3 and 10 by diluting the highest concentration of the mixed solution further to a certain concentration, respectively.

2.4.2. Precision, Stability, and Recovery

The analysis of intra- and interday precision were assessed by six repetitive injections of the standard solution at three different (low, medium, and high) concentrations in the same day and on three consecutive days, respectively. The precision was expressed as relative standard deviations (RSDs). The reference solution at three different concentrations was determined by replicate injection at 2, 4, 6, 8, 12, and 24h to evaluate the stability. The recovery was calculated by comparing the experimental group with the controlled group. The experimental group solutions were prepared by spiking 10 μ L 4-MU at three different (low, medium, and high) concentrations into the centrifugal tubes with 10 μ L of 25U mL⁻¹ NA and 280 μ L phosphate buffer, respectively. The controlled group solutions consisted of 10 μ L 4-MU at three different (low, medium, and high) concentrations and 290 μ L phosphate buffer, respectively. All the solutions were in six replicates. Then, the mixed solutions were analyzed according to the conditions listed above. The recoveries at different concentrations of 4-MU were determined by the formula recovery (%) = (experimental group amount – controlled group amount) $\times$ 100%.

2.5. Bioassay

2.5.1. Determination of Half Maximal Inhibitory Concentration (IC₅₀)

The linear regression equation, expressed as $y = ax + b$, was used to determine the amount of product (4-MU) after the reaction of NA and 4-MuNeu5Ac. The experiments are conducted in 96-well plates. 70 μ L phosphate buffer, different amounts (2, 4, 6, 8, and 10 μ L) of NA at the concentration of 0.25U mL⁻¹, different amounts (10, 8, 6, 4, and 2) of phosphate buffer, and 10 μ L 50% methanol (because all the screened compounds were diluted with 50% methanol) were added to 96-well plates in order to obtain the linear regression equation of NA and product. To screen the compounds which can inhibit NA, 70 μ L phosphate buffer, 10 μ L NA at the concentration of 0.25U mL⁻¹, and 10 μ L of screening compound solutions at eight aliquots of serial suitable dilutions were added to 96-well plates in order. The samples were then mixed for 1 minute and incubated for 2 minutes. The last step was adding 10 μ L 4-MuNeu5Ac to each well at the concentration of 25 μ g mL⁻¹ and shaking for 1 minute. After incubation for 30 minutes, the final fluorescence values were read by using a Flex Station 3 Microplate Reader [6]. All samples were in triplicate. The calculation of IC₅₀ values was performed on the program GraphPad Prism 5.01. After fluorescence detection, 200 μ L methanols were added into each well and the 96-well plates were read by using a microplate reader again after 0, 5, 20, and 30 minutes to prove that the reaction was indeed terminated. Then, all solutions were pipetted into the centrifugal tubes and centrifuged for 10min at 16650g. The supernatant was injected in the HPLC. It is worth noting that calibration curves were carried out in each plate and each well must be repeated three times.

2.5.2. Km Determinations

Neuraminidase (10 μ L), buffer solution (70 μ L), and ultrapure water (10 μ L) were added into 96-well fluorescent

enzyme plates according to the abovementioned methods. Then, a series of concentrations (0–284 μm) of neuraminidase fluorescent substrate (10 μL) were added, vibrated, and mixed again for 1 min. After incubation at 37°C for 30 min, 200 μL methanols were added into each well in the 96-well plates. Then, all solutions were pipetted into the centrifugal tubes and centrifuged for 10 min at 16650g. The supernatant was injected in the HPLC. The K_m value of neuraminidase was calculated by GraphPad prism software.

2.5.3. K_i Determination

Neuraminidase (10 μL) was mixed with a series of concentrations of peramivir (10 μL , 0–457 μm) and buffer solution (70 μL), incubated at 37°C for 2 min, and then, added with a series of concentrations of the neuraminidase fluorescent substrate (10 μL , 0–284 μm). After incubation at 37°C for 30 min, the fluorescence was detected by HPLC-FLD. The K_i value of peramivir was calculated by GraphPad prism software.

2.5.4. Statistical Analysis

All analysis of the bioassay experiments was measured in triplicates. The values were expressed in the form mean $\pm$ standard deviation (SD); IC_{50} values were calculated by the program GraphPad Prism 5.01.

3. Results and Discussion

3.1. Optimization of HPLC Separation Conditions

The substrate including 22 screened compounds and peramivir was separated from the product on a Hadera TM ODS column (4.6 $\times$ 250 mm, 5 μm). The mobile phase composition, different flow rates, and column temperatures were investigated in order to achieve good resolution and symmetrical peak shape in a shorter analysis time. The mobile-phase compositions were prepared by different concentrations of methanol and acetonitrile. The optimum conditions were as follows: isocratic elution with 55% methanol for 16 min. In addition, column temperatures (25, 35, and 40°C) and flow rates (0.8, 1.0, and 1.2 mL min⁻¹) were also investigated. Finally, column temperature and flow rate were optimal at 35°C and 1.0 mL min⁻¹, respectively. The result depicted in Figure 3 suggested that the product was 4-methylumbelliferone. Moreover, the HPLC-FLD method could be applied to screen NAIs due to the decreased fluorescence value of the product after reaction.

[figures omitted; refer to PDF]

3.2. Method Validation

3.2.1. Linearity, LODs, and LOQs

The linear regression equation of 4-methylumbelliferone (product 4-MU) is as follows: $Y = 0.6608x + 0.1247$. The linear range ranged from 0.16 ng mL⁻¹ to 100 ng mL⁻¹. A good linear relationship was obtained with $r^2 = 0.9997$. The LOD and LOQ value of 4-methylumbelliferone were 0.05 ng mL⁻¹ and 0.16 ng mL⁻¹, respectively. These results demonstrated that HPLC-FLD was highly sensitive in the determination of the product 4-methylumbelliferone.

3.2.2. Precision, Stability, and Recovery

The intraday and interday precision at different concentrations (low, medium, and high) are shown in Table 1. Their RSD values were less than 2.66% and 4.64%, respectively. The stability of 4-MU was analyzed within one day at 0, 2, 4, 6, 8, 12, and 24 h, the RSD values ranged from 2.95% to 4.88%, and the range of accuracy was 102% to 105%. The RSD values and recoveries were in the range of 1.38% to 2.58% and 100% to 102%, respectively (Table 2).

Table 1

Intraday and interday accuracy and precision and stability of 4-methylumbelliferone ($n=6$).

Compound	Concentration (ng/mL)	Interday	Intraday	Stability

RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	4-Methylumbelliferone	0.50
2.26	99.8	3.25	102	4.88	102	5.00	1.92
99.6	4.56	102	3.81	105	50.00	1.92	102

Table 2

The recovery of 4-methylumbelliferone ($n=3$).

Compound	Concentration (ng/mL)	Recovery	
RSD (%)	Accuracy (%)	4-Methylumbelliferone	0.50
2.01	102	5.00	1.38
100	50.00	2.58	100

3.3. Bioassay

3.3.1. Optimization of the Proportion of Enzyme and Substrate

Taking different proportions of NA and substrate into account, various concentrations of NA were reacted with a certain concentration of substrate each time. Three concentrations ($10\mu\text{g mL}^{-1}$, $25\mu\text{g mL}^{-1}$, and $50\mu\text{g mL}^{-1}$) of the substrate were investigated to evaluate the effect of different proportions on the fluorescence value and peak area of 4-MU, respectively. Figures 4(a) and 4(d) implied that the fluorescence values and peak areas of 4-MU increased linearly lower than 0.25 U mL^{-1} of NA. However, the trend curve was slowed down after concentration. Thus, the 0.25 U mL^{-1} of NA was chosen the optimum condition reacted with $10\mu\text{g mL}^{-1}$ of 4-MU. When the concentration of the substrate was $25\mu\text{g mL}^{-1}$, the result of optimization was as displayed in Figures 4(b) and 4(e)). The variation tendency tended to flatten out at the concentration of 0.25 U mL^{-1} of NA. $50\mu\text{g mL}^{-1}$ of the substrate was also assayed, and the result is listed in Figures 4(c) and 4(f)). The fluorescence values and peak areas of 4-MU increased significantly with the increase in the concentration lower than 0.5 U mL^{-1} of NA.

[figures omitted; refer to PDF]

When the concentrations of the substrate were $10\mu\text{g mL}^{-1}$, $25\mu\text{g mL}^{-1}$, and $50\mu\text{g mL}^{-1}$, the concentrations of NA were optimum at 0.25 U mL^{-1} , 0.25 U mL^{-1} , and 0.5 U mL^{-1} , respectively. It was found that there is no significant difference between the results determined by NA enzyme-based inhibitory assays and HPLC-FLD. In the experimental process, it is a fact that higher concentrations of substrate and NA result in lower inhibitory potential of the samples at the same concentration. In the contrary, the concentrations of the substrate and NA were lower; the ability of inhibition would be highly evaluated. Finally, the condition of $10\mu\text{g mL}^{-1}$ of the substrate and 0.25 U mL^{-1} NA was achieved to screen the inhibitors from TMs.

3.3.2. Inhibitor Screening

In order to validate the accuracy of the enzyme activity, the positive drug peramivir was selected to test the half-maximal inhibitory concentration (IC_{50}) by both NA enzyme-based inhibitory assays and the HPLC-FLD method. The results showed that the K_m value of neuraminidase was $93.23 \pm 9.03\mu\text{m}$ and the K_i value of peramivir was $47.01 \pm$

2.54 μm . IC₅₀ of peramivir was $290 \pm 30 \mu\text{m}$ by NA enzyme-based inhibitory assays and $270 \pm 40 \mu\text{m}$ by the HPLC-FLD method. It was demonstrated that the method to determine 4-methylumbelliferone (4-MU) by HPLC-FLD could accurately be used to determine the inhibitory activity of compounds on neuraminidase. Twenty-one natural compounds were determined by new methods. It was found that the NA inhibitory activity of seven compounds (genipin, geniposidic acid, 8-O-acetyl shanzhiside methyl ester, neochlorogenic acid, coumarin, notopterol, and xanthotoxin) was lower than 50% at test concentration ($500 \mu\text{g mL}^{-1}$). It was shown that these seven compounds have weak inhibitory activity on neuraminidase. The results in Table 3 displayed the IC₅₀ values of other 14 compounds (geniposide, Shanzhiside methyl ester, chlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A, isochlorogenic acid C, baicalin, baicalein, isoimpemtorin, psoralen, angelicin, xanthotol, bergapten, and osthole). The results indicated that fourteen natural compounds showed potential inhibition for NA. The inhibition potentials were ranked in the following order: shanzhiside methyl ester > baicalein > peramivir > baicalin > isoimpemtorin > psoralen > isochlorogenic acid A > xanthotol > isochlorogenic acid C > geniposide > chlorogenic acid > angelicin > osthole > cryptochlorogenic acid > bergapten. The ranking order of the inhibition potency of baicalein > baicalin was consistent with that shown in a previous paper [16]. Four TMs of *S. baicalensis*, *N. incisun*, *G. jasminoides*, and *L. japonica* were further used to screen inhibitory activity due to four TM extracts having the 21 components screened above, respectively. All extracts were diluted into 10 different concentrations to calculate the IC₅₀ values. The result displayed in Table 4 suggested that the four TMs extracts have good inhibitory activity to NA. The experimental results agreed with those in [17] except the *G. jasminoides* because there is no study published about it. *S. baicalensis*, *N. incisun*, and *L. japonica* extracts exhibited a significant inhibitory activity to NA at $40 \mu\text{g mL}^{-1}$. However, the IC₅₀ values of the three extracts were $218 \mu\text{g mL}^{-1}$, $4.44 \mu\text{g mL}^{-1}$, and $1392 \mu\text{g mL}^{-1}$, respectively. The different IC₅₀ values of the two studies might be caused by assaying with different systems. In summary, the facts that the four extracts of TCMs were highly effective to inhibit NA activity have been further confirmed. It was concluded that HPLC-FLD method was highly efficient, more stable, economical, and feasible to simultaneously screen NAIs from compounds and extract from TMs.

Table 3

The IC₅₀ values of 15 compounds assayed with NA enzyme-based inhibitory assays and the HPLC-FLD method (mM).

No.	Compounds	NA enzyme-based inhibitory assays	HPLC-FLD
1	Geniposide	1.01 ± 0.09	0.97 ± 0.09
2	Shanzhiside methyl ester	0.01 ± 0.00	0.01 ± 0.00
3	Chlorogenic acid	1.39 ± 0.13	2.56 ± 0.06
4	Cryptochlorogenic acid	1.59 ± 0.01	2.20 ± 0.01
5	Isochlorogenic acid A	0.71 ± 0.01	1.11 ± 0.07
6	Isochlorogenic acid C	0.80 ± 0.03	1.17 ± 0.05
7	Baicalin	0.32 ± 0.04	0.50 ± 0.04
8	Baicalein	0.06 ± 0.02	0.11 ± 0.01

9	Isoimpemtorin	0.43±0.03	0.66±0.16
10	Psoralen	0.70±0.06	1.00±0.63
11	Angelicin	1.46±0.31	2.55±0.38
12	Xanthotol	0.76±0.07	1.05±0.07
13	Bergapten	2.05±0.20	2.80±0.16
14	Osthole	1.50±0.18	2.14±0.15
15	Peramivir	0.29±0.03	0.27±0.04

Table 4

The IC₅₀ values of 4 extracts assayed with NA enzyme-based inhibitory assays and the HPLC-FLD method.

Extracts	IC ₅₀ (μg/mL)	
NA enzyme-based inhibitory assays	HPLC-FLD	<i>S. baicalensis</i>
187±10	218±19	<i>N. incisun</i>
3.53±0.58	4.44±0.91	<i>G. jasminoides</i>
2.37±0.60	3.22±0.59	<i>L. japonica</i>

Note. The extraction efficiency of *S. abaicalensis*, *N. incisun*, *G. jasminoides*, and *L. japonica* was 29.4%, 23.03%, 23.74%, and 28.2%, respectively.

4. Conclusions

A simple and effective HPLC-FLD method was established and validated to determining 4-methylumbelliferon for screening the natural neuraminidase inhibitors (NAIs) from traditional medicines. Compared with the results of NA enzyme-based inhibitory assays, the method was sensitivity, effective, and economical accounting for the extremely low LOD and LOQ values, high stability, and good accuracy. Additionally, the novel method can greatly minimize the false-positive results caused by the autofluorescence substances in NA enzyme-based inhibitory assays. It was concluded that the new method for determining 4-methylumbelliferone by HPLC-FLD is a promising screening tool for NAI traditional medicines.

Acknowledgments

This research was supported by the project of integration of traditional Chinese medicine and Western medicine by the Tianjin Health and Family Planning Commission (2015092).

References

- [1] A. Meijer, H. Rebelo-de-Andrade, V. Correia, "Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2012-2013," *Antiviral Research*, vol. 110, pp. 31-41, DOI: 10.1016/j.antiviral.2014.07.001, 2014.
- [2] A. S. Monto, D. M. Fleming, D. Henry, "Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza A and B virus infections," *The Journal of Infectious Diseases*, vol. 180 no. 2, pp. 254-261,

DOI: 10.1086/314904, 1999.

- [3] J. McKimm-Breschkin, T. Trivedi, A. Hampson, "Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir," *Antimicrobial Agents and Chemotherapy*, vol. 47 no. 7, pp. 2264-2272, DOI: 10.1128/aac.47.7.2264-2272.2003, 2003.
- [4] T. G. Sheu, V. M. Deyde, M. Okomo-Adhiambo, "Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008," *Antimicrobial Agents and Chemotherapy*, vol. 52 no. 9, pp. 3284-3292, DOI: 10.1128/aac.00555-08, 2008.
- [5] W. Liu, H. L. Wang, B. Zhu, "An activity-integrated strategy of the identification, screening and determination of potential neuraminidase inhibitors from radix scutellariae," *PLoS One*, vol. 12 no. 5, 2017.
- [6] L. J. An, X. A. Yu, W. Liu, J. Li, Y. X. Chang, "Identification and screening of natural neuraminidase inhibitors from reduning injection via one-step high-performance liquid chromatography-fraction collector and UHPLC/Q-TOF-MS," *International Journal of Analytical Chemistry*, vol. 2020, DOI: 10.1155/2020/8838025, 2020.
- [7] U. Grienke, M. Schmidtke, S. von Grafenstein, J. Kirchmair, K. R. Liedl, J. M. Rollinger, "Influenza neuraminidase: a druggable target for natural products," *Natural Product Reports*, vol. 29 no. 1, pp. 11-36, DOI: 10.1039/c1np00053e, 2012.
- [8] J. Liu, M. Zu, K. Chen, "Screening of neuraminidase inhibitory activities of some medicinal plants traditionally used in lingnan Chinese medicines," *BMC Complementary and Alternative Medicine*, vol. 18 no. 1, DOI: 10.1186/s12906-018-2173-1, 2018.
- [9] M. H. Park, S. Jung, H. J. Yuk, "Rapid identification of isoprenylated flavonoids constituents with inhibitory activity on bacterial neuraminidase from root barks of paper mulberry (*Broussonetia papyrifera*)," *International Journal of Biological Macromolecules*, vol. 174, pp. 61-68, DOI: 10.1016/j.ijbiomac.2021.01.140, 2021.
- [10] H. Zhao, Z. Chen, "Screening of neuraminidase inhibitors from traditional Chinese medicines by integrating capillary electrophoresis with immobilized enzyme microreactor," *Journal of Chromatography A*, vol. 1340, pp. 139-145, DOI: 10.1016/j.chroma.2014.03.028, 2014.
- [11] Y. Shahbazi, H. Malekinejad, H. Tajik, "Determination of naturally occurring estrogenic hormones in cow's and river buffalo's meat by HPLC-FLD method," *Journal of Food and Drug Analysis*, vol. 24 no. 3, pp. 457-463, DOI: 10.1016/j.jfda.2016.02.014, 2016.
- [12] J.-L. Wu, S.-Y. Yu, S.-H. Wu, A.-M. Bao, "A sensitive and practical RP-HPLC-FLD for determination of the low neuroactive amino acid levels in body fluids and its application in depression," *Neuroscience Letters*, vol. 616, pp. 32-37, DOI: 10.1016/j.neulet.2016.01.024, 2016.
- [13] O. Golge, F. Hepsag, B. Kabak, "Determination of aflatoxins in walnut sujuk and Turkish delight by HPLC-FLD method," *Food Control*, vol. 59, pp. 731-736, DOI: 10.1016/j.foodcont.2015.06.035, 2016.
- [14] FDA, Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, 2001.
- [15] Y.-X. Chang, X.-P. Ding, J. Qi, "Determination of phenolic acids in danshen preparations by LC with chemiluminescence detection," *Chromatographia*, vol. 69 no. 3-4, pp. 319-323, DOI: 10.1365/s10337-008-0876-z, 2009.
- [16] A.-L. Liu, H.-D. Wang, S. M. Lee, Y.-T. Wang, G.-H. Du, "Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities," *Bioorganic & Medicinal Chemistry*, vol. 16 no. 15, pp. 7141-7147, DOI: 10.1016/j.bmc.2008.06.049, 2008.
- [17] H. Ge, Y.-F. Wang, J. Xu, "Anti-influenza agents from traditional Chinese medicine," *Natural Product Reports*, vol. 27 no. 12, pp. 1758-1780, DOI: 10.1039/c0np00005a, 2010.

DETAILS

Subject:	Mass spectrometry; Herbal medicine; Fluorescence; Sodium; Inhibitors; Flavonoids; Influenza; Scientific imaging; Chromatography; High performance liquid chromatography; Flow velocity; Enzymes; Pigments; Screening; Bioassays
Location:	United States--US; China
Editor:	Pavel Nesterenko
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-11-10 (Received); 2021-04-10 (Revised); 2021-08-16 (Accepted); 2021-08-25 (Pub)
DOI:	https://doi.org/10.1155/2021/6694771
ProQuest documentID:	2569268676
Document URL:	https://www.proquest.com/scholarly-journals/evaluation-neuraminidase-inhibitory-activity/docview/2569268676/se-2?accountid=211160

Copyright:

Copyright © 2021 Guangying Yu and Dan Fang. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2023-10-02

Database:

Publicly Available Content Database

Document 22 of 59

Synthesis of Zearalenone Immunogen and Comparative Analysis of Antibody Characteristics

Wang, Yanan ¹

; Wang, Xiaofei ²

; Zhang, Haitang ³

; Jiang, Jinqing ³

; Fotina, Hanna ⁴

¹ College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, China; Faculty of Veterinary Medicine, Sumy National Agrarian University, Sumy 40021, Ukraine ² Xinke College, Henan Institute of Science and Technology, Xinxiang 453003, China ³ College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, China ⁴ Faculty of Veterinary Medicine, Sumy National Agrarian University, Sumy 40021, Ukraine

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Background. This study aimed to explore the zearalenone (ZEN) immunogen synthesis method, immunogenicity, and antibody characteristics and to lay a foundation for the establishment of immunoassay methods for ZEN single residue and ZEN and its analogs total residue. **Methods.** Based on the molecular structure and active sites of ZEN, oxime active ester (OAE), condensation mixed anhydride (CMA), formaldehyde (FA), and 1,4-butanediol diglycidyl ether method (BDE) were designed and used for immunogen (ZEN-BSA) synthesis. The immunogens were identified by infrared (IR) and ultraviolet (UV) spectra and gel electrophoresis (SDS-PAGE) and were then used to immunize Balb/c mice to prepare ZEN polyclonal antibody (ZEN pAb). The titers and sensitivity of the ZEN pAb were determined by indirect noncompetitive ELISA (inELISA) and indirect competitive ELISA (icELISA), respectively, and its specificity was assessed by the cross-reaction test (CR). **Results.** ZEN-BSA was successfully synthesized, and the molecular binding ratios of ZEN to BSA were 17.2:1 (OAE), 14.6:1 (CMA), 9.7:1 (FA), and 8.3:1 (BDE), respectively. The highest inELISA titers of ZEN pAb of each group were 1:(6.4×10³) (OAE), 1:(3.2×10³) (CMA), 1:(1.6×10³) (FA), and 1:(1.6×10³) (BDE), respectively. The 50% inhibition concentrations (IC₅₀) for ZEN by icELISA of each group were 11.67 µg/L (OAE), 16.29 µg/L (CMA), 20.92 µg/L (FA) and 24.36 µg/L (BDE), respectively. ZEN pAb from the mice immunized with ZEN-BSA (OAE) and ZEN-BSA (CMA) had class broad specificity to ZEN and its

analogs. The CRs of ZEN pAb with α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON were 36.53%, 16.98%, 64.33%, 20.16%, and 10.66%, respectively. ZEN pAb from the mice immunized with ZEN-BSA (FA) and ZEN-BSA (BDE) had high specificity for ZEN. The CRs of ZEN pAb with its analogs were all less than 1.0%. *Conclusion.* This study demonstrated that the preparation of the class broad-specificity antibodies of ZEN and its analogs can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the OAE method, while the preparation of highly specific antibodies can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the FA method. These findings lay the material and technical foundation for immunoassay of ZEN single residue and ZEN and its analogs total residue.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Zearalenone (ZEN) is a toxic secondary metabolite produced by members of the genus *Fusarium*, and ZEN mainly contaminates grains including corn, wheat, barley, rice, and oats or foods containing these grains, and its chemical name is (E)-(S)-2,4-dihydroxy-7-methyl-7,8,9,10,13,14-hexahydro-12h-6-oxa-benzocyclotetradecene-5,11-dione, also called F-2 toxin. The ZEN analogs produced under natural conditions mainly include α -zearalanol (α -ZAL), β -zearalanol (β -ZAL), α -zearalenol (α -ZOL), β -zearalenol (β -ZOL), and zearalanone (ZON) (Figure 1) [1, 2]. Owing to the reproductive toxicity, genotoxicity, immunotoxicity, endocrine toxicity, and carcinogenic toxicity of ZEN to the body [3, 4], most countries and regions globally have implemented the maximum residue limits (MRLs) of ZEN in food and feed. The EU stipulates that the MRL of ZEN in cereals and grain products is 2 mg/kg; the MRL of ZEN in corn by-products is 3 mg/kg; the MRL of ZEN in compound feeds for piglets and young sows are 0.1 mg/kg [5]. For instance, Italy stipulates that the MRL of ZEN in grains and cereal products is 0.1 mg/kg [6]; Australia stipulates that the MRL of ZEN in grains is 0.05 mg/kg [7]. The current ZEN MRL standard of food and agricultural products in China is "GB 2761-2017 Food Mycotoxin Limit," which strictly stipulates that the MRL of ZEN in wheat, wheat flour, corn, and corn flour is 0.06 mg/kg [8]. However, with people's increasing attention to food safety issues, researchers have extensively explored the occurrence and toxicity of ZEN and its analogs. Reports show that ZEN, α -ZOL, and β -ZOL often exist simultaneously in cereals infected by *Fusarium spp.* Notably, ZEN can be metabolized into α -ZOL, β -ZOL, and ZON. α -ZAL is the reducing product of ZEN and is metabolized into β -ZAL and ZON. Thus, ZEN and its analogs are similar, with toxic effects on the human body. However, α -ZOL has the strongest toxicity, which is 10–20 times higher than ZEN [9, 10]. Therefore, single ZEN detection cannot meet the needs of food and feed industry, and this has prompted immense research on detecting the total amount of ZEN and its analogs.

[figures omitted; refer to PDF]

Currently, there are two main methods for detecting ZEN residues, including physicochemical analysis and immunoassay. The main physicochemical analysis methods used in all countries are thin-layer chromatography (TLC) [11], high-performance liquid chromatography (HPLC) [12], gas chromatography-mass spectrometry (GC-MS) [13], and liquid chromatography/tandem mass spectrometry (LC-MS/MS) [14], among others. However, these techniques are expensive and lengthy and require complicated sample pretreatment procedures, expensive instruments, and skilled technicians. Therefore, they are not good enough to meet the needs of actual detection [15]. The immunoassay methods based on antigen-antibody reaction have the common characteristics of strong selectivity and high sensitivity and have become a hot topic in ZEN detection research, such as flexible pressure sensor (FPS) [16], self-powered temperature sensor (SPTS) [17], nano-hybrid-mediated photoelectrochemical immunoassay (NMPECIA) [18], and quantum dot-based photoelectrochemical immunoassay (QDPECIA) [19]. In addition, the established immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA) [20], gold immunochromatographic assay (GICA) [21], and the time-resolved fluoroimmunoassay (TRFIA) [22], also have the advantages of fast, low cost, and on-site detection [15], fluorescence polarization immunoassay (FPIA) [23], immunosensor (IS) [24], and antibody microarray (AMA) [25] have the advantage of the high-throughput detection.

These immunoassay techniques play an important role in the rapid detection of ZEN. However, there are associated drawbacks such as poor specificity in single ZEN immunoassay and poor broad-spectrum in total detection of ZEN and its analogs, which cannot meet the actual needs of detection. Such challenges may be attributed to low quality of the prepared antibody. Of note, high-quality antibodies are the core reagents for establishing immunoassay methods, and the production of highly specific antibodies and class broad-specificity antibodies depends on the design of hapten molecules and immunogen synthesis [26].

At present, there have been many reports on the research of ZEN antigen synthesis, antibody preparation, and immunoassay method establishment, but there are no reports about the research on the comparison and analysis of ZEN different antigen synthesis and antibody characteristics. In view of this, through the molecular design of ZEN hapten, immunogen synthesis and identification, preparation and characteristic comparison, and analysis of polyclonal antibody (pAb), we selected the best immunogen synthesis method, which laid the foundation for the specific single residual immunoassay of ZEN and the total amount residual immunoassay of ZEN and its analogs (Figure 1).

2. Materials and Methods

2.1. Chemicals and Reagents

ZEN standard (solvent-free), α -zearalanol (α -ZAL), β -zearalanol (β -ZAL), α -zearalenol (α -ZOL), β -zearalenol (β -ZOL), and zearalanone (ZON) standard solutions in methanol, carboxymethoxylamine hemihydrochloride (CMO), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide (EDC), hydroxylamine hydrochloride, succinic anhydride, 1,4-butanediol diglycidyl ether (BDE), phenacetin, 3,3',5,5'-tetra-methylbenzidine (TMB), urea peroxide, and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), and goat anti-mouse IgG conjugated with horseradish peroxidase (GaMlgG-HRP) were obtained from Pierce (Rockford, IL, USA). Dioxane, isobutyl chloroformate (IBCF), dimethylformamide (DMF), and formaldehyde (FA) were from Beijing Chemical Works (Beijing, China). Meanwhile, ethylenediamine (EDA) and tri-n-butylamine (TNBA) were from J & K Chemicals (Shanghai, China). All other solvents, reagents, and chemicals were standard commercial products of analytical grade or better.

2.2. Standard Solutions and Buffers

The standard solutions used were as follows: (1) stock solutions of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON at a concentration of 1000 ng/mL were prepared in methanol and diluted to a standard solution with 0.01 mol/L PBS; (2) cationized carrier protein solution, 200 mg (0.003 mmol) BSA (or 135 mg, 0.003 mmol, OVA), was dissolved in 10 mL PBS, and 7.2 mg (0.12 mmol) EDA and 11.5 mg (0.06 mmol) EDC was added sequentially. The solution was magnetically stirred at room temperature for 2 h. The reaction solution was dialyzed with PBS at 4°C for three days, and the dialysate was changed once a day to obtain cationized BSA (cBSA) or cOVA, stored at 4°C for subsequent use.

The buffers used included the following: (1) phosphate buffer saline (0.01 M PBS, pH 7.4), consisting of NaCl (137 mmol), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (10 mmol), KCl (2.68 mmol), and KH_2PO_4 (1.47 mmol); (2) for the coating buffer, carbonate buffer (0.05 M CBS, pH 9.6), consisting of Na_2CO_3 (15 mmol) and NaHCO_3 (35 mmol); (3) washing buffer consisting of PBS, containing 0.05% Tween-20 (PBST); (4) blocking buffer containing swine serum (5%, v/v) in PBST; (5) substrate buffer made from a mixture of part A (500 mL) and part B (500 mL) solutions. Part A contained (per 1 L water) 3.15 g citric acid, 6.966 g anhydrous sodium acetate, 0.08 g phenacetin, and 0.05 g urea peroxide adjusted to pH 5.0 with HCl. In contrast, Part B contained 1.27 g TMB dissolved in 500 mL methanol and 500 mL glycerol. (6) We used 2 M H_2SO_4 as the stopping solution.

2.3. Equipment and Instruments

A spectrophotometric microtiter reader (Multiskan MK3, Thermo Co., Shanghai, China) was used to measure the absorbance. The infrared (IR) spectra were obtained using a Bruker Tensor 27 spectrometer (Bruker, Germany), while the ultraviolet (UV) visible spectra were obtained with a DU-800 UV visible spectrophotometer (Beckman-Coulter, Fullerton, CA, USA). JY300C electrophoresis apparatus and gel imaging system were purchased from

Beijing Junyi Dongfang Electrophoresis Equipment Co., Ltd. (Beijing, China). In addition, a 303A-1 electric heating constant temperature incubator was purchased from Beijing Zhongxing Weiye Instrument Co., Ltd. (Beijing, China). Exceed DZG-303A ultrapure water polishing system was purchased from Chengdu Kangning Special Experiment Pure Water Equipment Factory (Chengdu, China). An LDZX-30KB vertical pressure steam sterilizer was purchased from Shanghai Shenan Medical Instrument Factory (Shanghai, China). Transparent 96-well polystyrene microtiter plates were from Boyang Experimental Equipment Factory (Jiangsu, China).

2.4. Experimental Animals

Six-week-old female Balb/c mice and feed were provided by the Experimental Animal Center of Medical College of Zhengzhou University (Zhengzhou, China). They were given tap water and a diet *ad libitum*. The room housing the animals was maintained at a temperature of $24 \pm 2^\circ\text{C}$, relative humidity of $50\% \pm 20\%$, and a 12h light/dark cycle.

2.5. Synthesis of Immunogen

According to the molecular structure and active site of ZEN, the immunogen and coating antigen were synthesized via oxime active ester (OAE) [27], condensation mixed anhydride (CMA) [28], formaldehyde (FA) [29], and 1,4-butanediol diglycidyl ether (BDE) techniques [30].

The basic principle of the OAE method was as follows. The C6' position carbonyl group of ZEN molecule was selected as the active site, and ZEN oxime (ZENO) was synthesized by oximation reaction with CMO. The carboxyl group of ZENO and amino group of BSA were coupled with a monoamide bond under the action of EDC to synthesize artificial immunogen ZEN-BSA. Briefly, according to the initial molar ratio of ZEN and BSA (50:1), 5mg (0.0157mmol) ZEN was dissolved in 2mL pyridine, followed by the addition of 10mg (0.09117mmol) CMO. After sealing, the reaction proceeded while stirring using a magnetic bar at room temperature for 24h to obtain the yellow solution product. The product was dried using nitrogen, and then, we added 3.0mL deionized water, adjusted pH to 8.0 using 0.1mol/L NaOH, and extracted with an equal volume of ethyl acetate thrice. The aqueous phase was discarded. The organic phase was collected and dried using nitrogen to obtain hapten ZENO, which was a light yellow oil. ZENO was dissolved in 2mL dioxane, after which 2.5mg NHS and 5mg EDC were added, and stirred at 4°C for 4h to prepare the hapten activation solution. To the hapten activation solution, 1mL cBSA activation solution was added at a concentration of 20mg/mL dropwise. The reaction solution was stirred at 4°C for 4h and then dialyzed with PBS at 4°C for three days. The dialysate was changed once a day and stored at 4°C for subsequent applications. The synthetic route of ZEN-BSA (OAE) is shown in Figure 2. The coating antigen ZEN-OVA (OAE) was prepared via the same method.

[figure omitted; refer to PDF]

The basic principle of the CMA method was as follows. The C6' position carbonyl group of ZEN molecule was selected as the active site, and the hydroxyl group was introduced via condensation with hydroxylamine hydrochloride. The hydroxyl group reacted with succinic anhydride to introduce the active group carboxyl group. The carboxyl groups react with isobutyl chloroformate in the presence of tri-*n*-butylamine to generate active intermediate mixed anhydride, which then reacts with primary amino groups on protein carriers to release an immunogen by monoamide coupling. Briefly, according to the initial molar ratio of ZEN and BSA (50:1), 5mg (0.0157mmol) ZEN was dissolved in 2mL pyridine, and 5.5mg (0.0785mmol) of hydroxylamine hydrochloride was added. The reaction was stirred at room temperature for 24h, after which pyridine and unreacted hydroxylamine hydrochloride were removed via rotary evaporation to obtain the primary product. The initial product was dissolved in 0.5mL pyridine, and 7.8mg (0.0785mmol) succinic anhydride was added, followed by 1mL tetrahydrofuran. The reaction was stirred at room temperature for 24h. The solvent was removed via rotary evaporation to obtain the hapten product, which was dissolved in 2mL of dioxane, and then, 15 μL of tri-*n*-butylamine and 45 μL of isobutyl chloroformate were added. The reaction was stirred for 0.5h at 0°C in an ice bath. Eventually, the hapten activation solution was formed, which was added dropwise to 1mL of cBSA activation solution at a concentration of 20mg/mL. The reaction was stirred at 4°C for 4h. We dialyzed the reaction with PBS at 4°C for three days. The dialysate was changed once a day. The resulting ZEN-BSA conjugate was stored at 4°C for subsequent use. The synthetic route of ZEN-BSA (CMA) is shown in Figure 3. Notably, the coating antigen ZEN-OVA (CMA) was prepared via the same method.

[figure omitted; refer to PDF]

The basic principle of the FA method was as follows: the artificial immunogen was synthesized by the Mannich condensation reaction between the hapten containing active hydrogen and the amino group of protein triggered by formaldehyde. We used the active hydrogen at the C7' position of ZEN as the active site. Briefly, the experiment was designed according to an initial molar ratio of 50:1 (ZEN to BSA). Exactly 5 mg (0.0157 mmol) ZEN was dissolved in 1 mL of dimethylformamide (DMF) and then added dropwise to 1 mL at a concentration of 20 mg/mL cBSA activation solution. 60 μ L 37% formaldehyde aqueous solution (containing formaldehyde 0.8 mmol) was added, and the reaction was magnetically stirred at room temperature for 24 h. The reaction solution was dialyzed with PBS at 4°C for three days, and the PBS was changed once a day. The dialysate was collected and stored at 4°C for subsequent use. The synthetic route of ZEN-BSA (FA) is shown in Figure 4. The coating antigen ZEN-OVA (FA) was prepared via the same method.

[figure omitted; refer to PDF]

The basic principle of the BDE method was as follows: using the active site of the C2 position hydroxyl group on the ZEN molecule, 1,4-butanediol diglycidyl ether contains an oxirane group at both ends. One end of the oxirane group reacts with the hydroxyl group of the hapten to form an ether bond. At the other end, the oxirane group reacts with the amino group of the carrier protein to form a secondary amine, which is coupled to synthesize an artificial immunogen through the BDE long-chain bond. Briefly, the experiment was designed according to the initial molar ratio of 50:1 (ZEN to BSA); 5 mg (0.0157 mmol) ZEN was dissolved in 0.5 mL dimethylformamide (DMF); 31 μ L (0.157 mmol) 1,4-butanediol diglycidyl ether (BDE) was dissolved in 0.5 mL of double-distilled water and added dropwise to the previously mentioned solution. Then, the pH was adjusted to 10.8 with 1 mol/L NaOH, and the solution was magnetically stirred at room temperature for 4 h. This formed the hapten activation solution. The hapten activation solution was added dropwise to 1 mL of cBSA activation solution with a 20 mg/mL concentration, the pH was adjusted to 10.8 with 1 mol/L NaOH, and the reaction was stirred at 4°C for 4 h. The reaction solution was dialyzed with PBS at 4°C for three days. The dialysate was changed once a day. After collection, the dialysate was stored at -20°C for subsequent use. The synthetic route is shown in Figure 5. The coating antigen ZEN-OVA (BDE) was prepared via the same method.

[figure omitted; refer to PDF]

Table 1

Molecular binding ratio of ZEN-BSA prepared via the four methods.

Synthesis methods	Initial molar ratio of ZEN to BSA	Molecular binding ratio of ZEN-BSA	Usage percentage of ZEN* (%)
OAE	50 : 1	17.2 : 1	34.4
CMA	50 : 1	14.6 : 1	29.2
FA	50 : 1	9.7 : 1	19.4
BDE	50 : 1	8.3 : 1	16.6

*Since the molecular weight of BSA (66446) was much larger than that of ZEN (318.36), the utilization rate of BSA was set to 100% when calculating the utilization rate of BSA and ZEN.

3.3. SDS-PAGE Identification

All the electrophoretic bands of the artificial immunogen ZEN-BSA synthesized via the four methods were lagging behind the BSA bands, indicating that the molecular weight of ZEN-BSA was greater than BSA. This proved the successful synthesis of ZEN-BSA (Figure 8). Detected by the gel imaging system, the molecular weight of BSA was

66446Da, whereas the molecular weight of ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) was 71803Da, 70994Da, 69482Da, and 699023Da, respectively. The molecular binding ratios of ZEN and BSA in ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) were 17.17:1, 14.57:1, 9.73:1, and 8.26:1, respectively, which concurred with the UV identification results.

[figure omitted; refer to PDF]

3.4. ZEN pAb Characteristic Analysis

3.4.1. Titers Measurement

After immunization, we selected one immunized mouse with the highest inELISA titers from each group for comparative analysis. The inELISA titers of immunized mice with ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) were $1:(6.4 \times 10^3)$, $1:(3.2 \times 10^3)$, $1:(1.6 \times 10^3)$ and $1:(1.6 \times 10^3)$, all of which attained beyond $1:(1 \times 10^3)$ (Figure 9). Following the previously mentioned results, the four artificial immunogens synthesized showed satisfactory immunogenicity. According to the inELISA titers, the immune effects of the four immunogens were evaluated in the order of ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (BDE), and ZEN-BSA (FA).

[figure omitted; refer to PDF]

3.4.2. Sensitivity Measurement

The icELISA curves of the four immunized mice with the highest inELISA titers selected by each group showed a good linear relationship (Figure 10). The regression equation, R^2 value, and IC50 value of the inhibition curve are shown in Table 2. The IC50 value for ZEN by icELISA of each immunogen ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) were 11.67 $\mu\text{g/L}$, 16.29 $\mu\text{g/L}$, 20.92 $\mu\text{g/L}$, and 24.36 $\mu\text{g/L}$, respectively. Using IC50 value for ZEN, in evaluating the immune effect of the four artificial immunogens, the order was reported as follows: ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE).

[figure omitted; refer to PDF]

Table 2

The regression equation, R^2 and IC50 value for ZEN via icELISA.

Group	Regression equation	R^2	IC50 ($\mu\text{g/L}$)
ZEN-BSA (OAE)	$y = -30.589x + 82.637$	0.9805	11.67
ZEN-BSA (CMA)	$y = -31.071x + 87.658$	0.9895	16.29
ZEN-BSA (FA)	$y = -31.894x + 92.116$	0.9918	20.92
ZEN-BSA (BDE)	$y = -32.173x + 94.599$	0.9922	24.36

3.4.3. Specificity Identification

ZEN pAb produced by immunizing mice with ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) could all recognize ZEN 100% (Table 3). The ZEN pAb from the mice immunized with ZEN-BSA (OAE) and ZEN-BSA (CMA) had similar characteristics; that is, it had class broad specificity for ZEN and its analogs. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (OAE) was 11.67 $\mu\text{g/L}$, and the CR values of α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON were 36.53%, 16.98%, 64.33%, 20.16%, and 10.66%, respectively. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (CMA) was 16.29 $\mu\text{g/L}$, and the CR values of α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON were 23.55%, 12.18%, 51.86%, 18.62%, and 9.64%, respectively. In terms of class broad-specificity, the ZEN pAb from mice immunized with ZEN-BSA (OAE) was more prominent. Furthermore, the ZEN pAb from the mice immunized with ZEN-BSA (FA) and ZEN-BSA (BDE) exhibited similar characteristics; that is, it had high specificity for ZEN but with poor class broad specificity for ZEN analogs. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-

BSA (FA) was 20.92 $\mu\text{g/L}$, and the CR values of α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON were all less than 1%. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (BDE) was 24.36 $\mu\text{g/L}$, except for the higher CR (36.57%) with ZON, and the CR values of α -ZAL, β -ZAL, α -ZOL, and β -ZOL were less than 3%. In terms of specificity, the ZEN pAb from mice immunized with ZEN-BSA (FA) was more prominent. These results demonstrated that the preparation of the class broad-specificity antibodies for ZEN and its analogs can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by OAE method, while the preparation of highly specific antibodies for ZEN can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the FA method.

Table 3

The cross-reaction of ZEN pAb with ZEN and its analogs.

Compound	ZEN pAb (OAE)		ZEN pAb (CMA)		ZEN pAb (FA)		ZEN pAb (BDE)	
IC50 ($\mu\text{g/L}$) ^{a,b}	CR (%) ^a	IC50 ($\mu\text{g/L}$) ^{a,b}	CR (%) ^a	IC50 ($\mu\text{g/L}$) ^{a,b}	CR (%) ^a	IC50 ($\mu\text{g/L}$) ^{a,b}	CR (%) ^a	ZEN
11.67	100	16.29	100	20.92	100	24.36	100	α -ZAL
31.95	36.53	69.17	23.55	2682.05	0.78	1441.42	1.69	β -ZAL
68.73	16.98	133.74	12.18	2582.72	0.81	2511.34	0.97	α -ZOL
18.14	64.33	31.41	51.86	3670.18	0.57	990.24	2.46	β -ZOL
57.89	20.16	87.49	18.62	4358.33	0.48	1331.15	1.83	ZON

^aAll of the data were calculated from triplicate assays. ^bThe compound standard solution was prepared in 70% methanol-PBS (7:3, v/v).

4. Discussion

4.1. Design of the ZEN Immunogen Synthesis Method

ZEN has a molecular weight of 318.36Da and is a small molecule compound. It has only reactogenicity but no immunogenicity. It cannot directly induce the body to produce specific antibodies. It must be combined with the carrier protein to form an immunogen to induce the body to produce specific antibodies. Therefore, the design of ZEN immunogen synthesis method is very important [37]. According to the hapten-carrier effect theory, in the immunogen synthesis method, the main factors affecting the characteristics of the antibody produced by the immunogen include the complexity of the hapten molecular structure, selection of the position of the active site, length of the introduced spacer, molecular space structure, and molecular binding ratio of hapten to carrier protein [38]. The complexity of hapten molecular structure refers to whether the hapten molecular structure contained benzene ring and number, heterocycle and number, and branch structure and number. Generally, the hapten with complex structure easily induces the body to produce antibodies, while the hapten with simple structure difficultly induces the body to produce antibodies [39]. Szurdoki et al. reported that the success rate of preparing antibodies with a benzene ring in the hapten molecular structure was 33.3%, while the success rate without a benzene ring was

only 9.1% [40]. Regarding the selection of active sites, on the one hand, different active sites will cause changes in the distribution of electrons in the hapten molecule, thereby changing its electrochemical properties, thereby changing its immune activity, and affecting the affinity of antibodies [33]. On the other hand, because different active sites will lead to different spatial structures of molecules, the exposed antigenic determinants are also different, which in turn affects the specificity of antibodies [41]. Therefore, we should try to use different sites to synthesize immunogens separately and then, through comparative analysis, screen out the best immunogen for target antibody preparation. The selection of the spacer arm includes the length and structure of the spacer arm. For the determination of the spacer arm length, Tuomola et al. and Jung et al. proposed that the optimal length of the spacer arm was three to six carbon atoms. Shortness of spacer arm was not conducive to full exposure of hapten, and too long spacer arm would cause the folding of alkyl chain due to hydrophobic effect, which would cause the hapten molecule to be covered by carrier protein, which was not conducive to be recognized by antigen-presenting cells [42, 43]. Regarding the choice of the spacer structure, Bruun et al. and Kolosova et al. considered that the spacer should not contain structures with high immunological activity such as aromatic rings, conjugated double bonds, or heterocycles, so as to reduce the production of antibodies. Overrecognition of the spacer arm reduced the ability to recognize the target molecule [44, 45]. Regarding the determination of the molecular binding ratio, Vikas et al. and Peterson et al. believed that when the molecular binding ratio was too large, the carrier protein was not conducive to the binding of the carrier protein and lymphocytes due to the excessive coverage of the hapten molecule, which weakened the carrier effect. If the molecular binding ratio was too small, the immunogen was not enough to cause the body to produce an immune response. The best molecular binding ratio of the hapten to the carrier protein should be 5–20:1 [46, 47].

In this study, based on the molecular structure characteristics of ZEN, and on the basis of a careful and comprehensive summary of previous studies, the C6' position carbonyl group, C7' position active hydrogen, and C2 position hydroxyl group were selected as active sites, and the spacer arm was introduced through different chemical reaction methods to realize the coupling of ZEN with carrier protein to synthesize immunogen.

4.2. Synthesis Methods of ZEN Immunogen and Characteristics of ZEN Antibody

ZEN belongs to the chemical compound of the resorcylic acid lactones, which contains the main characteristic structure of 2,4-dihydroxybenzene ring and macrocyclic lactone ring. ZEN and its five analogs are structurally identical apart from minor differences at C6' position carbonyl group or hydroxyl group and at C1'-C2' position single bond or double bond in the macrocyclic lactone ring. Therefore, the special chemical properties of ZEN lay a good foundation for the preparation of class broad-specificity antibodies of ZEN and its five analogs but meanwhile also bring difficulties for the preparation of high-specificity antibodies of ZEN [30]. The specificity of antibody depends on the recognition of immunogen determinants by immune cells. The spatial conformation and characteristic structure exposure of hapten on carrier protein are closely related to the immune recognition of immune cells. Therefore, in hapten design, its main characteristic structure must be exposed as a part of the immunogen to the maximum extent in order to obtain the desired target antibody [29, 48]. Because of this, hapten molecular design, immunogen synthesis and ZEN high-specificity antibody preparation and ZEN class broad-specificity antibody preparation are the key to realize ZEN residual immunoassay and ZEN and its analogs total residual immunoassay. Several recent reports have used immunogens developed using the OAE method to obtain ZEN class broad-specificity antibodies and using FA method to obtain ZEN highly specific antibodies. A comparison between ZEN pAbs in this study and other ZEN pAbs or ZEN mAbs reported in the recent literature is shown in Table 4.

Table 4

Comparison of IC₅₀ values and CR values of ZEN pAbs by the OAE method and FA method in this study and other previously reported ZEN pAbs and ZEN mAbs.

References	ZEN antibody	Coupling method	Immunoassay format	IC ₅₀ of ZEN (μg/L)	CR (%) ^a

α -ZAL	β -ZAL	α -ZOL	β -ZOL	ZON	This study (2021)	ZEN pAb	OA E	icEL ISA ^b	11.67
36.53	16.98	64.33	20.16	10.66	Dong et al. [49]	ZEN mAb 6C2	OA E	icEL ISA	114.0
99.2	55.5	89.5	39.3	—	Zhang et al. [23]	ZEN mAb 3D4	OA E	FPI A ^c	0.041
107.9	57.2	103.5	73.9	66	Liu et al. [50]	ZEN mAb 7B2	OA E	BA-ELISA ^d	0.18
46.7	39.2	60.5	24.7	59.5	Chai et al. [27]	ZEN mAb kk-ZEN	OA E	icEL ISA	131.3
108.1	119.3	114.1	130.3	—				dcELISA ^e	150.6
123.8	99.3	149.5	124.8	—	Thongruamee et al. [51]	ZEN mAb 2C5	OA E	dcELISA	1.79
121.5	65.3	21.5	18.9	—	This study (2021)	ZEN pAb	FA	icEL ISA	20.92
0.78	0.81	0.57	0.48	0.92	Burkin et al. [52]	ZEN pAb	FA	icEL ISA	31.7
0.12	—	0.15	0.02	—	Gao et al. [29]	ZEN pAb	FA	icEL ISA	233.35

2.25	5.65	3.14	1.96	6.79		ZEN mAb [#]	FA	icEL ISA	55.7 2

^aAll of the data were calculated using the CR of ZEN as 100%. ^bicELISA: indirect competitive enzyme-linked immunosorbent assay; ^cFPIA: fluorescence polarization immunoassay; ^dBA-ELISA: biotin-streptavidin amplified enzyme-linked immunosorbent assay; ^edcELISA: direct competitive enzyme-linked immunosorbent assay; —: no data. [#]No name.

The OAE method is a classic and commonly used bioconjugation method. In recent years, some scholars have used this method to synthesize immunogens and have successfully prepared ZEN antibodies with class broad specificity [23, 27, 28, 49, 51]. Dong et al. [49], Zhang et al. [23], and Cha et al. [27] hold the same view on this. They believe that the OAE method is to introduce the carboxyl group onto the macrolide ring of ZEN at the C6' position through the carbonyl oximation reaction, and then, the active ester is used to couple the carboxyl group with the amino group of carrier protein with a monoamide bond, so that the main characteristic structures of ZEN including 2,4-dihydroxybenzene ring, macrolide ring, carbonyl or hydroxyl group at C6' position, and double bond at C1'-C2' position can be fully exposed and recognized by immune cells, resulting in a strong immune response. The antibody produced can recognize ZEN and its five analogs. In addition, Liu et al. [28] and Dixon et al. [53] consider whether the double bond at the C6' position and C1'-C2' position of ZEN can be recognized by immune cells, and the degree of recognition determines the class broad specificity of ZEN antibody and has no direct relationship with the hydroxyl group at the C6' position.

The FA method is also one of the classic biological coupling methods. Burkin et al. [52] and Gao et al. [29] have used this method to synthesize immunogens and have successfully prepared ZEN antibodies with high specificity [29, 52]. Burkin et al. [52] believe that the FA method is to introduce formyl substituents into the macrolide ring of ZEN at C7' position through the formaldehyde condensation, so that the main characteristic structures of ZEN including the 2,4-dihydroxybenzene ring, macrolide ring, and carbonyl group at C6' position are fully exposed, which are easier to be recognized by immune cells, and the antibody produced is highly specific to ZEN. However, the CRs for ZEN five analogs are small. As for why immune cells cannot recognize the single bond and double bond at C1'-C2' position and the hydroxyl group at C6' position, the reaction mechanism is still obscure.

In this study, the ZEN antibody with class broad specificity for ZEN and its five analogs was prepared by the OAE method, and the IC₅₀ for ZEN was 11.67 µg/L, and the CRs for α-ZAL, β-ZAL, α-ZOL, β-ZOL, and ZON were 36.53%, 16.98%, 64.33%, 20.16%, and 10.66%, respectively. However, the ZEN pAb had a lower broad specificity than that of ZEN mAb 6C2 reported by Dong et al. [49], ZEN mAb 3D4 reported by Zhang et al. [23], and ZEN mAb kk-ZEN reported by Cha et al. [27]. Compared with the OAE method, the CMA method had the defects of poor broad-specificity antibody, more side reactions, and lower product yield. The ZEN antibody with high-specificity for ZEN was prepared by the FA method, and the IC₅₀ for ZEN was 20.92 µg/L, and the CRs for α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were all less than 1%. The antibody had high-specificity similar to that previously reported by Burkin et al. [52] and Gao et al. [29], but had better sensitivity. Compared with FA method, the antibody produced by the BDE method had too high CR with ZON, which could not meet the purpose of preparation of high-specificity antibody against ZEN. These results lay the material and technical foundation for the immunoassay of single residues for ZEN and total residues for ZEN and its analogs.

In addition, Li et al. believed that the specificity and broad specificity of antibodies not only depend on the synthesis method of immunogen but are also closely related to the animal immunization dose of the immunogen. Taking aflatoxin B1 as the research object, the effect of immunogen dose on antibody specificity and class broad specificity was discussed. The results showed that low-dose immunogen can improve the specificity of the antibody, and high-dose immunogen can increase the broad specificity of the antibody, and the research results provided reference for the development of new antibodies [54]. However, whether the results of this study are applicable to the preparation of antibodies to ZEN and other small molecule compounds remains to be further explored in the future.

5. Conclusion

In this study, according to the molecular structure and active sites of ZEN, four artificial immunogen synthesis methods were designed, and they were identified by IR, UV, SDS-PAGE, and comparative analysis of the characteristics of ZEN pAb produced by immunized animals. The results showed that the OAE method was the best method for preparing broad-specificity antibodies for ZEN and its homologs, and the FA method was the best method for preparing ZEN high-specificity antibodies, which laid a material and technical foundation for single immunoassay of ZEN residue and total amount immunoassay of ZEN and its analogs.

Ethical Approval

All experiments were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the Animal Ethics Committee of Henan Institute of Science and Technology. The approval number issue no. is 2020HIST047.

Authors' Contributions

J. J. and Y. W. were responsible for conceptualization. Y. W., X. W., and H. Z. provided software. X. W. was involved in formal analysis. Y. W. and X. W. were responsible for data curation. Y. W. was responsible for writing and original draft preparation. J. J., H. F., and H. Z. were responsible for writing and review and editing. J. J. was responsible for supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript. Jinqing Jiang and Hanna Fotina equally contributed to this article.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (31702263), 13th Five-Year National Key Research and Development Plan Food Safety Technology Research and Development Major Project (2019YFC1605705), and Program for Innovative Research Team (in Science and Technology) at the University of Henan Province (20IRTSTHN025).

References

- [1] A. Borzekowski, R. Anggriawan, M. Auliyati, "Formation of zearalenone metabolites in tempeh fermentation," *Molecules* (Basel, Switzerland), vol. 24 no. 15, DOI: 10.3390/molecules24152697, 2019.
- [2] A. Rogowska, P. Pomastowski, G. Sagandykova, B. Buszewski, "Zearalenone and its metabolites: effect on human health, metabolism and neutralisation methods," *Toxicon*, vol. 162, pp. 46-56, DOI: 10.1016/j.toxicon.2019.03.004, 2019.
- [3] T. Kuiper-Goodman, P. M. Scott, H. Watanabe, "Risk assessment of the mycotoxin zearalenone," *Regulatory Toxicology and Pharmacology*, vol. 7 no. 3, pp. 253-306, DOI: 10.1016/0273-2300(87)90037-7, 1987.
- [4] A. Zinedine, J. M. Soriano, J. C. Moltó, J. Mañes, "Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin," *Food and Chemical Toxicology*, vol. 45 no. 1, DOI: 10.1016/j.fct.2006.07.030, 2007.
- [5] EC, Setting Maximum Levels for Certain Contaminants in Foodstuffs. No. 1881 (2006), Official Journal of the European Union, 5–24, 2006
- [6] T. Bertuzzi, M. Camardo Leggeri, P. Battilani, A. Pietri, "Co-occurrence of type A and B trichothecenes and zearalenone in wheat grown in northern Italy over the years 2009–2011," *Food Additives & Contaminants: Part B*, vol. 7 no. 4, pp. 273-281, DOI: 10.1080/19393210.2014.926397, 2014.
- [7] D. C. Tan, G. R. Flematti, E. L. Ghisalberti, "Mycotoxins produced by *Fusarium* species associated with annual legume pastures and "sheep feed refusal disorders" in Western Australia," *Mycotoxin Research*, vol. 27 no. 2, pp. 123-135, DOI: 10.1007/s12550-010-0085-0, 2011.
- [8] Z. Jun-Nan, "Public proposals put forward by the national health and family planning commission in 2017," *Chinese Journal of Medical Library and Information Science*, vol. 27 no. 6, pp. 59-65, 2018.
- [9] A. Rai, M. Das, A. Tripathi, "Occurrence and toxicity of a fusarium mycotoxin, zearalenone," *Critical Reviews in Food Science and Nutrition*, vol. 60 no. 16, pp. 2710-2729, DOI: 10.1080/10408398.2019.1655388, 2020.
- [10] C.-K. Yang, Y.-H. Cheng, W.-T. Tsai, "Prevalence of mycotoxins in feed and feed ingredients between 2015 and 2017 in Taiwan," *Environmental Science and Pollution Research*, vol. 26 no. 23, pp. 23798-23806, DOI: 10.1007/s11356-019-05659-0, 2019.

- [11] M. S. Klarić, Z. Cvetnić, S. Pepeljnjak, I. Kosalec, "Co-occurrence of aflatoxins, ochratoxin A, fumonisins, and zearalenone in cereals and feed, determined by competitive direct enzyme-linked immunosorbent assay and thin-layer chromatography," *Arhiv Za Higijenu Rada i Toksikologiju*, vol. 60 no. 4, pp. 427-434, DOI: 10.2478/10004-1254-60-2009-1975, 2009.
- [12] P. Thongprapai, W. Cheewasedtham, K. F. Chong, T. Rujiralai, "Selective magnetic nanographene oxide solid-phase extraction with high-performance liquid chromatography and fluorescence detection for the determination of zearalenone in corn samples," *Journal of Separation Science*, vol. 41 no. 23, pp. 4348-4354, DOI: 10.1002/jssc.201800441, 2018.
- [13] S. Kinani, S. Bouchonnet, S. Bourcier, J. M. Porcher, S. Aït-Aïssa, "Study of the chemical derivatization of zearalenone and its metabolites for gas chromatography-mass spectrometry analysis of environmental samples," *Journal of Chromatography. A*, vol. 1190 no. 1-2, pp. 307-315, DOI: 10.1016/j.chroma.2008.02.115, 2008.
- [14] S. Sun, K. Yao, S. Zhao, "Determination of aflatoxin and zearalenone analogs in edible and medicinal herbs using a group-specific immunoaffinity column coupled to ultra-high-performance liquid chromatography with tandem mass spectrometry," *Journal of Chromatography B*, vol. 1092, pp. 228-236, DOI: 10.1016/j.jchromb.2018.06.012, 2018.
- [15] J. N. Selvaraj, Y. Wang, L. Zhou, "Recent mycotoxin survey data and advanced mycotoxin detection techniques reported from China: a review," *Food Additives & Contaminants: Part A*, vol. 32 no. 4, pp. 440-452, DOI: 10.1080/19440049.2015.1010185, 2015.
- [16] Z. Yu, G. Cai, X. Liu, D. Tang, "Pressure-based biosensor integrated with a flexible pressure sensor and an electrochromic device for visual detection," *Analytical Chemistry*, vol. 93 no. 5, pp. 2916-2925, DOI: 10.1021/acs.analchem.0c04501, 2021.
- [17] L. Huang, J. Chen, Z. Yu, D. Tang, "Self-powered temperature sensor with seebeck effect transduction for photothermal-thermoelectric coupled immunoassay," *Analytical Chemistry*, vol. 92 no. 3, pp. 2809-2814, DOI: 10.1021/acs.analchem.9b05218, 2020.
- [18] Z. Luo, Q. Qi, L. Zhang, R. Zeng, L. Su, D. Tang, "Branched polyethylenimine-modified upconversion nanohybrid-mediated photoelectrochemical immunoassay with synergistic effect of dual-purpose copper ions," *Analytical Chemistry*, vol. 91 no. 6, pp. 4149-4156, DOI: 10.1021/acs.analchem.8b05959, 2019.
- [19] J. Shu, D. Tang, "Current advances in quantum-dots-based photoelectrochemical immunoassays," *Chemistry-An Asian Journal*, vol. 12 no. 21, pp. 2780-2789, DOI: 10.1002/asia.201701229, 2017.
- [20] A. Gutzwiller, J. L. Gafner, P. Silacci, "Urinary zearalenone measured with ELISA as a biomarker of zearalenone exposure in pigs," *Mycotoxin Research*, vol. 30 no. 4, pp. 187-190, DOI: 10.1007/s12550-014-0198-y, 2014.
- [21] X. Zhang, K. He, Y. Fang, "Dual flow immunochromatographic assay for rapid and simultaneous quantitative detection of ochratoxin A and zearalenone in corn, wheat, and feed samples," *Journal of Zhejiang University-Science B*, vol. 19 no. 11, pp. 871-883, DOI: 10.1631/jzus.b1800085, 2018.
- [22] J. Zhang, L. Gao, B. Zhou, L. Zhu, Y. Zhang, B. Huang, "Simultaneous detection of deoxynivalenol and zearalenone by dual-label time-resolved fluorescence immunoassay," *Journal of the Science of Food and Agriculture*, vol. 91 no. 2, pp. 193-197, DOI: 10.1002/jsfa.4151, 2011.
- [23] X. Zhang, S. A. Eremin, K. Wen, "Fluorescence polarization immunoassay based on a new monoclonal antibody for the detection of the zearalenone class of mycotoxins in maize," *Journal of Agricultural and Food Chemistry*, vol. 65 no. 10, pp. 2240-2247, DOI: 10.1021/acs.jafc.6b05614, 2017.
- [24] A. Foubert, N. V. Beloglazova, M. Hedström, S. De Saeger, "Antibody immobilization strategy for the development of a capacitive immunosensor detecting zearalenone," *Talanta*, vol. 191, pp. 202-208, DOI: 10.1016/j.talanta.2018.08.062, 2019.
- [25] X. Zhang, Z. Wang, Y. Fang, "Antibody microarray immunoassay for simultaneous quantification of multiple mycotoxins in corn samples," *Toxins*, vol. 10 no. 10, DOI: 10.3390/toxins10100415, 2018.
- [26] T. Gefen, J. Vaya, S. Khatib, "The effect of haptens on protein-carrier immunogenicity," *Immunology*, vol. 144

no. 1, pp. 116-126, DOI: 10.1111/imm.12356, 2015.

[27] S.-H. Cha, S.-H. Kim, K. Bischoff, H.-J. Kim, S.-W. Son, H.-G. Kang, "Production of a highly group-specific monoclonal antibody against zearalenone and its application in an enzyme-linked immunosorbent assay," *Journal of Veterinary Science*, vol. 13 no. 2, pp. 119-125, DOI: 10.4142/jvs.2012.13.2.119, 2012.

[28] M. T. Liu, B. P. Ram, L. P. Hart, J. J. Pestka, "Indirect enzyme-linked immunosorbent assay for the mycotoxin zearalenone," *Applied and Environmental Microbiology*, vol. 50 no. 2, pp. 332-336, DOI: 10.1128/aem.50.2.332-336.1985, 1985.

[29] Y. Gao, M. Yang, C. Peng, X. Li, R. Cai, Y. Qi, "Preparation of highly specific anti-zearalenone antibodies by using the cationic protein conjugate and development of an indirect competitive enzyme-linked immunosorbent assay," *The Analyst*, vol. 137 no. 1, pp. 229-236, DOI: 10.1039/c1an15487g, 2012.

[30] Y. Sun, X. Hu, Y. Zhang, "Development of an immunochromatographic strip test for the rapid detection of zearalenone in corn," *Journal of Agricultural and Food Chemistry*, vol. 62 no. 46, pp. 11116-11121, DOI: 10.1021/jf503092j, 2014.

[31] Y. Wang, C. H. He, H. Zheng, H. B. Zhang, "Characterization and comparison of fumonisin B(1)-protein conjugates by six methods," *International Journal of Molecular Sciences*, vol. 13 no. 1, pp. 84-96, DOI: 10.3390/ijms13010084, 2012.

[32] J.-q. Jiang, H.-t. Zhang, H.-h. Zhang, Z.-l. Wang, X.-f. Yang, G.-y. Fan, "Development of an enzyme-linked immunosorbent assay for detection of clopidol residues in chicken tissues," *Journal of the Science of Food and Agriculture*, vol. 94 no. 11, pp. 2295-2300, DOI: 10.1002/jsfa.6557, 2014.

[33] M. K. Pedersen, N. S. Sorensen, P. M. Heegaard, N. H. Beyer, L. Bruun, "Effect of different hapten-carrier conjugation ratios and molecular orientations on antibody affinity against a peptide antigen," *Journal of Immunological Methods*, vol. 311 no. 1-2, pp. 198-206, DOI: 10.1016/j.jim.2006.02.008, 2006.

[34] L. Han, Y. Li, J. Jiang, "Preparation and characterisation of monoclonal antibodies against deoxynivalenol," *Italian Journal of Animal Science*, vol. 19 no. 1, pp. 560-568, DOI: 10.1080/1828051x.2020.1763861, 2020.

[35] N. Imtiaz, A. W. Yunus, "Comparison of some ELISA kits for aflatoxin M1 quantification," *Journal of AOAC International*, vol. 102 no. 2, pp. 677-679, DOI: 10.5740/jaoacint.18-0185, 2019.

[36] Ö. Ertekin, T. Kaymak, Ş. Ş. Piriñçi, E. Akçael, S. Öztürk, "Aflatoxin-specific monoclonal antibody selection for immunoaffinity column development," *Biotechniques*, vol. 66 no. 6, pp. 261-268, DOI: 10.2144/btn-2018-0143, 2019.

[37] F. A. Esteve-Turrillas, J. Parra, A. Abad-Fuentes, C. Agulló, A. Abad-Somovilla, J. V. Mercader, "Hapten synthesis, monoclonal antibody generation, and development of competitive immunoassays for the analysis of picoxystrobin in beer," *Analytica Chimica Acta*, vol. 682 no. 1-2, pp. 93-103, DOI: 10.1016/j.aca.2010.09.042, 2010.

[38] N. Olivier, P. Chappey, G. SandoukJean-Michel, "Monoclonal antibodies in hapten immunoassays," *Pharmaceutical Research*, vol. 9, pp. 1375-1379, 1992.

[39] A. Josten, M. Meusel, F. Spener, "Microbial transglutaminase-mediated synthesis of hapten-protein conjugates for immunoassays," *Analytical Biochemistry*, vol. 258 no. 2, pp. 202-208, DOI: 10.1006/abio.1998.2617, 1998.

[40] F. Szurdoki, H. K. M. Bekheit, M. P. Marco, M. H. Goodrow, B. D. Hammock, "Synthesis of haptens and conjugates for an enzyme immunoassay for analysis of the herbicide bromacil," *Journal of Agricultural and Food Chemistry*, vol. 40 no. 8, pp. 1459-1465, DOI: 10.1021/jf00020a034, 1992.

[41] Z.-L. Xu, G.-M. Xie, Y.-X. Li, "Production and characterization of a broad-specificity polyclonal antibody for O,O-diethyl organophosphorus pesticides and a quantitative structure-activity relationship study of antibody recognition," *Analytica Chimica Acta*, vol. 647 no. 1, pp. 90-96, DOI: 10.1016/j.aca.2009.05.025, 2009.

[42] F. Jung, S. J. Gee, R. O. Harrison, "Use of immunochemical techniques for the analysis of pesticides," *Pesticide Science*, vol. 26 no. 3, pp. 303-317, 1989.

[43] M. Tuomola, R. Harpio, H. Mikola, "Production and characterisation of monoclonal antibodies against a very small hapten, 3-methylindole," *Journal of Immunological Methods*, vol. 240 no. 1-2, pp. 111-124, DOI: 10.1016/s0022-1759(00)00179-4, 2000.

- [44] L. Bruun, C. Koch, B. Pedersen, M. H. Jakobsen, J. Aamand, "A quantitative enzyme-linked immunoassay for the detection of 2, 6-dichlorobenzamide (BAM), a degradation product of the herbicide dichlobenil," *Journal of Immunological Methods*, vol. 240 no. 1-2, pp. 133-142, DOI: 10.1016/s0022-1759(00)00190-3, 2000.
- [45] A. Y. Kolosova, J.-H. Park, S. A. Eremin, S.-J. Kang, D.-H. Chung, "Fluorescence polarization immunoassay based on a monoclonal antibody for the detection of the organophosphorus pesticide parathion-methyl," *Journal of Agricultural and Food Chemistry*, vol. 51 no. 5, pp. 1107-1114, DOI: 10.1021/jf025801v, 2003.
- [46] E. C. Peterson, M. D. Hambuchen, R. L. Tawney, "Simple radiometric method for accurately quantitating epitope densities of hapten-protein conjugates with sulfhydryl linkages," *Bioconjugate Chemistry*, vol. 25 no. 12, pp. 2112-2115, DOI: 10.1021/bc500456z, 2014.
- [47] K. V. Singh, P. Khanna, C. R. Suri, M. L. Garg, "Structural and immunogenic effects of multiple hapten loading on carrier protein," *Journal of AOAC International*, vol. 93 no. 1, pp. 59-65, DOI: 10.1093/jaoac/93.1.59, 2010.
- [48] R. López-Moreno, J. V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, "Immunoassays for trifloxystrobin analysis. Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies," *Food Chemistry*, vol. 152, pp. 230-236, DOI: 10.1016/j.foodchem.2013.11.150, 2014.
- [49] G. Dong, Y. Pan, Y. Wang, "Preparation of a broad-spectrum anti-zearalenone and its primary analogues antibody and its application in an indirect competitive enzyme-linked immunosorbent assay," *Food Chemistry*, vol. 247, DOI: 10.1016/j.foodchem.2017.12.016, 2018.
- [50] N. Liu, D. Nie, Z. Zhao, X. Meng, A. Wu, "Ultrasensitive immunoassays based on biotin-streptavidin amplified system for quantitative determination of family zearalenones," *Food Control*, vol. 57, pp. 202-209, DOI: 10.1016/j.foodcont.2015.03.049, 2015.
- [51] T. Thongrussamee, N. S. Kuzmina, W.-B. Shim, "Monoclonal-based enzyme-linked immunosorbent assay for the detection of zearalenone in cereals," *Food Additives & Contaminants: Part A*, vol. 25 no. 8, pp. 997-1006, DOI: 10.1080/02652030701823159, 2008.
- [52] A. A. Burkin, G. P. Kononenko, N. A. Soboleva, "Production and analytical properties of antibodies with high specificity to zearalenone," *Prikl Biokhim Mikrobiol*, vol. 38 no. 3, pp. 305-311, DOI: 10.1023/a:1015479626475, 2002.
- [53] D. E. Dixon, R. L. Warner, L. P. Hart, J. J. Pestka, B. P. Ram, "Hybridoma cell line production of a specific monoclonal antibody to the mycotoxins zearalenone and α -zearalenol," *Journal of Agricultural and Food Chemistry*, vol. 35 no. 1, pp. 122-126, DOI: 10.1021/jf00073a026, 1987.
- [54] P. Li, J. Wu, L. Zhang, "Doses of immunogen contribute to specificity spectrums of antibodies against aflatoxin," *Toxins*, vol. 9 no. 5, DOI: 10.3390/toxins9050172, 2017.

DETAILS

Subject:	Antibodies; Toxicity; Food; Reagents; Chemical synthesis; Molecular structure; Hogs; Scientific imaging; Chromatography; Cereals; Immunoassay; Analogs; Immunization; Mass spectrometry; Animals; Ultraviolet spectra; Condensates; Medical research; Butanediol; Residues; Electrophoresis; Chemicals; Antigens; Quantum dots; Zearalenone
Location:	Beijing China; United States--US; China
Editor:	Adil Denizli
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021

Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-05-06 (Received); 2021-06-20 (Revised); 2021-07-12 (Accepted); 2021-07-26 (Pub)
DOI:	https://doi.org/10.1155/2021/7109383
ProQuest document ID:	2559338753
Document URL:	https://www.proquest.com/scholarly-journals/synthesis-zearalenone-immunogen-comparative/docview/2559338753/se-2?accountid=211160
Copyright:	Copyright © 2021 Yanan Wang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-09-01
Database:	Publicly Available Content Database

Comparison of Essential Oils of *Houttuynia cordata* Thunb. from Different Processing Methods and

Harvest Seasons Based on GC-MS and Chemometric Analysis

Pan, Xue ¹

; Li, Haiying ¹

; Chen, Dingfang ²; Zheng, Jinjin ²; Yin, Longhua ²; Zou, Juan ²; Zhang, Yalun ²; Deng, Kaiwen ³;

Xiao, Meifeng ¹; Meng, Lei ¹

; He, Fuyuan ¹

¹ College of Pharmacy, Hunan University of Chinese Medicine, Changsha 410208, China; Hunan Provincial Key Laboratory of Drugability and Preparation Modification of TCM, Changsha 410208, China; Supramolecular Mechanism and Mathematic-Physics Characterization for Chinese Materia Medica, Hunan University of Chinese Medicine, Changsha 410208, China ² College of Pharmacy, Hunan University of Chinese Medicine, Changsha 410208, China ³ The First Affinity Hospital, Hunan University of Chinese Medicine, Changsha 410007, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Houttuyniae Herba (HH) refers to the dried aerial part of *Houttuynia cordata* Thunb. (DHC) or the fresh whole grass of *Houttuynia cordata* Thunb. (FHC), where DHC are harvested in summer and FHC around the year. However, harvest seasons and processing methods (i.e., medicinal parts and drying process) might affect the quality of HH. To compare the essential oils (EOs) of DHC and FHC and their two harvest seasons, GC-MS analysis combined with chemometric analysis was applied. The results showed that the oil yield of FHC ($0.076 \pm 0.030\%$) was higher than that of DHC ($0.038 \pm 0.029\%$), and oil yield was higher in summer than in autumn ($0.044 \pm 0.029\%$ for DHC1, $0.036 \pm 0.028\%$ for DHC2, $0.084 \pm 0.026\%$ for FHC1, and $0.067 \pm 0.033\%$ for FHC2, respectively). Moreover, hierarchical cluster analysis (HCA) and principal component analysis (PCA) successfully distinguished the chemical constituents of DHC and FHC oils. Additionally, according to orthogonal partial least squares discriminant analysis (OPLS-DA), eleven components were selected as chemical markers for discriminating DHC and FHC, and two and four chemical markers for discriminating two harvest seasons of DHC and FHC, respectively. Among these markers, the average contents of α -pinene, limonene, β -phellandrene, α -terpineol, 4-tridecanone, and ethyl decanoate were higher in FHC oils. In contrast, the average contents of nonanal, 1-nonanol, β -cyclocitral, *n*-hexadecanoic acid, and octadecanol were higher in DHC oils. Additionally, the contents of 4-tridecanone and ethyl decanoate were both higher in DHC1 oils than in DHC2 oils. Moreover, the contents of β -myrcene and β -phellandrene were higher in FHC1 oils, while the contents of 2,6-octadien-1-ol, 3,7-dimethyl-, acetate, and (z)-phytol were higher in FHC2 oils. For these reasons, this study provides a scientific basis for quality control and clinical medication.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Houttuynia cordata Thunb. (HC), a promising herbal medicine of the family Saururaceae, is mainly distributed in Asia, including China, India, Thailand, Japan, and Korea. It has a long history of clinical use in traditional Chinese medicine for its several functions, including antifebrile and detoxification, eliminating carbuncle and discharging pus, and promoting diuresis and relieving stranguria [1]. HC mainly contains essential oil (EO), organic acids, flavonoids, phenols, polysaccharides, and alkaloids. EO is one of the main effective parts of HC because it has a variety of pharmacological activities, such as antibacterial, anti-inflammatory, anticancer, antifungal, antiviral, antihistamine,

and antidiabetic activities [1–3]. Thus, investigating the chemical compositions of EO could be an effective approach to evaluate the quality of HC. To date, many researchers studied the chemical constituents of EO of HC. Some research teams revealed the main effective components of EO of HC were 2-undecanone, decanal, decanoyl acetaldehyde, α -pinene, camphene, β -pinene, β -myrcene, lauraldehyde, bornyl acetate, limonene, caryophyllene, 4-terpineol, nonanol, and caryophyllene oxide [3]. In addition, some investigators suggested that medicinal parts, production areas, harvest seasons, and processing methods might influence the chemical composition, which in turn affect the therapeutic efficacy of the herbs [1, 3–5].

According to the 2020 edition of Chinese pharmacopoeia, DHC and FHC are indiscriminately used as one herb medicine called Yuxingcao in Chinese (Houttuyniae Herba), and DHC are harvested in summer and FHC around the year. The different processing methods (i.e., medicinal parts and drying process) and harvest seasons might influence their chemical composition and quality. As DHC and FHC are indiscriminately used in clinic and EO is regarded as one of the main effective parts [1], it is necessary to compare the EOs of DHC and FHC and their two harvest seasons systematically and comprehensively. GC-MS fingerprinting technology is the routine method to analyze EO compounds of HC. However, according to our knowledge, only one study directly compared the content differences of 16 components. The authors found 5 components with obvious differences of 2 batches of DHC and 8 batches of FHC from Emei in China [1]. This intuitive comparison could find some components with obvious differences. However, conventional mutual chemical comparison cannot determine elements that result in quality variance. In recent years, chemical fingerprints combined with chemometric analysis such as HCA, PCA, and OPLS-DA have become powerful tools for the identification and quality assessment of traditional Chinese medicine [6–8]. In this study, GC-MS analysis combined with chemometric methods was first applied to compare EOs of DHC and FHC and their two harvest seasons systematically. HCA and PCA were utilized for the classification and distinction of their EOs. Then, OPLS-DA was further employed to identify the potential chemical markers responsible for the discrimination. In addition, the relative content difference of these chemical markers in DHC and FHC and their two harvest seasons were analyzed, and their pharmacological properties were considered. The aim of this study is to investigate the differences of EOs of DHC and FHC and their two harvest seasons and to provide scientific basis for quality control and clinical medication.

2. Materials and Methods

2.1. Plant Material and Isolation of Essential Oils

Twenty-one batches of DHC and twenty-three batches of FHC were collected from the main production areas in China such as Zhejiang, Guangxi, Jiangxi, Hunan, Guizhou, Sichuan, and Jiangsu, at two harvest seasons (summer season and autumn season). DHC were obtained from the aerial parts of fresh HC, which were dried naturally until the weight was unchanged, while FHC were obtained from the whole grass of fresh HC. The plants were identified by Professor Xiaojiang Zhou (School of pharmacy, Hunan University of Chinese Medicine, Changsha, China). Among all the samples, DHC were labeled as DHC (batches in summer labeled DHC1-1 to DHC1-11 and batches in autumn labeled DHC2-1 to DHC2-10), while FHC were labeled as FHC (batches in summer labeled FHC1-1 to FHC1-12 and batches in autumn labeled FHC2-1 to FHC2-11).

DHC (120g) or FHC (600g) were cut into small segments (2–3 cm) and subjected to hydrodistillation in a Clevenger-type apparatus for four hours for isolation of EOs. The oil yields (% v/w) were calculated in milliliters of oil per 100g of FHC and the aerial parts of fresh HC for preparing DHC. After being dehydrated over anhydrous sodium sulphate, it was stored at approximately 4°C, ready for GC-MS detection. Each EO was diluted 20 times with n-hexane before GC-MS analysis.

2.2. GC-MS Analysis

GC-MS analysis was performed on GC-MS-QP2010 (Shimadzu, Japan) equipped with the DB-5 capillary column (60m $\times$ 250 μ m $\times$ 0.25 μ m film thickness; Agilent). A volume of 1 μ L of each sample was injected. The carrier gas was He (purity > 99.999%) at 1.5 mL/min with a split ratio of 10:1 and the injector temperature was 250°C. The oven temperature was set at 80°C for 2 minutes and then increased to 125°C at a rate of 10°C/min and maintained for 5 min, to 165°C at a rate of 10°C/min and held for 10 min, to 185°C at a rate of 2°C/min, and finally to 240°C at a rate

of 10°C/min and lasted for 20 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV in a scan range of 35–500 m/z. The temperature of the ion source and transmission line was 250°C and 280°C, respectively. The solvent delay was 4 min.

2.3. Data Preprocessing

Peaks were picked up with the signal-to-noise ratio (S/N) > 6. Raw data of GC-MS were transformed into MZXML format by the Shimadzu postrun workstation and finally transformed into ABF format and then processed by Mass Spectrometry-Data Independent Analysis (MS-DIAL) software to detect volatile compounds features and align all the peaks. The components of EO were identified and screened by the MS-DIAL and National Institute of Standards and Technology (NIST) 17.0 Mass Spectra Database. The semiquantitative analysis of volatile compounds was performed by comparing their peak areas in the GC-MS total ion chromatogram. The percentage compositions of common compounds were calculated by the area normalization method.

2.4. Statistical Analysis

Heatmap and HCA were generated on the MetaboAnalyst5.0 (<https://www.metaboanalyst.ca/>) to display the overall difference of chemical compositions of DHC and FHC. PCA and OPLS-DA were applied to investigate the distinction of chemical compositions of DHC and FHC by SIMCA-P 15.0 (Umetrics AB, Umea, Sweden). The significant chemical markers were evaluated based on their p value and differing variable importance in projection (VIP) value calculated with OPLS-DA. One-way ANOVA was performed using SPSS 16.0 (Chicago, IL, USA).

3. Results and Discussion

3.1. EO Yields of DHC and FHC and Their Two Harvest Seasons

The EO yields (% v/w) of DHC and FHC and their two harvest seasons (DHC1 and DHC2; FHC1 and FHC2) are shown in Figure 1. The results showed that the EO yield of DHC ($0.038 \pm 0.029\%$) was significantly lower than that of FHC ($0.076 \pm 0.030\%$) ($p < 0.05$). This was probably due to the loss or decomposition of various volatile components during the drying process [1]. On the other hand, between the two harvest seasons, the EO yields of DHC1 ($0.044 \pm 0.029\%$) were a little higher than that of DHC2 ($0.036 \pm 0.028\%$), and so were FHC1 ($0.084 \pm 0.026\%$) and FHC2 ($0.067 \pm 0.033\%$), indicating that the content of EO was higher in summer than that in autumn. One potential explanation for this finding is that the flowering season of HC was in summer and flowers could produce the most oil [9].

[figure omitted; refer to PDF]

Table 1

Average relative content of sixty-nine common volatile constituents of DHC and FHC at two harvest seasons.

No.	Name/class	RT (min)	Similarity (%)	Average relative content (%)					
DHC (n=21)	FHC (n=23)	DHC 1 (n=11)	DHC2 (n=10)	FHC1 (n=12)	FHC 2 (n=11)	Aliphatic compounds			
1	Nonane	6.47	99	0.02 (0.02)	0.02 (0.03)	0.02 (0.01)	0.03 (0.03)	0.02 (0.03)	0.02 (0.02)
2	2-Nonanone	10.38	96	0.06 (0.10)	0.02 (0.02)	0.09 (0.13)	0.02 (0.01)	0.01 (0.01)	0.03 (0.03)

3	Undecane	10.59	98	0.04 (0.03)	0.02 (0.01)	0.04 (0.04)	0.04 (0.03)	0.01 (0.01)	0.02 (0.01)
4	Nonanal	10.75	90	0.67 (0.68)	0.07 (0.04)	0.81 (0.85)	0.50 (0.39)	0.05 (0.03)	0.08 (0.05)
5	1-Nonanol	12.75	99	3.10 (1.50)	0.49 (0.55)	2.73 (1.15)	3.51 (1.80)	0.31 (0.21)	0.69 (0.73)
6	Decanal	13.88	99	2.68 (6.35)	2.61 (5.48)	2.55 (7.32)	2.82 (5.47)	1.30 (3.26)	4.04 (7.08)
7	1-Decanol	15.53	99	0.22 (0.55)	0.14 (0.20)	0.29 (0.75)	0.13 (0.13)	0.07 (0.08)	0.22 (0.27)
8	2-Undecanone	16.25	92	44.92 (27.15)	48.49 (15.4 8)	44.23 (30.2 9)	45.67 (24.7 3)	45.94 (13.1 8)	51.27 (17.8 7)
9	Undecanal	16.5	99	0.19 (0.40)	0.09 (0.22)	0.25 (0.54)	0.14 (0.16)	0.02 (0.04)	0.17 (0.31)
10	1-Undecanol	18.31	99	0.60 (0.83)	0.17 (0.22)	0.68 (0.97)	0.52 (0.70)	0.17 (0.12)	0.18 (0.30)
11	2-Dodecanone	19.01	91	0.30 (0.31)	0.22 (0.17)	0.28 (0.28)	0.31 (0.36)	0.17 (0.12)	0.28 (0.21)
12	<i>n</i> -Decanoic acid	19.02	98	0.08 (0.05)	0.04 (0.03)	0.06 (0.03)	0.10 (0.07)	0.02 (0.01)	0.05 (0.03)
13	Dodecanal	19.48	99	0.12 (0.17)	0.30 (1.10)	0.07 (0.06)	0.17 (0.23)	0.04 (0.03)	0.58 (1.58)
14	Dodecanol	21.76	99	0.10 (0.19)	0.04 (0.07)	0.12 (0.26)	0.07 (0.04)	0.02 (0.02)	0.06 (0.09)

15	Vinyl decanoate	21.8	89	0.10 (0.05)	0.05 (0.03)	0.08 (0.04)	0.11 (0.05)	0.04 (0.01)	0.06 (0.03)
16	4-Tridecanone	22.66	89	0.55 (0.56)	1.78 (1.11)	0.76 (0.55)	0.32 (0.49)	2.03 (0.79)	1.52 (1.37)
17	2-Tridecanone	22.67	92	2.29 (2.16)	1.18 (1.10)	2.58 (2.20)	1.96 (2.20)	1.41 (1.41)	0.92 (0.58)
18	Ethyl decanoate	27.81	82	0.03 (0.02)	0.80 (0.46)	0.04 (0.02)	0.02 (0.02)	0.81 (0.26)	0.79 (0.63)
19	Tetradecanal	28.4	99	1.48 (1.19)	0.82 (0.38)	1.27 (1.12)	1.72 (1.27)	0.78 (0.39)	0.87 (0.37)
20	Isopentyl decanoate	36.36	78	0.06 (0.04)	0.11 (0.07)	0.06 (0.02)	0.06 (0.06)	0.09 (0.05)	0.13 (0.09)
21	<i>n</i> -Hexadecanoic acid	42.62	94	0.09 (0.06)	0.01 (0.01)	0.07 (0.03)	0.11 (0.07)	0.01 (0.00)	0.02 (0.01)
22	Octadecanol	45.57	99	0.36 (0.39)	0.04(0.03)	0.16 (0.09)	0.58 (0.48)	0.02 (0.01)	0.05 (0.04)
-									
<i>Aromatic compounds</i>									
23	Benzeneacetaldehyde	9.35	92	0.06 (0.05)	0.04 (0.04)	0.05 (0.03)	0.08 (0.06)	0.04 (0.05)	0.03 (0.02)
24	4-Isopropyl-1-methylcyclohex-2-enol	11.55	96	0.08 (0.10)	0.06 (0.08)	0.07 (0.06)	0.10 (0.13)	0.08 (0.10)	0.05 (0.05)
25	2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	19.96	85	0.05 (0.05)	0.02 (0.02)	0.03 (0.02)	0.08 (0.06)	0.01 (0.01)	0.03 (0.02)

26	1,4-Di-Tert-Butylbenzene	21.75	80	0.10 (0.12)	0.03 (0.04)	0.11 (0.17)	0.08 (0.05)	0.02 (0.01)	0.05 (0.06)
-									
<i>Monoterpene hydrocarbons</i>									
27	Bicyclo[3.1.0]hex-2-ene,2-methyl-5-(1-methylethyl)-	7.04	92	0.04 (0.05)	0.06 (0.06)	0.03 (0.04)	0.05 (0.06)	0.05 (0.06)	0.07 (0.07)
28	α -Pinene	7.23	99	0.63 (0.44)	2.18 (1.15)	0.66 (0.43)	0.59 (0.48)	2.24 (1.14)	2.11 (1.22)
29	Camphene	7.54	99	0.18 (0.14)	0.26 (0.08)	0.20 (0.16)	0.15 (0.13)	0.28 (0.08)	0.25 (0.09)
30	(1S)-(-)- β -Pinene	7.92	91	0.77 (1.05)	1.64 (1.79)	0.62 (0.95)	0.95 (1.17)	1.62 (1.97)	1.67 (1.65)
31	β -Pinene	8.09	99	9.95 (7.16)	8.67 (3.43)	10.59 (7.40)	9.25 (7.21)	9.23 (3.93)	8.07 (2.85)
32	β -Myrcene	8.14	99	17.68 (12.99)	18.36 (7.64)	18.85 (13.4 4)	16.39 (13.0 6)	21.33 (8.72)	15.12 (5.58)
33	α -Phellandrene	8.54	98	0.02 (0.02)	0.03 (0.02)	0.02 (0.02)	0.02 (0.01)	0.03 (0.01)	0.04 (0.03)
34	α -Terpinene	8.78	99	0.06 (0.08)	0.16 (0.17)	0.05 (0.07)	0.08 (0.08)	0.13 (0.14)	0.19 (0.20)
35	1-Isopropyl-4-Methylbenzene	8.94	95	0.09 (0.10)	0.08 (0.13)	0.09 (0.11)	0.09 (0.10)	0.06 (0.13)	0.10 (0.14)
36	Limonene	9.06	98	0.37 (0.22)	1.35 (0.55)	0.36 (0.20)	0.38 (0.24)	1.31 (0.52)	1.38 (0.60)

37	β -Phellandrene	9.13	93	0.53 (1.51)	1.82 (2.23)	0.99 (2.02)	0.03 (0.05)	2.82 (2.65)	0.73 (0.83)
38	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	9.31	96	0.07 (0.06)	0.10 (0.09)	0.06 (0.05)	0.08 (0.07)	0.11 (0.10)	0.09 (0.07)
39	2-Octene, 2-methyl-6-methylene-	9.47	80	0.08 (0.07)	0.01 (0.01)	0.09 (0.09)	0.07 (0.05)	0.01 (0.01)	0.02 (0.01)
40	Gamma-terpinene	9.73	99	0.22 (0.28)	0.44 (0.47)	0.19 (0.27)	0.25 (0.29)	0.32 (0.36)	0.56 (0.56)
41	Terpinolene	10.54	96	0.05 (0.04)	0.11 (0.08)	0.05 (0.04)	0.05 (0.04)	0.09 (0.07)	0.14 (0.10)
-									
<i>Sesquiterpene hydrocarbons</i>									
42	Caryophyllene	20.56	99	0.27 (0.13)	0.16 (0.08)	0.23 (0.10)	0.31 (0.15)	0.13 (0.04)	0.19 (0.11)
43	α -Guaiene	22.37	91	0.07 (0.05)	0.03 (0.01)	0.07 (0.05)	0.08 (0.05)	0.03 (0.01)	0.03 (0.02)
44	Azulene, 1,2,3,5,6,7,8,8a- octahydro-1,4-dimethyl-7-(1- methylethenyl)-, [1S- (1. α ,7. α ,8a. β)]-	23.5	91	0.14 (0.08)	0.07 (0.04)	0.13 (0.07)	0.16 (0.08 0	0.07 (0.05)	0.07 (0.03)
-									
<i>Diterpene hydrocarbons</i>									
45	3-Methylene-7,11,15-Trimethyl- 1,6,10,14-Hexadecatetraene	21.19	90	0.19 (0.12)	0.07 (0.04)	0.17 (0.0)	0.21 (0.13)	0.07 (0.04)	0.07 (0.03)
46	m-Camphorene	42.42	95	0.19 (0.18)	0.11 (0.08)	0.19 (0.15)	0.19 (0.21)	0.12 (0.09)	0.09 (0.06)

47	p-Camphorene	43.3	94	0.09 (0.09)	0.05 (0.04)	0.08 (0.07)	0.10 (0.11)	0.06 (0.05)	0.04 (0.04)
48	(E)- β -Farnesene	46.1	78	0.26 (0.27)	0.03 (0.02)	0.22 (0.20)	0.29 (0.33)	0.02 (0.01)	0.04 (0.02)
-									
<i>Oxygenated monoterpenes</i>									
49	(E)-3,7-Dimethyl-2,6-octadien-1-ol	9.06	90	1.55 (1.73)	2.44 (1.99)	1.59 (1.78)	1.51 (1.78)	3.04 (2.44)	1.80 (1.15)
50	(3E,5E)-2,6-Dimethylocta-3,5,7-trien-2-ol	10.62	85	0.07 (0.04)	0.03 (0.02)	0.06 (0.04)	0.09 (0.05)	0.03 (0.02)	0.04 (0.03)
51	2-Methyl-6-Methylene-2,7-octadien-4-ol	12.06	92	0.07 (0.05)	0.04 (0.03)	0.06 (0.04)	0.08 (0.06)	0.03 (0.02)	0.05 (0.03)
52	Isoborneol	13.03	96	0.09 (0.07)	0.05 (0.02)	0.10 (0.07)	0.07 (0.06)	0.04 (0.01)	0.06 (0.02)
53	4-Terpinenol	13.31	99	0.52 (0.55)	0.93 (1.06)	0.53 (0.61)	0.51 (0.51)	0.65 (0.77)	1.25 (1.26)
54	Neral	13.45	87	0.02 (0.02)	0.02 (0.03)	0.02 (0.01)	0.02 (0.03 0)	0.02 (0.03)	0.01 (0.01)
55	α -Terpineol	13.63	99	0.06 (0.03)	0.21 (0.13)	0.07 (0.03)	0.06 (0.02)	0.17 (0.09)	0.26 (0.16)
56	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-	14.06	91	0.06 (0.04)	0.03 (0.02)	0.06 (0.05)	0.05 (0.04)	0.03 (0.02)	0.04 (0.03)
57	β -Cyclocitral	14.36	91	0.12 (0.06)	0.01 (0.01)	0.09 (0.05)	0.15 (0.06)	0.01 (0.00)	0.02 (0.01)

58	3,7-Dimethyl-2,6-octadien-1-ol(trans)	15.1	99	0.18 (0.11)	0.03 (0.01)	0.18 (0.09)	0.17 (0.13)	0.02 (0.01)	0.03 (0.02)
59	4-(1-Methylethenyl)cyclohexene-1-methanol	15.33	86	0.08 (0.06)	0.04 (0.03)	0.05 (0.03)	0.12 (0.07)	0.03 (0.02)	0.05 (0.03)
60	Bornyl acetate	16.18	92	1.89 (0.92)	1.28 (0.26)	1.85 (1.06)	1.93 (0.80)	1.27 (0.22)	1.30 (0.31)
61	α -Terpinyl acetate	17.86	85	0.06 (0.03)	0.07 (0.04)	0.05 (0.03)	0.06 (0.03)	0.06 (0.04)	0.08 (0.04)
62	4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)-, acetate	18.02	90	0.11 (0.08)	0.06 (0.03)	0.09 (0.07)	0.12 (0.08)	0.04 (0.01)	0.07 (0.04)
63	Geranyl acetate	18.62	98	2.65 (1.80)	0.75 (0.46)	2.23 (0.94)	3.12 (2.41)	0.59 (0.30)	0.91 (0.56)
64	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	18.63	94	0.28 (0.21)	0.15 (0.10)	0.22 (0.12)	0.34 (0.27)	0.08 (0.03)	0.23 (0.09)
-									
<i>Oxygenated sesquiterpenes</i>									
65	<i>trans</i> -Nerolidyl formate	26.01	97	0.34 (0.35)	0.07 (0.06)	0.32 (0.27)	0.36 (0.45 0	0.07 (0.05)	0.07 (0.06)
66	Caryophyllene oxide	27.95	96	0.37 (0.25)	0.04 (0.02)	0.34 (0.25)	0.39 (0.27)	0.03 (0.01)	0.05 (0.03)
	Oxygenated diterpenes								
67	5,9,13,17-Tetramethyl 4,8,12,16-Octadecatetraenoic acid	34.51	88	0.08 (0.11)	0.03 (0.04)	0.06 (0.05)	0.11 (0.15)	0.02 (0.01)	0.04 (0.06)

68	Phytol	46.42	96	1.06 (0.94)	0.19 (0.21)	0.54 (0.20)	1.63 (1.11)	0.07 (0.03)	0.33 (0.24)
69	1,6,10,14-Hexadecatetraen-3-ol,3,7,11,15-tetramethyl-, (E,E)-	56.29	81	0.07 (0.05)	0.06 (0.03)	0.05 (0.03)	0.08 (0.07)	0.04 (0.01)	0.08 (0.03)
	Total identified (%)			95.62 (4.63)	98.41 (2.01)	94.88 (5.12)	96.43 (4.13)	99.09 (0.61)	97.67 (2.70)
<i>Class composition</i>									
	Nonterpene compounds			58.34 (23.28)	57.66 (12.3 9)	57.51 (25.6 8)	59.25 (21.6 7)	53.51 (12.0 4)	62.19 (11.6 2)
	Aliphatic compounds			58.04 (23.34)	57.50 (12.4 7)	57.25 (25.7 4)	58.91 (21.7 5)	53.36 (12.1 5)	62.03 (11.6 7)
	Aromatic compounds			0.30 (0.18)	0.15 (0.11)	0.25 (0.18)	0.34 (0.17)	0.15 (0.13)	0.16 (0.09)
	Terpenoids			41.66 (23.28)	42.34 (12.3 9)	42.49 (25.6 8)	40.75 (21.6 7)	46.49 (12.0 4)	37.81 (11.6 2)
	Terpene hydrocarbons			31.95 (21.79)	35.79 (10.9 5)	33.93 (23.1 5)	29.77 (21.2 0)	40.13 (10.0 6)	31.05 (10.2 5)
	Monoterpene hydrocarbons			30.74 (21.40)	35.27 (10.8 5)	32.84 (22.5 3)	28.42 (21.0 4)	39.62 (9.88)	30.52 (10.2 1)
	Sesquiterpene hydrocarbons			0.48 (0.22)	0.26 (0.11)	0.42 (0.19)	0.55 (0.24)	0.23 (0.08)	0.29 (0.13)
	Diterpene hydrocarbons			0.73 (0.62)	0.26 (0.17)	0.66 (0.51)	0.80 (0.75)	0.28 (0.19)	0.24 (0.14)

Oxygenated terpenes			9.72 (4.07)	6.55 (2.09)	8.56 (3.21)	10.99 (4.69)	6.36 (2.33)	6.76 (1.88)
Oil yield (% , v/w)			0.04 (0.03)	0.08 (0.03)	0.04 (0.03)	0.03 (0.03)	0.08 (0.03)	0.07 (0.03)
Total identified (%)			95.62 (4.63)	98.41 (2.01)	94.88 (5.12)	96.43 (4.13)	99.09 (0.61)	97.67 (2.70)

Note: RT represents retention time. Data were presented as mean (SD).

However, it was worth noting that oxygenated terpenoids in DHC (9.72%) were higher than in FHC (6.55%), whereas terpenoids in DHC (31.95%) were lower than in FHC (35.79%). One potential explanation is the loss of the terpenoids or its oxidation to oxygenated terpenoids during the drying process [11, 12]. Moreover, the content of some chemical constituents had intuitive differences and varied with DHC and FHC and two harvest seasons. The contents of the components such as α -pinene, (1S)-(-)- β -pinene, β -myrcene, (E)-3,7-dimethyl-2,6-octadien-1-ol, and β -phellandrene ($2.18 \pm 1.15\%$, $1.64 \pm 1.79\%$, 18.36 ± 7.64 , $2.44 \pm 1.99\%$, and $1.82 \pm 2.23\%$, respectively) were higher in FHC oil than in DHC oil ($0.63 \pm 0.44\%$, $0.77 \pm 1.05\%$, $17.68 \pm 12.99\%$, $1.55 \pm 1.73\%$, and $0.53 \pm 1.51\%$, respectively), while the contents of 1-nonanol, geranyl acetate, 2-tridecanone, tetradecanal, and bornyl acetate were higher in DHC oil ($3.10 \pm 1.50\%$, $2.65 \pm 1.80\%$, $2.29 \pm 2.16\%$, $1.48 \pm 1.19\%$, and $1.89 \pm 0.92\%$, respectively) than in FHC oil ($0.49 \pm 0.55\%$, $0.75 \pm 0.46\%$, $1.18 \pm 1.10\%$, $0.82 \pm 0.38\%$, and $1.28 \pm 0.26\%$, respectively). Compared with the results of DHC and FHC, the differences of oils between two harvest seasons (DHC1 and DHC2; FHC1 and FHC2) were not so obvious. Some constituents such as (E)-3,7-dimethyl-2,6-octadien-1-ol and β -phellandrene in FHC1 oil ($3.04 \pm 2.44\%$ and $2.82 \pm 2.65\%$, respectively) were higher than in FHC2 oil ($1.80 \pm 1.15\%$ and $0.73 \pm 0.83\%$, respectively), while decanal and dodecanal were lower in FHC1 ($1.30 \pm 3.26\%$ and $0.04 \pm 0.03\%$, respectively) than in FHC2 ($4.04 \pm 7.08\%$ and $0.58 \pm 1.58\%$, respectively). The percentage composition of β -phellandrene in DHC1 ($0.99 \pm 2.02\%$) was higher than in DHC2 ($0.03 \pm 0.05\%$), whereas phytol was lower in DHC1 ($0.54 \pm 0.20\%$) than in DHC2 ($1.63 \pm 1.11\%$). These differences between the samples might be attributed to the medicinal parts, drying process, and collection seasons [1, 3]. In order to further explore the distinctions between DHC and FHC and their two harvest seasons, chemometric methods were utilized.

3.3. Heatmap and HCA and PCA Analysis

To make the overall discrepancies in the chemical profiles of DHC and FHC oils more externally, heatmap and HCA analysis were further performed to explore the prominent distinctions between DHC and FHC. Heatmap is one of the most popular bioinformatic graphic displays for data visualization by color intensity. HCA is a clustering technique that measures either the difference or the similarity among the samples to be clustered. Based on the relative contents of each constituent in the EOs of the samples, the position of various samples will be crudely redistributed, and samples with close similarities will be classified into the same group by HCA. As illustrated in Figure 3, the blue box indicated that the content was lower, while the red box showed a greater content than the average level of the sample. It was noted that forty-four batches of samples were apparently classified into two categories; i.e., the first group included twenty-one batches of DHC and the second group twenty-three batches of FHC. Nevertheless, eleven batches of DHC1 and ten batches of DHC2 could not be well clustered; neither were twelve batches of FHC1 nor eleven batches of FHC2. In other words, although there was significant difference in chemical composition between DHC and FHC, the difference between DHC1 and DHC2 and FHC1 and FHC2 was not obvious.

[figure omitted; refer to PDF]

Moreover, another unsupervised PCA approach was employed to provide more information about the discrimination

of DHC and FHC (Figure 4(a)). The results showed that $R^2X=0.773$ and $Q^2=0.519$, which indicated that the model was reliable and had good predictive ability. The score plot showed that the DHC (both blue and green spots) and FHC (both red and yellow spots) were clearly separated. However, DHC1 (green spots) and DHC2 (blue spots) and FHC1 (red spots) and FHC2 (yellow spots) could not be classified obviously. The result was consistent with heatmap and HCA analysis. In general, HCA and PCA are both good classification tools that distinguished DHC and FHC. Still, they could not well classify the samples at two harvest seasons and could not find out the major chemical differential components between them. To find their chemical markers for discrimination, OPLS-DA analysis was applied.

[figures omitted; refer to PDF]

3.4. OPLS-DA Analysis and Identification of Chemical Markers

To further distinguish the major variations responsible for the differentiation and find the chemical markers, a supervised OPLS-DA approach was constructed to maximize sample separation. As indicated in Figure 4(b), the samples of DHC and FHC were distributed on two sides evidently. The variation in X (R^2X_{cum}) was 0.658 and the variation in Y (R^2Y_{cum}) was 0.975, predicting 91.9% of the variation in response to Y ($Q^2_{cum}=0.919$). The value of these parameters demonstrated that the model had high reliability and predictive abilities. To validate the model, 200 times permutation tests were performed (Figure 4(c)). Moreover, the p value and VIP value of the 69 common constituents were obtained. The constituents with VIP value >1.5 and p value <0.01 were regarded as potential chemical markers responsible for the distinctions. As a result, the eleven components including α -pinene, limonene, β -phellandrene, α -terpineol, 4-tridecanone, ethyl decanoate, nonanal, 1-nonanol, β -cyclocitral, n-hexadecanoic acid, and octadecanol had significant qualitative differences, which were screened as chemical markers between DHC and FHC.

Regarding the two harvest seasons of DHC, the samples of DHC1 and DHC2 were clearly classified and aggregated on the scatter plot (Figure 4(d)). The variation in X (R^2X_{cum}) was 0.617 and the variation in Y (R^2Y_{cum}) was 0.974, predicting 70.4% of the variation in response to Y ($Q^2_{cum}=0.704$). Using the same screening criteria as used above, two components, including 4-tridecanone and ethyl decanoate, were identified as chemical markers for DHC1 and DHC2. In addition, the samples of FHC1 and FHC2 were distributed on two sides obviously, as indicated in Figure 4(f). The variation in X (R^2X_{cum}) was 0.637 and the variation in Y (R^2Y_{cum}) was 0.956, predicting 83.3% of the variation in response to Y ($Q^2_{cum}=0.833$). Four components, including β -myrcene, β -phellandrene, 2,6-octadien-1-ol, 3,7-dimethyl-acetate, and (z)-phytol, were identified as chemical markers for FHC1 and FHC2. All the chemical markers are shown in Table 2.

Table 2

Pharmacological effects of chemical markers identified for DHC and FHC, DHC1 and DHC2, and FHC1 and FHC2 from the OPLS-DA validation plot.

Compound		Chemical formula	Classification	p value	VIP value	Pharmacological effects
DHC- FHC	α -Pinen e ^a	C ₁₀ H ₁₆	Monoterpene hydrocarbons	7.37 × 10 ⁻⁸	1.70	Anti-inflammatory [13], antioxidant [14], antitumor [14], antifungal [14], antiallergy, improving ulcer [14], antianxiety [14]

Limonene ^b	$C_{10}H_{16}$	Monoterpene hydrocarbons	8.43×10^{-11}	1.76	Anti-inflammatory [15], antidiabetic effects [16], immunomodulatory activity [17], analgesic [18], hypolipidemic and antioxidant activities [19], antimicrobial activity [20], anticancer activity [20], insecticidal activity [20]	β -Phellandrene ^c
$C_{10}H_{16}$	Monoterpene hydrocarbons	8.75×10^{-7}	2.19	Antitumor activity [21], antioxidant activities [22]	α -Terpineol ^d	$C_{10}H_{18}O$
Oxygenated monoterpenes	5.04×10^{-12}	1.60	Anti-inflammatory and analgesic [23], anticonvulsant [24], gastric protection effects [25]	4-Tridecanone ^e	$C_{13}H_{26}O$	Aliphatic compounds
7.85×10^{-5}	1.53	—	Ethyl decanoate ^f	$C_{12}H_{24}O_2$	Aliphatic compounds	1.23×10^{-16}

2.68	—	Nonanal ^g	$C_9H_{18}O$	Aliphatic compounds	1.03×10^{-7}	1.54
—	1-Nonanol ^h	$C_9H_{20}O$	Aliphatic compounds	8.58 ₉	1.68	—
β -Cyclocitral ⁱ	$C_{10}H_{16}O_6$	Oxygenated monoterpenes	2.07×10^{-12}	1.78	—	<i>n</i> -Hexadecanoic acid ^j
$C_{16}H_{32}O_2$	Aliphatic compounds	2.11×10^{-11}	1.67	Anti-inflammatory [26]	Octadecanol ^k	$C_{18}H_{38}O$
Aliphatic compounds	3.58×10^{-8}	1.68	—	-		
DHC1-DHC2	4-Tridecanone ^l	$C_{13}H_{26}O$	Aliphatic compounds	3.43×10^{-4}	3.34	—
Ethyl decanoate ^m	$C_{15}H_{32}O_4$	Oxygenated sesquiterpenes	2.39×10^{-3}	2.56	—	.
FHC1-FHC2	β -Myrcene ⁿ	$C_{10}H_{16}$	Monoterpene hydrocarbons	4.70×10^{-8}	1.67	Antioxidant [27], liver monooxygenase induction effects [28]
β -Phellandrene ^o	$C_{10}H_{16}$	Monoterpene hydrocarbons	7.60×10^{-3}	2.56	Antitumor activity [21], antioxidant activities [22]	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)- ^p

$C_{12}H_{20}O_2$	Oxygenated monoterpenes	5.29×10^{-5}	2.15	—	Phytol ^q	$C_{20}H_{40}O$

Note: VIP > 1.5 and $p < 0.01$. The symbol “—” stands for none.

3.5. Content Comparison of Chemical Markers and Their Pharmacological Effects

Given that the EO of HC exerts various pharmacological activities [2, 3], we explored whether the above chemical markers possess these activities. As present in Table 2, five chemical markers (α -pinene, limonene, β -phellandrene, α -terpineol, and *n*-hexadecanoic acid) between DHC and FHC and three chemical markers (β -myrcene, β -phellandrene, and phytol) in FHC at two harvest seasons exhibit a wide range of pharmacological activities including anti-inflammatory, ant-oxidant, antiallergy, antibacterial, antiviral, immunologic, anticancer, antianxiety, analgesic, and antidiabetic effects [13–31]. Therefore, the different contents of these chemical markers in DHC and FHC and the two harvest seasons of FHC might influence the quality and efficacy.

As shown in Figure 5, scatter plots were applied to further intuitively compare the content differences of the chemical markers in the three groups (DHC and FHC; DHC1 and DHC2 FHC1 and FHC2). Comparing DHC and FHC, the average contents of α -pinene^a ($0.63 \pm 0.44\%$ and $2.18 \pm 1.15\%$, respectively), limonene^b ($0.37 \pm 0.22\%$ and $1.35 \pm 0.55\%$, respectively), β -phellandrene^c ($0.53 \pm 1.51\%$ and $1.82 \pm 2.23\%$, respectively), α -terpineol^d ($0.06 \pm 0.03\%$ and $0.21 \pm 0.13\%$, respectively), 4-tridecanone^e ($0.55 \pm 0.56\%$ and $1.78 \pm 1.11\%$, respectively), and ethyl decanoate^f ($0.03 \pm 0.02\%$ and $0.80 \pm 0.46\%$, respectively) were higher in FHC oils, while the average contents of nonanal^g ($0.67 \pm 0.68\%$ and $0.07 \pm 0.04\%$, respectively), 1-nonanol^h ($3.10 \pm 1.50\%$ and $0.49 \pm 0.55\%$, respectively), β -cyclocitralⁱ ($0.12 \pm 0.06\%$ and $0.01 \pm 0.01\%$, respectively), *n*-hexadecanoic acid^j ($0.09 \pm 0.06\%$, and $0.01 \pm 0.01\%$, respectively), and octadecanol^k ($0.36 \pm 0.39\%$ and $0.04 \pm 0.03\%$, respectively) from DHC oils were higher than those from FHC oils. Among these markers, α -pinene^a, limonene^b, β -phellandrene^c, and α -terpineol^d higher in FHC had several pharmacological activities, especially anti-inflammatory [13, 15], antioxidant [14, 19], antimicrobial [20], and anticancer activities [14, 20, 21]. Meanwhile, only one chemical marker, *n*-hexadecanoic acid^j, with anti-inflammatory activity [26] was higher in DHC. This result suggested that FHC should be considered as the first choice when it was used as an anti-inflammatory, antioxidant, anticancer, and antibacterial herb. The suggestion was consistent with previous reports [1]. Additionally, the contents of 4-tridecanone^l ($0.76 \pm 0.55\%$ and $0.32 \pm 0.49\%$, respectively) and ethyl decanoate^m ($0.04 \pm 0.02\%$ and $0.02 \pm 0.02\%$, respectively) were higher in DHC1 oils than DHC2 oils. The contents of β -myrceneⁿ ($21.33 \pm 8.72\%$ and $15.12 \pm 5.58\%$, respectively) and β -phellandrene^o ($2.82 \pm 2.65\%$ and $0.73 \pm 0.83\%$, respectively) higher in FHC1 oils had antitumor [21] and antioxidant activities [22, 27], while the contents of 2,6-octadien-1-ol, 3,7-dimethyl-, acetate^p ($0.08 \pm 0.03\%$ and $0.23 \pm 0.09\%$, respectively), and (z)-phytol^q ($0.07 \pm 0.03\%$ and $0.33 \pm 0.24\%$, respectively) with anti-inflammatory and antibacterial activities [29, 30] were higher in FHC2 oils. [figures omitted; refer to PDF]

4. Conclusions

In this work, GS-MS analysis and chemometric methods were applied to compare the oils of DHC and FHC in two harvest seasons. The results showed that EO yields of FHC were higher than DHC, and EO yields of DHC1/FHC1 collected in summer were higher than of DHC2/FHC2 collected in autumn. GC-MS fingerprints showed that DHC and FHC were similar in general and 69 common chemical constituents were characterized. 2-Undecanone, β -myrcene, and β -pinene (accounting for about 70% of the total) were the three most abundant components in both DHC and FHC. Nevertheless, the contents of some constituents were significantly different such as α -pinene, β -phellandrene, 1-nonanol, and geranyl acetate. Moreover, the results of chemometric analysis including HCA, PCA, and OPLS-DA indicated obvious distinction between DHC and FHC and the two harvest seasons of DHC (DHC1 and DHC2) and FHC (FHC1 and FHC2), and OPLS-DA further revealed 11, 2, and 4 components as their potential

chemical markers, respectively. Through this study, we found that the processing methods (i.e., medicinal parts and drying process) and harvest seasons can directly affect the chemical composition of HC and their quality. Future studies are needed to verify whether the differences between them would influence the pharmacological effects to provide a better reference for clinical medication.

Authors' Contributions

Dingfang Chen, Kaiwen Deng, Lei Meng, and Meifeng Xiao were responsible for data curation; Yalun Zhang.; Methodology, Jinjin Zheng, Ronghua Yin, and Fuyuan He conducted the investigation; Juan Zou acquired the resources; Xue Pan and Haiying Li wrote the original draft. All authors have read and agreed to the published version of the manuscript. Xue Pan and Haiying Li contributed equally to this work.

Acknowledgments

This work was financially supported by the Pharmaceutical Open Fund of Domestic First-Class Disciplines of Hunan Province (2018YX06 and 2020YX13) and Changsha Bureau of Science and Technology (kq1907137).

References

- [1] J. Meng, K. S. Leung, Z. Jiang, X. Dong, Z. Zhao, L. J. Xu, "Establishment of GC-MS fingerprint of fresh *Houttuynia cordata*," *Chemical & Pharmaceutical Bulletin*, vol. 53 no. 12, pp. 1484-1489, DOI: 10.1248/cpb.53.1484, 2005.
- [2] O. Se, "An effective quality control of pharmacologically active volatiles of *Houttuynia cordata* Thunb by fast gas chromatography-surface acoustic wave sensor," *Molecules*, vol. 20 no. 6, pp. 10298-10312, 2015.
- [3] R. S. Verma, N. Joshi, R. C. Padalia, "Chemical composition and allelopathic, antibacterial, antifungal, and antiacetylcholinesterase activity of fish-mint (*Houttuynia cordata* Thunb.) from India," *Chemistry & Biodiversity*, vol. 14, DOI: 10.1002/cbdv.201700189, 2017.
- [4] Y. Asakawa, K. Tomiyama, K. Sakurai, Y. Kawakami, Y. Yaguchi, "Volatile compounds from the different organs of *Houttuynia cordata* and *litsea cubeba* (L. Citriodora)," *Journal of Oleo Science*, vol. 66 no. 8, pp. 889-895, DOI: 10.5650/jos.ess17049, 2017.
- [5] J. Chen, W. Wang, C. Shi, J. Wan, L. Deng, J. Fang, "Determination of four volatile compounds with anti-inflammatory biological activity in *Houttuynia chordata* Thunb. by gas chromatography and gas chromatography-mass Spectrometry," *Analytical Letters*, vol. 47 no. 5, pp. 730-741, DOI: 10.1080/00032719.2013.858260, 2014.
- [6] H. Ao, J. Wang, L. Chen, S. Li, C. Dai, "Comparison of volatile oil between the fruits of *Amomum villosum* Lour. and *Amomum villosum* Lour. var. *xanthioides* T. L. Wu et Senjen based on GC-MS and chemometric techniques," *Molecules* (Basel, Switzerland), vol. 24 no. 9, DOI: 10.3390/molecules24091663, 2019.
- [7] Y. Hu, W. Kong, X. Yang, L. Xie, J. Wen, M. Yang, "GC-MS combined with chemometric techniques for the quality control and original discrimination of *Curcuma longae* rhizome: analysis of essential oils," *Journal of Separation Science*, vol. 37 no. 4, pp. 404-411, DOI: 10.1002/jssc.201301102, 2014.
- [8] C. Wang, C. X. Zhang, C. F. Shao, "Chemical fingerprint analysis for the quality evaluation of deepure instant Pu-erh tea by HPLC combined with chemometrics," *Food Analytical Methods*, vol. 9 no. 12, DOI: 10.1007/s12161-016-0524-4, 2016.
- [9] W. F. Yang, Y. Chen, J. Y. Cheng, "Study on the volatile components of different parts of *Houttuynia cordata*," *Chinese Traditional and Herbal Drugs*, vol. 37, pp. 1149-1151, 2006.
- [10] H. Lu, X. Wu, Y. Liang, J. Zhang, "Variation in chemical composition and antibacterial activities of essential oils from two species of *Houttuynia* THUNB," *Chemical and Pharmaceutical Bulletin*, vol. 54 no. 7, pp. 936-940, DOI: 10.1248/cpb.54.936, 2006.
- [11] H. M. Lu, Y. Z. Liang, X. J. Wu, L. Z. Yi, S. Chen, "Comparative study of fingerprints of *Houttuynia cordata* injection made of fresh and dry raw material," *Chinese Journal of Analytical Chemistry*, vol. 34 no. 6, pp. 813-816, 2006.
- [12] L. H. Lan, A Comparative Study between the Active Components of Fresh and Dry Medicinal *Houttuynia* Thunb, 2012.
- [13] S.-Y. Nam, C.-K. Chung, J.-H. Seo, S.-Y. Rah, H.-M. Kim, H.-J. Jeong, "The therapeutic efficacy of α -pinene in

- an experimental mouse model of allergic rhinitis," *International Immunopharmacology*, vol. 23 no. 1, pp. 273-282, DOI: 10.1016/j.intimp.2014.09.010, 2014.
- [14] F. H. Zhu, C. Zhang, F. X. Wei, "Overview of research on pharmacology and application of alpha pinene," *Hans Journal of Medicinal Chemistry*, vol. 3 no. 3, pp. 23-28, 2015.
- [15] S. Chaudhary, M. Siddiqui, M. Athar, M. S. Alam, "D-Limonene modulates inflammation, oxidative stress and Ras-ERK pathway to inhibit murine skin tumorigenesis," *Human & Experimental Toxicology*, vol. 31 no. 8, pp. 798-811, DOI: 10.1177/0960327111434948, 2012.
- [16] M. R. Saravanan, "Antidiabetic effect of d-limonene, a monoterpene in streptozotocin-induced diabetic rats," *Biomedicine & Preventive Nutrition*, vol. 2 no. 4, pp. 269-275, 2012.
- [17] T. J. Raphael, G. Kuttan, "Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillid acid," *Immunopharmacology and Immunotoxicology*, vol. 25 no. 2, pp. 285-294, DOI: 10.1081/iph-120020476, 2003.
- [18] J. F. do Amaral, M. I. G. Silva, M. R. A. de Aquino Neto, "Antinociceptive effect of the monoterpene R-(+)-Limonene in mice," *Biological and Pharmaceutical Bulletin*, vol. 30 no. 7, pp. 1217-1220, DOI: 10.1248/bpb.30.1217, 2007.
- [19] S. Ahmad, Z. H. Beg, "Hypolipidemic and antioxidant activities of thymoquinone and limonene in atherogenic suspension fed rats," *Food Chemistry*, vol. 138 no. 2-3, pp. 1116-1124, 2012.
- [20] P. Erasto, A. M. Viljoen, "Limonene—a review: biosynthetic, ecological and pharmacological relevance," *Natural Product Communications*, vol. 3 no. 7, pp. 1193-1202, DOI: 10.1177/1934578x0800300728, 2008.
- [21] G. Y. Zhang, L. L. Gong, R. M. Dang, "Analysis on main components and antitumor activities of essential oil from *fissistigma cavaleriei* leaves," *Guizhou Agricultural Sciences*, vol. 40 no. 9, pp. 67-69, 2012.
- [22] J. J. Xu, J. Q. Lu, X. S. Li, "Study on spectrum-effect relationship between fingerprints of essential oil from *Shennong Xiangju* and the in vitro antioxidant activities," *Chinese Journal of Pharmaceutical Analysis*, vol. 36 no. 10, pp. 1823-1830, 2016.
- [23] D. O. P. D. Sousa, F. Nóbrega, L. Morais, R. Almeida, "Evaluation of the anticonvulsant activity of terpinen-4-ol," *Zeitschrift Fur Naturforschung C*, vol. 64 no. 1-2, DOI: 10.1515/znc-2009-1-201, 2009.
- [24] J. S. Batista, D. D. Sousa, J. P. Silva, "Gastroprotective activity of α -terpineol in two experimental models of gastric ulcer in rats," *Daru Journal*, vol. 19 no. 4, pp. 277-281, 2011.
- [25] M. Oliveira, R. B. Marques, M. Santana, " α -terpineol reduces mechanical hypernociception and inflammatory response," *Basic & Clinical Pharmacology & Toxicology*, vol. 111 no. 2, pp. 120-125, DOI: 10.1111/j.1742-7843.2012.00875.x, 2012.
- [26] V. Aparna, K. V. Dileep, P. K. Mandal, P. Karthe, C. Sadasivan, M. Haridas, "Anti-inflammatory property of n-hexadecanoic acid: structural evidence and kinetic assessment," *Chemical Biology & Drug Design*, vol. 80 no. 3, pp. 434-439, DOI: 10.1111/j.1747-0285.2012.01418.x, 2012.
- [27] O. Ciftci, I. Ozdemir, S. Tanyildizi, S. Yildiz, H. Oguzturk, "Antioxidative effects of curcumin, β -myrcene and 1,8-cineole against 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced oxidative stress in rats liver," *Toxicology and Industrial Health*, vol. 27 no. 5, pp. 447-453, DOI: 10.1177/0748233710388452, 2011.
- [28] A. C. A. X. De-Oliveira, L. F. Ribeiro-Pinto, S. S. Otto, A. Gonçalves, F. J. R. Paumgartten, "Induction of liver monooxygenases by β -myrcene," *Toxicology*, vol. 124 no. 2, pp. 135-140, DOI: 10.1016/s0300-483x(97)00144-3, 1997.
- [29] R. O. Silva, F. B. M. Sousa, S. R. B. Damasceno, "Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress," *Fundamental & Clinical Pharmacology*, vol. 28 no. 4, pp. 455-464, DOI: 10.1111/fcp.12049, 2014.
- [30] M. T. Ghaneian, M. H. Ehrampoush, A. Jebali, S. Hekmatimoghaddam, M. Mahmoudi, "Antimicrobial activity, toxicity and stability of phytol as a novel surface disinfectant," *Environmental Health Engineering and Management Journal*, vol. 2 no. 1, pp. 13-16, 2015.
- [31] J. D. Moraes, R. D. Oliveira, J. P. Costa, "Phytol, a diterpene alcohol from chlorophyll, as a drug against

neglected tropical disease schistosomiasis mansoni," Plos Neglected Tropical Diseases, vol. 8 no. 1,DOI: 10.1371/journal.pntd.0002617, 2014.

DETAILS

Subject:	Markers; Cluster analysis; Summer; Quality control; Principal components analysis; Seasons; Globular clusters; Essential oils; Discriminant analysis; Chinese medicine; Palmitic acid; Oils & fats; Chemometrics; Terpineol
Location:	China; Japan
Editor:	Pavel Nesterenko
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-04-09 (Received); 2021-06-29 (Revised); 2021-07-05 (Accepted); 2021-07-17 (Pub)
DOI:	https://doi.org/10.1155/2021/8324169
ProQuest document ID:	2554895061
Document URL:	https://www.proquest.com/scholarly-journals/comparison-essential-oils-i-houttuynia-cordata/docview/2554895061/se-2?accountid=211160

Copyright:

Copyright © 2021 Xue Pan et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2021-07-26

Database:

Publicly Available Content Database

Document 24 of 59

Simultaneous Determination of 25 Ginsenosides by UPLC-HRMS via Quantitative Analysis of Multicomponents by Single Marker

Jia, Xiujuan ¹

; Hu, Chenxing ² ; Zhu, Xuepeng ² ; Ye Yuan ²

; Zhou, Yifa ²

¹ National Demonstration Center for Experimental Biology Education, Northeast Normal University, Changchun 130024, China; School of Life Sciences, Northeast Normal University, Changchun 130024, China ² School of Life Sciences, Northeast Normal University, Changchun 130024, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

A method using UPLC-HRMS has been developed for a rapid, simultaneous qualitative and quantitative analysis of twenty-five ginsenosides. Chromatographic separation was achieved on a C₁₈ analytical column with an elution gradient comprising 0.1% aqueous formate/acetonitrile as the mobile phase. HRMS detection acquired full mass data for quantification and fullms-ddms² (i.e., data-dependent scan mode) yielded product ion spectra for identification. Furthermore, quantitative analysis of multiginsenosides by single marker (QAMS) was developed and validated using a relative correction factor. Under optimal conditions, we could simultaneously separate eight groups of isomers of the 25 ginsenosides. Good linearity was observed over the validated concentration range for each analyte ($r^2 > 0.9924$), showing excellent sensitivity (LODs, 0.003–0.349 ng/mL) and lower limit quantification (LOQs, 0.015–1.163 ng/mL). The LC-MS external standard method (ESM) and QAMS were compared and successfully applied to analyze the ginsenoside content from *Panax ginseng* roots. Overall, our UPLC-HRMS/QAMS approach provides high precision, stability, and reproducibility and can be used for high-throughput analysis of complex ginsenosides and quantitative analysis of multiple components and quality control of traditional Chinese medicines (TCM).

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer) has been used as an herbal medication for centuries. Ginsenosides, the major pharmacologically active ingredient in ginseng, have a wide range of pharmacological and therapeutic properties, e.g., improving brain function, preventing cancer, enhancing immune responses, and exhibiting antiaging, antiobesity, and antidiabetic effects [1, 2]. Up to now, about 300 ginsenosides have been detected from ginseng [3]. However, it has remained problematic to quantify all ginsenosides, difficult to distinguish among ginsenosides with the same molecular weight, and hard to acquire sufficient numbers of ginsenoside standards for quantification of their chemical and physical properties.

Several methods have been developed to assess ginsenoside content in ginseng. Among these approaches, high-performance liquid chromatography (HPLC) and HPLC-MS are by far the most employed analytical methods [4–8]. However, HPLC-UV has always shown considerable baseline noise and relatively poor sensitivity due to the weak UV absorption of ginsenosides. Moreover, ginsenosides that have been reported were not well separated, leading to inaccurate quantitation. HPLC-MS is a powerful tool for determining ginsenosides in diverse ginseng extracts [9, 10]. For instance, thirteen ginsenosides were simultaneously and quantitatively determined by HPLC-MS [9]. Among the various LC approaches, ultra-performance LC (UPLC) has provided better separations over short periods of time and increased sample throughput and sensitivity [11]. Among different MS analyzers, Orbitrap has provided much better accuracy, precision, and greater mass resolution [12].

In our present study, we report a novel UPLC-Orbitrap-HRMS approach that has been validated for rapid, simultaneous qualitative/quantitative analysis of twenty-five ginsenosides. UPLC and HRMS methods were optimized to obtain excellent peak separations and maximum signal in the MS detector. Quantitative analysis of complex ginsenosides is difficult in the absence of reference standards. Therefore, a quantitative analysis of the multiginsenoside by single marker (QAMS) was developed and validated using a relative correction factor. The LC-MS external standard method (ESM) and QAMS were compared and successfully applied to analyzing ginsenoside content from *Panax ginseng* root. The present work has provided a sensitive and accurate approach for elucidating ginsenoside constituents that can also be used for multicomponent quality control of TCMs.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC-grade acetonitrile and methanol were purchased for Fisher Scientific (Waltham, MA); ultrapure water (18.25 M Ω /cm) was prepared by a Mili-Q water system (Millipore, Bedford, USA). Reference ginsenosides, including ginsenosides Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, Rb3, F1, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2, and PPD, were isolated or transformed from ginseng and purified by a series of chromatography procedures in our laboratory. The purity of the twenty-five standards was >95%.

2.2. Liquid Chromatography

Ultra-performance liquid chromatography (UPLC) was performed using an UPLC system (Dionex Ultimate 3000, Thermo Scientific, USA) equipped with a binary gradient pump, an autosampler, and a thermostatic column compartment. Chromatographic separation was achieved on a HyperSil GOLD C18 column (2.1 mm $\times$ 50 mm, 1.9 μ m) at a flow rate of 0.35 mL/min. The gradient elution system consisted of water/0.1% formic acid (A) and acetonitrile (B) using the following gradient program: 0–4 min, 23–30% B; 4–8 min, 30% B; 8–8.5 min, 30–35% B; 8.5–12 min, 35% B; 12–12.5 min, 35–39% B; 12.5–16 min, 39% B; 16–17.5 min, 39–41% B; 17.5–20 min, 41–80% B; 20–21 min, 80–95% B; 21–21.5 min, 95–23% B; 21.5–25 min, 23% B. Column temperature was set at 30°C. The temperature of the autosampler was set at 15°C, and the injection volume was 5 μ L.

2.3. Mass Spectrometry

The UPLC system coupled to a Q-Orbitrap HRMS (Thermo-Fisher, USA) with ESI operating in the negative ion mode and using full mass and fullms-ddms² scan types. The UPLC-Q-Orbitrap-HRMS acquired full MS data for quantification over the scan range of m/z 100–1500. The MS resolution was set at 70,000 FWHM, and the AGC target and the maximum inject time were set at 1.0 e⁶ and 100 ms, respectively. The fullms-ddms² (i.e., data-dependent scan mode) provided product ion spectra for identification using the mass inclusion list. The data-

dependent- (dd-) ms^2 resolution was set at 17500 FWHM, and the AGC target was set at 2.0×10^5 with a maximum injection time of 100 ms. The top 5 most intense ions were selected to perform MS/MS acquisition. Precursor ions were filtered by the quadrupole, which operates at an isolation window of m/z 4 Da. The normalized collision energy (NCE) of each ginsenoside was optimized by injecting a working mix standard solution at a concentration of 1 $\mu\text{g/mL}$. UPLC-HRMS data were controlled using Thermo Xcalibur 4.3 software (Thermo Finnigan, San Jose, CA, USA). In addition, the spray voltage was set at 2.8 kV, the capillary temperature was 320°C , and the S-lens RF level was fixed at 50 to obtain the best experimental conditions. Nitrogen was used as the sheath gas with a flow rate set at 40, and the aux gas flow rate set at 10. The aux gas heater temp was 300°C to achieve the highest signal intensity.

2.4. Preparation of Calibration Standards

The standard stock solutions of ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, Rb3, F1, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2 and PPD were prepared by dissolution in methanol at a final concentration of 1.0 mg/mL . Appropriate aliquots of the 25 stock solutions were then mixed to prepare a final standard solution. The stock solution was diluted with methanol to achieve serial working solutions.

2.5. Ginseng Sample Solutions

Freeze-dried ginseng root powder (purchased from Ningbo Gianon Biotech Co., Ltd.) was dissolved in 1 mL methanol and filtered through a $0.22 \mu\text{m}$ filter for further UPLC-Q-Orbitrap-MS analysis.

2.6. Method Validation

2.6.1. Selectivity

The chromatogram of a blank methanol sample was compared with a mixed ginsenoside standard solution and sample solution to investigate whether the target analytes had the same retention time with other unrelated components present in the sample.

2.6.2. Calibration Curve and Sensitivity

The mixed solution of 25 kinds of single standards with concentrations of 0.001, 0.002, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 $\mu\text{g/mL}$ for mass spectrometer detector was injected in sextuplicate ($n=6$). A standard regression line was drawn with the standard sample concentration (x) and peak area (y). The areas under analyte peaks of standards and noise around corresponding analyte peaks were measured by injection samples in sextuplicate ($n=6$). The limit of detection (LOD) had a signal-to-noise ratio of 3 ($S/N=3$), and the limit of quantitation (LOQ) had a signal-to-noise ratio of 10 ($S/N=10$).

2.6.3. Precision and Accuracy

0.2 $\mu\text{g/mL}$ of mixed ginsenoside standard solutions was prepared and injected six consecutive times ($n=6$). The precision obtained by calculating the relative standard deviation (RSD) (set to be less than $\pm 15\%$) and the accuracy were expressed as a relative error (RE) between measured and targeted values required to be within $\pm 15\%$.

2.6.4. Stability

To determine sample stability, solutions were prepared for sequential injections at 0, 2, 6, 12, 24, and 48 h ($n=6$). Specific peaks over accurate mass ranges were identified, and the area under each ginsenoside peak was recorded. The stability was expressed as RSD%.

2.6.5. Reproducibility

The reproducibility of the method was assessed by preparing 0.2 $\mu\text{g/mL}$ of mixed ginsenoside standard solutions in six replicates ($n=6$). After injection, the exact peak with accurate mass range area of each ginsenoside was recorded, and the RSD% was calculated.

2.7. Calculation of Relative Response Factors of Ginsenosides

When a single reference was used to determine multiple components in samples, the concentration of each analyte (C_x) was calculated as the ratio between the peak area of the analyte in the sample solution (A_x) and the peak area of a chosen reference analyte in a standard solution as a unit concentration (A_s/C_s) and then calibrated by the relative response factor (RCF) of each analyte (F_x). The formula is as follows: (1) $C_x = A_x A_s / C_s \times F_x$.

The relative response factor (F_x) for each ginsenoside was calculated as the ratio of the peak area in a unit

concentration between standards (A_s/C_s) and the analyte (A_x/C_x): $(2)F_x = A_s/C_s A_x/C_x$.

It is worth mentioning that the final value of RCF is the average value of multiple RCFs determined using a series of concentrations of the internal reference sample.

For comparison of this new QAMS approach with an ESM, the standard method difference (SMD) was calculated according to the following equations: $(3)SMD = \frac{C_{ESM} - C_{QAMS}}{C_{ESM}} \times 100\%$, where C_{ESM} and C_{QAMS} are the concentrations of analytes assayed by the external standard and QAMS methods, respectively.

3. Results and Discussion

3.1. Optimization of UPLC Conditions

The separation of mixtures of ginsenosides by chromatography is very difficult. This is especially true for the simultaneous separation of eight groups of isomers (Rg1/Rf, Re/Rd/GXVII, Rh1/F1, Rc/Rb2/Rb3, nFe/CO/nFd, Rg2/G75/Rg3/F2, Mc/CY/CMx, and CK/Rh2). The use of an appropriate chromatographic column is a key factor for separating ginsenosides. Therefore, two chromatographic columns, including HyperSil GOLD C18 (2.1 mm × 100 mm, 1.9 μm) and HyperSil GOLD C18 (2.1 mm × 50 mm, 1.9 μm) columns, were employed and compared as shown in Figure S1 in the Supplementary Materials for comprehensive image analysis. Our results demonstrate that the HyperSil GOLD C18 (2.1 mm × 50 mm, 1.9 μm) column not only exhibited better resolution and higher peak capacity but also could separate ginsenoside isomers Rg3 and G75. Therefore, the HyperSil GOLD C18 (2.1 mm × 50 mm, 1.9 μm) column was chosen for this study.

To improve sensitivity and resolution, weak acid has usually been added to the water phase [13, 14]. Using acetonitrile as the organic phase, the effect of formic acid as an additive to the aqueous phase was investigated using the HyperSil GOLD C18 (2.1 mm × 50 mm, 1.9 μm) column. Our results show that the addition of 0.1% aqueous formate/acetonitrile enhanced the intensity of mass signals and improved peak shape, thus providing the optimal choice (Figure S2 in the Supplementary Materials).

The effects of varying flow rate from 0.2 mL/min to 0.35 mL/min were assessed by observing the resolution of each analyte (Figure S3 in the Supplementary Materials). Increasing the flow rate significantly shortened analysis time and improved resolution. At an optimal flow rate of 0.35 mL/min, all isomeric compounds were effectively separated over a short period of time, except for ginsenosides Rg3 and G75. Finally, column temperatures of 30°C, 35°C, and 40°C were investigated. As shown in Figure S4 in Supplementary Materials, increasing the column temperature slightly influenced the retention time and resolution of each ginsenoside. In general, a temperature of 30°C was optimal due to a shorter retention time and better resolution for ginsenosides Rg3 and G75.

Overall, optimal chromatograms for the 25 analytes were obtained within 25 min and showed stable baselines and high resolutions.

3.2. Optimization of ESI-HRMS Conditions

In this study, ESI positive and negative modes were compared. All of the investigated ginsenosides exhibited strong $[M-H]^-$ ions in the negative ion mode but comparatively low-abundance $[M+H]^+$ ions and $[M+Na]^+$ ions in the positive ion mode. Furthermore, the negative ion mode provided more direct structural information, consistent with previous studies [15, 16]. In order to rapidly acquire MS/MS fragmentation data for the identification of chemical constituents, the fullms-ddms² data acquisition mode was used to simultaneously collect the information from both precursor ion and their related fragment ions in a single run. Mass parameters of the spray voltage (2.0–4.0 kV), the capillary temperature (250°C–350°C), and S-lens RF level (30–70) were optimized by infusing standard solutions to achieve maximum responses of precursor ions. Moreover, the sheath gas flow rate (20–40), aux gas flow rate (5–10), and aux gas heater temperature (250°C–350°C) were also optimized manually to achieve the greatest signal intensity. Furthermore, to make optimization easier, the stepped normalized collision energies (NCE) of (10V, 15V, and 20V), (10V, 20V, and 35V), and (20V, 30V, and 40V) were used to obtain representative product ion spectra for each compound.

3.3. Method Validation

To assess the selectivity of this method, chromatograms of a blank methanol sample, ginsenoside standards, and the ginseng sample itself are shown in Figure 1. All ginsenoside isomers were adequately separated on the column

with elution times of 1.46 and 4.08 min for Rg1 and Rf (m/z 799), 1.46, 9.25, and 10.07 min for Re, Rd, and GXVII (m/z 945), 5.02 and 6.73 min for Rh1 and F1 (m/z 637), 6.09, 7.13, and 7.59 min for Rc, Rb2, and Rb3 (m/z 1077), 10.8, 11.61, and 12.01 min for nFe, CO, and nFd (m/z 915), 5.08, 13.68, 15.63, and 15.89 min for Rg2, F2, G75, and Rg3 (m/z 783), 17.29, 18.02, and 18.46 min for Mc, CY, and CMx (m/z 753), 19.31 and 19.6 min for CK and Rh2 (m/z 621), 14.75 min for PPT, and 20.99 min for PPD. There was no interference peak near the corresponding retention time of each target peak, which shows that this method has reasonably good specificity.

[figures omitted; refer to PDF]

In addition, characteristics of calibration curves, including the range of linearity, the square of correlation coefficient (r^2), limit of quantification (LOQ), and limit of detection (LOD) of each ginsenoside, are listed in Table 1.

Table 1

Calibration curves, limit of quantification (LOQ), and limit of detection (LOD) of UPLC-MS methods for determination of 25 ginsenosides.

Ginsenoside	RT	Calibration curve	Test range (ug/mL)	r^2	LOQ (ng/mL)	LOD (ng/mL)
Rg1	1.46	$Y=64984X+980287$	0.02-2	0.9990	0.106	0.032
Re	1.46	$Y=41225.1X+688583$	0.02-2	0.9959	0.164	0.049
Rf	4.08	$Y=42146.9X-44017.1$	0.01-0.5	0.9994	0.116	0.035
Rh1	5.02	$Y=238413X-153145$	0.002-0.2	0.9994	0.025	0.007
Rb1	5.44	$Y=6240.95X+85602.4$	0.02-2	0.9964	1.163	0.349
Rg2	5.08	$Y=52298.5X-612776$	0.05-1	0.9924	0.012	0.003
Rc	6.09	$Y=22275X-24796.1$	0.01-1	0.9991	0.182	0.055
F1	6.73	$Y=292355X-175557$	0.002-0.2	0.9995	0.029	0.009
Rb2	7.13	$Y=20778.7X-36514.3$	0.01-1	0.9994	0.313	0.094
Rb3	7.59	$Y=20695.1X+48835.8$	0.05-2	0.9994	0.513	0.154
Rd	9.25	$Y=61029.8X-558838$	0.1-2	0.9989	0.015	0.005
G17	10.07	$Y=85248.5X-57819.7$	0.002-0.2	0.9998	0.217	0.065
nFe	10.8	$Y=29919.9X-7299.02$	0.002-0.2	0.9996	0.192	0.058
CO	11.61	$Y=31396.7X-13591.7$	0.001-0.2	0.9990	0.217	0.065
nFd	12.01	$Y=20835.8X-25094.8$	0.01-0.2	0.9959	0.833	0.250

F2	13.68	$Y=212189X-115446$	0.002-0.2	0.9998	0.029	0.009
G75	15.63	$Y=231218X-99660.6$	0.001-0.2	0.9991	0.042	0.013
Rg3	15.89	$Y=149379X-82845.1$	0.002-0.2	0.9993	0.154	0.046
PPT	14.75	$Y=27249.2X-50845.3$	0.002-0.05	0.9984	0.150	0.045
Mc	17.29	$Y=58309.3X-14125.5$	0.001-0.2	0.9995	0.294	0.088
CY	18.02	$Y=41233.6X+36194$	0.001-0.2	0.9993	0.175	0.053
CMx	18.46	$Y=80902.7X-22662.5$	0.002-0.2	0.9990	0.189	0.057
CK	19.31	$Y=140955X+96724.4$	0.001-0.2	0.9998	0.018	0.005
Rh2	19.6	$Y=316346X-188172$	0.002-0.2	0.9996	0.016	0.005
PPD	20.99	$Y=3099.89X-4843.21$	0.002-0.2	0.9990	0.535	0.160

All compounds showed excellent linearity over a relatively wide concentration range, with correlation coefficients (r^2) of all calibration curves ranging from 0.9924 to 0.9998. The range of LOQs was within the range of 0.015–1.163 ng/mL. The range of LODs fell within the range of 0.003–0.349 ng/mL. In the stability test, analytes did not significantly degrade after storage of test solutions at room temperature for 48 h ($RSD \leq 3.77\%$). Additionally, variation in RSD reproducibility was less than 3.86%. In terms of precision, RSDs ranged from 0.52% to 2.37%, and accuracy (RE%) ranged from 0.06% to 3.92%. These results are shown in Table 2, with all values being within an acceptable range.

Table 2

Instrument precision, accuracy, sample stability, and repeatability of the assay ($n=6$).

Ginsenoside	Precision (RSD%)	Accuracy (RE%)	Stability (RSD%)	Repeatability (RSD%)
Rg1	0.88	0.06	1.05	0.92
Re	0.97	0.87	1.23	1.26
Rf	0.78	0.11	0.84	1.10
Rh1	0.63	0.25	0.68	1.41
Rb1	0.93	1.06	1.89	1.24
Rg2	0.52	3.92	1.71	1.45
Rc	1.76	2.13	1.56	1.58

F1	0.91	0.93	0.95	1.03
Rb2	1.38	2.24	1.77	1.14
Rb3	1.55	1.07	2.71	2.00
Rd	1.60	1.64	0.70	0.78
GXVII	0.79	0.31	2.14	2.01
nFe	1.93	0.22	3.77	3.31
CO	0.97	0.11	1.54	2.48
nFd	1.55	0.22	2.15	1.74
F2	0.74	1.71	0.65	1.34
G75	1.16	2.60	1.06	3.40
Rg3	1.85	3.65	0.71	1.58
PPT	1.63	0.36	1.32	3.86
Mc	1.10	0.73	1.84	2.15
CY	1.02	0.19	2.87	1.86
CMx	0.67	0.17	1.36	1.39
CK	0.85	2.07	1.76	1.79
Rh2	1.42	2.25	1.13	1.17
PPD	2.37	0.94	1.08	1.23

RSD, relative standard deviation, RE, relative error.

3.4. Qualitative Analysis of Ginsenosides

Twenty-five authentic standards and ginsenoside mixture from *Panax ginseng* root were determined using UPLC-Orbitrap-HRMS under optimized conditions. Typical total ion chromatograms are shown in Figure 1. In the present study, the use of the fullms-ddms² acquisition mode with scans ranging from 100 to 1500 m/z in negative ion mode combined with MS/MS fragmentation for the top 5 ions was proposed to aid in the structural identification of the components. The formula data, retention time, and experimental molecular mass and MS/MS fragment information are shown in Table 3. This provided abundant information that can be used as the basis for identifying constituents from *Panax ginseng* roots. Here, a total of 43 ginsenosides were identified. Among the 43 ginsenosides found, 25 ginsenosides (including peaks 3, 4, 5, 8, 9, 11, 12, 15, 17, 18, 21, 22, 23, 24, 25, 30, 31, 33, 34, 36, 37, 38, 39, 42,

and 43) were identified as Rg1, Re, Rf, Rh1, Rg2, Rb1, Rc, F1, Rb2, Rb3, Rd, GXVII, nFe, CO, nFd, F2, PPT, G75, Rg3, Mc, CY, CMx, CK, Rh2, and PPD, by comparing retention times and high-resolution accurate mass and ion fragment data with the reference to available standard ginsenosides. In addition, the other 18 ginsenosides were tentatively identified using high-resolution accurate mass and ion fragments and the retention sequence by comparing values with those from the literature [3, 17–19].

Table 3

Characterization of compounds in *Panax ginseng* roots using UPLC-HRMS.

No.	Identification	Formula	T_R (min)	$[M+COOH]^-$ (m/z)	$[M-H]^-$ (m/z)	MS ² fragment ions (m/z)
1	20-Glc-ginsenoside Rf	C ₄₈ H ₈₂ O ₁₉	1.05	1007.5447	961.5384	799.4883[M-H-Glc] ⁻ 637.4325[M-H-2Glc] ⁻ 475.3785[M-H-3Glc] ⁻
2	Notoginsenoside R1 (nR1)	C ₄₇ H ₈₀ O ₁₈	1.17	977.5344	931.5288	799.4872[M-H-Xyl] ⁻ 637.4320[M-H-Xyl-Glc] ⁻ 475.3792[M-H-Xyl-2Glc] ⁻
3*	Ginsenoside Rg1	C ₄₂ H ₇₂ O ₁₄	1.42	845.4910	799.4869	637.4324[M-H-Glc] ⁻ 475.3803[M-H-2Glc] ⁻
4*	Ginsenoside Re	C ₄₈ H ₈₂ O ₁₈	1.46	991.5502	945.5451	799.4931[M-H-Rha] ⁻ 783.4944[M-H-Glc] ⁻ 637.4338[M-H-Glc-Rha] ⁻ 475.3784[M-H-2Glc-Rha] ⁻
5*	Ginsenoside Rf	C ₄₂ H ₇₂ O ₁₄	4.05	845.4898	799.4855	637.4332[M-H-Glc] ⁻ 475.3791[M-H-2Glc] ⁻
6	Ginsenoside Ra3	C ₅₉ H ₁₀₀ O ₂₇	4.33	1285.6436	1239.6372	1107.6023[M-H-Xyl] ⁻ 945.5419[M-H-Xyl-Glc] ⁻ 783.4863[M-H-Xyl-2Glc] ⁻ 621.4360[M-H-Xyl-3Glc] ⁻
7	Ginsenoside F3/F5	C ₄₁ H ₇₀ O ₁₃	4.48	815.4810	769.4754	637.4333[M-H-Ara] ⁻ 475.3789[M-H-Ara-Glc] ⁻
8*	Ginsenoside Rh1	C ₃₆ H ₆₂ O ₉	4.96	683.4381	637.4322	475.3778[M-H-Glc] ⁻
9*	Ginsenoside Rg2	C ₄₂ H ₇₂ O ₁₃	5.03	829.4963	783.4898	637.4306[M-H-Rha] ⁻ 475.3807[M-H-Rha-Glc] ⁻
10	Ginsenoside Ra2	C ₅₈ H ₉₈ O ₂₆	5.23	1255.6328	1209.6276	1077.5858[M-H-Xyl] ⁻ 945.5414[M-H-Xyl-Ara (f)] ⁻ 783.4915[M-H-Xyl-Ara (f)-Glc] ⁻ 621.4380[M-H-Xyl-Ara (f)-2Glc] ⁻ 459.3847[M-H-Xyl-Ara (f)-3Glc] ⁻

11*	Ginsenoside Rb1	C ₅₄ H ₉₂ O ₂₃	5.40	1153.6014	1107.5 956	945.5421[M-H-Glc] ⁻ 783.4899[M-H-2Glc] ⁻ 621.4374[M-H-3Glc] ⁻ 459.3840[M-H-4Glc] ⁻
12*	Ginsenoside Rc	C ₅₃ H ₉₀ O ₂₂	6.06	1123.5912	1077.5 854	945.5413[M-H-Ara (f)] ⁻ 783.4907[M-H-Ara (f)-Glc] ⁻ 621.4377[M-H-Ara (f)-2Glc] ⁻ 459.3847[M-H-Ara (f)-3Glc] ⁻
13	Ginsenoside Ra2-isomer	C ₅₈ H ₉₈ O ₂₆	6.27	1255.6329	1209.6 266	1077.5870[M-H-Xyl] ⁻ 945.5418[M-H-Xyl-Ara (f)] ⁻ 783.4917[M-H-Xyl-Ara (f)-Glc] ⁻ 459.3863[M-H-Xyl-Ara (f)-3Glc] ⁻
14	Ginsenoside Ro	C ₄₈ H ₇₆ O ₁₉	6.44	-	955.49 15	793.4398[M-H-Glc] ⁻ 631.3837[M-H-2Glc] ⁻ 455.3540[M-H-2Glc-GlcA] ⁻
15*	Ginsenoside F1	C ₃₆ H ₆₂ O ₉	6.64	683.4385	637.42 88	475.3827[M-H-Glc] ⁻
16	Ginsenoside Ra1	C ₅₈ H ₉₈ O ₂₆	6.68	1255.6334	1209.6 271	1077.5790[M-H-Xyl] ⁻ 783.4831[M-H-Xyl-Ara (p)-Glc] ⁻ 621.4320[M-H-Xyl-Ara (p)-2Glc] ⁻
17*	Ginsenoside Rb2	C ₅₃ H ₉₀ O ₂₂	7.09	1123.5912	1077.5 859	945.5415[M-H-Ara (p)] ⁻ 783.4908[M-H-Ara (p)-Glc] ⁻ 621.4387[M-H-Ara (p)-2Glc] ⁻ 459.3847[M-H-Ara (p)-3Glc] ⁻
18*	Ginsenoside Rb3	C ₅₃ H ₉₀ O ₂₂	7.48	1123.5916	1077.5 838	945.5406[M-H-Xyl] ⁻ 783.4916[M-H-Xyl-Glc] ⁻ 621.4372[M-H-Xyl-2Glc] ⁻ 459.3855[M-H-Xyl-3Glc] ⁻
19	Ginsenoside Ra1-isomer	C ₅₈ H ₉₈ O ₂₆	8.03	1255.6338	1209.6 284	1077.5868[M-H-Xyl] ⁻ 945.5466[M-H-Xyl-Ara (p)] ⁻ 783.4907[M-H-Xyl-Ara (p)-Glc] ⁻ 621.4337[M-H-Xyl-Ara (p)-2Glc] ⁻ 459.3868[M-H-Xyl-Ara (p)-3Glc] ⁻
20	Chikusetsusaponin Iva (CS-Iva)	C ₄₂ H ₆₆ O ₁₄	8.91	-	793.43 95	631.3858[M-H-Glc] ⁻ 455.3555[M-H-Glc-GlcA] ⁻
21*	Ginsenoside Rd	C ₄₈ H ₈₂ O ₁₈	9.21	991.5497	945.54 38	783.4911[M-H-Glc] ⁻ 621.4377[M-H-2Glc] ⁻ 459.3850[M-H-3Glc] ⁻
22*	Gypenoside XVII	C ₄₈ H ₈₂ O ₁₈	10.0 1	991.5506	945.54 32	783.4890[M-H-Glc] ⁻ 621.4405[M-H-2Glc] ⁻
23*	Notoginsenoside Fe (nFe)	C ₄₇ H ₈₀ O ₁₇	10.7 4	961.5374	915.53 48	783.4901[M-H-Ara (f)] ⁻ 621.4399[M-H-Ara (f)-Glc] ⁻ 459.3845[M-H-Ara (f)-2Glc] ⁻
24*	Compound O (CO)	C ₄₇ H ₈₀ O ₁₇	11.1 7	961.5401	915.53 27	783.4915[M-H-Ara (p)] ⁻ 621.4379[M-H-Ara (p)-Glc] ⁻ 459.3847[M-H-Ara (p)-2Glc] ⁻

25*	Notoginsenoside Fd (nFd)	C47H 80O17	11.5 4	961.5401	915.53 30	783.4908[M-H-Xyl] ⁻ 621.4370[M-H-Xyl-Glc] ⁻ 459.3854[M-H-Xyl-2Glc] ⁻
26	Ginsenoside Rg6	C42H 70O12	12.0 9	811.4872	765.47 98	619.4215[M-H -Rha] ⁻
27	Ginsenoside Rk3	C36H 60O8	12.5 1	665.4287	619.33 49	—
28	Ginsenoside F4	C42H 70O12	12.9 0	811.4871	765.47 86	619.4219[M-H-Rha] ⁻ 457.3643[M-H-Rha-Glc] ⁻
29	Ginsenoside Rh4	C36H 60O8	13.2 4	665.4284	619.33 44	—
30*	Ginsenoside F2	C42H 72O13	13.6 2	829.4974	783.48 61	621.4400[M-H-Glc] ⁻ 459.3816[M-H-2Glc] ⁻
31*	PPT	C30H 52O4	14.6 9	521.3853	475.37 92	—
32	Glycoside D3a	C42H 66O14	14.7 0	-	793.43 92	613.3735[M-H-Glc-H ₂ O] ⁻ 455.3517[M-H-Glc-GlcA] ⁻
33*	Gypenoside LXXV (G75)	C42H 72O13	15.5 1	829.4975	783.49 13	621.4393[M-H-Glc] ⁻ 459.3844[M-H-2Glc] ⁻
34*	Ginsenoside Rg3	C42H 72O13	15.7 9	829.4951	783.49 12	621.4393[M-H-Glc] ⁻ 459.3845[M-H-2Glc] ⁻
35	20 (R)-Rg3	C42H 72O13	16.2 8	829.4974	783.49 05	621.4382[M-H-Glc] ⁻ 459.3864[M-H-2Glc] ⁻
36*	Ginsenoside Mc	C41H 70O12	17.3 1	799.4866	753.47 93	621.4371[M-H-Ara (f)] ⁻ 459.3869[M-H-Ara (f)-Glc] ⁻
37*	Compound Y (CY)	C41H 70O12	17.9 4	799.4868	753.48 04	621.4374[M-H-Ara (p)] ⁻ 459.3866[M-H-Ara (p)-Glc] ⁻
38*	Compound Mx (CMx)	C41H 70O12	18.4 2	799.4869	753.47 70	621.4406[M-H-Xyl] ⁻ 459.3858[M-H-Xyl-Glc] ⁻
39*	Compound K (CK)	C36H 62O8	19.3 0	667.4473	621.43 92	459.3828[M-H-Glc] ⁻

40	Ginsenoside Rk1	C42H 70O12	19.4 6	811.4865	765.48 03	603.4224[M-H-Glc] ⁻
41	Ginsenoside Rg5	C42H 70O12	19.5 5	811.4869	765.48 43	603.4230[M-H-Glc] ⁻
42*	Ginsenoside Rh2	C53H 90O22	19.5 9	667.4438	621.43 72	459.3831[M-H-Glc] ⁻
43*	PPD	C30H 52O3	20.9 9	505.3909	459.38 43	—

*Identified with a standard reference.

The production of adduct ions depends on the modifier added in the mobile phase [20, 21]. In this study, we added 0.1% formic acid in water that facilitated formation of the [M+COOH]⁻ solvent adduct ion. The [M-H]⁻ ion was generated from the [M+COOH]⁻ ion, following the loss of one HCOOH unit. The ginsenosides were grouped structurally as having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. The glycosidic bond was easily broken and then the common fragmentation patterns of ginsenosides were simultaneously or successively lost as glycosidic units until the formation of [Aglycon-H]⁻ ions. The species and amount of sugar moieties were detected in MS/MS data, where mass differences of 162 Da and 146 Da suggested the presence of a β -D-glucose and α -L-rhamnose, respectively. The mass difference of 132 Da indicated the presence of a pentose (β -D-xylose or α -L-arabinose (pyranose or furanose)) fragment. Based on the structural characteristics of ginsenosides, PPTs and PPDs generated aglycon ions at m/z 475 and m/z 459, respectively. As shown in Table 3, there are nine PPT ginsenosides, including compounds 1, 2, 3, 4, 5, 7, 8, 9, and 15 and twenty-three PPDs ginsenosides such as compounds 6, 10, 11, 12, 13, 16, 17, 18, 19, 21, 22, 23, 24, 25, 30, 33, 34, 35, 36, 37, 38, 39, and 42. As an example, peak 11 (RT=5.40), identified as ginsenoside Rb1, produced [protopanaxadiol-H]⁻ at m/z 459.3840 in the MS/MS spectrum by successive loss of four Glc-H₂O (162 Da) groups. As another example, peak 4 (RT=1.46), identified as ginsenoside Re, produced [protopanaxatriol-H]⁻ at m/z 475.3784 via successive elimination of one Rha, one Glc, and two Glc, respectively. Moreover, the OAs displayed an aglycone ion m/z 455 corresponding to [oleanolic acid-H]⁻. There are three OAs (e.g., compounds 14, 20, and 32). Peak 20 was identified as Chikusetsusaponin Iva by comparing retention times and ion fragments with those previously reported [22]. This peak displayed a [M-H]⁻ ion at m/z 793.4395. In the fullms-ddms² experiment, two major fragment ions at m/z 631.3858 and 455.3555 were observed, indicating that the structure of saponins contains one glucose and one glucuronate group. The fragment ion at m/z 455.3555 indicated the presence of an oleanolic acid aglycon moiety that lost all linked glycosidic units. These results are shown in Figure 2.

[figures omitted; refer to PDF]

3.5. Quantitative Analysis of Ginsenosides by QAMS

3.5.1. Relative Response Factors of Ginsenosides

Selecting a proper internal standard is vital for the accuracy of multicomponents using QAMS. Using Rb2 as an internal reference (1.00), the relative response factors (RCF) against ginsenoside Rb2 for all of the ginsenoside standards for the m/z signals of the exact peaks with accurate mass ranges in the full scan mode were established by using five different concentrations. The average RCF of each compound, calculated using Equation (2), can be seen in Table 4. It should be noted that the RSDs of the RCFs of all ginsenosides were less than 5.08%, suggesting that the RCFs obtained on the same instrument at different concentrations have good reproducibility.

Table 4

Relative response factor (RCF) and relative standard deviation (RSD) values of 25 ginsenosides.

Ginsenoside	Concentration (ng/mL)					Mean	RSD (%)
10	20	50	100	200	Rb2	1.00	1.00
1.00	1.00	1.00	—	—	Rg1	0.32	0.30
0.31	0.30	0.30	0.31	2.92	Re	0.48	0.46
0.47	0.48	0.46	0.47	2.13	Rf	0.48	0.48
0.52	0.48	0.50	0.49	3.64	Rb1	3.45	3.25
3.05	3.18	3.22	3.23	4.48	Rg2	0.44	0.43
0.46	0.42	0.42	0.43	3.86	Rh1	0.09	0.09
0.09	0.09	0.08	0.09	5.08	Rc	0.93	0.88
0.94	0.94	0.92	0.92	2.70	F1	0.07	0.08
0.08	0.08	0.08	0.08	2.83	Rb3	0.97	0.97
0.98	0.99	0.98	0.98	0.86	Rd	0.36	0.34
0.35	0.34	0.34	0.35	2.59	GXVII	0.24	0.24
0.25	0.23	0.24	0.24	2.95	nFe	0.62	0.67
0.66	0.69	0.68	0.66	4.07	CO	0.60	0.62
0.63	0.66	0.65	0.63	3.78	nFd	1.05	1.00
1.05	0.99	1.05	1.03	2.95	F2	0.10	0.10
0.09	0.10	0.10	0.10	4.56	G75	0.09	0.09
0.09	0.08	0.09	0.09	5.08	Rg3	0.14	0.14
0.14	0.13	0.14	0.14	3.24	PPT	1.00	0.97
0.95	0.99	1.01	0.98	2.45	Mc	0.33	0.34
0.34	0.36	0.34	0.34	3.20	CY	0.47	0.48
0.50	0.49	0.47	0.48	2.71	CMx	0.24	0.25

0.25	0.26	0.25	0.25	2.83	CK	0.14	0.13
0.14	0.15	0.14	0.14	5.05	Rh2	0.06	0.07
0.07	0.07	0.07	0.07	3.24	PPD	6.48	6.95

3.5.2. Reproducibility of RCFs and Relative Retention Times

The effects of using various column temperatures (30°C, 35°C, and 40°C) and flow rates (0.25, 0.3, and 0.35 mL/min) on RCF were investigated in order to evaluate the reproducibility of the QAMS approach. Results are shown in Table 5. RSDs measured under different conditions were all less than 5.97%, indicating that the RCF calculated by the established QAMS method has good reproducibility. When the content of analytes is determined by QAMS, the correct localization of the peaks is crucial. The relative retention time (RT_R) between measured components and the internal reference (Rb2) was calculated here using the ratio of t_{Rk} to t_{Rs} . RSDs of RT_R were calculated to evaluate the influence of column temperature (30°C, 35°C, and 40°C) and flow rate (0.25, 0.3, and 0.35 mL/min). Our results demonstrated that RSDs measured at different temperatures were less than 1.79%, and at different flow rates were less than 7.60%. This indicates that RT_R is reproducible at different temperatures when the flow rate is constant. Thus, RT_R can be used to accurately locate the chromatographic peak of each analyte.

Table 5

Effects of column temperature and flow rate upon relative response factor (RCF) and relative retention time (RT_R).

		Effects of column temperature				Effects of flow rate		
RCF		RT_R		RCF		RT_R		Ginse nosid e
Mean	RSD%	Mean	RSD %	Mean	RSD %	Mean	RSD %	Rg1
0.29	3.45	0.21	0.00	0.30	3.33	0.22	5.33	Re
0.47	2.44	0.21	0.00	0.49	2.37	0.22	4.55	Rf
0.48	1.21	0.56	1.79	0.48	1.19	0.58	1.00	Rb1
3.08	3.83	0.77	0.75	3.19	1.58	0.76	2.02	Rg2
0.34	4.45	0.70	1.43	0.35	1.63	0.72	0.81	Rh1
0.09	3.94	0.70	1.66	0.09	3.32	0.71	0.00	Rc
0.91	3.34	0.86	0.67	0.92	0.00	0.85	1.18	F1
0.08	0.00	0.95	0.61	0.08	0.00	0.96	1.04	Rb3

0.98	3.11	1.06	0.00	0.99	1.01	1.07	0.54	Rd
0.32	3.13	1.29	0.45	0.33	1.73	1.24	5.26	GXVII
0.25	4.00	1.41	0.41	0.25	2.28	1.36	4.80	nFe
0.67	1.49	1.52	0.38	0.68	0.85	1.46	4.12	CO
0.64	2.40	1.64	0.71	0.63	2.44	1.58	3.16	nFd
1.06	1.44	1.69	0.34	1.05	1.45	1.64	3.37	F2
0.10	5.97	1.92	0.52	0.10	0.00	1.95	6.48	G75
0.09	3.27	2.19	0.00	0.09	4.61	2.12	3.55	Rg3
0.13	4.56	2.21	0.90	0.13	0.00	2.16	3.50	PPT
1.03	2.03	2.05	1.49	1.02	1.96	1.96	5.61	Mc
0.34	2.94	2.43	0.24	0.35	1.67	2.33	4.54	CY
0.45	4.44	2.53	0.40	0.45	3.37	2.41	6.08	CMx
0.26	3.85	2.59	1.68	0.28	5.52	2.45	7.54	CK
0.11	5.41	2.69	1.14	0.10	5.59	2.53	7.50	Rh2
0.07	5.09	2.72	1.18	0.07	3.36	2.57	7.60	PPD

3.5.3. Quantitative Measurement of *Panax ginseng* Roots

In this study, a new UPLC-HRMS approach combined with the external standard method (ESM) and QAMS was developed to simultaneously determine 25 ginsenosides from *Panax ginseng* root, with calibration curve-based quantification provided in Table 6. The comparison of the two methods on the precision and accuracy is listed in Table S1 (in Supplementary Materials). The results indicated that the precision (RSD%) range of ESM and QAMS was 0.52%–2.37% and 0.45%–3.44%, respectively, and the accuracy (RE%) range was 0.06%–3.92% and 0.03%–5.01%, respectively. Both the ESM method and QAMS method had good precision and accuracy for the contents of 25 ginsenosides within the allowable error range. Using the ESM method, the amounts of Rg1, Re, Rf, Rh1, Rg2, and F1, which are all PPTs type, were calculated as 53.85, 151.28, 29.34, 1.39, 29.22, and 0.55 mg/g, respectively. The amounts of Rb1, Rc, Rb2, Rb3, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, Mc, CY, CMx, CK, and Rh2, which are PPDs type, were detected as 324.15, 97.77, 112.03, 17.94, 97.66, 2.56, 3.92, 2.22, 1.75, 1.25, 0.11, 2.95, 0.91, 2.42, 0.38, 1.42, and 0.33 mg/g, respectively. The aglycon of PPT and PPD was detected as 1.03 and 0.47 mg/g, respectively. Among them, Re, Rb1, Rc, Rb2, and Rd had the highest content in *Panax ginseng* root. Moreover, the content of F1, G75, PPT, Mc, CMx, Rh2, and PPD was generally low.

Table 6

Comparative experiment results of the external standard method (ESM) and quantitative analysis of

multiginsenosides by single marker method (QAMS) (mg/g).

No.	Ginsenoside	ESM	QAMS	SMD%
1	Rg1	53.85	53.80	0.10
2	Re	151.28	143.83	4.92
3	Rf	29.34	29.47	0.46
4	Rb1	324.15	316.05	2.50
5	Rg2	29.22	30.21	3.38
6	Rh1	1.39	1.35	2.54
7	Rc	97.77	96.82	0.97
8	F1	0.55	0.57	2.52
9	Rb2	112.03	112.03	-
10	Rb3	17.94	17.88	0.38
11	Rd	97.66	98.03	0.38
12	GXVII	2.56	2.54	0.91
13	nFe	3.92	3.71	5.33
14	CO	2.22	2.20	0.52
15	nFd	1.75	1.76	0.50
16	F2	1.25	1.24	0.71
17	G75	0.11	0.10	5.45
18	Rg3	2.95	2.86	3.05
19	PPT	1.03	1.01	1.53
20	Mc	0.91	0.88	3.69
21	CY	2.42	2.42	0.21

22	CMx	0.38	0.37	1.40
23	CK	1.42	1.41	0.18
24	Rh2	0.33	0.32	4.12
25	PPD	0.47	0.46	2.09

At present, QAMS, as an alternative approach for the quality control of TCMs, has attracted increasing interest in terms of quality assessment and control of complex multicomponent systems [23, 24]. In this study, the content of 25 compounds was determined using the QAMS method. Using Rb2 as internal reference (1.00), RCFs of Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rc, F1, Rb3, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2, and PPD were 0.31, 0.47, 0.49, 3.23, 0.43, 0.09, 0.92, 0.08, 0.98, 0.35, 0.24, 0.66, 0.63, 1.03, 0.10, 0.09, 0.14, 0.98, 0.34, 0.48, 0.25, 0.14, 0.07, and 6.71, respectively. To validate the feasibility of using QAMS for quantitative analysis of ginsenosides, the standard method difference (SMD) was established between the QAMS and ESM. As shown in Table 6, there were no significant differences between results from QAMS and ESM methods according to the SMD% falling between 0.1% and 5.45%, illustrating that the use of QAMS in our approach is reliable and accurate for detecting the concentration of each component.

4. Conclusions

In this study, we report an innovative approach based on UPLC-HRMS combined with QAMS and ESM for the simultaneous determination of multiple ginsenosides. By optimizing detection conditions, we can acquire for each ginsenoside within 25min a high-resolution UPLC chromatogram with excellent sensitivity and stability. The QAMS method established using relative response factors shows minimal difference (SMDs below 5.45%) compared to the external standard method, implying that the UPLC-MS-QAMS approach can be used as a replacement for the external standard method when standard substances are absent. For these 25 different ginsenosides, data reproducibility was very good at low concentrations for MS detection. Furthermore, our approach is simple to use, highly sensitive and accurate, and applicable for all kinds of ginseng samples and for quality control of ginseng containing products.

Acknowledgments

The authors wish to acknowledge Professor of Guohui Huang for the kind assistance with the paper from the National Demonstration Center for Experimental Biology Education, Northeast Normal University. This study was supported by Jilin Provincial Department of Science and Technology Medicine and Health Project (20200504005YY).

References

- [1] A. S. Attele, J. A. Wu, C.-S. Yuan, "Ginseng pharmacology," *Biochemical Pharmacology*, vol. 58 no. 11, pp. 1685-1693, DOI: 10.1016/s0006-2952(99)00212-9, 1999.
- [2] K. C. Huang, *The Pharmacology of Chinese Herbs*, 1993.
- [3] W. Z Yang, Y. Hu, W. Y. Wu, M. Ye, D. A. Guo, "Saponins in the genus *Panax* L. (Araliaceae): a systematic review of their chemical diversity," *Phytochemistry*, vol. 106, DOI: 10.1016/j.phytochem.2014.07.012, 2014.
- [4] Q. Mao, Y. Li, S. L. Li, "Chemical profiles and anticancer effects of saponin fractions of different polarity from the leaves of *Panax notoginseng*," *Chinese Journal of Natural Medicines*, vol. 12, pp. 30-37, 2014.
- [5] Z. Ying, J. Wei, R. Liu, F. Zhao, Y. Yu, X. Tian, "An UPLC-MS/MS method for determination of osimertinib in rat plasma: application to investigating the effect of ginsenoside Rg3 on the pharmacokinetics of osimertinib," *International Journal of Analytical Chemistry*, vol. 2020, DOI: 10.1155/2020/8814214, 2020.
- [6] Q. Mao, J. Yang, X.-M. Cui, "Target separation of a new anti-tumor saponin and metabolic profiling of leaves of *Panax notoginseng* by liquid chromatography with electrospray ionization quadrupole time-of-flight mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 59, pp. 67-77, DOI:

10.1016/j.jpba.2011.10.004, 2012.

[7] L. P. Christensen, "Ginsenosides chemistry, biosynthesis, analysis, and potential health effects," *Advances in Food and Nutrition Research*, vol. 55, DOI: 10.1016/S1043-4526(08)00401-4, 2009.

[8] N. Fuzzati, "Analysis methods of ginsenosides," *Journal of Chromatography B*, vol. 812 no. 1-2, pp. 119-133, DOI: 10.1016/s1570-0232(04)00645-2, 2004.

[9] J. J. Jo, P. J. Cho, S. Lee, "Simultaneous quantification of 13 ginsenosides by LC-MS/MS and its application in diverse ginseng extracts," *Mass Spectrometry Letters*, vol. 9 no. 2, pp. 41-45, 2018.

[10] X. Sun, P. Chen, S. L. Cook, G. P. Jackson, J. M. Harnly, P. B. Harrington, "Classification of cultivation locations of *Panax quinquefolius* L samples using high performance liquid chromatography-electrospray ionization mass spectrometry and chemometric analysis," *Analytical Chemistry*, vol. 84 no. 8, pp. 3628-3634, DOI: 10.1021/ac2032832, 2012.

[11] H. W. Park, S. T. HanIn, M.-W. Lee, "Simultaneous determination of 30 ginsenosides in *Panax ginseng* preparations using ultra performance liquid chromatography," *Journal of Ginseng Research*, vol. 37 no. 4, pp. 457-467, DOI: 10.5142/jgr.2013.37.457, 2013.

[12] X. Huang, Y. Liu, N. Zhang, "UPLC orbitrap HRMS analysis of *Panax quinquefolium* L. For authentication of *Panax* genus with chemometric methods," *Journal of Chromatographic Science*, vol. 56 no. 1, pp. 25-35, DOI: 10.1093/chromsci/bmx077, 2018.

[13] S.-Y. Han, C. Liang, K. Zou, J.-Q. Qiao, H.-Z. Lian, X. Ge, "Influence of variation in mobile phase pH and solute pKa with the change of organic modifier fraction on QSRs of hydrophobicity and RP-HPLC retention of weakly acidic compounds," *Talanta*, vol. 101, pp. 64-70, DOI: 10.1016/j.talanta.2012.08.051, 2012.

[14] J. C. Heaton, J. J. Russell, T. Underwood, R. Boughtflower, D. V. McCalley, "Comparison of peak shape in hydrophilic interaction chromatography using acidic salt buffers and simple acid solutions," *Journal of Chromatography A*, vol. 1347, pp. 39-48, DOI: 10.1016/j.chroma.2014.04.026, 2014.

[15] J.-B. Wan, Q.-W. Zhang, S.-J. Hong, P. Li, S.-P. Li, Y.-T. Wang, "Chemical investigation of saponins in different parts of *Panax notoginseng* by pressurized liquid extraction and liquid chromatography-electrospray ionization-tandem mass spectrometry," *Molecules*, vol. 17 no. 5, pp. 5836-5853, DOI: 10.3390/molecules17055836, 2012.

[16] F. Liu, N. Ma, C. He, "Qualitative and quantitative analysis of the saponins in *Panax notoginseng* leaves using ultra-performance liquid chromatography coupled with time-of-flight tandem mass spectrometry and high performance liquid chromatography coupled with UV detector," *Journal of Ginseng Research*, vol. 42 no. 2, pp. 149-157, DOI: 10.1016/j.jgr.2017.01.007, 2018.

[17] H.-M. Zhang, S.-L. Li, H. Zhang, "Holistic quality evaluation of commercial white and red ginseng using a UPLC-QTOF-MS/MS-based metabolomics approach," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 62, pp. 258-273, DOI: 10.1016/j.jpba.2012.01.010, 2012.

[18] Q. Mao, M. Bai, J.-D. Xu, "Discrimination of leaves of *Panax ginseng* and *P. quinquefolius* by ultra high performance liquid chromatography quadrupole/time-of-flight mass spectrometry based metabolomics approach," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 97, pp. 129-140, DOI: 10.1016/j.jpba.2014.04.032, 2014.

[19] J. Lee, B.-R. Choi, Y.-C. Kim, "Comprehensive profiling and quantification of ginsenosides in the root, stem, leaf, and berry of *Panax ginseng* by UPLC-QTOF/MS," *Molecules*, vol. 22 no. 12, pp. 2147-2212, DOI: 10.3390/molecules22122147, 2017.

[20] N. Fuzzati, B. Gabetta, K. Jayakar, R. Pace, F. Peterlongo, "Liquid chromatography-electrospray mass spectrometric identification of ginsenosides in *Panax ginseng* roots," *Journal of Chromatography A*, vol. 854 no. 1-2, pp. 69-79, DOI: 10.1016/s0021-9673(99)00463-x, 1999.

[21] Y. Jin, T. Tian, Y. Ma, H. Xu, Y. Du, "Simultaneous determination of ginsenoside Rb1, naringin, ginsenoside Rb2 and oridonin in rat plasma by LC-MS/MS and its application to a pharmacokinetic study after oral administration of Weifuchun tablet," *Journal of Chromatography B*, vol. 1000, pp. 112-119, DOI: 10.1016/j.jchromb.2015.06.027, 2015.

[22] Z. Du, J. Li, X. Zhang, J. Pei, L. Huang, "An integrated LC-MS-based strategy for the quality assessment and

discrimination of three Panax species," *Molecules*, vol. 23 no. 11, pp. 2988-3020, DOI: 10.3390/molecules23112988, 2018.

[23] C.-Q. Wang, X.-H. Jia, S. Zhu, K. Komatsu, X. Wang, S.-Q. Cai, "A systematic study on the influencing parameters and improvement of quantitative analysis of multi-component with single marker method using notoginseng as research subject," *Talanta*, vol. 134, pp. 587-595, DOI: 10.1016/j.talanta.2014.11.028, 2015.

[24] J. Xie, J. Li, J. Liang, P. Luo, L.-S. Qing, L.-S. Ding, "Determination of contents of catechins in Oolong teas by quantitative analysis of multi-components via a single marker (QAMS) method," *Food Analytical Methods*, vol. 10 no. 2, pp. 363-368, DOI: 10.1007/s12161-016-0592-5, 2016.

DETAILS

Subject:	Standards; Qualitative analysis; Acetonitrile; Accuracy; Markers; Data acquisition; Quality control; Signal to noise ratio; Traditional Chinese medicine; Isomers; Quantitative analysis; Stability analysis; Methods; Chromatography; Reproducibility
Location:	United States--US
Editor:	David M Lubman
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-05 (Received); 2021-06-15 (Accepted); 2021-07-01 (Pub)
DOI:	https://doi.org/10.1155/2021/9986793

ProQuest document ID: 2550175168

Document URL: <https://www.proquest.com/scholarly-journals/simultaneous-determination-25-ginsenosides-uplc/docview/2550175168/se-2?accountid=211160>

Copyright: Copyright © 2021 Xiujuan Jia et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-07-11

Database: Publicly Available Content Database

Document 25 of 59

Determination of Pendimethalin Dynamic Residual Distribution in Crucian Carp Tissues and Associated Risk Assessment

Lu, Qiao ¹ ; Yuan, Na ² ; Han, Gang ¹ ; Cheng, Bo ¹ ; Zhang, Defu ² ; Song, Jinlong ¹ ; Mu, Yingchun ¹

¹ Key Laboratory of Control of Quality and Safety for Aquatic Products (Ministry of Agriculture and Rural Affairs), Chinese Academy of Fishery Sciences, Beijing 100141, China ² College of Food Science and Engineering, Bohai University, Jinzhou, Liaoning 121000, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Pendimethalin has been considered a moderately to extremely toxic compound for fish and aquatic organism. This study developed the determination of dynamic residual distribution for pendimethalin in crucian carp tissues (muscle, liver, kidney, gill, and blood) under semistatic exposure system by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method. The pendimethalin residues in various fish tissues increased initially and then decreased, and the residue amount of pendimethalin varied from tissue to tissue of crucian carp. Particularly, the pendimethalin accumulation in most fish tissues made significant decreases at two-time points. Pendimethalin was initially absorbed and enriched by fish body, and then partial pendimethalin was discharged into the outside environment through the metabolism function of crucian carp. The residue levels of pendimethalin distributed in crucian carp were ranked in the following decreasing order: liver>kidney>gill or muscle>blood, attributed to the fact that pendimethalin tends to accumulate in lipid-rich tissues of fish. Risk assessment results indicated that the chronic risk from dietary exposure to pendimethalin through crucian carp consumption for Chinese residents was acceptable, along with a lower estimated exposure dose (EED) than acceptable daily intake (ADI) and risk quotient (RQ) <1. This study performed the first analysis for pendimethalin residual distribution in crucian carp tissues under semistatic exposure condition and provided a reference for pollution control and risk assessment of

pendimethalin aimed at aquatic products.

FULL TEXT

DETAILS

Subject:	Mass spectrometry; Risk management; Muscles; Blood; Organisms; Kidneys; Aquatic ecosystems; Fish; Quotients; Risk assessment; Agriculture; Carp; Herbicides; Experiments; Pesticides; Exposure; Pollution control; Residues; Lipids; High performance liquid chromatography; Liver; Bioaccumulation
Business indexing term:	Subject: Risk assessment
Location:	United States--US; Germany; China; Japan
Editor:	David Touboul
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-17 (Received); 2021-06-21 (Accepted); 2021-07-02 (Pub)
DOI:	https://doi.org/10.1155/2021/9984230
ProQuest document ID:	2550174661

Document URL: <https://www.proquest.com/scholarly-journals/determination-pendimethalin-dynamic-residual/docview/2550174661/se-2?accountid=211160>

Copyright: Copyright © 2021 Lu Qiao et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-07-11

Database: Publicly Available Content Database

Document 26 of 59

Phytochemical Composition, Antioxidant, Antiacetylcholinesterase, and Cytotoxic Activities of *Rumex crispus* L.

Saoudi, Mohamed Marouane ¹

; Bouajila, Jalloul ²

; Rahmani, Rami ³

; Alouani, Khaled ⁴

¹ Laboratoire de Chimie Analytique et Électrochimie, Faculté des Sciences de Tunis, Université de Tunis El Manar, Campus Universitaire, Tunis 2092, Tunisia; Laboratoire de Génie Chimique, Université Paul Sabatier, CNRS, INPT, UPS, Toulouse, France ² Laboratoire de Génie Chimique, Université Paul Sabatier, CNRS, INPT, UPS, Toulouse, France ³ Laboratoire de Génie Chimique, Université Paul Sabatier, CNRS, INPT, UPS, Toulouse, France; Unité de Recherche "Valorisation des Biomolécules Actives", Institut Supérieur de Biologie Appliquée de Médénine, Route El Jorf—km 22.5, Medenine 4119, Université de Gabès, Gabès, Tunisia ⁴ Laboratoire de Chimie Analytique et Électrochimie, Faculté des Sciences de Tunis, Université de Tunis El Manar, Campus Universitaire, Tunis 2092, Tunisia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Rumex crispus L. (*R. crispus*) is regarded as an aromatic plant. It was used for its excellent biological properties in traditional medicine. The aerial part was extracted successively by maceration with three solvents increasing polarity (cyclohexane (CYH), dichloromethane (DCM), and methanol (MeOH)) to evaluate their chemical compositions and biological activities. The extracts were rich in phenolic compounds (13.0 to 249.8 mg GAE/g of dry weight (dw)). The MeOH extract has presented remarkable IC_{50} = 6.2 μ g/mL for anti-DPPH and 31.6 μ g/mL for anti-AChE. However, the DCM extract has the highest cytotoxic activity against the two cancer cells (HCT-116 and MCF-7) (69.2 and 77.2% inhibition at 50 μ g/mL, respectively). Interestingly, GC-MS analysis enabled to identify three new compounds in *R. crispus* extracts, such as L-(–)-arabitol (5), D-(–) fructopyranose (7) detected only in MeOH extract, and 2, 5-

dihydroxyacetophenone (3) detected in all extracts. For HPLC chromatograms, cardamonin (8), 5-hydroxy-3'-methoxyflavone (17), and 3'-hydroxy-b-naphthoflavone (18) showed the highest concentrations of 74.0, 55.5, and 50.4 mg/g of dw, respectively, among others who are identified. Some phenolic compounds were identified and quantified by HPLC in more than one organic extract, such as 4', 5-dihydroxy-7-methoxyflavone (13), 4', 5-dihydroxy-7-methoxyflavone (14), 5-hydroxy-3'-methoxyflavone (17), and 3'-hydroxy-b-naphthoflavone (18), were found for the first time in the *R. crispus* extracts. Our results showed that the biological activities of this plant might be linked to their phenolic compounds and that the polar extracts could be considered as new natural supplements to be used in food and pharmaceuticals.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Excessive free radical production and lipid peroxidation represent an important role in some pathogenesis of serious diseases, such as diabetes, acquired immunodeficiency syndrome, neurodegenerative diseases, cardiovascular disease, cancer, cirrhosis of the liver, ischemia, atherosclerosis, and aging [1, 2]. Approximately 95% of the pathologies detected in humans over the age of 35 are associated with the production and accumulation of free radicals [3]. These toxic radicals can be obtained either through biochemical or physiological processes or through pollution and other endogenous sources. In fact, they are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and certain molecules, leading to bad cellular consequences [4].

Plants can give several antioxidant compounds such as flavonoids, phenolic acids, and α -tocopherol, which help the human body to neutralize free radicals in a synergistic and interactive way. For this reason, these compounds are more considered as important dietary factors [2, 5]. In fact, it has been noted that foods rich in antioxidants have an inverse relationship with the incidence of human disease [6]. Since the use of synthesized antioxidants promotes negative health effects, a vision has led researchers to replace them with natural antioxidants [7] to valorize the raw materials of certain plants by identifying their new powerful compounds, which can be used as an herbal medicine for certain diseases. Tunisia has a wide variety of species with multiple interests, including popular therapeutic practices. Some plants for each species have not been subjected to chemical studies [8]. Approximately 200 species of plants in the genus *Rumex* are characterized by their edible and medicinal virtues [9]. These species contain many bioactive compounds with complex chemical structures. The aerial and roots parts of different plants belong to the Polygonaceae family, including *Rumex*, have been used as a natural source of traditional medicines in several therapeutic uses, such as anti-inflammatory, antioxidants, antianalgesic, and antimicrobial activities [10, 11]. They are also used for the treatment of certain diseases like cancer, tumor, and diseases related to liver and urinary/kidney functions [12, 13]. There are some reports in the literature about the evaluation of some species of *Rumex* which have exhibited health-promoting effects and have been used as traditional foods and herbal remedy. For example, stems, leaves, and roots of *R. abyssinicus* L., *R. bequaertii* L., and *R. usambarensis* L. are used to treat coughs, pneumonia, abscesses, smallpox, and upset stomach [14]. In addition, *R. nepalensis* L. roots have shown significant antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* [15]. In a recent study, roots from *R. hastatus* D. showed interesting antimicrobial, cytotoxic, and antitumor activities [16]. Moreover, in the spring, the aerial parts of many species (*R. crispus*, *R. acetosa*, *R. pseudonatronatus*, *R. patientia*, and *R. acetosella*) are collected and used as vegetables [17].

Rumex crispus, commonly named "curled dock" because of the wavy and curly leaves, is a perennial plant which growing wild (native) in the most of European area, Central and East Asia, and North African regions, including Tunisia [18, 19]. It grows well in disturbed soil, roadsides, shorelines, and forest edges and can reach 1.20m of tall with a fleshy taproot [20, 21]. Despite being considered an invasive plant in several countries (North American, New Zealand, and Australia) [22], the curly dock is regularly used in traditional medicine as a useful alternative in Siberian and Turkish traditional medicine. It is useful as a drug intended for the treatment of venereal diseases and used

synonymously with “blood purifier” [18]. Furthermore, young leaves of *R. crispus* are edible, and they can be consumed eaten as a potherb or raw as a vegetable, especially in the spring, added to the salads [21]. However, the physiological or chemical evidence supporting any claim of therapeutic value for *R. crispus* that we can find in Tunisia and in some Mediterranean countries [23].

Therefore, in this work, phytochemical screening (TPC, HPLC, and GC-MS) and antioxidant evaluation and the biological activities (anti-AChE and cytotoxicity) of *R. crispus* extracts were carried out.

2. Materials and Methods

2.1. Chemicals Used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma, Aldrich: Ascorbic acid, Acetic acid, acetylcholinesterase (AChE), acetylthiocholine (ACTH), acetonitrile (ACN), N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), cyclohexane (CYH), dichloromethane (DCM), Dulbecco's modified eagle medium (DMEM), dimethyl sulfoxide (DMSO), 1-1-diphenyl-2-picryl hydrazyl (DPPH), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), Folin-Ciocalteu reagent (2N), methanol (MeOH), human colon cancer cells (HCT116), human breast cancer cells (MCF-7), 3- [4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT), Roswell Park Memorial Institute (RPMI), Chlorotrimethylsilane (TMCS), and tetrahydrofuran (THF).

The analytical standards used for the identification and quantification of the principal phenolic compounds found in the extracts: 3-amino-4-hydroxybenzoic acid, Gallic acid, 3, 4-dihydroxy-5 methoxybenzoic acid, 7-hydroxycoumarin-3-carboxylic acid, rutin hydrate, Butyl gallate, 4-hydroxytamoxifen, cardamonin, phenoxodiol, pinostilbene hydrate, 3-benzyloxy-4, 5-dihydroxy-benzoic acid methyl ester, ethyl trans-2-hydroxycinnamate, 4', 5-dihydroxy-7-methoxyflavone, pinosylvan monomethyl ether, 3, 6, 3'-trimethoxyflavone, shikonin, 5-hydroxy-3'-methoxyflavone, and 3'-hydroxy-b-naphthoflavone were also purchased from Sigma, Aldrich.

2.2. Plant Material

The aerial part (stems, leaves, and flowers) of *R. crispus* was collected from the locality of Borj-Cedria (Northern Tunisia). The plant was harvested in September 2014, corresponding to its full bloom period. Then, the harvested plant was identified at the center of biotechnology of Borj-Cedria (CBBC), and a voucher specimen was placed at the herbarium of the laboratory of the ecoprocesses in the same center cited.

2.3. Preparation of Different Extracts

The plant material was dried in the shade at room temperature. Grinding was carried out to obtain a fine powder. For the extraction of the metabolites, fifty grams (50g) were subjected to successive exhaustion using solvents of increasing polarity (cyclohexane (CYH), dichloromethane (DCM), and methanol (MeOH)) by maceration and stirring at room temperature in a volume of 500 mL and for 2 hours for each solvent. After filtration using Whatman N°2 filter paper (Fisher, France), the filtrates were evaporated using a vacuum rotary evaporator at 35°C (IKA, Germany). This protocol enabled us to obtain the CYH, DCM, and MeOH dry extracts. These extracts were stored at -4°C until further analysis (phytochemical and biological analysis). The extraction yield was characterized as follows: $(1) R\% = \frac{m_{\text{residue}}}{m} \times 100$, where m_{residue} : weight of residue (g); m : weight of plant material (g).

2.4. Determination of Total Phenolic Content

According to the Folin–Ciocalteu colorimetric method, the total phenolic compound content in the extract was evaluated, and some modifications were made [24]. In short, add 100 µL of Folin–Ciocalteu reagent (0.2N) to 20 µL of diluted extract (0.5 mg/mL). After incubating for 5 minutes at room temperature (20–27°C), add 80 µL of sodium carbonate solution (75 g/L in water). The blend was then incubated for 15 minutes, and the absorbance was measured at 765 nm, using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland). Gallic acid was used as a standard reference substance for the calibration curve (0–115 mg/L). The results were expressed as mg of gallic acid equivalents (GAE)/g of dw.

2.5. DPPH Scavenging Activity of Free Radicals

The 1-1-diphenyl-2-picryl hydrazyl (DPPH) test was used to measure antioxidant activity. The quantitative estimate of the scavenging capacity of free radicals was determined according to the method described by Yahyaoui et al. [24]. This mixture was homogenized and incubated 25 min in the dark at room temperature. Then, the absorbance of

all samples was measured at 524 nm. The percentage of inhibition percent of the free radical scavenging activity of each sample was calculated as follows: $(2)\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$, where A_{blank} is the absorbance of the negative control reaction without extract. A_{sample} is the absorbance of the test sample. This activity was also expressed as IC_{50} (mg/L), which represents the concentration of test material required to promote a 50% decrease in the initial concentration of DPPH. All measurements were performed in triplicate using ascorbic acid as a reference.

2.6. Chromatographic Analysis

2.6.1. HPLC-DAD Analysis

Using the method of Yahyaoui et al. [24] with a C18 column (25 cm $\times$ 4.6 mm $\times$ 5 μ m), the different extracts of *R. crispus* were analyzed on analytical HPLC-DAD (Thermo Fisher Scientific, USA). Using a mobile phase composed of acidified water (pH=2.65) (solvent A) and water/acetonitrile (20:80 v/v, pH=2.65) (solvent B), elution was performed at a flow rate of 1.2 mL/min. The limit of detection (LOD) of the method was defined from 0.01–0.1 mg/L. The gradient proceeds as follows: from 0.1% to 30% B in 35 minutes, from 30% to 50% B in 5 minutes, from 50% to 99.9% B in 5 minutes, and finally from 99.9% to 0.1% B in 15 minutes. To detect most compounds, all samples were prepared at the same concentration (20 mg/mL). After injecting twenty microliters (20 μ L) of each sample, they were detected at 280 nm. The phenolic compounds can be determined by comparing the retention time of the unknowns with the standards with known retention time.

2.6.2. GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out using Varian Saturn 2000 (Les Ulis, France) ion trap GC/MS and CP-3800 GC system equipped with fused silica capillary DB-5MS column (5% phenylmethyl Polyoxane, 30 $\times$ 0.25 mm, film Thickness 0.25 μ m) [25]. Chromatographic conditions were 60–260°C, and the temperature was increased in a gradient of 5°C/min and continued for 15 minutes under isothermal conditions of 260°C. Apply a second gradient to reach 340°C at a rate of 40°C/min. The temperature of the trap was 250°C and the temperature of the transmission line was 270°C. Perform quality scans from 40 to 650 m/z. The extract was dissolved in its extraction solvent in an amount of 5 mg/mL, and 2 μ L was injected. The molecules were identified by comparing the retention index (RI) obtained on a nonpolar DB-5MS column versus C5–C24 n-alkanes with compounds provided in the literature and comparing their mass spectra with the NIST 08 (National Institute of Standards and Technology) database.

(1) *Derivatization Method*. Rahmani et al. [25] described the derivatization method and made some modifications. In a 2 mL vial, mix 150 μ L of 99% N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) + 1% of chlorotrimethylsilane (TMCS) with 1 mL of extract (5 mg/mL in tetrahydrofuran (THF) solvent). After that, the blend was mixed for 30 seconds to increase solubility. The reaction mixture was kept at 40°C for 15 minutes. Then, ten microliters (10 μ L) of each derivative solution was injected into the same GC-MS equipment and analyzed as described in the previous section to identify the molecules of each extract.

2.7. Biological Activities

2.7.1. Antiacetylcholinesterase (AChE) Activity

The anti-AChE activity was determined according to the Ellman method [25]. In short, 25 μ L of each extract was mixed with 50 μ L of sodium phosphate buffer (0.1 M; pH=8), 25 μ L of AChE solution and 125 μ L of 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) (3 mM, pH=7). The mixture was added to a 96-well microplate and incubated at 25°C for 15 minutes. After that, 25 μ L of ACTHI iodide solution (15 mM) was added and the final mixture was incubated again at 25°C for 10 minutes. The absorbance of the yellow complex (5-thio-2-nitrobenzoate anion) was read at 412 nm. The concentration of the extract that caused 50% inhibition of AChE activity (IC_{50}) was calculated by nonlinear regression analysis. The percentage of inhibition was calculated as follows: $(3)\% \text{ inhibition} = \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{sample}}} \times 100$, where A_{sample} and A_{control} were the absorbance of sample with the test material and of sample without the enzyme, respectively. Galantamine was used as a reference.

2.7.2. Cytotoxic Activity

The *in vitro* cytotoxic activity of different extracts on two different human cell lines, MCF-7 (human breast cancer

cells) and HCT-116 (human colon cancer cells), was evaluated according to the method as described by Rahmani et al. [25]. This activity was estimated by MTT colorimetric test. Cells were distributed in 96-well plates at 3×10^4 cells/well in 100 μL and added 100 μL of the corresponding culture medium (DMEM, Sigma Aldrich, USA) for MCF-7 or RPMI-1640 (Sigma Aldrich, USA) for HCT-116 to each well, which contains samples of various concentrations. The plate was then incubated for 48 h at 37°C. Next, the supernatant was then eliminated, and cells were treated with 50 μL of MTT solution during 40 min of incubation at 37°C. Then after the elimination of the MTT solution, 50 μL of dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan crystal. The absorbance was measured at 605 nm. The tamoxifen was used as a positive reference. The cytotoxic effect of the extract was estimated based on the percentage of growth inhibition and calculated as follows: $(4)\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$.

2.8. Statistical Analysis

All measurements were performed in quadruplicate by one-way analysis of variance (ANOVA) using the SPSS (Version 20.0) for the significance calculation and the Tukey's test was used to estimate the statistical differences between the solvents used in the study. The linear correlation coefficient (R^2) was examined to determine the rapport between the biological activities or antioxidant and the (TPC). Finally, the Principal Component Analysis (PCA) was also performed using XLSTAT (version 5.03) to visualize the difference between all the parameters. The reliability limits were set at $p \leq 0.05$.

3. Results and Discussion

3.1. Extraction Yields and Total Phenolic Content (TPC)

In this study, three solvents with different polarities, namely: CYH, DCM, and MeOH were used for the extraction of the aerial part of *R. crispus*. Table 1 showed the yield obtained from different extracts. While the MeOH was highlighted the highest yield with 12.2%, the extraction yield of both CYH and DCM does not exceed 1.0%. These results were in agreement with those found by Idris et al. [21], who reported that maximum extract yield from *R. crispus*, collected in South Africa, was obtained with MeOH solvent. This yield was lower than that found in this study. The difference in the extract yield from the different extracts might be related to the availability of the extractable components.

Table 1

Yields and TPC in the aerial part of *R. crispus* extracts.

Samples	Yields (%)	TPC (mg GAE/g of dw)
CYH	1.0 ^b	13.0 $\pm$ 0.3 ^b
DCM	0.3 ^c	21.6 $\pm$ 0.5 ^b
MeOH	12.2 ^a	249.8 $\pm$ 9.9 ^a

CYH: cyclohexane; DCM: dichloromethane; MeOH: methanol. nd: not detected. Data are the mean of three repetitions $\pm$ SD. Different letters indicate significant differences according to Tukey test ($p \leq 0.05$).

As for the TPC of *R. crispus* extracts, it was ranged between 13.0 and 249.8 mg GAE/g of dw. A significant difference ($p \leq 0.05$) between the different solvents used. The difference in TPC noticed between different solvents may be due to the polarity of phenolic compounds. A low TPC was obtained with the CYH and DCM extracts, which showed a content of 13.0 and 21.6 mg GAE/g of dw, respectively. However, the highest content was obtained with MeOH extract (249.8 mg GAE/g of dw). This result was higher than those measured in the MeOH extract (56.3 mg GAE/g of dw) in the study of Coruh et al. [26] when working with the aerial part of *R. crispus* growing wild in Turkey.

3.2. Antioxidant Activity

DPPH is one of the most used radicals to indicate the antioxidant activity possessed by both food and extracts. The antioxidant capacity of extracts from the aerial part of *R. crispus* was measured by the method of DPPH. The results

were reported as the percentage of scavenged DPPH free radical and as the IC_{50} (Table 2). Statistically, there was a significant difference ($p \leq 0.05$) between the different extracts in terms of antioxidant activity. Similar to the total phenolics, MeOH extract presented a major DPPH scavenging activity. This extract showed a high antioxidant activity of 93.5% at the concentration of 50 $\mu\text{g/mL}$ with an IC_{50} value of 6.2 $\mu\text{g/mL}$. The results were compared to the commercial antioxidant (ascorbic acid) ($IC_{50} = 3.9 \mu\text{g/mL}$). However, DCM and CYH extracts showed a feeble ability to neutralize DPPH free radicals, with an inhibition percentage that did not exceed 30%. These results indicate that the highest DPPH activity of the MeOH extract is closely related to the high content of phenolics. This was validated in our work by a high correlation between TPC and DPPH activity ($R^2 = 0.98$).

Table 2

Antioxidant activity of the areal part of *R. crispus* extracts.

Samples	Antioxidant activity (DPPH)	
% Inhibition (50 $\mu\text{g/mL}$)	IC_{50} ($\mu\text{g/mL}$)	CYH
14.3 $\pm$ 2.0 ^c	>50	DCM
29.3 $\pm$ 1.6 ^b	>50	MeOH
93.5 $\pm$ 1.0 ^a	6.2 $\pm$ 3.0	Ascorbic acid

CYH: cyclohexane; DCM: dichloromethane; MeOH: methanol. Data are the mean of three repetition $\pm$ SD. Different letters indicate significant differences according to the Tukey's test ($p \leq 0.05$).

Compared to the literature, the present results were higher compared to those found by Ćebović et al. [27]. They found that the water extract of *R. crispus* fruit has low DPPH activity, with a high $IC_{50} = 46 \mu\text{g/mL}$. However, the study of Elzaawely et al. [28], when working with *Rumex japonicas* aerial part, was not in accordance with our findings. They found that the EtOAc extract (moderately polar solvent) revealed the highest antioxidant activity.

3.3. Chemical Composition of *R. crispus* Extracts

3.3.1. HPLC-DAD Analysis

The identification of the phenolic compounds in the different extracts of the aerial part of *R. crispus* was made using the HPLC-DAD (Figure 1). For each chromatogram and based on the comparison of the retention time and DAD spectra of the standard compound analyzed under the same conditions, many phenolic compounds were identified. In total, 18 phenolic compounds were identified and quantified in all the extracts by means of their relative retention time (Table 3). Interestingly, the compounds which showed the highest concentrations belong to flavone and cardamonin classes. These compounds are cardamonin (**8**), 5-hydroxy-3'-methoxyflavone (**17**), and 3'-hydroxy-b-naphthoflavone (**18**), detected in the DCM extract, with a concentration of 74.0, 55.5, and 50.4 mg/g of dw, respectively. Besides, with moderate concentrations, we found other compounds belong to flavone and benzoic acid classes such as 4', 5-dihydroxy-7-methoxyflavone (**13**) (25.0 mg/g of dw), 3, 6, 3'-trimethoxyflavone (**15**) (19 mg/g of dw), and 3, 4-dihydroxy-5 methoxybenzoic acid (**3**) (15.3 mg/g of dw). All these phenolic compounds were found for the first time in *R. crispus* extracts.

[figure omitted; refer to PDF]

Table 3

Phenolic compounds contents identified in various extracts of *R. crispus* aerial parts by HPLC-DAD.

N°	Compounds and chemical structure compounds	Rt (min)	Concentration (mg/g of dw)	References

CY H	DCM	MeOH	1	3- amino- 4- hydroxy benzoic acid	2.1	nd
nd	2.0	Suzuki et al. [29]	2	Gallic acid	3.5	nd
nd	0.9	Nayee m and Asdaq [30]	3	3, 4- dihydro xy-5 methox ybenzoi c acid	7.7	nd
15.3	nd	Huyut et al. [31]	4	7- hydroxy coumari n-3- carboxy lic acid	19.8	nd
nd	7.2	Wahd an et al. [32]	5	Rutin hydrate	22.7	nd
nd	2.1	Karak as et al. [33]	6	Butyl gallate	43.0	nd
1.3	nd	Park et al. [34]	7	4- hydroxy tamoxif en	43.0	nd
1.1	nd	Shin and Choi [35]	8	Cardam onin	44.2	nd
74.0	nd	Gonça lves et al. [36]	9	Phenox odiol	44.3	0.3

nd	nd	Souza et al. [37]	10	Pinostilbene hydrate	44.6	1.2
nd	nd	Armstrong and Greder [38]	11	3-benzoyloxy-4, 5-dihydroxy-benzoic acid methyl ester	45.0	nd
0.6	nd	International Bureau [39]	12	Ethyl trans-2-hydroxycinnamate	45.3	nd
0.9	nd	Weitekamp et al. [40]	13	4', 5-dihydroxy-7-methoxyflavone	46.3	26.4
25.0	nd	Rudrapaul et al. [41]	14	Pinosylvin monomethyl ether	47.0	0.3
0.5	nd	Gabaston et al. [42]	15	3, 6, 3'-trimethoxyflavone	48.0	nd
19.0	nd	Aritomi et al. [43]	16	Shikonin	48.0	3.2
nd	nd	Shi et al. [44]	17	5-hydroxy-3'-methoxyflavone	49.3	3.5

55.5	nd	Shafa ghat et al. [45]	18	3'- hydroxy -b- naphtho flavone	49.8	7.9

CYH: cyclohexane; DCM: dichloromethane; dw: dry weight; MeOH: methanol. RT: retention time; nd: not detected. The HPLC chromatograms showed that the chemical composition changes significantly according to the polarity of the solvent used. Therefore, the CYH extract contains more nonpolar phenolic compounds and their elution process has occurred at the end of acquisition. For the DCM extract, both the polar and nonpolar compounds can be identified as having a maximum intensity equal to 110mV.

The polar extract (MeOH) had more polar compounds eluted between 2 and 20min compared to the other two extracts with a maximum intensity equal to 650mV. It can be deduced that the intensity of the peaks increased with the polarity of the solvent, which can confirm the results of TPC (Table 1). In other words, the HPLC-DAD results were strongly correlated with the results found by the colorimetry test and confirm the richness of the extract by the phenolic compounds. By comparing with the literature, some phenolic compounds found in the extract of *R. crispus* were discovered for the first time, such as 4', 5-dihydroxy-7-methoxyflavone (**13**), 5-hydroxy-3'-methoxyflavone (**17**), and 3'-hydroxy-b-naphthoflavone (**18**). However, other compounds were previously detected, such as gallic acid detected in *R. acetosa* (Kucekova et al. [46], and cardamonin detected in *Polygonum ferrugineum* [47]. Cardamonin has received serious attention from scientific researchers due to the expectation that it is beneficial to human health [36].

In summary, these characteristic variations in the chemical composition between the different organic extracts could be due to the extractability of each solvent and the influence of environmental factors in the phenolic composition.

3.3.2. Identification of the Volatile Compounds by GC-MS Analysis

Gas chromatography coupled with mass spectrometry (GC-MS) was used to identify the volatile compounds in the organic extracts of *R. crispus* aerial part. Only two compounds (vitamin E and α -sitosterol) were detected without derivatization. While α -sitosterol was found in CYH and DCM extracts, the vitamin was detected only in the DCM one. Hence, in order to identify other volatile compounds, GC-MS derivatization was done to yield derivatized compounds with chromatographic characteristics and volatility. Overall, this was leading to the identification of 10 compounds in the three extracts (Table 4). These compounds were distributed as follows: 2 compounds in the CYH and DCM extracts, each, and 8 compounds in the MeOH extracts. Three compounds were detected in more than one extract. The characteristics of volatile compounds from different extracts indicate the presence of three families of organic compounds. Sterols, sugars, and phenolic acids. Some of these compounds were identified for the first time in *R. crispus* extracts, such as D-(-) fructopyranose, L-(-) arabitol, and 2, 5-dihydroxyacetophenone. Interestingly, the MeOH contained a variety of phenolic compounds, which are known for their antioxidant activity [59]. This finding was in agreement with the high antioxidant activity found in the current study. Moreover, several studies suggested the potent activity of some phenolic compounds against cancer cells, especially against MCF-7 and HCT-116 cell lines [25, 60]. Furthermore, β -sitosterol, the only compound detected with and without derivatization, is known for its antioxidant activity and its medicinal one (improve cholesterol levels in the human organism) [61, 62].

Table 4

Volatil compounds identified, by GC-MS, in the different extracts of *R. crispus* aerial parts.

N°	Volatil compounds and chemical structure	Rt (min)	Peak area ($\times 10^6$)	References

CYH	DCM	MeOH	Without derivatization			
1	Vitamin E	26.95	nd	5.9	nd	Farina et al. [48]
2	β -sitosterol	29.35	30.6	32.7	nd	Sen et al. [49]
With derivatization						
3	2, 5-dihydroxyacetophenone	10.87	1.9	4.0	18.9	Bowman et al. [50]
4	β -D-xylopyranose	13.40	3.6	0.7	nd	Huang et al. [51]
5	L-(-)-arabitol	14.58	nd	nd	14.6	Shomo et al. [52]
6	D-(-) fructofuranose	15.27	nd	nd	78.6	Yu et al. [53]
7	D-(-) fructopyranose	15.33	nd	nd	78.6	Yu et al. [53]
8	D-glucose	15.88	nd	nd	36.4	McComsey et al. [54]
9	Gallic acid	16.42	nd	nd	53.5	Nguyen et al. [55]
10	Myo-inositol	17.17	nd	nd	17.3	Maldonado et al. [56]
11	Sucrose	20.36	nd	nd	51.1	Durand et al. [57]
12	D-(+)-turanose	20.51	nd	nd	7.4	Pereira et al. [58]

CYH: cyclohexane; DCM: dichloromethane; MeOH: methanol. RT: retention time; nd: not detected.

3.4. Biological Activities

3.4.1. AChE Inhibitory Activity

The anti-AChE activity of *R. crispus* aerial part has not been evaluated previously. Nevertheless, MeOH extract showed the highest inhibition (83.7%) with an $IC_{50} = 31.6 \mu\text{g/mL}$ when compared to galanthamine, while CYH and DCM extract showed no AChE inhibitory activity (Table 5). This study showed that the anti-AChE activity of the MeOH extract was higher than that of the other extracts obtained. Indeed, Patil et al. [63] proved that the presence of some phenolic compounds such as Rutin hydrate (with $IC_{50} = 1.1 \times 10^3 \mu\text{g/mL}$) could contribute to the inhibition of AChE. In addition, the present results were better than those found by Ahmad et al. [62] when working with *R. hastatus* extracts. They found that the different extracts with different polarities (polar, moderately polar, and apolar) present an IC_{50} ranging between 75 and $1420 \mu\text{g/mL}$. Recent studies showed that there was a relation between antioxidant property and cholinesterase inhibitory activity [64]. This can validate our findings where we found a high correlation value between TPC and anti-AChE activity ($R^2=0.99$).

Table 5

Anti-acetylcholinesterase activity of *R. crispus* extracts.

Samples	Anti-acetylcholinesterase activity	
% Inhibition (50 μ g/mL)	IC ₅₀ (μ g/mL)	CYH
na	>50	DCM
na	>50	MeOH
83.7 $\pm$ 2.2 ^a	31.6 $\pm$ 3.7	Galanthamine

CYH: cyclohexane; DCM: dichloromethane; MeOH: methanol. na: not active.

3.4.2. Cytotoxic Activity

In this study, the cytotoxic effect of the different extracts of *R. crispus* against two cancer cell lines (HCT-116 and MCF-7) and its potential to inhibit their growth were evaluated. All the extracts were found active against both cell lines, with DCM extract more dominant, as shown in Figure 2. When taking each cell line separately, a significant difference ($p \leq 0.05$) was noticed between the different extracts in terms of effect against each cell line. The CYH extract has a weak effect against HCT-116 less with an inhibition that does not exceed 20%, while it showed good activity against the MCF-7 cell line. Furthermore, the MeOH extract has almost an equal effect against both cell lines (HCT-116 and MCF-7) with inhibition of 58.3 and 57.3% at the concentration of 50mg/L, respectively. In addition, for both cell lines, HCT-116 and MCF-7, the DCM extract revealed the highest cytotoxic potential with almost 69.2 and 77.2% of inhibition, respectively. This inhibition may be due to the presence of some phenolic compounds, which are known for their important cytotoxic activities, especially cardamonin with IC₅₀ equal to 8.1 and 8.6 μ g/mL, respectively, against the two cell lines (MCF-7 and HCT-116) [65]. Other studies suggested potent activity against cancer cells, including MCF-7 and HCT-116 cell lines, among which shikonin molecule (IC₅₀ = 0.83 and 0.53 (μ g/ml)), respectively [66], which confirms our obtained results. We also compared the results of our study with those found by Wang et al. [67]. They showed that methanolic extract of *R. crispus* species, collected from Rafha region, Kingdom of Saudi Arabia, had no cytotoxic activity (0%) against the MCF-7 cell line. In a previous study, Shaik and Raja [68] reported that the polysaccharide characterized from the aqueous extract of *Polygonum equisetiforme* (Polygonaceae) has low activity against both MCF-7 and HCT-116, with 11.3 and 14.5%, respectively.

[figure omitted; refer to PDF]

3.5. Principal Components Analysis (PCA)

Principal component analysis was done to understand the relationship among the measured antioxidant, biological activities, and TPC. The various results of PCA are shown in Figure 3. The main components (*F1* and *F2*) explaining 93.99% of the total data variance. According to this analysis, the inertial axis was withheld, as shown in Table 6. The loadings in the PCA curve not only express the degree of correlation of the factors with the initial variables but also indicate the correlation between the different activities (DPPH, AChE, MCF-7, and HCT-116) and TPC. While the first principal component (*F1*) correlated well with DPPH, AChE, and TPC having loadings 0.98, 1.0, and 0.99, respectively, the second principal component (*F2*) was related to the cytotoxic activity with both cell lines (MCF-7 and HCT-116) with loadings of 0.62 and 0.98, respectively (Table 7). Figure 3 also presented good correlations between AChE activity, DPPH radical scavenging activity, and TPC.

[figure omitted; refer to PDF]

Table 6

Contribution of variable factors to the principal component's analysis (%).

	<i>F1</i>	<i>F2</i>

Total phenolic content (TPC)	27.5	0.2
Radical scavenging activity (DPPH)	26.5	2.9
Anti-acetylcholinesterase activity (AChE)	27.9	0.1
Cytotoxic activity (MCF-7)	17.0	27.8
Cytotoxic activity (HCT-116)	1.4	69.1

Table 7

Correlations between variables and factors.

	<i>F</i> 1	<i>F</i> 2
Total phenolic content (TPC)	0.99	0.052
Radical scavenging activity (DPPH)	0.98	0.19
Anti-acetylcholinesterase activity (AChE)	1.00	0.02
Cytotoxic activity (MCF-7)	-0.79	0.62
Cytotoxic activity (HCT-116)	0.22	0.98

Using the biplot figure (Figure 4), it seems that the extract relative to TPC and biological activity were positioned according to its chemical composition. The MeOH extract with the highest TPC was located close to DPPH and AChE activity. Therefore, it may be to suggest that polyphenols compounds help to inhibit these two activities. Likewise, the DCM extract was located close to MCF-7. This extract contains several compounds at medium polarity with a major compound: cardamon is known according to the literature by its cytotoxic properties [69, 70].
[figure omitted; refer to PDF]

4. Conclusion

Our findings revealed that the TPC and the antioxidant activity of *R. crispus* were higher compared with those of other species and varied with the polarity of the solvent of extraction. While a moderate correlation was found between the TPC, anti-AChE, and cytotoxic activities, there was a strong correlation between the TPC and antioxidant results of the different extracts. As a result of the HPLC-DAD analysis conducted on *R. crispus* extracts, our findings demonstrate that the dominant compounds belong to the cardamonin and flavone classes. Besides, GC-MS analysis showed that the MeOH extracts contain the majority of the volatile compounds which belong to the sugar class.

Based on the evidence of significant AChE inhibition and free radical scavenging on various samples of *R. crispus*, it can be inferred that our species may be the best source of antioxidants and anticholinesterase compounds. These important biological activities indicate that *R. crispus* could be a potential source of the active molecules intended for use in the pharmaceutical industry. Further work needs to be established to identify other molecules responsible for such activities using for example the flash chromatography.

Authors' Contributions

Experiments and the manuscript writing were done by Mohamed Marouane Saoudi, under the guidance of the

coauthors. Khaled Alouani and Bouajila Jalloul validated the experiments, proofread, and refined the manuscript to be ready for publication. Rami Rahmani performed the statistical analysis.

Acknowledgments

This work has been supported financially by the Tunisian Minister of Higher Education and Scientific Research, which provided a Ph.D. scholarship for the student Mohamed Marouane Saoudi.

- [1] F. A. Matough, S. B. Budin, Z. A. Hamid, N. Alwahaibi, J. Mohamed, "The role of oxidative stress and antioxidants in diabetic complications," *Sultan Qaboos University Medical Journal*, vol. 12 no. 1, pp. 556-569, DOI: 10.12816/0003082, 2012.
- [2] L. Machu, L. Misurcova, J. Vavra Ambrozova, "Phenolic content and antioxidant capacity in algal food products," *Molecules*, vol. 20 no. 1, pp. 1118-1133, DOI: 10.3390/molecules20011118, 2015.
- [3] G. Gunalan, N. Myla, R. Balabhaskar, "Research article in vitro antioxidant analysis of selected coffee bean varieties," *Journal of Chemical and Pharmaceutical Research*, vol. 4 no. 4, pp. 2126-2132, DOI: 10.1097/FPC.0000000000000202, 2012.
- [4] V. Lobo, A. Patil, A. Phatak, N. Chandra, "Free radicals, antioxidants and functional foods: impact on human health," *Pharmacognosy Reviews*, vol. 4 no. 8, pp. 118-126, DOI: 10.4103/0973-7847.70902, 2010.
- [5] S. U. Rakesh, P. R. Patil, S. R. Mane, "Use of natural antioxidants to scavenge free radicals: a major cause of diseases," *International Journal of PharmTech Research*, vol. 2 no. 2, pp. 1074-1081, 2010.
- [6] S. S. Naphade, S. S. Khadabadi, S. L. Deore, N. S. Jagtap, S. P. Hadke, "Antioxidant activity of different extracts of plant *Tricholepis glaberrima* DC (asteraceae)," *International Journal of PharmTech Research*, vol. 1 no. 3, pp. 502-505, 2009.
- [7] O. Aiyegoro, A. Okoh, "Phytochemical screening and polyphenolic antioxidant activity of aqueous crude leaf extract of *Helichrysum pedunculatum*," *International Journal of Molecular Sciences*, vol. 10 no. 11, pp. 4990-5001, DOI: 10.3390/ijms10114990, 2009.
- [8] S. Mariem, F. Hanen, J. Inès, S. Mejdi, K. Riadh, "Phenolic profile, biological activities and fraction analysis of the medicinal halophyte *Retama raetam*," *South African Journal of Botany*, vol. 94, pp. 114-121, DOI: 10.1016/j.sajb.2014.06.010, 2014.
- [9] M. Mohammadi-Sichani, P. Sadeghzadeh, M. Madani, "Evaluation of antibacterial activity of extract of *Rumex alveolatus* leaf against *Staphylococcus aureus* and *Pseudomonas aeruginosa*," *Zahedan Journal of Research in Medical Sciences*, vol. 15 no. 6, pp. 58-61, 2013.
- [10] L. Tavares, D. Carrilho, M. Tyagi, "Antioxidant capacity of macaronesian traditional medicinal plants," *Molecules*, vol. 15 no. 4, pp. 2576-2592, DOI: 10.3390/molecules15042576, 2010.
- [11] A.-L. Molan, A. M. Faraj, "The effects of condensed tannins extracted from different plant species on egg hatching and larval development of *Teladorsagia circumcincta* (nematoda: trichostrongylidae)," *Folia Parasitologica*, vol. 57 no. 1, pp. 62-68, DOI: 10.14411/fp.2010.008, 2010.
- [12] M. Saleem, B. Ahmed, M. Karim, "Hepatoprotective effect of aqueous methanolic extract of *Rumex dentatus* in paracetamol-induced hepatotoxicity in mice," *Bangladesh Journal of Pharmacology*, vol. 9 no. 3, pp. 284-289, DOI: 10.3329/bjp.v9i3.18874, 2014.
- [13] Q. C. Xie, Y. P. Yang, "Anti-proliferative of physcion 8-O- β -glucopyranoside isolated from *Rumex japonicus* Houtt. on A549 cell lines via inducing apoptosis and cell cycle arrest," *BMC Complementary and Alternative Medicine*, vol. 14 no. 1, DOI: 10.1186/1472-6882-14-377, 2014.
- [14] J. Ogweni Midiwo, A. Yenesew, B. F. Juma, "Bioactive compounds from some Kenyan ethnomedicinal plants: Myrsinaceae, Polygonaceae and *Psiadia punctulata*," *Phytochemistry Reviews*, vol. 1 no. 3, pp. 311-323, DOI: 10.1023/a:1026029609500, 2002.
- [15] L. Ghosh, J. R. Gayen, S. Sinha, S. Pal, M. Pal, B. P. Saha, "Antibacterial efficacy of *Rumex nepalensis* Spreng. roots," *Phytotherapy Research*, vol. 17 no. 5, pp. 558-559, DOI: 10.1002/ptr.1162, 2003.
- [16] S. Sahreen, M. R. Khan, R. A. Khan, T. B. Hadda, "Evaluation of phytochemical content, antimicrobial, cytotoxic and antitumor activities of extract from *Rumex hastatus* D. don roots," *BMC Complementary and Alternative*

Medicine, vol. 15 no. 1, DOI: 10.1186/s12906-015-0736-y, 2015.

- [17] A. Vasas, O. Orbán-Gyapai, J. Hohmann, "The genus rumex: review of traditional uses, phytochemistry and pharmacology," *Journal of Ethnopharmacology*, vol. 175, pp. 198-228, DOI: 10.1016/j.jep.2015.09.001, 2015.
- [18] Z. Maksimović, N. Kovačević, B. Lakušić, T. Čebović, "Antioxidant activity of yellow dock (*Rumex crispus* L., Polygonaceae) fruit extract," *Phytotherapy Research*, vol. 25 no. 1, pp. 101-105, DOI: 10.1002/ptr.3234, 2011.
- [19] P. Feduraev, G. Chupakhina, P. Maslennikov, N. Tacenko, L. Skrypnik, "Variation in phenolic compounds content and antioxidant activity of different plant organs from *Rumex crispus* L. and *Rumex obtusifolius* L. at different growth stages," *Antioxidants*, vol. 8 no. 7, DOI: 10.3390/antiox8070237, 2019.
- [20] J. G. Zaller, "Ecology and non-chemical control of *Rumex crispus* and *R. obtusifolius* (Polygonaceae): a review," *Weed Research*, vol. 44 no. 6, pp. 414-432, DOI: 10.1111/j.1365-3180.2004.00416.x, 2004.
- [21] O. A. Idris, O. A. Wintola, A. J. Afolayan, "Phytochemical and antioxidant activities of *Rumex crispus* L. in treatment of gastrointestinal helminths in eastern Cape province, south Africa," *Asian Pacific Journal of Tropical Biomedicine*, vol. 7 no. 12, pp. 1071-1078, DOI: 10.1016/j.apjtb.2017.10.008, 2017.
- [22] T. Olckers, "Invasive species: vectors and management strategies," *African Journal of Aquatic Science*, vol. 29 no. 2, DOI: 10.2989/16085910409503826, 2004.
- [23] E. Le Floc'h, L. Boulos, E. Vela, "*Rumex Crispus*," *Catalogue Synonymique Commenté de la Flore de Tunisie*, 2010.
- [24] M. Yahyaoui, J. Bouajila, S. Cazaux, M. Abderrabba, "The impact of regional locality on chemical composition, antioxidant and biological activities of *Thymelaea hirsuta* L. extracts," *Phytomedicine*, vol. 41, pp. 13-23, DOI: 10.1016/j.phymed.2018.01.010, 2018.
- [25] R. Rahmani, S. Beaufort, S. A. Villarreal-Soto, P. Taillandier, J. Bouajila, M. Debouba, "Kombucha fermentation of African mustard (*Brassica tournefortii*) leaves: chemical composition and bioactivity," *Food Bioscience*, vol. 30, DOI: 10.1016/j.fbio.2019.100414, 2019.
- [26] I. Coruh, A. Gormez, S. Ercisli, M. Sengul, "Total phenolic content, antioxidant, and antibacterial activity of *Rumex crispus* grown wild in Turkey," *Pharmaceutical Biology*, vol. 46 no. 9, pp. 634-638, DOI: 10.1080/13880200802182240, 2008.
- [27] T. Čebović, D. Jakovljević, Z. Maksimović, S. Djordjević, S. Jakovljević, D. Četojević-Simin, "Antioxidant and cytotoxic activities of curly dock (*Rumex crispus* L., Polygonaceae) fruit extract," *Vojnosanitetski Pregled*, vol. 77 no. 3, pp. 308-316, DOI: 10.2298/vsp170713084c, 2020.
- [28] A. A. Elzaawely, T. D. Xuan, S. Tawata, "Antioxidant and antibacterial activities of *Rumex japonicus* HOUTT. aerial parts," *Biological and Pharmaceutical Bulletin*, vol. 28 no. 12, pp. 2225-2230, DOI: 10.1248/bpb.28.2225, 2005.
- [29] H. Suzuki, Y. Ohnishi, Y. Furusho, S. Sakuda, S. Horinouchi, "Novel benzene ring biosynthesis from C3 and C4 primary metabolites by two enzymes," *Journal of Biological Chemistry*, vol. 281 no. 48, pp. 36944-36951, DOI: 10.1074/jbc.M608103200, 2006.
- [30] N. Nayeem, S. M. B. Asdaq, "Gallic acid: a promising lead molecule for drug development," *Journal of Applied Pharmacy*, vol. 08 no. 2, DOI: 10.4172/1920-4159.1000213, 2016.
- [31] Z. Huyut, Ş. Beydemir, I. Gülçin, "Antioxidant and antiradical properties of selected flavonoids and phenolic compounds," *Biochemistry Research International*, vol. 2017, DOI: 10.1155/2017/7616791, 2017.
- [32] O. Wahdan, S. Aly Badr, M. Abdelfattah, "Phytochemical analysis, antibacterial and anticancer activities of the *Physalis peruviana* calyces growing in Egypt," *Food & Nutrition Journal*, vol. 4 no. 1, DOI: 10.29011/2575-7091.100097, 2019.
- [33] F. P. Karakas, A. B. Yidirim, R. Bayram, M. Z. Yavuz, A. Gepdiremen, A. U. Turker, "Antiproliferative activity of some medicinal plants on human breast and hepatocellular carcinoma cell lines and their phenolic contents," *Tropical Journal of Pharmaceutical Research*, vol. 14 no. 10, pp. 1787-1795, DOI: 10.4314/tjpr.v14i10.8, 2015.
- [34] D. J. Park, H. J. Jung, C. H. Park, T. Yokozawa, J. C. Jeong, "Root bark of *Paeonia suffruticosa* extract and its component methyl gallate possess peroxynitrite scavenging activity and anti-inflammatory properties through NF- κ

- B inhibition in LPS-treated mice," *Molecules*, vol. 24 no. 19, DOI: 10.3390/molecules24193483, 2019.
- [35] S.-C. Shin, J.-S. Choi, "Effects of epigallocatechin gallate on the oral bioavailability and pharmacokinetics of tamoxifen and its main metabolite, 4-hydroxytamoxifen, in rats," *Anti-Cancer Drugs*, vol. 20 no. 7, pp. 584-588, DOI: 10.1097/cad.0b013e32832d6834, 2009.
- [36] L. M. Gonçalves, I. M. Valente, J. A. Rodrigues, "An overview on cardamonin," *Journal of Medicinal Food*, vol. 17 no. 6, pp. 633-640, DOI: 10.1089/jmf.2013.0061, 2014.
- [37] E. N. F. Souza, E. M. Williamson, J. A. Hawkins, "Which plants used in ethnomedicine are characterized? phylogenetic patterns in traditional use related to research effort," *Frontiers in Plant Science*, vol. 9, DOI: 10.3389/fpls.2018.00834, 2018.
- [38] C. P. Armstrong, A. J. Gregor, "Brief communications," *Psychiatry*, vol. 27 no. 1, pp. 69-72, DOI: 10.1080/00332747.1964.11023377, 1964.
- [39] V. P. Shipov, E. S. Pigarev, E. I. Fedoros, "Benzene polycarboxylic acid compounds and their use as drug," 2017. United States Patent, Patent No: (US 9644074)
- [40] P. Weitkamp, K. Vosmann, N. Weber, "Highly efficient preparation of lipophilic hydroxycinnamates by solvent-free lipase-catalyzed transesterification," *Journal of Agricultural and Food Chemistry*, vol. 54 no. 19, pp. 7062-7068, DOI: 10.1021/jf0611973, 2006.
- [41] P. Rudrapaul, M. Gruner, H. J. Knölker, B. Dinda, "Flavones and triterpenes from the leaves of *Vitex peduncularis*," *Indian Journal of Chemistry—Section B Organic and Medicinal Chemistry*, vol. 54B no. 2, pp. 279-282, 2015.
- [42] J. Gabaston, T. Richard, S. Cluzet, "Pinus pinaster knot: a source of polyphenols against *Plasmopara viticola*," *Journal of Agricultural and Food Chemistry*, vol. 65 no. 40, pp. 8884-8891, DOI: 10.1021/acs.jafc.7b04129, 2017.
- [43] M. Aritomi, T. Kawasaki, "Three highly oxygenated flavone glucuronides in leaves of *Spinacia oleracea*," *Phytochemistry*, vol. 23 no. 9, pp. 2043-2047, DOI: 10.1016/s0031-9422(00)84967-5, 1984.
- [44] J. Shi, J. Ming, W. Sun, "Passivation and chloride-induced corrosion of a duplex alloy steel in alkali-activated slag extract solutions," *Construction and Building Materials*, vol. 155, pp. 992-1002, DOI: 10.1016/j.conbuildmat.2017.08.117, 2017.
- [45] A. Shafaghat, F. Pirfarshi, M. Shafaghatlonbar, "Luteolin derivatives and antimicrobial activity of *Achillea tenuifolia* Lam. methanol extract," *Industrial Crops and Products*, vol. 62, pp. 533-536, DOI: 10.1016/j.indcrop.2014.09.016, 2014.
- [46] Z. Kucekova, J. Mlcek, P. Humpolicek, O. Rop, P. Valasek, P. Saha, "Phenolic compounds from *Allium schoenoprasum*, *Tragopogon pratensis* and *Rumex acetosa* and their antiproliferative effects," *Molecules*, vol. 16 no. 11, pp. 9207-9217, DOI: 10.3390/molecules16119207, 2011.
- [47] S. N. López, R. L. E. Furlan, S. A. Zacchino, "Detection of antifungal compounds in *Polygonum ferrugineum* Wedd. extracts by bioassay-guided fractionation. Some evidences of their mode of action," *Journal of Ethnopharmacology*, vol. 138 no. 2, pp. 633-636, DOI: 10.1016/j.jep.2011.09.038, 2011.
- [48] N. Farina, D. Llewellyn, I. Mgekn, N. Tabet, "Vitamin E for Alzheimer's dementia and mild cognitive impairment (review) summary of findings for the main comparison," *Cochrane Database of Systematic Reviews*, vol. 4 no. 4, DOI: 10.1002/14651858.CD002854.pub5, 2017.
- [49] A. Sen, P. Dhavan, K. K. Shukla, S. Singh, G. Tejovathi, "Analysis of IR, NMR and antimicrobial activity of β -sitosterol isolated from *Momordica charantia*," *Science Secure Journal of Biotechnology*, vol. 1 no. 1, 2012.
- [50] A. S. Bowman, S. O. Asare, B. C. Lynn, "Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis for characterization of lignin oligomers using cationization techniques and 2, 5-dihydroxyacetophenone (DHAP) matrix," *Rapid Communications in Mass Spectrometry*, vol. 33 no. 8, pp. 811-819, DOI: 10.1002/rcm.8406, 2019.
- [51] Y. Huang, N. Li, J.-B. Wan, D. Zhang, C. Yan, "Structural characterization and antioxidant activity of a novel heteropolysaccharide from the submerged fermentation mycelia of *Ganoderma capense*," *Carbohydrate Polymers*, vol. 134, pp. 752-760, DOI: 10.1016/j.carbpol.2015.08.067, 2015.

- [52] S. Shomo, H. Shifa, C. Senthilraja, M. G. Reddy, V. Paranidharan, R. Velazhahan, "Analysis of metabolite profiles in leaf tissues of transgenic groundnut plants modified for resistance against tobacco streak virus," *Biochemical and Cellular Archives*, vol. 16 no. 2, pp. 245-250, 2016.
- [53] S. Yu, X. Wang, T. Zhang, "Identification of a novel di-D-fructofuranose 1, 2': 2, 3' dianhydride (DFA III) hydrolysis enzyme from *arthrobacter aureus* SK8.001," *PLoS One*, vol. 10 no. 11, DOI: 10.1371/journal.pone.0142640, 2015.
- [54] D. F. McComsey, B. E. Maryanoff, "Improved synthesis of pseudo-.beta.-D-fructopyranose, a carbocyclic monosaccharide from (-)-quinic acid," *The Journal of Organic Chemistry*, vol. 59 no. 9, pp. 2652-2654, DOI: 10.1021/jo00088a065, 1994.
- [55] H. T. L. Nguyen, N. Panyoyai, V. D. Paramita, N. Mantri, S. Kasapis, "Physicochemical and viscoelastic properties of honey from medicinal plants," *Food Chemistry*, vol. 241, pp. 143-149, DOI: 10.1016/j.foodchem.2017.08.070, 2018.
- [56] J. Maldonado, Á. Fernández, L. Cabeza, "Corrosion assessment of myo-inositol sugar alcohol as a phase change material in storage systems connected to fresnel solar plants," *Molecules*, vol. 24 no. 7, DOI: 10.3390/molecules24071383, 2019.
- [57] M. Durand, B. Porcheron, N. Hennion, L. Maurousset, R. Lemoine, N. Pourtau, "Water deficit enhances C export to the roots in *Arabidopsis thaliana* plants with contribution of sucrose transporters in both shoot and roots," *Plant Physiology*, vol. 170 no. 3, pp. 1460-1479, DOI: 10.1104/pp.15.01926, 2016.
- [58] C. Pereira, J. C. M. Barreira, R. C. Calhelha, "Is honey able to potentiate the antioxidant and cytotoxic properties of medicinal plants consumed as infusions for hepatoprotective effects?," *Food & Function*, vol. 6 no. 5, pp. 1435-1442, DOI: 10.1039/c4fo01206b, 2015.
- [59] M. D'Archivio, C. Filesi, R. Vari, B. Scazzocchio, R. Masella, "Bioavailability of the polyphenols: status and controversies," *International Journal of Molecular Sciences*, vol. 11 no. 4, pp. 1321-1342, DOI: 10.3390/ijms11041321, 2010.
- [60] S. M. A. Moustafa, B. M. Menshawi, G. M. Wassel, K. Mahmoud, M. M. Marwa, "Screening of some plants in Egypt for their cytotoxicity against four human cancer cell lines," *International Journal of PharmTech Research*, vol. 6 no. 3, pp. 1074-1084, 2014.
- [61] N. Ammar, N. Ayoub, S. El-Ahmady, L. El-Kassem, E. Zeid, "Phytochemical and cytotoxic studies of *Rumex pictus* forssk. and *Rumex vesicarius* L. (family Polygonaceae), growing in Egypt," *European Journal of Medicinal Plants*, vol. 10 no. 3, DOI: 10.9734/ejmp/2015/19830, 2015.
- [62] S. Ahmad, F. Ullah, M. Ayaz, A. Sadiq, M. Imran, "Antioxidant and anticholinesterase investigations of *Rumex hastatus* D. don: potential effectiveness in oxidative stress and neurological disorders," *Biological Research*, vol. 48 no. 1, DOI: 10.1186/s40659-015-0010-2, 2015.
- [63] D. N. Patil, S. A. Patil, S. Sistla, J. P. Jadhav, "Comparative biophysical characterization: a screening tool for acetylcholinesterase inhibitors," *PLoS One*, vol. 14 no. 5, pp. e0215291-e0215328, DOI: 10.1371/journal.pone.0215291, 2019.
- [64] B. Z. Kurt, I. Gazioglu, E. Sevgi, F. Sonmez, "Anticholinesterase, antioxidant, antiaflatoxigenic activities of ten edible wild plants from Ordu area, Turkey," *Iranian Journal of Pharmaceutical Research: IJPR*, vol. 17 no. 3, pp. 1047-1056, DOI: 10.22037/ijpr.2018.2248, 2018.
- [65] M. Abdelwahab, S. Sangi, H. Arafat, E. Ragab, "New phytochemical constituent and bioactivities of *Horwoodia dicksoniae* and *Rumex cypricus*," *Pharmacognosy Magazine*, vol. 12 no. 47, pp. 165-170, DOI: 10.4103/0973-1296.186348, 2016.
- [66] I. Khlifi, E. A. Hayouni, S. Cazaux, R. Ksouri, J. Bouajila, "Evaluation of in vitro biological activities: antioxidant; anti-inflammatory; anticholinesterase; anti- xanthine oxidase, anti-superoxyde dismutase, anti- α -glucosidase and cytotoxic of 19 bioflavonoids," *Cellular and Molecular Biology*, vol. 66 no. 1, DOI: 10.14715/cmb/2019.66.1.2, 2020.
- [67] F. Wang, X. Yao, Y. Zhang, J. Tang, "Synthesis, biological function and evaluation of shikonin in cancer therapy," *Fitoterapia*, vol. 134, pp. 329-339, DOI: 10.1016/j.fitote.2019.03.005, 2019.

- [68] T. A. Ibrahim, A. A. El-Hela, "Polysaccharide from the roots of polygonum equisetiforme Sibth. & Sm: isolation, chemical composition, antioxidant potential and cytotoxic activity," International Journal of Pharma and Bio Sciences, vol. 3 no. 4, pp. 478-492, 2012.
- [69] L. Berning, L. Scharf, E. Aplak, "In vitro selective cytotoxicity of the dietary Chalcone cardamonin (CD) on melanoma compared to healthy cells is mediated by apoptosis," PLoS One, vol. 14 no. 9, DOI: 10.1371/journal.pone.0222267, 2019.
- [70] J. Jin, S. Qiu, P. Wang, "Cardamonin inhibits breast cancer growth by repressing HIF-1 α -dependent metabolic reprogramming," Journal of Experimental & Clinical Cancer Research: CR, vol. 38 no. 1, DOI: 10.1186/s13046-019-1351-4, 2019.

DETAILS

Subject:	Cancer; Physiology; Antioxidants; Acids; Dichloromethane; Reagents; Cytotoxicity; Free radicals; Phytochemicals; Solvents; Cyclohexane; Phenols; Biological properties; Chromatography; Chemical composition
Location:	Tunisia; France
Editor:	Antonio V Herrera-Herrera
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-12-28 (Received); 2021-06-14 (Revised); 2021-06-22 (Accepted); 2021-07-03 (Pub)

DOI: <https://doi.org/10.1155/2021/6675436>

ProQuest document ID: 2550174660

Document URL: <https://www.proquest.com/scholarly-journals/phytochemical-composition-antioxidant/docview/2550174660/se-2?accountid=211160>

Copyright: Copyright © 2021 Mohamed Marouane Saoudi et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. <https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-07-11

Database: Publicly Available Content Database

Document 27 of 59

The Use of Sugarcane Bagasse to Remove the Organic Dyes from Wastewater

Kerrou, Meryem

; Bouslamti, Najia; Raada, Abdelaziz; Elanssari, Abdellah; Mrani, Driss; My Sliman Slimani

[ProQuest document link](#)

ABSTRACT (ENGLISH)

In the present study, the potential of sugarcane bagasse (SCB) was evaluated by methylene blue (MB) retention. The selected low-cost adsorbent was characterized by scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), BET method, and determination of the point of zero charge (pHzpc). Batch kinetic and isothermal studies were performed to examine the effects of contact time, initial dye concentration, adsorbent dose, pH, and temperature. The results show that the kinetic study of MB adsorption on sugarcane bagasse is very fast; the equilibrium is reached after only 20 minutes. The kinetic model of pseudo-second-order and the Langmuir isotherm model perfectly explain the adsorption process of MB with a monolayer adsorption capacity equal to $49.261 \text{ mg} \cdot \text{g}^{-1}$ activation parameters' values such as free energy (ΔG°), enthalpy (ΔH°), and entropy (ΔS°) also determined as $-4.35 \text{ kJ} \cdot \text{mol}^{-1}$, $-31.062 \text{ kJ} \cdot \text{mol}^{-1}$, and $-0.084 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, respectively. Besides, the thermodynamic parameters of the methylene blue sugarcane bagasse system indicate that the exothermic adsorption process is spontaneous.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Industrial wastewater pollution has become a common problem in most countries [1] and one of the biggest environmental problems in recent decades. Industrial discharges include dyes used in different areas such as printing, food, cosmetics, and clinical products, but particularly in the textile industry [2]. The discharge of these colored effluents in nature affects not only humans and water but the whole environment. More than 14 million chemical products can be found in the environment; this number is gradually increasing which poses a negative impact on the biosphere and threatens the balance of the natural ecosystem. Indeed, the toxic chemical products dumped into the water are genotoxic and mutagenic which might cause hereditary diseases that can be transmitted to the future generation [3]. Moreover, this will also provoke the increase of the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) which inhibit and decrease the rate of photosynthesis and plants' growth [4]. In this context, a wide variety of physical, chemical, and biological techniques have been developed and tested in the treatment of these effluents loaded with dyes. Therefore, adsorption remains one of the easy-to-implement technologies, as it is widely used for water treatment [5]. Nowadays, adsorption has been accepted as a suitable removal technology, particularly for developing regions, because of its simple operation, the potential for regeneration. The adsorption process can be described as "the tendency of chemical species existing in a phase to adhere onto a solid" [6]. Technically, in adsorption science, the solid surface providing the adsorption sites is called adsorbent, and substances that are adsorbed at the solid surface are known as adsorbate [6]. The removal of dyes by adsorption on selective adsorbents such as low-cost adsorbents (sugarcane bagasse) as shown in Figure 1 has been synthesized and tested for dye removal.

[figure omitted; refer to PDF]

From the literature, we can find that the use of activated carbon is considered a good adsorbent due to its high adsorption capacity for organic materials. However, activated carbon is not only expensive but also difficult to regenerate. In this regard, there is a growing interest among researchers in the last few years towards the use of adsorbents that are based and prepared from natural materials with an organic phase such as cellulose, hemicellulose, and lignin contents [7] which are marked by their efficiency and low-cost and their abundance in large quantities in developing countries such as Lgharb region in Morocco. Recently, quite a good number of research articles have been published on the utilization of low-cost adsorbents derived from biomass for wastewater treatment such as banana waste, moringa seed and lemon seed, straws, cotton, and palm fibers; rice (*Oryza sativa*) and coffee (*Coffea Arabica*) husk wastes were used [8–10].

Therefore, the present study aims to investigate the MB dye removal efficiency using cost-effective sustainable solid biomass sugarcane bagasse (SCB) adsorbent materials using batch and the close circuit under various physiochemical process conditions which may be applicable in actual large scale industrial treatment operation.

2. Materials and Methods

2.1. Materials

The materials are methylene blue $C_{16}H_{18}ClN_3S$, sodium chloride (NaCl purity 99, 8%), chloride acid (HCl purity 37%), sodium hydroxide (NaOH purity 98%), and sugarcane bagasse (SCB).

2.2. Adsorbate

Methylene blue (MB) is an organic dye that is chosen for this study for its very high degree of purity (99%). It was used without any prior purification. Its characteristics are grouped in Table 1.

Table 1

Chemical characterization of MB.

Dye	Methylene blue
Structure	
Molecular formula	$C_{16}H_{18}ClN_3S$

Molecular weight (g/mol)	319.85
Maximum wavelength λ_{max} (nm)	665

2.3. Adsorbent

Sugarcane bagasse is a natural waste abundantly available in El Ghareb region, Morocco. The sugarcane bagasse was collected from various local “sugarcane juice” venders; then, it was treated as follows: first, it was washed several times to remove the impurities, then dried in the oven at a temperature of 105°C for 24h, and grind and sieved to a particle size equal to 250 μm .

3. Characterization of SCB

This adsorbent’s physical and chemical characteristics are viewed to possess high rates of efficiency of pollutants removal [11]. These characteristics are discussed in the following part.

3.1. Textural Characterization

The measurement of the specific surface of pore diameters and pore volume of SCB was obtained by using the nitrogen adsorption-desorption isotherm curve (Micromeritics ASAP 2010) using the Brunauer–Emmett–Teller (BET) method. Morphological analysis was made by scanning electron microscopy (SEM) type (VEGA3 TESCAN) coupled with EDX and quantitative analysis of the elemental composition of SCB was employed using X-ray energy dispersion spectroscopy (EDX).

3.2. Chemical Characterization

The chemical functions of the molecules present in the SCB were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) type (Shimadzu, JASCO 4100). IR spectra were recorded over a wavelength range of 400 and 4000 cm^{-1} . The zero point charge where pH_{zpc} was determined to clarify the net charge was carried by the surface of the SCB.

3.3. Adsorption Procedure

The adsorption tests were conducted in the batch reactor, at ambient temperature; the colored synthetic solution of MB in the presence of the adsorbent SCB is stirred for an hour; and homogenization of the mixtures was carried out by a Shaker type incubator agitator (Jisico, model J-NSIL-R) with a stirring speed equal to 127 rpm. The adsorbate-adsorbent separation was performed using a 0.45 μm diameter Wothman-type filtration system, and the supernatant absorbance was measured by a UV-Visible type spectrophotometer (Shimadzu 1601) at a wavelength corresponding to the maximum absorbance of MB ($\lambda_{\text{max}}=665$). Then, the residual dye concentration is determined from a calibration curve by Beer Lambert’s law.

The adsorption capacity of MB by SCB is calculated by the following formula: (1) $q_t = C_0 - C_t \cdot V/m$, where q_t ($\text{mg} \cdot \text{g}^{-1}$) is the amount adsorbed at time t (min), C_0 and C_t ($\text{mg} \cdot \text{L}^{-1}$) are the initial concentration and the concentration at time t in the dye, V (L) is the volume of the solution, and m (g) is the amount of the adsorbent in solution.

4. Results and Discussion

4.1. Adsorbent Characterization

4.1.1. Nitrogen Adsorption-Desorption Isotherm

The nitrogen adsorption-desorption isotherm is obtained at a temperature of 77.35K after degassing at 80°C. The BET S_{BET} surface area was determined using the Brunauer–Emmett–Teller (BET) equation, the pore volume at saturation V_P is the volume of nitrogen corresponding to the highest relative pressure of $P/P_0=0.99$, and the pore diameter DP is calculated by the relation $4 V_P/S_{\text{BET}}$ [12]. The nitrogen adsorption-desorption isotherm obtained on sugarcane bagasse powder is shown in Figure 2.

[figure omitted; refer to PDF]

According to the classification of isotherms (IUPAC), this curve has a sigmoidal shape, classified as type II, frequently found in fruits with a high sugar content [13], which means that the medium is nonporous or macroporous. The adsorption isotherm obtained shows that the SCB has a nonporous structure; this is confirmed by the textural measurements grouped in Table 2.

Table 2

Results of the analyses of the porous network by nitrogen adsorption.

Sample	Pore diameter (Å)	Specific surface (m ² /g)	Pore volume (cm ³ /g)
Sugarcane bagasse	57.285	0.7340	0.000679

4.1.2. Scanning Electron Microscopy (SEM)

The SEM images (Figure 3) illustrate the morphological structure of the SCB.

[figures omitted; refer to PDF]

The observations obtained show that sugarcane bagasse has a fibrous structure, each elementary fiber has a compact structure that is aligned in the direction of the fiber axis, and sugarcane bagasse has a smooth and continuous surface [14, 15].

4.1.3. Energy-Dispersive X-Ray Spectroscopy (EDX)

The surface elemental analysis of the SCB (Figure 4) indicates the presence of different chemical elements, and those results are grouped in Table 3. Which reveal the important presence of carbon and oxygen with a percentage of 51.10% and 48.85%, respectively, compared to the other chemical elements (S,...) which confirms the organic nature of our material [16], and the high oxygen content suggests that the surface of the adsorbent is more acidic.

[figure omitted; refer to PDF]

Figure 5 shows that the broadband at 3412.92 cm^{-1} is related to the elongation vibration of the O-H bond [17], mainly due to the presence of cellulose molecules [18]. The peak observed at 2922.93 is attributed to the C-H elongation and bending vibration of the CH_3 methyl group [19]. The peak at 1736 cm^{-1} is due to the elongation of the carbonyl group of aldehydes and ketones [20]. The 1605 band is due to aromatic skeletal elongation vibrations existing in the lignin structure [21]. The 1053.31 cm^{-1} band is attributed to stretching vibrations in steric $\text{O}=\text{C}-\text{O}-\text{C}$ compounds due to the existence of hemicellulose [22, 23], and the 608.93 cm^{-1} band corresponds to the bending modes of aromatic compounds [24].

4.3. Determination of Zero Point Charge (ZPC)

Like many physiochemical variables such as pH, temperature, and ZPC, the latter is considered to be a significant factor that helps in determining the biosorption capacity of the biosorbent and the nature of binding sites [25, 26]. In this study, this method consists of preparing a series of 20ml of a 5.10–2M NaCl solution, after adjusting the initial pH (pH_i) of each to values between 2 and 12, by the addition of HCl (0.1M) or NaOH (0.1M), a 0.2g mass of the SCB is then added to the different solutions. All of this is left to stir at ambient temperature for 48h until the final pH (pH_f) has stabilized. The intersection of the curve ΔpH ($\text{pH}_f - \text{pH}_i$) as a function of pH_i with the x-axis determines the ZPC (Figure 6).

[figure omitted; refer to PDF]

The ZPC of the SCB is equal to 4.69, which expresses that the surface of the SCB is positively charged at a pH below 4.69 and negatively charged at a pH above 4.69. The low ZPC value also indicates the acidity of the adsorbent surface, which confirms the results of the elemental chemical composition which shows high oxygen content [26].

4.4. Effect of Reaction Parameters

4.4.1. Effects of Adsorbent Amount

Biosorbent dosage has a major role in the dye removal process [26]. This study was conducted by varying the mass of the SCB between 0.05 and 0.5g, keeping the other parameters constant: an ambient temperature, a solution pH of 6.4, an initial dye concentration of $25\text{ mg}\cdot\text{L}^{-1}$, and a stirring speed of 127rpm. Figure 7 shows the effect of the adsorbent amount on dye removal.

[figure omitted; refer to PDF]

According to the results obtained, the percentage of dye removal increases with the increase of the adsorbent

amount from 80.27% for 0.05g up to 98.49% for 0.5g. This phenomenon is due to the increase of the specific surface area and the high availability of the adsorption sites [1]. For the continuation of the studies, we have chosen an amount equal to 0.2g with an elimination percentage equal to 95.86%. The chosen amount minimizes the consumption of the amount of the SCB to half compared to 0.5g with a percentage of 98.49% close to that of 0.2g.

4.4.2. Effect of Contact Time

The experiments were conducted in different contact times, and the variation in the amount of MB adsorbed by the SCB in the function of contact time (5–120 min) was observed as we set three initial concentrations of dye 5, 15, and $25\text{ mg}\cdot\text{L}^{-1}$ as Figure 8 illustrates.

[figure omitted; refer to PDF]

The results obtained show that the equilibrium time is independent of the initial dye concentration and that the amount of dye fixed on the adsorbent increases with the contact time. Figure 8 reveals the existence of two phases during the adsorption of the MB by the SCB: the first one is the fastest taking 5 minutes, which can be explained by the existence of a high affinity between the cations of the dye and the adsorbent [17], and the second is a slow phase where equilibrium is gradually reached after 20 minutes. This may be due to the saturation of the active sites in the support [27]. The contact time was set at 60 min for further studies.

4.4.3. Effect of the Initial Dye Concentration

The effect of the initial concentration of MB on its retention rate by the SCB was studied at different initial concentrations, ranging from 5 to $100\text{ mg}\cdot\text{L}^{-1}$, and a constant mass of the SCB of 0.2g at ambient temperature for 60 minutes (Figure 9).

[figure omitted; refer to PDF]

Figure 9 shows that the percentage of MB removal increases for low concentrations, up to a maximum obtained at 97% for a concentration of $25\text{ mg}\cdot\text{L}^{-1}$. This can be explained by the availability of active sites which are much higher than the amount of dye introduced. However, at high concentrations, the percentage decreases from 97% to 56% due to a lack of available active sites.

4.4.4. Effect of pH

The solution pH plays a significant role in the sorption process as it can affect the surface charge of the adsorbent and the molecular state of the dye molecule. In other words, it interrupts both the solution's chemistry of dyes and functional groups of the adsorbents. Meanwhile, it seems that the adsorption capacity of the dye depends on the pH of the solution [25, 28]. The variation of biosorption capacity of SCB for the removal of MB dye was studied in the pH range of 2 to 12 by adding HCl (chloride acid) purity 37% (0.1 M) or NaOH (sodium hydroxide) purity 98% (0.1 M), keeping other variables constant (0.2g/50 ml biosorbent dose, 25°C temperature, 127 rpm, and 60 min contact time). Figure 10 shows the effect of pH solution on dye removal.

[figure omitted; refer to PDF]

The results indicate that the basic pH was favorable for the removal of MB. The optimum pH for maximum dye removal of SCB was found to be 10, (dye removal=99.30%).

4.4.5. Effect of Ionic Strength

Wastewater contains various elements such as salts, organic, and metal ions. The presence of those ions is the reason for a high ionic strength which affects the performance of the adsorption process significantly [29]. The study of the influence of this parameter on the adsorption phenomenon is carried out by adding variable amounts of NaCl (sodium chloride, purity 99.8%) of concentration ranging from 0 to 0.1 M to solutions of the dye of initial concentration of $25\text{ mg}\cdot\text{L}^{-1}$ and a mass of 0.2g of the adsorbent. The influence of the initial concentration of NaCl on the rate of removal of MB by SCB is shown in Figure 11.

[figure omitted; refer to PDF]

According to these results, we notice a great decrease in the rate of elimination of the dye with the increase of the NaCl concentration from 93.63% to 77.71%. Above a concentration of 0.04 M NaCl, there is always a decrease but at a very low rate. This phenomenon can be attributed to the fact that Na^{+} ions accumulate in greater numbers next to the surface and thus screen the fixation sites [30].

4.4.6. Effect of Temperature

The influence of the temperature on the adsorption phenomenon was carried out by adding 0.2g/50ml of SCB to a solution of MB at a concentration of 25mg/L, at a temperature varying between 15 and 75°C (Figure 12).

[figure omitted; refer to PDF]

Figure 11 shows that the MB removal rate on the SCB decreases from 98.75% to 89.22% with increasing temperature from 15 to 75°C. This decrease can be explained by the destruction of the adsorption sites [31], which means that the increase in temperature adversely affects the adsorption mechanism, so the reaction is exothermic in nature.

4.5. Kinetics Study

MB adsorption kinetics data from the SCB are modeled by three models: pseudo-first-order, pseudo-second-order, and intraparticle diffusion.

The pseudo-first-order kinetic model, evaluated by the Lagergren relation [32], is described by the following equation: (2) $\frac{dq}{dt} = K_1(q_e - q_t)$.

After the integration for the boundary conditions $q_t = 0$ to $t = 0$ and $q_t = q_t$ to $t = t$, the equation becomes (3) $\ln(q_e - q_t) = \ln(q_e - K_1 t)$.

The quantity adsorbed at equilibrium q_e and the velocity constant k_1 are obtained from the intercept and the slope of the curve $\ln(q_e - q_t)$ versus time t , respectively.

The application of Blanchard's model allows us to define the pseudo-second-order of the reaction in a sorption process [33]: (4) $\frac{dq}{dt} = K_2(q_e - q_t)^2$.

The integration of the equation for the boundary conditions $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$ gives (5) $\frac{t}{q_t} = \frac{1}{K_2} \times \frac{1}{q_e^2} + \frac{t}{q_e}$.

The quantity adsorbed at equilibrium q_e and the velocity constant K_2 are determined, respectively, from the slope and the intercept at the origin of the t/q_t curve as a function of time t .

The intraparticle diffusion (internal transport) is also used to identify the diffusion mechanism. It is presented by the following equation: (6) $q_t = K_{id} \cdot t^{1/2} + C$.

By tracing q_t as a function of $t^{1/2}$, the constants K_{id} and C are deduced from the slope and intercept, respectively.

The best model that describes adsorption kinetics is the one with the highest R^2 linear regression coefficient.

Figure 13 presents the t/q_t curve in the function of time t at different initial MB concentrations of the pseudo-second-order model for MB adsorption by the SCB and shows improved linearity.

[figure omitted; refer to PDF]

Table 4 summarizes the results of the three models of MB adsorption kinetics by the SCB. According to this table, the adsorption kinetics is described perfectly with the pseudo-second-order model and the intraparticle diffusion model, with a correlation coefficient which is equal to 1 for the pseudo-second-order kinetic model and 0.96 for the intraparticle diffusion model; the calculated value of the adsorbed quantity at the equilibrium $q_{e,cal}$ is very close to the experimental value $q_{e,exp}$. On the other hand, the pseudo-first-order model represents a poor correlation. The velocity constant K_2 shows that the retention of MB by the SCB is quite fast [27].

Table 4

Kinetic parameters related to the adsorption of MB on the SCB.

	Dye concentration (mg·L ⁻¹)	5	15	25
$q_{e,exp}$ (mg·g ⁻¹)	2.621	6.285	7.047	.
Pseudo-first-order kinetic model	k_1 (min ⁻¹)	2.1591	1.4277	1.1817
$q_{e,cal}$ (mg·g ⁻¹)	0.968	0.991	0.9814	R^2

0.66	0.092	0.55	.	
Pseudo-second-order kinetic model	k_2 (g·mg ⁻¹ ·min ⁻¹)	2.329	10.156	0.991
q _{e,cal} (mg·g ⁻¹)	2.621	6.153	7.032	R ²
1	0.997	1	.	
Intraparticle diffusion model	K_{id1} (g·mg ⁻¹ ·min ^{-1/2})	0.8769	1.9524	2.2958
C (mg·g ⁻¹)	0.12	0.2071	0.3122	R ²
0.94	0.96	0.93	K_{id2} (g·mg ⁻¹ ·min ^{-1/2})	0.0061
0.1168	0.1142	C (mg·g ⁻¹)	2.5538	5.5959
6.4559	R ²	0.76	0.69	0.99

4.6. Adsorption Isotherms

Some of the important roles of the adsorption isotherms are that they allow the description of how the adsorbate interacts with the adsorbent, illustrate the type of accumulation of adsorbate on the adsorbent, and analyze the adsorption equilibrium [34]. Experimental data for the MB adsorption isotherms in the SCB were modeled using two models: the Langmuir model and the Freundlich model illustrated below.

4.7. Langmuir Model

Langmuir's model supposes that the adsorption comes from the monolayer coverage of the adsorbate on a homogeneous surface; that is, once a dye molecule takes up at a site, no further adsorption can take place at that site [35], and it is illustrated that (7) $q_e = K_{eq} C_e / (1 + K_{eq} C_e)$.

The linear transform of this model has the following equation [36]: (8) $C_e/q_e = 1/q_m \times K_L + C_e/q_m$.

With q_e and q_m (mg·g⁻¹) being the amount adsorbed at equilibrium and the maximum amount adsorbed at saturation of the monolayer, C_e (mg·L⁻¹) is the equilibrium concentration and K_L (L·mg⁻¹) is the Langmuir constant. By tracing C_e/q_e according to C_e , the Langmuir model is checked if a straight line of slope $1/q_m$ and ordinate at the origin $1/q_m K_L$ is obtained.

We can check whether adsorption is favorable or not by the equilibrium parameter R_L given as (9) $R_L = 1 / (1 + K_L C_0)$.

The adsorption is irreversible ($R_L = 0$), favorable ($0 < R_L < 1$), and unfavorable ($R_L > 1$) [37].

4.8. Freundlich Model

Freundlich's model is based on an empirical equation used to model adsorption isotherms on energetically heterogeneous surfaces [38]. It is expressed by the following relationship [39]: (10) $q_e = K_F C_e^{1/n}$, where q_e (mg·g⁻¹) is the equilibrium amount adsorbed, K_F and n are Freundlich's constants, and C_e (mg·L⁻¹) is the equilibrium concentration of the solute.

The logarithmic model of this relationship makes it possible to verify its linear transformation [36]: (11) $\ln q_e = 1/n \ln C_e + \ln K_F$.

The values of K_F and n are determined experimentally by drawing $\ln q_e$ according to $\ln C_e$.

The isotherms of MB adsorption by the SCB at 25°C are presented according to the Langmuir model (Figure 14) and the Freundlich model (Figure 15).

[figure omitted; refer to PDF]

Table 5 presents the values of the adsorption equilibrium parameters according to the Langmuir and Freundlich model.

Table 5

Adsorption parameters of the MB on the SCB according to the Langmuir and Freundlich models.

	Parameters	Values
Langmuir model	K_L ($L \cdot mg^{-1}$)	0.1225
q_{max} ($mg \cdot g^{-1}$)	49.261	RL
0.246	R^2	0.98
–		
Freundlich model	KF	10.0071
1/n	0.172	R^2

According to these results, the correlation coefficient of the Langmuir model is equal to 0.98, as it is close to the Freundlich correlation coefficient of 0.97. This means that the process of MB adsorption by SCB is perfectly described by both Langmuir and Freundlich models and the separation factor $RL < 1$ indicates that the adsorption is favorable.

4.9. Thermodynamic Study

Thermodynamic parameters such as enthalpy ΔH° , entropy ΔS° , and free enthalpy ΔG° were calculated at different temperatures of 15, 35, 55, and 75°C to describe the reaction of MB adsorption by the SCB from the following equations [40]: $(12) \ln K_d = \Delta S^\circ / R - \Delta H^\circ / RT$, $\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ$.

$K_d = q_e / C_e$ is the distribution coefficient; q_e ($mg \cdot g^{-1}$) is the amount adsorbed at equilibrium; R is the perfect gas constant and T (K) is the temperature of the solution; and C_e ($mg \cdot L^{-1}$) is the equilibrium concentration.

The thermodynamic parameters play a significant role as they provide information about the spontaneity and endo- or exothermicity of the adsorption process and the increment or decrease of randomness at the solid-liquid interface [41].

The plot of $\ln K_d$ as a function of $1/T$ (Figure 16) gives a straight line of slope $-\Delta H^\circ / R$ and an intercept at the origin $\Delta S^\circ / R$. The results obtained are grouped in Table 6.

[figure omitted; refer to PDF]

Table 6

Thermodynamic parameters related to the adsorption of the MB dye on the SCB.

Thermodynamic parameters	Temperature (°K)			
288	308	328	348	ΔG° ($kJ \cdot mol^{-1}$)

-6.87	-5.19	-3.51	-1.83	ΔS° (kJ·mol ⁻¹ ·K ⁻¹)
-0.084				ΔH° (kJ·mol ⁻¹)

These results show that the adsorption process of MB on the SCB is exothermic in nature and can be qualified as physical adsorption since the value of ΔH° is negative and greater than $-40\text{ kJ}\cdot\text{mol}^{-1}$; the negative value of ΔS° indicates that the molecules of the dye are more organized at the solid/liquid interface than in solution, hence a decrease in molecular disorder. Negative values of ΔG° indicate that adsorption is spontaneous and the increase in these values as the temperature increases from 15 to 75°C shows that the feasibility of adsorption decreases at elevated temperatures [42].

5. Conclusion

The adsorption process of MB on the SCB was the aim of this study. The results obtained show that the removal rate of MB increases from 80.27% to 98.49% with the increase of the mass of adsorbent from 0.05g to 0.5g due to the increase in specific surface area. The maximum removal of dye was observed at pH 10 and the biosorption process has reached equilibrium at 60min. The contact time effect indicated that the equilibrium time is independent of the initial MB concentration, adsorption is very fast after 5min, and equilibrium time is reached at 20min. On the other hand, the rate of elimination decreases with increasing temperature. Therefore, an exothermic reaction occurs. The kinetic and isothermal studies of the adsorption mechanism are perfectly described by pseudo-second-order kinetics and both Langmuir and Freundlich models as they perfectly present the adsorption of MB on the SCB. Finally, the thermodynamic studies have shown that the adsorption of MB by the SCB is exothermic, feasible, and spontaneous.

To conclude, SCB is found to be a good biosorbent for MB removal which makes it a great alternative for wastewater and dye effluents treatment, especially today, as we need to protect the environment using environment-friendly processes like this.

[1] A. Ahmad, M. Rafatullah, O. Sulaiman, M. H. Ibrahim, R. Hashim, "Scavenging behaviour of meranti sawdust in the removal of methylene blue from aqueous solution," *Journal of Hazardous Materials*, vol. 170 no. 1, pp. 357-365, DOI: 10.1016/j.jhazmat.2009.04.087, 2009.

[2] H. B. Mansour, O. Boughzala, d. Dridi, D. Barillier, L. Chekir-Ghedira, R. Mosrati, "Les colorants textiles sources de contamination de l'eau: CRIBLAGE de la toxicité et des méthodes de traitement," *Revue des Sciences de L'eau*, vol. 24 no. 3, pp. 209-238, DOI: 10.7202/1006453ar, 2011.

[3] M. Iqbal, "Vicia faba bioassay for environmental toxicity monitoring: a review," *Chemosphere*, vol. 144, pp. 785-802, DOI: 10.1016/j.chemosphere.2015.09.048, 2016.

[4] D. Dahiya, P. S. Nigam, "Waste management by biological approach employing natural substrates and microbial agents for the remediation of dyes' wastewater," *Applied Sciences*, vol. 10 no. 8, DOI: 10.3390/app10082958, 2020.

[5] N. Fayoud, S. A. Younssi, S. Tahiri, A. Albizane, "Kinetic and thermodynamic study of the adsorption of methylene blue on wood ashes," *Journal of Materials and Environmental Science*, vol. 11, pp. 3295-3306, 2015.

[6] M. A. Alaei Shahmirzadi, S. S. Hosseini, J. Luo, I. Ortiz, "Significance, evolution and recent advances in adsorption technology, materials and processes for desalination, water softening and salt removal," *Journal of Environmental Management*, vol. 215, pp. 324-344, DOI: 10.1016/j.jenvman.2018.03.040, 2018.

[7] S. V. Vassilev, D. Baxter, L. K. Andersen, C. G. Vassileva, T. J. Morgan, "An overview of the organic and inorganic phase composition of biomass," *Fuel*, vol. 94, DOI: 10.1016/j.fuel.2011.09.030, 2012.

[8] S. B. Patil, P. Sabale, S. A. Patil, "Experimental study on dye removal of textile wastewater by using natural adsorbent," *Resinap Journal of Science & Engineering*, vol. 3, pp. 526-533, 2019.

[9] O. Abdelwahab, S. M. Nasr, W. M. Thabet, "Palm fibers and modified palm fibers adsorbents for different oils,"

- Alexandria Engineering Journal, vol. 56 no. 4, pp. 749-755, DOI: 10.1016/j.aej.2016.11.020, 2017.
- [10] T. Ahmad, M. Danish, "Prospects of banana waste utilization in wastewater treatment: a review," *Journal of Environmental Management*, vol. 206, pp. 330-348, DOI: 10.1016/j.jenvman.2017.10.061, 2018.
- [11] M. Wakkal, B. Khiari, F. Zagrouba, "Textile wastewater treatment by agro-industrial waste: equilibrium modelling, thermodynamics and mass transfer mechanisms of cationic dyes adsorption onto low-cost lignocellulosic adsorbent," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 96, pp. 439-452, DOI: 10.1016/j.jtice.2018.12.014, 2019.
- [12] C. Balogoun, M. Bawa, S. Osseni, M. Aina, "Préparation des charbons actifs par voie chimique à l'acide phosphorique à base de coque de noix de coco," *International Journal of Biological and Chemical Sciences*, vol. 9 no. 1, DOI: 10.4314/ijbcs.v9i1.48, 2015.
- [13] A. Ferradji, A. Malek, "Isothermes d'adsorption des abricots secs à 25 C et 45 C," *Renewable & Sustainable Energy Reviews*, vol. 8, 2005.
- [14] A. Mandal, D. Chakrabarty, "Isolation of nanocellulose from waste sugarcane bagasse (SCB) and its characterization," *Carbohydrate Polymers*, vol. 86 no. 3, pp. 1291-1299, DOI: 10.1016/j.carbpol.2011.06.030, 2011.
- [15] G. J. M. Rocha, A. R. Gonçalves, B. R. Oliveira, E. G. Olivares, C. E. V. Rossell, "Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production," *Industrial Crops and Products*, vol. 35 no. 1, pp. 274-279, DOI: 10.1016/j.indcrop.2011.07.010, 2012.
- [16] S. Boumchita, A. Lahrichi, Y. Benjelloun, S. Lairini, V. Nenov, F. Zerrouq, "Removal of cationic dye from aqueous solution by a food waste: potato peel," *Journal of Materials and Environmental Science*, vol. 7 no. 1, pp. 73-84, 2016.
- [17] F. Mekhalef Benhafsa, S. Kacha, A. Leboukh, K. D. Belaid, "Étude comparative de l'adsorption du colorant victoria bleu basique à partir de solutions aqueuses sur du carton usagé et de la sciure de bois," *Revue des Sciences de L'eau*, vol. 31 no. 2, pp. 109-126, DOI: 10.7202/1051695ar, 2018.
- [18] H. Yang, R. Yan, H. Chen, D. H. Lee, C. Zheng, "Characteristics of hemicellulose, cellulose and lignin pyrolysis," *Fuel*, vol. 86 no. 12-13, pp. 1781-1788, DOI: 10.1016/j.fuel.2006.12.013, 2007.
- [19] R. Han, L. Zhang, C. Song, M. Zhang, H. Zhu, L. Zhang, "Characterization of modified wheat straw, kinetic and equilibrium study about copper ion and methylene blue adsorption in batch mode," *Carbohydrate Polymers*, vol. 79 no. 4, pp. 1140-1149, DOI: 10.1016/j.carbpol.2009.10.054, 2010.
- [20] C. R. Teixeira Tarley, M. A. Zezzi Arruda, "Biosorption of heavy metals using rice milling by-products. Characterisation and application for removal of metals from aqueous effluents," *Chemosphere*, vol. 54 no. 7, pp. 987-995, DOI: 10.1016/j.chemosphere.2003.09.001, 2004.
- [21] S. H. Ghaffar, M. Fan, "Structural analysis for lignin characteristics in biomass straw," *Biomass and Bioenergy*, vol. 57, pp. 264-279, DOI: 10.1016/j.biombioe.2013.07.015, 2013.
- [22] S. Naik, V. V. Goud, P. K. Rout, K. Jacobson, A. K. Dalai, "Characterization of Canadian biomass for alternative renewable biofuel," *Renewable Energy*, vol. 35 no. 8, pp. 1624-1631, DOI: 10.1016/j.renene.2009.08.033, 2010.
- [23] B.-L. Xue, J.-L. Wen, F. Xu, R.-C. Sun, "Structural characterization of hemicelluloses fractionated by graded ethanol precipitation from *Pinus yunnanensis*," *Carbohydrate Research*, vol. 352, pp. 159-165, DOI: 10.1016/j.carres.2012.02.004, 2012.
- [24] U. K. Garg, M. P. Kaur, V. K. Garg, D. Sud, "Removal of hexavalent chromium from aqueous solution by agricultural waste biomass," *Journal of Hazardous Materials*, vol. 140 no. 1-2, pp. 60-68, DOI: 10.1016/j.jhazmat.2006.06.056, 2007.
- [25] A. Kausar, M. Iqbal, A. Javed, "Dyes adsorption using clay and modified clay: a review," *Journal of Molecular Liquids*, vol. 256, pp. 395-407, DOI: 10.1016/j.molliq.2018.02.034, 2018.
- [26] S. Noreen, H. N. Bhatti, M. Iqbal, F. Hussain, F. M. Sarim, "Chitosan, starch, polyaniline and polypyrrole biocomposite with sugarcane bagasse for the efficient removal of acid black dye," *International Journal of Biological Macromolecules*, vol. 147, pp. 439-452, DOI: 10.1016/j.ijbiomac.2019.12.257, 2020.
- [27] H. B. Senturk, D. Ozdes, C. Duran, "Biosorption of rhodamine 6G from aqueous solutions onto almond shell

- (*Prunus dulcis*) as a low cost biosorbent," *Desalination*, vol. 252 no. 1-3, pp. 81-87, DOI: 10.1016/j.desal.2009.10.021, 2010.
- [28] Y. Song, J. Tan, G. Wang, L. Zhou, "Superior amine-rich gel adsorbent from peach gum polysaccharide for highly efficient removal of anionic dyes," *Carbohydrate Polymers*, vol. 199, pp. 178-185, DOI: 10.1016/j.carbpol.2018.07.010, 2018.
- [29] A. K. Kushwaha, N. Gupta, M. C. Chattopadhyaya, "Removal of cationic methylene blue and malachite green dyes from aqueous solution by waste materials of *Daucus carota*," *Journal of Saudi Chemical Society*, vol. 18 no. 3, pp. 200-207, DOI: 10.1016/j.jscs.2011.06.011, 2014.
- [30] Z. Reddad, C. Gérente, Y. Andrès, P. Le Cloirec, "Valorisation d'un sous-produit de l'industrie sucrière: mise en œuvre dans un procédé de traitement d'eaux usées industrielles," *Déchets, Sciences et Techniques*, vol. 39 no. 39, DOI: 10.4267/dechets-sciences-techniques.1966, 2005.
- [31] N. Tazerouti, M. Amrani, "Adsorption du Cr (VI) sur la lignine activée," *Revue des Sciences de L'eau*, vol. 23 no. 3, pp. 233-245, DOI: 10.7202/044687ar, 2010.
- [32] D. Mohan, K. Singh, V. Singh, "Trivalent chromium removal from wastewater using low cost activated carbon derived from agricultural waste material and activated carbon fabric cloth," *Journal of Hazardous Materials*, vol. 135 no. 1-3, pp. 280-295, DOI: 10.1016/j.jhazmat.2005.11.075, 2006.
- [33] T. Chouchane, S. Chouchane, A. Boukarai, A. Balaska, M. E. Samar, "Elimination du plomb en solution par la sciure de bois," *Revue des Énergies Renouvelables*, vol. 14, pp. 613-626, 2011.
- [34] G. Rivard, "Cinétique d'adsorption des contaminants de l'eau blanche sur les fines," 2011. Ph. D. thesis
- [35] Y. Miyah, A. Lahrichi, M. Idrissi, A. Khalil, F. Zerrouq, "Adsorption of methylene blue dye from aqueous solutions onto walnut shells powder: equilibrium and kinetic studies," *Surfaces and Interfaces*, vol. 11, pp. 74-81, DOI: 10.1016/j.surfin.2018.03.006, 2018.
- [36] A. B. Karim, B. Mounir, M. Hachkar, M. Bakasse, A. Yaacoubi, "Élimination du colorant basique "Bleu de Méthylène" en solution aqueuse par l'argile de Safi," *Revue Des Sciences De L'Eau*, vol. 23 no. 4, pp. 375-388, DOI: 10.7202/045099ar, 2010.
- [37] M. T. Yagub, T. K. Sen, S. Afroze, H. M. Ang, "Dye and its removal from aqueous solution by adsorption: a review," *Advances in Colloid and Interface Science*, vol. 209, pp. 172-184, DOI: 10.1016/j.cis.2014.04.002, 2014.
- [38] H. M. F. Freundlich, "Over the adsorption in solution," *Journal of Physical Chemistry*, vol. 57 no. 1906, pp. 385-470, 1906.
- [39] J. Avom, J. K. Mbadcam, M. R. L. Matip, P. Germain, "Adsorption isotherme de l'acide acétique par des charbons d'origine végétale," *African Journal of Science and Technology*, vol. 2 no. 2, DOI: 10.4314/ajst.v2i2.44663, 2001.
- [40] Z. Bouberka, S. Kacha, M. Kameche, S. Elmaleh, Z. Derriche, "Sorption study of an acid dye from an aqueous solutions using modified clays," *Journal of Hazardous Materials*, vol. 119 no. 1-3, pp. 117-124, DOI: 10.1016/j.jhazmat.2004.11.026, 2005.
- [41] L. Bulgariu, L. B. Escudero, O. S. Bello, "The utilization of leaf-based adsorbents for dyes removal: a review," *Journal of Molecular Liquids*, vol. 276, pp. 728-747, DOI: 10.1016/j.molliq.2018.12.001, 2019.
- [42] A. Mittal, V. Thakur, J. Mittal, H. Vardhan, "Process development for the removal of hazardous anionic azo dye congo red from wastewater by using hen feather as potential adsorbent," *Desalination and Water Treatment*, vol. 52 no. 1-3, pp. 227-237, DOI: 10.1080/19443994.2013.785030, 2014.

DETAILS

Subject:	Adsorbents; Lignin; Chemical elements; Chemical oxygen demand; Dyes; Binding sites; Adsorption; Methylene blue; Environmental impact; Enthalpy; Free energy; Wastewater; Fourier transforms; Activated carbon; Infrared spectroscopy; Efficiency; Scanning electron microscopy; Nitrogen; Spectrum analysis; Sugarcane; Bagasse; Cellulose; Mathematical models; Parameters; Morphology
Location:	Morocco
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	20 21
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-02 (Received); 2021-06-01 (Revised); 2021-06-14 (Accepted); 2021-06-29 (Pub)
DOI:	https://doi.org/10.1155/2021/5570806
ProQuest document ID:	2550174582
Document URL:	https://www.proquest.com/scholarly-journals/use-sugarcane-bagasse-remove-organic-dyes/docview/2550174582/se-2?accountid=211160

Copyright:

Copyright © 2021 Meryem Kerrou et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2021-07-11

Database:

Publicly Available Content Database

Document 28 of 59

A Fast and Validated HPLC Method for the Simultaneous Analysis of Five 5-HT₃ Receptor Antagonists via the Quantitative Analysis of Multicomponents by a Single Marker

Chen, Fuchao ¹
; Fang, Baoxia ²
; Li, Peng ²
; Wang, Sicen ¹

¹ Sinopharm Dongfeng General Hospital, Hubei University of Medicine, Shiyan, Hubei 442008, China; School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, Shanxi, China ² Sinopharm Dongfeng General Hospital, Hubei University of Medicine, Shiyan, Hubei 442008, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

In this study, a new strategy for the simultaneous quantization of five serotonin 5-hydroxytryptamine receptor antagonists—ondansetron, azasetron, ramosetron, granisetron, and tropisetron—either in infusion samples or in injection dosage form was first established based on high-performance liquid chromatography combined with a quantitative analysis of multiple components by a single marker. The quantitative analysis of multicomponents by a single marker method was conducted with ondansetron as an internal reference substance and performed using relative retention time and ultraviolet spectral similarity as the double indicator. The quantitative analysis of the 5-HT₃ receptor antagonists was calculated and investigated based on the relative correction factors. Chromatographic separation was achieved using a C₁₈ column (150 mm × 4.6 mm, 5.0 μm), and the mobile phase was composed of acetonitrile-0.05 mol·L⁻¹ potassium dihydrogen phosphate (pH 4.0) (25:75) at a flow rate of 1.0 mL·min⁻¹ and detection wavelengths of 307 nm (ondansetron, azasetron, ramosetron), 302 nm (granisetron), and 285 nm (tropisetron). In addition, the accuracy of the quantitative analysis of multicomponents by a single marker method was compared with an external standard method, and no significant difference was observed between the two methods. The established method is rapid, is easy, and does not require many reference substances, and it can be successfully applied as part of the quality control of the five 5-HT₃ receptor antagonists in their injection dosage form and infusion sample drugs in hospitals.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

The effects of chemotherapy, radiation, irritable bowel syndrome, opioid analgesic drugs, anesthesia, and postoperative-induced nausea and vomiting are among the most distressful side effects in patients. These side effects of nausea and vomiting can significantly cause poor appetite, bodyweight loss, decreased social skills, and more severe clinical consequences such as dehydration, electrolyte imbalances, and prolonged hospital stays [1]. The 5-hydroxytryptamine (5-HT₃) receptor antagonists, informally known as “setrons,” are a class of antiemetic medications that inhibit the release of 5-HT and vagal 5-HT receptors in the central nervous system. Serotonin 5-HT₃ receptor antagonists, including ondansetron (ODT), granisetron (GNT), tropisetron (TPT), azasetron (AZT), and ramosetron (RMT), are particularly effective in the treatment and prevention of nausea and vomiting [2–6]. The chemical structures of the five 5-HT₃ receptor antagonists are shown in Figure 1.

[figures omitted; refer to PDF]

At present, the rapid detection of drugs mainly involves infrared spectroscopy. However, near-infrared spectroscopy technology is only applicable to the initial screening of drugs, and a quantitative model is more complex and rare, resulting in the quantitative requirements of rapid drug detection not being met. HPLC is commonly used to verify rapid detection systems due to its attractive features, such as high peak efficiencies, great resolution, high sensitivity, good repeatability, and wide application range. HPLC analytic methods for the detection of 5-HT₃ receptor antagonists such as ODT, GNT, TPT, AZT, and RMT are already mature, and most of them use retention data in the qualitative analysis, while the external standard method (ESM) is applicable in quantitative analysis [7–24]. However, some of these methods may take a long time, and the specific drugs to be tested must correspond to reference substances, which cannot meet the requirements of rapid drug detection.

To solve the above problems, a unique quantitative analysis of multicomponents by a single marker (QAMS) analytical method was adopted in this paper. The QAMS method is able to simultaneously identify and quantify itself and the other analytes by a single reference standard, which greatly reduces the cost and analysis time of the experiment [25–32]. To date, there are no reports concentrating the QAMS or ESM method on the simultaneous quantification of ODT, GNT, TPT, AZT, and RMT in infusion samples and in injection dosage form. Thus, the purpose of this study was to establish a rapid HPLC-QAMS method for the detection of five 5-HT₃ receptor antagonists by using two indexes of relative retention time and UV spectrum similarity for qualitative analysis and a relative correction factor for quantitative analysis. This method was successfully employed for the routine quality control of 5-HT₃ receptor antagonist injection, infusion products, and the preliminary screening of unknown drugs in hospitals.

2. Experimental Procedure

2.1. Chemicals and Reagents

Six reference substances (ODT, GNT, TPT, AZT, RMT, and urine pyrimidine) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Each of six reference substances had a purity of more than 99.5%. HPLC-grade acetonitrile and AR-grade potassium dihydrogen phosphate, phosphoric acid, and triethylamine were supplied by the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Ultrapure water was obtained from a Millipore Milli-Q system (Millipore, USA).

2.2. Pharmaceutical Formulations

The following dosage forms were analyzed: commercial injection of ODT hydrochloride from Qilu Pharmaceutical Ltd. (Shandong, China) claimed to contain 2mg of ODT per mL. Commercial injection of GNT hydrochloride from Cinkate Pharmaceutical (Suzhou, China) claimed to contain 1 mg of GNT per mL. Commercial injection of TPT hydrochloride from Qilu Pharmaceutical (Shandong, China) claimed to contain 5mg of TPT per mL. Commercial injection of AZT hydrochloride from Wanma Pharmaceutical (Zhejiang, China) was labeled to contain 5mg of AZT

per mL. Commercial injection of RMT hydrochloride from Cisen Pharmaceutical (Shandong, China) was labeled to contain 0.3 mg of RMT per mL.

2.3. Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed on an HPLC system (Dionex, Germany) consisting of an UltiMate® 3000 quaternary pump, an autosampler, a column-heating compartment, and an ultraviolet detector (DAD).

Chromatographic data were collected and analyzed using Chromeleon® 7.2 software. The simultaneous separation of five 5-HT₃ receptor antagonists was performed using an InertSustain C₁₈ column (4.6 mm × 150 mm, 5 μm) supplied by SHIMADZU (China) Co., Ltd. The mobile phase consisted of acetonitrile-0.05 mol·L⁻¹ potassium dihydrogen phosphate (0.1% phosphoric acid, pH 4.0) (25:75, v/v) with a flow rate of 1.0 mL·min⁻¹ for 15 min. All determinations were carried out at ambient temperature 30°C. The detector wavelengths for ODT, GNT, TPT, AZT, and RMT were 307, 302, 285, 307, and 307 nm, respectively.

2.4. Preparation of Standard Solutions

Stock solutions were prepared by weighing accurately and dissolving the five 5-HT₃ receptor antagonists' standard references at 5.0 mg each and immersing them in ultrapure water in a 10 mL volumetric flask with a concentration of approximately 0.5 mg/mL as the reference standard. A standard stock solution of urine pyrimidine (20 μg/mL) was prepared by dissolving 2.0 mg of urine pyrimidine with ultrapure water in a 100 mL volumetric flask. Mixed 5-HT₃ receptor antagonist working standard solutions were prepared by appropriate dilution of the stock solutions with ultrapure water to the required concentrations for plotting the calibration curves. All standard solutions were stored at -20°C until use and sonicated for 10 min for injection.

2.5. Method Validation

The method was validated according to the International Conference of Harmonization (ICH) guidelines [33]. The following parameters were investigated: linearity, precision, stability, accuracy, limit of detection, and robustness. The limit of detection for each 5-HT₃ receptor antagonist was determined at a signal-to-noise ratio (S/N) of 3. For the limit of quantification, the ratio considered was 10:1 with an RSD% value less than 10% [34].

2.5.1. Construction of Calibration Graphs

The calibration graphs of the method were evaluated with a series of different concentrations of working standards (mixture of all five 5-HT₃ receptor antagonists). The concentration range was selected at six different concentrations, viz 5, 10, 20, 50, 75, and 100 μg/mL for ODT, GNT, TPT, and AZT and 0.5, 3, 6, 20, 30, and 50 μg/mL for RMT. A 20 μL aliquot of each working solution was injected in triplicate into a chromatographic system (*n*=3). The peak areas of the five 5-HT₃ receptor antagonists were plotted against the corresponding concentrations of each drug to obtain the calibration curve.

2.5.2. Precision

For method precision, three concentration levels of mixed standard solutions (10.0, 20.0, and 50.0 μg/mL for ODT, GNT, TPT, AZT; 1.0, 3.0, and 6.0 μg/mL for RMT) were assessed in triplicate during a single day and three consecutive days. The percent relative standard deviation (% RSD) of five analytes was calculated according to the peak area of each component in a single day and on different days to obtain the intraday precision and interday precision.

2.5.3. Stability

The sample solutions of the five 5-HT₃ receptor antagonists were prepared with ultrapure water stored at room temperature and injected into HPLC at 0, 1, 2, 4, 6, and 8 h after being prepared. The stability of the sample solutions was investigated by the RSD of variation in the peak area of the five analytes.

2.5.4. Accuracy

The accuracy of the method was determined as the recoveries of known added amounts of five 5-HT₃ receptor antagonist reference substance into the previously analyzed commercial injections in triplicate using three concentration levels.

2.5.5. Robustness Test

The robustness test was conducted by four deliberate variations to some chromatographic parameters such as the

column temperature, acetonitrile percent, flow rate, and pH value of the mobile phase. The acetonitrile percent, flow rate, and pH value of the mobile phase were changed by ± 2 , ± 0.02 , and ± 0.2 , respectively. The column temperature was altered to $\pm 1^\circ\text{C}$ (from 29 to 31°C). The separation degree and RSD% of the five 5-HT₃ receptor antagonists were investigated.

2.6. Qualitative Investigation

2.6.1. UV Spectral Similarity

The mixed working standard solutions of the five 5-HT₃ receptor antagonists were injected into the HPLC system according to the chromatographic conditions given in Section 2.3. Then, the spectral and 1st UV spectra were recorded. The cosine of the vector angle was calculated for similarity evaluation of UV spectra among the five 5-HT₃ receptor antagonists. The angle cosine formula is expressed in equation (1) in which X_{ki} and X_{kj} are the peak point absorbances of the reference and test samples, respectively. The similarity values can quantitatively reflect the similarity degree of the different UV spectra. When the similarity value is close to 1, the similarity degree of the different UV spectra is high. In addition, when the similarity value is close to 0, the difference between the UV spectra is large. (1) $C_{ij} = \frac{\sum_{k=1}^n X_{ki} X_{kj}}{\sqrt{\sum_{k=1}^n X_{ki}^2} \sqrt{\sum_{k=1}^n X_{kj}^2}}$.

2.6.2. Relative Retention Time

The same mixed standard solutions of the five analytes and urine pyrimidine were tested under the chromatographic conditions as described in Section 2.3 to identify the relative retention time (RRT) and the RSD%. The RRTs are calculated by the following equation: (2) $RRT = \frac{t_x - t_0}{t_i - t_0}$, where t_0 , t_i , and t_x represent the retention time of urine pyrimidine, ODT, and analyte, respectively.

2.7. Calculation of Relative Correction Factors Using the QAMS Method

In this study, due to the low price, availability, chemical stability, good separation, and chromatographic peaks having no interference with other 5-HT₃ receptor antagonists, ODT was chosen as the internal standard substance for the QAMS method. The relative correction factors (RCF, f) between ODT and the other 5-HT₃ receptor antagonists were calculated using equation (3). The concentration of each 5-HT₃ receptor antagonist in the sample solution can be calculated using equation (4) [25–32]. (3) $RCF_x = \frac{f_x f_i}{A_x / C_x A_i / C_i}$, (4) $C_x = \frac{C_i RCF_x \times A_x A_i}{A_i}$, where A_i is the peak area of an internal reference substance (ODT) under test, and C_i is the concentration of internal reference substance under test. A_x is the peak area of other investigated components, and C_x is the concentration of other investigated components in the sample solution.

2.8. Sample Analysis

2.8.1. Analysis of the Five 5-HT₃ Receptor Antagonists in Infusion Samples

All infusion samples of the five 5-HT₃ receptor antagonists were prepared under aseptic conditions in laminar flow hoods by licensed central intravenous additive services in hospitals. The most commonly submitted infusion preparations for ODT, GNT, TPT, AZT, and RMT were 80.0, 30.0, 50.0, 100, and 3.0 $\mu\text{g/mL}$ diluted in 0.9% sodium chloride injection. Accurate volumes of each 5-HT₃ receptor antagonist's infusion preparations were transferred into a set of 10 mL volumetric flasks and then diluted to volume with ultrapure water to keep the concentrations of the drugs within the linear ranges.

2.8.2. Analysis of the Five 5-HT₃ Receptor Antagonists in Commercial Injections

Commercially available injections of the five 5-HT₃ receptor antagonists were prepared with ultrapure water and injected into HPLC under the chromatographed conditions described above. Subsequently, the chromatographic peak area of each of the five 5-HT₃ receptor antagonists was recorded. Then, the content of the five 5-HT₃ receptor antagonists was calculated by the ESM and QAMS methods.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

According to the literature [7–24], acetonitrile and acidic water media were selected to optimize the mobile phase composition. A series of concentrations, pH values, the ratio of aqueous potassium dihydrogen phosphate solution, and different chromatographic column types were studied to ensure good resolution and appropriate retention time of the five 5-HT₃ receptor antagonists. The HPLC chromatograms are shown in Figures 2 and 3. The best result was

achieved by comparing the peak shapes and resolutions of the investigated drugs at a pH of 4.0 and acetonitrile-50 mM KH_2PO_4 buffer (25:75; v/v) at a flow rate of 1.0 mL/min.

[figure omitted; refer to PDF]

The qualitative HPLC method is usually compared with the retention time of the reference substance. The retention time is influenced by several factors such as column packing, mobile phase, and instrument. It is difficult for the five 5-HT₃ receptor antagonists with similar physical properties or structures to identify accurately using only the retention time. In this study, the relative retention time (RRT) was used to qualitatively determine the different 5-HT₃ receptor antagonists. The reproducibility of the data could be improved by correcting the dead volume. In this experiment, the effects of different LC instruments (UltiMate 3000, Agilent 1260, and SHIMADZU LC-20A), brand columns (Agilent Zorbax Extend C₁₈, InertSustain C₁₈ column, and Kromasil C₁₈), temperatures ($\pm 1^\circ\text{C}$), pH values (± 0.2), and flow rates (± 0.02) of the mobile phase on RRTs were investigated. The results are shown in Table 5. The results showed that the RRTs of the five 5-HT₃ receptor antagonists were significantly different, but the changes in pH of the mobile phase and the chromatographic column had some effects on the RRTs. The differences between RRTs can mainly be attributed to different manufacturers being associated with the properties and preparation of packing materials. To this end, we herein restricted conditional parameters and performed spectral similarity as a double indicator to qualify the qualitative analysis.

Table 5

Effects of instrument, column, flow rate, pH value of mobile phase, and column temperature upon RRT of the five 5-HT₃ receptor antagonists.

Compound	RRT	RSD (%)				
Instrument	Column	Flow rate	Temperature	pH value	Azasetron	0.431
0.3	4.2	0.9	0.5	2.3	Granisetron	0.679
0.7	2.8	1.3	0.9	1.9	Tropisetron	0.812
0.3	2.1	0.4	1.2	2.5	Ondansetron	1.000
0	0	0	0	0	Ramosetron	1.132

3.4. Robustness Test of QAMS

To evaluate the robustness of the RCFs, the influence of different LC instruments (UltiMate 3000, Agilent 1260, and SHIMADZU LC-20A), brand columns (Agilent Zorbax Extend C₁₈, InertSustain C₁₈ column, and Kromasil C₁₈), temperatures ($\pm 1^\circ\text{C}$), pH values (± 0.2), and flow rates (± 0.02) of the mobile phase on the RCFs was investigated. The RCFs of the five 5-HT₃ receptor antagonists are shown in Table 6. The experimental results show that the values of RCF have good repeatability (RSDs ranging from 0.3% to 4.2%) under different experimental conditions. These results ensure that the HPLC-QAMS method can be well applied to routine analysis.

Table 6

Effects of HPLC column, flow rate, pH value of mobile phase, and column temperature upon RCF of the five 5-HT₃ receptor antagonists.

Compound	RCF	RSD (%)				
Instrument	Column	Flow rate	Temperature	pH value	Azasetron	0.340
0.9	2.8	1.6	0.3	2.1	Granisetron	0.374
1.2	3.0	1.0	0.6	1.7	Tropisetron	0.572
0.8	2.3	1.5	1.2	2.2	Ondansetron	1.000
0	0	0	0	0	Ramosetron	1.247

3.5. Sample Analysis

The developed HPLC-based QAMS method and ESM analytical method were applied to determine the five 5-HT₃ receptor antagonists in their infusion samples and injection dosage form. The amounts of individual 5-HT₃ receptor antagonists in injection dosage form and in infusion samples were calculated, and the results are listed in Table 7. From the comparative analysis results, we can conclude that there was no significant difference between the two analytical methods (using a *T*-test, *p*>0.05), and the RSD values were <2.5%. Meanwhile, the contents of individual 5-HT₃ receptor antagonists were also determined by HPLC methods described in the Chinese Pharmacopoeia (2015 Edition), and the results showed no significant difference between the above methods.

Table 7

The results obtained by the QAMS and ESM methods (*n*=3).

Compound	The QAMS methods		The external standard methods	
Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	<i>The five 5-HT₃ receptor antagonists in injection dosage form</i>
Azasetron	97.3	1.5	97.4	1.8

Granisetron	102.1	2.1	101.8	1.0
Tropisetron	101.7	0.9	102.1	1.6
Ondansetron	99.2	1.7	99.4	0.8
Ramosetron	101.3	2.4	100.9	1.4
–				
<i>The five 5-HT₃ receptor antagonists in infusion samples</i>				
Azasetron	94.9	1.3	95.1	1.5
Granisetron	100.3	0.8	99.8	0.7
Tropisetron	97.1	2.2	97.2	1.3
Ondansetron	103.6	1.9	103.8	1.2
Ramosetron	98.8	1.5	99.0	0.9

4. Conclusions

The developed HPLC-based QAMS and external standard analytical method are fast, selectively convenient, and sensitive to the simultaneous determination of ODT, GNT, TPT, AZT, and RMT in their injection dosage form and infusion samples. The comparative analysis results show no apparent distinction between the assay results of the two methods. The QAMS analysis method can provide reliable results, save reference materials, and shorten the analysis time. This method has great potential and can play an enhanced role in hospital-based quality control and quality assurance programs.

Authors' Contributions

Fuchao Chen and Baoxia Fang contributed equally to this work and should be considered equal first authors.

Acknowledgments

This work was financially supported by the Science and Technology Research Program of Shiyao, China (number 18Y73).

References

- [1] R. M. Navari, M. Aapro, "Antiemetic prophylaxis for chemotherapy-induced nausea and vomiting," *New England Journal of Medicine*, vol. 374 no. 14, pp. 1356-1367, DOI: 10.1056/nejmra1515442, 2016.
- [2] A. C. Tricco, C. Soobiah, E. Blondal, "Comparative efficacy of serotonin (5-HT₃) receptor antagonists in patients undergoing surgery: a systematic review and network meta-analysis," *BMC Medicine*, vol. 13 no. 1, DOI: 10.1186/s12916-015-0371-y, 2015.
- [3] A. C. Tricco, E. Blondal, A. A. Veroniki, "Comparative safety and effectiveness of serotonin receptor antagonists in patients undergoing chemotherapy: a systematic review and network meta-analysis," *BMC Medicine*, vol. 14 no. 1, 2015.
- [4] S. Weibel, G. Rücker, L. H. Eberhart, "Drugs for preventing postoperative nausea and vomiting in adults after general anaesthesia: a network meta-analysis," *Cochrane Database of Systematic Reviews*, vol. 19 no. 10, 2020.
- [5] H. Y. Lee, H.-K. Kim, K. H. Lee, "A randomized double-blind, double-dummy, multicenter trial of azasetron versus ondansetron to evaluate efficacy and safety in the prevention of delayed nausea and vomiting induced by

- chemotherapy," *Cancer Research and Treatment*, vol. 46 no. 1, pp. 19-26, DOI: 10.4143/crt.2014.46.1.19, 2014.
- [6] A. Yokoi, T. Mihara, K. Ka, T. Goto, "Comparative efficacy of ramosetron and ondansetron in preventing postoperative nausea and vomiting: an updated systematic review and meta-analysis with trial sequential analysis," *PLoS One*, vol. 12 no. 10, DOI: 10.1371/journal.pone.0186006, 2017.
- [7] F. C. Chen, J. Zhu, B. Li, F. J. Yuan, L. H. Wang, "Stability of tramadol with three 5-HT₃ receptor antagonists in polyolefin bags for patient-controlled delivery systems," *Drug Design, Development and Therapy*, vol. 13 no. 10, pp. 1869-1875, 2016.
- [8] F. Bourdon, M. Lecoeur, P. Odou, C. Vaccher, C. Foulon, "Complementarity of UV-PLS and HPLC for the simultaneous evaluation of antiemetic drugs," *Talanta*, vol. 120, pp. 274-282, DOI: 10.1016/j.talanta.2013.12.015, 2014.
- [9] S. Bauer, E. Störmer, R. Kaiser, P.-B. Tremblay, J. Brockmöller, I. Roots, "Simultaneous determination of ondansetron and tropisetron in human plasma using HPLC with UV detection," *Biomedical Chromatography*, vol. 16 no. 3, pp. 187-190, DOI: 10.1002/bmc.125, 2002.
- [10] A. Heda, D. Gadade, J. Kathiriya, P. Puranik, "Development and validation of RP-HPLC method for simultaneous determination of granisetron and dexamethasone," *Indian Journal of Pharmaceutical Sciences*, vol. 73 no. 6, pp. 696-699, DOI: 10.4103/0250-474x.100255, 2011.
- [11] S. Y. Um, S. W. Chae, H. J. Park, M. W. Chung, S. O. Choi, H. J. Lee, "Simple determination of azasetron in rat plasma by column-switching high-performance liquid chromatography," *Journal of Separation Science*, vol. 23 no. 23-24, pp. 3638-3643, DOI: 10.1002/jssc.201000401, 2010.
- [12] F. C. Chen, H. Xiong, H. M. Liu, B. X. Fang, P. Li, "Compatibility of butorphanol with granisetron in 0.9% sodium chloride injection packaged in glass bottles or polyolefin bags," *American Journal of Health-System Pharmacy: AJHP: Official Journal of the American Society of Health-System Pharmacists*, vol. 72 no. 16, pp. 1374-1378, DOI: 10.2146/ajhp140824, 2015.
- [13] J. Maksić, A. Tumpa, A. Stajić, M. Jovanović, T. Rakić, B. Jančić-Stojanović, "Hydrophilic interaction liquid chromatography in analysis of granisetron HCl and its related substances. Retention mechanisms and method development," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 10 no. 123, pp. 93-103, 2016.
- [14] M. A. Tantawy, S. Alweshahy, D. A. Elshabasy, N. F. Youssef, "Simultaneous determination of co-administrated deflazacort, aprepitant and granisetron in dosage forms and spiked human plasma by RP-HPLC/PAD," *Journal of Chromatographic Science*, vol. 57 no. 9, pp. 790-798, DOI: 10.1093/chromsci/bmz062, 2019.
- [15] C. Huang, H. Wan, J. Zhang, "Quantification of ondansetron, granisetron and tropisetron in goat plasma using hydrophilic interaction liquid chromatography-solid phase extraction coupled with hydrophilic interaction liquid chromatography-triple quadrupole tandem mass spectrometry," *Journal of Chromatography B*, vol. 1095, pp. 50-58, DOI: 10.1016/j.jchromb.2018.07.009, 2018.
- [16] Y. Jiang, M. Lin, G. Fan, "Rapid determination of granisetron in human plasma by liquid chromatography coupled to tandem mass spectrometry and its application to bioequivalence study," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 42 no. 4, pp. 464-473, DOI: 10.1016/j.jpba.2006.05.001, 2006.
- [17] R. V. S. Nirogi, V. N. Kandikere, M. Shukla, K. Mudigonda, S. Maurya, R. Boosi, "Quantification of granisetron in human plasma by liquid chromatography coupled to electrospray tandem mass spectrometry," *Biomedical Chromatography*, vol. 20 no. 9, pp. 888-897, DOI: 10.1002/bmc.613, 2006.
- [18] Y. E. Chong, M. Chiang, K. Deshpande, S. Haroutounian, L. Kagan, J. B. Lee, "Simultaneous quantification of ondansetron and tariquidar in rat and human plasma using a high performance liquid chromatography-ultraviolet method," *Biomedical Chromatography: BMC*, vol. 33 no. 11, DOI: 10.1002/bmc.4653, 2019.
- [19] M. Espinosa Bosch, A. J. Ruiz Sánchez, F. Sánchez Rojas, C. Bosch Ojeda, "Review of analytical methodologies for the determination of 5-HT₃ receptor antagonists," *Microchemical Journal*, vol. 132 no. 1, pp. 341-350, DOI: 10.1016/j.microc.2017.02.023, 2017.
- [20] F. C. Chen, L. H. Wang, J. Guo, X. Y. Shi, B. X. Fang, "Simultaneous determination of dexamethasone, ondansetron, granisetron, tropisetron, and azasetron in infusion samples by HPLC with DAD detection," *Journal of*

Analytical Methods in Chemistry, vol. 2017,DOI: 10.1155/2017/6749087, 2017.

[21] B.-X. Fang, F.-C. Chen, D. Zhu, J. Guo, L.-H. Wang, "Stability of azasetron-dexamethasone mixture for chemotherapy-induced nausea and vomiting administration," *Oncotarget*, vol. 8 no. 63, pp. 106249-106257, DOI: 10.18632/oncotarget.22174, 2017.

[22] P. Chen, F. Chen, B. H. Zhou, "Compatibility and stability of dezocine and tropisetron in 0.9% sodium chloride injection for patient-controlled analgesia administration," *Medicine*, vol. 97 no. 50,DOI: 10.1097/MD.00000000000013698, 2018.

[23] G. He, F. Zeng, K. Lei, "Compatibility of dexamethasone sodium phosphate with 5-HT 3 receptor antagonists in infusion solutions: a comprehensive study," *European Journal of Hospital Pharmacy*, vol. 24 no. 3, pp. 162-166, DOI: 10.1136/ejhpharm-2016-000980, 2017.

[24] H. Miura, T. Takeshige, S.-I. Kobayashi, S. Higuchi, "A simple method for the determination of YM060 in plasma and urine by high performance liquid chromatography," *Biomedical Chromatography*, vol. 8 no. 2, pp. 103-104, DOI: 10.1002/bmc.1130080212, 1994.

[25] H. Yi, J. Zhou, X. Shang, "Multi-component analysis of Ilex Kudingcha C. J. Tseng by a single marker quantification method and chemometric discrimination of HPLC fingerprints," *Molecules*, vol. 23 no. 4,DOI: 10.3390/molecules23040854, 2018.

[26] Y. Li, Y. Zhang, Z. Zhang, Y. Hu, X. Cui, Y. Xiong, "Quality evaluation of gastrodia elata tubers based on HPLC fingerprint analyses and quantitative analysis of multi-components by single marker," *Molecules*, vol. 24 no. 8,DOI: 10.3390/molecules24081521, 2019.

[27] C. Zhu, X. Li, B. Zhang, Z. Lin, "Quantitative analysis of multi-components by single marker-a rational method for the internal quality of Chinese herbal medicine," *Integrative Medicine Research*, vol. 6 no. 1,DOI: 10.1016/j.imr.2017.01.008, 2017.

[28] L. H. Chen, Y. Wu, Y. M. Guan, C. Jin, W. F. Zhu, M. Yang, "Analysis of the high-performance liquid chromatography fingerprints and quantitative analysis of multicomponents by single marker of products of fermented *Cordyceps sinensis*," *Journal of Analytical Methods in Chemistry*, vol. 2018,DOI: 10.1155/2018/5943914, 2018.

[29] S. Wang, Y. Xu, Y. Wang, "Simultaneous determination of six active components in *Oviductus ranae* via quantitative analysis of multicomponents by single marker," *Journal of Analytical Methods in Chemistry*, vol. 2017,DOI: 10.1155/2017/9194847, 2017.

[30] C.-Q. Wang, X.-H. Jia, S. Zhu, K. Komatsu, X. Wang, S.-Q. Cai, "A systematic study on the influencing parameters and improvement of quantitative analysis of multi-component with single marker method using notoginseng as research subject," *Talanta*, vol. 134, pp. 587-595, DOI: 10.1016/j.talanta.2014.11.028, 2015.

[31] A. Chen, L. Sun, H. Yuan, A. Wu, J. Lu, S. Ma, "A holistic strategy for quality and safety control of traditional Chinese medicines by the "iVarious" standard system," *Journal of Pharmaceutical Analysis*, vol. 7 no. 5, pp. 271-279, DOI: 10.1016/j.jpha.2017.07.008, 2017.

[32] F. Chen, X. He, B. Fang, S. Wang, "Simultaneous quantitative analysis of six proton-pump inhibitors with a single marker and evaluation of stability of investigated drugs in polypropylene syringes for continuous infusion use," *Drug Design, Development and Therapy*, vol. 14, pp. 5689-5698, DOI: 10.2147/dddt.s279302, 2020.

[33] International Conference on Harmonization (ICH), Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 2005.

[34] F. C. Chen, B. X. Fang, S. C. Wang, "A fast and validated HPLC method for simultaneous determination of dopamine, dobutamine, phentolamine, furosemide, and aminophylline in infusion samples and injection formulations," *Journal of Analytical Methods in Chemistry*, vol. 2021,DOI: 10.1155/2021/8821126, 2021.

DETAILS

Subject:	Receptors; Accuracy; Markers; Quality control; Investigations; Calibration; KDP crystals; Urine; Chromatography; Potassium; Dosage; Quantitative analysis; Drug dosages; Vomiting; Acetonitrile; Nausea; Spectrum analysis; Pharmaceuticals; Potassium phosphates; Retention; High performance liquid chromatography; Serotonin; Flow velocity
Location:	Beijing China; China
Editor:	Amr M Mahmoud
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-02-10 (Received); 2021-04-27 (Revised); 2021-06-01 (Accepted); 2021-06-28 (Pub)
DOI:	https://doi.org/10.1155/2021/5533646
ProQuest document ID:	2550174529
Document URL:	https://www.proquest.com/scholarly-journals/fast-validated-hplc-method-simultaneous-analysis/docview/2550174529/se-2?accountid=211160

Copyright:

Copyright © 2021 Fuchao Chen et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2022-05-11

Database:

Publicly Available Content Database

Document 29 of 59

Ultraviolet-Visible Spectroscopy and Chemometric Strategy Enable the Classification and Detection of Expired Antimalarial Herbal Medicinal Product in Ghana

Mensah, Jacob N ¹ ; Brobbey, Abena A ¹ ; Addotey, John N ¹
; Ayensu, Isaac ¹ ; Asare-Nkansah, Samuel ¹ ; Opuni, Kwabena F M ²
; Adutwum, Lawrence A ²

¹ Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana ² Department of Pharmaceutical Chemistry, School of Pharmacy, College of Health Sciences, University of Ghana, Accra, Ghana

[ProQuest document link](#)

ABSTRACT (ENGLISH)

To meet the growing demand for complementary and alternative treatment for malaria, manufacturers produce several antimalarial herbal medicinal products. Herbal medicinal products regulation is difficult due to their complex chemical nature, requiring cumbersome, expensive, and time-consuming methods of analysis. The aim of this study was to develop a simple spectroscopic method together with a chemometric model for the classification and the identification of expired liquid antimalarial herbal medicinal products. Principal component analysis model was successfully used to distinguish between different herbal medicinal products and identify expired products. Principal component analysis showed a clear class separation between all five herbal medicinal products (HMP) studied, with explained variance for first and second principal components as 37.51% and 26.38%, respectively, while the third principal component had 18.74%. Support vector machine classification gave specificity and accuracy of 1.00 (100%) for training set data for all the products. The validation set HMP1, HMP2, and HMP3 had sensitivity, specificity, and accuracy of 1.00. HMP4 and HMP5 had sensitivity and specificity of 0.90 and 1.00, respectively, and an accuracy of 0.98. The support vector machine classification and principal component analysis models were successfully used to identify expired herbal medicinal products. This strategy can be used for rapid field detection of expired liquid antimalarial herbal medicinal products.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

The worldwide mortality attributed to malaria in 2019 was 409,000 out of which 384,000 occurred in Africa, with most of them being children and pregnant women [1]. These mortality cases in Africa represent over 93% of all malaria-related deaths worldwide. Of the 87 malaria endemic regions in the world, 28 African countries and India accounted for 95% of malaria cases reported globally [1]. Over the years, concerns have risen about the effectiveness and safety of orthodox antimalarial drugs, coupled with the development of resistance to drugs used in the treatment of malaria [2]. Artemisinin combination therapy was introduced to reduce the rate of development of resistance. However, the associated side effects and adverse drug reactions have made it unattractive for some patients [3], as well as cost of treatment, treatment failures, and accessibility.

In such situations, plant medicines have provided a viable alternative to orthodox medicines for the treatment of malaria. In Ghana, plant medicines remain a major source of antimalaria therapy, as a large number of Ghanaians are observed to patronize herbal antimalaria remedies. Such preference stems from the lower costs of products, perceived better efficacy and reduced side effects, and acceptability based on peer recommendation. Treatment outcomes from the use of these herbal remedies have been positive in a number of cases because medicinal plants have been sources of many bioactive compounds including natural scaffolds for antimalarial drugs such as quinine and artemisinin [4, 5]. These plant medicines may circumvent the challenges of parasite resistance and toxicity by the synergistic activity of the several constituent secondary metabolites [6].

Plant medicines are normally formulated as herbal medicinal products (HMPs) with optimum pH and minimal toxicity due to internal buffering effect [5] and relatively low concentration of constituent phytochemicals, respectively. Currently, the prevalence of use of HMPs in Ghana is about 76% [7]. Considering the debilitating nature of malaria and the potential for development of organ and neurological complications in severe cases, there is the need for surveillance and continuous quality monitoring of HMPs to safeguard the integrity of the products in order to safeguard the general public. However, the efficient quality monitoring of HMPs still remains a major challenge. In the case of orthodox medicines, an assay can be performed to determine the levels of active ingredients and other impurities that may be present. On the other hand, HMPs usually have several phytochemical constituents that nearly make it impossible to identify all the bioactive compounds and accordingly have them quantified, more especially when the products are polyherbal. In spite of this, variations in the levels of phytoconstituents in plant materials collected from different sources and at different times are a major concern for the monitoring of HMPs for content uniformity, especially in situations where the products are used for the treatment of infectious diseases such as malaria. It is already known that the levels of plant secondary metabolites are influenced by factors such as growth conditions, time and method of harvesting, and storage condition, as well as the geographical location [8–11]. Since at the moment a lot of these factors influence the levels of phytoconstituents in medicinal plants, and the manufacturing practices are not standardized, it becomes important in the interest of the HMP patrons to develop simple, cost-effective, and efficient system to assure the quality of HMPs.

In view of the difficulty associated with the development of assay methods for HMPs because of the myriad of secondary metabolites, the approach by the Food and Drugs Authority, Ghana, to approve and register HMPs has mainly focused on microbial load, toxicity, pH, and some physicochemical parameters such as ash value and acid value. It is however necessary to have a technique where the protocol involves measurement of some phytochemical components of the HMPs. Some of the challenges associated with such desired methods in resource constrained environments are the cost of equipment, accessories, and maintenance. In addition, the methods would be expected to target detection and quantification of almost all the phytochemical components of the polyherbal HMPs or selected markers [12]. These methods usually involve techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and capillary electrophoresis (CE) which are difficult to find in countries such as Ghana for routine chemical quality monitoring of HMPs. Besides the

instrumental challenges are tedious sample preparations with high volume of solvent and solvent-wasting separation methods [12, 13].

The ultraviolet-visible (UV-Vis) spectroscopy as a simple, cost-effective, and nondestructive technique has found applications in environmental, pharmaceutical, and other related fields. For example, the British and United States Pharmacopoeias employ UV-Vis-based methods for the assay of some pharmaceutical products, as well as adjunct method for the identification of certain active pharmaceutical ingredients. The technique has also been reported to be useful for the analysis of liquid HMPs [14], and the inherent advantages are that the methods are easy to use, enabling laboratories to adopt and effectively implement the protocols without any extensive training of technical staff. In UV-Vis spectroscopy, it has been observed that spectra obtained from complex mixtures such as liquid HMPs are usually highly convoluted. This is because the absorption spectra of several components within the complex samples are superimposed on each other. The authors, however, envisaged that the application of chemometric techniques to the complex UV-Vis data can lead to useful analyses with relevant conclusions on the quality of HMPs.

Chemometric methods involve the use of mathematical and statistical tools to extract useful information from complex data [15]. These methods have been around for a while but have become popular lately due to the availability of easy-to-use software and statistical packages [16, 17]. Chemometric methods are generally used for machine learning and optimization of experiments (i.e., design of experiment) [18, 19]. This work will focus on the former as the latter is beyond the scope of this paper. Machine learning methods are usually termed as supervised or unsupervised [20]. Unsupervised learning methods such as principal component analysis (PCA) are arguably the most used chemometric method [20, 21]. PCA is a dimensionality reduction technique which reveals inherently hidden patterns in data. In a PCA model, samples of the same class, which have similar attributes, are clustered closer to each other. Thus, a confidence ellipse generated around a cluster of samples would show high variability in the score space. Samples deviating from the standard product will be seen projecting further away from the center of that cluster. In the context of our study, this can provide guidance in the detection of unwholesome or expired HMPs. Supervised learning methods, such as support vector machines (SVM), can also be used to classify liquid HMPs in order to distinguish them from other products. Thus, two different antimalarial HMPs are expected to be classified into two different groups. In addition, variations in the chemical composition due to expiration of the product can also be determined as the UV-Vis chemical fingerprint of the product becomes altered. Such alteration, detectable with PCA or SVM, can be employed to identify expired HMPs.

Therefore, in this study, we propose a routine for the classification of liquid antimalarial HMPs using SVM and UV-Vis spectroscopy. We further demonstrate the use of PCA and SVM models to monitor the variations in liquid HMPs and to identify expired products. This approach has the potential to be expanded to the detection of adulterants in liquid antimalarial HMPs and by extension, other HMPs.

2. Experimental

2.1. Sample Preparation

Five liquid antimalarial HMPs were obtained from pharmacies and herbal shops in Accra and Kumasi, Ghana. The antimalarials used for this study were packaged in 500mL amber colored plastic bottles. To ensure anonymity, the samples were coded HMP1, HMP2, HMP3, HPM4, and HMP5. Three different batches each were obtained for each sample. A fourth batch consisting of expired HMP4 labelled HMP4X was also obtained from a herbal shop. A 5mL portion of each sample was pipetted into a 50mL volumetric flask. Distilled water was added to the volumetric flask to make up to the 50mL mark. This yielded 5% (v/v) concentration of the liquid herbal antimalarial in distilled water.

2.2. Data Collection

UV-Vis spectra were collected using a JENWAY 7315 UV-Vis spectrophotometer (Jenway, UK) equipped with a Perkin Elmer Spectrum (Spectrum Two, Version 10.03.09, Serial Number 94133, Waltham, USA). The samples were analyzed using a 1mL fused silica cuvette and at a wavelength range of 200 to 700nm using distilled water as blank/reference. At least ten repeated scans were made for each sample. The raw data from the spectrum was

imported into MATLAB R2020b (The MathWorks®, Natick, MA, USA). PCA and SVM models were generated using PLS Toolbox 8.9 (Eigenvector Research Inc., Manson, WA, USA).

2.3. Data Processing and Analysis

The data was organized into a matrix of samples in rows and wavelength in columns. The dataset matrix consisted of 167 × 501 (samples × wavelengths). Spectral data was smoothed with a moving average filter using a window of five. The spectral data was subsequently decluttered using generalized least squares- (GLS-) based weighting strategy using an alpha value of 0.02 [22]. Each column of the data matrix was mean centered while the rows were normalized to 1.

The data was further split into two main groups: 2/3 for model training and optimization and a third for external validation sets. The training and validation set data consisted of 111 and 56 samples, respectively.

Discriminant variable (DIVA) test was performed on the training set data to identify regions of the spectra providing information about the important regions of the data.

The PCA model was generated with the training and optimizing set data. In the PCA model, the variance explained by the model by each component is represented as a percentage. It is used to demonstrate measure of discrepancy between the model and the data. Thus, a higher explained variance is desired. The validation data were subsequently projected into the PCA model.

The training data was used to generate SVM classification models for the 5 classes of samples. The validation data was then projected into the model to evaluate the performance on an external validation set. The evaluation was based on the model's sensitivity, specificity, and accuracy [23]. A model's ability to predict positive samples is true positive rate/sensitivity (sensitivity = true positives (TP)/number of positives (NP)). Specificity measures the model's ability to correctly identify negative samples, also known as true negative rate (specificity = true negatives (TN)/number of negatives (NN)). Accuracy measures the overall true predicting power (accuracy = (TP + TN)/(NP + NN)). These metrics are scaled from zero to 1, with 0 and 1 being the worst and best model, respectively.

The SVM model was further tested on the HP4X, which are the expired HP4 samples. PCA models were also generated and evaluated with the training and validation sets, respectively.

3. Results and Discussion

The assessment of quality of HMPs in Ghana has largely been based on some organoleptic and physicochemical parameters including but not limited to color, pH, and microbial load. However, it is necessary to develop advanced but simple strategies that target the phytoconstituents of HMPs in the evaluation of chemical quality. It is known that variation in these secondary metabolites supposed to be responsible for the biological activities of HMPs exists as a result of factors including environment, harvesting, manufacturing, storage, and stability. Due to the presence of myriad of chemical compounds in polyherbal products and the lack of adequate robust analytical methods to check batch-to-batch consistency in levels of phytoconstituents and identify expired or decomposed HMPs that may not show perceptible physical changes, unscrupulous persons could rebottle and sell substandard and unwholesome products to the general public. Therefore, this study has explored the application of UV-Vis spectroscopy and chemometric analysis in dealing with the problem. Antimalarial HMPs were chosen as a test case due to their high demand and over-the-counter usage.

Generally, all the UV-Vis spectra obtained from the analyses of the liquid antimalarial HMPs showed maximum absorbance around 230nm, 280nm, and 375nm (Figure 1(a)), which suggests the presence of compounds with conjugated systems or chromophores and the suitability of our choice of technique. This agrees with other findings that show the presence of chromophoric compounds in plant products [12].

[figures omitted; refer to PDF]

Due to noise in the dataset, a moving window smoothing algorithm was implemented (Figure 1(b)), which showed similar spectra characteristics relative to the raw spectra. In order to identify the more informative regions of the spectra, discriminant variable analysis was performed as previously described [24] to generate a variable selectivity ratio (SR) plot (Figure 2). In this analysis, an SR value less than 10 was considered as one with low discrimination power and, thus, was eliminated from the data. The threshold shows the variables which were above the set limit

and reduced the number of variables from 501 to 324. It must be emphasized that a fewer number of descriptors with better discriminating power are much more desirable as they lead to simpler models [25, 26].

[figure omitted; refer to PDF]

Next, principal component analysis was performed using the training set data (only the 324 variables). The results show a PCA score plot of PC1 vs. PC2 vs. PC3 (Figure 3). The explained variances for PC1 and PC2 were 37.51% and 26.38%, respectively, while the third principal component had 18.74%. Thus, a total explained variance of 82.63% was observed. It can be seen in this three-dimensional score space that the various antimalarial HMPs are all clustered in separate groups. A 95% confidence ellipse generated around each cluster shows quite a few of the training set samples straying out of the cluster. This demonstrates that UV-Vis spectra and PCA models can be used to distinguish various liquid antimalarial HMPs. Subsequently, the external validation set of each HMP class was projected in the model (Figure 4, where HMP1 are red circles, HMP2 are purple squares, HMP3 are green diamonds, HMP4 are red pentagrams, and HMP5 are blue triangles). In Figure 4, samples used for the training and validation sets are represented by hollow and filled markers, respectively. Here it is also apparent that those samples that were not used in training the model are also projected into the correct subgroups.

[figure omitted; refer to PDF]

The ability of the PCA model generated to identify expired products was also evaluated. Expired products from HMP4, labelled as HMP4X, were also projected into the model as shown in Figure 4. These expired products were clustered into a different score space (represented as black triangles). This further demonstrates that, using the UV-Vis spectra and PCA, products that are expired can be easily detected. This is of high importance due to the fact that liquid HMPs could go bad without showing perceptible variations in their physical appearance and taste.

SVM classification models were generated using the 324 features obtained from the DIVA test. The model was generated using a radial basis function kernel with cost and gamma values of 100 and 0.1, respectively. A venetian blind cross validation was employed due to structure of the data and it gave consistent results. The model was generated with the training set data and validated using an external validation set. The class predicted probability plots for all the 5 products (HMP1, HMP2, HMP3, HMP4, and HMP5) are shown in Figures 5(a)–5(e), where HMP1 are red circles, HMP2 are purple squares, HMP3 are green diamonds, HMP4 are red pentagrams, and HMP5 are blue triangles, red dash lines are discrimination barrier, and training and validation sets are represented by hollow and filled markers, respectively. In the SVM of Figure 5, class predicted probability closer to zero (black dash lines) indicated less likelihood of samples belonging to the class being predicted. On the other hand, a class predicted probability close to 1.00 (green dash line) indicates that the sample may belong to the class being predicted. A class discrimination boundary is represented by a red dashed line. The numerical results from Figure 5 are shown in Table 1. There were no false negative or false positives leading to classification sensitivity, specificity, and accuracy of 1.00 (denoting 100%), which demonstrate the power of combination of UV-Vis spectrum and SVM for HMPs classification. However, in the validation set, two false positives were identified from HMP4 and HMP5. It is normal for a classification model to perform better on a training set data than a validation set.

[figures omitted; refer to PDF]

Table 1

Table of results for SVM classification for training and validation sets for all herbal products.

Product ID	True positive	False negative	True negative	False positive	Sensitivity	Specificity	Accuracy
<i>Training set</i>							
HMP1	24	0	87	0	1.00	1.00	1.00

HMP2	24	0	87	0	1.00	1.00	1.00
HMP3	24	0	87	0	1.00	1.00	1.00
HMP4	20	0	91	0	1.00	1.00	1.00
HMP5	19	0	92	0	1.00	1.00	1.00
–							
<i>Validation set</i>							
HMP1	12	0	44	0	1.00	1.00	1.00
HMP2	12	0	44	0	1.00	1.00	1.00
HMP3	12	0	44	0	1.00	1.00	1.00
HMP4	9	0	46	1	0.90	1.00	0.98
HMP5	9	0	46	1	0.90	1.00	0.98

The ability of the SVM model to detect expired products was evaluated using the spectra of HMP4X. The samples in HMP4X were projected into the model to check, if indeed, the model will predict it as HMP4 or others. It can be seen that HMP4X samples are not predicted to be in any of the 5 HMP groups (Figure 6). Thus, the class predicted probability is below 0.5 for all five classes of HMPs. This is because these products have undergone some chemical changes even though there is no perceptible physical change which has altered their UV-VIS spectra. This demonstrates that, using UV-Vis spectra and SVM classification, expired herbal products can be detected. The study scope would be expanded to include batch-to-batch consistency and stability of HMPs.

[figures omitted; refer to PDF]

4. Conclusion

A simple spectroscopic method (UV-Vis spectroscopy) together with chemometric models was successfully developed and applied to the assessment of liquid antimalarial HMPs. This method demonstrates the ability of PCA to distinguish between different HMPs. In addition, we applied SVM models to UV-VIS spectra of liquid HMPs to classify different antimalarials. Prediction sensitivity, specificity, and accuracy of 1.00 (100%) were observed for training set data for all the products. With respect to the validation set, sensitivity, specificity, and accuracy of prediction were 1.00 in all cases for HMP1, HMP2, and HMP3. However, sensitivity and accuracy for HMP4 and HMP5 were 0.90 and 0.98, respectively. The SVM method also demonstrated its ability to distinguish between wholesome and expired products.

References

- [1] World Health Organization, World Malaria Report 2020, 2021.
- [2] F. Nosten, N. J. White, "Artemisinin-based combination treatment of falciparum Malaria," American Society of Tropical Medicine and Hygiene, vol. 77 no. 6, pp. 181-192, DOI: 10.4269/ajtmh.2007.77.181, 2007.
- [3] R. Adisa, T. O. Fakeye, D. Dike, "Evaluation of adverse drug reactions to artemisinin-based combination therapy in a Nigeria University community," Tropical Journal of Pharmaceutical Research, vol. 7 no. 2, pp. 937-944, DOI: 10.4314/tjpr.v7i2.14680, 2008.
- [4] H. A. Pawar, "Natural product as a source of lead to the design of new drugs," Natural Products Chemistry and

Research, vol. 2 no. 6, DOI: 10.4172/2329-6836.1000156, 2014.

- [5] E. G. Azeh, "Malaria and treatment: herbal antimalarials as alternative to conventional medicine," Research Journal of Phytomedicine, vol. 1, 2015.
- [6] S. M. Abdel-Aziz, A. Aeron, T. A. Kahil, "Health benefits and possible risks of herbal medicine," Microbes in Food and Health, vol. 36, pp. 97-116, 2016. <http://paperpile.com/b/rSJ4hz/cZL7> 10.1007/978-3-319-25277-3_6
- [7] I. A. Kretchy, A. Koduah, K. F. M. Opuni, "Prevalence, patterns and beliefs about the use of herbal medicinal products in Ghana: a multi-centre community-based cross-sectional study," Tropical Medicine & International Health, vol. 26 no. 4, pp. 410-420, DOI: 10.1111/tmi.13541, 2021.
- [8] D. Donno, R. Boggia, P. Zunin, "Phytochemical fingerprint and chemometrics for natural food preparation pattern recognition: an innovative technique in food supplement quality control," Journal of Food Science and Technology, vol. 53 no. 2, pp. 1071-1083, DOI: 10.1007/s13197-015-2115-6, 2016.
- [9] H. C. Andola, K. S. Gaira, R. S. Rawal, M. S. M. Rawat, I. D. Bhatt, "Habitat-dependent variations in berberine content of berberis asiatica Roxb. ex. DC. in Kumaon, Western Himalaya," Chemistry & Biodiversity, vol. 7 no. 2, pp. 415-420, DOI: 10.1002/cbdv.200900041, 2010.
- [10] I. Zribi, F. Omezzine, R. Haouala, "Variation in phytochemical constituents and allelopathic potential of nigella sativa with developmental stages," South African Journal of Botany, vol. 94, pp. 255-262, DOI: 10.1016/j.sajb.2014.07.009, 2014.
- [11] A. Lavola, A. Salonen, V. Virjamo, R. Julkunen-Tiitto, "Phytochemical variation in the plant-part specific phenols of wild crowberry (*Empetrum hermaphroditum* Hagerup) populations," Phytochemistry Letters, vol. 21, pp. 11-20, DOI: 10.1016/j.phytol.2017.05.016, 2017.
- [12] D. Kumadoh, K. O. Kwakye, N. Kuntworbe, O. Adi-Dako, J. A. Appenahier, "Determination of shelf life of four herbal medicinal products using high-performance liquid chromatography analyses of markers and the systat sigmaplot software," Journal of Applied Pharmaceutical Science, vol. 10 no. 6, pp. 72-80, DOI: 10.7324/japs.2020.10610, 2020.
- [13] M. A. Archer, D. Kumadoh, G. N. Yeboah, "Formulation and evaluation of capsules containing extracts of cassia sieberiana for improved therapeutic outcome," Scientific African, vol. 10, DOI: 10.1016/j.sciaf.2020.e00609, 2020.
- [14] K. Kucharska-Ambrożej, J. Karpinska, "The application of spectroscopic techniques in combination with chemometrics for detecting adulteration of some herbs and spices," Microchemical Journal, vol. 153, pp. 104-278, DOI: 10.1016/j.microc.2019.104278, 2020.
- [15] I. E. Frank, B. R. Kowalski, "Chemometrics," Analytical Chemistry, vol. 54 no. 5, pp. 232-243, DOI: 10.1002/chin.198249366, 1982.
- [16] J. Camacho, E. Saccenti, "Chemometrics analysis of big data," Comprehensive Chemometrics, vol. 36, pp. 437-458, DOI: 10.1016/b978-0-12-409547-2.14602-5, 2020.
- [17] H. Martens, "Quantitative big data: where chemometrics can contribute," Journal of Chemometrics, vol. 30 no. 9, DOI: 10.1002/cem.2823, 2016.
- [18] R. G. Brereton, Applied Chemometrics for Scientists, 2007.
- [19] M. Otto, Chemometrics: Statistics and Computer Application in Analytical Chemistry, 2016.
- [20] M. Alloghani, D. Al-Jumeily, J. Mustafina, A. Hussain, A. J. Aljaaf, "A systematic review on supervised and unsupervised machine learning algorithms for data science," Unsupervised vs Semi-supervised Learning, vol. 47, DOI: 10.1007/978-3-030-22475-2_1, 2020.
- [21] C. Christophe, "PCA: the basic building block of chemometrics," Analytical Chemistry, vol. 29, DOI: 10.5772/51429, 2012.
- [22] Q. Liu, Y. Okui, Y. Yoshimura, "Generalized least squares model averaging," Economic Review, vol. 35 no. 8, pp. 1692-1752, DOI: 10.1080/07474938.2015.1092817, 2016.
- [23] T. W. Loong, "Understanding sensitivity and specificity with the right side of the brain," BMJ, vol. 327 no. 7417, pp. 716-719, 2003.
- [24] T. Rajalahti, R. Arneberg, A. C. Kroksveen, M. Berle, K. M. Myhr, O. M. Kvalheim, "Discriminating variable test

and selectivity ratio plot: quantitative tools for interpretation and variable (biomarker) selection in complex spectral or chromatographic profiles," *Analytical Chemistry*, vol. 81 no. 7, pp. 2581-2590, 2009.

[25] A. Amirteimoori, S. Kordrostami, A. Masoumzadeh, M. Maghbouli, "Increasing the discrimination power of data envelopment analysis," *International Journal of Operational Research*, vol. 19 no. 2, DOI: 10.1504/ijor.2014.058950, 2014.

[26] L. Angulo-Meza, M. P. E. Linz, "Review of methods for increasing discrimination in data envelopment analysis," *Annals of Operations Research*, vol. 116 no. 1-4, pp. 225-242, DOI: 10.1023/a:1021340616758, 2002.

DETAILS

Subject:	Machine learning; Malaria; Design of experiments; Principal components analysis; Classification; Herbal medicine; Sensitivity; Support vector machines; Phytochemicals; Pharmaceuticals; Mortality; Drugs; Chromatography; Metabolites; Chemometrics
Business indexing term:	Subject: Machine learning
Location:	United States--US; Africa; Ghana
Editor:	Spas D Kolev
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-02-02 (Received); 2021-06-18 (Accepted); 2021-06-25 (Pub)

DOI: <https://doi.org/10.1155/2021/5592217>

ProQuest document ID: 2548294689

Document URL: <https://www.proquest.com/scholarly-journals/ultraviolet-visible-spectroscopy-chemometric/docview/2548294689/se-2?accountid=211160>

Copyright: Copyright © 2021 Jacob N. Mensah et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-07-04

Database: Publicly Available Content Database

Document 30 of 59

Development of a Gas Chromatography-Time-of-Flight Method for Detecting Glucosinolate Metabolites and Volatile Organic Compounds in Kimchi

Ho Jin Kim; Mi Jin Lee; Hur, Suel Hye; Jeong, Min Hee

[ProQuest document link](#)

ABSTRACT (ENGLISH)

This study examined the volatile organic compounds (VOCs) and metabolites of glucosinolate (GLS) contained in kimchi and analyzed GLS using myrosinase. The analysis was conducted using gas chromatography-time of flight (GC-TOF), and VOC and the metabolite quantities were detected and analyzed. Based on 22 samples, tests were conducted, and 12 metabolites and 52 VOCs were found. When the detected metabolites were compared in general, the rate of isothiocyanate, which is well known for its anticancer effects and various other activities, was the highest. A total of 52 VOCs, including 15 aliphatic hydrocarbons, 7 acids, and 6 alcohols, were detected by GC-TOF. Therefore, the analytical methods provide a good basis to examine VOC and GLS metabolites; furthermore, the methods are of great help to secure excellent kimchi and evaluate its quality.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Traditional fermented food, kimchi, was certified by Codex Alimentarius in July 2001 and is recognized as a global dish that is distinct from other types of pickles [1]. It is made by adding various ingredients (chili pepper powder, garlic, ginger, spring onion, radish, etc.) and salted seafood to cabbage, the main ingredient, which is then fermented at low temperature based on lactic acid bacteria [2–4]. The unique flavor of kimchi is derived from the ingredients and produced during the fermentation process. Kimchi is rich in vitamins and minerals, and it is a low-calorie food that plays an important role in reducing cholesterol. One of the effects of kimchi is its anticancer effect, which is promoted by minor ingredients of kimchi, namely, garlic and dried red pepper powder [5].

Kimchi contains glucosinolate (GLS), which is one of the main components of Cruciferae, metabolites, and volatile organic compounds (VOCs). First, GLS and its decomposition products are known for excellent effects. As shown in Figure 1, GLS decomposes into isothiocyanate (a bioactive substance), nitrile (a toxic compound), thiocyanate, oxazolidine-2-thiones, and thiocyanates, as well as epithionitriles and glucosevia, the myrosinase enzyme [6].
[figure omitted; refer to PDF]

Isothiocyanates are involved in the defense mechanism of plants, offering strong antibacterial and insecticidal attributes, as well as strong flavor; isothiocyanates also help prevent cancers, such as liver, lung, and stomach cancers in human beings [7, 8]. Particularly, sulforaphane (the isothiocyanate of glucoraphanin) and indole-3-carbinol (the isothiocyanate of glucobrassicin) are excellent contributors to the anticancer effect of kimchi [9–11]; according to experimental and statistical results reported in other studies, the prevalence of breast cancer can be reduced with the consumption of crucifers [12]. In animal testing, tumors causing skin cancer were suppressed by increasing consumption of crucifers [13]. Furthermore, sulforaphane reduces the size and occurrence of tumors, as well as delays their growth [14]. Sulforaphane is also known to promote defense mechanisms against insects and pathogens and to be involved in sulfur, nitrogen, and glucose metabolism [15]. In addition, sulforaphane influences hormone auxin levels, and it influences colon, lung, and prostate cancers in human beings with anticancer compounds [16]. Many pharmacological and physiological activities have been reported, among many other research studies on these compounds in kimchi [9–11]. Accordingly, for GLS, various studies have been conducted on the VOC composition and discrimination of GLS decomposition products in crucifers [17]; one study on GLS in serum, using LC-MS/MS and its decomposition products, was also completed [18]. However, studies on GLS metabolites are lacking.

VOCs are known to have the most powerful effects on the sourness, organoleptic properties, and palatability of kimchi [19]. There are various types of VOCs, such as dimethyl sulfides, sulfide, isothiocyanates, aldehydes, ketones, and alcohols. In relevant research, 25 VOCs collected by SPME [20], 40 types of sulfides [21], and GLS-derived VOCs [6] were identified.

Many analytical instruments have been recently developed to provide many advantages, including decreasing analysis time, saving sample and extraction solvent amounts, detecting minute amounts with high sensitivity, separating mixtures, identifying structures, and analyzing minute samples. Moreover, the appearance of TOF recently is advantageous to measure the exact mass with high resolution, high sensitivity, and strengthened selectivity [22–25]. Because properties that could not be analyzed before have since become resolvable using these analytical instruments, it has become possible to develop new analytical methods.

This study investigated GLS and VOC metabolites in myrosinase-treated kimchi using GC-TOF. The manufacturers and ingredients of various kimchi samples were used to analyze the GLS metabolites and VOCs. The findings can be used to verify the benefits of kimchi and the use of various cruciferous vegetables.

2. Materials and Methods

2.1. Samples, Chemicals, and Reagents

Tests were made with various kinds of kimchi purchasable in local marts; a total of 22 samples were used. In the experiment, 22 kimchi were sliced and prefrozen for 48 hours at -40°C . After that, it is freeze-dried in a dryer at -70°C for 24 hours. Freeze-dried kimchi was pulverized using a food-mixer blender and filtered with a 3 mesh size. Water was purified using a Milli-Q® RiOsTM/Elix® water purification system (Millipore, Bedford, MA, USA). Myrosinase enzymes and L-ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Dichloromethane was

purchased from Merck KGaA (Darmstadt, Germany). Anhydrous sodium sulfate was purchased from Dae Jung.

2.2. Sample Preparation

5g of the lyophilized sample was placed in a volumetric flask, and 250mL of distilled water, 5 units of myrosinase enzyme, and 5mg of L-ascorbic acid were added to the mixture. The mixture was then kept at room temperature for 2 hours. Then, 100mL of dichloromethane was added and stirred for 30 minutes. The layers were separated by centrifugation at 3,500rpm for 15 minutes. The separated organic solvent layer was passed through anhydrous sodium sulfate, and the sample was then concentrated in a rotary evaporator until 0.5mL of the sample remained. After the concentrate temperature reached -20°C, the sample was tested.

2.3. GC-TOF Analysis

GC was performed on an HP 7890GC (Agilent Technologies, Santa Clara, CA, USA), and the injector was used on a rail system (CTC, Gerstel, LEAP). The injection temperature was set at 250°C, and the injection type was set at split 10:1 mode. The other experimental parameters were set as follows: carrier gas He, flow rate 1.5mL/min, column RTX-5MS 30m×0.25mm×0.25µm, and transfer line temperature (260°C). Detection was performed on a LECO®PegasusHT® TOF-MS and controlled using a Chroma TOF (LECO, St. Joseph, MI, USA). The other experimental parameters were set as follows: acquisition delay 130s, mass range (5–650µ, acquisition rate 10 spectra/s, detector voltage 1650V, electron energy 70eV, and ion source temperature 250°C.

3. Results and Discussion

Tests were conducted based on 22 samples purchasable in marts and repeated three times. VOC and metabolites of GLS ingredients were detected in all 22 samples (Figure 2) with an S/N ratio of more than 50. The results are shown in Sections 3.1 and 3.2.

[figures omitted; refer to PDF]

3.1. Composition Analysis of Glucosinolate Metabolite in Kimchi

GLS metabolites can be largely divided into the following five types: isothiocyanate, oxazolidin-2-thione, nitrile, epithionitrile, and thiocyanate. In this study, 7 istiocyanates, 1 oxazolidin-2-thione, and 4 nitriles were detected. The 12 GLS metabolites were classified based on the GC library, and the sample area was confirmed. First, there were 7 isothiocyanates including 1-isothiocyanato heptane, 2-isothiocyanato butane, and 2-isothiocyanatoethyl benzene, and sulforaphane was identified as a glucoraphanin metabolite, which is a type of GLS (found in the GLS comparative study). This suggests that samples containing sulforaphane have glucoraphanin. On average, 4-isothiocyanato-1-butene was the largest at 55,822,360, especially in samples 17 and 21, which had values of 229,507,199 and 278,971,384, respectively. In addition, in samples 17 and 21, 2-isothiocyanatoethyl benzene was also found in large quantities of 37,200,818 and 39,458,110, respectively; however, the compound was present in all samples. The average area of oxazolidin-2-thione class metabolites was 13,905,958, and the area was observed only in samples 17 and 21. Among nitrile metabolites, cyclopropylacetonitrile, benzenepropanenitrile, 2-(5-methoxy-2-oxo-1,3-dihydroindol-3-yl) acetonitrile, and 1H-indole-3-acetonitrile were found. Benzenepropanenitrile was observed in the highest content with an area of 0–39,724,396 (average 14,958,208). More detailed results are presented in Figure 3, Table 1, and Table S1 [20, 21, 26–29].

[figure omitted; refer to PDF]

Table 1

Structure of glucosinolate metabolites in kimchi.

3.2. Composition Analysis of Kimchi VOC

Table 2 classifies VOCs detected in the 22 samples according to class. In total, 52 VOCs were observed, specifically aliphatic hydrocarbons (15), acids (7), alcohol (6), alkane (5), ketone (5), aldehyde (4), S-containing compounds (4), terpene hydrocarbons (5), and a miscellaneous compound (1) (Table 2). The largest areas were occupied by 2, 3, 4-trimethyl hexane (214,485,249), 4, 6-dimethyl dodecane (176,577,662), 1,3-bis (1,1-dimethylethyl), and benzene (139,454,474), in that order. A comparison of the sample areas showed that samples 11 and 18 of 2,3,4-trimethyl hexane had the largest areas (275,021,365 and 263,894,944, respectively). Among the 52 compounds, only 21 were observed in all samples, with the average area of 145,926–214,485,249. The overall results are presented in Figure 4 and Table S2. The mass spectrum and structure are shown in Table 2 and Figure S1.

Table 2

Classified VOCs.

NO.	Compound	Chemical group	Reference
1	1-Penten-3-one	Ketone	[26]
2	2-Methyl-2-butenal	Aldehyde	[26]
3	cis-3-Hexenal	Aldehyde	[26]
4	Di-2-propenyltrisulfide,	S-containing compounds	[21]
5	Diallyldisulphide	S-containing compounds	[21]
6	Dimethyl tetrasulphide	S-containing compounds	[21]
7	Farnesene	Terpene hydrocarbons	[21, 28]
8	Sesquiphellandrene	Terpene hydrocarbons	[21, 28]
9	Sabinene	Terpene hydrocarbons	[21, 28]
10	(-)-Pinene	Terpene hydrocarbons	[28]
11	Camphene	Terpene hydrocarbons	[28]
12	2,4-Dimethyl- heptane	Aliphatic hydrocarbons	[28]
13	4,6-Dimethyl- dodecane	Aliphatic hydrocarbons	[28]
14	Tetradecane	Aliphatic hydrocarbons	[28]
15	Hexadecane	Aliphatic hydrocarbons	[28]

16	1,3-bis (1,1-Dimethylethyl)- benzene	Miscellaneous compounds	[28]
17	Butanoic acid	Acids	[27]
18	iso-Valeric acid	Acids	[27]
19	2-Methylbutanoic acid	Acids	[27]
20	n-Hexadecanoic acid	Acids	[27]
21	3-Methyl-1-butanol,	Alcohols	[27]
22	3-Methyl-2-buten-1-ol	Alcohols	[27]
23	Linalool	Alcohols	[27]
24	Eugenol	Alcohols	[27]
25	(R)- 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2 (4H)-benzofuranone	Ketone	[27]
26	Dodecane	Aliphatic hydrocarbons	[27]
27	1-Dodecane	Aliphatic hydrocarbons	[27]
28	1-Tetradecene	Aliphatic hydrocarbons	[27]
29	Benzeneacetaldehyde	Aldehyde	[29]
30	Nonadecane	Alkane	[29]
31	Octadecane	Alkane	[29]
32	Hexadecanoic acid	Acids	[29]
33	Pentadecane	Alkane	[29]
34	Dimethyl disulfide,	S-containing compounds	[20]
35	Hexanoic acid	Acids	[20]
36	Octanoic acid	Acids	[20]
37	4-Methyl-1-penten-3-ol	Ketone	[26]

38	3-Methyl-2-butenal	Aldehyde	[26]
39	1-Hexen-3-ol	Alcohols	[26]
40	2-Pentanone	Ketone	[26]
41	2,3,5-Trimethyl- hexane	Aliphatic hydrocarbons	[28]
42	2,3,4-Trimethyl- hexane	Aliphatic hydrocarbons	[28]
43	4,4,5-Trimethyl-2-Hexene,	Aliphatic hydrocarbons	[28]
44	3,5,5-Trimethyl-2-Hexene,	Aliphatic hydrocarbons	[28]
45	3,5,5-Trimethyl-1-Hexene,	Aliphatic hydrocarbons	[28]
46	2,3,3-Trimethyl-1-hexene	Aliphatic hydrocarbons	[28]
47	4,4-Dimethyl- heptane,	Aliphatic hydrocarbons	[28]
48	2,3-Dimethyl- heptane,	Aliphatic hydrocarbons	[28]
49	3-Penten-2-ol	Ketone	[27]
50	3-Methyl-3-buten-1-ol	Alcohols	[27]
51	2-Octene	Alkene	[27]

[figure omitted; refer to PDF]

4. Conclusions

The cell walls of Brassicaceae plants contain an enzyme called myrosinase. When external animals and insects destroy the cell wall or the cell wall is destroyed by microorganisms or bacteria, an enzyme called mirosinase is released. The enzymes in myrosinase break down GLS, resulting in the production of secondary metabolites. In kimchi, 51 compounds of VOCs and GLS metabolite were detected by GC-TOF. This analysis technique was used for analyzing VOCs and GLS metabolites contained in kimchi, based on myrosinase treatment. For the analysis, GC-TOF was used in comparison with the library and to identify the compounds. As a result, in 22 samples, 12 GLS metabolites and 51 VOCs were observed. The findings of GLS metabolites and the GC-TOF analysis technique in this study can provide useful data for relevant analyses and quality assessments, as well as provide evidence of the excellence and reliability of kimchi.

Authors' Contributions

Ho Jin Kim and Mi Jin Lee contributed equally to this work.

- [1] S.-W. Moon, H.-K. Shin, G.-E. Gi, "Effects of xylitol and grapefruit seed extract on sensory value and fermentation of baechu kimchi," Korean Journal of Food Science and Technology, vol. 35, pp. 246-235, 2003.
- [2] W.-S. Park, I.-S. Lee, Y.-S. Han, Y.-J. Koo, "Kimchi preparation with brined Chinese cabbage and seasoning mixture stored separately," Korean Journal of Food Science and Technology, vol. 26, pp. 231-238, 1994.
- [3] M.-K. Lee, "A survey of research papers on Korean kimchi and R and D trends," Journal of the Korean Society of

Food Culture, vol. 6, pp. 104-114, 2007.

- [4] I.-S. Lee, "Changes in some characteristics of brined Chinese cabbage of fall cultivars during storage," Korean Journal of Food Science and Technology, vol. 26, pp. 239-245, 1994.
- [5] E. J. Cho, S. H. Kim, K. Y. Park, S. H. Rhee, "in vitro anticancer effect of active compounds from Chinese cabbage kimchi," Journal of Korean Association of Cancer Prevention, vol. 2, pp. 98-103, 2004.
- [6] I. Winde, U. Wittstock, "Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system," Phytochemistry, vol. 72 no. 13, pp. 1566-1575, DOI: 10.1016/j.phytochem.2011.01.016, 2011.
- [7] P. Talalay, "Mechanisms of induction of enzymes that protect against chemical carcinogenesis," Advances in Enzyme Regulation, vol. 28, pp. 237-250, DOI: 10.1016/0065-2571(89)90074-5, 1989.
- [8] Y. Zhang, T. W. Kensler, C. G. Cho, G. H. Posner, P. Talalay, "Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates," Proceedings of the National Academy of Sciences, vol. 91 no. 8, pp. 3147-3150, DOI: 10.1073/pnas.91.8.3147, 1994.
- [9] H. J. Kim, M. J. Lee, M. H. Jeong, J. E. Kim, "Identification and quantification of glucosinolates in kimchi by liquid chromatography-electrospray tandem mass spectrometry," International Journal of Analytical Chemistry, vol. 2017, DOI: 10.1155/2017/6753481, 2017.
- [10] P. J. Thornalley, "Isothiocyanates: mechanism of cancer chemopreventive action," Anti-Cancer Drugs, vol. 13 no. 4, pp. 331-338, DOI: 10.1097/00001813-200204000-00001, 2002.
- [11] J. D. Hayes, M. O. Kelleher, I. M. Eggleston, "The cancer chemopreventive actions of phytochemicals derived from glucosinolates," European Journal of Nutrition, vol. 47 no. 2, pp. 73-88, DOI: 10.1007/s00394-008-2009-8, 2008.
- [12] P. Terry, A. Wolk, I. Persson, C. Magnusson, "Brassica vegetables and breast cancer risk," JAMA, vol. 285 no. 23, pp. 2975-2977, DOI: 10.1001/jama.285.23.2975, 2001.
- [13] A. T. Dinkova-Kostova, S. N. Jenkins, J. W. Fahey, "Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts," Cancer Letters, vol. 240 no. 2, pp. 243-252, DOI: 10.1016/j.canlet.2005.09.012, 2006.
- [14] J. W. Fahey, X. Haristoy, P. M. Dolan, "Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[a]pyrene-induced stomach tumors," Proceedings of the National Academy of Sciences, vol. 99 no. 11, pp. 7610-7615, DOI: 10.1073/pnas.112203099, 2002.
- [15] P. J. Thornalley, "Glycation in diabetic neuropathy: characteristics, consequences, causes, and therapeutic options," International Review of Neurobiology, vol. 50, pp. 37-57, DOI: 10.1016/s0074-7742(02)50072-6, 2002.
- [16] K. A. Moy, J.-M. Yuan, F.-L. Chung, "Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms and gastric cancer risk: a prospective study of men in Shanghai, China," International Journal of Cancer, vol. 125 no. 11, pp. 2652-2659, DOI: 10.1002/ijc.24583, 2009.
- [17] A. Luca, P. V. Mahajan, M. Edelenbos, "Changes in volatile organic compounds from wild rocket (*Diplotaxis tenuifolia* L.) during modified atmosphere storage," Postharvest Biology and Technology, vol. 119, 2015.
- [18] H. E. Sun, "Determination of bioactive compounds and anti-cancer effect from extracts of Korean cabbage and cabbage," The Korean Journal of Food and Nutrition, vol. 25, pp. 259-265, 2012.
- [19] J. H. Seung, "Major odor components of raw kimchi materials and changes in odor components and sensory properties of kimchi during ripening," Journal of the Korean Society of Food Culture, vol. 25, pp. 607-614, 2010.
- [20] J. Ha, "Analysis of volatile organic compounds in kimchi absorbed in SPME by GC-AED and GC-MSD," Journal of the Korean Society of Food Science and Nutrition, vol. 31 no. 3, pp. 543-545, DOI: 10.3746/jkfn.2002.31.3.543, 2002.
- [21] Y.-S. Lee, H.-S. Sohn, J.-O. Rho, "Study on sensory properties and volatile flavor compounds of kimchi added with backryeoncho (*Opuntia ficus-indica* var. *saboten*) extracts," Journal of the East Asian Society of Dietary Life, pp. 506-513, 2012.
- [22] B. Ma, X. Li, Q. Zhang, "Metabonomic profiling in studying anti-osteoporosis effects of strontium fructose 1,6-diphosphate on estrogen deficiency-induced osteoporosis in rats by GC/TOF-MS," European Journal of

Pharmacology, vol. 718 no. 1-3, pp. 524-532, DOI: 10.1016/j.ejphar.2013.06.030, 2013.

[23] J. Ji, P. Zhu, F. Pi, "GC-TOF/MS-based metabolomic strategy for combined toxicity effects of deoxynivalenol and zearalenone on murine macrophage ANA-1 cells," *Toxicon*, vol. 120, pp. 175-184, DOI: 10.1016/j.toxicon.2016.08.003, 2016.

[24] J. G. Meher, N. P. Yadav, J. J. Sahu, P. Sinha, "Determination of required hydrophilic-lipophilic balance of citronella oil and development of stable cream formulation," *Drug Development and Industrial Pharmacy*, vol. 39 no. 10, pp. 1540-1546, DOI: 10.3109/03639045.2012.719902, 2013.

[25] X. Wei, X. Shi, I. Koo, "MetPP: a computational platform for comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry-based metabolomics," *Bioinformatics*, vol. 29 no. 14, pp. 1786-1792, DOI: 10.1093/bioinformatics/btt275, 2013.

[26] L. Bell, N. D. Spadafora, C. T. Müller, C. Wagstaff, H. J. Rogers, "Use of TD-GC-TOF-MS to assess volatile composition during post-harvest storage in seven accessions of rocket salad (*Eruca sativa*)," *Food Chemistry*, vol. 194, pp. 626-636, DOI: 10.1016/j.foodchem.2015.08.043, 2016.

[27] J. Y. Kim, E.-Y. Park, Y.-S. Kim, "Characterization of volatile compounds in low-temperature and long-term fermented baechu kimchi," *Journal of the Korean Society of Food Culture*, vol. 21, pp. 319-324, 2006.

[28] M. K. Yoon, S. M. Lee, J. W. Kim, "Characterization of volatile Components according to fermentation Periods in gamdongchotmoo kimchi," *Korean Journal of Food Science and Technology*, vol. 40 no. 5, pp. 497-502, 2008.

[29] Y.-H. Pyo, "Volatile compounds of mustard leaf (*Brassica juncea*) kimchi and their changes during fermentation," *Korean Journal of Food Science and Technology*, vol. 32 no. 1, pp. 56-61, 2000.

DETAILS

Subject:	Cancer; Volatile organic compounds--VOCs; Time of flight method; Acids; Hydrocarbons; Ingredients; Gas chromatography; Glucosinolates; Anticancer properties; Decomposition; Alcohols; Metabolites; Tumors; Aliphatic hydrocarbons; Enzymes; Kimchi
Location:	United States--US
Editor:	Barbara Bojko
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760

e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-22 (Received); 2021-05-25 (Revised); 2021-06-03 (Accepted); 2021-06-18 (Pub)
DOI:	https://doi.org/10.1155/2021/9978251
ProQuest document ID:	2545429193
Document URL:	https://www.proquest.com/scholarly-journals/development-gas-chromatography-time-flight-method/docview/2545429193/se-2?accountid=211160
Copyright:	Copyright © 2021 Ho Jin Kim et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-06-27
Database:	Publicly Available Content Database

Document 31 of 59

Synthesis and Characterization of Chitosan Particles Loaded with Antioxidants Extracted from Chia (*Salvia hispanica* L .) Seeds

Morales-Olán, Gema ¹

; Luna-Suárez, Silvia ¹

; De Dios Figueroa-Cárdenas, Juan ²

; Corea, Monica ³

; Rojas-López, Marlon ¹

¹ Instituto Politécnico Nacional, Centro de Investigación en Biotecnología Aplicada, Ex Hacienda De San Juan Molino, Carretera Estatal Santa Ines, Tecuexcomac-Tepetitla. Km. 1.5, Tepetitla, Tlaxcala 90700, Mexico ² CINVESTAV Unidad Querétaro, Libramiento Norponiente No. 2000, Fracc Real de Juriquilla, Querétaro, Qro 76230, Mexico ³ Instituto Politécnico Nacional, Escuela Superior de Ingeniería Química e Industrias Extractivas, UPALM, Zacatenco, Ciudad de México 07732, Mexico

ABSTRACT (ENGLISH)

Chia (*Salvia hispanica* L.) seeds contain antioxidants with great benefits for health and are widely used in the food industry. Antioxidants can be degraded by environmental factors, decreasing their biological activity. Their encapsulation in chitosan (CH) particles represents an alternative to protect them and increases their application. The encapsulation efficiency (%EE) of the antioxidants in the CH particles depends on the synthesis conditions. In this study, two methods for encapsulation of chia extract in chitosan particles were evaluated: method A, 0.05% CH in 1% acetic acid was mixed with 0.07% of tripolyphosphate (TPP) and method B, 0.3% CH in 2% acetic acid was mixed with 1% TPP. The results showed that the %EE decreased with the concentration of the extract, and the FTIR analysis suggested that the compounds of the extract were adsorbed on the surface of the particles. Dynamic light scattering and zeta potential analysis showed that the particles of method A are unstable and with a tendency to agglomerate, and the particles of method B are stable. The highest %EE was obtained with 0.2mg·mL⁻¹ (method A) and 1.0mg·mL⁻¹ (method B) of the extract. The higher loading capacity (%LC) (16–72%) was exhibited by the particles of method A. The best particle yield (62–69%) was observed for method B. The particles with the extract adsorbed showed antioxidant activity (5–60%) at 25°C; however, in the particles with the extract encapsulated, the activity increased after subjecting to acidic conditions at 40°C due to the breakdown of the particles. The results obtained will allow choosing the appropriate conditions for the synthesis of chitosan particles loaded with chia extracts with specific characteristics (%EE, %LC, size, and type) according to their future applications. The particles could be used in food and pharmaceutical industries and even in edible films for food packaging.

FULL TEXT

DETAILS

Subject:	Antioxidants; Encapsulation; Food packaging industry; Zeta potential; Chitosan; Food packaging; Nanoparticles; Acetic acid; Seeds; Methods; Biological activity; Synthesis; Variance analysis; Ethanol; Photon correlation spectroscopy
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760

e-ISSN: 16878779

Source type: Scholarly Journal

Language of publication: English

Document type: Journal Article

Publication history :

Milestone dates: 2021-02-26 (Received); 2021-05-31 (Accepted); 2021-06-15 (Pub)

DOI: <https://doi.org/10.1155/2021/5540543>

ProQuest document ID: 2545429115

Document URL: <https://www.proquest.com/scholarly-journals/synthesis-characterization-chitosan-particles/docview/2545429115/se-2?accountid=211160>

Copyright: Copyright © 2021 Gema Morales-Olán et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-06-27

Database: Publicly Available Content Database

Document 32 of 59

Fluorescence Spectroscopy and ^{13}C NMR Spectroscopy Characteristics of HA in Black Soil at Different Corn Straw Returning Modes

Zheng, Shuang; Sen Dou
; Duan, HongMei; Zhang, BoYan; Bai, Yue

[ProQuest document link](#)

ABSTRACT (ENGLISH)

A three-year field experiment was conducted to analyze the effects of straw enrichment and deep incorporation on the humus composition and the structure of humic acid (HA) in black soil. The differences in the HA structure between different straw returning methods were detected by three-dimensional fluorescence spectroscopy and ^{13}C NMR technology. The purpose of this paper is to provide a theoretical basis and data support for improving the straw

returning system. Four different treatments, including no straw applied (CK), straw mulching (SCR), straw deep ploughing (MBR), and straw enrichment and deep incorporation (SEDI: harvested the corn straw from four rows together with a finger-plate rake and then crushed and buried them in one row in the 20–40 cm deep level in the subsoil with a wind-driven input cylindrical plough), were used in this study. Our results showed that compared to CK treatment, SEDI significantly increased the contents of organic carbon (SOC), soil humic acid carbon (HAC), fulvic acid carbon (FAC), and humin C content (HM-C) in the subsurface soil layer by 27.47%, 34.33%, 19.66%, and 31.49%, respectively. Among all the straw returning treatments, SEDI treatment had the most significant effect in increasing the contents of HEC, HAC, and FAC. Straw returning not only reduced the degree of condensation and oxidation of the HA structure but also increased the proportion of alkyl C and enhanced the hydrophobicity of the HA structure in subsurface soil. Moreover, SEDI treatment significantly increased the proportion of aliphatic C/aromatic C of the HA structure in subsurface soil and improved the aliphatic property of HA, which had a significant effect on the HA structure compared to other treatments.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

In recent years, due to long-term intensive agricultural production and soil erosion, the soil organic content and humus quality of black soil have decreased significantly in Northeast China, resulting in shallower soil layers [1] and poor cultivability of land. Moreover, corn straw incorporation was effective in increasing soil organic carbon [2] and humus C content [3], deepening the degree of soil humification [4], and improving the HA structure. Chen et al. showed that straw returning increases the aliphatic group of HA molecular structure, reduces the degree of condensation and oxidation of HA structure, and makes HA structure more lipidic and simple [5].

Soil organic carbon (SOC) pool is the largest terrestrial carbon pool, and 70–80% of SOC is composed of humus [6]. Humic acid (HA) is the main component of humus [7]. Furthermore, the composition, structure, and properties of HA were related to the fertilization characteristics of the soil. Returning straw to the field can improve soil fertility and increase the content of soil organic matter. Different straw returning methods have different effects on the composition of soil humus and the structure of HA. Studies have shown that straw mulching is beneficial to the accumulation of organic carbon and humic substance C content [8] and the enhancement of functional groups such as aliphatic, hydroxyl, methoxy, and carboxyl groups on the surface soil [9], but straw mulching also affects seedling emergence and makes tillage difficult [10]. A new straw returning method had been developed, namely, straw enrichment and deep incorporation (SEDI), consisting in (1) raking the corn stalk in the field together into rows at a ratio of 4:1 with a finger-plate rake; (2) crushing the corn straw and burying it into subsoil, 20–40 cm deep along designated strips with a wind-driven input cylindrical plough; (3) sowing seeds into the strips with no straw buried in between the strips with straw buried in a normal way with a nontillage seeder, to realize separation of the seeded strips (narrow rows) from the strips (wide rows) with straw buried in a wide-and-narrow row alternating cultivation mode. Zuber et al. showed that straw deep ploughing has a better effect on the accumulation of soil organic matter than that of straw mulching [11] and also increases the organic carbon content of HE and HA [12]. Straw deep incorporation can also significantly increase the content of soil subsurface active organic carbon [13], deepen the degree of soil humification [14], reduce the degree of HA structure condensation and oxidation, and increase the content of aliphatic chain hydrocarbons and aromatic carbon. Zhang et al. have shown that straw deep incorporation accumulates soil organic carbon and humus component carbon content in different soil layers and improved the aromaticity and hydrophobicity of HA molecules [15]. Al et al. compared the changes in the structure of soil humic acid after applying crop straw, jujube leaf, and animal manure by solid-state-¹³C NMR. Their results indicated that crop straw has the highest aliphatic content and the strongest aliphatic content [16]. Chen Xi et al. showed that straw returning could increase the relative content of alkoxy carbon in soil organic carbon through ¹³C NMR research [17]. Nowadays, three-dimensional fluorescence spectroscopy and ¹³C NMR techniques were used to study the effects of straw returning on humic composition and HA structure. Previous research mainly focused on straw mulching and

straw shallow application on the accumulation of soil organic carbon and humus C in the surface layer [8]. However, the effects of straw enrichment and deep incorporation (SEDI) on soil humus composition and HA structure are yet poorly understood, along with the differences of HA structure between SEDI and other returning methods. To address the issue, we conducted a 3-year field experiment to (i) determine the SOC content, (ii) characterize humus composition and C content, and (iii) compare the changes of HA structure from different return methods by three-dimensional fluorescence spectroscopy and ^{13}C CPMAS NMR technology. In order to provide a theoretical basis and data support for the improvement of straw returning system, this paper focused on discussing the impact of the hydrophilicity and hydrophobicity of the HA structure on soil stability.

2. Materials and Methods

2.1. Experimental Site

The experiment was located at the Experimental Station of Agricultural Technology Extension Center in Jiutai District, Changchun City, Jilin Province (44°08'N, 125°50'E). The experimental soil was classified as Argiudolls. The basic properties of the sampled soil are shown in Table 1.

Table 1

Basic properties of the tested soil.

Depth (cm)	Organic matter (g/kg)	Total N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	pH
0~20	12.20	78.73	95.86	235.80	6.6
20~40	12.05	76.52	70.17	204.75	6.3

2.2. Experimental Design

The experiment adopted wide-and-narrow row planting. The ridge height was 12 cm, and the row spacing was 40 cm. The planting density was 65000/hm². Four treatments were set randomly with three replications. CK: no straw was applied; SCR: corn straw was evenly placed on the soil surface; MBR: corn straw was evenly placed on the soil surface, and straw deep turning and returning to the field to 25–30 cm soil layer; SEDI: the corn straw was harvested from four rows together with a finger-plate rake and then crushed and buried them in one row in the 20–40 cm deep level in the subsoil with a wind-driven input cylindrical plough. The corn straw was mechanically crushed in all three corn straw treatments. The experiment selected field planting; each treatment area was 1334 m². Each plot had the same fertilizer application rate: 200 kg·N·ha⁻¹, 100 kg·P·ha⁻¹, and 100 kg·K·ha⁻¹.

2.3. Soil Sampling and Analysis

Soil samples were collected after the maize harvest in November 2019. For each treatment, the soil samples were collected from three points in each plot replicate. The sampling depth was 0–20 cm and 20–40 cm. Each soil sample was air-dried and passed through a 2 mm sieve to remove plant residues for extracting soil HA.

2.4. Laboratory Analysis

SOC was determined using the K₂Cr₂O₇ external heating method. Alkalytic N was measured using the alkali diffusion method; available P was measured using sodium bicarbonate-molybdenum antimony colorimetry; available K was measured using a flame photometer, which was measured by a pH meter [18].

Humus composition was analyzed following the International Humic Substances Society method [19]. Briefly, 5 g of soil sample was sequentially extracted with 30 mL of distilled water, then with 30 mL of 0.1 N mixed alkali solution (NaOH+Na₄P₂O₇) under continuous shaking at 70 °C for one hour. The supernatant was humic extractable (HE) substance. 30 mL HE was acidified to pH 1 to separate HA from humic fulvic (FA). The precipitation was retained as HA and FA remained in the solution. HA was redissolved with 0.05 mol·L⁻¹NaOH.

HA isolation and purification were processed using the procedure described by the International Humic Substances Society procedure described by Kuwatsuka et al. [20]. Briefly, 50 g of soil sample was decalcified with HCl; then, residues were extracted by NaOH solution and allowed to stand overnight. The supernatant was soaked in a mixture

of HF and HCL solution to remove ash, dialysed to electrodialysis, and freeze-dried afterwards.

2.5. Characterization of HA

HA element composition, such as C, H, N, and O, was determined by a Vario EL III elemental analyzer. The fluorescence spectra were obtained at a concentration of 100mgL^{-1} (pH was adjusted to 8.0 with 0.05M NaOH). The EEM spectra were recorded with emission wavelength between 300 and 600nm, while the excitation wavelength was increased sequentially from 250 to 550nm. The emission-excitation slit was fixed at the 5nm bandwidth and the scanning speed was set at $12,000\text{nmmin}^{-1}$. The solid-state ^{13}C NMR spectra of soil samples were obtained on an AVANCE III 400 WB spectrometer at 100.6MHz with a spinning rate of 8kHz, an acquisition time of 34ms, a recycle time of 5s, and a contact time of 2ms. Chemical shift values were externally referenced to the methylene resonance of the adamantane standard at 38.4ppm. Semiquantification was performed by integration using Mestre Nova 14.0 software [21].

2.6. Statistical Analysis

Analysis of variance was performed using SPSS 21.0 software. Statistical significance differences among corn straw returning mode means were evaluated using the least significant difference test at a level of $p < 0.05$.

3. Results

3.1. SOC and Humic C

Data on the C contents of soil for each treatment are presented in Figure 1. Compared with CK treatment, SCR, MBR, and SEDI treatments increased SOC content in the surface soil layer by 12.65%, 10.07%, and 8.38%, respectively. In the 20–40cm soil depth, the SOC contents increased in the following trend: $\text{SEDI} > \text{MBR} > \text{SCR} > \text{CK}$. All corn straw treatments increased SOC content in the subsurface soil layer by 2.89%, 18.29%, and 27.47%. Thus, it could be seen that for the different straw returning treatments, the SEDI had a more significant cumulative effect on the subsurface organic carbon content.

[figure omitted; refer to PDF]

Data on the humic fractions for each treatment are presented in Table 2. For the humic fractions, SEDI treatment significantly increased the content of humic fraction (HEC) in the 20–40cm, soil humic acid carbon (HAC), fulvic acid carbon (FAC), and humin C content (HM-C) by 34.33%, 19.66%, and 31.49%, respectively, compared with CK. PQ value is the proportion of HA in humic substances, which can reflect the degree of humification of SOC. Compared with CK, the treatment of straw returning to the field increased the PQ value of the soil to varying degrees. The SEDI treatment increased the PQ value of subsurface soil the most, which increased from 62.71% to 65.39% compared with CK.

Table 2

Effect of different treatments of corn straw on humic substances in black soil.

Depth (cm)	Treatment	HA-C	FA-C	HM-C	PQ (%)
0~20	CK	3.19 ± 0.10^d	1.89 ± 0.10^b	5.31 ± 0.16^c	62.78 ± 0.01^b
SCR	4.24 ± 0.80^a	2.22 ± 0.08^a	6.49 ± 0.46^a	65.67 ± 0.01^a	MBR
3.96 ± 0.18^b	2.20 ± 0.13^a	6.16 ± 0.12^{ab}	64.25 ± 0.02^a	SEDI	3.68 ± 0.12^c
2.08 ± 0.14^a	5.85 ± 0.25^b	63.85 ± 0.02^a	20~40	CK	3.00 ± 0.16^c
1.78 ± 0.15^a	5.05 ± 0.13^c	62.71 ± 0.03^a	SCR	3.22 ± 0.22^{bc}	1.86 ± 0.20^a
5.80 ± 0.19^b	63.41 ± 0.04^a	MBR	3.60 ± 0.10^b	2.03 ± 0.20^a	6.01 ± 0.21^b

63.90±0.04 ^a	SEDI	4.03±0.14 ^a	2.13±0.20 ^a	6.64±0.38 ^a	65.39±0.03 ^a

Note. CK: no straw application; SCR: straw mulching; MBR: straw deep ploughing; SEDI: straw enrichment and deep incorporation. There were significant differences between different letters representing different treatments ($p < 0.05$).

3.2. Elemental Composition of HA

It can be seen from Table 3 that the elements of HA are mainly composed of C and O, in which the C content ranges from 482.1 g kg⁻¹ to 512.9 g kg⁻¹, and the O content ranges from 422.5 g kg⁻¹ to 479 g kg⁻¹. After corn straw returning, the content of C, H, and N in HA increased, while the content of O decreased. Among all the treatments, the SEDI treatment significantly increased the C, H, and N contents in the 20–40 cm depth. In the topsoil, the H/C ratio increased in the trend of SCR>MBR>SEDI>CK, while the O/C ratio decreased in the trend of CK>SEDI>MBR>SCR. In the subsoil, the H/C ratio increased in the trend of SEDI>MBR>SCR>CK, while the O/C ratio decreased in the trend of CK>SCR>MBR>SEDI.

Table 3

Effect of straw returning to the field on HA element composition in soil.

Depth (cm)	Treatment	Element content (g·kg ⁻¹)				O/C	H/C
C	H	N	O	0~20	CK	487.3±0.8 ^d	44.99±0.30 ^d
22.71±0.28 ^d	477.0±0.8 ^a	0.734±0.002 ^a	1.108±0.009 ^c	SCR	512.9±0.7 ^a	49.54±0.06 ^a	27.88±0.10 ^a
422.5±0.5 ^d	0.618±0.001 ^d	1.159±0.001 ^b	MBR	495.1±0.9 ^b	47.97±0.10 ^b	25.70±0.12 ^b	435.2±0.9 ^c
0646±0.001 ^c	1.140±0.002 ^a	SEDI	491.9±0.8 ^c	46.43±0.08 ^c	23.42±0.09 ^c	467.5±0.4 ^b	0.713±0.001 ^b
1.133±0.002 ^a	20~40	CK	482.1±0.9 ^d	43.21±0.90 ^d	21.52±0.10 ^c	479.0±0.9 ^a	0.745±0.001 ^a
1.076±0.024 ^c	SCR	486.8±0.5 ^c	44.56±0.08 ^c	22.90±0.66 ^b	475.0±0.7 ^b	0.732±0.002 ^b	1.099±0.001 ^{bc}
MBR	488.7±0.6 ^b	45.5±0.17 ^b	23.20±0.44 ^b	453.1±0.9 ^c	0.695±0.001 ^c	1.117±0.005 ^b	SEDI

Note. CK: no straw application; SCR: straw mulching; MBR: straw deep ploughing; SEDI: straw enrichment and deep incorporation. There were significant differences between different letters representing different treatments ($p < 0.05$).

3.3. Three-Dimensional Fluorescence EEM Spectra of HA

The three-dimensional fluorescence spectroscopy of straw returning modes is displayed in Figures 2 and 3. All of the EEM fluorescence spectra of HA exhibited three fluorophores (peaks A–C). The three fluorophores were centered at the Em/Ex wavelength of 430–500/470–550 nm for peak A, 340–370/490–550 nm for peak B, and 270–340/460–550 nm for peak C, which may be related with the amount of humic substances. Among all the

treatments, the highest fluorescence intensities of peaks A-C were recorded under SEDI in the 20–40cm, and the lowest under CK in the 0–20cm. Generally, higher fluorescence intensity indicates greater proportions of hydroxyl, alkoxyl, and methoxyl [22]. The results showed that SEDI treatment had a slight increase in FI as compared with CK, implying that straw enrichment and deep incorporation were more conducive to improving the straw decomposition and soil HA formation than other treatments (Table 4).

[figures omitted; refer to PDF]

[figures omitted; refer to PDF]

Table 4

Excitation (Ex)/emission (Em) wavelength and fluorescence intensity (FI) of peaks in humic acid after different corn straw return modes.

Soil depth (cm)	Treatment	Peak A		Peak B		Peak C	
Ex/Em	Intensity (a.u.)	Ex/Em	Intensity (a.u.)	Ex/Em	Intensity (a.u.)	0~20	CK
460/524	115.02	370/504	105.00	290/503	117.84	SCR	460/531
154.52	370/523	136.33	290/508	153.54	MBR	460/523	149.63
370/498	135.81	290/521	141.71	SEDI	460/528	130.56	370/495
117.72	290/523	129.32	20~40	CK	460/528	112.86	370/511
112.61	290/515	114.30	SCR	460/529	125.33	370/493	114.19
290/520	120.14	MBR	460/537	144.73	370/505.6	126.12	300/522
135.99	SEDI	460/535	172.34	370/506	143.47	290/509	164.00

Note. CK: no straw application; SCR: straw mulching; MBR: straw deep ploughing; SEDI: straw enrichment and deep incorporation.

3.4. ¹³C NMR Spectra of HA

The ¹³C NMR spectra of HA for each treatment are reported in Figure 4. The spectrum can be divided into 4 main resonance regions, namely, alkyl C (0~50ppm), alkoxy C (50~110ppm), aromatic C (110~160ppm), and carboxyl C (160~200ppm) [23]. The peaks at 30ppm in the alkyl C region were assigned as-CH₂-. The peak at 55~56ppm in the O-alkyl C region was assigned as methoxyl C in lignin. The peaks at 70~73ppm and 104~105ppm were carbohydrate C and hydrogen peroxide in polysaccharide, respectively. The peak at 128~130ppm in the aromatic C region was ascribed to aryl C. The peak at 171~173ppm in the carbonyl C region was indicative of carboxylic acid, amide, and ester [24]. The shape of the spectrum of all treatments of HA shows similar tendencies; however, the absorption peak intensity is significantly different. It indicated that although HA has a similar structure, the HA structure changed after straw returning.

[figure omitted; refer to PDF]

The relative intensity of the HA groups for different treatment is shown in Table 5. Compared with the CK treatment,

all the straw returning treatments increased the alkyl C, whereas the O-alkyl C decreased. SCR, MBR, and SEDI treatments were 0.77%, 4.39%, and 16.80% higher than CK treatment in alkyl C and 3.11%, 5.26%, and 8.24% lower in O-alkyl C. These results showed that corn straw return was disadvantageous of accumulation for O-alkyl C; nevertheless, SEDI was beneficial to the formation of alkyl C. Moreover, SEDI treatments decreased aromatic C contents of soil HA by 1.72%. Furthermore, compared with CK, SEDI treatments had no significant difference for carbonyl C; SCR treatment was 2.10% higher than CK treatment. However, MBR treatment decreased by 1.72%. For the ratio of alkyl/O-alkyl and hydrophobic/hydrophilic, all the corn straw return treatments were higher than CK, and SEDI had a higher increasing rate (27.28% and 5.87%). The higher aliphatic C/aromatic C ratio indicated that HA is more aliphatic, and SEDI is more beneficial to improve the straw decomposition and HA formation than the other treatments.

Table 5

Relative intensity (%) of different chemical shift intervals from ^{13}C CPMAS NMR spectra of HA examined.

Treat ment	Carbonyl C (%) (160–230 ppm)	Aromatic C (%) (110–160 ppm)	O-Alkyl C (%) (50–110 ppm)	Alkyl C (%) (0–50 ppm)	Alkyl C/O-alkyl C	Aliphatic C/aromatic C	Hydrophobic C/hydrophilic C
CK	18.58	53.98	14.45	12.98	0.898	0.508	2.027
SCR	18.97	53.96	14.00	13.08	0.934	0.502	2.034
MBR	18.26	54.49	13.69	13.55	0.99	0.500	2.129
SEDI	18.53	53.05	13.26	15.16	1.143	0.536	2.146

Note. CK: no straw application; SCR: straw mulching; MBR: straw deep ploughing; SEDI: straw enrichment and deep incorporation.

4. Discussion

4.1. Corn Straw Returning Increased SOC and Humus C Contents

Soil stores most of the ecosystem carbon in the form of organic matter [25], and humus is the most abundant and important component of soil organic matter [7]. Thus, humus is a significant indicator to evaluate soil fertility [26]. Zhang et al. [27] observed that straw returning could increase the content of SOC and humus and improve soil fertility.

After three years, corn straw returning significantly increased SOC, HAC, FA-C, and HM-C contents in both soil depths (Table 2), consistent with previous studies [28–30]. Straw contains carbon, nitrogen, phosphorus, potassium, other nutrients [31] and a certain number of humus fractions [1]. Therefore, its application increases the content of humus C through stem and root exudates [32], which reduces the direct mineralization of soil organic carbon content. Lhadi et al. reported that the degradation of corn straw leads to the formation of humus [33]. The humus C persisted in the soil for a certain time, and then increased the soil carbon sequestration and humus composition content. It led to the increase of the HA/FA ratio, which indicated the humification degree of soil was deepened [3]. Among all the treatments, SEDI was more conducive to increasing SOC, HAC, and FA-C contents in the subsoil (Table 2). Previous studies have shown that the organic carbon content of MBR is higher than that of SCR in the subsoil [34], due to the fact that the corn straw could not be fully mixed with soil under SCR treatment, and decreases its decomposition rate by soil microorganisms [35]. Compared with MBR, the process of deep burying of straw under the SEDI treatment disturbs the subsurface layer of the soil and stimulates microbial activity [36], increasing their metabolic rate [22] and increasing the soil easily oxidizable carbon [37]. The latter processes increase soil carbon sources. Straw returning to the field can promote a significant increase in the carbon content of

HA, FA, and HM, while the PQ value increases slightly, but the change is not significant, indicating that the degree of soil maturation and fertility state of the soil transforms in the appropriate direction after straw returning [15].

4.2. Corn Straw Returning Decreased the Oxidation Degree and Condensation Degree of HA

The results showed that corn straw returning increased the H/C molar ratio of HA and decreased the O/C molar ratio of HA (Table 3), which indicated that straw returning reduced the condensation degree and oxidation degree of HA structure and simplified the structure of HA. Chen et al. reported that the condensation degree and oxidation degree of HA structure in soil decreased after straw application [5], due to the promotion of microbial activity after straw application, and during the process of microbial metabolic decomposition, HA with complex structure in soil was decomposed, which reduced the stability of HA structure [38]. In addition, the number of newly formed HA oxygen-containing functional groups in the process of straw decomposition was less [39], which reduced the degree of oxidation of HA and made the structure of HA be simpler and younger.

4.3. Corn Straw Returning Increased the Hydrophobicity of HA

Fluorescence spectroscopy can be used to measure the structure and properties of compounds and determine the structure of functional groups of humus [40, 41]. Our research has shown that the hydrophobicity of HA structure of the soil was enhanced after the straw returning. In our study, straw application compared with CK increased the intensities of peaks A to C, which indicates that the application of corn straw contains more electron-donating substituents such as hydroxyl, methoxy, and amino [42], and they can increase the fluorescence intensity by increasing the transition probability between the singlet state and the ground state [43]. Gao et al. have shown that straw returning to the field can improve the soil and found that the soil had a higher content of alkoxy carbon and methyl carbon [22, 44], while methyl C was converted from lignin polymerization [45] and was a hydrophobic substance. Therefore, returning straw enhances the hydrophobicity of HA.

Considering the complex peak assignment in the elemental analysis and fluorescence spectroscopy of HA, we evaluated the HA structural characteristics further using the solid-state ^{13}C CPMAS NMR technique. Different ways of returning straw to the field led to different changes in the structure of functional groups, mainly manifested as changes in aromatic carbon, alkyl carbon, alkoxy carbon, and carboxyl carbon. Alkyl C (aliphatic compound, methyl C, etc.) is a carbon compound difficult to degrade in soil. Thus, the application of straw results in the increase of alkyl C community and microbial structure [46]. The decomposition of O-alkyl C is mainly due to the fact that cellulose and hemicellulose in plant residues are easily metabolized by microorganisms to utilize organic carbon functional groups. The relative content of alkoxy C slightly increases after straw returning, while during the rapid phase of corn stalk decomposing, the O-alkyl C in the plant residues will be quickly lost into the soil, resulting in a relatively low content of alkoxy C [47]. Aromatic C (mainly from tannin and lignin) is relatively stable. The decrease of the structure in SEDI may be due to the aerobic degradation of lignin by white rot and brown rot fungi through dehydration, demethylation, or cleavage of $\beta\text{-O-4}$ bonds [48]. Carboxyl carbons mainly come from carboxylic acids, amides, and esters [49]. The increase in carboxyl C under SCR treatment might be related to the oxidation of lignin side chains and polysaccharides [39]. The hydrophobicity of HA was determined by the ratio of hydrophobic C (aromatic C and alkyl C) to hydrophilic C (alkoxy C and carboxyl C). The higher the ratio, the stronger the hydrophobicity and the stronger the stability of soil HA [50].

4.4. Effects of Different Returning Methods on the Soil HA Structure

Studies have shown that hydrophobicity was meaningful for maintaining the stability of soil organic carbon [50]. Our results showed that straw return enhances the hydrophobicity of HA structure, and SEDI treatment was the most effective (Table 5). Studies have shown that the ratio of high alkyl C and low aromatic C makes the soil organic matter younger [51]. This implies that HA is younger under SEDI treatment. The higher aliphatic C/aromatic C ratio showed (Table 5) that HA was highly aliphatic under SEDI treatment, which made the structure of HA simpler. On the contrary, the aromaticity was enhanced under the MBR and SCR treatment, demonstrating a more complex HA structure. Therefore, straw buried in the subsurface of soil increases the soil oxidizable carbon, stimulates the microbial activity, speeds up the metabolic rate of microorganisms, and promotes the decomposition of HA.

5. Conclusion

In this 3-year study, ^{13}C NMR spectroscopy and three-dimensional fluorescence spectroscopy were adapted to analyze the structure of soil HA after straw returning. The study concludes the following:

(i) Adding corn straw into soil significantly increased SOC content and humus composition. Compared to all the rest treatments, SEDI accumulated the largest quantities of SOC and C contents of humic acid, especially in the subsurface soil layer.

(ii) Among the four treatments, SEDI was the most conducive way to enhance the hydrophobicity of the HA structure, which made it more stable, and improve the soil carbon sequestration capacity.

Acknowledgments

This study was supported by the National Key Research and Development Program of China (2017YFD0200601), the Key Research and Development Program of Jilin Province (20200402098NC), and the National Natural Science Foundation of China (42077022). The authors thank Dr. L. C. Ming and Zhang. Y. Feng for their valuable advice on ^{13}C NMR spectroscopy analysis.

References

- [1] X. Zhang, S. Dou, B. S. Ndzelu, X. W. Guan, B. Y. Zhang, Y. Bai, "Effects of different corn straw amendments on humus composition and structural characteristics of humic acid in black soil," *Communications in Soil Science and Plant Analysis*, vol. 51 no. 1, pp. 107-117, DOI: 10.1080/00103624.2019.1695827, 2020.
- [2] A. Chatterjee, "Annual crop residue production and nutrient replacement costs for bioenergy feedstock production in United States," *Agronomy Journal*, vol. 105 no. 3, pp. 685-692, DOI: 10.2134/agronj2012.0350, 2013.
- [3] P. Cong, *Fertilization Effect and Mechanism of Subsoil under High Dosage Straw Returning in Black Soil of Northeast China*, 2019.
- [4] H. Zou, "Effect of different straw return years on humus composition of soil," *Chinese Journal of Soil Science*, vol. 44 no. 6, pp. 1398-1402, 2013.
- [5] X. Chen, J. Wu, Y. Opoku-Kwanowaa, "Effects of returning granular corn straw on soil humus composition and humic acid structure characteristics in saline-alkali soil," *Sustainability*, vol. 12 no. 3, DOI: 10.3390/su12031005, 2020.
- [6] F. Adani, M. Spagnol, K. G. J. Nierop, "Biochemical origin and refractory properties of humic acid extracted from maize plants: the contribution of lignin," *Biogeochemistry*, vol. 82 no. 1, pp. 55-65, DOI: 10.1007/s10533-006-9052-4, 2007.
- [7] G. Brunetti, C. Plaza, C. E. Clapp, N. Senesi, "Compositional and functional features of humic acids from organic amendments and amended soils in Minnesota, USA," *Soil Biology and Biochemistry*, vol. 39 no. 6, pp. 1355-1365, DOI: 10.1016/j.soilbio.2006.12.012, 2007.
- [8] Y. Qu, B. Feng, "Straw mulching improved yield of field buckwheat (*Fagopyrum*) by increasing water-temperature use and soil carbon in rain-fed farmland," *Acta Ecologica Sinica*, vol. 11, 2020.
- [9] B. S. Ndzelu, S. Dou, X. Zhang, "Changes in soil humus composition and humic acid structural characteristics under different corn straw returning modes," *Soil Research*, vol. 58 no. 5, pp. 452-460, DOI: 10.1071/sr20025, 2020.
- [10] L. Zhuang, B. Huang, F. Jiang, "Straw returning and its effective returning method: research progress," *Chinese Agricultural Science Bulletin*, vol. 35 no. 22, pp. 38-41, 2019.
- [11] S. M. Zuber, G. D. Behnke, E. D. Nafziger, M. B. Villamil, "Crop rotation and tillage effects on soil physical and chemical properties in Illinois," *Agronomy Journal*, vol. 107 no. 3, pp. 971-978, DOI: 10.2134/agronj14.0465, 2015.
- [12] P. Jiang, M. Rong, Q. Zhang, "Effects of increasing soil fertility and maize output by manure the maize straw with different returning ways to upland soil," *Soil Fertilizer*, vol. 3, 2004.
- [13] X. Liu, "Effects of deep loosening and deep straw returning on soil organic carbon and its. Active fractions in black soil," *Chinese Journal of Soil Science*, vol. 50 no. 03, pp. 602-608, 2019.
- [14] T. Cui, Z. Li, S. Wang, "Effects of in-situ straw decomposition on composition of humus and structure of humic acid at different soil depths," *Journal of Soils and Sediments*, vol. 17 no. 10, pp. 2391-2399, DOI: 10.1007/s11368-017-1704-6, 2017.

- [15] Y. Zhang, "Effect of deep incorporation of corn stover combined chemical fertilizer on composition of soil humus and structure of humic acid in soil," *Acta Pedologica Sinica*, vol. 53 no. 03, pp. 694-702, 2016.
- [16] Y. S. S. Al-Faiyz, "CPMAS ¹³C NMR characterization of humic acids from composted agricultural Saudi waste," *Arabian Journal of Chemistry*, vol. 10, pp. 839-853, DOI: 10.1016/j.arabjc.2012.12.018, 2017.
- [17] X. Chen, *Effect of Straw Incorporation on Elemental Composition and Chemical Structure of Soil Organic Matter*, 2016.
- [18] R. Lu, *Soil Agricultural Chemistry Analysis Method*, 2000.
- [19] T. Anđelković, D. Anđelković, J. Perović, "Decrease of oxygen interference on humic acid structure alteration during isolation," *Facta Universitatis-Series: Physics, Chemistry and Technology*, vol. 2 no. 3, pp. 163-171, 2001.
- [20] S. Kuwatsuka, A. Watanabe, K. Itoh, S. Arai, "Comparison of two methods of preparation of humic and fulvic acids, IHSS method and NAGOYA method," *Soil Science and Plant Nutrition*, vol. 38 no. 1, pp. 23-30, DOI: 10.1080/00380768.1992.10416948, 1992.
- [21] P. Conte, "Quantitative differences in evaluating soil humic substances by liquid-and solid-state ¹³C-NMR spectroscopy," *Geoderma*, vol. 80 no. 3-4, pp. 339-352, DOI: 10.1016/s0016-7061(97)00059-1, 1997.
- [22] J. Hu, J. Wu, X. Qu, J. Li, "Effects of organic wastes on structural characterizations of humic acid in semiarid soil under plastic mulched drip irrigation," *Chemosphere*, vol. 200, pp. 313-321, DOI: 10.1016/j.chemosphere.2018.02.128, 2018.
- [23] M. Schnitzer, C. M. Preston, "Analysis of humic acids by solution and solid-state carbon-13 nuclear magnetic resonance," *Soil Science Society of America Journal*, vol. 50 no. 2, pp. 326-331, DOI: 10.2136/sssaj1986.03615995005000020015x, 1986.
- [24] L. Li, W. Huang, P. a. Peng, G. Sheng, J. Fu, "Chemical and molecular heterogeneity of humic acids repetitively extracted from a peat," *Soil Science Society of America Journal*, vol. 67 no. 3, pp. 740-746, DOI: 10.2136/sssaj2003.0740, 2003.
- [25] D. H. Wall, "Biodiversity and ecosystem functioning," *Bioscience*, vol. 49 no. 2, pp. 107-108, DOI: 10.2307/1313535, 1999.
- [26] S. Salmon, "Changes in humus forms and soil animal communities in two developmental phases of Norway spruce on an acidic substrate," *Forest Ecology & Management*, vol. 237 no. 1-3, DOI: 10.1016/j.foreco.2006.09.089, 2006.
- [27] J. Zhang, Y. Wei, J. Liu, "Effects of maize straw and its biochar application on organic and humic carbon in water-stable aggregates of a Mollisol in Northeast China: a five-year field experiment," *Soil and Tillage Research*, vol. 190, DOI: 10.1016/j.still.2019.02.014, 2019.
- [28] N. Liu, "Depth of straw incorporation significantly alters crop yield, soil organic carbon and total nitrogen in the North China Plain," *Soil and Tillage Research*, vol. 205, DOI: 10.1016/j.still.2020.104772, 2021.
- [29] A. L. Hammerbeck, S. J. Stetson, S. L. Osborne, T. E. Schumacher, J. L. Pikul, "Corn residue removal impact on soil aggregates in a No-till corn/soybean rotation," *Soil Science Society of America Journal*, vol. 76 no. 4, pp. 1390-1398, DOI: 10.2136/sssaj2011.0421, 2012.
- [30] J. Liu, "Effects of returning stalks into field on soil humus composition of continuous cropping cotton field," *Scientia Agricultura Sinica*, vol. 48 no. 2, pp. 293-302, 2015.
- [31] P. Wang, "Effect of sweet corn straw returning to the field on soil fertility, yield and benefit," *Meteorological & Environmental Research*, vol. 7 no. 4, 2016.
- [32] S. Ma, Z. Kan, J. Qi, H. Zhang, "Effects of straw return mode on soil aggregates and associated carbon in the north China plain," *Agronomy*, vol. 10 no. 1, DOI: 10.3390/agronomy10010061, 2020.
- [33] E. K. Lhadi, H. Tazi, M. Aylaj, P. L. Genevini, F. Adani, "Organic matter evolution during co-composting of the organic fraction of municipal waste and poultry manure," *Bioresource Technology*, vol. 97 no. 16, pp. 2117-2123, DOI: 10.1016/j.biortech.2005.09.012, 2006.
- [34] N. Liang, G. Cai, F. Jiang, "Effect of different maize straw-returning modes on the fertility of black soil," *Journal of Maize Sciences*, vol. 24 no. 6, pp. 107-113, 2016.

- [35] H. Wang, D. Wang, H. Tian, "Effect of straw-returning on the storage and distribution of different active fractions of soil organic carbon," *Chinese Journal of Applied Ecology*, vol. 25 no. 12, pp. 3491-3498, 2014.
- [36] I. Lobe, "Aggregate dynamics and associated soil organic matter contents as influenced by prolonged arable cropping in the South African Highveld," *Geoderma*, vol. 162 no. 3-4, pp. 251-259, DOI: 10.1016/j.geoderma.2011.02.001, 2011.
- [37] C. Li, L. Gao, P. Jiang, "Research on mechanized Technology of returning corn stalks to field," *Modern Agricultural Machinery*, vol. 5, pp. 43-44, 2019.
- [38] S. Abiven, S. Menasseri, C. Chenu, "The effects of organic inputs over time on soil aggregate stability - a literature analysis," *Soil Biology and Biochemistry*, vol. 41 no. 1, DOI: 10.1016/j.soilbio.2008.09.015, 2009.
- [39] M. Tamura, V. Suseela, M. Simpson, B. Powell, N. Tharayil, "Plant litter chemistry alters the content and composition of organic carbon associated with soil mineral and aggregate fractions in invaded ecosystems," *Global Change Biology*, vol. 23 no. 10, pp. 4002-4018, DOI: 10.1111/gcb.13751, 2017.
- [40] D. M. B. P. Milori, L. Martin-Neto, C. Bayer, J. Mielniczuk, V. S. Bagnato, "Humification degree of soil humic acids determined by fluorescence spectroscopy," *Soil Science*, vol. 167 no. 11, pp. 739-749, DOI: 10.1097/00010694-200211000-00004, 2002.
- [41] L. Pospíšilová, "Effect of selected organic materials on soil humic acids chemical properties," *Environmental Research*, vol. 187, 2020.
- [42] W. Zech, "Factors controlling humification and mineralization of soil organic matter in the tropics," *Ganoderma*, vol. 79 no. 1, pp. 117-161, DOI: 10.1016/s0016-7061(97)00040-2, 1997.
- [43] N. Senesi, T. M. Miano, M. R. Provenzano, G. Brunetti, "Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy," *Soil Science*, vol. 152 no. 4, pp. 259-271, DOI: 10.1097/00010694-199110000-00004, 1991.
- [44] J. Gao, S. Dou, Z. Wang, "Structural analysis of humic acid in soil at different corn straw returning modes through fluorescence spectroscopy and infrared spectroscopy," *International Journal of Analytical Chemistry*, vol. 2019, DOI: 10.1155/2019/1086324, 2019.
- [45] K. Wang, D. Xu, F. Ding, "Process of plant residue transforming into soil organic matter and mechanism of its stabilization: a review," *Acta Pedologica Sinica*, vol. 56 no. 3, pp. 528-540, 2019.
- [46] G. Wu, H. Wang, M. Jiang, "Study on humic acids in the soil applied with corn stalk by spectroscopy measurements," *Scientia Agricultura Sinica*, vol. 38 no. 7, 2005.
- [47] N. J. Mathers, Z. Xu, "Solid-state ¹³C NMR spectroscopy: characterization of soil organic matter under two contrasting residue management regimes in a 2-year-old pine plantation of subtropical Australia," *Geoderma*, vol. 114 no. 1-2, pp. 19-31, DOI: 10.1016/s0016-7061(02)00339-7, 2003.
- [48] S. Derenne, C. Largeau, "A review of some important families of refractory macromolecules: composition, origin, and fate in soils and sediments," *Soil Science*, vol. 166 no. 1, DOI: 10.1097/00010694-200111000-00008, 2001.
- [49] N. Li, M. Sheng, Y. You, "Advancement in research on application of ¹³C NMR techniques to exploration of chemical structure of soil organic matter," *Acta Pedologica Sinica*, vol. 56 no. 4, pp. 796-812, 2019.
- [50] S. Bai, M. Luo, H. Li, "Chemical composition and structural features of soil organic carbon (SOC) in plantation," *Journal of Southwest Minzu University (Natural Science Edition)*, vol. 43 no. 5, pp. 474-479, 2017.
- [51] M. von Lützow, I. Kögel-Knabner, K. Ekschmitt, "SOM fractionation methods: relevance to functional pools and to stabilization mechanisms," *Soil Biology and Biochemistry*, vol. 39 no. 9, pp. 2183-2207, DOI: 10.1016/j.soilbio.2007.03.007, 2007.

DETAILS

Subject:	Soils; Software; Humus; Agricultural production; Spectrum analysis; Fluorescence; Experiments; Soil structure; Hydrophobicity; Carbon; NMR spectroscopy; Aliphatic compounds; Organic carbon; Oxidation; Methods; Humic acids; Soil improvement; Soil layers
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-07 (Received); 2021-05-21 (Revised); 2021-05-31 (Accepted); 2021-06-07 (Pub)
DOI:	https://doi.org/10.1155/2021/9940116
ProQuest document ID:	2543215094
Document URL:	https://www.proquest.com/scholarly-journals/fluorescence-spectroscopy-sup-13-c-nmr/docview/2543215094/se-2?accountid=211160
Copyright:	Copyright © 2021 Shuang Zheng et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/

Document 33 of 59

A Brief Review of Analytical Methods for the Estimation of Allopurinol in Pharmaceutical Formulation and Biological Matrices

Sharma, Hemraj ¹; Hari Prasad Sapkota ¹; Dangi, Nim Bahadur ²

¹ Department of Pharmacy, Shree Medical and Technical College, Bharatpur, Chitwan, Nepal ²
Pharmaceutical Sciences Program, School of Health and Allied Sciences, Faculty of Health Sciences,
Pokhara University, Kaski, Nepal

[ProQuest document link](#)

ABSTRACT (ENGLISH)

This review article represents the collection and discussion of various analytical methods available in the literature for the determination of allopurinol (ALLP) in pharmaceutical and biological samples consisting of HPLC, UV-visible method, near-IR spectroscopy, spectrofluorometry, capillary electrophoresis, polarography, voltammetry, and hyphenated techniques such as LC-MS, LC-MS/MS, UPLC-MS/MS, and GC-MS. The anticipated review provides details about the comparative utilization of various analytical techniques for the determination of ALLP. The present review article can be effectively explored to conduct future analytical investigation for the estimation of ALLP.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Hyperuricemia is a condition characterized by abnormally elevated levels of serum urate in which there is major increase in purine metabolism subsequent to the fast lysis of malignant cells occurring in patients with large tumor burden, both spontaneously and after aggressive chemotherapy [1]. It results from the impairment with uric acid renal clearance, among patients with kidney diseases or iatrogenic adverse events. This metabolic complication may be potentially dangerous since uric acid, via precipitating in renal tubules, can cause acute renal failure [2]. The drugs used for hyperuricemia are used with the intent of lowering the quantity of uric acid in blood, and this may be obtained by reducing the formation of uric acid or by increasing the clearance of formed uric acid. ALLP has structural similarity with hypoxanthine (natural purine base) and acts by inhibiting the production of uric acid [3].

2. Experimental

2.1. Physiochemical Properties

ALLP is chemically 1H-pyrazolo[3,4-d]pyrimidin-4-ol (Figure 1). It is a tautomeric mixture of 1H-pyrazolo[3,4-d]pyrimidin-4-ol and 1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one [4]. The molecular weight and molecular formula

of ALLP are $136.11 \text{ g mol}^{-1}$ and $\text{C}_5\text{H}_4\text{N}_4\text{O}$, respectively. It is sparingly soluble in water and in ethanol (95%) and practically insoluble in chloroform and in ether. It is soluble in dilute alkali hydroxides solutions. It is white to almost white, crystalline powder [5].

[figure omitted; refer to PDF]

2.2. Mechanism of Action

ALLP interferes with the catabolism of purines by interfering with xanthine oxidase enzyme and inhibits its effect which is responsible for the interconversion of hypoxanthine to xanthine and too uric acid. Interestingly, ALLP is not only a blocker, but also a substrate of xanthine oxidase, and the deriving metabolite, oxypurinol (OXP), is a potent inhibitor of xanthine oxidase itself and may be considered responsible for much of its pharmacological effect [6]. The reduction in the production of uric acid lowers its plasma and urinary levels and favors the formation of hypoxanthine and xanthine, which can be considered as its precursors [7]. Hypoxanthine is more soluble in water, whereas xanthine is yet less soluble compared to uric acid [8]; hence, the use of ALLP may potentially amplify the threat of precipitating xanthine in renal tubules [9].

2.3. Pharmacokinetics and Pharmacodynamics

ALLP can be administered either orally or intravenously. The oral bioavailability is about 67 to 90% with a peak plasma concentration occurring within one hour; the volume of distribution is approximately 1.6 L kg^{-1} [10, 11]. It is principally metabolized by aldehyde oxidase to the active compound OXP. The peak plasma concentration of OXP occurs within 3–5 hours. Mean elimination plasma half-life ranges between 0.7 and 1.5 hours for ALLP and 18–40 hours for OXP [12]. ALLP is excreted in urine (less than 10% unchanged, 70% as OXP) and in feces (20%). The main objective of the present work is to describe various simple and sophisticated analytical methods for determining ALLP in various formulations and matrices. The compiled data may be explored for the studies on analysis of ALLP. The major analytical methods include UV-visible spectrophotometric method, IR spectroscopy, spectrofluorimetric method, chromatographic methods like high-performance liquid chromatography (HPLC) and gas chromatography (GC), hyphenated techniques like gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), and miscellaneous methods.

2.4. Pharmaceutical Methods of ALLP

2.4.1. Pharmacopeial Methods

ALLP is an official drug in the Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), and United States Pharmacopoeia (USP). IP and BP have reported an HPLC procedure for the analysis of ALLP using UV-visible spectrophotometer by measuring the absorbance of drug at 250 nm. The pharmacopeia has used 563 as the specific absorbance of ALLP at 250 nm. The drug was extracted with sodium hydroxide, followed by HCl, and HCl was used as a blank [13, 14]. USP describes the analysis of ALLP using chromatographic method. HPLC analysis was done using reversed-phase column (30 cm × 4 mm), using mobile phase composed of 0.05 M solution of monobasic ammonium phosphate at a flow rate of 1.5 mL min^{-1} , and detection of wavelength was made at 254 nm [15].

3. Analytical Methods

3.1. UV-Visible Spectrophotometric and Spectrofluorimetric Methods (UV-Vis Methods)

Up till now, a number of UV spectrophotometric methods have been reported for determining ALLP. Khayoon has developed rapid, cheap, reliable, and simple spectrophotometric method for the quantitative analysis of ALLP in tablet formulation based on the reaction of ALLP with catechol reagent and Fe(II) to form a blue soluble complex (Figure 2) which was measured at λ_{max} 580 nm.

[figures omitted; refer to PDF]

Optimization of the developed method was studied on various parameters like the order of addition of reagent, volume of ferrous solution, catechol solution, and time of stability. The results stated that adding catechol followed by ALLP and ferrous solution gave good result. 2 ml of ferrous solution and 3 ml of catechol gave high absorbance whereas the authors found that the color complex was stable up to 2 hours in stability study. The drug was linear over the concentration range $2\text{--}10 \mu\text{g mL}^{-1}$ with a good recovery of 100% and RSD of 1.0%–1.3% [16].

Patel et al. established spectroscopic method for simultaneous estimation of ALLP and α -lipoic acid (LA) in

combined tablet dosage forms using AUC and absorbance correction method. ALLP is estimated by absorption correction method at 250nm whereas α -lipoic acid was determined by AUC area at 310nm to 390nm. The drugs seem to be linear at the concentration range of 10–50 $\mu\text{g mL}^{-1}$ with a mean recovery of 101.35% and 101.41% for ALLP and LA, respectively. The limit of detection (LOD) was 0.16 $\mu\text{g mL}^{-1}$ and 2.55 $\mu\text{g mL}^{-1}$ for ALLP and α -lipoic acid, respectively. The limit of quantification (LOQ) was 0.5 $\mu\text{g mL}^{-1}$ and 7.74 $\mu\text{g mL}^{-1}$ for ALLP and α -lipoic acid, respectively. Precision of the developed method was evaluated as per interday and intraday and was found to be <2% [17].

Refat et al. have established a spectrophotometric micro determination of ALLP via charge-transfer formation by using 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) and 3,6-dichloro-2,5-dihydroxy-p-benzoquinone (p-CLA) reagents as shown in Figure 3. The absorbance was measured at λ_{max} of 450 and 515nm for ALLP-DDQ and ALLP-p-CLA CT complexes, respectively, against reagent blanks.

[figures omitted; refer to PDF]

The drug obeyed Beer's limit over the concentration of 2.50–60.00 $\mu\text{g mL}^{-1}$ for DDQ and 5.00–50.00 $\mu\text{g mL}^{-1}$ for p-CLA method, respectively, with a mean recovery of 98.40–100.7% and 98.20–100.4% for ALLP-DDQ and ALLP-p-CLA CT complexes, respectively. Precision of the developed method was evaluated as per interday and intraday and was found to be <2% (0.12–0.94%). The LOD was 7.96 $\mu\text{g mL}^{-1}$ and 1.70 $\mu\text{g mL}^{-1}$ for ALLP-DDQ and ALLP-p-CLA CT complexes, respectively. LOQ was 26.53 $\mu\text{g mL}^{-1}$ and 5.68 $\mu\text{g mL}^{-1}$ for ALLP-DDQ and ALLP-p-CLA CT complexes, respectively [18].

Abdel-Hay et al. have derived a derivative spectrophotometry for the in vitro determination of ALLP and uric acid (UA) mixtures in urine. UA was determined by measuring the second derivative (2D) value at 293nm in 0.1N hydrochloric acid, while ALLP was determined by the first derivative (1D) value at 284nm in 0.1N sodium hydroxide. The drug obeyed Beer's limit over the concentration of 0.2–1.2 mg dL^{-1} for both drugs with a mean recovery of 100.14% and 100.23 for ALLP and uric acid, respectively. The interday precision was evaluated through replicate analysis of urine samples spiked with ALLP at different concentration levels, and the % RSD was 1.39, indicating the high precision of the method [19].

Shoukrallah et al. published a research article, stating the quantitative analysis of ALLP and flucytosine (FC), in commercial tablets using differential UV spectrophotometric method. Because the active ingredients (ALLP and FC) exhibit different UV spectra according to the pH of the solutions to be analyzed, it was favorable to use the differential spectroscopy. Both drugs showed linear relationships obeying Beer's law over a concentration range of 0.25–3.5 $\mu\text{g mL}^{-1}$. The mean recoveries were found to be 99.75 and 99.84% for ALLP and FC, respectively. The coefficients of variation worked out to 0.80 and 0.94% for AP and FC, respectively [20].

Mohamed et al. have established simple and accurate spectrophotometric method that does not require many steps of mathematical equations or software for processing of the recommended data for ALLP and Lesinurad (LSD) in recently approved FDA pharmaceutical preparation. LSD was analyzed by zero-order spectrophotometric method at 290nm, and ratio difference and ratio derivative spectrophotometric methods were applied for quantitative analysis of ALLP. For ratio difference method, the wavelengths 252 and 228nm were plotted against the corresponding ALLP concentrations, and ratio derivative spectrophotometric method was analyzed by first-order method using $\Delta\lambda=2$ and scaling factor=10 at 240nm. The drugs obeyed Beer–Lambert law over the concentration range 3–45 $\mu\text{g mL}^{-1}$ and 1–16 $\mu\text{g mL}^{-1}$ with a mean recovery of 100.27% and 99.56–99.68% for LSD and ALLP, respectively. The LOD was 0.201 $\mu\text{g mL}^{-1}$ and 0.203 $\mu\text{g mL}^{-1}$ for ALLP by ratio difference and ratio derivative method and 0.903 $\mu\text{g mL}^{-1}$ for LSD. The LOQ was 0.610 $\mu\text{g mL}^{-1}$ and 0.615 $\mu\text{g mL}^{-1}$ for ALLP by ratio difference and ratio derivative method and 2.736 $\mu\text{g mL}^{-1}$ for LSD. The repeatability and intermediate precision was evaluated through replicate analysis of ALLP at different concentration levels, and the % RSD was <2% (0.458–0.951%), indicating the high precision of the method [21].

Bedair et al. demonstrated a spectrofluorimetric method for determination of cimetidine (CM), thiabendazole (TB), carbimazole (CB), and ALLP on the basis of fluorescence quenching of mercurochrome (MER) in an aqueous alkaline medium and measuring the MER fluorescence (λ_{ex} =365nm and λ_{em} =535nm) before (F_0) and after (F)

the addition of quencher [22] using the corresponding buffer as blank and calculating $\Delta F(=F_0-F)$ and F_0/F ratio for the analysis. The mean recovery was found to be in the limit of 99.75–101.04% [23].

Attia et al. have established highly sensitive, selective, and accurate fluorescence spectroscopic methods for quantitative analysis of LSD and ALLP in pharmaceutical formulations and in human plasma. For LSD, λ_{ex} was established at 288nm and λ_{em} at 343nm, whereas ALLP was analyzed with λ_{ex} of 465nm and λ_{em} of 535nm. Calibration graphs were linear up to $0.25\text{--}4.0\text{ }\mu\text{g mL}^{-1}$ for LSD and $0.2\text{--}20\text{ }\mu\text{g mL}^{-1}$ for ALLP. The mean recovery for tablet formulation was found to be $99.55\pm 0.99\%$ and $100.18\pm 1.84\%$ for LSD and ALLP, respectively, and for spiked human plasma it was found to be $97.19\pm 0.85\%$ and $95.79\pm 1.82\%$ for LSD and ALLP, respectively. The LOD was $0.056\text{ }\mu\text{g mL}^{-1}$ for ALLP and $0.069\text{ }\mu\text{g mL}^{-1}$ for LSD, whereas LOQ was found to be $0.171\text{ }\mu\text{g mL}^{-1}$ and $0.210\text{ }\mu\text{g mL}^{-1}$ for ALLP and LSD, respectively. The repeatability and intermediate precision were evaluated through replicate analysis of ALLP at different concentration levels, and the % RSD was $<2\%$ ($0.573\text{--}1.051\%$), indicating the high precision of the method. The authors also established the robustness of the developed method by using constancy of the fluorescence intensity with small variations in the optimum conditions such as pH (± 0.2) and buffer volume ($\pm 0.2\text{ mL}$) of LSD and ALLP in addition to 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) volume ($\pm 0.2\text{ mL}$) and heating temperature ($\pm 10^\circ\text{C}$) for ALLP only. It was confirmed that there was no considerable effect on the fluorescence intensity of LSD and ALLP resulted from minor changes of procedure parameters [24]. All of the UV-visible and spectrofluorimetric methods of ALLP are summarized in Table 1.

Table 1

UV-visible and spectrofluorimetric methods for the determination of allopurinol.

S. no.	Drugs	Pharmaceutical matrix	Wavelength (nm)	Linearity ($\mu\text{g}\cdot\text{mL}^{-1}$)	Assay (%)	Accuracy of the study (%)	Ref.
<i>UV-visible spectrophotometric methods</i>							
1.	ALLP	Tablet	580	2–10	98.2–102.5	100	[16]
2.	ALLP and LA	Tablet	250 and 310–390	10–50	101.2 and 100.69	101.18–101.61 and 100.81–101.84	[17]
3.	ALLP	Tablet	450 and 515	10–70	101.1 and 101.4	98.40–100.7 and 98.20–100.4	[18]
4.	ALLP and UA	Urine	284 and 293	0.2–1.2mg/100ml	99.25 and 98.97	100.14 and 100.23	[19]
5.	ALLP and FC	Tablet	281 and 275	0.25–3.5	99.01–102.24 and 99.88–101.89	99.75 and 99.84	[20]

6.	ALLP and LSD	Tablet	252–228, 240, and 290	1–16 $\mu\text{g mL}^{-1}$ and 3–45 $\mu\text{g mL}^{-1}$	99.98–100.23 and 100.05	99.77–100.22 and 100.26	[2 1]
–							
<i>Spectrofluorimetry</i>							
7.	CM, TB, CB, and ALLP	Tablet	535/365	1–6 for CM and CB, 0.75–6 for TB, and 4–16 for ALLP	—	99.75–101.04	[2 3]
8.	LSD and ALLP	Tablet and human plasma	288/3434 65/535	0.25–4.0 $\mu\text{g mL}^{-1}$ for LSD and 0.2–20 $\mu\text{g mL}^{-1}$ for ALLP	100.41 and 99.28	99.55 and 100.18	[2 4]

3.2. High-Performance Liquid Chromatography Method

Several HPLC methods were reported for the analysis of ALLP in combination with other drugs. Dastiagiramma has developed HPLC method of ALLP and Lesinurad (LSD) in the API and marketed formulation. Separation of ALLP was accomplished on a Zorbax C18 column by using methanol: phosphate buffer as the mobile phase at a flow rate of 1.0 ml/min. Further, UV (ultraviolet) detection of drug was measured at 255 nm. The application of the developed method in marketed formulation showed mean recoveries in the range of 99%–100.1% while LOD and LOQ were found to be 0.07 $\mu\text{g mL}^{-1}$ and 0.2 $\mu\text{g mL}^{-1}$, respectively [25].

Khader et al. have established HPLC method for ALLP and LSD in API and pharmaceutical formulation. Separation was carried out on a Inertsil ODS by using 0.1% trifluoroacetic acid and methanol as the mobile phase at a flow rate of 1.0 mL min⁻¹. Further, UV (ultraviolet) detection of drug was measured at 255 nm. The application of the developed method in marketed formulation showed mean recoveries in the range of 100.48–100.96% for ALLP and 100.20–100.75% for LSD. LOD and LOQ were established by S/N method. LOD was found to be 3.03 and 2.98 and LOQ 10.02 and 9.98, respectively [26].

Reinders et al. have reported HPLC method for ALLP and oxypurinol (OXP) in human serum using reversed-phase LiChrospher 100 RP-18 column and 0.02 M sodium acetate as the mobile phase at a flow rate of 1.0 mL min⁻¹, an injection volume of 40 μL , and UV detection at 254 nm. The application of developed method was established on the serum of 66 patients which showed <0.5–4.3 mg L⁻¹ for ALLP and <1.0–39.2 mg L⁻¹ for OXP, respectively, for prescribed 300 mg/day dose of drug. LOD was found to be 0.1 mg L⁻¹ (4 ng) and 0.2 mg L⁻¹ (8 ng) for ALLP, and LLOQ was found to be 0.4 mg L⁻¹ (16 ng) for ALLP and 0.6 mg L⁻¹ (24 ng) for OXP [27].

Rajkumar et al. have established HPLC method for simultaneous quantitative estimation of ALLP and alpha-lipoic acid (LPA) in tablets, using Enable C18 G (250 × 4.6 mm; 5 μ) as a stationary phase (50:50 v/v) and acetonitrile: 0.02 M ammonium acetate buffer (pH adjusted to 4.6) as a mobile phase with flow rate of 0.8 mL min⁻¹, and the detection was done at 210 nm using a UV detector. Percentage mean recoveries were in the range of 98–102%, and LOD was found to be 3 ng mL⁻¹ for ALLP and 0.5 $\mu\text{g mL}^{-1}$ for LPA, while LOQ was obtained as 10 ng mL⁻¹ for ALLP and 1 $\mu\text{g mL}^{-1}$ for LPA [28].

Fadul has developed an UV and HPLC method for determination of ALLP in tablet dosage form using C8 column and 70% buffer solution (monobasic ammonium phosphate 0.05 M with 30% acetonitrile and methanol 1:1) as the mobile phase at a flow rate of 1.0 mL min⁻¹. The drug was analyzed at 250 nm. Analytical method development results indicated good regression value with assay value of 98.43%. The LOD and LOQ were found to be 0.051 $\mu\text{g mL}^{-1}$ and 0.156 $\mu\text{g mL}^{-1}$, respectively [29].

dos Santos et al. have developed stability-indicating assay method for the simultaneous determination of ALLP and ketoconazole (KTZ) in the pharmaceutical form of capsules, the combination of which was used for the treatment of

canine leishmaniasis. InertSustain® C18 column ($4.6 \times 100 \text{ mm} \times 3 \mu\text{m}$) was used as stationary phase and acetonitrile: water (52:48 v/v) with pH adjusted to 3.0 as a mobile phase, with a flow rate of 0.45 mL min^{-1} . ALLP was detected at 250 nm and KTZ at 225 nm. The LOD obtained was 0.0858 and $0.2599 \mu\text{g mL}^{-1}$ for ALLP and KTZ, respectively. The LOQ was 0.0331 and $0.1004 \mu\text{g mL}^{-1}$ for ALLP and KTZ, respectively. Additionally, robustness was indicated by the Plackett–Burman model, and the method was not significantly influenced by any of the variations [30].

Palmisano et al. have developed HPLC method with polarographic and voltammetric anodic detection: simultaneous determination of ALLP, OXP, and uric acid (UA) in body fluids by using a reversed-phase column (PerkinElmer RP-8, $10 \mu\text{m}$, $250 \times 4.6 \text{ mm}$) as a stationary phase and 0.025 M phosphate buffer pH 6.1 with 6–8% of methanol as a mobile phase, at a flow rate of 1.5 mL min^{-1} . The current-concentration plots of samples were straight-lined over the range 2–2000 ng using the oxidative mode, with lowest detectability of $0.2 \mu\text{g mL}^{-1}$. Patients taking 300 mg/day ALLP were analyzed by voltammetry and polarographic method. Glassy carbon wall jet detector operating at +1.2 V vs. SCE was used for voltammetry analysis and oxidative mode with applied potential +0.24 V vs. Ag/AgCl as reference electrode, and dropping mercury electrode was used for polarography analysis. Uric acid showed good resolution when the operating potential was dropped from +1.2 V to +0.7 V by voltammetry method [31].

Brown and Bye have developed HPLC method with ion exchange chromatographic detection of ALLP and OXP in biological samples like human plasma and urine. This method is based on high-performance ion-exchange chromatography with proficient sample purification using resin (Chelex-100) in the cuprous form. ALLP has good linear calibration curves in the range of 0.068 – $1.36 \mu\text{g mL}^{-1}$ in plasma and 0.68 – $136 \mu\text{g mL}^{-1}$ in urine, and OXP has 0.076 – $15.2 \mu\text{g mL}^{-1}$ and 15.2 – $304 \mu\text{g mL}^{-1}$ in plasma and urine, respectively. The analysis was carried out by using HPLC precolumn and columns with configuration of stainless steel tubes 6.35 mm ($1/4 \text{ in.}$) OD, 4.5 mm ID, 30 mm and 70 mm long, respectively, packed with Aminex A-27 (12 – $15 \mu\text{m}$) anion-exchange resin. The analysis was carried out at 254 nm with isocratically using ammonium acetate pH 8.7 at a flow rate of 1 mL min^{-1} . The use of ligand exchange resins shortens the retention time to 25–30 min by isocratic elution when compared to gradient elution [32].

Tada et al. have developed HPLC method with UV detection of ALLP and OXP in human serum in the presence of sulfanilamide as internal standard. Separation was achieved by using C18 (particle size $10 \mu\text{m}$, Waters Co., Milford, MA, USA) with RCM 8×10 (Waters compressing modulator) connected to a precolumn ($3.9 \times 20 \text{ mm i.d.}$, Waters guard column) and packed with Resolve C18 which was used for HPLC. The mobile phase was 2% (v/v) acetonitrile solution containing 100 mM potassium phosphate solution (pH 4.0) and 0.5 mM tetra-*n*-butylammonium hydrogen sulphate. The analysis was carried out at 260 nm, and the mobile phase was pumped at a flow rate of 2.0 mL min^{-1} . The recovery of this method was obtained (97.4–101% for ALLP and 93.2–98.1% for OXP, respectively). The % RSD of this method was <5.1% for ALLP and <5.6% for OXP intraday. The % RSD of interday was <6.6% for ALLP over the range of 0.5 – $5.0 \mu\text{g mL}^{-1}$ and <5.2% for OXP over the range of 0.4 – $20 \mu\text{g mL}^{-1}$. The LOQ was 6 ng for ALLP and 4.8 ng for OXP. To evaluate potential interference by compounds derived from xanthines, samples were spiked with uric acid, hypoxanthine, xanthine, theophylline, theobromine, 1,7-dimethylxanthine, and caffeine, and the result revealed that there were no interfering peaks at retention times corresponding to those of ALLP and OXP [33].

Putterman et al. have developed HPLC and GC method for simultaneous analysis of hypoxanthine (HPX), xanthine (XN), ALLP, OXP, and UA in standard mixtures and physiological fluids. For HPLC, separation was carried out isocratically with an Altex Model 310 (Altex Instruments, Berkeley, Calif.) high-pressure liquid chromatograph equipped with a dual wavelength (254 and 280 nm) detector with mobile phase of acetonitrile-buffer (1:1). For GC, separation was carried on SE-30 (4% on 100/120 mesh SUPELCOPORT) glass column, $0.2 \times 180 \text{ cm}$, with flow of nitrogen as carrier gas at 20 mL min^{-1} . The injection temperature was 200°C , and the flame ionization detector temperature was maintained at 300°C . HPLC requires no prior derivatization, uses isocratic elution with a buffer containing no organic solvent, and has 50- to 100-fold greater sensitivity than GC, whereas the interference effect was minimal in GC as some metabolite present in the urine sample interfered with HPLC. However, with isocratic elution employing a completely aqueous buffer at pH 4.5, the authors analyzed the compounds of interest without

interference from compounds such as pseudouridine which is known to be present at high levels in the urine of cancer patients. With two sensitive techniques, GC and HPLC, the identities of all five compounds of interest were highly possible. Prepurification for HPLC was done by deproteinization, whereas for GC it was done by using Sephadex G-10 equilibrated with 0.156M triethyl ammonium acetate, pH 5.0. The recoveries of the five compounds of interest were in the following ranges: UA, 89–108%; hypoxanthine, 95–110%; xanthine, 88–106%; OXP, 97–110%, and ALLP, 95–105%. The application of the developed method has been established by analyzing urine sample from cancer patients having ALLP by both methods. Excellent correlation was obtained for each of the compounds except ALLP in HPLC, but GC showed low but measurable levels of ALLP in these samples [34]. Boulieu et al. have developed an HPLC method for simultaneous analysis of HPX, XN, ALLP, OXP, and UA in physiological fluids. For HPLC, separation was carried out using ODS column (15cm×4.6mm ID) and a precolumn (5cm×4.6mm ID), used as a guard column. The mobile phase consisted of 0.02M potassium dihydrogen phosphate, the pH of which was adjusted to 3.65 with orthophosphoric acid. The flow rate was 1.5mL min⁻¹, and the detection was carried out at 254nm. Calibration curves were linear from 0.15 to 20mgL⁻¹ for ALLP and OXP and from 0.50 to 50μmolL⁻¹ for HPX and XN. LOD was found to be 1.5ng for ALLP and OXP, 2.5 pmol for HPX, and 5.0 pmol for XN. Precision in terms of reproducibility and accuracy were determined on spiked plasma. The interassay coefficient of variation for the analysis of ALLP and OXP over the concentration range 0.5–5mgL⁻¹ was found to be about 3% for both ALLP and OXP, for HPX and XN it was found to be 1.5%, and recovery was almost 100%. The authors observed that the HPX and XN levels in plasma and especially in urine from patients under ALLP therapy are higher than those obtained in their study of healthy subjects [35].

Hung et al. have developed HPLC method with UV detection of ALLP and OXP in human serum in the presence of sulfanilamide as internal standard. Separation was achieved by using 100mm×4.6mm ID and slurry packed with 5μm Hypersil ODS (Shandon, London, UK). The mobile phase was 20mM disodium hydrogen phosphate dihydrate and was adjusted to pH 2.0 with orthophosphoric acid. The analysis was carried out at 254nm, and the mobile phase was pumped at a flow rate of 2.0mL min⁻¹. Trichloroacetic acid and perchloric acid were found to be quite more effective in the removal of interfering substances than when either was used alone. The recovery of this method was obtained (97.4–101% for ALLP and 93.2–98.1% for OXP, respectively). The % RSD of this method was <8% for ALLP over the range of 0.05–5.0μg mL⁻¹. The LOQ for ALLP and OXP was 30ng mL⁻¹ [36]. All of the HPLC/UPLC methods of ALLP are summarized in Table 2.

Table 2

HPLC/UPLC methods for the determination of allopurinol.

S. no.	Drugs	Pharmaceutical or biological matrix	Stationary phase	Chromatographic conditions	Linearity (μg/ml)	Wavelength (nm)	Ref.
1	ALLP and LSD	Tablet	Zorbax C18 (4.6×150 mm×5μm), injection volume=10μL	Methanol: Phosphate buffer pH 3.9 (55:45v/v), flow rate=1.0 mL min ⁻¹	100–500μg mL ⁻¹ for ALLP and 1–5μg mL ⁻¹ for LSD	255	[25]
—							

2	ALLP and LSD	Tablet	Inertsil ODS (4.6 × 250mm, 5mm), injection volume=20 μL	Trifluoroacetic acid: methanol (40:60v/v), flow rate=1.0mL min^{-1}	30–150 $\mu\text{g mL}^{-1}$ for ALLP and 20–100 $\mu\text{g mL}^{-1}$ for LSD	255	[26]
–							
3	ALLP and OXP	Human plasma	Reversed-phase LiChrospher 100 RP-18 column (5m; 250 × 4mm; Merck, Darmstadt, Germany), flow rate=1.0mL min^{-1} , injection volume=40 μL	0.02M sodium acetate adjusted with acetic acid 30% to pH 4.5, flow rate=1.0mL min^{-1}	0.5–10 mg L^{-1} for ALLP and 1.0–40 mg L^{-1} for OXP	254	[27]
–							
4	ALLP and LPA	Tablet	Reversed-phase, Enable C18G (250X4.6X5), injection volume=20 μL	Acetonitrile: 0.02M ammonium acetate buffer adjusted to pH 4.6 in the proportion of 50:50 v/v, flow rate=0.8mL min^{-1}	50–175 $\mu\text{g mL}^{-1}$ for both drugs	210	[28]
–							
5	ALLP	Tablet	C8 (250 × 4.6mm, 5mm), injection volume=10 μL	70% buffer solution (monobasic ammonium phosphate 0.05M with 30% acetonitrile and methanol 1:1), flow rate=1 mL min^{-1}	10–50 $\mu\text{g mL}^{-1}$	250	[29]
–							
6	ALLP and KTZ	Capsule	InertSustain® C18 column (4.6 × 100mm) × 3 μm , injection volume=20 μL	Acetonitrile: water (52:48 v/v) with pH adjusted to 3.0, flow rate of 0.45mL min^{-1}	2.0 to 16.0 $\mu\text{g mL}^{-1}$ for ALLP and 1.0 to 15.0 $\mu\text{g mL}^{-1}$ for KTZ	ALLP at 250, KTZ at 225	[30]
–							
7	ALLP, OXP, and UA	Urine and serum	RP (PerkinElmer RP-8, 10 μm , 250 × 4.6mm), injection volume=20 μL	0.025M phosphate buffer pH 6.1 with 6–8% of methanol, flow rate of 1.5mL min^{-1}	2–2000ng in all matrices	+1.2V vs. SCE for voltammetry, +0.24 V vs. Ag/AgCl for polarography,	[31]

-							
8	ALLP and OXP	Human plasma and urine	Stainless steel tubes 6.35mm (1/4 in.) OD, 4.5mm ID, 30mm and 70mm long, packed with Aminex A-27 (12–15 μ m) anion-exchange resin	Ammonium acetate pH 8.7 at a flow rate of 1 mL min ⁻¹	For ALLP, 0.068–1.36 μ g mL ⁻¹ , 0.68–136 μ g mL ⁻¹ ; for OXP, 0.076–15.2 μ g mL ⁻¹ , 15.2–304 μ g mL ⁻¹ in plasma and urine, respectively	254	[32]
-							
9	ALLP and OXP	Human serum	C18 (particle size 10 μ m, Waters co., Milford, MA, USA)	2% (v/v) acetonitrile solution-100mm potassium phosphate (pH 4.0) and 0.5mm tetra-n-butylammonium hydrogen sulphate	For ALLP, 0.5–5.0 μ g mL ⁻¹ ; for OXP, 0.4–20 μ g mL ⁻¹	260	[33]
-							
10	HPX, XN, ALLP, OXP, and UA	Urine	HPLC: C18 Altex Model 310 (Altex Instruments, Berkeley, Calif.), GC: SE-30 (4% on 100/120 mesh SUPELCOPORT) glass column, 0.2 × 180cm	HPLC: acetonitrile-buffer (1:1), GC: nitrogen as carrier gas at 20 mL min ⁻¹	40 to 500 μ g mL ⁻¹ for all samples	254 and 280	[34]
-							
11	ALLP, OXP, HPX, XN	Urine and human plasma	ODS column (15cm × 4.6mm ID) and the precolumn (5cm × 4.6 mm ID)	0.02M potassium dihydrogen phosphate, pH adjusted to 3.65 with orthophosphoric acid	0.15 to 20 mg L ⁻¹ for ALLP and OXP; 0.50 to 50 μ mol L ⁻¹ for HPX and XN	254	[35]
-							
12	ALLP, OXP	Human plasma	100mm × 4.6mm ID and slurry packed with 5 μ m Hypersil ODS	20mM disodium hydrogen phosphate dehydrate, pH 2.0 with orthophosphoric acid	0.05–5.0 μ g mL ⁻¹ for both drugs	254	[36]

3.3. Gas Chromatography

Milleret al. have developed a gas chromatography method for the analysis of ALLP in phenanthrene tablet as an internal standard. ALLP was analyzed by derivatization technique, which involves silylation reaction consisting of

stationary phases of 3% OV-101 and 3% OV-17 on Chromosorb W/HP AW-DMCS (100-120 mesh). The temperature of column was maintained at 150°C for analysis of ALLP. Qualitative analysis was carried out in terms of retention indices on both columns with OV-101 and OV-17 stationary phases. All the substances yielded single symmetrical elution peaks on both columns, for which ALLP was chromatographed at 1612.6 for OV-101 and 1723.6 for OV-17, respectively. The amount of ALLP in a tablet was found to be 97.44 ± 0.83 mg [37]. No validation parameters were analyzed in this method.

3.4. HPTLC-Densitometry Method

Pandya et al. have developed highly sensitive high-performance thin layer chromatography (HPTLC) method for the simultaneous determination of ALLP and OXP in human plasma and ALLP in tablet dosage form. Separation was achieved on aluminium plates precoated with silica gel 60G F254, using methanol: chloroform: ammonia (2.0:7.9:0.1, v/v/v) as the developing solvent system. Densitometric measurements were established at 206 nm, and the results of retention factors (R_f) were 0.38 ± 0.01 and 0.65 ± 0.01 for ALLP and OXP, respectively. The regression plots were linear ($r^2 > 0.9993$) over the concentration range of 100–700 ng/band for both analytes. The LOD and LOQ of the method were 19.56 and 59.29 ng/band for ALLP and 19.01 and 57.59 ng/band for OXP, respectively, for plasma samples. For spiked plasma samples, protein precipitation with formic acid in acetonitrile afforded mean recovery of 84.67% and 86.21% for ALLP and OXP, respectively. The recovery of ALLP and OXP in tablet formulation was in the range of 99.68–101.72% [38].

3.5. Hyphenated Techniques

3.5.1. LC-MS

Kasawar et al. have developed highly sensitive LC-MS/MS method for determination of ALLP and OXP in human plasma using lamivudine as an internal standard. Chromatography separation was carried out in Waters Symmetry Shield RP₈, 150 mm × 3.9 mm, 5 μm columns using a mixture of 0.01% formic acid in water and acetonitrile in the ratio of 95:05 (v/v) as the mobile phase. Negative electrospray was used for detecting and quantifying the analyte by mass spectrometry. The drug was linear up to $0.01\text{--}10 \mu\text{g mL}^{-1}$ with a lower limit of quantification of $0.01 \mu\text{g mL}^{-1}$ for both ALLP and OXP. In this research, dilution integrity test, hemolysis and anticoagulant effect, and matrix effect studies were reported [39].

Rathod et al. reported a pharmacokinetic/bioequivalence study based on LC-MS/MS method for the simultaneous determination of ALLP and its active metabolite, OXP in human plasma. The analytes were separated on Hypersil Gold (150 mm × 4.6 mm, 5 μm) column using 0.1% formic acid-acetonitrile (98:2, v/v) as the mobile phase. Positive electrospray was used for the detection and quantification of the analyte by mass spectrometry. The proposed method obeyed good calibration curve in the range of 60.0 to 6000 ng mL⁻¹ for ALLP and 80.0–8000 ng mL⁻¹ for OXP. Moreover, the absence of matrix interference was confirmed by IS-normalized matrix factors and concentration of drugs from different plasma source [40].

Liu et al. also reported a pharmacokinetic/bioequivalence study based on LC-MS/MS method for the simultaneous determination of ALLP and its active metabolite, OXP in human plasma and urine, using 2,6-dichloropurine as the internal standard (IS). Simple and rapid liquid–liquid extraction was adopted, and ethyl acetate was used as the extraction agent. Ethyl acetate was used to extract analytes (0.5 ml aliquots of plasma or urine) and IS and separated on an Agilent Eclipse Plus C18 column using methanol and ammonium formate–formic acid buffer containing 5 mM ammonium formate and 0.1% formic acid (95:5, v/v) as the mobile phase (A) for ALLP or methanol: 5 mM ammonium formate aqueous solution (95:5, v/v) as the mobile phase (B) for OXP. ALLP was detected in positive ion mode, and the analysis time was about 7 min. The drug ALLP was linear from $0.05\text{ to }5 \text{ g mL}^{-1}$ in plasma and $0.5\text{--}30 \text{ g mL}^{-1}$ in urine. The lower limit of quantification (LLOQ) was 0.05 g mL^{-1} in plasma and 0.5 g mL^{-1} in urine. The intra- and interday precision and relative errors of quality control (QC) samples were $\leq 11.1\%$ for plasma and $\leq 8.7\%$ for urine. OXP was detected in negative mode with an analysis time of about 4 min. The calibration curve was linear from $0.05\text{ to }5 \text{ g mL}^{-1}$ in plasma (LLOQ, 0.05 g mL^{-1}) and from 1 to 50 g mL^{-1} in urine (LLOQ, 1 g mL^{-1}). The intra- and interday precision and relative errors were $\leq 7.0\%$ for plasma and $\leq 9.6\%$ for urine. This method was then successfully applied to investigate the pharmacokinetics of ALLP and OXP in humans [41].

3.5.2. GC-MS

Pechlivanis et al. have established GC-MS method to study the metabonomic investigation of the effects of physical exercise with ALLP administration in Wistar rats. Analysis was performed on an Agilent 7890A GC coupled to a 5975C Inert XL EI/CI MSD with Triple-Axis Detector MS and a CTCCH 4222 autosampler, using helium as a carrier gas at a flow of 3 mL min^{-1} . Methoxyamine hydrochloride (MeOX) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) are two techniques that the authors used for the derivatization of metabolite in this study. The researchers have studied the influence of allopurinol on the metabolic profile of blood plasma of rats that had undergone exhaustive swimming, and it was investigated by GC-MS. The programmed temperature vaporizing injector was ramped by $720^{\circ}\text{C min}^{-1}$ from 100 to 270°C , where it was kept for 1 min, and then from 270 to 350°C , where it was kept for 5 min. The GC oven was ramped by $5^{\circ}\text{C min}^{-1}$ from 70°C (2 min, initial time) to 200°C (1 min, stable) and from 200 to 320°C (5 min, stable). The total run time was 45 min per sample. Separation of the study groups as per the exercise was mainly due to lactic acid, pyruvic acid, 2-hydroxybutyric acid, uracil, oxalic acid, pyroglutamic acid, and stearic acid $p < 0.05$. Lactic and pyruvic acids, indicating increased carbohydrate breakdown, and also 2-hydroxybutyric and pyroglutamic acids, stating increased glutathione synthesis in response to oxidative stress, were among the major differentiators of exercise from the resting state. Inosine, hypoxanthine, xanthine, xanthosine, and uric acid, indicating xanthine oxidase inhibition, as well as methionine, proline, and leucine, indicating increased protein synthesis, were among the major differentiators of ALLP administration from placebo. Results reveal that, despite having effects on metabolism and the redox status, ALLP does not seem to modulate the metabolic responses to exercise. Electron ionization source $50\text{--}800\text{ m/z}$ was used for the detection and quantification of the analyte by mass spectrometry [42].

3.5.3. UPLC-MS/MS

Iqbal et al. have developed ultraperformance hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry for simultaneous determination of ALLP, OXP, and LSD in rat plasma. Liquid-liquid extraction using ethyl acetate as extracting agent was used for sample extraction procedure. Acquity UPLC HILIC column ($100\text{ mm} \times 2.1, 1.7\text{ }\mu\text{m}$) was used as stationary phase in the presence of internal standard (5-fluorouracil). The mobile phase, consisting of acetonitrile, water, and formic acid (95:5:0.1, v/v/v), was eluted at 0.3 mL min^{-1} flow rate having total chromatographic run time of 3 min per sample. The analytes were detected on Acquity Triple Quadrupole Mass Spectrometer equipped with Z-Spray Electrospray Ionization (ESI). The calibration curve was found to be linear between 22 and 8000 ng mL^{-1} for ALLP, $33\text{--}12000\text{ ng mL}^{-1}$ for OXP, and $25\text{--}9000\text{ ng mL}^{-1}$ for LSD, respectively. The intra and interday precision (% RSD) for ALLP, OXP, and LES were found to be ≤ 10.54 , ≤ 13.98 , and $\leq 14.84\%$, respectively, whereas the intra and interday accuracy were within the range of $90.40\text{--}111.21\%$, $95.16\text{--}111.13\%$, and $91.92\text{--}108.30\%$, respectively. The mean absolute recovery of ALLP, OXP, and LES was found to be 79.42 , 66.89 , and 55.95% , respectively [43].

3.6. Miscellaneous Methods

3.6.1. Electrophoresis

Pérez-Ruiz et al. have developed capillary zone electrophoresis method with UV absorbance detection for ALLP and its metabolite OXP, using 15 mM buffer adjusted at pH 8.8 as electrophoretic electrolyte equipped with P/ACE 5000 coupled to a diode array detector as electrophoresis instrument. The voltage and temperature during the analysis were 15 kV and 30°C , respectively. In this method, the researchers have optimized the electrophoresis conditions for ALLP and OXP, and they have also studied the effects of pH, type of buffer and its concentration, voltage effect on mobility, resolution, sensitivity, and speed. They found that the electrophoretic mobility of ALLP and OXP decreased continuously with the increase in the pH of buffer. The LOD obtained was $0.08\text{ }\mu\text{g mL}^{-1}$ and $0.12\text{ }\mu\text{g mL}^{-1}$ for ALLP and OXP, respectively. The LOQ was 0.58 and $0.67\text{ }\mu\text{g mL}^{-1}$ for ALLP and OXP, respectively. The drug was linear over the concentration of $0.68\text{--}96\text{ }\mu\text{g mL}^{-1}$ for ALLP and $0.77\text{--}154\text{ }\mu\text{g mL}^{-1}$ for OXP. The usefulness of this method is demonstrated by the excellent results obtained in the determination of ALLP and OXP in human serum and ALLP in different pharmaceutical formulations [44].

Sun et al. have established another electrophoresis method for end-column amperometric detection based on the

responses of ALLP and OXP at carbon fiber electrode. The optimal conditions for the separation include buffer composed of 15mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ at a pH 9.55, electrokinetic injection 7s at 5kV, separation voltage at 15kV, and detection potential at 1.20V. Effects of pH, separation voltage, buffer concentration, injection voltage, and time were analyzed. Validation of analytical parameters was as per ICH guidelines. LOD was found to be $1 \times 10^{-8} \text{ mol L}^{-1}$ for ALLP and OXP, and drug was found to be linear over the concentration of 2×10^{-7} to $1 \times 10^{-4} \text{ mol L}^{-1}$ and 1×10^{-7} to 1×10^{-4} for ALLP and OXP, respectively. The applicability of the developed method was established by spiking diluted urine sample ($V_{\text{urine}}:V_{\text{buffer}} = 1:8$) with ALLP and OXP, which produced favorable result [45].

Eman has developed electrophoresis method for the separation and determination of LSD and ALLP in their bulk, combined dosage form and in the presence of their degradation products under different stress conditions. Additionally, this is the first article to study the forced degradation and degradation kinetics to investigate the stability and half-life of the mixture at room temperature. Separation was carried out by using fused silica capillary (55cm $\times$ 50 μm id) using 50mM borate buffer adjusted to pH 10 with 0.5M NaOH. The drugs were found to be linear over the concentration of 5–50 and 10–100 $\mu\text{g mL}^{-1}$ for LSD and ALLP, respectively. Force degradation study of drugs showed that LSD was more liable for acidic and alkaline degradation and that ALLP degraded more by oxidation. The applicability of the developed method was established on tablet formulation which showed good result [46].

3.6.2. Polarographic Method

Tommaso and Cataldi have developed anodic polarographic detection for ALLP at a mercury electrode using 0.05M borate buffer. The flow injection-anodic polarographic detection method provided an accurate and sensitive drug determination with a detection limit of 1.8 μM and a relative standard deviation of 3.1% at the 28 μM level. The applicability of the developed method was established in commercially available ALLP tablets, and the recovery was found to be 99.6% with an RSD of 1.1% [47].

3.6.3. Electrochemical Sensor Method

Ladmakhi et al. have developed a sensitive, selective, and precise electrochemical sensor based on Fe_3O_4 @GO/OMC hybrid film on a carbon paste electrode for the determination of ALLP. The results showed sphere shape Fe_3O_4 nanoparticles with a diameter in the range 17–22nm on composite. Modification of carbon paste electrode (CPE) with Fe_3O_4 @GO/OMC (Fe_3O_4 @GO/OMC-CPE) allowed the ultrasensitive and selective detection of ALO at oxidation potential of 1.05V with linear range of 0.05–7 $\mu\text{mol L}^{-1}$, limit of detection of 47 nmol L^{-1} , and sensitivity of 708 $\mu\text{Ammol}^{-1}\text{L}$. The proposed sensor offers a simple and fast way for ALLP sensing in clinical samples within short analysis time, making the concept of interest. 500 μL of human serum plasma or 1 mg of tablet was taken for analysis. The determinations of samples were also carried out by UV-visible spectroscopy, and characterizations were done by Raman and X-ray diffraction. Raman analysis of GO and Fe_3O_4 @GO revealed the introduction of defects in the graphene framework after decoration of GO by Fe_3O_4 nanoparticles. The D and the G bands of GO appeared at 1323 and 1577 cm^{-1} with the intensity ratio of D and G bands (ID/IG) of 1.14. X-ray diffractogram revealed that the diffraction peaks at 32.1°, 35.8°, 37.4°, 43.8°, 52.4°, 57.3°, and 63.5 of Fe_3O_4 @GO composite are in good agreement with the face-centered cubic spinel structure of Fe_3O_4 nanoparticles [48].

3.6.4. Colorimetric Paper-Based Analytical Method

Pratiwi et al. have established a novel design and optimization of colorimetric paper-based analytical device for rapid detection of ALLP in herbal medicine. In this work, nine colorimetric reagents were screened to find the best colorimetric reagent for ALLP detection. These nine colorimetric reagents were chosen based on the reaction between the functional group on ALLP and a general reagent that was used as a colorimetric reagent. They are Dragendorff reagent, ferric chloride, Folin–Ciocalteu reagent, sodium nitroprusside, p-DAB reagent, Schiff reagent, potassium chlorate, Tollens reagent, and sodium nitrite. To calculate the levels of ALLP in herbal sample, standard curve of ALLP with concentrations of 6–16 $\mu\text{g mL}^{-1}$ was made and measured at 252nm wavelength, and the concentration of ALLP in sample was determined (7.44 $\mu\text{g mL}^{-1}$). The developed paper-based analytical device was successful in detecting ALLP in herbal medicine sample which also agrees with TLC and spectrophotometry data [49].

3.6.5. Near-Infrared Spectroscopy

Smetisko and Miljanic have developed a NIR spectroscopic method for assessment of drug dissolution from ALLP immediate release tablets. Thirty-three different batches of ALLP immediate release tablets containing constant amount of the active ingredient, but varying in excipient content and physical properties, were introduced in a PLS calibration model. The dissolution values were measured by UV-Vis method, and the data extracted from the NIR spectra, values of correlation coefficient, bias, slope, residual prediction determination, and root mean square error of prediction (0.9632, 0.328%, 1.001, 3.58, 3.75%) were evaluated. The obtained values revealed that the NIR diffuse reflectance spectroscopy can serve as a faster and simpler substitute to the conventional dissolution procedure, even for the tablets with a very fast dissolution rate (>85% in 15 minutes). The spectral region from 7158.9cm^{-1} to 5484.9cm^{-1} was chosen for the final calibration model, and the broad bands at 6872, 6493, and 6265cm^{-1} resulted from lactose monohydrate. The distinctive ALLP bands were observed at 6092 and 6060cm^{-1} and were allocated to the first overtone of the =CH stretching. Despite the large differences in the samples included in the calibration model, the developed method is not complicated and shows acceptable accuracy [50].

4. Conclusion

The present review provides a summary of various analytical methods reported in the literature for the determination of ALLP in bulk, pharmaceutical formulations and also in various biological matrices like blood plasma and urine. Analytical methods consisting of chromatography, spectroscopy, hyphenated techniques, and electrochemical methods were employed for determination of ALLP in bulk, pharmaceutical dosage forms and biological matrix. The primary objective of the compilation of review is to collect maximum information available on analytical methods of ALLP and study it in detail. From this survey, it is revealed that a handful of analytical methods are obtainable on HPLC and UV-visible spectrophotometry and very few articles are available based on hyphenated methods and electrochemical methods.

The reported data for analysis of ALLP revealed that HPLC with UV detection is the most frequent technique employed for the determination of ALLP in pharmaceutical matrix. For analysis of ALLP in biological matrices like blood plasma, urine HPLC with UV detection is appropriate since this strategy gives precise outcomes and minimal effort. Furthermore, employing MS techniques in LC offered unique selectivity and sensitivity as well as a choice of method for analysis of ALLP and its metabolites in biological samples. Hyphenated techniques such as GC-MS, LC-MS, LC-MS/MS, and UPLC-MS/MS methods are also reported for quantification of ALLP in plasma and other biological fluids.

Acknowledgments

The authors are thankful to Shree Medical and Technical College, Bharatpur, and School of Health and Allied Sciences, Pokhara University, for providing the necessary facilities to complete this review article.

References

- [1] C. L. Benn, P. Dua, R. Gurrell, "Physiology of hyperuricemia and urate-lowering treatments," *Frontiers in Medicine*, vol. 5, DOI: 10.3389/fmed.2018.00160, 2018.
- [2] T. Bardin, P. Richette, "Definition of hyperuricemia and gouty conditions," *Current Opinion in Rheumatology*, vol. 26 no. 2, pp. 186-191, DOI: 10.1097/bor.000000000000028, 2014.
- [3] F. Pea, "Pharmacology of drugs for hyperuricemia," *Hyperuricemic Syndromes: Pathophysiology and Therapy*, vol. 147, pp. 35-46, 2005.
- [4] S. A. Benezra, T. R. Bennett, "Allopurinol," *Analytical Profiles of Drug Substances*, DOI: 10.1016/s0099-5428(08)60087-8, 1978.
- [5] J. S. Changdeo, M. Vinod, K. B. Shankar, C. A. Rajaram, "Physicochemical characterization and solubility enhancement studies of allopurinol solid dispersions," *Brazilian Journal of Pharmaceutical Sciences*, vol. 47 no. 3, pp. 513-523, DOI: 10.1590/s1984-82502011000300009, 2011.
- [6] R. W. Rundles, "The development of allopurinol," *Archives of Internal Medicine*, vol. 145 no. 8, pp. 1492-1503, DOI: 10.1001/archinte.145.8.1492, 1985.
- [7] G. Elion, "The purine path to chemotherapy," *Science*, vol. 244 no. 4900, pp. 41-47, DOI: 10.1126/science.2649979, 1989.

- [8] J. R. Klinenberg, S. E. Goldfinger, J. E. Seegmiller, "The effectiveness of the xanthine oxidase inhibitor allopurinol in the treatment of gout," *Annals of Internal Medicine*, vol. 62 no. 4, pp. 639-647, DOI: 10.7326/0003-4819-62-4-639, 1965.
- [9] P. R. Band, D. S. Silverberg, J. F. Henderson, "Xanthine nephropathy in a patient with lymphosarcoma treated with allopurinol," *New England Journal of Medicine*, vol. 283 no. 7, pp. 354-357, DOI: 10.1056/nejm197008132830708, 1970.
- [10] S. J. Appelbaum, M. Mayersohn, R. T. Dorr, D. Perrier, "Allopurinol kinetics and bioavailability," *Cancer Chemotherapy and Pharmacology*, vol. 8 no. 1, pp. 93-98, DOI: 10.1007/bf00292878, 1982.
- [11] H. Breithaupt, M. Tittel, "Kinetics of allopurinol after single intravenous and oral doses," *European Journal of Clinical Pharmacology*, vol. 22 no. 1, pp. 77-84, DOI: 10.1007/bf00606429, 1982.
- [12] H. Jaeger, "Comparative study of the bioavailability and the pharmacodynamic effect of five allopurinol preparations (author's transl)," *Arzneimittel-Forschung*, vol. 32 no. 4, 1982.
- [13] Government of India, "Ministry of health and family welfare, I.P.," *Assay of Allopurinol*, 2007.
- [14] B. Pharmacopoeia, HM Stationery Office, 2007.
- [15] The United States Pharmacopeia and National Formulary, "The Official Compendia of Standards," USP 30-NF 25 The United States, 2007.
- [16] W. S. Khayoon, "Spectrophotometric determination of Allopurinol in tablet formulation," *Journal of Physical Science*, vol. 19 no. 2, pp. 23-30, 2008.
- [17] D. J. Patel, V. C. Jain, H. A. Raj, "Development and validation of spectroscopic absorbance correction method for simultaneous estimation of Allopurinol and α -lipoic acid in combination tablet," *International Journal of Pharmaceutical Sciences*, vol. 5 no. 2, pp. 129-140, 2014.
- [18] M. S. Refat, G. G. Mohamed, A. Fathi, "Spectrophotometric determination of allopurinol drug in tablets: spectroscopic characterization of the solid CT complexes," *Bulletin of the Korean Chemical Society*, vol. 31 no. 6, pp. 1535-1542, DOI: 10.5012/bkcs.2010.31.6.1535, 2010.
- [19] M. H. Abdel-Hay, M. H. Barary, M. A. Elsayed, E. M. Hassan, "Use of derivative spectrophotometry for the in vitro determination of allopurinol and uric acid mixtures," *Analytical Letters*, vol. 24 no. 9, pp. 1517-1530, DOI: 10.1080/00032719108052990, 1991.
- [20] I. Shoukrallah, A. Sakla, R. Wintersteiger, "Determination of allopurinol and flucytosine in tablets by differential UV spectroscopy," *Analytical Letters*, vol. 23 no. 5, pp. 843-861, DOI: 10.1080/00032719008052486, 1990.
- [21] A. A. Mohamed, A. El-Olemy, S. Ramzy, A. H. Abdelazim, M. K. M. Omar, M. Shahin, "Spectrophotometric determination of lesinurad and allopurinol in recently approved FDA pharmaceutical preparation," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 247, DOI: 10.1016/j.saa.2020.119106, 2021.
- [22] C. Gnecco, S. J. Carlan, J. McWhorter, L. Ge, D. Sanchez, M. Madruga, "Burkitt's lymphoma with placental invasion diagnosed at cesarean delivery: a case report," *Journal of Medical Case Reports*, vol. 12, DOI: 10.1186/s13256-017-1548-0, 2018.
- [23] M. M. Bedair, M. A. Korany, M. A. Elsayed, O. T. Fahmy, "Spectrofluorimetric determination of three pharmaceutical thiocompounds and allopurinol using mercurochrome," *Spectroscopy Letters*, vol. 23 no. 2, pp. 161-173, DOI: 10.1080/00387019008054044, 1990.
- [24] K. A. M. Attia, A. El-Olemy, S. Ramzy, "Application of different spectrofluorimetric methods for determination of lesinurad and allopurinol in pharmaceutical preparation and human plasma," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 244, DOI: 10.1016/j.saa.2020.118871, 2021.
- [25] D. Dastiagiramma, "Simultaneous estimation of lesinurad and allopurinol by using reverse phase high performance liquid chromatography in api and marketed formulation," *International Journal of Medical & Pharmaceutical Sciences*, vol. 3 no. 7, 2018.
- [26] S. Khader, A. K. Begum, D. Ramakrishna, "Development and validation of reverse phase HPLC method for simultaneous estimation of allopurinol and lesinurad in its API and pharmaceutical dosage form," *International Journal of Applied Pharmaceutical Sciences and Research*, vol. 4 no. 4, pp. 50-57, DOI: 10.21477/ijapsr.4.4.1, 2019.

- [27] M. K. Reinders, L. C. Nijdam, E. N. van Roon, "A simple method for quantification of allopurinol and oxipurinol in human serum by high-performance liquid chromatography with UV-detection," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 45 no. 2, pp. 312-317, DOI: 10.1016/j.jpba.2007.08.002, 2007.
- [28] B. Rajkumar, T. Bhavya, A. Ashok Kumar, "Reverse phase HPLC method development and validation for the simultaneous quantitative estimation of alpha lipoic acid and allopurinol in tablets," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 6, pp. 307-312, 2014.
- [29] M. A. S. Fadul, *Development and Validation of SOME Analytical Methods for Quantitative Determination of Allopurinol Drug*, 2016.
- [30] P. E. dos Santos, A. C. Lucca, M. Serrou do Amaral, N. Mohamad Kassab, "Development and validation of a stability-indicating analytical method for simultaneous determination of drugs employed in canine leishmaniasis treatment," *Drug Analytical Research*, vol. 4 no. 2, pp. 24-30, DOI: 10.22456/2527-2616.102740, 2020.
- [31] F. Palmisano, E. Desimoni, P. O. Zamboni, "High-performance liquid chromatography with polarographic and voltammetric anodic detection: simultaneous determination of allopurinol, oxipurinol and uric acid in body fluids," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 306, pp. 205-214, DOI: 10.1016/s0378-4347(00)80883-x, 1984.
- [32] M. Brown, A. Bye, "The determination of allopurinol and oxipurinol in human plasma and urine," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 143 no. 2, pp. 195-202, DOI: 10.1016/s0378-4347(00)81824-1, 1977.
- [33] H. Tada, A. Fujisaki, K. Itoh, T. Suzuki, "Facile and rapid high-performance liquid chromatography method for simultaneous determination of allopurinol and oxypurinol in human serum," *Journal of Clinical Pharmacy and Therapeutics*, vol. 28 no. 3, pp. 229-234, DOI: 10.1046/j.1365-2710.2003.00488.x, 2003.
- [34] G. J. Putterman, B. Shaikh, M. R. Hallmark, C. G. Sawyer, C. V. Hixson, F. Perini, "Simultaneous analysis of substrates, products, and inhibitors of xanthine oxidase by high-pressure liquid chromatography and gas chromatography," *Analytical Biochemistry*, vol. 98 no. 1, pp. 18-26, DOI: 10.1016/0003-2697(79)90700-0, 1979.
- [35] R. Boulieu, C. Bory, P. Baltassat, C. Gonnet, "Simultaneous determination of allopurinol, oxipurinol, hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 307, pp. 469-474, DOI: 10.1016/s0378-4347(00)84124-9, 1984.
- [36] C. T. Hung, A. R. Zoest, D. G. Perrier, "Analysis of allopurinol and oxipurinol in plasma by reversed phase HPLC," *Journal of Liquid Chromatography*, vol. 9 no. 11, pp. 2471-2483, DOI: 10.1080/01483918608076879, 1986.
- [37] V. Miller, V. Pacáková, E. Smolková, "Gas chromatographic analysis of pharmaceuticals based on pyrimidine and purine substances," *Journal of Chromatography A*, vol. 123 no. 1, pp. 216-219, DOI: 10.1016/s0021-9673(00)81118-8, 1976.
- [38] J. J. Pandya, M. Sanyal, P. A. Shah, P. S. Shrivastav, "HPTLC-densitometric determination of allopurinol and its metabolite oxypurinol in human plasma and allopurinol in tablet dosage form," *Analytical Chemistry Letters*, vol. 8 no. 4, pp. 537-551, DOI: 10.1080/22297928.2018.1445556, 2018.
- [39] G. Kasawar, M. Razzak, Z. Zaheer, M. Farooqui, "Validated RP-LC-MS/MS method for the simultaneous determination of allopurinol and its major metabolite, oxypurinol, in human plasma," *Journal of Liquid Chromatography & Related Technologies*, vol. 34 no. 1, pp. 26-37, DOI: 10.1080/10826076.2011.534692, 2010.
- [40] D. M. Rathod, K. R. Patel, H. N. Mistri, A. G. Jangid, P. S. Shrivastav, M. Sanyal, "Simultaneous analysis of allopurinol and oxypurinol using a validated liquid chromatography-tandem mass spectrometry method in human plasma," *Journal of Pharmaceutical Analysis*, vol. 7 no. 1, pp. 56-62, DOI: 10.1016/j.jpba.2016.05.005, 2017.
- [41] X. Liu, X.-J. Ni, D.-W. Shang, "Determination of allopurinol and oxypurinol in human plasma and urine by liquid chromatography-tandem mass spectrometry," *Journal of Chromatography B*, vol. 941, pp. 10-16, DOI: 10.1016/j.jchromb.2013.09.028, 2013.
- [42] A. Pechlivanis, A. C. Chatziioannou, A. S. Veskoukis, D. Kouretas, V. Mougios, G. A. Theodoridis, "GC-MS

analysis of blood for the metabonomic investigation of the effects of physical exercise and allopurinol administration on rats," Journal of Chromatography B, vol. 966, pp. 127-131, DOI: 10.1016/j.jchromb.2014.02.005, 2014.

[43] M. Iqbal, E Ezzeldin, R. N Herqash, O Alam, "Ultra-performance hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry for simultaneous determination of allopurinol, oxypurinol and lesinurad in rat plasma: application to pharmacokinetic study in rats," PLoS One, vol. 14 no. 3, DOI: 10.1371/journal.pone.0213786, 2019.

[44] T. Pérez-Ruiz, C. Martínez-Lozano, V. Tomás, R. Galera, "Development of a capillary electrophoresis method for the determination of allopurinol and its active metabolite oxypurinol," Journal of Chromatography B, vol. 798 no. 2, pp. 303-308, DOI: 10.1016/j.jchromb.2003.10.002, 2003.

[45] X. Sun, W. Cao, X. Bai, X. Yang, E. Wang, "Determination of allopurinol and its active metabolite oxypurinol by capillary electrophoresis with end-column amperometric detection," Analytica Chimica Acta, vol. 442 no. 1, pp. 121-128, DOI: 10.1016/s0003-2670(01)01135-7, 2001.

[46] I. Eman, "Green stability-indicating capillary electrophoretic method for simultaneous determination of Lesinurad and Allopurinol in tablet dosage form: degradation kinetics investigation," Microchemical Journal, vol. 158, 2020.

[47] R. I. Tommaso, F. P. a.P. G. Z. Cataldi, "Flow injection with anodic polarographic detection for the determination of allopurinol in pharmaceutical formulations," Analyst, vol. 114 no. 11, pp. 1449-1452, 1989.

[48] H. B. Ladmakhi, F. Chekin, S. Fathi, J. B. Raoof, "Electrochemical sensor based on magnetite graphene oxide/ordered mesoporous carbon hybrid to detection of allopurinol in clinical samples," Talanta, vol. 211, DOI: 10.1016/j.talanta.2020.120759, 2020.

[49] R. Pratiwi, R. N. Septyani, R. Febriany, F. A. Saputri, R. F. Nuwarda, "Design and optimization of colorimetric paper-based analytical device for rapid detection of allopurinol in herbal medicine," International Journal of Analytical Chemistry, vol. 2019, DOI: 10.1155/2019/4682839, 2019.

[50] J. Smetisko, S. Miljanic, "Dissolution assessment of allopurinol immediate release tablets by near infrared spectroscopy," Journal of Pharmaceutical and Biomedical Analysis, vol. 145, pp. 322-330, 2017.

DETAILS

Subject:	Mass spectrometry; Plasma; Mathematical analysis; Reagents; Lysergic acid diethylamide--LSD; Pharmaceuticals; Uric acid; Drugs; Urine; Quantitative analysis; Biological properties; Electrophoresis; Scientific imaging; Methods; Chromatography; Caustic soda; Polarography; Infrared spectroscopy
Editor:	Richard G Brereton
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York

Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-01-24 (Received); 2021-04-19 (Revised); 2021-05-31 (Accepted); 2021-06-07 (Pub)
DOI:	https://doi.org/10.1155/2021/5558651
ProQuest document ID:	2543214341
Document URL:	https://www.proquest.com/scholarly-journals/brief-review-analytical-methods-estimation/docview/2543214341/se-2?accountid=211160
Copyright:	Copyright © 2021 Hemraj Sharma et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-06-20
Database:	Publicly Available Content Database

Document 34 of 59

Extraction of Pregabalin in Urine Samples Using a Sulfonated Poly(ether ether ketone) Membrane

Basheer, Chanbasha ¹

¹ Department of Chemistry, King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia; Membranes and Water Security, Interdisciplinary Research Center, KFUPM, Dhahran 31261, Saudi Arabia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

In this work, a simple polymer-assisted microextraction technique was developed to determine pregabalin (an anticonvulsant drug) in the urine sample. A sulfonated poly(ether ether ketone) membrane was used as a sorbent for pregabalin extraction, and the extraction performance was compared with that of the conventional polydimethylsiloxane membrane. The extraction device is free moving and tumbles continuously throughout the stirred sample solution during extraction to enhance the extraction efficiency. The electrostatic interactions between the sulfonic-acid-functionalized polymeric membrane and the amine group in the pregabalin molecule facilitate higher preconcentration factor at a shorter extraction time. Optimizing conditions of the extraction method were investigated to obtain higher extraction efficiency. The developed method exhibited good linearity in the range of 0.05 to 2 µg/mL with a correlation of determination (r^2) 0.9998, acceptable limits of detection, limits of quantification, and preconcentration factor of 105-fold. The within-day and between-day precisions of pregabalin were lower than 7% relative standard deviations. Pregabalin was extracted from urine samples with recoveries of >92%, and no significant matrix effects were observed.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Pregabalin is used as adjunctive therapy for partial seizures with or without secondary generalization in adults. Pregabalin (S-3-(aminomethyl)-5-methyl hexanoic acid) is a structural derivative of the inhibitory neurotransmitter aminobutyric acid. In recent research, pregabalin has been confirmed for adjunctive treatment of partial seizures to treat neuropathic pain from postherpetic neuralgia and diabetic neuropathy in adults in both the United States and Europe [1, 2]. The chemical structure of pregabalin is shown in Figure 1(a). Pregabalin is available under the trade name Lyrica (Pfizer, New York, NY) for use for treating epilepsy, diabetic neuropathy pain, postherpetic neuralgia [3], effective at fibromyalgia [4], and spinal cord injury [5].

[figures omitted; refer to PDF]

The precise mode of action of pregabalin has not been fully elucidated. However, it does interact with the same binding site and has a similar pharmacologic profile as gabapentine (1-[aminomethyl] cyclohexane acetic acid) [6, 7]. Pregabalin is minimally metabolized and primarily excreted in urine as unchanged drugs, and studies in healthy volunteers indicate oral bioavailability to be approximately 90% [8]. It is available in 25mg, 50mg, 75mg, 150mg, and 300mg capsules, and this variation allows it to be easier to prescribe when the medication is being introduced. In clinic, dizziness and somnolence are the most frequently reported adverse events, with dizziness experienced by 29% of pregabalin-treated patients compared with 9% with placebo and somnolence experienced by 22% of pregabalin-treated patients. Decreased concentration, increased appetite and weight gain, dry mouth, and vomiting are other side effects [9].

On top of that, increasing slow-wave sleep in healthy volunteers is another side effect correlated with the sleep's restorative aspect.

A relatively uncomplicated and more cost-effective method such as spectrophotometry or spectrofluorometry is the basic requirement for routine analysis of the drug in bulk powder and pharmaceutical preparations, particularly in research laboratories and the pharmaceutical industry. Literature review revealed that conventional direct analysis or liquid-liquid extraction methods were used to analyze pregabalin in tablets or biological matrices. After extraction, analyses were performed by gas chromatography-mass spectrophotometry (GC-MS), high-performance liquid chromatography (HPLC)-MS-MS [10, 11], HPLC [11–16], and fluorometry [17] without any derivatization.

However, to improve the direct analysis's sensitivity, various derivatization reagents were used; for example, cyclodextrins were added before capillary electrophoresis and nuclear magnetic resonance analysis [18]. Other chromogenic reagents such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 7,7,8,8-tetracyanoquinodimethane (TCNQ) or 2,4-dinitrofluorobenzene and 2,3,5,6-tetrachloro-1,4-benzoquinone and

ninhydrin were used for UV/visible spectrophotometry or spectrofluorometry analysis [2, 19–21].

The derivatization method requires additional tedious derivatization steps, which further dilute the analytes' concentrations [11, 22] and are challenging for trace-level quantitation. Unfortunately, the excess derivatization reagents often result in resolution and detection problems such as difficulty to separate a trace target derivative in the presence of a significant excess of an unreacted reagent. The excessive reagent might interfere with the chromatographic separation process [23].

It is well known that, in sorptive processes, higher extraction efficiencies are obtained when the analytes are in their nonionic form. Various sorptive extraction techniques using polydimethylsiloxane (PDMS) have been reported for the extraction drug samples in urine samples which include solid-phase microextraction [24] and stirrer bar sorptive microextraction (SBSE) [25, 26]. Recently, functionalized polymers were used to extract a wide range of organic compounds with high selectivity and enrichment [27]. The selective extraction mechanism of analytes was explained by various interactions, which includes chelation [28, 29], chiral recognition [30–32], ion-exchange through electrostatic interactions [33–35], creation of recognition sites for target analytes using molecularly imprinted polymers [36–38], and immunoaffinity through antibody-antigen interactions [39–42].

In this study, for the first time, we have developed polymer-assisted microextraction (PME) using a sulfonated poly(ether ether ketone) (SPEEK) functionalized polymer membrane as an adsorbent. After extraction, the extract was injected into HPLC (without any derivatization). The developed method required only small amounts of sample and solvent.

2. Materials

2.1. Chemicals

Sulfonated poly(ether ether ketone) (Fumatech, ion-exchange capacity=1.6 mmole/g) and sodium hydroxide were purchased from Fluka (AG, Switzerland). Pregabalin (99.5%) was obtained from Symed Laboratories (AP, India). HPLC-grade organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The polydimethylsiloxane (PDMS) polymer comprising the Slygard 184 silicone elastomer and a curing agent was purchased from Dow Corning Corporation (Midland, MI, USA). Ultrapure water was used for the preparation of all standard solutions and mobile phase. Stock standard solution of pregabalin was prepared by dissolving 50mg of pregabalin into 25mL distilled water. Standard solutions of pregabalin (2.0, 1.5, 1, 0.5, 0.1, and 0.05 $\mu\text{g/mL}$) were prepared by subsequent dilution.

2.2. Urine Samples

Urine samples were collected from a healthy volunteer at the university campus. The samples were taken in amber glass bottles previously rinsed with methanol and ultrapure water, and the samples were stored in the dark at 4°C for a maximum of 48 hours. Before their analysis, urine samples were filtered using cellulose acetate membranes (0.45 μm pore size). In order to test the accuracy of the method, the drug-free urine samples were spiked with a known amount of pregabalin standard.

3. Instrument

The Waters HPLC system was used in the study with a μ -Bondapak C₁₈ column (3.9×300mm) (USA). The flow rate was adjusted to 1 mL/min, and the injection volume used was 10 μL with a retention time of 15 mins. A Waters 2996 Photodiode Array was used as the detector, and the wavelength of detection was 210nm. The mobile phase was prepared by dissolving 1.2g of monobasic potassium phosphate in 940mL water. Then, the pH of the resultant solution was adjusted to 6.90 (by using 5N NaOH solution), then 60mL of acetonitrile was added, and the solution was degassed.

4. Membrane Preparation

In this study, sulfonated poly(ether ether ketone) (SPEEK), with the chemical structure shown in Figure 1(b), is used as the acid-functionalized polymer to prepare the corresponding acid-functionalized membrane.

Dry SPEEK polymer was dissolved in dimethylacetamide (DMAc) solvent to form an approximately 10 wt.% solution. Next, the solution was poured into a glass plate, the solvent was slowly evaporated, and the resulted membrane was peeled off by immersing in deionized water for a few hours. The residual solvent was then removed by drying the

obtained membrane in a vacuum oven. The dried membrane was conditioned by soaking in 0.5 M H_2SO_4 , washed with deionized water, and finally, dried at 60°C for 3 hours.

The attached sulfonic acid functionality on the poly(ether ether ketone) backbone makes the polymer surface more hydrophilic. It provides robust, accessible acidic ion-exchange sites for possible interaction with analytes containing basic nitrogen functionality such as pregabalin, as illustrated in Figure 2. Moreover, the extraction performance of PME was compared for polymeric adsorbents using SPEEK and the commercial polydimethylsiloxane (PDMS) membrane.

[figure omitted; refer to PDF]

5. Polymeric-Membrane-Assisted Microextraction (PME)

50mg of the SPEEK membrane was placed into a 10mL drug-free urine sample spiked with various pregabalin concentrations, and the sample was agitated with a stir bar for 2min. Then, the polymeric sorbent was removed from the sample vial and wiped softly with tissue.

The pregabalin-extracted SPEEK membrane was desorbed in 500 μL of 0.1 M NaOH solution via ultrasonication for 2min. Finally, the membrane was removed from the HPLC autosampler vial, and 10 μL of the extract was injected into HPLC. The SPEEK membrane was conditioned again with a large volume of NaOH solution for 10min to check the carryover effect; no analyte was detected after the second desorption. This indicates that the membrane is suitable for subsequent extraction. To condition and regenerate the SPEEK membrane, the membrane was ultrasonicated with 50mL of 0.5M H_2SO_4 for 5min and used for further extraction.

6. Results and Discussion

6.1. Optimization of PME

The extraction principle of PME is based on ion-pair partitioning of pregabalin with the SPEEK membrane. The ion-exchange extraction mechanism of SPEEK provides a convenient pathway to the simultaneous sampling, sample preparation, and preconcentration of pregabalin. The extraction parameters affecting PME, such as extraction time, desorption time, desorption solvent, and sample pH, were optimized. These are the factors that were considered to play an essential role in determining extraction efficiency.

6.2. Selection of Polymeric Sorbent

The extraction performance of PDMS and SPEEK membranes were tested at identical conditions (2min extraction time, 0.1 M NaOH desorption solvent with 2min desorption time). Compared with PDMS, SPEEK showed improved extraction ability for the pregabalin analyte, as shown in Figure 3. The enhanced extraction ability of the SPEEK membrane could be attributed to the hydrophilicity of the acid-functionalized polymer as well as the presence of electrostatic interactions between the grafted sulfonic acid moiety on the polymer backbone ($\text{P}K_a = 10.6$), as illustrated in Figure 2. The significant $\text{P}K_a$ difference allows for fast and effective ionic crosslinking and successful electrostatic interaction between the SPEEK and the $-\text{NH}_2$ functionality on the pregabalin molecules. Hence, the SPEEK membrane was further selected as the functionalized extraction sorbent for method development.

[figure omitted; refer to PDF]

6.3. Extraction Time

The extraction was monitored over the range of 2 to 20min. Figure 4 shows the behavior of pregabalin under different extraction periods. The HPLC signals decreased to an extraction time of 2min, and extraction efficiency remains approximately constant after 10 minutes. Faster mass transfer of the analyte was achieved due to the functionality of the SPEEK membrane. Thus, 2min extraction time was selected for further analysis.

[figure omitted; refer to PDF]

6.4. Desorption Solvent

After extraction, the SPEEK-containing analyte was desorbed with an organic solvent via ultrasonication. Two important factors should be considered before selecting a suitable solvent for desorption of the analyte from SPEEK: (i) the polymer should be insoluble in the desorbing solvent, and (ii) analytes must be soluble in the solvent. In the present case, the SPEEK membrane with ion-exchange capacity ($\text{IEC} = 1.6$) is soluble in polar solvents such as methanol, ethanol, and acetonitrile. However, since the adsorption mechanism is based on electrostatic

interactions and ion pair formation, the desorption solvent shall be able to break the complexed ion pairs by neutralization of the charged polymeric functionalities ($-\text{SO}_3\text{H}/-\text{CO}_2\text{H}$) as illustrated in Figure 2. Based on the abovementioned discussion, a 0.1 M NaOH solution was selected as the desorption solvent for pregabalin elution from the SPEEK membrane. Finally, NaOH extract was injected directly into the HPLC system.

6.5. Desorption Time

The effects of the desorption period (ultrasonication time using a 0.1 M NaOH solution as a desorption solvent) on the pregabalin extraction were studied. According to the literature, the desorption time required using a solvent is usually no longer than 20 min, and mechanisms to facilitate the desorption process may be ultrasonication or agitation [43]. On this basis, desorption time between 2 and 20 min was investigated. Since the extraction process is based on an ion-exchange mechanism, the analyte was quickly desorbed in a shorter time (2 min). After each extraction and desorption, the SPEEK membrane was rinsed with acetone for 2 min to avoid any analyte carryover problem.

6.6. Effect of Sample pH

The effect of sample pH on the pregabalin's extraction efficiency was evaluated in the pH range of 2–12. The highest response was obtained at the lowest pH 2. The obtained highest response in the acidic environment could be attributed to the efficient ion pair electrostatic interactions between the SPEEK membrane and pregabalin analyte. In other words, when the medium becomes less acidic, the ion-exchange capacity of SPEEK polymer decreases, which means that the number of free acidic protons (H^+) decreases; thus, the number of free acidic sites available for interaction with the amine groups of pregabalin became less, which led to a slight reduction in the instrument response. Although the maximum obtained response at $\text{pH}=2$ is about one order of magnitude higher than the lowest response at $\text{pH}>6$, this observation supports the mechanism of electrostatic interaction and ion pair formation in the studied PME system. In comparison, when the medium became alkaline ($\text{pH}>7$), the sulfonic acid groups neutralized and become negatively charged, which makes them inaccessible for the formation of ion pairs with basic ($-\text{NH}_2$) functional groups of the pregabalin analyte. Regardless, the obtained constant response in the alkaline pH range could be attributed to the improved surface hydrophilicity (Figure 1(b)) of the polymeric membrane due to the grafted polar functional groups ($-\text{SO}_3^-$). Hence, in the alkaline medium, the adsorption-desorption mechanism could be suggested due to both the pregabalin analyte and SPEEK membrane's hydrophilic nature.

6.7. Effect of Salt Addition

The addition of salt to the sample solution may decrease the polar analyte's solubility and increase the partition coefficient, and this process is called the salting-out effect. To assess the salting-out effect on the extraction of pregabalin from urine samples, various NaCl concentrations were added to urine samples in the range of 1–20% wt/vol. The addition of salt to the urine samples does not significantly improve the extraction (data not shown).

To illustrate, when NaCl is added in high concentration to the analyzed urine samples, H^+ protons of the sulfonic acid groups in SPEEK polymer are exchanged with Na^+ protons. As a result, HCl is liberated.

Hence, the sulfonic acid groups became neutralized by sodium counterions and unable to form ion pairs with basic functionalities ($-\text{NH}_2$). This result provided evidence for the critical role of the enhanced surface hydrophilicity of the SPEEK membrane, which facilitates the adsorption-desorption of hydrophilic analytes with polar functionalities such as pregabalin. Therefore, no salt was added to the urine samples in the reported experiments.

6.8. Quantitative Analysis

Based on the experiments discussed above, the optimal PME conditions were: as follows 50 mg of SPEEK was used, sample pH of 2, and an extraction time of 2 min. After the extraction, the analytes were desorbed using 500 μL of 0.1 M NaOH by ultrasonication for 2 min. 10 μL of the extract was injected into the HPLC system. To evaluate the PME technique, repeatability, linearity, and detection limits under the optimal extraction conditions were investigated. The repeatability in peak areas was studied for three replicate experiments (3 separate pieces of SPEEK). The relative standard deviations (RSDs) were lower than 7%. Extraction of pregabalin exhibited good linearity over the concentration range 0.05–2 $\mu\text{g}/\text{mL}$ under optimum conditions. Coefficients of correlation (r^2) better than 0.999 were obtained. LOD and LOQ were calculated as 3.3 σ /s and 10 σ /s, respectively, as per International

Conference on Harmonization (ICH) definitions, where σ is the mean, standard deviation of replicate determination values under the same conditions as the sample analysis in the absence of the analyte (blank determination), and s is the sensitivity, namely the slope of the calibration graphs. LOD values for pregabalin were 0.03 ng/mL and LOQ 0.09 ng/mL, respectively. The PME method obtained 105-fold enrichment.

The PME method was compared with already reported methods in the literature and summarized in Table 1. The results demonstrate the applicability of the method for routine trace-level analysis of pregabalin from a urine sample. The PME method's recovery was investigated with a healthy person's urine samples, and the samples were spiked with the known concentration of pregabalin, 0.5 and 1.5 μ g/mL, respectively. The extraction recoveries were calculated and reported in Table 2, and recoveries varied between 90 and 92%, respectively, which indicates that SPEEK polymer can successfully extract the pregabalin from urine samples more effectively. These results demonstrate the absence of significant matrix effects on the efficiency of PME. Figure 5 shows the HPLC chromatograms of (a) the pregabalin standard sample with a concentration of 5 μ g/mL and (b) the SPEEK extract drug-free urine sample spiked with 0.1 μ g/mL pregabalin.

Table 1

Quantitative parameter of PME.

	Spiked	Detected	Recovery	Spiked	Detected	Recovery (%)
Concentration (mg/L) ($n=3$)			Concentration (mg/L) ($n=3$)			Pregabalin

Table 2

Comparison of the methods reported in the literature for pregabalin.

Method	SV (ml)	ET	LOD	%RSD	RR	Ref
LLE (manual)	50	10 min	4.8 ng/mL	0.17	99.5–101	[44]
LLE (ultrasonication)	50	~15 min	66.9 ng/mL	0.068–0.167	97.12–98.86	[13]
LLE (manual)	15	3 min	1 ng/mL	11.4%.	69.8–72%	[45]
PME	20	2 min	0.03 ng/mL	6.8%	90–92	Current study

SV: sample volume (mL). ET: extraction time (min). LOD: limit of detection (ng/mL). RSD: relative standard deviation (%). RR: relative recovery (%).

[figures omitted; refer to PDF]

7. Conclusions

In this work, a polymer-assisted microextraction coupled with HPLC is developed to rapidly determine the amount of pregabalin in urine samples. We reported the potential use of the acid-functionalized SPEEK as an ion-pair low-cost sorbent for selective sample enrichment of pregabalin from urine samples. The PME device was allowed to tumble freely in the sample solution to enhance the extraction efficiency. A small amount of low-cost sorbent, reusability of the sorbent up to 50 times without loss of analytes after consecutive extractions, and the extreme simplicity of the procedure are the main advantages of this technique. Also, the method permits the determination of analytes at low concentrations showing good performance over the commercial PDMS membrane. [46]

Ethical Approval

The urine samples were obtained from the University Medical Clinic with consent from a volunteer and the medical

director.

Acknowledgments

The author gratefully acknowledges the support of King Fahd University of Petroleum and Minerals.

- [1] M. J. Brodie, "Pregabalin as adjunctive therapy for partial seizures," *Epilepsia*, vol. 45 no. s6, pp. 19-27, DOI: 10.1111/j.0013-9580.2004.455004.x, 2004.
- [2] A. Bali, P. Gaur, "A novel method for spectrophotometric determination of pregabalin in pure form and in capsules," *Chemistry Central Journal*, vol. 5, DOI: 10.1186/1752-153x-5-59, 2011.
- [3] R. H. Dworkin, P. Kirkpatrick, "Pregabalin," *Nature Reviews Drug Discovery*, vol. 4 no. 6, pp. 455-456, DOI: 10.1038/nrd1756, 2005.
- [4] L. J. Crofford, M. C. Rowbotham, P. J. Mease, "Pregabalin for the treatment of fibromyalgia syndrome: results of a randomized double-blind placebo-controlled trial," *Arthritis & Rheumatism*, vol. 52 no. 4, pp. 1264-1273, DOI: 10.1002/art.20983, 2005.
- [5] P. J. Siddall, M. J. Cousins, A. Otte, T. Griesing, R. Chambers, T. K. Murphy, "Pregabalin in central neuropathic pain associated with spinal cord injury: a placebo-controlled trial," *Neurology*, vol. 67 no. 10, pp. 1792-1800, DOI: 10.1212/01.wnl.0000244422.45278.ff, 2006.
- [6] G. Sills, "The mechanisms of action of gabapentin and pregabalin," *Current Opinion in Pharmacology*, vol. 6 no. 1, pp. 108-113, DOI: 10.1016/j.coph.2005.11.003, 2006.
- [7] M. A. Rose, P. C. A. Kam, "Gabapentin: pharmacology and its use in pain management," *Anaesthesia*, vol. 57 no. 5, pp. 451-462, DOI: 10.1046/j.0003-2409.2001.02399.x, 2002.
- [8] D. Wesche, H. Bockbrader, J. Bron, G. L. Gibson, H. N. Bockbrader, "Pregabalin drug interaction studies: lack of effect on the pharmacokinetics of carbamazepine, phenytoin, lamotrigine, and valproate in patients with partial epilepsy," *Epilepsia*, vol. 46, pp. 1407-1413, 2005.
- [9] D. R. P. Guay, "Pregabalin in neuropathic pain: a more "pharmaceutically elegant" gabapentin?," *The American Journal of Geriatric Pharmacotherapy*, vol. 3 no. 4, pp. 274-287, DOI: 10.1016/j.amjopharm.2005.12.008, 2005.
- [10] U. Mandal, A. K. Sarkar, K. V. Gowda, "Determination of pregabalin in human plasma using LC-MS-MS," *Chromatographia*, vol. 67 no. 3-4, pp. 237-243, DOI: 10.1365/s10337-007-0440-2, 2008.
- [11] A. S. Jadhav, D. B. Pathare, M. S. Shingare, "Validated enantioselective LC method with precolumn derivatization with marfey's reagent for analysis of the antiepileptic drug pregabalin in bulk drug samples," *Chromatographia*, vol. 65 no. 3-4, pp. 253-256, DOI: 10.1365/s10337-006-0152-z, 2007.
- [12] D. Berry, C. Millington, "Analysis of pregabalin at therapeutic concentrations in human plasma/serum by reversed-phase HPLC," *Therapeutic Drug Monitoring*, vol. 27 no. 4, pp. 451-456, DOI: 10.1097/01.ftd.0000158874.54100.1a, 2005.
- [13] R. S. Gujral, S. K. M. Haque, S. Kumar, "A novel method for the determination of pregabalin in bulk pharmaceutical formulations and human urine samples," *African Journal of. Pharmacy and Pharmacology*, vol. 3, pp. 327-334, 2009.
- [14] G. B. Kasawar, M. N. Farooqui, "Development and validation of HPLC method for the determination of pregabalin in capsules," *Indian Journal of Pharmaceutical Sciences*, vol. 72, pp. 517-519, DOI: 10.4103/0250-474X.73935, 2010.
- [15] G. Shah, C. Ghosh, B. Thaker, "Determination of pregabalin in human plasma by electrospray ionisation tandem mass spectroscopy," *Journal of Advanced Pharmaceutical Technology & Research*, vol. 1 no. 3, pp. 354-357, DOI: 10.4103/0110-5558.72423, 2010.
- [16] M. Ashu, S. Parmar, K. Nagarajan, V. Singh, "Development and validation of rapid HPLC method for determination of Pregabalin in bulk drug and capsule dosage forms," *Der Pharma Chemica*, vol. 3, pp. 482-489, 2011.
- [17] T. Vermeij, P. Edelbroek, J. Chromatogr, "Simultaneous high-performance liquid chromatographic analysis of pregabalin gabapentin and vigabatrin in human serum by precolumn derivatization with o-phthalaldehyde and fluorescence detection," *Journal of Chromatography B*, vol. 810 no. 2, pp. 297-303, DOI: 10.1016/s1570-

0232(04)00662-2, 2004.

- [18] S. Béni, T. Sohajda, G. Neumajer, R. Iványi, L. Szente, B. Noszál, "Separation and characterization of modified pregabalins in terms of cyclodextrin complexation using capillary electrophoresis and nuclear magnetic resonance," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 51 no. 4, pp. 842-852, DOI: 10.1016/j.jpba.2009.10.010, 2010.
- [19] R. S. Gujrak, S. M. Haque, P. Shanker, "A sensitive spectrophotometric method for the determination of pregabalin in bulk, pharmaceutical formulations and in human urine samples," *International Journal of Biomedical Science*, vol. 5, pp. 175-180, 2009.
- [20] A. Oenal, O. Sagirli, "Spectrophotometric and spectrofluorimetric methods for the determination of pregabalin in bulk and pharmaceutical preparation," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 72, pp. 68-71, DOI: 10.1016/j.saa.2008.08.009, 2009.
- [21] R. A. Shaalan, "Spectrofluorimetric and spectrophotometric determination of pregabalin in capsules and urine samples," *International Journal of Biomedical Science: IJBS*, vol. 6, pp. 260-267, 2010.
- [22] M. I. Walash, F. Belal, N. El-Enany, M. H. El-Maghrabey, "Simple and sensitive spectrofluorimetric method for the determination of pregabalin in capsules through derivatization with fluorescamine," *Luminescence*, vol. 26 no. 5, pp. 342-348, DOI: 10.1002/bio.1235, 2011.
- [23] J. M. Rosenfeld, J. L. Crocco, "Specific pentafluorobenzoylation of phenols in a biphasic system," *Analytical Chemistry*, vol. 50 no. 6, pp. 701-704, DOI: 10.1021/ac50027a011, 1978.
- [24] S. D. Brown, D. J. Rhodes, B. J. Pritchard, "A validated SPME-GC-MS method for simultaneous quantification of club drugs in human urine," *Forensic Science International*, vol. 171 no. 2-3, pp. 142-150, DOI: 10.1016/j.forsciint.2006.10.015, 2007.
- [25] B. Tienpont, F. David, T. Benijts, P. Sandra, "Stir bar sorptive extraction-thermal desorption-capillary GC-MS for profiling and target component analysis of pharmaceutical drugs in urine," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 32 no. 4-5, pp. 569-579, DOI: 10.1016/s0731-7085(03)00164-x, 2003.
- [26] P. L. Kole, J. Millership, J. C. McElney, "Determination of diclofenac from paediatric urine samples by stir bar sorptive extraction (SBSE)-HPLC-UV technique," *Talanta*, vol. 85 no. 4, pp. 1948-1958, DOI: 10.1016/j.talanta.2011.07.016, 2011.
- [27] M. Ulbricht, "Advanced functional polymer membranes," *Polymer*, vol. 47 no. 7, pp. 2217-2262, DOI: 10.1016/j.polymer.2006.01.084, 2006.
- [28] Y. Kalyan, S. Das, A. K. Pandey, G. R. K. Naidu, P. K. Sharma, A. V. R. Reddy, "Solid phase preconcentration and determination of mercury and uranyl ions using an itaconic acid functionalized adsorptive membrane," *Analytical Methods*, vol. 3 no. 9, pp. 2017-2024, DOI: 10.1039/c1ay05125c, 2011.
- [29] L. Zhang, Z. Li, X. Du, X. Chang, "Activated carbon functionalized with 1-amino-2-naphthol-4-sulfonate as a selective solid-phase sorbent for the extraction of gold(III)," *Microchimica Acta*, vol. 174 no. 3-4, pp. 391-398, DOI: 10.1007/s00604-011-0643-y, 2011.
- [30] K. Singh, H. C. Bajaj, P. Ingole, A. Bhattacharya, "Comparative study of enantioseparation of racemic tryptophan by ultrafiltration using BSA-immobilized and BSA-interpenetrating network polysulfone membranes," *Separation Science and Technology*, vol. 45 no. 3, pp. 346-354, DOI: 10.1080/01496390903423253, 2010.
- [31] M. Hatanaka, Y. Nishioka, M. Yoshikawa, "Polyurea with L-lysiny residues as components: application to membrane separation of enantiomers," *Macromolecular Chemistry and Physics*, vol. 212 no. 13, pp. 1351-1359, DOI: 10.1002/macp.201100054, 2011.
- [32] Z. Wang, C. Cai, Y. Lin, Y. Bian, H. Guo, X. Chen, "Enantioselective separation of ketoconazole enantiomers by membrane extraction," *Separation and Purification Technology*, vol. 79 no. 1, pp. 63-71, DOI: 10.1016/j.seppur.2011.03.013, 2011.
- [33] M. Lavén, T. Alsberg, Y. Yu, M. Adolfsson-Erici, H. Sun, "Serial mixed-mode cation- and anion-exchange solid-phase extraction for separation of basic neutral and acidic pharmaceuticals in wastewater and analysis by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry," *Journal of Chromatography A*,

vol. 1216 no. 1, pp. 49-62, DOI: 10.1016/j.chroma.2008.11.014, 2009.

[34] T.-Y. Wang, G.-L. Chen, C.-C. Hsu, S. Vied, E. D. Conte, S.-Y. Suen, "Octadecyltrimethylammonium surfactant-immobilized cation exchange membranes for solid-phase extraction of phenolic compounds," *Microchemical Journal*, vol. 96 no. 2, pp. 290-295, DOI: 10.1016/j.microc.2010.04.004, 2010.

[35] A. Razaq, G. Nystroem, M. Stromme, A. Mihranyan, L. Nyholm, *PLoS One*, vol. 6, DOI: 10.1371/journal.pone.0029243, 2011.

[36] S. A. Piletsky, H. Matuschewski, U. Schedler, "Surface functionalization of porous polypropylene membranes with molecularly imprinted polymers by photograft copolymerization in water," *Macromolecules*, vol. 33 no. 8, pp. 3092-3098, DOI: 10.1021/ma991087f, 2000.

[37] T. A. Sergeev, H. Matuschewski, S. A. Piletsky, J. Bendig, U. Schedler, M. Ulbricht, "Molecularly imprinted polymer membranes for substance-selective solid-phase extraction from water by surface photo-grafting polymerization," *Journal of Chromatography. A*, vol. 907, pp. 89-99, DOI: 10.1016/s0021-9673(00)01053-0, 2001.

[38] M. Bryjak, I. Duraj, "Molecularly imprinted membranes for removal of bisphenol A," *Solvent Extraction and Ion Exchange*, vol. 29 no. 3, pp. 432-439, DOI: 10.1080/07366299.2011.573446, 2011.

[39] T. R. Dombrowski, G. S. Wilson, E. M. Thurman, "Investigation of anion-exchange and immunoaffinity particle-loaded membranes for the isolation of charged organic analytes from water," *Analytical Chemistry*, vol. 70 no. 9, pp. 1969-1978, DOI: 10.1021/ac971081t, 1998.

[40] L. Castilho, W. D. Deckwer, F. B. Anspach, "Influence of matrix activation and polymer coating on the purification of human IgG with protein A affinity membranes," *Journal of Membrane Science*, vol. 172 no. 1-2, pp. 269-277, DOI: 10.1016/s0376-7388(00)00343-4, 2000.

[41] N. A. Guzman, "Improved solid-phase microextraction device for use in on-line immunoaffinity capillary electrophoresis," *Electrophoresis*, vol. 24 no. 21, pp. 3718-3727, DOI: 10.1002/elps.200305647, 2003.

[42] S. Almeda, L. Arce, M. Valcárcel, "Combined use of supported liquid membrane and solid-phase extraction to enhance selectivity and sensitivity in capillary electrophoresis for the determination of ochratoxin A in wine," *Electrophoresis*, vol. 29 no. 7, pp. 1573-1581, DOI: 10.1002/elps.200700372, 2008.

[43] L. P. Melo, A. M. Nogueira, F. M. Lanças, M. E. C. Queiroz, "Polydimethylsiloxane/polypyrrole stir bar sorptive extraction and liquid chromatography (SBSE/LC-UV) analysis of antidepressants in plasma samples," *Analytica Chimica Acta*, vol. 633 no. 1, pp. 57-64, DOI: 10.1016/j.aca.2008.11.042, 2009.

[44] M. Dousa, P. Gibala, K. Lemr, "Liquid chromatographic separation of pregabalin and its possible impurities with fluorescence detection after postcolumn derivatization with o-phthalaldehyde," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 53, pp. 717-722, DOI: 10.1016/j.jpba.2010.04.008, 2010.

[45] R. Nirogi, V. Kandikere, K. Mudigonda, "Liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry method for the quantification of pregabalin in human plasma," *Journal of Chromatography B*, vol. 877 no. 30, pp. 3899-3906, DOI: 10.1016/j.jchromb.2009.10.004, 2009.

DETAILS

Subject:	Laboratories; Cellulose acetate; Polymers; Diabetes; Acids; Convulsions & seizures; Reagents; Solvents; Binding sites; Anticonvulsants; Urine; Polydimethylsiloxane; Pharmaceutical industry; Chromatography; Membranes; Diabetic neuropathy; Sorbents

Business indexing term:	Subject: Pharmaceutical industry

Location:	United States--US

Editor:	Amr M Mahmoud

Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-04-14 (Received); 2021-05-06 (Revised); 2021-05-20 (Accepted); 2021-06-01 (Pub)
DOI:	https://doi.org/10.1155/2021/3439242
ProQuest document ID:	2540406761
Document URL:	https://www.proquest.com/scholarly-journals/extraction-pregabalin-urine-samples-using/docview/2540406761/se-2?accountid=211160
Copyright:	Copyright © 2021 Chanbasha Basheer. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-06-13
Database:	Publicly Available Content Database

An Effective and Efficient Sample Preparation Method for 2-Methyl-Isoborneol and Geosmin in Fish and Their Analysis by Gas Chromatography-Mass Spectrometry

Liang-liang, Tian ¹
; Han, Feng ¹
; Essy Kouadio Fodjo ²
; Zhai, Wenlei ³
; Xuan-Yun, Huang ¹
; Kong, Cong ¹
; Yong-Fu, Shi ¹
; You-Qiong Cai ¹

¹ Key Laboratory of East China Sea Fishery Resources Exploitation, Ministry of Agriculture and Rural Affairs, East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai 200090, China ² Laboratory of Constitution and Reaction of Matter, UFR SSMT, Université Felix Houphouet Boigny, 22 BP 582, Abidjan 22, Côte d'Ivoire ³ Beijing Research Center for Agricultural Standards and Testing, No. 9 Middle Road of Shuguanghuayuan, Haidian District, Beijing 100097, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

The intensive aquaculture strategy and recirculating aquaculture system often lead to the production of off-flavor compounds such as 2-methyl-isoborneol (2-MIB) and Geosmin (GSM). The regular purge and trap extraction followed by analysis with gas chromatography-mass spectrometry (GC-MS) usually involve a complicated assembly of facilities, more working space, long sample preparation time, and headspace solid-phase microextraction (SPME). In this work, a method with easier sample preparation, fewer and simplified facilities, and without SPME on GC-MS analysis is developed for the determination of 2-MIB and GSM in fish samples. Unlike previous methods, solvent extract from samples, QuEChERS-based cleanup, and solid-phase extraction for concentration are applied. The LOD (S/N>3) and LOQ (S/N>10) of this method were validated at 0.6 µg/kg and 1.0 µg/kg for both 2-MIB and GSM, which are under the sensory limit (1 µg/kg). Application of this method for incurred fish samples demonstrated acceptable analytical performance. This method is suitable for large-scale determination of 2-MIB and GSM in fish samples, owing to the use of simple facility and easy-to-operate procedure, rapid sample preparation, and shorter time for GC-MS analysis without SPME.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

The continuous increase in aquaculture production provides more than 50% of aquatic product for global demand. To support the nutrition and food security around the world, the intensive aquaculture strategy is adopted in many farm areas [1]. In parallel, the recirculating aquaculture system (RAS) is also developed to avoid the contamination

of water system, and to better control the environment [2] for farming and to save water [3]. However, the intensive farming and RAS [4, 5] can result in the occurrence of off-flavor compounds and the tainting of fishery product, which adversely impact the fish-farming industry [6]. The earthy-musty off-flavor tainting in the fish product can lead to complaints from consumers and the decrease of its market value and to be perceived as unfit for retail [7]. Removal of the off-flavor in fishery product leads to profit reduction [8].

One of the most popular sources of off-flavors originates from the secondary metabolic products in the environmental microbial, such as cyanobacteria [9], actinomycetes [10, 11], or fungi [12]. The dominant objectionable compounds are identified as 2-methyl-isoborneol (2-MIB) and geosmin (GSM), which are responsible for the earthy-musty off-flavor in water or fish product [13]. Usually, only trace amounts of the two taste and odor compounds persist in fish flesh. However, the sensory thresholds for human organoleptic response are quite sensitive and are reported to be no more than 1 µg/kg in different fishes [14]. This tiny residue can lead to unpalatable taste for consumers, decreasing the quality of fish product, and leading to subsequent loss for farmers. There are instruments or methods based on organoleptic test for the evaluation of the tainting status of off-flavors in fish [15]. A panel of trained staffs needs to be prepared for complicated and time-consuming assessment and scores [16]. The sensory threshold varies, depending on the flavors of fish [17]. Moreover, the off-flavors can only be assessed without the identification of their cause and quantification [18]. To determine the exact origin and extend of off-flavor by these two most common compounds, methods for their determination in fish are reasonably required [19].

Many methods have been developed for the determination of 2-MIB and GSM in water environments over the past 30 years [20]. Relatively fewer methods are reported for their determination in fish flesh. The prevalent methods are carried out on gas chromatography-mass spectrometer, regardless of the water or fish flesh. The extraction [10] from matrix often involves the purge and trap process [21], with many different assisted distillation and extraction techniques [22]. Moreover, the trapped residues usually undergo enrichment before GC-MS analysis [23] such as solid-phase microextraction [24]. When it comes to the large-scale determination of samples, the purge and trap process involve assembly of complicated devices and require more working space for parallel operation. Furthermore, it requires large sampling weight for these methods, and the enrichment [25]. The headspace sampling for concentrating analytes from fish matrix demands extra time for recleaning for the next sample analysis. Besides, owing to the semivolatile properties of 2-MIB and GSM, the headspace sampling [26] or purge-trap process can lead to a poor reproducibility for repeated analysis [27]. Therefore, these methods are labor-intensive and time-consuming and rely on more consumables, facilities, and spaces for large-scale sample analysis.

In this study, we aimed to develop a method for large-scale determination of 2-MIB and GSM, with simple solvent extraction, cleaning, enrichment, and sampling process, as well as fewer facilities usage and easier operation, as shown in Figure 1. To overcome the shortages by purge and trap extraction process, a liquid extraction from fish flesh matrix is investigated. QuEChERS method, as a popular and rapid sample extract cleaning method in pesticide and veterinary detection, has been examined for the cleaning of the extract in this work. SPE method is applied for the enrichment of analytes and solvent replacement. GC-MS analysis of these two compounds is performed without SPME for concentration. The performance of this method is evaluated on the limit of detection, recovery, and linear range. Furthermore, it is validated with incurred samples.

[figure omitted; refer to PDF]

2. Materials and Methods

2.1. Reagents and Materials

N-hexane (HPLC grade), ethyl acetate ester, and acetone were supplied by J. T. Baker. 2-Methyl-isoborneol (100 µg/mL, purity: >98%) and geosmin (100 µg/mL, purity: >98%) were obtained from Dr. Ehrenstorfer. They were diluted to 1 µg/mL using *n*-hexane and stored at -18°C in the dark before use. Sodium sulfate anhydrous and magnesium sulfate anhydrous were provided by Aladdin Industrial Corporation. Solid-phase extract cartridge (Silica 500mg/3 mL) and dispersive solid-phase extract (C18, graphitized black carbon, PSA) were purchased from Agela Technologies. Grass carp and crucian carp were bought from local farm produce market in Shanghai.

2.2. Instrument

Gas chromatography coupled to mass spectrometer (TSQ QUANTUM GC, Thermofisher Scientific) was used for GC-MS analysis. Balance (0.01 g, Sartorius company, Germany), solid-phase extraction device (Supelco company), and ultrasonic cleaner (Branson) were also used during the experiment.

2.3. QuEChERS Materials Adsorption Profile Test

0.5 g of graphitized black carbon, C18, PSA, sodium sulfate anhydrous, silica, alumina (neutral or alkaline), florisil, chitosan, and diatomite were added to 5 mL of these solutions, respectively. The solutions were stirred and set quiet to collect the supernatant, which was prepared for GC-MS detection. Each adsorbent was examined with 2 replicates.

2.4. Extraction and Enrichment of 2-MIB and GSM from Fish

15 mL of *n*-hexane was added into a plastic centrifuge tube containing 5 g of sample, which was sealed immediately to avoid volatile loss. This mixture was vortexed for 5 min at 2500 rpm and treated with ultrasonic bath for 15 min. To get the supernatant collected, the sample was centrifuged at 5000 $\times$ g for 8 min. The remaining sample was extracted with 10 mL *n*-hexane again. The extract was combined in a 50 mL plastic centrifuge tube. This extract was mixed with 0.5 g of C18 adsorbent, 0.5 g of graphitized black carbon, and 2 g of magnesium sulfate anhydrous and was vortexed for 7 min, at 2500 rpm, followed by centrifugation at 1400 $\times$ g for 5 min to obtain the supernatant. The silica SPE cartridge was preconditioned with 10 mL *n*-hexane before the passthrough of extract, which was rinsed with 3 mL *n*-hexane after loading, and dried by vacuum. The analyte was eluted with 2 mL of *n*-hexane/ethyl acetate (3/1), and the cartridge was blown to dry during eluting. The eluate was then collected in a vial and vortexed for GC-MS analysis.

2.5. GC-MS Determination

The HP-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.5 μ m) was used for GC separation with constant nitrogen gas flow at 1.0 mL/min. The programmable temperature procedure was initially set at 60°C for 1 min, increased to 120°C at the rate of 5°C/min, and kept for 3 min. Then, it was increased to 170°C at the rate of 5°C/min and kept for 1 min. Subsequently, it went to 280°C with 20°C/min and was kept for 5 min. 1 μ L of the sample solution was injected into the sampling port at 250°C without diverting.

In the mass spectrometry analysis, electron ionization (EI) source was used with a source temperature at 250°C, MS transfer line temperature at 280°C, and electron energy at 70 eV. Selective ion monitoring (SIM) was used for data acquisition. The quantitative ion for 2-MIB is *m/z* 94.9, and its qualitative ions are *m/z* 94.9, 107.9, and 134.9, while the quantitative ion for GSM is *m/z* 111.9, and its qualitative ions are *m/z* 111.9, 124.9, and 96.9.

2.6. Real Incurred Samples Preparation

Crucian carp and grass carp were used for incurred sample preparation. Before incurring, 5 crucian carp (ca. 500 g/each) and 5 grass carp (1500 g/each) were adaptively cultured in a 300-liter tank for 3 days with regular feeding and bubbling. Half of the water was replaced every day during culture. For incurring with 2-MIB and GSM, 300 liters of water was prepared by spiking 100 μ g/L of the two contaminants. And then, the fishes were bathed for 8 hours in the contaminated water before transferring to clean water. After depuration in clean water for 2 hours, the incurred fish was sampled immediately by cutting off the fillets and storing in refrigerator.

3. Results and Discussion

3.1. Adsorption of Different QuEChERS Materials

In the beginning, to use the QuEChERS materials for cleanup of the extract, the adsorption profiles of different materials under various solvents at the concentration of 50 ng/mL of 2-MIB and GSM in *n*-hexane, ethyl acetate, acetone, and acetonitrile are examined. These solvents are intended to extract 2-MIB and GSM from flesh samples. Results of the recoveries of the analytes after adsorption by different adsorbents in different solvents for 2-MIB and GSM are shown in Figure 2. In the solvent of *n*-hexane, C18, graphitized black carbon and magnesium sulfate show less than 10% loss of the two analytes, while PSA, alumina (alkaline), florisil, diatomite, and chitosan show 10–80% adsorption of these analytes. In contrast, the alumina (neutral) and silica adsorb all the analytes. In the solvent of ethyl acetate, graphitized black carbon, magnesium sulfate anhydrous, silica, alumina (neutral or alkaline), florisil,

and chitosan do not adsorb the analytes. C18 and PSA adsorbed 10–40% of these analytes. In acetone, C18 and PSA also adsorb 10–40% of the analytes and other materials show no significant adsorption. In acetonitrile, except magnesium sulfate anhydrous, diatomite, florisol, and chitosan, other materials show around 10–70% adsorption for both analytes.

[figures omitted; refer to PDF]

The above four solvents can dissolve various disturbing components when used to extract analytes from fish flesh. The combined use of these materials was preferred for better cleaning of these extracts. Besides, we would like to do analyte enrichment after cleanup. It is noticed in Figure 2 that silica can be an adsorbent for enrichment when analytes are dissolved in *n*-hexane. *N*-hexane is chosen as it can extract the analytes with good solubility [4, 11], undergo QuEChERS cleanup with low analytes loss, and promise feasibility for following concentration process. Therefore, we checked the combined use of QuEChERS materials that shows less than 10% adsorption of analytes in *n*-hexane. Among these material combinations, 0.5g of C18, 0.5g of graphitized black carbon, and 2g of magnesium sulfate anhydrous can keep more than 95% recovery after QuEChERS cleaning. Therefore, they are chosen as QuEChERS materials for extract cleaning. Furthermore, the performance of *n*-hexane as an extract solvent for the analytes is also examined as shown in Figure S1 and Table S1 (see Supplementary Materials).

3.2. Concentrate of Analytes

To increase the sensitivity of detection with small sampling weight, the concentration process is necessary and expected to be most feasible. According to Figure 2, 2-MIB and GSM can be adsorbed by silica when dissolved in *n*-hexane and have no adsorption when dissolved in ethyl acetate and acetone. Therefore, the extract of the two analytes with a large volume of *n*-hexane can be enriched on silica cartridge and can further be eluted with small volume of solvent mixture. The adsorption rate of these two analytes is examined with silica cartridge (500mg, 3 mL). The result shows no residue in the collected solvent, indicating the complete adsorption (100%) of 2-MIB and GSM on the silica cartridge.

Furthermore, the elution solvent for the release of adsorbed analytes from the silica cartridge was investigated. After washing with *n*-hexane, the loading cartridge was dried under nitrogen blow and eluted with different solvent mixture. Initially, the solvent mixture with different ratio of *n*-hexane and acetone was examined. As displayed in Figure 3, high percentage of acetone can increase the elution rate on the cartridge. However, the stability of the eluted analytes is quite low, with an obvious difference between 2-MIB and GSM. Under the ratio of 1:1 between *n*-hexane and acetone, the elution rate can reach around 100%. To find a highly efficient elute solvent, the solvent mixture with different ratio of *n*-hexane and ethyl acetate was further examined. As shown in Figure 3, the pure ethyl acetate does not produce higher elution rate, while the ratio of 1:1 and 3:1 between *n*-hexane and ethyl acetate can elute almost 100% of 2-MIB and GSM. However, further increase in the ratio of *n*-hexane leads to the decrease of elution rate and even results in no elution at all. When applied in real samples, it should be noted that there is more impurity peak on the chromatogram for 2-MIB and GSM in the collected elute with the mixture of *n*-hexane and acetone than with the mixture of *n*-hexane and ethyl acetate as displayed in Figure S6 (Supplementary Materials). Finally, the mixture of *n*-hexane and ethyl acetate (3/1) is selected as an ideal elute solvent with high elution rate, less impurity peak on the chromatogram, and good stability.

[figures omitted; refer to PDF]

3.3. Matrix Effect

The matrix effect of this method was evaluated with grass carp, crucian carp, and carp samples as they have been frequently reported to accumulate these two off-flavors [9]. For this purpose, 100ng of 2-MIB and GSM were spiked in 1 mL of the blank matrix solution, which were obtained by extracting from the fish samples, followed by cleaning with QuEChERS method and eluting with silica cartridge. The matrix effect was calculated through the ratio between the response areas of analytes in the spiked solution and in the solvent at the same concentration, which is further reduced by one and is multiplied by 100%. As displayed in Figure 4, the matrix effect for 2-MIB is no more than 5% in the three matrices, and for GSM, the matrix effect is the weakest in crucian carp at 1%–3% and the strongest in grass carp at 11%. According to these results, the matrix effect for the two analytes in all these matrices is

acceptable for calibration with solvent standards and quantification with no apparent bias.

[figure omitted; refer to PDF]

3.4. Method Validation

After optimization of several parameters of the method, its performance was validated with linearity, LOD, LOQ, and recovery. The result shows linearity for standard solutions of 6 different concentrations of 2–500 ng/mL with a relative coefficient of 0.9997 for both 2-MIB and GSM. The LOD is confirmed with grass carp at the concentration when the ratio of signal to noise ≥ 3 and LOQ is confirmed when the ratio of signal to noise ≥ 10 , with the accuracy of 70–120% and an imprecision $< 20\%$. As a result, both 2-MIB and GSM are confirmed with LOD at 0.6 $\mu\text{g/kg}$, and LOQ of both at 1.0 $\mu\text{g/kg}$, which are below the sensory limit of humans, indicating the practicability for 2-MIB and GSM detection. The recoveries were obtained in grass carp and crucian carp with 2.0, 10.0, and 20.0 $\mu\text{g/kg}$ of 2-MIB and GSM spiked in the samples. Table S3 and Table S4 (Supplementary Materials) show the results of recoveries at these spiking levels. All the average recoveries for these different spiking levels are higher than 80% with a relative standard deviation of no more than 8% in both grass carp and crucian carp. These results indicate good recoveries and stability of the method for detecting 2-MIB and GSM in fish samples.

To further test the practicability of the method, the real incurred sample, grass carp, and a liver sample of grass carp were analyzed for their content of these two analytes. These samples show the presence of off-flavor compounds through organoleptic evaluation. As shown in Table 1, one of the grass carp has a residue of 2-MIB of about 10 times the sensory limit and a residue of 3 times the sensory limit of GSM. The other grass carp accumulates a much higher amount of 2-MIB and GSM, which are around 40 and 15 times the other grass carp. In the liver sample, residues of 2-MIB and GSM are found, which are much higher than their residue in flesh samples. A typical chromatogram of 2-MIB and GSM in grass carp 1 is displayed in Figure 5. This result demonstrates good separation of analytes and interference for real samples. The relative RSDs for flesh samples are less than 5%, indicating good stability in these matrices. However, the detected residue in the liver varies even in the same sample. It can be attributed to the less sampling amount that results in the poor homogeneity of samples and volatile loss during sample preparation. The above results further demonstrate the feasibility of this method for 2-MIB and GSM determination in off-flavor fish samples.

Table 1

Results of the 2-MIB and GSM residues in the incurred samples.

Sample	2-MIB ($\mu\text{g/kg}$)	Average ($\mu\text{g/kg}$)	RSD (%)	GSM ($\mu\text{g/kg}$)	Average ($\mu\text{g/kg}$)	RSD (%)
Flesh 1	11.7/11.8/12.2	11.9	2.22	3.43/3.15/3.19	3.26	4.65
Flesh 2	441.5/507.5/358.4	435.8	17.1	45.3/56.0/40.2	47.2	17.1
Liver	704.6/781.2/1063	849.6	22.2	224.9/253.2/318.8	265.6	18.1

[figures omitted; refer to PDF]

4. Conclusions

In general, a method for large-scale determination of 2-MIB and GSM in fish samples has been developed through this work. The extraction mechanism, enrichment, and injection mode are different from the previous reported purge and trap method [8, 21]. The method allows straightforward liquid extract from fish flesh instead of purge and trap extraction, followed by cleanup with optimized QuEChERS material and further concentration with silica cartridge. A sampling process without SPME has further simplified the GC-MS analysis process. The performance of this method is evaluated and validated with incurred samples, showing its practicability. The LOD of this method is under the sensory limit of fish samples. Besides, this method involves no complicated facility and easy-to-operate procedure and rapid sample preparation. It allows large-scale determination of 2-MIB and GSM in fish samples and can also

find extended application in other semivolatile compound determination.

Acknowledgments

This work was supported by the special research fund for the national nonprofit institutes (East China Sea Fisheries Research Institute) (2014T14), the National Natural Science Foundation of China (31701698), Shanghai Key Laboratory of Forensic Medicine (Academy of Forensic Science) (KF1910), and Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2020TD72).

References

- [1] K. K. Schrader, J. W. Davidson, A. M. Rimando, S. T. Summerfelt, "Evaluation of ozonation on levels of the off-flavor compounds geosmin and 2-methylisoborneol in water and rainbow trout *Oncorhynchus mykiss* from recirculating aquaculture systems," *Aquacultural Engineering*, vol. 43 no. 2, pp. 46-50, DOI: 10.1016/j.aquaeng.2010.05.003, 2010.
- [2] Z. Bai, A. Pilote, P. K. Sarker, G. Vandenberg, J. Pawliszyn, "In vivo solid-phase microextraction with in vitro calibration: determination of off-flavor components in live fish," *Analytical Chemistry*, vol. 85 no. 4, pp. 2328-2332, DOI: 10.1021/ac3033245, 2013.
- [3] K. Schrader, S. A. Rubio, R. Piedrahita, A. Rimando, "Geosmin and 2-methylisoborneol cause off-flavors in cultured largemouth bass and white sturgeon reared in recirculating-water systems," *North American Journal of Aquaculture*, vol. 67 no. 3, pp. 177-180, DOI: 10.1577/a04-070.1, 2005.
- [4] A. Angioni, A. Cau, P. Addis, "Gas chromatographic mass spectrometry determination of geosmin and 2-methylisoborneol off-flavor in *Mugil cephalus* roe," *Food Analytical Methods*, vol. 8 no. 6, pp. 1484-1489, DOI: 10.1007/s12161-014-0025-2, 2015.
- [5] M. C. J. Verdegem, R. H. Bosma, J. A. J. Verreth, "Reducing water use for animal production through aquaculture," *International Journal of Water Resources Development*, vol. 22 no. 1, pp. 101-113, DOI: 10.1080/07900620500405544, 2006.
- [6] C. C. Grimm, S. W. Lloyd, R. Batista, P. V. Zimba, "Using microwave distillation-solid-phase microextraction—gas chromatography—mass spectrometry for analyzing fish tissue," *Journal of Chromatographic Science*, vol. 38 no. 7, pp. 289-296, DOI: 10.1093/chromsci/38.7.289, 2000.
- [7] S. W. Lloyd, C. C. Grimm, "Analysis of 2-methylisoborneol and geosmin in catfish by microwave distillation—solid-phase microextraction," *Journal of Agricultural and Food Chemistry*, vol. 47 no. 1, pp. 164-169, DOI: 10.1021/jf980419x, 1999.
- [8] S. Houle, K. K. Schrader, N. R. Le François, "Geosmin causes off-flavour in arctic charr in recirculating aquaculture systems," *Aquaculture Research*, vol. 42 no. 3, pp. 360-365, DOI: 10.1111/j.1365-2109.2010.02630.x, 2011.
- [9] D. Varga, Z. Sándor, C. Hancz, I. Csengeri, Z. Jeney, Z. Papp, "Off-flavour compounds in common carp (*Cyprinus carpio* L.) flesh in context of type of fish pond," *Acta Alimentaria*, vol. 44 no. 2, pp. 311-315, DOI: 10.1556/066.2015.44.0008, 2015.
- [10] R. L. Bristow, I. S. Young, A. Pemberton, J. Williams, S. Maher, "An extensive review of the extraction techniques and detection methods for the taste and odour compound geosmin (trans-1, 10-dimethyl-trans-9-decalol) in water," *TrAC Trends in Analytical Chemistry*, vol. 110, pp. 233-248, DOI: 10.1016/j.trac.2018.10.032, 2019.
- [11] S. Yarnpakdee, S. Benjakul, P. Penjamras, H. G. Kristinsson, "Chemical compositions and muddy flavour/odour of protein hydrolysate from Nile tilapia and broadhead catfish mince and protein isolate," *Food Chemistry*, vol. 142, pp. 210-216, DOI: 10.1016/j.foodchem.2013.07.043, 2014.
- [12] S. E. Jensen, C. L. Anders, L. J. Goatcher, T. Perley, S. Kenefick, S. E. Hrudey, "Actinomycetes as a factor in odour problems affecting drinking water from the north Saskatchewan river," *Water Research*, vol. 28 no. 6, pp. 1393-1401, DOI: 10.1016/0043-1354(94)90306-9, 1994.
- [13] P. C. Lindholm-Lehto, J. Vielma, "Controlling of geosmin and 2-methylisoborneol induced off-flavours in recirculating aquaculture system farmed fish—a review," *Aquaculture Research*, vol. 50, DOI: 10.1111/are.13881, 2019.

- [14] P. Howgate, "Tainting of farmed fish by geosmin and 2-methyl-iso-borneol: a review of sensory aspects and of uptake/depuration," *Aquaculture*, vol. 234 no. 1–4, pp. 155-181, DOI: 10.1016/j.aquaculture.2003.09.032, 2004.
- [15] C. C. Grimm, S. W. Lloyd, P. V. Zimba, "Instrumental versus sensory detection of off-flavors in farm-raised channel catfish," *Aquaculture*, vol. 236 no. 1–4, pp. 309-319, DOI: 10.1016/j.aquaculture.2004.02.020, 2004.
- [16] E. D. Conte, C.-Y. Shen, P. W. Perschbacher, D. W. Miller, "Determination of geosmin and methylisoborneol in catfish tissue (*Ictalurus punctatus*) by microwave-assisted distillation–solid phase adsorbent trapping," *Journal of Agricultural and Food Chemistry*, vol. 44 no. 3, pp. 829-835, DOI: 10.1021/jf9505032, 1996.
- [17] R. F. Robertson, K. Jauncey, M. C. M. Beveridge, L. A. Lawton, "Depuration rates and the sensory threshold concentration of geosmin responsible for earthy-musty taint in rainbow trout, *Onchorhynchus mykiss*," *Aquaculture*, vol. 245 no. 1–4, pp. 89-99, DOI: 10.1016/j.aquaculture.2004.11.045, 2005.
- [18] H. Zhang, P. Ma, J. Shu, B. Yang, J. Huang, "Rapid detection of taste and odor compounds in water using the newly invented chemi-ionization technique coupled with time-of-flight mass spectrometry," *Analytica Chimica Acta*, vol. 1035, pp. 119-128, DOI: 10.1016/j.aca.2018.07.051, 2018.
- [19] C. S. Tucker, "Off-flavor problems in aquaculture," *Reviews in Fisheries Science*, vol. 8 no. 1, pp. 45-88, DOI: 10.1080/10641260091129170, 2000.
- [20] C. Li, M. H. Ngai, K. K. Reddy, S. C. Y. Leong, Y. W. Tong, C. L. L. Chai, "A fluorescence-displacement assay using molecularly imprinted polymers for the visual, rapid, and sensitive detection of the algal metabolites, geosmin and 2-methylisoborneol," *Analytica Chimica Acta*, vol. 1066, pp. 121-130, DOI: 10.1016/j.aca.2019.03.041, 2019.
- [21] X. Deng, P. Xie, M. Qi, "Microwave-assisted purge-and-trap extraction device coupled with gas chromatography and mass spectrometry for the determination of five predominant odors in sediment, fish tissues, and algal cells," *Journal of Chromatography A*, vol. 1219, pp. 75-82, DOI: 10.1016/j.chroma.2011.11.031, 2012.
- [22] M. Zhu, F. J. Aviles, E. D. Conte, D. W. Miller, P. W. Perschbacher, "Microwave mediated distillation with solid-phase microextraction: determination of off-flavors, geosmin and methylisoborneol, in catfish tissue," *Journal of Chromatography A*, vol. 833 no. 2, pp. 223-230, DOI: 10.1016/s0021-9673(98)01010-3, 1999.
- [23] E. Wright, H. Daurie, G. A. Gagnon, "Development and validation of an SPE-GC-MS/MS taste and odour method for analysis in surface water," *International Journal of Environmental Analytical Chemistry*, vol. 94 no. 13, pp. 1302-1316, DOI: 10.1080/03067319.2014.974586, 2014.
- [24] M. A. Petersen, G. Hyldig, B. W. Strobel, N. H. Henriksen, N. O. G. Jørgensen, "Chemical and sensory quantification of geosmin and 2-methylisoborneol in rainbow trout (*Oncorhynchus mykiss*) from recirculated aquacultures in relation to concentrations in basin water," *Journal of Agricultural and Food Chemistry*, vol. 59 no. 23, pp. 12561-12568, DOI: 10.1021/jf2033494, 2011.
- [25] E. D. Ruan, J. L. Aalhus, S. T. Summerfelt, J. Davidson, B. Swift, M. Juárez, "Determination of off-flavor compounds, 2-methylisoborneol and geosmin, in salmon fillets using stir bar sorptive extraction-thermal desorption coupled with gas chromatography-mass spectrometry," *Journal of Chromatography A*, vol. 1321, pp. 133-136, DOI: 10.1016/j.chroma.2013.10.038, 2013.
- [26] M. Bedner, K. Saito, "Development of a liquid chromatography atmospheric pressure chemical ionization mass spectrometry method for determining off-flavor compounds and its application toward marine recirculating aquaculture system monitoring and evaluation of aeration as a depuration approach," *Journal of Chromatography A*, vol. 1609, DOI: 10.1016/j.chroma.2019.460499, 2020.
- [27] X. Ma, N. Gao, B. Chen, Q. Li, Q. Zhang, G. Gu, "Detection of geosmin and 2-methylisoborneol by liquid-liquid extraction-gas chromatograph mass spectrum (LLE-GCMS) and solid phase extraction-gas chromatograph mass spectrum (SPE-GCMS)," *Frontiers of Environmental Science & Engineering in China*, vol. 1 no. 3, pp. 286-291, DOI: 10.1007/s11783-007-0048-7, 2007.

DETAILS

Subject:	Aquaculture; Mass spectrometry; Farming; Fisheries; Gas chromatography; Aroma compounds; Carbon; Solvents; Adsorption; Spectroscopy; Scientific imaging; Methods; Fishing; Chromatography; Solvent extraction; Fish; Carp; Solid phases
Editor:	Victoria F Samanidou
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-08 (Received); 2021-04-26 (Accepted); 2021-05-10 (Pub)
DOI:	https://doi.org/10.1155/2021/9980212
ProQuest document ID:	2530720411
Document URL:	https://www.proquest.com/scholarly-journals/effective-efficient-sample-preparation-method-2/docview/2530720411/se-2?accountid=211160
Copyright:	Copyright © 2021 Liang-liang Tian et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-05-23

Document 36 of 59

A RP-HPLC Method for the Analysis of Neostigmine Methylsulfate and Process-Related Impurities, Forced Degradation Studies, in the Injection Formulation

Parab, Manali ¹ ; Shirsat, Vaishali A ¹¹ ; Kodgule, Yogita M ² ; Kodgule, Mandar ² ¹ Bombay College of Pharmacy (Autonomous), Kalina, Santacruz East, Mumbai 400098, India ² IQGEN-X Pharma Pvt. Ltd., A-165, Khairane Road, Sector 2, Kopar Khairane, Navi Mumbai 400710, India[ProQuest document link](#)

ABSTRACT (ENGLISH)

Neostigmine methylsulfate is an anticholinesterase agent and is clinically used for treating myasthenia gravis. It is also used for reversing nondepolarising neuromuscular blocking agents. Neostigmine methylsulfate may be administered by intravenous, intramuscular, or subcutaneous injection. In this research paper, a distinct stability-indicating reverse phase HPLC method was developed and validated for the quantitative determination of related impurities and degradation impurities in neostigmine methylsulfate API and injection formulation. The specific objective was to improve the resolution between European Pharmacopoeia listed impurity A and impurity B and degradation impurity of neostigmine methylsulfate API and injection formulation. The analysis was performed using Kromasil C₁₈ column at 30°C of column oven temperature with phosphate-buffer/acetonitrile in a gradient mode. The RP-HPLC method was developed and validated for in-house neostigmine methylsulfate synthesis process sample and injection formulation. The injection formulation sample was studied for accelerated stability, temperature cycling stability, and photostability. The validation studies for neostigmine methylsulfate synthesis process API were studied using impurity A, impurity B, and impurity C. The analytical method validation parameters studied were specificity, precision, linearity, limit of detection, limit of quantitation, accuracy, and robustness. The API and the injection formulation were subjected to forced degradation under acid, alkali, oxidation, and photolytic and thermal conditions. The proposed method showed a significantly improved RRT (Relative Retention Time) of impurity A and impurity B with a resolution greater than 1.5. The developed method eliminates the use of an ion-pairing agent and thereby a good performance of column was established.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Stability-indicating HPLC method for determination of process and degradation-related impurities in Neostigmine methyl sulfate drug substance and drug product (injection). Stress testing of the drug substance can help to identify the possible degradation products, the stability of the molecule, elucidate degradation pathways, determine the intrinsic stability of drug molecule, and also validate the stability, selectivity, and specificity of the analytical

procedures followed. It also provides evidence on how the quality of a drug substance varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, which are necessary for the recommendation of storage conditions, retesting periods, and shelf-life establishment. These degradation products can be separated and identified by developed and validated stability-indicating methods. Degradation products formed due to drug excipients interaction or drug-drug interaction can be analysed by stressing samples of API, formulation, and placebo separately and comparing the impurity profiles. Forced degradation studies can be helpful to determine the appropriate packaging to reduce or avoid the formation of degradation products. The knowledge acquired from stress testing aid in the improvement of the manufacturing process. Literature provides suggestions for the experimental conditions to conduct the forced degradation testing. Stability data available in the literature suggests the conditions at which the drug molecule is labile. These given sets of conditions can be repeated to establish the new stability studies for the new dosage form. Typical degradation studies include acidic and alkaline hydrolysis, oxidative degradation, photolytic degradation, and thermal degradation [1–13].

Neostigmine methylsulfate (3-(dimethylcarbamoyloxy) phenyl)-trimethylazanium: methyl sulfate is well known quaternary ammonium compound and is used as a competitive cholinesterase inhibitor. It decreases the breakdown of acetylcholine in the synaptic cleft, thus, increasing the levels of the same. This acetylcholine competes for the binding sites as a nondepolarising neuromuscular blocking agent and reverses the neuromuscular blockade. This intensifies the nicotinic and muscarinic effects. It activates the skeletal muscles. Neostigmine methylsulfate does not readily cross the blood-brain barrier and hence has no significant effect on the central nervous system. Due to the ionic nature of neostigmine methylsulfate, it gets poorly absorbed through the gastrointestinal tract. Hence, it is administered as a parenteral injection. This is clinically used for the treatment of myasthenia gravis, treatment or prevention of postoperative and nonobstructive abdominal distention, and reversal of nondepolarising neuromuscular blocking agents [14, 15].

Several analytical methods have been reported for the detection of neostigmine methylsulfate. Two HPLC methods were developed for neostigmine methylsulfate content determination in injection by Yue et.al. [16] Pavani Peddi et al. [17] developed a stability-indicating HPLC method for estimation of related substances in neostigmine. This method included the quantitation and validation of only impurity A and impurity B. This method failed to detect and quantify impurity Jogi et al. [18] developed a stability-indicating RP-HPLC method for the estimation of glycopyrrolate and neostigmine in bulk and tablet dosage form. This method failed to detect the impurities present in tablet formulation.

European pharmacopoeia (EP) specifies a RP-HPLC method for the determination of the related substance in neostigmine methylsulfate API. The specified limits for impurities in neostigmine methylsulfate API are as follows: impurity B (NMT 0.01%), other unspecified impurities—impurity A and impurity C (NMT 0.1%)—and total impurities (NMT 0.2%). Impurity A is a degradation product while impurities B and C are process impurities [19].

The chemical structures of EP reported impurities and neostigmine methylsulfate is given in Table 1. The mobile phase specified in EP comprises sodium dihydrogen phosphate (pH=3.2) along with sodium dodecylsulfate as an aqueous phase and acetonitrile as an organic phase in the ratio 89:11 v/v. The Relative Retention Time (RRT) of impurity A and impurity B given is 0.56 and 0.61, which fails to give a resolution more than 1.5. The repeated use of ion-pairing agents like sodium dodecyl sulfate affects column chemistry [20-23]. The objective of the study was to improve the resolution between impurity A and impurity B without the use of the ion-pairing reagent. Thus, in this paper, a distinct stability-indicating RP-HPLC method was developed and validated for the quantitative determination of related substance which includes related impurities and degradation impurity in neostigmine methylsulfate API and injection formulation (0.5mg/mL).

Table 1

Chemical structures of neostigmine methylsulfate and its EP reported impurities [19].

Impurity A (3-hydroxy-N, N, N-trimethylanilinium)	Impurity B (3-(dimethylamino)phenol)
Impurity C (3-(dimethylamino)phenyl dimethylcarbamate)	Neostigmine methylsulfate (3-(dimethylcarbamoyloxy)phenyl)-trimethylazanium; methyl sulfate

2. Materials and Methods

2.1. Chemicals and Reagents

Neostigmine methylsulfate API (NMS synthesis process sample) was obtained by IQGENX Pharma Private Limited (Kopar Khairane, Navi Mumbai, Maharashtra). Neostigmine methylsulfate injection (0.5 mg/mL) was manufactured by IQGENX Pharma Private Limited (Kopar Khairane, Navi Mumbai, Maharashtra). Neostigmine methylsulfate API (99.9%), impurity A (99.73%), impurity B (98.96%), and impurity C (96.9%) reference standards were obtained from IQGENX Pharma Private Limited. HPLC grade sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), orthophosphoric acid, and acetonitrile were purchased from Merck. Milli-Q-water, purified using a Milli-Q-Water System, Merck Millipore water system (Dubuque, IA, USA), was used to prepare the mobile phase, sample, and standard solutions.

2.2. HPLC Instrumentation and Chromatographic Conditions

Chromatographic separations were performed on HPLC system with Waters Alliance e2695, Quaternary system, separation module equipped with a Waters 2998 photodiode array detector, and 2489 UV/Vis Detector. The integration of output signals was carried out using an Empower 4 data handling system (Waters Corporation, Milford, MA, USA). The analysis was carried using Kromasil C18 (250 mm × 4.6 mm, 5 μ) column at 30°C of column oven temperature and 15°C of sample cooler temperature with phosphate-buffer/acetonitrile in gradient mode flowing at a rate of 1.00 mL/min during 45 min analysis time. The mobile phase was prepared using a phosphate-buffer solution (Na_2HPO_4 , pH 3.0 ± 0.05, 10.00 mM). The pH of the buffer solution was adjusted with 25% orthophosphoric acid (Emplura, Merck). The buffer was filtered through 0.45 μ m PVDF membrane filter. The phosphate-buffer solution was used as Mobile phase A and acetonitrile as Mobile phase B and was run in a linear gradient elution program for the determination of related substances in neostigmine methylsulfate API and neostigmine methylsulfate Injection. The linear gradient program is shown in Table 2.

Table 2

Gradient program.

Sr. no.	Time (min)	% of mobile phase A	% of mobile phase B
1	0.01	95	5
2	10.00	85	15
3	20.00	65	35
4	35.00	65	35
5	38.00	95	5
6	45.00	95	5

The UV detection was done at 215nm. The injection volume was fixed as 20 μ l for NMS API and formulation analysis. Water and acetonitrile were used in the of ratio 90:10 v/v as a diluent.

2.3. Standard Stock Solutions

The standard stock solution of neostigmine methylsulfate (1000 μ g/mL) was prepared by dissolving the drug in the diluent. The standard solution of 10 μ g/mL of neostigmine methylsulfate was prepared from the standard stock solution. The individual “impurity standard stock” solutions were prepared in the diluent to give 100 μ g/mL concentration of each impurity. The specification limits set by EP were considered for validation studies and are as follows: 0.1% for Imp-A, 0.1% Imp-C, and 0.01% for Imp-B. Thus, the individual impurity standard stock solutions and NMS were finally diluted to give the following concentrations of the impurities and NMS as follows: impurity A: 10 μ g/mL; impurity B: 1 μ g/mL, and impurity C: 10 μ g/mL and NMS 10 μ g/mL. This final diluted solution was considered as a “working standard solution” and was used for the validation studies.

2.4. Sample Solution

A stock solution of the NMS synthesis process sample (1000 μ g/mL) was prepared in the diluent. The injection formulation (0.5mg/mL) was manufactured by IQGENX Pharma Pvt. Ltd. and is further referred to as “NMS Injection.” Each mL of the injection contains neostigmine methylsulfate (0.5mg), phenol (4.5mg) (used as a preservative), and sodium acetate trihydrate (0.2mg) in water for injection. The pH of the injection solution was adjusted with either acetic acid or sodium hydroxide to achieve a value of pH=5.5. A sufficient amount of injection sample was transferred directly into the HPLC vials for HPLC automated analysis.

2.5. Stability Samples

2.5.1. Accelerated Stability Study

The stability studies were performed as per the ICH guidelines [24–26]. The accelerated stability of the NMS API was determined using Thermolab Stability Chambers (TH 400/G) maintained at 40°C/75% RH, 25°C/60% RH for 6 months. Injection formulations were subjected to 40°C/75% RH, 25°C/60% RH, and 2–8°C for 6 months, packed in a clear glass vial USP type I with flip-off seal and rubber closure. The stability study was carried out as per ICH guidelines.

2.5.2. Temperature Cycling Study

In this study, the inverted injection vials were incubated at –20°C along with a placebo for a period of 2 days. After 2 days, the vials were placed in a stability chamber maintained at 40°C/75% RH. The studies were repeated for three-time change in the mentioned temperature cycle.

2.5.3. Photostability Study

The formulated injection was kept in Suntest Photostability Chamber (Model no. XLS+) providing an overall illumination of not less than 1.2 million lux hours and integrated near ultraviolet energy of not less than 200-watt hours/square meter in 3 packings for the duration of 10 days. This study was performed in three pack types with vials placed in inverted orientation. The primary pack of injection formulation was directly exposed to light by keeping the vials in USP type I clear glass vials (10mL). For the secondary pack (market pack), the injection vials were enclosed in a carton and then placed in the photostability chamber. The control sample consisted of the primary pack covered with aluminium foil.

3. Method Validation

The validation parameters were designed as per the specification limits of individual impurities. The “working standard solution” was diluted as per the requirement of validation studies to obtain impurity A, 0.1 μ g/mL; impurity C, 0.1 μ g/mL; impurity B, 0.01 μ g/mL at the specification limit; and NMS API, 1 μ g/mL.

Similarly, the EP specified limit for impurity A and impurity C is 0.1% of NMS injection which corresponds to 0.5 μ g/mL concentrations and for impurity B, the specification limit is 0.01% of NMS injection which corresponds to 0.05 μ g/mL concentration. These solutions were prepared by diluting the injection sample and subsequently the impurities were spiked in this injection sample. These concentrations were considered during the conduct of validation studies.

3.1. Specificity

3.1.1. For NMS API

All forced degradation samples of neostigmine methylsulfate API were analysed at an initial concentration of 1000 μ g/mL of NMS API by the developed HPLC method. For degradation studies of NMS API, 25mg of API was accurately weighed and transferred to each of the 4 different volumetric flasks (25ml). In the first volumetric flask, 5 ml of 0.1N NaOH was added for studying the alkaline degradation. For acid degradation studies, 2ml of 1N HCl and 5ml of hydrogen peroxide (30% v/v) for oxidative degradation were added separately in two volumetric flasks and placed in a water bath at a temperature of 60°C for 30min. The solutions were quenched, cooled, dissolved, and made up to the volume with diluent. Since NMS showed complete degradation on heating in presence of 5mL of 0.1 N NaOH, the 4th volumetric flask was kept at room temperature on the benchtop. All the solutions were diluted up to the mark using the diluent.

3.1.2. For NMS Injection

All forced degradation samples of neostigmine methylsulfate injections were analysed at an initial concentration of 400 μ g/mL of NMS injection using the aforementioned HPLC conditions. For degradation studies of NMS injection, 8 mL of injection was accurately transferred to each of the 4 different volumetric flasks (10ml). In the first volumetric flask, 1 ml of 1N NaOH was added for studying the alkaline degradation. For acid degradation studies, 1 ml of 1N HCl and 1 ml of hydrogen peroxide (30% v/v) for oxidative degradation were added separately in two volumetric flasks and placed in a water bath at a temperature of 60°C for 30min. The last solution was quenched, cooled, dissolved, and made up to the volume with diluent.

The method specificity for process-related impurities was assessed by diluting working standard solution and spiking into the NMS API and NMS injection at specification level concentration. The resulting chromatograms were compared with individual samples of standards of drug and impurities (Imp-A, Imp-B, and Imp-C) at those specification levels.

3.2. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The standard solution of neostigmine methylsulfate was diluted quantitatively to obtain 10 μ g/mL and 5 μ g/mL of NMS and the solutions were injected six times ($n=6$). For the method precision study, sample solutions of NMS API (1000 μ g/mL) were injected six times ($n=6$). The injection samples of the same batch from six different vials were directly filled in six HPLC vials and injected.

3.3. Linearity

The calibration curve was obtained by plotting the graph of varying concentrations of analyte versus their corresponding detector signals obtained. Method linearity was evaluated by determining the correlation coefficient (r^2), slope, and intercept values of calibration curves. The working standard solution was diluted quantitatively to carry out the linearity studies at seven different concentration levels, that is, 50, 80, 100, 120, 150, 200, and 300%. For each concentration level, solutions were prepared and injected in series of triplicate ($n=3$). The equations of linear regression were performed using regression analysis.

3.4. LOD and LOQ

The LOD and LOQ studies were carried out to establish the sensitivity of the proposed method.

This study was carried out by residual standard deviation method and by visual evaluation method. The LOD and LOQ are expressed as LOD=3.3 σ /S and LOQ= 10 σ /S, respectively, where σ =the standard deviation of the response and S=the slope of the calibration curve. The slope S was estimated from the calibration curve of the respective analyte. The estimate of σ was carried out by the residual standard deviation method. The LOD and LOQ estimation by the visual method was done by injecting the diluted working standard solution. The precision was carried out at LOQ level with six injections ($n=6$).

3.5. Accuracy

Recovery studies were performed to assess the accuracy of the method.

3.5.1. Accuracy Studies for NMS API

Accuracy studies were performed by analysing spiked impurity standard stock solution in NMS API (1000 μ g/mL) at

50%, 100%, and 150% of specification concentration limits of impurities.

3.5.2. Accuracy Studies for NMS Injection

Accuracy studies were performed by analysing spiked impurity standard stock solution in NMS injection (0.5 mg/mL) at 50%, 100%, and 150% of specification concentration limits of impurities.

The standard deviation (SD) and (%) RSD were calculated at each level and the results obtained were expressed as the percentage of impurities recovered.

3.6. Robustness

The method robustness was performed to determine how system suitability would be affected by variations in experimental conditions. Therefore, the standard and sample solutions were subjected to deliberate variations in chromatographic conditions which include pH (3.1 and 2.9), temperature (35°C), flow rate (0.8 mL/min and 1.2 mL/min), and wavelength (213 nm and 217 nm). Evaluation of results was carried out by determining any change in retention time and peak area for all impurities and standard solution. The robustness studies were performed by spiking impurities in the standard solution of NMS API, whereas the robustness study for the injection sample was performed without the addition of any impurities.

4. Result and Discussion

4.1. Optimization of Chromatographic Conditions

The related substance method given in the EP monograph demonstrates that the RRT of impurity A (0.56) and impurity B (0.61) are very close. The first trial experiment performed as per the EP method showed the merging of impurities A and B resulting in a single peak signal. Thus, the EP method failed to meet the system suitability requirement for resolution. Thus, the resolution between impurity A and impurity B was less than 1.5. Impurity B is genotoxic and thus accurate quantitation is essential.

The regular use of sodium dodecylsulfate as an ion-pairing agent will affect the column chemistry. The isocratic mobile phase mentioned in EP consisted of acetonitrile: sodium dihydrogen phosphate buffer and did not give adequate resolution for the detection of all impurities. Thus, there was a need to develop a precise, sensitive, and convenient method for the quantitative determination of NMS and its impurities. Parameters such as column selection, buffer concentration, buffer pH, and mode of elution were studied.

Neostigmine methylsulfate is a polar molecule and has a pKa value of 5. In order to reduce unwanted silanol interaction, it is advisable by EP to use base deactivated silica columns for separation. Initial trials were carried by keeping the chromatographic conditions mentioned in EP with the use of base deactivated silica columns—Hypersil BDS C8 (250 × 4.6 mm, 5 μ) column. The chromatograms revealed the coelution of impurity A and impurity B. Moreover, the repeatability of system suitability parameters was not achieved. Hence, change in column chemistry was opted by using X bridge column C18 (150 × 4.6 mm, 3.5 μ) having BEH technology (Ethylene Bridged Hybrid technology). By use of this column, the impurities (A and B) eluted close to the retention time of mobile phase peak and within 3 minutes. Hence, this column was found not to be suitable for analysis. Thus, the Kromasil column was selected for further development.

Initially, the isocratic mobile phase specified by EP consisting of acetonitrile: sodium dihydrogen phosphate buffer (pH 3.2) (89:11% v/v) with Kromasil column was used for the analysis. However, the impurity C remained undetected. Hence, it was decided to develop a gradient elution technique. The various proportions of acetonitrile and sodium dihydrogen phosphate buffer with different gradient elution patterns and run times were optimized to get the total run time of analysis. The most critical observation was with respect to the pH of the buffer. Phosphate buffer of pH varying from 2.9 to 3.1 was tried for optimization of the mobile phase A. The phosphate buffer of pH=3.00 was very important for the identification and quantification of all impurities. The concentrations of all the impurities were observed to be less than LOD when the pH of the buffer was changed by ± 1 unit from pH=3. Thus, pH=3 was a critical parameter for the simultaneous determination of neostigmine methylsulfate and all impurities. The optimized gradient elution mobile phase composition is shown in Table 2. The flow rate of the mobile phase was 1.0 mL/min and the detection wavelength was 215 nm.

4.2. Method Validation

The analytical validation parameters such as specificity, linearity, range, precision, accuracy, limit of detection, and limit of quantitation were validated according to ICH Q2 (R1) guidelines [27].

4.2.1. Specificity

The chromatograms of the individual standard of neostigmine methylsulfate and impurities were compared with spiked sample solutions of NMS API and injection. All impurity peaks were well separated in the spiked sample of API and formulation with a resolution more than 2. In the placebo chromatogram, no coeluting peaks were observed at the RTs of neostigmine methylsulfate and known impurities. Placebo peaks were well separated from the impurity peaks. Hence, it was deduced that the placebo solution showed no interference with standard as well as impurities (Figures 1–4).

[figure omitted; refer to PDF]

The results of all degradation studies showed that neostigmine methylsulfate API and injection degrade in alkaline conditions. Impurity A was formed as the main degradation product due to acidic, alkaline, and oxidative hydrolysis. In case of API, the highest degradation of 31.58% was observed in alkaline hydrolysis (5 mL of 0.1 N NaOH at 60°C and 30 min). While in the injection solution, the highest 6.13% degradation was observed in presence of 1 mL of 1 N NaOH for 30 minutes at 60°C (Tables 3 and 4). The chromatograms showing control sample, acid hydrolysis, alkali hydrolysis, and oxidative hydrolysis of neostigmine methylsulfate API are depicted in Figures 5–9 and for neostigmine methylsulfate injection are shown in Figures 10–13.

Table 3

Forced degradation data of neostigmine methylsulfate API.

Condition	Impurity A (% w/w)	Total unknown impurities (% w/w)	Total impurities (% w/w)
Acid hydrolysis (1 mL of 1 N HCl) 60°C and 30 min	0.03	0.14	0.17
Alkali hydrolysis (1 mL of 1 N NaOH) 60°C and 30 min	6.03	0.10	6.13
Alkali hydrolysis (1 mL of 0.1 N NaOH) benchtop	0.20	0.01	0.21
Oxidative hydrolysis (1 mL of 30% v/v H ₂ O ₂) 60°C and 30 min	0.05	0.26	1.60
Oxidative hydrolysis (2 mL of 30% H ₂ O ₂) 60°C and 30 min	0.07	0.07	0.14

Table 4

Forced degradation data of neostigmine methylsulfate injection.

Condition	Impurity A (% w/w)	Total unknown impurities (% w/w)	Total impurities (% w/w)
Acid hydrolysis (2 mL of 1 N HCl) 60°C and 30 min	0.86	0.03	0.89

Alkali hydrolysis (5mL of 0.1N NaOH) benchtop	9.98	0.02	10.00
Alkali hydrolysis (5mL of 0.1N NaOH) 60°C and 30min	31.54	0.04	31.58

[figure omitted; refer to PDF][figure omitted; refer to PDF][figure omitted; refer to PDF][figure omitted; refer to PDF][figure omitted; refer to PDF][figure omitted; refer to PDF][figure omitted; refer to PDF]

The chromatogram of impurity A obtained from forced degradation studies was compared with the standard chromatogram of impurity A. Determination of peak purity was performed using a photo diode array detector which confirmed the spectral homogeneity of the peak. The purity angle of neostigmine methylsulfate and Impurity A was found to be less than the purity threshold in the chromatograms of the stress degradation sample, thus indicating the peak purity of Impurity A. The chromatograms of the individual standard of neostigmine methylsulfate and impurities were compared with spiked sample solutions of NMS API and injection. All impurity peaks were well separated in the spiked sample of API and formulation with a resolution of more than 2 (Figures 5–13). The purity threshold of neostigmine methylsulfate and known impurities peaks were found to be less than the purity angle. The system suitability parameters for neostigmine methylsulfate impurities in API and impurity A in neostigmine methylsulfate injection are given in Tables 5 and 6, respectively. The retention time of neostigmine methylsulfate in API and in injection was found to be 10.405min and 10.319min, respectively.

Table 5

System suitability parameters for neostigmine methylsulfate impurities in API.

Component names	RT (min)	RRT	Area	Theoretical plates	Tailing factor	Resolution	Purity angle	Purity threshold
Impurity A	4.841	0.4689	39322	21703	1.23	6.29	1.791	2.365
Impurity B	7.882	0.7602	3170	33200	1.20	18.99	16.099	19.496
Impurity C	29.606	2.8623	61058	91925	1.05	7.78	1.150	2.096

Table 6

System suitability parameters for impurity A in NMS injection.

Component names	RT (min)	RRT	Area	Theoretical plates	Tailing factor	Resolution	Purity angle	Purity threshold
Impurity A	4.891	0.4739	13357	20867	1.24	6.25	1.723	2.678

4.2.2. Precision Study

For the precision study, the (%) RSD of system precision should ideally be less than 2%. The in-house limits specify that the RSD of method precision should be less than 10%. In case of system precision study, the RSD of peak area

of neostigmine methylsulfate standard was found to be 0.23% and 0.37% for NMS API and NMS injection, respectively, while in the method precision study for Impurity A calculated as (%) w/w, the RSD was found to be 3.89% and 0.88% for NMS API and NMS injection, respectively. These results thus demonstrate that the method is precise.

4.2.3. Linearity

The seven-point calibration curves for neostigmine methylsulfate, impurity A, and impurity C were prepared in the range of 0.20–3.00 µg/mL. The range for impurity B was 0.02–0.30 µg/mL. The data was subjected to the linear-regression analysis.

The regression equations obtained were as follows:

Neostigmine methylsulfate: $y=23578x-157.98$, impurity A: $y=25765x-708.1$, impurity B: $y=32200x-41.603$, and impurity C: $y=66632x-894.28$. The calibration curves were linear with good correlation coefficients (r^2) of 0.999 for all the compounds.

4.2.4. LOD and LOQ Studies

The LOD for neostigmine methylsulfate API, impurity A, impurity B, and impurity C was found to be 0.0978 µg/mL, 0.0357 µg/mL, 0.0040 µg/mL, and 0.0149 µg/mL, respectively. The LOQ for neostigmine methylsulfate API, impurity A, impurity B, and impurity C was found to be 0.2965 µg/mL, 0.1083 µg/mL, 0.0121 µg/mL, and 0.0454 µg/mL, respectively. The (%) RSD at LOQ concentration was found to be less than 10%. This indicates that the method is sensitive enough to quantitate very small concentrations of the impurities in API and injection.

4.2.5. Accuracy

Accuracy was established across the analytical range of neostigmine methylsulfate and all impurities. Reproducible peak shapes were obtained under each condition. At each level, the (%) recovery for all impurities was found to be NLT 98.0% and NMT 102.0% for NMS API. Similarly, for the NMS injection, (%) recovery of all impurities was found to be NLT 80.0% and NMT 120.0%. This data suggests that the developed method is accurate for the quantification of impurities in both API and injection.

4.2.6. Robustness

The results of the robustness study for NMS API and NMS injection are summarised in Tables 7 and 8.

Table 7

Robustness study of NMS API.

Component	Mean RT (min)±SD	(%) RSD of RT	Mean of peak area±SD	(%) RSD of peak area	Mean RT (min)± SD	% RSD of RT	Mean of peak area±SD	(%) RSD of peak area
Robustness study of NMS API at wavelength=215±2nm								
At wavelength=217 nm					At wavelength=213nm			
NMS	10.387± 0.086	0.828	1776887 3± 106476.7	0.599	10.304 ±0.062	0.60 02	2560286 0±50635	0.198
Impurity A	4.856± 0.043	0.887	39372± 51.08	0.130	4.861± 0.0257	0.52 89	37201± 19	0.051

Impurity B	7.871± 0.047	0.603	3246± 6.81	0.210	7.872± 0.019	0.24 31	3146± 9.17	0.291
Impurity C	29.622± 0.067	0.226	45084± 27.30	0.061	29.608 ±0.068	0.23 07	7475± 65.73	0.088
-								
Robustness study of NMS API at flow rate= 1.0±0.2mL/min								
At flow rate= 1.2mL/min					At flow rate=0.8mL/min			
NMS	9.097± 0.009	0.093 9	2244329 7± 40747.54	0.182	11.92± 0.014	0.11 9	3363981 4± 79924.44	0.238
Impurity A	4.071± 0.027	0.654 2	40086± 55.08	0.137	6.038± 0.023	0.37 9	58861± 45.09	0.077
Impurity B	7.318± 0.060	0.813 6	2072± 9.29	0.448	9.621± 0.040	0.41 6	4708± 20.07	0.426
Impurity C	27.016± 0.001	0.003 7	55508± 66.16	0.119	33.659 ±0.049	0.14 5	81426± 41.19	0.051
-								
Robustness study of NMS API for mobile phase A (phosphate buffer) pH=3.0±0.1								
At pH=3.1					At pH=2.9			
NMS	10.491± 0.0151	0.144 1	2282651 4± 5858.3	0.026	10.579 ±0.070	0.65 7	2282477 8±62157	0.272
Impurity A	4.911± 0.0557	1.133 6	31870± 92.089	0.289	4.835± 0.081	1.67 3	32536± 81.28	0.250
Impurity B	8.457± 0.0716	0.847 0	3416± 12.22	0.358	7.207± 0.065	0.90 5	2820± 24.79	0.879
Impurity C	30.651± 0.0675	0.220 2	105084± 150.44	0.143	30.611 ±0.138	0.45 2	21815± 50.34	0.231

Table 8

Robustness study of NMS Injection.

Component	Mean RT (min)±SD	(%) RSD of RT	Mean of peak area ±SD	(%) RSD of peak area	Mean RT (min)± SD	(%) RSD of RT	Mean of peak area ±SD	(%) RSD of peak area
Robustness study of NMS injection at wavelength=215±2nm								
At wavelength=217 nm					At wavelength=213 nm			
NMS	10.384± 0.024	0.230	1777451 7± 22268.68	0.130	10.416 ±0.011	0.100	25738094 ± 48861.32	0.190
Impurity A	4.889± 0.014	0.277	38521± 66.55	0.173	4.880± 0.025	0.104	37613± 61.20	0.163
Impurity B	7.881± 0.006	0.071	3133± 11.24	0.359	7.847± 0.034	0.428	2677± 23.09	0.863
Impurity C	29.605± 0.016	0.065	47303± 14.01	0.030	29.615 ±0.008	0.025	75631± 149.721	0.198
-								
Robustness study of NMS injection at flow rate=1.0±0.2mL/min								
At flow rate=1.2mL/min					At flow rate=0.8mL/min			
NMS	9.082± 0.023	0.255	2245111 5± 38468.14	0.171	11.954 ±0.034	0.281	33595014 ±216906	0.646
Impurity A	4.041± 0.042	1.039	40158± 75.35	0.188	6.052± 0.040	0.165	58851± 64.29	0.109
Impurity B	7.347± 0.047	0.642	2052.33± 12.50	0.609	9.646± 0.023	0.247	4702± 23.46	0.499
Impurity C	27.09± 0.027	0.100	55498± 90.59	0.163	33.645 ±0.046	0.136	81454± 46.11	0.057
-								
Robustness study of NMS injection at mobile phase pH=3.0±0.1								
At pH=3.1					At pH=2.9			

NMS	10.502± 0.038	0.158	2306276 9± 277.10	0.001	10.55± 0.032	0.306	23079307 ±29289	0.127
Impurity A	4.950± 0.004	0.088	4211± 18.90	0.449	4.924± 0.008	0.153	4500± 61.27	1.362
Impurity B	8.456± 0.016	0.188	Below detection limit	-----	7.139± 0.009	0.130	Below detection limit	-----
Impurity C	30.413± 0.042	0.138	20941± 71.99	0.344	30.727 ±0.031	0.100	20889± 228.7	1.095

From the results of robustness, it can be concluded that, for a slight change in the wavelength detection and flow rate, the proposed HPLC method was robust within the acceptable limits of (%) RSD less than 2.0. However, the control of the pH of the mobile phase composition A (phosphate buffer) having pH=3.00 was imperative for the detection and quantification of impurities A, B, and C. The merging of small unknown impurities was observed at the retention time of impurity B and impurity C when the pH of the phosphate buffer was changed by 1 unit.

4.2.7. Accelerated Stability Studies

As per the accelerated stability study, it was seen that NMS API and injection degrade gradually (Tables 9–12). By the end of the six-month study, the following was concluded:

- (i) The (%) w/w of impurity A increased in injection sample as well as in API
- (ii) In case of API, the highest degradation—impurity A—was found to be 0.06% at 25°C/60% RH for 3 months and 40°C/75% RH for 3 months
- (iii) The highest total impurities in API were found to be 0.25% at 40°C/75% RH, 3 months
- (iv) In case of injection, the highest degradation—impurity A—was found to be 0.07% at 40°C/75% RH, 6 months when kept in inverted and upright condition
- (v) The highest total impurities in injection were found to be the same, that is, 0.12% in both the studies conducted for 3 months and 6 months, by maintaining the parameters such as 40°C/75% RH, positioned in inverted as well as upright condition

Table 9

Accelerated stability study data of NMS API.

Conditions	Neostigmine methylsulfate API	
Impurity A (% w/w)	Total impurities (% w/w)	40°C/75% RH, 1M
0.06	0.21	25°C/60% RH, 3M
0.06	0.23	40°C/75% RH, 3M

0.06	0.25	25°C/60% RH, 6M
0.05	0.10	40°C/75% RH, 6M

Table 10

Accelerated stability study of NMS injection studied from 1–6 months at various temperature and humidity conditions.

	25°C/60% RH (1 month)	40°C/75% RH (1 month)	25°C/60% RH (3 months)	40°C/75% RH (3 months)	2–8°C (3 months)	25°C/60% RH (6 months) (upright)	25°C/60% RH (6 months) (inverted)
(%) w/w of Impurity A	0.02	0.04	0.02	0.04	0.02	0.03	0.03
(%) w/w of unknown impurities	0.02	0.05	0.07	0.07	0.07	0.04	0.05
(%) w/w of total impurities	0.04	0.09	0.09	0.11	0.09	0.07	0.08

Table 11

Accelerated stability study of NMS injection studied for 6 months at various temperature and humidity conditions and temperature cycling study.

	40°C/75% RH for 6 months (upright)	40°C/75% RH for 6 months (inverted)	2–8°C for 6 months (upright)	2–8°C for 6 months (inverted)	Temperature cycling study
(%) w/w of Impurity A	0.07	0.07	0.02	0.02	0.07
(%) w/w of unknown impurities	0.04	0.05	0.05	0.05	0.14
(%) w/w of total impurities	0.11	0.12	0.07	0.07	0.21

Table 12

Accelerated photostability study of NMS injection studied for 10 days using three pack types.

NMS injection formulation	Controlled pack	Primary pack	Secondary pack
(%) w/w of Impurity A	0.10	0.10	0.02
(%) w/w of unknown impurities	0.13	0.13	0.06
(%) w/w of total impurities	0.24	0.23	0.09

Any injection formulation product requires temperature-controlled distribution channels for the sake of transportation. This puts product quality at risk, when transportation time and temperature control during the same cannot be maintained. To study the effects of change in temperature during transportation and storage, temperature cycling studies were carried out. According to the study, (%) w/w of impurity A and that of total impurities were within the specification limits. In case of photostability study of injection, (%) w/w of impurity A and that of total impurities were found to be constant at every stage of analysis. It was thus observed that the injection remained stable during photostability analysis.

5. Conclusion

The European Pharmacopoeia has specified a method for separation, identification, and quantitation of impurities using an ion-pairing agent—sodium dodecylsulfate. Columns are at a high risk of deterioration due to their continuous exposure to sodium dodecylsulfate, as it alters the column chemistry during prolonged use, which in turn affects the retention time, area, resolution, and other system suitability parameters pertaining to the peaks of the desired analyte. The method developed in this study does not require the use of an ion-pairing agent, thus successfully eliminating the aforementioned risk of damage to the column during its use. This method not only establishes the good performance of the column but also improves the column life. The proposed method shows a significantly improved RRT of impurity A and impurity B with a resolution greater than 1.5. Validation was performed according to the ICH Q2 (R1) guidelines, and the method was verified to be selective, specific, precise, accurate, and robust and gave linear responses to the concentration gradation. The developed stability-indicating method can be simultaneously used for quality control, forced degradation, and accelerated stability study analysis of the neostigmine methylsulfate drug substance and injection formulation.

Acknowledgments

The authors are thankful to the Department of Science and Technology (Ministry of Science and Technology), Government of India, and the Indian Pharmaceutical Association, Maharashtra State Branch, for providing research fund to perform this work under the project entitled “National Facility for Research and Training in Integrated Analytical Strategies for Discovery, Development and Testing of Drugs, Pharmaceuticals and Nutraceuticals” DPRP scheme to Bombay College of Pharmacy, Mumbai, Project Sanction Order no. VI-D &P/552/2016–17/TDT(G), dated 07.03.2017. Special thanks are to Dr. Vinayak Godbole, IQGENX Pharma Pvt Ltd., Navi Mumbai, for his continuous guidance in the conduct of work.

- [1] R. Simon, H. Hicks, "Forced degradation to develop stability-Indicating methods," Pharmaceutical Outsourcing, 2012.
- [2] T. Rawat, I. P. Pandey, "Forced degradation studies for drug substances and drug products- scientific and regulatory considerations," Journal of Pharmaceutical Science and Research, vol. 7 no. 5, pp. 238-241, 2015.
- [3] S. Venkataraman, M. Manasa, "Forced degradation studies: regulatory guidance, characterization of drugs, and their degradation products—a review," Drug Invention Today, vol. 10 no. 2, pp. 137-146, 2018.
- [4] C. Rack, "An introduction to the accelerated stability assessment program," American Pharmaceutical Review, vol. 20 no. 5, pp. 86-90, 2017.
- [5] S. Bajaj, S. Singh, "Stability Testing of Pharmaceuticals, Series Title-Methods in Pharmacology and Toxicology," 2018.
- [6] B. M. Rao, M. K. Srinivasu, K. P. Kumar, "A stability indicating LC method for rivastigmine hydrogen tartrate,"

- [7] A. Arunachalam, M. Shankar, "Stability studies: a review," Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry, vol. 1 no. 4, pp. 184-195, 2013.
- [8] S. Gorog, M. Babjak, G. Balogh, "Drug impurity profiling strategies," Talanta, vol. 44 no. 9, pp. 1517-1526, DOI: 10.1016/s0039-9140(96)02179-0, 1997.
- [9] S. W. Baertschi, "Analytical methodologies for discovering and profiling degradation-related impurities," TrAC Trends in Analytical Chemistry, vol. 25 no. 8, pp. 758-767, DOI: 10.1016/j.trac.2006.05.012, 2006.
- [10] ICH Q3A (R2), Impurities in New Drug Substance, 2006.
- [11] ICH Q3B (R2), Impurities in New Drug Products, 2006.
- [12] ICH Q3C (R7), Guideline for Residual Solvents, 2018.
- [13] ICH S2 (R1), Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, 2011.
- [14] Food and Drug Administration, BLOXIVERZ™ (Neostigmine Methylsulfate Injection), 2013.
- [15] J. Liu, "Neostigmine," Reference Module in Biomedical Sciences, 2017.
- [16] C.-I. Yue, L.-j. Mao, Z. Guo, "Study on RP-HPLC content determination of neostigmine methylsulfate for injection," Chinese Journal of Pharmaceutical Analysis, vol. 25 no. 10, pp. 1264-1265, 2005.
- [17] P. Peddi, T. Raja Rajeswari, S. L. Tulasi, "Development and validation of stability indicating HPLC method for estimation of related substances in neostigmine," Proceedings of the Knowledge Sharing, Technological Advancements and Sustainable Development (IMC2k18), .
- [18] K. Jogi, "Development and validation of stability indicating RP- HPLC method for the estimation of glycopyrrolate and neostigmine in bulk and tablet," International Journal of Scientific & Engineering Research, vol. 7 no. 12, pp. 1236-1242, 2016.
- [19] European Pharmacopoeia (EP) by Council of Europe; European Pharmacopoeia Commission; European Directorate for the Quality of Medicines & HealthCare, Council of Europe: European Directorate for the Quality of Medicines and HealthCare (EDQM), Strasbourg, France
- [20] L. R. Snyder, J. J. Kirland, Practical HPLC Method Development, 1997.
- [21] A. P. Schellinger, P. W. Carr, "Isocratic and gradient elution chromatography: a comparison in terms of speed, retention reproducibility and quantitation," Journal of Chromatography A, vol. 1109 no. 2, pp. 253-266, DOI: 10.1016/j.chroma.2006.01.047, 2006.
- [22] J. W. Dolan, L. R. Snyder, Gradient Elution Chromatography, Encyclopedia of Analytical Chemistry, 2006.
- [23] J. W. Dolan, "How fast can a gradient be run," LC/GC Europe, vol. 24, pp. 406-410, 2011.
- [24] ICH Q1A (R2), Stability Testing of New Drug Substances and Products, 2003.
- [25] ICH Q1B, Stability Testing: Photostability Testing of New Drug Substances and Products, 1996.
- [26] ICH Q1E, Evaluation of Stability Data, 2003.
- [27] ICH Q2 (R1), Validation of Analytical Procedures: Text and Methodology, 2005.

DETAILS

Subject:	Acetonitrile; Sodium; Scientific papers; Binding sites; Oxidation; Impurities; Methods; Stability; Phenols; Chromatography; Degradation; Synthesis; Validation studies; Drug dosages

Location:	United States--US; Mumbai India

Editor:	Victoria F Samanidou

Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-01-14 (Received); 2021-04-10 (Revised); 2021-04-25 (Accepted); 2021-05-12 (Pub)
DOI:	https://doi.org/10.1155/2021/5570173
ProQuest document ID:	2530720399
Document URL:	https://www.proquest.com/scholarly-journals/rp-hplc-method-analysis-neostigmine-methylsulfate/docview/2530720399/se-2?accountid=211160
Copyright:	Copyright © 2021 Manali Parab et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-05-23
Database:	Publicly Available Content Database

Techniques for Detection of Clinical Used Heparins

Li, Binjie ¹

; Zhao, Huimin ²

; Yu, Mingjia ¹

¹ Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing, China ² College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Heparins and sulfated polysaccharides have been recognized as effective clinical anticoagulants for several decades. Heparins exhibit heterogeneity depending on the sources. Meanwhile, the adverse effect in the clinical uses and the adulteration of oversulfated chondroitin sulfate (OSCS) in heparins develop additional attention to analyze the purity of heparins. This review starts with the description of the classification, anticoagulant mechanism, clinical application of heparins and focuses on the existing methods of heparin analysis and detection including traditional detection methods, as well as new methods using fluorescence or gold nanomaterials as probes. The in-depth understanding of these techniques for the analysis of heparins will lay a foundation for the further development of novel methods for the detection of heparins.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Heparins are highly sulfated linear glycosaminoglycans, exhibiting prominent heterogeneity. Pharmaceutical heparins are generally obtained from bovine lung and porcine mucosa [1, 2]. The chemical properties of heparins derived from different sources including molecular mass distribution, pattern of sulfation, and purity will lead to discrepancies in biological activities and clinical drug safety. As one of the most widely used clinical anticoagulants [3], the relationship between curative effect and dose is not clear. Furthermore, it is the fact that the adverse effects during the clinical uses and the contamination of heparins such as oversulfated chondroitin sulfate (OSCS) [4] have become common in clinical use, which reveals that the precise methods for the detection and analysis of heparins are needed to be developed.

2. Classifications of Heparins

Heparin was first found by McLean and Howell at Johns Hopkins University [5] as a successful anticoagulant for over 80 years and its main chemical structure was described as a highly sulfated linear polysaccharide belonging to the family of heparan sulfate (HS) in glycosaminoglycan (GAG) [6, 7], which is composed of sulfated repeating 14-linked disaccharide units, like β -D-uronic acid and D-N-glucosamine (GlcN). Clinical used heparins can be divided into the following three categories by their molecular weight (with an average MW of 12–15 kDa and a MW range of 5–40 kDa) [8], including heparin, low-molecular-weight heparin (LMWH), and ultralow-molecular-weight heparin (ULMWH) [9, 10].

2.1. Heparin/Unfractionated Heparin (UH)

2.1.1. Nature Product of Heparin/Unfractionated Heparin (UH)

Heparins can be extracted from many animal sources [11]. The first clinical used heparin was extracted from bovine

lung and then shifted into other animal tissues, owing to the lack of bovine lung in World War II when beef had become a food source [12]. Nowadays, most pharmaceutical heparin is extracted from porcine mucosa. It has been reported that heparin derived from porcine might be more effective in anticoagulant activity than heparin derived from bovine after structural and functional analysis and less likely leading to adverse effect of bleeding [2, 13]. Due to the risk of bleeding complications in heparin derived from bovine, the detection and analysis of heparins have been hotspots of the current research in order to remove heparins derived from other animal sources as a high anticoagulant risk factor in heparin products.

2.1.2. Biosynthesis of Heparin/Unfractionated Heparin (UH)

The biosynthetic heparin is made from nonanimal sources [14, 15], which relies on a chemoenzymatic system. Mainly, the biosynthetic pathway of heparin has been divided into the following three parts [16]. The first part is using polysaccharide synthases to polymerize activated monosaccharides into heparosan, which is the starting polysaccharide in heparin biosynthesis with a repeating 14-linked β -D-glucuronic acid (GlcA) and D-N-acetylglucosamine (GlcNAc) [17] disaccharide unit. The second part includes the chemical transformation from an N-acetyl intermediate to an N-deacetylation and N-sulfonation intermediate with proper molecular weight, which is similar to commercial heparin and appropriate composition. The third part involves several groups of enzymes to modify the heparosan into HS/heparin, including C5-epimerase, 2-O-sulfotransferase (2-OST), 3-O-sulfotransferase (3-OST), and 6-O-sulfotranferase (6-OST). The most common sequence of HS/heparin is the iduronic acid (IdoA)2S (14) GlcNS6S residue, trisulfated disaccharide, while the less commonly occurring sequence is IdoA/GlcA (or very rarely a GlcA2S) 14 linked to a GlcNAc or GlcNAc6S (or rarely a GlcNS3S6S) residue (Figure 1). HS is structurally similar to heparin [18].

[figure omitted; refer to PDF]

Although there are various advantages on biosynthesis of heparin, including extraction from nonlimited species, the product free from animal viruses' contamination and operations for better control, some problems still exist. The nonnegligible one is to match the clinical used products' chain length and structure with the standard heparins.

2.2. Low-Molecular-Weight Heparin (LMWH)

Low-molecular-weight heparin is a general term for heparin with an average molecular weight between 4500 and 5000Da [19] prepared by depolymerization of UH through chemical or enzymatic pathways. Since LMWH's chains are smaller than UH's, it shows several superiorities. For instance, compared to UH, LMWH has relatively lower binding performance with thrombin, macrophages, and endothelial cells, leading to a lower ability to decrease thrombin activity but a longer half-life in plasma [20]. Meanwhile, LMWH has better bioavailability at low doses and is more predictable between dose and response characteristics. Therefore, LMWH has been widely used in clinical practice.

A variety of the clinical used LMWH [21] is prepared from UH by chemical or enzymatic ways. There are four main methods of depolymerization, including nitrous acid depolymerization (e.g., nadroparin calcium, dalteparin sodium, reviparin sodium), benzylation followed by alkaline depolymerization (e.g., enoxaparin sodium), peroxidative depolymerization (e.g., ardeparin sodium), and heparinase enzymatic depolymerization (e.g., tinzaparin sodium). Although LMWH's chains are smaller than UH's, there are many chains that are not sufficient for liver's elimination, which might give rise to a hemorrhagic tendency. Hence, the analysis of LMWH's content and concentration is indispensable.

2.3. Ultralow-Molecular-Weight Heparin (ULMWH)

ULMWH has even smaller chain lengths in the range of 1.5 to 3.5kDa. Hence, ULMWH has a lower bleeding risk, longer plasma half-life, and better bioavailability as well as the ability to penetrate the blood-brain barrier. Clinical commonly used ULMWH such as fondaparinux sodium is usually chemically synthesized through one-pot synthesis [22]. Chemical synthesis can make the product purer and its structure more predictable, whereas by-products still exist so that the amounts of them are required to be quantified before launched on the market or clinical used.

3. Anticoagulant Mechanism of Heparins

Blood freely circulates in healthy individuals' arteries and veins. Nevertheless, as soon as the hemostatic system is

triggered, the biochemical cascade reactions leading to the hemostatic effects will take place with the aggregation of platelets and fibrin. Heparins have been regarded as effective anticoagulants for several decades. This part mainly elaborates the anticoagulant mechanism of heparins.

3.1. The Process of Hemostasis

Primary process of hemostasis is defined as the platelet plug's formation after blood vessel's damage. The platelet aggregation is mainly mediated by fibrinogen which binds to the platelet surface receptors, principally including glycoprotein (GP) receptor Ib. Furthermore, the connection between the platelet surface receptors and Von Willebrand factor (vWF) [23] on the endothelial collagen has put platelet and endothelium together, leading to the platelet adhesion. Meanwhile, the activated platelet's degranulation releases different kinds of factors including adenosine diphosphate (ADP), thromboxane A₂ (TXA₂) [24], and serotonin [25, 26] (Figure 2) that might have different impact on platelet including the platelet aggregation as well as vasoconstriction.

[figure omitted; refer to PDF]

Secondary process of hemostasis is defined as the insoluble fibrin's formation mediated by biochemical cascade reactions (Figure 2). There are two pathways in secondary hemostasis cascade reactions including the intrinsic and extrinsic pathways. The intrinsic pathway is triggered by coagulation factors XII and XI. Factor XII's local concentration increases when factor XII is attached to the damaged vessels' negatively charged surface where phospholipids exposed. Then, factor XII is activated to become factor XIIa by endodermal collagen. The extrinsic pathway, also called tissue factor pathway, is the prior in plasma-mediated hemostasis. The membrane protein tissue factor (TF) contacts with factor VII in plasma and then triggers the extrinsic pathway leading to the formation of TF-VIIa complex. TF-VIIa complex and factor XIIa coactivate and catalyze the transformation of factor from XI to IXa, while the TF-VIIa complex and factor IXa catalyze the conversion of factor X to factor Xa. Then, factor Xa catalyzes the formation of prothrombin (factor II) to thrombin (factor IIa). As the final serine protease cascade reactions starts, thrombin has multiple functions in coagulation including activating platelets, factors V, VIII, IX, protein C, and fibrinolysis inhibitor in order to promote the coagulation cascade reactions as well as convert fibrinogen into fibrin. The last step in the clotting process is the conversion from fibrin to insoluble fibrin clot, which is mediated by factor XIIIa [20] (Figure 3).

[figure omitted; refer to PDF]

3.2. Heparins' Anticoagulation

Antithrombin (AT) is a glycoprotein and a serine protease inhibitor produced by the liver, which can inhibit various activated proteases associated with coagulation, including TF-VIIa complex [27], factor IXa [28], factor Xa [29], and thrombin by covalently binding to their serine residues. Moreover, together with heparins, AT plays a key role in the inhibition of serine protease by forming a thrombin-AT-heparin ternary complex with the presence of thrombin. UH is commonly used in clinic which is extracted from animal tissues, mainly from porcine intestinal mucosa with the average molecular weight from 10 to 20kDa. UH's anticoagulation activity is mainly determined by its unique pentasaccharide sequence which gives it high affinity to bind with AT, whereas only about one-third of UH has the unique structure that could bind to AT, leading to only one-third of UH administered to patients having anticoagulant activity mediated by AT. UH accelerates the inhibition of factor Xa by endogenous AT. The close integration between UH and AT/thrombin gives rise to a more significant inhibition of thrombin activity. The clinical used LMWH is mostly prepared by UH's depolymerization containing the pentasaccharide sequences like UH, which leads to the same anticoagulant mechanism as UH. However, the heparins contain less than 18 saccharides making it unable to bind thrombin [30]. Above all, compared with UH and LMWH, ULMWH exerts effects by catalyzing the AT-mediated inhibition of factor Xa's activity. In addition to the combination with AT, heparins bind to heparin cofactor II and platelets leading to the inactivation of factor IIa [19].

4. Heparins' Therapeutic Applications

Heparins are suitable for many clinical indications, and the therapeutic applications can be mainly divided into the following two categories: anticoagulant [11] and anti-inflammatory.

4.1. Heparins' Therapeutic Application as Anticoagulants

Heparins' major clinical application is for the treatment of venous thromboembolism (VTE) including pulmonary embolism (PE) [31, 32] and deep venous thrombosis (DVT). UH has been the standard medication for the treatment of VTE for more than half a century, whereas it had been demonstrated that LMWH' dose-determined treatment was as safe and efficient [33] as UH's dose-adjusted treatment for PE [34] and DVT [35]. Hence, LMWH is the preferred drug of initial therapy in VTE. Dose-determined LMWH administration for once or twice daily shows the convenience that pharmacokinetic characteristics are more predictable without the routine laboratory monitoring [33], whereas there are several situations that UH may be more suitable, such as when patients are at high risk of bleeding or with impaired renal function. When using UH, the anticoagulant effect can be terminated more quickly as soon as the infusion is stopped and if necessary, protamine's reverse effect on coagulation is more significant in patients at risk for bleeding. Moreover, LMWH's drug clearance tends to be reduced, which would make LMWH prone to bleeding, especially in impaired-liver-function patients [12, 36]. During the treatment of VTE, UH or LMWH is given via intravenous or subcutaneous injection. Depending on the severity of VTE, the therapeutic approach and dose would be different. For example, the initial treatment for patients at moderate risk of PE [37] requires intravenous or subcutaneous UH, or subcutaneous LMWH for the first 5 to 10 days, a dosage varying from 300IU/kg for UH or 170–200IU/kg for LMWH, respectively.

There are special cases in patients with VTE, who have pregnancy or suffer from cancer. Compared with nonpregnant women, the risk of VTE increases approximately fivefold in pregnant women and the risk remains increased until about 12 weeks postpartum, leading to about 10% maternal deaths in developed countries [38]. Both UH and LMWH are safe for the treatment and prophylaxis of VTE [39] in patients with pregnancy, because they do not pass the blood brain barrier (BBB), whereas the use of vitamin K antagonists (VKAs) capable of crossing the BBB will cause teratogenicity in the first trimester and intracranial hemorrhage in the third trimester in infants. Furthermore, compared with UHs, LMWHs are the preferred agents and could be injected subcutaneously twice a day, for they are less likely to cause the side effects such as thrombocytopenia or osteoporosis [20, 40].

The risk of VTE increases approximately fourfold in patients with cancer when compared with other patients. Furthermore, the mechanism of antitumor property in heparins is mainly due to the inhibition in the formation of blood vessels around tumors, since the growth of tumor larger than 1 mm³ is dependent on angiogenesis [20]. LMWH is the primary therapeutic agents of VTE therapy for cancer patients, including the treatment of catheter-related thrombus [40]. The initial duration of treatment with LMWH will last for 3–6 months, and the treatment could be continued indefinitely if the cancer is not cured for cancer patients.

4.2. Heparins' Therapeutic Application as Anti-Inflammatory Agents

Heparins can be administered intranasally besides intravenous and subcutaneous medication, which could work as anti-inflammation drugs. Heparins could inhibit the release of inflammatory cell-derived mediators and the recruitment of inflammatory cells to tissues by modulating the interaction between leukocytes and vascular endothelial cells, leading to the inhibition of nasal airway pressure [12], leukocyte infiltration, eosinophilic cell migration, and eosinophilic cationic protein. Hence, it has been demonstrated that the intranasal administration of heparin might be used in the treatment of allergic rhinitis and asthma [20].

Despite the widespread application of heparins, several adverse reactions still exist, such as hemorrhage, heparin-induced thrombocytopenia [41], osteoporosis [42], elevation of transaminases, and hypersensitivity [20]. Hence, the clinical analysis and trials of heparins are indispensable.

5. Analysis of Heparins

5.1. Preclinical Samples Analysis

Heparins have been widely used as anticoagulant drugs, whereas heparins are extracted or synthesized from different sources, with different molecular weight. And there are heparins-induced adverse reactions existing during the clinical treatment. Furthermore, the adulteration of OSCS (Figure 4(a)) in heparin (Figure 4(b)) gives rise to anaphylactic shock, resulting in a global crisis [4]. Hence, the detection and quantification of heparins before being applied to clinical practice is indispensable. There are several methods for the analysis of heparins, including single technique and multiple combinations of techniques for detection and analysis (Table 1).

[figures omitted; refer to PDF]

Table 1

The characteristics of routine heparin analysis and detection methods.

Meth od	Samples	Purpose	Loading quantity	LOD/LOQ	Ref nr
NMR	Standard s	To quantify the degree of porcine derived heparin adulteration with bovine derived heparin	70mg/mL	2% w/w	[43]
MS	Tissue/st andards	To identify the disaccharide components retaining the differential isomerization configuration of the parental heparin chain	—	—	[44]
CE	Standard s	To quantify the degree of heparin adulteration with OSCS	5-10mg/mL	0.05% w/w	[45]
PAG E	Tissue/st andards	To quantify the degree of heparin adulteration with OSCS	0.16-4 mg/mL	0.1–5% w/w	[45]
SAX - HPL C	Standard s	To analyze the structure and content of oligosaccharides after digesting heparin by heparinases	2.5mg/mL	LOD 0.1% LOQ 0.3%	[46]
WAX - HPL C	Standard s	To isolate and quantify the degree of heparin adulteration with OSCS	1 mg/mL	0.025–0.0 75% w/w	[47]
SEC	Standard s	To quantitatively analyze the molecular weight of UH and LMWH	0.02mg/mL	—	[48]
LC- MS	Standard s	To detect heparin mixture derived from different sources by analyzing the composition difference of disaccharide and tetrasaccharide	Disaccharide: 1 mg/mL. Tetrasaccharide: 2mg/mL	—	[49]
HPL C- UV	Tissue/st andards	To analyze the disaccharide and tetrasaccharide composition of heparin oligosaccharides	Disaccharide: 250 µg/mL tetrasaccharide: 5mg/mL	—	[50]
CE- MS	Standard s	To analyze the disaccharide components of heparin oligosaccharides	100 µg/mL	—	[51]

5.1.1. Single Techniques for Detection and Analysis of Heparins

(1). *Nuclear Magnetic Resonance (NMR)*. NMR is a physical process in which the spin level of a nucleus with a nonzero magnetic moment shows Zeeman splitting under the action of an external magnetic field, and a certain

radio frequency of saccharide is absorbed by resonance. NMR spectroscopy has been recognized as a powerful analytical method for over 60 years. NMR spectral parameters such as the chemical shift, the J-coupling, nuclear Overhauser effects, and residual dipolar couplings convert into angular or distance data and then provide conformation at the atomic level [52]. In heparins' analysis, samples are prepared by dissolving in D₂O and lyophilizing to remove exchangeable hydrogen from the backbone of the structure. Then, the samples are redissolved in D₂O to minimize the HDO peak in the NMR spectrum [49, 50]. Through one-dimensional (1D) ¹H-NMR spectra and two-dimensional (2D) heteronuclear single quantum coherence spectroscopy- (HSQC-) NMR, St Ange et al. [50] verified the structural similarities and differences between bovine and porcine heparins. By the method of NMR, it reveals that fact of the glucosamine substitution and chiral properties of uronic acid residues in heparin and LMWH [53].

(2). *Mass Spectrometry*. In the process of mass spectrometry, each component of the samples is ionized in the ion source to generate charged ions with different ratios of charge to mass, and the ion beam is formed under the action of the accelerating electric field and then entering the mass analyzer [54]. In the mass analyzer, electric and magnetic fields are used to generate the opposite velocity dispersion, and they are, respectively, focused to obtain the mass spectrum, so as to determine the molecular weight of the components. Ola et al. [55] developed a method which combined electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MSⁿ). In the study, heparins were depolymerized by heparin lyases, and the mixture was compatible with ESI-MS and MSⁿ without any further purification. Through the analysis of the substance of mixture and standard by using the combination of ESI-MS and MSⁿ techniques, it obtained the composition analysis and quantification of eight commercially available disaccharides derived from bovine and porcine heparins.

(3). *Electrophoresis*. Electrophoresis is a technique in which charged particles are separated by moving at different speeds in an electric field. Since the adulteration of heparins with OSCS, methods for heparins purity analysis have been developed rapidly [4], including capillary electrophoresis (CE) and polyacrylamide gel electrophoresis [12]. The mechanism of CE separation is based on the ratio of molecular charge to volume which is particularly effective for the analysis of polyanions such as heparin and OSCS [45]. Somsen et al. [56] replaced sodium ions with Tris in the high concentration buffer, realizing the effective separation of heparin and OSCS. CE is also an effective method to characterize heparin-bovine serum albumin (BSA) interaction, which has been carried out by microfluidic chip [57]. PAGE uses polyacrylamide gel as the supporting medium which has a network structure, and molecular sieve effect. Hence, PAGE becomes an efficient method to separate heparins and their impurity, mainly OSCS, by exploiting the different properties in their molecular sizes, conformations, and charges [45].

(4). *High-Performance Liquid Chromatography (HPLC)*. HPLC takes liquid as the mobile phase and uses a high-pressure infusion system to pump single solvent with different polarity or mixed solvents and buffers or other mobile phases into a column equipped with a chromatographic column. After each component in the column is separated, it enters the detector for inspection, realizing the analysis of sample. There are two main types of HPLC which are involved in the analysis of heparins including strong anion-exchange (SAX)-HPLC and weak anion-exchange (WAX)-HPLC. In SAX-HPLC, because of the reversible ionic interaction between a charged analyte and oppositely charged stationary phase, the analyte with low charge and small size will be eluted first. In heparins' analysis, highly negatively charged GAG is preserved on anion-exchange columns and then eluted with gradient inorganic salt solution with high ionic strength [47]. Miller et al. [58] developed a SAX method using volatile salt buffer in the process of UH analysis, realizing the purification of heparin oligosaccharides and the subsequent MS analysis. The mechanism of WAX-HPLC is similar to that of SAX-HPLC; the main difference is that the interaction between the analyte and the stationary phase is relatively weaker. Hashii et al. [59] isolated heparins and OSCS from heparin sodium and heparin calcium by WAX.

(5). *Size Exclusion Chromatography (SEC)*. SEC is a chromatography technique in which molecules are separated according to the size of the samples. In the field of heparins' analysis, Ouyang et al. [48] used multiple angle laser scattering (MALS) and refractive index (RI) in SEC to detect the difference in light scattering intensity between the background and the chromatographic analytes, which is vital to calculate the accurate molecular weight of UH and

LMWH. Gel permeation chromatography (GPC) also belongs to the method of SEC, which uses organic solvents as the mobile phase passing the measured polymer solution through a column with different pore sizes to measure the molecular weight of heparins [50].

5.1.2. Multiple Combinations of Techniques for Detection and Analysis of Heparins

(1). *Liquid Chromatography- (LS-) MS*. LS-MS is a combination of LS and MS, LS can effectively separate the organic compounds in the samples, and MS is able to analyze the separated organic compounds in order to obtain the information on their molecular weight, structures, and concentrations. It is worth noting that, before the LS-MS heparin analysis, the samples need to be boiled and centrifuged to remove the heparinase; then the supernatants required to be lyophilized and redissolved [49, 53]. Ouyang et al. [48] developed LC-MC analysis method which can detect adulterant of 10% bovine or ovine heparin in porcine heparin.

(2). *HPLC-Ultraviolet Spectroscopy*. HPLC is combined with spectroscopy in the form of the UV detector, turning into a powerful detection technique [60]. The mechanism of UV detector is to absorb ultraviolet light to determine the individual quantities of each component in the samples. The amount of UV light absorbed by each component is measured by passing UV light through the eluted samples. St Ange et al. [50] developed a method combined with HPLC-UV spectroscopy to analyze the disaccharide composition after the depolymerization of heparins derived from bovine and porcine.

(3). *CE-MS*. CE is a technique of electrophoresis based on the separated differences of components in mobility and distribution behavior. MS is a technique for quantitative and structural analysis by measuring the mass and strength of the ions in the samples. Combined techniques CE-MS has shown high efficiency and sensitivity for the analysis of heparin oligosaccharides and LMWH [61, 62]. Ouyang et al. [49] developed a CE-MS method, in which the jobs involved with the removal of sulfonic group and the selective chemical derivation of oligosaccharides are not required, compared to LS-MS. Furthermore, the oligosaccharides of heparin have a large negative charge, which are ideal preconditions for tandem mass spectrometry analysis [51]. Researches have also shown that, by using a novel electrokinetic CE-MS interface, the analysis of GAG oligosaccharides from heparin disaccharide to LMWH efficiency has been greatly improved, leading to the generation of high sensitivity and throughput analysis for heparins [61].

5.1.3. New Techniques for Detection and Analysis of Heparins

(1). *Fluorescent Probe*. Fluorescence detection of biomolecules has attracted great interest because of its high sensitivity [63], high selectivity [64], high throughput [65], and low cost [66]. Short peptides composed of less than three to four amino acid residues can self-assemble in aqueous solution to form definite nanostructures. Lee et al. [67] synthesized a heparin-induced self-assembly fluorescent peptide probe with an aggregation-induced emission fluorophore, which was able to form nanoscale aggregates binding with heparins in order to measure the amount of heparins. Since OSCS has an inhibitory effect on heparinase, Mehta et al. [68] had developed a method which was able to detect as low as 0.0001% of OSCS contamination in heparins by adding heparinase into the mixture of heparins and detected the fluorescent change. If heparins were contaminated by OSCS, the changes in fluorescence intensity were reduced compared to the uncontaminated standard.

Cheng et al. [69] developed a composite which was prepared from graphite carbon nitride quantum dots, and silver nanoparticles. The fluorescence of the composite was strongly decreased under the coating of polyethylenimine (PEI), whereas PEI had a higher affinity for heparins than the composite, so the fluorescence of the system was enhanced after adding heparins. Moreover, the composite was also applied to the fluorometric determination of heparins, such as the detection of heparin in human serum samples.

There are also other types of visible fluorescence immunoassay impregnated on paper that may have great potential for heparin detection. Huang et al. [70] developed a paper-based analytical device (PAD) that Ag^+ fluorescently quenched CdTe quantum dots (QD) on nitrocellulose films by distance dependent silver ion exchange reaction, followed by Sandwich immune reactions between silver nanoparticles labelled secondary antibodies and the microtitration pores coated with primary antibodies to test human serum samples. Meanwhile, Lv et al. [71] produced wet NH_3 by sandwich immunoassay using highly functionalized gold nanoparticles with glutamate dehydrogenase

and secondary antibody. The wet NH_3 could trigger the structural change of the paper impregnation analysis device coated with NH2-MIL-125 (Ti), which showed good visible fluorescence intensity. Moreover, Qiu et al. [72] developed an integrated PAD that combined DNA-gated mesoporous silica nanocontainers with biological controlled release systems for the visual fluorescence detection of CdTe/CdSe QD-enzyme impregnated paper.

(2). *Gold Nanomaterial Labelled Sensors*. There are mainly two kinds of gold nanomaterials for the detection of heparins, including gold nanoparticles and gold nanorods. Gold nanomaterials have unique optical properties and their light scattering is clearly stronger than that the emission organic dyes [73].

Fu et al. [74] developed an ultrasensitive detection method based on the self-assembly of gold nanoparticles (AuNPs) on the surface of graphene oxide (GO) with the polycationic protamine as medium. The electrostatic interaction between protamine and gold nanoparticles induced the self-assembly of gold nanoparticles (AuNPs) on the surface of GO accompanied by AuNPs turning into blue. Due to the strong affinity with protamine, heparins with polyanion were interfered with the self-assembly of AuNPs. Hence, with the increase of heparins concentration, the number of AuNPs self-assembly decreased and the color of AuNPs color changed from blue to red. Wang et al. [73] developed the plasmonic gold nanorods (AuNRs) as light scattering probes to detect the concentration of heparins. In the reaction, the Au atoms in AuNRs were oxidized by Fe^{3+} to Au^+ , which then formed AuTu_2 with thiourea. Then, the reaction was promoted by heparins to form a complex with AuTu_2 due to the strong electrostatic interaction. During this process, the light of AuNRs scattered from red to green. And the time taken for light scattering depended on the concentration of heparins.

AuNPs are able to greatly improve the sensitivity of detection by interacting with other materials, which can be used for reference to the future application of heparin detection.

Tang Group [75] developed a label-free adapter sensor based on the change in color/absorbance induced by salt-induced aggregation of AuNPs in order to detect the samples of adenosine 5'-triphosphate (ATP). After the addition of the target ATP, the aptamer-based hairpin probe would expose a new sticky end for the strand-displacement reaction with another complementary hairpin leading to the decreasing single-stranded DNA and the formation of double-stranded DNA, which would release more AuNPs, triggering a red-to-blue color change by salt-induced aggregation of AuNPs. The sensor with high sensitivity was able to detect the concentration of ATP as low as 1.0 nM. Furthermore, this technique could be applied to the quantitative detection of other biomolecules by changing the sequence of the corresponding aptamer.

Clinical drug detection is mainly aiming at the quantitative analysis of drug content in serum. Cai et al. [76] developed a sensor that could detect sensitive photoelectrochemical (PEC) aptasensing from human serum samples by exciton plasma interaction between graphene nanosheets (GN) coated with AuNPs and CdS QDs. This sensor has good reproducibility and high sensitivity with the limit of detection of antigen in 0.52 pg/mL.

Zhuang et al. [77] developed a PEC sensing platform to detect the T4 polynucleotide kinase activity based on AuNPs decorated $\text{g-C}_3\text{N}_4$ nanosheets after the deoxyribozyme-mediated catalytic precipitation amplification. Meanwhile, due to the introduction of AuNPs on the surface of $\text{g-C}_3\text{N}_4$, the surface plasmon resonance enhanced the light collection and the separation of the photo-generated e^-/h^+ pairs leading to its highly sensitive activity detection. Furthermore, Lv et al. [78] developed the sensor which was assembled on the 3D printing device based on the rolling ring amplification. By using D-A F8BT/ $\text{g-C}_3\text{N}_4$ type II heterojunction nanocomposite as the photoelectrode material, this sensor improved the photocurrent effect of $\text{g-C}_3\text{N}_4$, enhanced the built-in electric field, and promoted the generation of electron holes with the local plasma resonance of AuNPs, which further improved the photoelectric effect leading to a highly sensitive detection.

5.2. Clinical Sample Analysis of Heparins

The usual samples for clinical heparins' analysis are patients' plasma or serum. The measurable indicators mainly include D-dimer and activated partial thromboplastin time (APTT). D-dimer is a reliable and sensitive indicator of fibrin deposition and stabilization, which is also a sign of thrombosis. The concentration of D-dimer measured by enzyme linked immunosorbent assay (ELISA) can be used to semiquantitatively analyze heparins in plasma, whereas D-dimer also presents a high concentration in certain conditions unrelated to thrombosis, leading to a false

positive result without symptoms of thrombosis, which might give an inaccurate analysis of heparins [79]. The APTT assay is performed on platelet poor plasma (PPP) and then heated to 37°C by adding to preheated phospholipid and a contact activator. Subsequently, the mixture was incubated for 3–5 minutes to activate the contact pathway; then, calcium was added to begin clotting. APTT is the time from adding calcium to the formation of fibrin clots. Furthermore, there is a good correlation between APTT and heparin dose; hence, APTT can roughly define the plasma heparin level [30].

Furthermore, there are new detection methods applied in clinic. Jang et al. [80] developed the self-assembled conjugated fluorescent micelles which amplified the fluorescence response to heparins and had been applied to the highly sensitive detection of heparins in serum. Meanwhile, AuNPs with the same principle as the above gold nanomaterials had been applied in the clinical detection of heparins. Yuan et al. [81] developed a high sensitivity SPR sensor to detect heparins in serum in a portable optical fiber SPR sensing system. Hence, the sensor is expected to be a practical tool for real-time monitoring of heparins.

6. Conclusions

There are many effective methods for clinical used heparins' analysis, including both single techniques: NMR, MS, CE, PAGE, HPLC, and SEC and multiple combinations of techniques: LS-MS, HPLC-UV, and CE-MS, which mainly detect the D-dimer concentration and APTT in the analysis of clinical samples. Despite the fact that several new technologies including fluorescent probe and gold nanomaterial labelled sensors have been developed to accurately detect heparin, there is still plenty of room to investigate more high-sensitive, high-throughput, and rapid clinical methods for analysis of heparins. Furthermore, heparins have not only been shown to have powerful therapeutic effects in coagulation related diseases, but also been regarded as a therapy for COVID-19 through antiviral and anti-inflammatory effects [82, 83]. Hence, component analysis of heparin in patients' body fluids and comprehensive efficacy assessment are the top priority for follow-up work.

Authors' Contributions

Binjie Li and Huimin Zhao contributed equally to this work by writing the first draft.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31961133004), the Swedish Research Council (2018-02503 and 2018-06016), projects funded by the China Postdoctoral Science Foundation (2020M670109 and 2020TQ0029), and Beijing Advanced Innovation Center for Soft Matter Science and Engineering. Figures 1, 2, and 3 were created with tools obtained from BioRender.com.

References

- [1] T. W. Barrowcliffe, "History of heparin," *Heparin - A Century of Progress*, vol. 207, DOI: 10.1007/978-3-642-23056-1_1, 2012.
- [2] A. M. Tovar, L. A. Teixeira, S. M. Rembold, M. Leite, J. R. Lugon, P. A. Mourão, "Bovine and porcine heparins: different drugs with similar effects on human haemodialysis," *BMC Research Notes*, vol. 6 no. 1, DOI: 10.1186/1756-0500-6-230, 2013.
- [3] C. S. Ong, J. A. Marcum, K. J. Zehr, D. E. Cameron, "A century of heparin," *The Annals of Thoracic Surgery*, vol. 108 no. 3, pp. 955-958, DOI: 10.1016/j.athoracsur.2019.03.104, 2019.
- [4] S. Beni, J. F. K. Limtiaco, C. K. Larive, "Analysis and characterization of heparin impurities," *Analytical and Bioanalytical Chemistry*, vol. 399 no. 2, pp. 527-539, DOI: 10.1007/s00216-010-4121-x, 2011.
- [5] E. Jorpes, S. Bergström, "On the relationship between the sulphur content and the anticoagulant activity of heparin preparations," *Biochemical Journal*, vol. 33 no. 1, pp. 47-52, DOI: 10.1042/bj0330047, 1939.
- [6] J. Casale, J. S. Crane, *Biochemistry, Glycosaminoglycans*, 2020.
- [7] B. Dudas, K. Semeniken, "Glycosaminoglycans and neuroprotection," *Heparin - A Century of Progress*, vol. 207, pp. 325-343, DOI: 10.1007/978-3-642-23056-1_14, 2012.
- [8] T. C. Laurent, A. Tengblad, L. Thunberg, M. Höök, U. Lindahl, "The molecular-weight-dependence of the anti-coagulant activity of heparin," *Biochemical Journal*, vol. 175 no. 2, pp. 691-701, DOI: 10.1042/bj1750691, 1978.
- [9] O. Achour, N. Poupard, N. Bridiau, "Anti-heparanase activity of ultra-low-molecular-weight heparin produced by

- physicochemical depolymerization," *Carbohydrate Polymers*, vol. 135, pp. 316-323, DOI: 10.1016/j.carbpol.2015.08.041, 2016.
- [10] X. Zhou, L. Li, R. J. Linhardt, J. Liu, "Neutralizing the anticoagulant activity of ultra-low-molecular-weight heparins using N-acetylglucosamine 6-sulfatase," *FEBS Journal*, vol. 280 no. 10, pp. 2523-2532, DOI: 10.1111/febs.12169, 2013.
- [11] H. Nader, C. Lopes, H. Rocha, E. Santos, C. Dietrich, "Heparins and heparinoids: occurrence, structure and mechanism of antithrombotic and hemorrhagic activities," *Current Pharmaceutical Design*, vol. 10 no. 9, pp. 951-966, DOI: 10.2174/1381612043452758, 2004.
- [12] B. Mulloy, J. Hogwood, E. Gray, R. Lever, C. P. Page, "Pharmacology of heparin and related drugs," *Pharmacological Reviews*, vol. 68 no. 1, pp. 76-141, DOI: 10.1124/pr.115.011247, 2016.
- [13] G. R. C. Santos, A. M. F. Tovar, N. V. M. Capillé, M. S. Pereira, V. H. Pomin, P. A. S. Mourão, "Structural and functional analyses of bovine and porcine intestinal heparins confirm they are different drugs," *Drug Discovery Today*, vol. 19 no. 11, pp. 1801-1807, DOI: 10.1016/j.drudis.2014.07.004, 2014.
- [14] J. Kreuger, L. Kjellén, "Heparan sulfate biosynthesis," *Journal of Histochemistry & Cytochemistry*, vol. 60 no. 12, pp. 898-907, DOI: 10.1369/0022155412464972, 2012.
- [15] P. Carlsson, L. Kjellén, "Heparin biosynthesis," *Heparin-A Century of Progress*, vol. 207, pp. 23-41, DOI: 10.1007/978-3-642-23056-1_2, 2012.
- [16] U. Bhaskar, E. Sterner, A. M. Hickey, "Engineering of routes to heparin and related polysaccharides," *Applied Microbiology and Biotechnology*, vol. 93 no. 1, DOI: 10.1007/s00253-011-3641-4, 2012.
- [17] N. E. Ustyuzhanina, M. I. Bilan, N. E. Nifantiev, A. I. Usov, "Structural analysis of holothurian fucosylated chondroitin sulfates: degradation versus non-destructive approach," *Carbohydrate Research*, vol. 476, DOI: 10.1016/j.carres.2019.02.008, 2019.
- [18] U. Lindahl, L. Kjellén, "Heparin or heparan sulfate - what is the difference?," *Thrombosis and Haemostasis*, vol. 66 no. 01, pp. 044-048, DOI: 10.1055/s-0038-1646372, 1991.
- [19] J. Hirsh, S. S. Anand, J. L. Halperin, V. Fuster, "Guide to anticoagulant therapy: heparin," *Circulation*, vol. 103 no. 24, pp. 2994-3018, DOI: 10.1161/01.cir.103.24.2994, 2001.
- [20] A. Onishi, K. St Ange, J. S. Dordick, R. J. Linhardt, "Heparin and anticoagulation," *Frontiers in Bioscience (Landmark Edition)*, vol. 21, pp. 1372-1392, DOI: 10.2741/4462, 2016.
- [21] K. Norrby, "Low-molecular-weight heparins and angiogenesis," *APMIS-cta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 114 no. 2, pp. 79-102, DOI: 10.1111/j.1600-0463.2006.apm_235.x, 2006.
- [22] Z. Liu, S. Ji, J. Sheng, F. Wang, "Pharmacological effects and clinical applications of ultra low molecular weight heparins," *Drug Discoveries & Therapeutics*, vol. 8 no. 1, DOI: 10.5582/ddt.8.1, 2014.
- [23] J. O'Donnell, V. W. F. Low, "Insights into pathogenesis, diagnosis, and clinical management," *Blood Advances*, vol. 4 no. 13, pp. 3191-3199, DOI: 10.1182/bloodadvances.2020002038, 2020.
- [24] D. Rucker, A. S. Dhamoon, *Physiology, Thromboxane A2*, 2020.
- [25] D. J. David, A. M. Gardier, "Les bases de pharmacologie fondamentale du système sérotoninergique: application à la réponse antidépressive," *L'Encéphale*, vol. 42 no. 3, pp. 255-263, DOI: 10.1016/j.encep.2016.03.012, 2016.
- [26] G. Fouquet, T. Coman, O. Hermine, F. Côté, "Serotonin, hematopoiesis and stem cells," *Pharmacological Research*, vol. 140, pp. 67-74, DOI: 10.1016/j.phrs.2018.08.005, 2019.
- [27] L. Spiezia, E. Campello, F. D. Valle, B. Woodhams, P. Simioni, "Factor VIIa-antithrombin complex: a possible new biomarker for activated coagulation," *Clinical Chemistry and Laboratory Medicine (CCLM)*, vol. 55 no. 4, pp. 484-488, DOI: 10.1515/cclm-2016-0399, 2017.
- [28] T. Zhang, P. Andre, T. J. Bateman, "Development of a novel class of potent and selective FIXa inhibitors," *Bioorganic & Medicinal Chemistry Letters*, vol. 25 no. 21, pp. 4945-4949, DOI: 10.1016/j.bmcl.2015.04.057, 2015.
- [29] A. N. Khadse, M. K. Sharma, P. R. Murumkar, S. J. Rajput, M. R. Yadav, "Advances in the development of novel factor Xa inhibitors: a patent review," *Mini-Reviews in Medicinal Chemistry*, vol. 18 no. 16, pp. 1332-1353,

DOI: 10.2174/1389557518666180424120726, 2018.

- [30] I. Baluwala, E. J. Favaloro, L. Pasalic, "Therapeutic monitoring of unfractionated heparin - trials and tribulations," *Expert Review of Hematology*, vol. 10 no. 7, pp. 595-605, DOI: 10.1080/17474086.2017.1345306, 2017.
- [31] S. V. Konstantinides, G. Meyer, C. Becattini, "2019 ESC Guidelines for the diagnosis and management of acute pulmonary embolism developed in collaboration with the European respiratory society (ERS)," *European Respiratory Journal*, vol. 54 no. 3, DOI: 10.1183/13993003.01647-2019, 2019.
- [32] S. V. Konstantinides, A. Torbicki, G. Agnelli, "ESC guidelines on the diagnosis and management of acute pulmonary embolism," *European Heart Journal*, vol. 35 no. 43, pp. 3033-3069, DOI: 10.1093/eurheartj/ehu283, 2014.
- [33] R. G. Holzheimer, "Low-molecular-weight heparin (LMWH) in the treatment of thrombosis," *European Journal of Medical Research*, vol. 9 no. 4, pp. 225-239, 2004.
- [34] J. Leentjens, M. Peters, A. C. Esselink, Y. Smulders, C. Kramers, "Initial anticoagulation in patients with pulmonary embolism: thrombolysis, unfractionated heparin, LMWH, fondaparinux, or DOACs?," *British Journal of Clinical Pharmacology*, vol. 83 no. 11, pp. 2356-2366, DOI: 10.1111/bcp.13340, 2017.
- [35] P. C. Kruger, J. W. Eikelboom, J. D. Douketis, G. J. Hankey, "Deep vein thrombosis: update on diagnosis and management," *Medical Journal of Australia*, vol. 210 no. 11, pp. 516-524, DOI: 10.5694/mja2.50201, 2019.
- [36] F. Atiq, P. M. L. A. van den Bemt, F. W. G. Leebeek, T. van Gelder, J. Versmissen, "A systematic review on the accumulation of prophylactic dosages of low-molecular-weight heparins (LMWHs) in patients with renal insufficiency," *European Journal of Clinical Pharmacology*, vol. 71 no. 8, pp. 921-929, DOI: 10.1007/s00228-015-1880-5, 2015.
- [37] A. T. Cohen, M. Dobromirski, M. M. P. Gurwith, "Managing pulmonary embolism from presentation to extended treatment," *Thrombosis Research*, vol. 133 no. 2, pp. 139-148, DOI: 10.1016/j.thromres.2013.09.040, 2014.
- [38] A. L. Marshall, "Diagnosis, treatment, and prevention of venous thromboembolism in pregnancy," *Postgraduate Medicine*, vol. 126 no. 7, pp. 25-34, DOI: 10.3810/pgm.2014.11.2830, 2014.
- [39] K. Kleinschmidt, R. Charles, "Pharmacology of low molecular weight harparins," *Emergency Medicine Clinics of North America*, vol. 19 no. 4, pp. 1025-1049, DOI: 10.1016/s0733-8627(05)70233-9, 2001.
- [40] Y. Dargaud, L. Rugeri, M. C. Vergnes, "A risk score for the management of pregnant women with increased risk of venous thromboembolism: a multicentre prospective study," *British Journal of Haematology*, vol. 145 no. 6, pp. 825-835, DOI: 10.1111/j.1365-2141.2009.07698.x, 2009.
- [41] P. Lingamaneni, S. Gonakoti, K. Moturi, I. Vohra, M. Zia, "Heparin-induced thrombocytopenia in COVID-19," *Journal of Investigative Medicine High Impact Case Reports*, vol. 8, DOI: 10.1177/2324709620944091, 2020.
- [42] O. Topal, M. Çina Aksoy, İ. M. Ciriş, D. K. Doğuç, S. Sert, S. Çömlekçi, "Assessment of the effect of pulsed electromagnetic field application on the healing of bone defects in rats with heparin-induced osteoporosis," *Electromagnetic Biology and Medicine*, vol. 39 no. 3, pp. 206-217, DOI: 10.1080/15368378.2020.1762636, 2020.
- [43] Y. B. Monakhova, B. W. K. Diehl, J. Fareed, "Authentication of animal origin of heparin and low molecular weight heparin including ovine, porcine and bovine species using 1D NMR spectroscopy and chemometric tools," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 149, pp. 114-119, DOI: 10.1016/j.jpba.2017.10.020, 2018.
- [44] T. Zhang, X. Liu, H. Li, "Characterization of epimerization and composition of heparin and dalteparin using a UHPLC-ESI-MS/MS method," *Carbohydrate Polymers*, vol. 203, pp. 87-94, DOI: 10.1016/j.carbpol.2018.08.108, 2019.
- [45] N. Volpi, F. Maccari, J. Suwan, R. J. Linhardt, "Electrophoresis for the analysis of heparin purity and quality," *Electrophoresis*, vol. 33 no. 11, pp. 1531-1537, DOI: 10.1002/elps.201100479, 2012.
- [46] F. Spelta, L. Liverani, A. Peluso, "SAX-HPLC and HSQC NMR spectroscopy: orthogonal methods for characterizing heparin batches composition," *Frontiers in Medicine*, vol. 6, DOI: 10.3389/fmed.2019.00078, 2019.
- [47] J. M. Fasciano, N. D. Danielson, "Ion chromatography for the separation of heparin and structurally related glycoaminoglycans: a review," *Journal of Separation Science*, vol. 39 no. 6, pp. 1118-1129, DOI:

10.1002/jssc.201500664, 2016.

[48] Y. Ouyang, Y. Zeng, L. Yi, "Qualitative and quantitative analysis of heparin and low molecular weight heparins using size exclusion chromatography with multiple angle laser scattering/refractive index and inductively coupled plasma/mass spectrometry detectors," *Journal of Chromatography A*, vol. 1522, pp. 56-61, DOI: 10.1016/j.chroma.2017.09.040, 2017.

[49] Y. Ouyang, X. Han, Y. Yu, "Chemometric analysis of porcine, bovine and ovine heparins," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 164, pp. 345-352, DOI: 10.1016/j.jpba.2018.10.052, 2019.

[50] K. St Ange, A. Onishi, L. Fu, "Analysis of heparins derived from bovine tissues and comparison to porcine intestinal heparins," *Clinical and Applied Thrombosis/Hemostasis*, vol. 22 no. 6, pp. 520-527, DOI: 10.1177/1076029616643822, 2016.

[51] L. Lin, X. Liu, F. Zhang, "Analysis of heparin oligosaccharides by capillary electrophoresis-negative-ion electrospray ionization mass spectrometry," *Analytical and Bioanalytical Chemistry*, vol. 409 no. 2, pp. 411-420, DOI: 10.1007/s00216-016-9662-1, 2017.

[52] D. Marion, "An introduction to biological NMR spectroscopy," *Molecular & Cellular Proteomics*, vol. 12 no. 11, pp. 3006-3025, DOI: 10.1074/mcp.O113.030239, 2013.

[53] X. Liu, K. St Ange, X. Wang, "Parent heparin and daughter LMW heparin correlation analysis using LC-MS and NMR," *Analytica Chimica Acta*, vol. 961, pp. 91-99, DOI: 10.1016/j.aca.2017.01.042, 2017.

[54] W. Bittremieux, D. L. Tabb, F. Impens, "Quality control in mass spectrometry-based proteomics," *Mass Spectrometry Reviews*, vol. 37 no. 5, pp. 697-711, DOI: 10.1002/mas.21544, 2018.

[55] O. M. Saad, J. A. Leary, "Compositional analysis and quantification of heparin and heparan sulfate by electrospray ionization ion trap mass spectrometry," *Analytical Chemistry*, vol. 75 no. 13, pp. 2985-2995, DOI: 10.1021/ac0340455, 2003.

[56] G. W. Somsen, Y. H. Tak, J. S. Toraño, P. M. J. M. Jongen, G. J. de Jong, "Determination of oversulfated chondroitin sulfate and dermatan sulfate impurities in heparin by capillary electrophoresis," *Journal of Chromatography A*, vol. 1216 no. 18, pp. 4107-4112, DOI: 10.1016/j.chroma.2009.02.063, 2009.

[57] X. Liu, X. Liu, A. Liang, "Studying protein-drug interaction by microfluidic chip affinity capillary electrophoresis with indirect laser-induced fluorescence detection," *Electrophoresis*, vol. 27 no. 15, pp. 3125-3128, DOI: 10.1002/elps.200500890, 2006.

[58] R. L. Miller, S. E. Guimond, M. Shivkumar, "Heparin isomeric oligosaccharide separation using volatile salt strong anion exchange chromatography," *Analytical Chemistry*, vol. 88 no. 23, pp. 11542-11550, DOI: 10.1021/acs.analchem.6b02801, 2016.

[59] N. Hashii, N. Kawasaki, S. Itoh, "Heparin identification test and purity test for OSCS in heparin sodium and heparin calcium by weak anion-exchange high-performance liquid chromatography," *Biologicals*, vol. 38 no. 5, pp. 539-543, DOI: 10.1016/j.biologicals.2010.04.002, 2010.

[60] O. Guvench, A. D. MacKerell, "Comparison of protein force fields for molecular dynamics simulations," *Methods in Molecular Biology*, vol. 443, pp. 63-88, DOI: 10.1007/978-1-59745-177-2_4, 2008.

[61] X. Sun, L. Lin, X. Liu, "Capillary electrophoresis-mass spectrometry for the analysis of heparin oligosaccharides and low molecular weight heparin," *Analytical Chemistry*, vol. 88 no. 3, pp. 1937-1943, DOI: 10.1021/acs.analchem.5b04405, 2016.

[62] P. Sanderson, M. Stickney, F. E. Leach, "Heparin/heparan sulfate analysis by covalently modified reverse polarity capillary zone electrophoresis-mass spectrometry," *Journal of Chromatography A*, vol. 1545, pp. 75-83, DOI: 10.1016/j.chroma.2018.02.052, 2018.

[63] Y. Kondo, S. Higa, T. Iwasaki, "Sensitive detection of fluorescence in western blotting by merging images," *PLoS One*, vol. 13 no. 1, DOI: 10.1371/journal.pone.0191532, 2018.

[64] J. Yang, W. Feng, K. Liang, C. Chen, C. Cai, "A novel fluorescence molecularly imprinted sensor for Japanese encephalitis virus detection based on metal organic frameworks and passivation-enhanced selectivity," *Talanta*, vol. 212, DOI: 10.1016/j.talanta.2020.120744, 2020.

- [65] Q. He, D. Yu, M. Bao, "High-throughput and all-solution phase African Swine Fever Virus (ASFV) detection using CRISPR-Cas12a and fluorescence based point-of-care system," *Biosensors and Bioelectronics*, vol. 154, DOI: 10.1016/j.bios.2020.112068, 2020.
- [66] G. Mowatt, S. Zhu, M. Kilonzo, "Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer," *Health Technology Assessment*, vol. 14 no. 4, DOI: 10.3310/hta14040, 2010.
- [67] H. Lee, B. In, P. K. In, M. Y. L. N. Kishore, K.-H. Lee, "Dual role of a fluorescent peptidyl probe based on self-assembly for the detection of heparin and for the inhibition of the heparin-digestive enzyme reaction," *ACS Applied Materials & Interfaces*, vol. 10 no. 3, pp. 2282-2290, DOI: 10.1021/acsami.7b15411, 2018.
- [68] P. K. Mehta, H. Lee, K.-H. Lee, "Highly sensitive ratiometric detection of heparin and its oversulfated chondroitin sulfate contaminant by fluorescent peptidyl probe," *Biosensors and Bioelectronics*, vol. 91, pp. 545-552, DOI: 10.1016/j.bios.2017.01.002, 2017.
- [69] Q. Cheng, Y. He, Y. Ge, J. Zhou, G. Song, "Ultrasensitive detection of heparin by exploiting the silver nanoparticle-enhanced fluorescence of graphitic carbon nitride (g-C₃N₄) quantum dots," *Microchimica Acta*, vol. 185 no. 7, DOI: 10.1007/s00604-018-2864-9, 2018.
- [70] L. Huang, J. Wang, Q. Wang, D. Tang, Y. Lin, "Distance-dependent visual fluorescence immunoassay on CdTe quantum dot-impregnated paper through silver ion-exchange reaction," *Microchimica Acta*, vol. 187 no. 10, DOI: 10.1007/s00604-020-04546-7, 2020.
- [71] S. Lv, Y. Tang, K. Zhang, D. Tang, "Wet NH₃-triggered NH₂-MIL-125(Ti) structural switch for visible fluorescence immunoassay impregnated on paper," *Analytical Chemistry*, vol. 90 no. 24, pp. 14121-14125, DOI: 10.1021/acs.analchem.8b04981, 2018.
- [72] Z. Qiu, J. Shu, D. Tang, "Bioresponsive release system for visual fluorescence detection of carcinoembryonic antigen from mesoporous silica nanocontainers mediated optical color on quantum dot-enzyme-impregnated paper," *Analytical Chemistry*, vol. 89 no. 9, pp. 5152-5160, DOI: 10.1021/acs.analchem.7b00989, 2017.
- [73] J. Wang, H. Z. Zhang, J. J. Liu, D. Yuan, R. S. Li, C. Z. Huang, "Time-resolved visual detection of heparin by accelerated etching of gold nanorods," *The Analyst*, vol. 143 no. 4, pp. 824-828, DOI: 10.1039/c7an01923h, 2018.
- [74] X. Fu, L. Chen, J. Li, "Ultrasensitive colorimetric detection of heparin based on self-assembly of gold nanoparticles on graphene oxide," *The Analyst*, vol. 137 no. 16, pp. 3653-3658, DOI: 10.1039/c2an35552c, 2012.
- [75] Z. Gao, Z. Qiu, M. Lu, J. Shu, D. Tang, "Hybridization chain reaction-based colorimetric aptasensor of adenosine 5'-triphosphate on unmodified gold nanoparticles and two label-free hairpin probes," *Biosensors and Bioelectronics*, vol. 89 no. 2, pp. 1006-1012, DOI: 10.1016/j.bios.2016.10.043, 2017.
- [76] G. Cai, Z. Yu, R. Ren, D. Tang, "Exciton-plasmon interaction between AuNPs/graphene nanohybrids and CdS quantum dots/TiO₂ for photoelectrochemical aptasensing of prostate-specific antigen," *ACS Sensors*, vol. 3 no. 3, pp. 632-639, DOI: 10.1021/acssensors.7b00899, 2018.
- [77] J. Zhuang, W. Lai, M. Xu, Q. Zhou, D. Tang, "Plasmonic AuNP/g-C₃N₄ nanohybrid-based photoelectrochemical sensing platform for ultrasensitive monitoring of polynucleotide kinase activity accompanying DNAzyme-catalyzed precipitation amplification," *ACS Applied Materials & Interfaces*, vol. 7 no. 15, pp. 8330-8338, DOI: 10.1021/acsami.5b01923, 2015.
- [78] S. Lv, K. Zhang, Q. Zhou, D. Tang, "Plasmonic enhanced photoelectrochemical aptasensor with D-A F8BT/g-C₃N₄ heterojunction and AuNPs on a 3D-printed device," *Sensors and Actuators B: Chemical*, vol. 310, DOI: 10.1016/j.snb.2020.127874, 2020.
- [79] A. Tripodi, "D-dimer testing in laboratory practice," *Clinical Chemistry*, vol. 57 no. 9, pp. 1256-1262, DOI: 10.1373/clinchem.2011.166249, 2011.
- [80] Y. J. Jang, B. Kim, E. Roh, H. Kim, S. H. Lee, "Micellization-induced amplified fluorescence response for highly sensitive detection of heparin in serum," *Scientific Reports*, vol. 10 no. 1, DOI: 10.1038/s41598-020-66360-8, 2020.
- [81] H. Yuan, W. Ji, S. Chu, "Au nanoparticles as label-free competitive reporters for sensitivity enhanced fiber-optic SPR heparin sensor," *Biosensors and Bioelectronics*, vol. 154, DOI: 10.1016/j.bios.2020.112039, 2020.

[82] J. A. Hippensteel, W. B. LaRiviere, J. F. Colbert, C. J. Langouët-Astrié, E. P. Schmidt, "Heparin as a therapy for COVID-19: current evidence and future possibilities," American Journal of Physiology-Lung Cellular and Molecular Physiology, vol. 319 no. 2, pp. L211-L217, DOI: 10.1152/ajplung.00199.2020, 2020.

[83] B. Buijsers, C. Yanginlar, M. L. Maciej-Hulme, Q. de Mast, J. van der Vlag, "Beneficial non-anticoagulant mechanisms underlying heparin treatment of COVID-19 patients," EBioMedicine, vol. 59, DOI: 10.1016/j.ebiom.2020.102969, 2020.

DETAILS

Subject:	Polysaccharides; Plasma; Glycoproteins; Acids; Heparin; Fluorescence; Sodium; Biosynthesis; Anticoagulants; Molecular weight; Blood platelets; Heparan sulfate; Heterogeneity; Nanomaterials; Bioavailability
Editor:	Eladia M Pen a Mendez
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-02-24 (Received); 2021-04-16 (Revised); 2021-04-29 (Accepted); 2021-05-07 (Pub)
DOI:	https://doi.org/10.1155/2021/5543460
ProQuest document ID:	2527980006

Document URL: <https://www.proquest.com/scholarly-journals/techniques-detection-clinical-used-heparins/docview/2527980006/se-2?accountid=211160>

Copyright: Copyright © 2021 Binjie Li et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-05-16

Database: Publicly Available Content Database

Document 38 of 59

Bismuth-Remaining Cupellation Fire Assay Preconcentration Combined with Inductively Coupled Plasma Mass Spectrometry for the Simultaneous Determination of Ultratrace Au, Pt, Pd, Ru, Rh, and Ir in Geologic Samples

Ni, Wenshan ¹
; Mao, Xiangju ¹
; Yao, Mingxing ¹
; Sun, Qiliang ¹
; Guo, Xiaorui ¹
; Zhang, Hongli ¹
; Liu, Lu ¹

¹ Zhengzhou Institute of Multipurpose Utilization of Mineral Resources, CAGS, Zhengzhou 450006, China; China National Engineering Research Center for Utilization of Industrial Minerals, Zhengzhou 450006, China; Key Laboratory of Evaluation and Multipurpose Utilization of Polymetallic Ores Ministry of Natural Resources, Zhengzhou 450006, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

In this work, a novel method of bismuth fire assay (Bi-FA) combined with inductively coupled plasma mass spectrometry (ICP-MS) simultaneous determination of ultratrace gold (Au), platinum (Pt), palladium (Pd), ruthenium (Ru), rhodium (Rh), and iridium (Ir) in geologic samples was established. Bismuth oxide (Bi_2O_3) was used as noble metal elements fire assay collector, and Bi-remaining protection cupellation was employed to generate Bi granule. After the Bi granule was microwave-digested by aqua regia (40%, v/v), ^{197}Au , ^{195}Pt , ^{106}Pd , ^{101}Ru , ^{103}Rh , and ^{193}Ir in the sample solution were determined by ICP-MS. Using Bi as cupellation protector, volatile Ru could be collected

effectively and without volatilization loss during microwave digestion and decompression. Moreover, the toxicity of Bi was exceptionally low compared to toxic nickel oxide and lead oxide in nickel sulfide/lead fire assay; thus Bi-FA was a green environmental analysis method. The mineral composition and decomposition character of chromite, black shale, and polymetallic ore were investigated, and pretreatment procedures were optimized for such special samples. Besides, the influence of mass spectrum interference of coexisting elements was discussed. Under the optimal conditions, excellent curve fittings of Au, Pt, Pd, Ru, Rh, and Ir were obtained between 0.01 and 100 ng·mL⁻¹, with the correlation coefficients exceeding 0.9995. The detection limits were from 0.002 ng·g⁻¹ to 0.025 ng·g⁻¹. The developed method was applied to analyze the Chinese Certified Reference Materials (CRMs) and the determined values were in good agreement with the certified values.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Au, Pt, Pd, Ru, Rh, and Ir are noble metal elements, due to their high melting point, good conductivity, corrosion resistance, thermal stability, and catalytic activity, known as the “industrial vitamin,” which are widely applied in industrial catalysis [1, 2], military industry [3], energy, and biomedical fields [4, 5]. Mineral resource reserves of Au, Pt, Pd, Ru, Rh, and Ir are limited, and their abundances in the crust are extremely low (mass fraction is between 10⁻⁸ and 10⁻⁹) and their distributions are uneven [6–8]; thus the accurate determination of noble metal elements has always been a challenge to geoanalysts.

The ordinary chemical analysis methods with small amount of sample weight (<3g) and low sensitivity could not meet the demands of ultratrace analysis; thus methods with large amount of sample weight were developed to preconcentrate and detect noble metal elements. Fire assay was the application of metallurgical principles and techniques to analytical chemistry, which was an important method for precious metal elements enrichment. The target precious metals in rock, soil, metallurgy, and other samples were concentrated by Pb/Ni/Sb/Sn/Cu/Bi-containing collectors and successfully separated from the sample matrix. Fire assay methods by weighing 10–40g samples with good sampling representative and high enrichment factor combined with high sensitivity graphite furnace atomic absorption spectrometry (GFAAS) and ICP-MS were used to determine noble elements [9–13]. The commonly used methods include lead fire assay (Pb-FA) [13, 14], nickel sulfide fire assay (NiS-FA) [9, 10, 12, 15–17], antimony fire assay (Sb-FA) [18], and tin fire assay (Sn-FA) [19, 20]. Pb-FA was the earliest classical method, and it could quantitatively preconcentrate noble metal elements. However, due to the high blank and high toxicity of collector reagent, as well as the volatilization loss of Ru and osmium (Os) during cupellation, the application in determination of ultratrace noble metal elements in geological samples was seriously restricted. The high blank problem was also existing for NiS-FA; then the collector should be purified tediously, or highly toxic nickel carbonyl was employed [15, 16]. Moreover, the capture efficiency of NiS-FA for Au was not ideal. For simple matrix samples, Sb-FA could replace Pb-FA to quantitatively enrich noble metal elements. However, impurity elements such as Cu, Pb, Bi, Ni, and Co could also be collected into Sb granule, which was prone to disturb the determination of noble metal elements. Sn-FA was effective for black shale, but the volatilization loss of Ru and Os during HCl digestion was inescapable [21].

Au, Pt, Pd, Ru, Rh, and Ir could form a series of alloys or metal intercompounds with Bi at high temperatures; thus Bi could quantitatively collect the noble metal elements in geological samples [22]. In this work, a novel method of Bi-remaining cupellation fire assay preconcentration combined with ICP-MS simultaneous determination of ultratrace Au, Pt, Pd, Ru, Rh, and Ir in geologic samples was established. Minimal toxic Bi₂O₃ was used as fire assay collector, and Bi-remaining protection cupellation was employed to enrich the noble metal elements into Bi granule. After the Bi granule was microwave-digested by aqua regia, the target analytes in the sample solution were determined by ICP-MS. It was worth mentioning that volatile Ru could be accurately determined by the proposed Bi-FA method. Compared with Pb-FA and NiS-FA, the reagent blank of Bi₂O₃ was relatively low and could be directly employed to

collect noble metal elements without purifying. Moreover, the harm of toxic collector to the analyst and environment was avoided by using the nontoxic Bi_2O_3 . The developed method was applied to analyze the Chinese Certified Reference Materials and the determined values were in good agreement with the certified values.

2. Materials and Methods

2.1. Instrumentation

A quadrupole (Q) ICP-MS (Model iCAP RQ, Thermo Scientific, USA) with a polyfluoroalkoxy (PFA) concentric nebulizer was used in all experiments for the determination of Au, Pt, Pd, Ru, Rh, and Ir. The operating parameters are summarized in Table 1. The MWS-3⁺ microwave digestion system (BERGHOF, Germany) was employed for sample digestion.

Table 1

ICP-MS operation conditions.

<i>ICP-MS plasma</i>	
Rf power	1550W
Sampling depth	5.0mm
Nebulizer gas flow	1.00L·min ⁻¹
Auxiliary gas flow	0.80L·min ⁻¹
Cool gas flow	14.0L·min ⁻¹
Sampler/skimmer diameter orifice	Nickel 1.0mm/0.5mm
Peristaltic pump speed	40rpm
—	
<i>Time-resolved data acquisition</i>	
Survey runs	Jump
Channels	1
Number of sweeps	40
Dwell time	10ms
Measurement mode	STD
Isotopes	¹⁹⁷ Au, ¹⁹⁵ Pt, ¹⁰⁶ Pd, ¹⁰¹ Ru, ¹⁰³ Rh, ¹⁹³ Ir, and ¹⁸⁵ Re

The scanning electron microscopy and energy spectrum graph of chromite slag was characterized by scanning electron microscope (FEI Quanta 650F, Australia) and electron energy disperse spectroscopy (Bruker QUANTAX, Germany). X-ray powder polycrystal diffractometer (SmartLab SE, Rigaku Corporation, Japan) was used for

component analysis of chromite slag.

2.2. Standard Solutions and Reagents

The multielement calibration standard solutions (Au, Pt, Pd, Ru, Rh, and Ir $10\mu\text{g}\cdot\text{mL}^{-1}$, respectively) in 10% aqua regia were bought from SPEX CertiPrep Group (USA). 8% (v/v) aqua regia was used as diluent and mixed standard solution of Au, Pt, Pd, Ru, Rh, and Ir ($100\text{ng}\cdot\text{mL}^{-1}$) was employed as stock solution. Rhenium (Re) standard solution ($50\text{ng}\cdot\text{mL}^{-1}$) was used as internal standard.

Bi_2O_3 , glass powder, sodium carbonate anhydrous (Na_2CO_3), sodium borate ($\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$), and flour were of analytical reagent (AR) grade and purchased from Tianjin Commie Company. HCl and nitric acid (HNO_3) were of excellent reagent grade. Deionized water was used throughout the whole experiments.

For covering agent, Na_2CO_3 , $\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$, glass powder, Bi_2O_3 , and flour were mixed well at 55g: 15g: 20g: 40g: 4g.

2.3. Sample Pretreatment

2.3.1. Fire Assay Recipes and Melting Process for Geochemical Samples

Regarding refractory chromite, chromite was difficult to melt and therefore presintering was required. 10g chromite sample was mixed well with 30g CaO and 40g Na_2O_2 , and then it was put on the porcelain dish padded with quartz sand, which was sintered at about 700°C for 1.5 hours. Once cooled, the sample was smashed and transferred into a 500mL ingredient bottle. The following fusing and cupellation procedure were the same for the common geological samples.

For common geochemical samples, the fire assay recipes of rock, soil, river sediment, chromite, black shale, and polymetallic ore were mixed according to the mineral composition characteristics, which were shown in Table 2. Raw materials according to Table 2 were mixed well in ingredient bottle and transferred into a 500mL fire-clay crucible and 20g of covering agent was uniformly covered onto the recipes. Then the crucible was fused in a furnace preheated at 1000°C and gradually heated to 1060°C and kept for 40min. The melts in the crucible were poured into a cast iron mold. Once cooled, the Bi bead was separated from the slag.

Table 2

Bismuth fire assay recipes for geochemical samples.

Sample type	Recipe (g)						
Sample weight	$\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$	Glass powder	Na_2CO_3	Bi_2O_3	Flour	KNO_3	Silicate rock
10–20	15	15	55	40	4	—	Carbon ate rock
10–20	15	20	50	40	4	—	Sulfide rock*
10–20	15	20	50	40	4.5	—	Soil
10–20	15	15	55	40	4	—	Stream sediment
10–20	15	15	55	40	4	—	Black shale

10	20	20	50	80	7	5	Polymetallic ore
10	15	20	50	100	5	—	Chromite

*Sample was placed into the furnace maintained at 600°C for 2 hours.

2.3.2. Bi-Remaining Cupellation and Microwave Digestion

Bi-remaining protection cupellation was employed to generate Bi granule. The Bi bead was cupellated for 30min in magnesia cupel at 850°C until it became a small granule (~5mg). The blank sample was also subjected to this procedure.

The Bi granule was put into a microwave digestion tank, and 10mL aqua regia (40%, v/v) newly prepared was added. Then the digestion tank was microwave-digested by the following microwave program: 100°C, rising 5min, keeping 5min; 105°C, rising 2min, keeping 5min; 110°C, rising 2min, keeping 10min; and 100°C, rising 1min, keeping 5min. After natural cooling, the closed tank was opened and stabilized to 50mL volumetric bottle. Re (50 ng·mL⁻¹) was online-added as the internal standard element; thence Au, Pt, Pd, Ru, Rh, and Ir in the sample solution were determined by ICP-MS.

3. Results and Discussion

3.1. Covering Agent

The mixed reagent with almost the same formula as the fire assay recipes was used as covering agent, which could not only prevent the splash loss of the target analytes during sample melting process but also secondary collect the remaining Au, Pt, Pd, Ru, Rh, and Ir in the slag when Bi₂O₃ in the covering agent was reduced to Bi; thus the collecting rates of target analytes were improved.

3.2. Bi-Remaining Cupellation

Ag was an effective fire assay cupellation protector for Au, Pt, and Pd, but it did not work for Ru, Rh, and Ir due to the fact that it could not form alloys with Ru, Rh, and Ir. In this work, Bi was used as fire assay collector and cupellation protector, and Bi-remaining cupellation was employed to enrich Au, Pt, Pd, Ru, Rh, and Ir into ~5mg Bi granule. It was notable that the magnesia cupel with Bi granule after cupellation could not be directly immersed into the water (Figures 1(a) and 1(b)), but it should be half-dipped to make the Bi granule gradually condense (Figures 1(c) and 1(d)), because the hot liquid Bi might be lost by splash in contact with water as shown in Figure 1(b).

[figures omitted; refer to PDF]

3.3. Microwave Digestion

It was found that Ru had no volatilization loss under the digestion conditions of closed high temperature and high pressure [17]. Therefore, microwave digestion apparatus was used in this experiment to completely dissolve Bi granule. While ensuring the complete dissolution of Bi granule, it is necessary to reduce the dissolution temperature and time as much as possible to protect personnel safety and prolong the instrument service life. Through experiments, Bi granule could be completely dissolved by using the heating procedure.

3.4. Pretreatment of Special Samples

3.4.1. Refractory Chromite

Chromite is one of the refractory minerals with strong acid oxide and high hardness, which could not be completely melt by general fusing procedure [14]; there were still a few chromite particles entrained into slag lead to lower recoveries of noble metal elements. The significant chromite characteristic peaks before and after Bi-FA were shown in the X-ray powder polycrystal diffraction graph (Figure 2); it could be seen that the refractory characteristic peaks still existed in the slag though the peak height decreased significantly after Bi-FA. In order to enhance the recoveries of noble metal elements, presintering of chromite samples with CaO and Na₂O₂ at 600°C was carried out to facilitate subsequent melting.

[figure omitted; refer to PDF]

3.4.2. Black Shale

Black shale is a complex mineral containing noble metal elements. The mineral composition of black shale was analyzed by electron energy disperse spectroscopy (Fig. S1) and scanning electron microscope (Fig. S2). It could be concluded that black shale was a complex mineral composed of organic carbon, molybdenite, pyrite, arsenite, nickel, apatite, quartz, gypsum, orthoclase, and barite, which also contained metallic and nonmetallic elements such as F, Cl, Br, Cu, Pb, Zn, Mn, Se, Sb, V, rare earth, U, Zr, Re, Au, Ag, Pt, Pd, Ru, Rh, Os, and Ir.

The burning of carbon and sulfur in black shale sample would cause volatile loss of Au, Pt, Pd, Ru, Rh, and Ir. Besides, the sulfur of black shale would form stable sulfide with the target noble metal elements and then further formed solid solution with sulfide of Cu, Ni, and Co and entered the slag, which would still further lower the results of Au, Pt, Pd, Ru, Rh, and Ir. Therefore, proper amount of KNO_3 was added to oxide the sulfur to sulfate into the slag, and then the formation of metal sulfides was prevented so that Au, Pt, Pd, Ru, Rh, and Ir in the sample could be completely collected by Bi bead. The recovery ratios of Au, Pt, Pd, Ru, Rh, and Ir in the black shale sample by Bi-FA with and without KNO_3 were compared, and the results were shown in Table 3. It could be seen that the sample content increased significantly with KNO_3 . Therefore, KNO_3 was added for black shale sample.

Table 3

The analytical results of different pretreatment procedures for black shale ($\text{ng}\cdot\text{g}^{-1}$).

Sample	Without KNO_3 *						With KNO_3					
Au	Pt	Pd	Ru	Rh	Ir	Au	Pt	Pd	Ru	Rh	Ir	Black shale 1
174 ± 2.5	473 ± 31	456 ± 17	1.22 ± 0.09	0.31 ± 0.02	1.51 ± 0.12	209 ± 18	505 ± 34	520 ± 28	7.42 ± 0.54	17.1 ± 1.22	3.78 ± 0.21	Black shale 2

Note: all the values were determined by Bi-FA ICP-MS. *Sample was placed into the furnace burn at 700°C .

3.4.3. Polymetallic Ore

Polymetallic ores are generally rich in Cu, Pb, Zn, Sb, Bi, Ni, Co, and Sn and usually accompany noble metal elements. For the pretreatment of polymetallic ores, more Bi_2O_3 was needed, some of Bi_2O_3 was reduced into Bi granule to enrich precious metals, another compound state of Bi_2O_3 was used to collect associated impurity metals into the slag, and then 100g of Bi_2O_3 was added to minimize the interferences caused by associated impurity metals.

3.5. Measured Isotopes and Interferences of Polyatomic Molecular Ion

The isotopes of the determined noble metal elements were shown in Table S1, and ^{197}Au , ^{195}Pt , ^{106}Pd , ^{101}Ru , ^{103}Rh , ^{193}Ir , and ^{185}Re were selected as measuring isotopes and internal standard isotope with the principle of high abundance and free from isobars interference.

While precious metal elements were enriched during Bi-FA, trace amounts of Cu, Pb, Sb, Ni, Co, Sn, Se, Te, and As also were collected into the Bi granule. Os, As, and Sb were oxidized into OsO_4 , As_2O_3 , and Sb_2O_3 and completely volatilize in the cupellation process, and trace oxides of Cu, Pb, Ni, Co, Sn, Se, and Te in Bi granule could also be absorbed into the magnesia cupel. Therefore, most of the interferences were effectively eliminated through Bi-FA, and only Bi, Au, Ag, Pt, Pd, Ru, Rh, Ir, N, H, O, and Cl were coexistent in the final sample solution after microwave digestion. The possible interferences of polyatomic molecular ion were shown in Table S2. Despite the high concentration of H in the sample solution, the contents of ^{196}Pt , ^{194}Pt , ^{105}Pd , ^{100}Ru , ^{102}Ru , ^{102}Pd , and ^{192}Pt that make up polyatomic molecular ions in the solution were at $\text{ng}\cdot\text{mL}^{-1}$ level, so there were no effective polyatomic molecular ion interferences to the measured isotopes of ^{197}Au , ^{195}Pt , ^{106}Pd , ^{101}Ru , ^{103}Rh , and ^{193}Ir .

3.6. Analytical Performance

The mixed standard solutions for Au, Pt, Pd, Ru, Rh, and Ir were prepared in the medium of 8% (v/v) aqua regia with

the concentrations of 0.01 to 100 ng·mL⁻¹. At the optimum conditions, the intensities of Au, Pt, Pd, Rh, Ru, and Ir were detected by ICP-MS, and Re (50 ng·mL⁻¹) was online-added as internal standard element.

Then the analytical performances of the proposed Bi-FA ICP-MS method for the determination of Au, Pt, Pd, Rh, Ru, and Ir were evaluated, and the analytical results including linear ranges and limits of detection (LODs) are listed in Table 4. The dynamic linear range was covering four orders of magnitudes with the correlation coefficients (R^2) ranging from 0.9995 to 0.9999 and low background equivalent concentration (BEC) from 0.002 to 0.085 ng·mL⁻¹. According to the definition of IUPAC, the LODs (3σ) were in the range of 0.002–0.025 ng·g⁻¹. The enrichment factor, defined as the specific value of the sample weight (10g) before Bi-FA and the weight of Bi granule after Bi-FA (5 mg), was calculated as 2000.

Table 4

Analytical performance data by the established Bi-FA ICP-MS method for Au, Pt, Pd, Ru, Rh, and Ir.

Isotopes	Linear range (ng·mL ⁻¹)	Linear equation	R^2	BEC (ng·mL ⁻¹)	LOD* (ng·g ⁻¹)	Enrichment factor**
¹⁹⁷ Au	0.01~100	$y=98397x+3170$	0.9997	0.032	0.025	2000
¹⁹⁵ Pt	0.01~100	$y=70732x+1006$	0.9999	0.014	0.008	2000
¹⁰⁶ Pd	0.01~100	$y=65527x+12474$	0.9995	0.085	0.024	2000
¹⁰¹ Ru	0.01~100	$y=42747x+1066$	0.9999	0.025	0.005	2000
¹⁰³ Rh	0.01~100	$y=242359x+621$	0.9999	0.003	0.002	2000
¹⁹³ Ir	0.01~100	$y=198777x+456$	0.9995	0.002	0.003	2000

*LOD=3*standard deviation of background/slope of calibration curve, for 10g sample. **Enrichment factor was the specific value of the sample weight (10g) before Bi-FA and the weight of Bi granule after Bi-FA (5mg).

4. Sample Analysis

At the optimum conditions, the developed Bi-FA ICP-MS method was validated by the analysis of a series of CRMs such as soil (GBW07288, GBW07294, and GBW07340) stream sediment GBW07289, olivine GBW07290, pyroxene peridotite GBW07291, chromite GBW07292, palladium poor ore GBW07293, palladium ore (GBW07341 and GBW07342), and gold geological samples (GBW07228, GBW07229 and GBW07230), the determined values are shown in Table 5, and t -test was applied to the CRMs and the $t_{0.05,4}$ values for Au, Pt, Pd, Rh, Ru, and Ir in the sample are also listed in Table 5. It could be seen that the determined values were in good agreement with the certified values and t -test results were satisfactory, which showed that this method is suitable for trace noble metal elements analysis of soil, rock, and mineral samples.

Table 5

Comparison of analytical data and recommended values for Au, Pt, Pd, Ru, Rh, and Ir in certified reference materials samples by the proposed Pb-FA LA-ICP-MS method ($n=5$, ng·g⁻¹).

Element		Au	Pt	Pd	Ru	Rh	Ir
GBW07288	Found	1.1±0.2	0.23±0.03	0.30±0.04	0.062±0.014	0.022±0.005	0.038±0.008

Soil	Certified	0.9±0.2	0.26±0.05	0.26±0.05	(0.05)*	0.017±0.006	0.032±0.012
<i>t-test</i>		2.24	2.24	2.24	1.92	2.24	1.68
GBW07289	Found	12±2	1.4±0.2	2.5±0.3	0.12±0.02	0.099±0.021	0.07±0.02
Stream sediment	Certified	10±2	1.6±0.3	2.3±0.2	(0.10)*	0.095±0.012	0.05±0.01
<i>t-test</i>		2.24	2.24	1.49	2.24	0.43	2.24
GBW07290	Found	1.3±0.2	5.6±0.4	5.1±0.4	16.2±2.8	1.1±0.2	4.7±0.3
Olivine	Certified	1.1±0.1	6.4±0.9	4.6±0.6	14.8±2.7	1.3±0.3	4.3±0.5
<i>t-test</i>		2.24	4.47	2.80	1.12	2.24	2.98
GBW07291	Found	4.7±0.4	61±5	67±5	2.7±0.3	4.9±0.4	5.5±0.4
Pyroxene peridotite	Certified	4.3±0.3	58±5	60±9	2.5±0.2	4.3±0.8	4.7±1.1
<i>t-test</i>		2.24	1.34	3.13	1.49	3.35	4.47
GBW07292	Found	—	23±4	10.1±0.7	501±54	12±2	144±11
Chromite	Certified	—	20±4	11.3±1.5	527±91	10±2	136±10
<i>t-test</i>			1.68	3.83	1.08	2.24	1.63
GBW07293	Found	49±4	432±24	597±42	15±2	24±2	33±5
Palladium poor ore	Certified	(45)*	440±37	568±51	13±1	22±3	28±7
<i>t-test</i>		2.24	0.75	1.54	2.24	2.24	2.24
GBW07294	Found	2.1±0.4	13.1±2.6	17.1±2.4	0.63±0.15	0.92±0.18	1.5±0.2
Soil	Certified	(1.8)*	14.7±2.5	15.2±2.3	0.66±0.20	1.1±0.2	1.2±0.3
<i>t-test</i>		1.68	1.38	1.77	0.30	2.24	3.35
GBW07340	Found	2.6±0.3	0.61±0.06	0.72±0.09	0.47±0.09	0.070±0.007	0.18±0.03
Soil	Certified	(2.3)*	0.66±0.08	0.66±0.08	0.43±0.16	0.066±0.008	0.16±0.03
<i>t-test</i>		2.24	1.86	1.49	0.99	1.28	1.49

GBW07341	Found	—	1947±122	591±43	68±6	5.76±0.45	26±4
Palladium ore	Certified	—	1900±200	570±50	74±7	(6)*	28±6
<i>t</i> -test			0.86	1.09	2.24	1.19	1.12
GBW07342	Found	—	5988±422	1755±145	1.7±0.2	1.7±0.3	2.6±0.3
Palladium ore	Certified	—	5700±500	1670±130	(2)*	1.5±0.5	2.1±0.7
<i>t</i> -test			1.53	1.31	3.35	1.49	3.73
GBW07201	Found	—	11.5±1.8	7.5±0.8	328±12	13.3±2.2	95±8
Chromite	Certified	—	10±1	7±1	305±6	12±1	90±6
<i>t</i> -test			1.86	1.40	4.29	1.32	1.40
GBW07228	Found	3.5±0.3	—	—	—	—	—
Gold geological sample	Certified	3.4±0.2	—	—	—	—	—
<i>t</i> -test		0.75					
GBW07229	Found	54±4	—	—	—	—	—
Gold geological sample	Certified	52±3	—	—	—	—	—
<i>t</i> -test		1.12					
GBW07230	Found	1362±28	—	—	—	—	—
Gold geological sample	Certified	1330±20	—	—	—	—	—
<i>t</i> -test		2.56					

*The value in brackets is the reference value. $t_{0.05,4}=2.77$, $t_{0.01,4}=4.60$.

5. Conclusions

In this work, the method of Bi-remaining cupellation fire assay ICP-MS for the simultaneous determination of Au, Pt, Pd, Rh, Ru, and Ir was established. Lower toxicity Bi_2O_3 was used as fire assay collector, and Bi protection cupellation was employed. The Bi granule was microwave-digested by aqua regia and the target noble metal elements were determined by ICP-MS. Volatile Ru could be accurately determined by the proposed Bi-FA method. Compared with toxic Pb-FA and NiS-FA, Bi-FA was green and environmental protection, and it was effective for

volatile Ru by using Bi as cupellation protector. The developed method was applied to analyze the multiple types of CRMs and the determined values were in good agreement with the certified values; the proposed method was suitable for the determination of trace and ultratrace Au, Pt, Pd, Rh, Ru, and Ir in geochemical samples on a large scale.

Disclosure

Wenshan Ni and Xiangju Mao were co-first authors.

Authors' Contributions

Wenshan Ni and Xiangju Mao contributed equally to this work.

References

- [1] T. T. Li, Y. B. Shen, X. X. Zhong, "Effect of noble metal element on microstructure and NO₂ sensing properties of WO₃ nanoplates prepared from a low-grade scheelite concentrate," *Journal of Alloys and Compounds*, vol. 818, DOI: 10.1016/j.jallcom.2019.152927, 2020.
- [2] Z. Hou, W. Pei, X. Zhang, "Rare earth oxides and their supported noble metals in application of environmental catalysis," *Journal of Rare Earths*, vol. 38 no. 8, pp. 819-839, DOI: 10.1016/j.jre.2020.01.011, 2020.
- [3] M. H. Kaye, B. J. Lewis, W. T. Thompson, "Thermodynamic treatment of noble metal fission products in nuclear fuel," *Journal of Nuclear Materials*, vol. 366 no. 1-2, DOI: 10.1016/j.jnucmat.2006.11.014, 2007.
- [4] N. B. Saber, A. Mezni, A. Alrooqi, T. Altalhi, "A review of ternary nanostructures based noble metal/semiconductor for environmental and renewable energy applications," *Journal of Materials Research and Technology*, vol. 9, 2020.
- [5] Y. Yao, L. Y. Lan, X. X. Liu, Y. B. Ying, J. F. Ping, "Spontaneous growth and regulation of noble metal nanoparticles on flexible biomimetic MXene paper for bioelectronics," *Biosensors and Bioelectronics*, vol. 148, DOI: 10.1016/j.bios.2019.111799, 2020.
- [6] L. Pattou, J. P. Lorand, M. Gros, "Non-chondritic platinum-group element ratios in the Earth's mantle," *Nature*, vol. 379 no. 6567, pp. 712-715, DOI: 10.1038/379712a0, 1996.
- [7] J. E. Snow, G. Schmidt, "Constraints on earth accretion deduced from noble metals in the oceanic mantle," *Nature*, vol. 391 no. 6663, pp. 166-169, DOI: 10.1038/34396, 1998.
- [8] J. Amossé, "Determination of platinum-group elements and gold in geological matrices by inductively coupled plasma-mass spectrometry (ICP-MS) after separation with selenium and tellurium carriers," *Geostandards and Geoanalytical Research*, vol. 22 no. 1, pp. 93-102, DOI: 10.1111/j.1751-908x.1998.tb00548.x, 1998.
- [9] I. Shazali, L. Van't Dack, R. Gijbels, R. Gijbels, "Determination of precious metals in ores and rocks by thermal neutron activation/ γ -spectrometry after preconcentration by nickel sulphide fire assay and coprecipitation with tellurium," *Analytica Chimica Acta*, vol. 196, pp. 49-58, DOI: 10.1016/s0003-2670(00)83069-x, 1987.
- [10] C. Li, C. Chai, X. Li, X. Mao, "Determination of platinum-group elements and gold in two Russian Candidate Reference Materials SCHS-1 and SLg-1 by ICP-MS after nickel sulfide fire assay preconcentration," *Geostandards and Geoanalytical Research*, vol. 22 no. 2, pp. 195-197, DOI: 10.1111/j.1751-908x.1998.tb00692.x, 1998.
- [11] A. R. Date, A. E. Davis, Y. Y. Cheung, "The potential of fire assay and inductively coupled plasma source mass spectrometry for the determination of platinum group elements in geological materials," *The Analyst*, vol. 112 no. 9, DOI: 10.1039/an9871201217, 1987.
- [12] S. E. Jackson, B. J. Fryer, W. Gosse, D. C. Healey, H. P. Longerich, D. F. Strong, "Determination of the precious metals in geological materials by inductively coupled plasma-mass spectrometry (ICP-MS) with nickel sulphide fire-assay collection and tellurium coprecipitation," *Chemical Geology*, vol. 83 no. 1-2, pp. 119-132, DOI: 10.1016/0009-2541(90)90144-v, 1990.
- [13] C. I. Cerceau, C. D. F. Carvalho, A. C. S. Rabelo, C. G. dos Santos, S. M. D. Gonçalves, E. V. V. Varejão, "Recovering lead from cupel waste generated in gold analysis by Pb-Fire assay," *Journal of Environmental Management*, vol. 183, pp. 771-776, DOI: 10.1016/j.jenvman.2016.08.052, 2016.
- [14] W. Ni, X. Mao, H. Zhang, L. Liu, X. Gao, F. Xiao, "Lead fire assay preconcentration and high resolution continuum source graphite furnace atomic absorption spectrometry for the determination of ultra-trace amounts of

- Au, Ir, Pd, Pt and Rh in rocks and minerals," *Spectrochimica Acta Part B: Atomic Spectroscopy*, vol. 158, DOI: 10.1016/j.sab.2019.105643, 2019.
- [15] Y. Sun, X. Guan, A. Du, "Determination of platinum group elements by inductively coupled plasma-mass spectrometry combined with nickel sulfide fire assay and tellurium coprecipitation," *Spectrochimica Acta Part B: Atomic Spectroscopy*, vol. 53 no. 10, pp. 1463-1467, DOI: 10.1016/s0584-8547(98)00167-0, 1998.
- [16] W. Ni, X. Mao, H. Zhang, "Determination of ultra-trace platinum, palladium, ruthenium, rhodium, and iridium in rocks and minerals by inductively coupled-plasma mass spectrometry following nickel sulfide fire assay preconcentration and open mixed acid digestion," *Analytical Letters*, vol. 52 no. 11, pp. 1699-1710, DOI: 10.1080/00032719.2019.1566348, 2019.
- [17] W. Ni, H. Zhang, X. Mao, L. Liu, F. Xiao, "Determination of ultra-trace osmium and ruthenium in geological samples by ICP-MS combined with nickel sulfide fire assay pre-concentration and microwave digestion," *Microchemical Journal*, vol. 150, DOI: 10.1016/j.microc.2019.104187, 2019.
- [18] W. Ni, H. Zhang, X. Mao, "Simultaneous determination of ultra-trace Au, Pt, Pd, Ru, Rh, Os and Ir in geochemical samples by KED-ICP-MS combined with Sb-Cu fire assay and microwave digestion," *Microchemical Journal*, vol. 158, DOI: 10.1016/j.microc.2020.105197, 2020.
- [19] P. E. Moloughney, J. A. Graham, "Determination of silver in ores and metallurgical concentrates by a combination of fire-assay preconcentration (using tin as collector) and atomic-absorption spectrophotometry," *Talanta*, vol. 18 no. 5, pp. 475-479, DOI: 10.1016/0039-9140(71)80075-9, 1971.
- [20] P. Moloughney, G. H. Faye, "A rapid fire-assay/atomic-absorption method for the determination of platinum, palladium and gold in ores and concentrates: a modification of the tin-collection scheme," *Talanta*, vol. 23 no. 5, pp. 377-381, DOI: 10.1016/0039-9140(76)80050-1, 1976.
- [21] W. Ni, X. Mao, H. Zhang, L. Liu, X. Guo, Q. Sun, "Simultaneous determination of ultra-trace Pt, Pd, Rh and Ir in geochemical samples by inductively coupled plasma mass spectrometry following tin fire assay preconcentration and microwave digestion," *Current Analytical Chemistry*, vol. 17 no. 4, pp. 552-563, DOI: 10.2174/1573411016999200715160650, 2021.
- [22] S. L. Zhang, H. M. Tu, "Study on enrichment of precious metals in ore by bismuth assay," *Mineral Resources and Geology*, vol. 2, 1981.

DETAILS

Subject:	Mass spectrometry; Bismuth trioxide; Reagents; Geology; Ruthenium; Bismuth oxides; Rhodium isotopes; Scientific imaging; Chromite; Pretreatment; Nickel sulfide; Toxicity; Gas flow; Correlation coefficients; Platinum; Lead oxides; Gold; Curve fitting; Assaying; Iridium; Palladium; Methods; Recipes; Noble metals; Rhodium; Granular materials; Inductively coupled plasma mass spectrometry; Emission spectroscopy
Location:	United States--US; Germany
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021

Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-03-08 (Received); 2021-04-25 (Revised); 2021-04-29 (Accepted); 2021-05-06 (Pub)
DOI:	https://doi.org/10.1155/2021/9960673
ProQuest document ID:	2527980005
Document URL:	https://www.proquest.com/scholarly-journals/bismuth-remaining-cupellation-fire-assay/docview/2527980005/se-2?accountid=211160
Copyright:	Copyright © 2021 Wenshan Ni et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2023-02-06
Database:	Publicly Available Content Database

Document 39 of 59

Remarkable Phytochemical Characteristics of Chi-Nan Agarwood Induced from New-Found Chi-Nan Germplasm of *Aquilaria sinensis* Compared with Ordinary Agarwood

Yu, Meng ¹

; Liu, Yangyang ¹

; Feng, Jian ²; Chen, Deli ³; Yang, Yun ²; Liu, Peiwei ²; Yu, Zhangxin ²; Wei, Jianhe ¹

¹ Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education & National Engineering Laboratory for Breeding of Endangered Medicinal Materials, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, China; Hainan Provincial Key Laboratory of Resources Conservation and Development of Southern Medicine & Key Laboratory of State Administration of Traditional Chinese Medicine for Agarwood Sustainable Utilization, Hainan Branch of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Haikou 570311, China ² Hainan Provincial Key Laboratory of Resources Conservation and Development of Southern Medicine & Key Laboratory of State Administration of Traditional Chinese Medicine for Agarwood Sustainable Utilization, Hainan Branch of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Haikou 570311, China ³ Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education & National Engineering Laboratory for Breeding of Endangered Medicinal Materials, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Wild Chi-Nan agarwood is regarded as the highest quality agarwood from *Aquilaria* spp. However, the comprehensive research on chemical composition of wild Chi-Nan agarwood is limited. An integrated strategy using SHS-GC-MS and UPLC-Q/Tof-MS was applied to explore the phytochemical characteristics of a kind of agarwood induced from a newly identified germplasm of Chi-Nan *A. sinensis*. Progenesis Q1 and MS-Dial were used to preprocess the UPLC-Q/Tof-MS and GC-MS raw data, respectively. Principle component analysis (PCA) and orthogonal partial least squares to latent structure-discriminant analysis (OPLS-DA) models were built to discriminate Chi-Nan agarwood from ordinary agarwood and to screen potential distinguishing components between them. In this study, we clarified the distinguishing differences between Chi-Nan agarwood and ordinary agarwood. The difference is mainly manifested in the average contents of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxybenzene)ethyl]chromone, which are 170 and 420 times higher in Chi-Nan agarwood than in ordinary agarwood, respectively, while the contents of 5,6,7,8-diepoxy-2-(2-phenylethyl)chromones (DEPECs), 5,6-epoxy-2-(2-phenylethyl)chromones (EPECs), and 5,6,7,8-tetrahydro-2-(2-phenylethyl)chromones (THPECs) such as agarotetrol are extremely low. The content of the main sesquiterpenes in Chi-Nan agarwood was higher than that in ordinary agarwood, especially in regard to guaiane and eudesmane derivatives. In addition, there were significant differences in the contents of low-molecular-weight aromatic compounds such as 2-methyl-4H-1-benzopyran-4-one, 4-methoxybenzaldehyde, and 2-hydroxybenzaldehyde between Chi-Nan agarwood and ordinary agarwood. All the mentioned main chemical characteristics of this new Chi-Nan agarwood were coincident with those of the rare wild Chi-Nan agarwood from *A. malaccensis*, *A. sinensis*, and *A. crassna*. We reported differences in 2-(2-phenylethyl)chromones, sesquiterpenes, and low-molecular-weight aromatic compounds between Chi-Nan agarwood and ordinary agarwood from *A. sinensis* for the first time; it is necessary to evaluate the agarwood from the new-found Chi-Nan germplasm.

FULL TEXT

1. Introduction

Agarwood is resinous wood obtained from wounded *Aquilaria* tree, which is a genus belonging taxonomically to the Thymelaeaceae family [1]. The main constituents in agarwood are volatile constituents and semi-volatile components [2]. The former include low-molecular-weight aromatic compounds and sesquiterpene derivatives, and the latter principally consist of 2-(2-phenylethyl)chromone derivatives [3]. Sesquiterpenes, including agarofurans, cadinanes, eudesmanes, eremophilanes, guaianes, and agarospiroanones, are considered to be the prominent contributors to agarwood aroma [4]. 2-(2-phenylethyl)chromone monomers can be divided into four categories based on the A ring of 2-(2-phenylethyl)chromones, namely, 5,6,7,8-tetrahydro-2-(2-phenylethyl)chromones (THPECs), diepoxy-tetrahydro-2-(2-phenylethyl)chromones (DEPECs), epoxy-tetrahydro-2-(2-phenylethyl)chromones (EPECs), and flindersia type 2-(2-phenylethyl)chromones (FTPECs) [2, 5].

Chi-Nan agarwood (CNA), a high-quality agarwood, is also called Qi-Nan or Jar-Nan in China, *Kanankoh* or *Kyara* in Japan, and *Tagara* in India [6]. In China, wild CNA is considered representative of high-quality agarwood, whose price is increasing up to thousands of RMB yuan per gram [7, 8]. Wild Chi-Nan agarwood is valued for its mysterious and elegant oriental odour that could be obviously smelt without heating, which make it discriminate from other kinds of agarwood. Investigations on wild CNA have rarely been reported, in contrast to abundant reports related to ordinary agarwood. In 1985, Hashimoto proved that benzaldehyde and 4-methoxybenzaldehyde originated from the pyrolysis of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxyphenyl)ethyl]chromone, respectively, in the neutral part of *Kyara* [9]. Ishihara discovered that the relative contents of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxyphenyl)ethyl]chromone from *Kanankoh* (*A. agallocha*, synonym: *A. malaccensis*) [10] smoke were much higher than those from Jinkoh [11]. In addition, taking four types of wild CNA (*A. sinensis* and *A. agallocha*) as materials, Dai showed that there were abundant 2-(2-phenylethyl)chromones and 2-[2-(4'-methoxyphenyl)ethyl]chromones [6, 12]. It is not difficult to notice that previous studies took only the 2-(2-phenylethyl)chromones into account. In addition, Chinese pharmacologist Xie Zongwan described the morphological characteristics of CNA but failed to provide information on the original plant [13]. Due to the confusion regarding the original plant, relevant information on wild CNA has seldom been described in the mentioned studies.

In recent years, a kind of special agarwood germplasm of *A. sinensis* has been introduced and domesticated from the wild population and propagated by grafting with ordinary germplasm of *A. sinensis*. Agarwood with more than 40% of the alcohol soluble extractive content can be obtained after this kind of tree is wounded with the drilling method over one year. However, for ordinary germplasm of *A. sinensis*, it is almost impossible to obtain agarwood with more than 10% of the alcohol soluble extractive content with the same inducing method [14]. Because of the similarity of characteristics such as appearance, form, texture, color, smell, and taste to wild CNA, this new-found agarwood is also called CNA. To date, there has been only one report by Li [15] in which the chemical properties of this kind of CNA have been tested by TG-FTIR and HS-GC-MS, and these authors named it *Kynam*. However, the authors did not clarify the specific components to distinguish CNA in terms of fragrance and the chemical characteristics of CNA. Here, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) were used to analyze the differences between CNA induced from new-found germplasm and ordinary agarwood (OA) to obtain a deep knowledge of phytochemical characteristics.

2. Materials and Methods

2.1. Agarwood Materials

Thirteen agarwood samples were analyzed as shown in Table 1 and Figure S1. They are divided into CNA (Chi-Nan agarwood) and OA (ordinary agarwood) group. The seven CNA samples were collected from new-introduced germplasms *A. sinensis* tree that were provided by three farmers from different planting bases of Hainan and Guangdong province in China. As for the OA group, we chose six ordinary agarwood induced with three kinds of common agarwood-inducing methods, including whole-tree agarwood-inducing technique (Agar-Wit) [16], burning-chisel-drilling (BCD), and wild.

Table 1

Sample information.

Nu m.	Speci es	Agarwood induction method	Place of production	Description	Sin kag e
C N A1	<i>A. sinensis</i>	3-year-old Chi-Nan germplasm by drilling for 15 months	Ding'an, Hainan province	Irregular pieces, black brown resin bands alternate with yellow white wood stripes, sufficient resin, rich of aromas, acrid in taste, soft and glutinous	x
C N A2	<i>A. sinensis</i>	3-year-old Chi-Nan germplasm by drilling for 14 months	Ding'an, Hainan province	Irregular strips, brown resin bands and white wood are distributed alternately, strong fragrance, cool feeling, bitter in taste, hard texture, slightly sticky	x
C N A3	<i>A. sinensis</i>	5-year-old Chi-Nan germplasm by drilling for 18 months	Ding'an, Hainan province	Irregular pieces, black brown resin scatter like spots, adequate resin, the aroma is thick, numb the tongue, hard texture, sticky	√
C N A4	<i>A. sinensis</i>	10-year-old Chi-Nan germplasm by drilling for 12 months	Maoming, Guangdong province	Irregular strips, apparent brown resin spread throughout the surface, intense aroma, taste peppery, hard texture, sticky	√
C N A5	<i>A. sinensis</i>	3-year-old Chi-Nan germplasm by drilling for 12 months	Maoming, Guangdong province	Irregular pieces, black brown resin scatter like spots, saturated with resin, fragrance is elegant, spicy and numb, tough, sticky	x
C N A6	<i>A. sinensis</i>	20-year-old Chi-Nan germplasm by drilling for 18 months	Maoming, Guangdong province	Irregular pieces, black brown resin scatter like spots or stripe, quite strong aroma, little spicy and numb, tough, sticky	√
C N A7	<i>A. sinensis</i>	3-year-old Chi-Nan germplasm by drilling for 12 months	Maoming, Guangdong province	Irregular pieces, obvious black brown resin scatter like spots or stripe, cool feeling, taste peppery and numb, tough, soft and glutinous	√
O A1	<i>A. sinensis</i>	6-year-old trees induced by Agar-Wit ^a for 18 months	Haikou, Hainan province	Irregular slices, brown resin bands alternate with yellow white wood stripe, pleasant fragrance, crisp	x
O A2	<i>A. sinensis</i>	6-year-old trees induced by Agar-Wit for 8 months	Danzhou, Hainan province	Irregular thin slices, saturated with resin brown resin, sweet fragrance, crisp	x

O A3	<i>A. sinensis</i>	Wild agarwood	Hainan province	Irregular pieces, massive protrusions and patches distribute throughout the appearance, slight aroma, soft	x
O A4	<i>A. sinensis</i>	Wild agarwood	Hainan province	Irregular pieces, many protrusions and patches distribute throughout the appearance, tawny resin scatter like spots, slight aroma, crisp	x
O A5	<i>A. sinensis</i>	5-year-old trees induced by BCD ^b for 12 months	Maoming, Guangdong province	Irregular pieces or slices, tawny resin and white wood are distributed alternately, many fibers in the cross section, slight aroma, resilient	x
O A6	<i>A. sinensis</i>	5-year-old trees induced by BCD for about 12 months	Maoming, Guangdong province	Irregular pieces, brown resin scatter like spots, cheerful, aroma, crisp	x

^aThe abbreviation of whole-tree agarwood-inducing technique is Agar-Wit. ^bThe abbreviation of burning-chisel-drilling is BCD. “√”: the agarwood sample could sink in water. “x”: the agarwood sample could not sink in water.

2.2. Chemical Reagent

Al₂O₃ powder as dispersant (High purity, Tianjin Guangfu Fine Chemical Research Institute, China) and retention index marker alkanes (AccuStandard, USA, C9–C40) were used in GC-MS, as well as anhydrous alcohol (AR, Xilong Science Co., Ltd, China), and acetonitrile (Merck HPLC grade, Darmstadt, Germany).

2.3. Sample Preparation

For all the samples described in Section 1, agarwood material sections, chips, or blocks were ground into powder with liquid nitrogen. Agarwood powder (0.2g) was extracted with 10mL of 50% ethanol by means of sonication at room temperature for 30min. Then, the supernatant was used as the sample reserve solution. Through preexperiments, we found that the content of 2-(2-phenylethyl)chromones and 2-[2-(4'-methoxyphenyl)ethyl]chromones in CNA was too high to analyze, so we diluted the test solution 10 times before analysis.

Agarwood powder was put into a 20ml headspace bottle, 0.2g Al₂O₃ was added as the dispersant [17], and the solution was analyzed in the headspace sampler according to the following conditions: headspace heating temperature: 180°C; transmission line temperature: 185°C; injection probe temperature: 190°C; headspace heating time: 40min; and N₂ purging time: 30s. For each of the samples, 1 ml of headspace gas was used for GC-MS analysis. The retention index marker alkanes (C9–C40) were analyzed by the same program in GC-MS.

2.4. LC-Q/Tof-MS Analysis Condition

LC-MS was performed using an ultrahigh-performance liquid chromatography (UPLC) system (H-class, Waters, USA) coupled with a quadrupole time-of-flight tandem spectrometer (Xevo G2-XS, Waters, USA). Separation was performed using a Waters Acquity UPLC BEH C18 column (3.0mm×100mm, 1.7 μm, Waters, USA). The column temperature was 30°C. The detection wavelength was set at 196nm for all the tested compounds, and the mobile phases were acetonitrile (A) and water (B). A gradient elution was used: 0min, 20% (v/v) A; 20min, 50% (v/v) A; 30 min, 20% (v/v) A. The mobile phase was established at a flow rate of 0.2mL·min⁻¹, and the injection volume was 5 μL.

The nebulization gas was set to 600L·h⁻¹ at a temperature of 350°C, and the cone gas was set to 50L·h⁻¹. The source temperature was set to 110°C. The capillary voltage and cone voltage were set to 3500V and 30V, respectively. The data acquisition rate was set to 0.3s with a 0.1 s interscan delay. Data between *m/z* 50 and 1200

were recorded in positive ion mode. The quality axis was corrected by sodium formate, and the quality of leucine enkephalin was corrected in real time.

2.5. SHS-GC-MS Analysis Conditions

GC-MS analysis was performed with a TSQ GC system (Agilent Technologies, USA). The chromatographic separation was conducted with a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness, Agilent Technologies, USA). The GC/MS interface temperature was maintained at 250°C. Ultrahigh purity helium (99.9995%) was used as the carrier gas at a flow rate of 1 mL·min⁻¹. The column temperature program was set as follows: 1 min at the initial temperature of 50°C, which was subsequently ramped to 143°C at a rate of 15°C·min⁻¹; 10 min at 143°C, which was ramped to 155°C at a rate of 1°C·min⁻¹; 0 min at 155°C, which was ramped to 225°C at a rate of 25°C·min⁻¹; 7 min at 225°C, which was ramped to 300°C at a rate of 25°C·min⁻¹; then the temperature was held at 300°C for 5 min. The ratio of gas flowing out of the chromatographic system and gas flowing into the chromatographic column after sample gasification was 5:1.

2.6. Data Preprocessing and Statistical Analysis

UPLC-Q/ToF-MS raw data were imported into Progenesis QI (Waters, USA) for preprocessing, while GC-MS raw data were converted and preprocessed by MS-Dial (NSF-JST, Japan). Statistical analysis was carried out by SIMCA-P (version 14.1, Umetrics, Umea, Sweden) on the basis of data matrixes. Unsupervised principal component analysis (PCA) was used to identify similarities or latent differences between groups. Data plotted using PC revealed intersample relationships via their spatial proximity. Orthogonal projection to latent structure discriminant analysis (OPLS-DA) was then implemented to detect maximum information from the data set and to distinguish the components induced by different groups. Distinguished compounds were screened by analyzing the Variable Importance in the Projection (VIP) and S-plot. Finally, Student's *t*-test (*p* < 0.05) was used to screen the significant variables with SPSS R26 (IBM, USA).

3. Results

3.1. Phytochemical Characteristics of CNA Based on LC-MS

Based on the high-resolution mass spectrometry and fragment ion information obtained in the experiment, thirty 2-(2-phenethyl)-chromones in thirteen batches of samples were identified according to the methods used in previous studies [18–20], as shown in Table S1. They were distributed into three parts of the total ion chromatograms in Figure 1. It is noteworthy that, between 0 and 10 min (zone *a*), the main components detected were THPECs, while EPECs/DEPECs flowed out from 10–15 min (zone *b*). The FTPECs and dimers flowed out after 15 min of the elution gradient (zone *c*). Historically, the composition of CNA is relatively simple, especially before 15 min, which indicates that FTPECs are the predominant type of 2-(2-phenethyl)chromones in CNA.

[figure omitted; refer to PDF]

Thus, we carried out PCA to obtain an objective understanding of LC-MS data, and the results are shown in Figure 2(a). The two PCs described 72.9% variance, of which the first PC (PC1) accounted for 57.3% and PC2 accounted for 15.6% of the total variance. The CNA mainly gathered on the positive side of PC1, and the OA was distributed on the negative side of PC1 which proved that the two groups had obvious differences in the composition of 2-(2-phenethyl)chromones. Then, supervised OPLS-DA was subsequently used. As depicted in Figure 2(b), the CNA samples are clearly separated from the OA samples [*R*²_X (cum) = 0.727; *R*²_Y (cum) = 0.977; *Q*² (cum) = 0.952]. One orthogonal component was calculated. The validation plot obtained from 200 permutation tests confirmed the validity of this OPLS-DA model. The criteria for validity include the following: all the permuted *R*² and *Q*² values to the left are lower than the original points to the right, and the regression line of the *Q*² points intersects the vertical axis (on the left) at or below zero.

[figures omitted; refer to PDF]

3.2. Phytochemical Characteristics of CNA Based on GC-MS

To obtain a chemical profile of the volatile constituents of agarwood, an analytical method based on SHS-GC-MS was developed. The TIC chromatographs of representative samples (OA1 and CNA1) and the plot corresponding to the temperature program are shown in Figure 3. With the application of Agilent MassHunter Quantitative Analysis

10.0, 101 compounds were identified by searching NIST14 in the MS data and retention index. Tentative identification with similarity over 80% and retention index within ± 20 is listed in Table S2. Figure 3 shows that low-molecular-weight aromatic compounds, sesquiterpenes, and chromones flow out in order during different temperatures. Among them, mainly low-molecular-weight aromatic compounds were detected when the temperature was above 50°C (zone *a*). When the temperature ranges from 142°C to 225°C (zone *b*), sesquiterpenes and their thermal cracking fragments flow out of the column. Finally, low-polarity 2-(2-phenethyl)-chromones can be detected when the temperature rises up to 225°C until 38.6 min (zone *c*).

[figure omitted; refer to PDF]

Mutual projections of factor scores for the first two PCs are presented in Figure 4(a), and these scores described 35.8% (PC1) and 16.7% (PC2) of the variability in the data when ellipse hoteling was set as 95% (70.1% can be explained by the four components). Thirteen batches of agarwood samples could be readily divided into two different groups, which indicated that the content and distribution of components were different between CNA and OA. The CNA samples were clustered closer to each other than to the OA samples on the negative side of PC1, which indicates that the differences between the groups are relatively minor.

[figures omitted; refer to PDF]

To identify distinguishing compounds, the data were further processed with orthogonal partial least squared discriminant analysis (OPLS-DA). The classification results are shown in Figure 4(b). The CNA group could be clearly separated from the OA group. The values of R^2X (cum) and R^2Y (cum) are 0.628 and 1, respectively, which is an important parameter to show how much data contributed to creating the model. In addition, Q^2 (cum) was 0.956, indicating that the OPLS-DA model had high predictability. To guard against model overfitting, permutation tests with 200 iterations were carried out.

Furthermore, we analyzed the distribution of different types of sesquiterpenes in the samples. Finally, a total of seventeen types of sesquiterpenes were identified in thirteen batches of samples. Among them, seven predominant types of sesquiterpenes, including α -santalanes, agarospiranes, guaianes, eremophilanes, eudesmanes, cadinanes, and agarofurans, were used for successive analysis. Following the logarithmic conversion of the total peak area of various sesquiterpenes, Figure 5 reflects the distribution of different configurations of sesquiterpenes between CNA and OA. The stars on the boxes represent mean values, and the results indicate that CNA possesses a higher concentration of all sesquiterpenes than OA. The length of the box, which indicates the interquartile range, clearly shows that CNA possesses greater dispersion. Obviously, eudesmane derivatives are most abundant in CNA, while eremophilane derivatives comprise the majority of sesquiterpenes in OA. Comparatively, the concentrations of guaianes and eudesmanes were significantly different ($p < 0.05$) between the two groups.

[figure omitted; refer to PDF]

3.3. The Distinguishing Components between CNA and OA

To determine the differences in the composition of 2-(2-phenethyl)-chromones, 13 potential distinguishing components were identified, as shown in Table 2. The S-plot derived from the OPLS-DA model comparing the two groups is shown in Figure 6(a). The VIP value reflects the influence of every compound's ion on the classification. Variables with a VIP value > 1 have an above-average influence on the explanation of the *Y* matrix. In our research, we adjusted VIP up to 5 to evaluate significant variables according to the practical situation. The qualified variables that meet the above criteria are the best possible distinguishing components labeled by a gray square. Subsequently, these components were used for Student's *t*-test.

Table 2

Thirteen distinguished compounds between CNA and OA in terms of 2-(2-phenylethyl)chromones.

Variable ID	Molecular formula	p value	Group	Proposed compound		
Types	A ring	B ring	6455	$C_{19}H_{18}O_4$	0.014	OA

FTPECs	1 OCH ₃	1 OCH ₃	4531	C ₁₈ H ₁₆ O ₅	0.024	OA
DEPECs		1 OCH ₃	6289	C ₂₀ H ₂₀ O ₅	0.028	OA
FTPECs	2 OCH ₃	1 OCH ₃	5685	C ₁₉ H ₁₈ O ₅	0.001	OA
FTPECs	1 OCH ₃	1 OH, 1 OCH ₃	3225	C ₁₆ H ₁₆ O ₆	0.005	OA
THPECs	2 OH	1 OH, 1 OCH ₃	4729	C ₁₇ H ₁₄ O ₄	0.034	OA
DEPECs	2 -O-		2045	C ₁₇ H ₁₈ O ₆	0.005	OA
THPECs	Agarotetrol		6964	C ₁₈ H ₁₆ O ₃	***	CNA
FTPECs		1 OCH ₃	7234	C ₁₇ H ₁₄ O ₂	***	CNA
FTPECs	2-(2-Phenylethyl)chromone		5255	C ₁₈ H ₁₆ O ₄	***	CNA
FTPECs		1 OH, 1 OCH ₃	6936	C ₃₅ H ₂₈ O ₇	***	CNA
Dimeric-PECs			7180	C ₃₆ H ₃₀ O ₈	***	CNA
Dimeric-PECs			5072	C ₁₈ H ₁₆ O ₄	***	CNA

***indicates a significant difference between two groups (p<0.001).

[figures omitted; refer to PDF]

Table 2 shows seven identified significant components, including two DEPECs, two THPECs, and three FTPECs with higher contents in OA; these components are highlighted with blue stars. The p values of compounds **2045**, **3225**, and **5685** were less than 0.01, which means that the differences in these constituents were significant. Likewise, all 6 identified significant components were FTPECs and dimeric PECs in CNA. Moreover, all the constituents' p values were below 0.001. It should be noted that the average peak areas of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxybenzene)ethyl]chromone are 170 and 420 times higher in CNA than in OA, respectively, as shown in Table S2.

We applied the same method to select the distinguishing volatile constituents from agarwood. According to the specific conditions of this experimental variable, the VIP threshold was adjusted to 4. Based on the above

conditions, the compounds in the rectangular frame, as shown in Figure 6(b), are highlighted. Student's *t*-test was performed in succession, and variables without significant differences between the two groups ($p>0.05$) were eliminated. The remaining compounds (marked with stars in Figure 6(b)) were selected for identification. Detailed information on the twelve distinguishing components is listed in Table 3. The most noteworthy differences between CNA and OA are in low-weight aromatic compounds and 2-(2-phenylethyl)chromones. Regarding low-weight aromatic compounds, compounds **53**, **64**, **180**, **334**, and **570** were abundant in OA, while compounds **223**, **286**, **304**, **361**, and **614** were abundant in CNA. Remarkably, the *p* values of 2-(2-phenethyl)-chromone and 2-[2-(4'-methoxyphenyl)ethyl]-chromone were below 0.001, which indicates that the contents of these two compounds are significantly different between CNA and OA.

Table 3

Twelve distinguished compounds between CNA and OA in terms of volatile constituents.

Peak	RI	Molecular formula	p value	Group	Proposed compound
570	1504	$C_{11}H_{14}O_2$	***	OA	4-(4-methoxyphenyl)-2-butanone
53	733	$C_2H_4O_2$	0.002	OA	acetic acid
334	1248	$C_{10}H_{12}O$	0.022	OA	4-phenyl-2-butanone
64	750	$C_3H_6O_2$	0.008	OA	1-hydroxy-2-propanone
180	966	C_7H_6O	0.047	OA	benzaldehyde
614	1526	$C_{10}H_8O_2$	***	CNA	2-methyl-4H-1-benzopyran-4-one
304	1207	$C_8H_8O_2$	0.009	CNA	4-methoxybenzaldehyde
361	1290	$C_8H_{10}O_2$	***	CNA	4-methoxybenzenemethanol
223	1051	$C_7H_6O_2$	***	CNA	2-hydroxybenzaldehyde
286	1172	$C_8H_8O_2$	0.001	CNA	1-(2-hydroxyphenyl)ethanone
1589	2618	$C_{17}H_{14}O_2$	***	CNA	2-(2-phenethyl)chromone
1557	2352	$C_{18}H_{16}O_3$	***	CNA	2-[2-(4'-methoxyphenyl)ethyl]chromone

***indicates a significant difference between two groups ($p<0.001$).

4. Discussion

4.1. The Differences in 2-(2-Phenylethyl)chromones between CNA and OA

As the results demonstrate, the 2-(2-phenylethyl)chromones in CNA induced from new-found Chi-Nan germplasm are relatively simple and all belong to FTPECs. Noticeably, the contents of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxybenzene)ethyl]-chromone are hundreds of times higher in CNA than in OA. Additionally, Japanese researchers discovered that the relative content of two constituents accounted for 5.83% and 1.59% in *Kanankoh* (wild CNA), while they accounted for only 0.28% and below 0.05% in *Jinkoh* (OA) [11]. Dai reported that the relative

contents of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxyphenyl)ethyl]-chromone were extremely high in the ether extract of four kinds of wild CNA. Therefore, we considered 2-(2-phenylethyl)-chromone and 2-[2-(4'-methoxyphenyl)ethyl]-chromone as phytochemical characteristic constituents to distinguish CNA. Such characteristics can also account for the high alcohol soluble extractive content, as described in previous studies [15]. However, some 2-(2-phenylethyl)chromones, such as DEPECs, EPECs, and THPECs, exist in agarwood apart from FTPECs, especially THPECs, which are specific compounds that have so far been detected only in agarwood [21]. We found that the amount and content of THPECs and DEPECs in OA, such as compounds **2045**, **4531**, **3225**, and **4729**, especially agarotetrol (**2045**), were higher. Agarotetrol has been designated as an index component in the quality control of content determination in medicinal agarwood according to the *Chinese Pharmacopoeia* 2020. In contrast, the content of THPECs in CNA was extremely low, especially in agarotetrol. Thus, CNA fails to reach the requirement of the *Chinese Pharmacopoeia* 2020 on the basis of the content of agarotetrol.

4.2. Distinguishing Differences in Sesquiterpenes and Low-Weight Aromatic Compounds between CNA and OA

Agarwood is processed into perfumes and incenses in addition to being used as a raw material in traditional and modern medicines. Sesquiterpene volatile constituents and low-weight aromatic compounds are the major volatile constituents in agarwood [2]. Therefore, SHS was used as the pretreatment method to analyze the volatile components of agarwood to replicate the fumigation. In our results, 69 sesquiterpenes were detected and identified from CNA and 43 sesquiterpenes from OA. However, it is noteworthy that the contents of guaianes and eudesmanes derivatives in CNA were superior to those in OA. It has been reported that guaiane and eudesmane derivatives accounted for the majority of sesquiterpenes in wild CNA according to investigations of Ishihara et al. [22] and Yang et al. [23]. Even more importantly, many of them were reported to possess a pleasant fragrance [2, 12]. Guaianes and eudesmanes seem to contribute to the gorgeous and elegant character of CNA by contributing to their odoriferous properties [18]. Consequently, guaianes and eudesmanes may be significant contributors to the unique fragrance of CNA. Of course, we need to carry out relevant studies to specify the characteristics in terms of sesquiterpenes so that we can explain the fragrance of the CNA more perspicuously.

It is meaningful that we found that several low-weight aromatic compounds are similar to the partial structure of FTPECs, such as 4-methoxybenzene-methanol (**361**), 2-hydroxybenzaldehyde (**223**), and 1-(2-hydroxyphenyl)ethenone (**286**). Moreover, the contents of these compounds were higher than those in OA, so it is reasonable for us to speculate that the above low-weight aromatic compounds partly originate from the pyrolysis of 2-(2-phenylethyl)-chromone and 2-[2-(4'-methoxybenzene)ethyl]-chromone. Similarly, the high content of 4-methoxybenzaldehyde (**304**) in CNA has the same explanation. In fact, Hashimoto found that 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxyphenyl)ethyl]chromone could pyrolyze at 150°C for 6 h to produce benzaldehyde and 4-methoxybenzaldehyde, respectively [9]. It may be difficult to understand the origin of abundant benzaldehyde in OA. However, Takamatsu proved that agarotetrol could produce pyrolysis products such as 4-phenyl-2-butanone and benzaldehyde with HS-SPME-GC-MS analysis when heated to 190°C–200°C [19]. Ishihara et al. found that smoke from agarwood contained a small amount of pulp wood pyrolysis products such as acetic acid, benzaldehyde, and vanillin when *Kanankoh* (CNA in Japanese) was heated at 180–210°C by an alcohol lamp [11]. Therefore, we can hypothesize that the non-resinous part and agarotetrol in OA results in high concentrations of benzaldehyde (**180**) and acetic acid (**53**). In addition, we discovered that the content of 4-phenyl-2-butanone (**334**) in OA is nearly 1.5 times as high as that in CNA by calculating the peak area shown in Table S2. This is probably related to the biosynthesis of 2-(2-phenylethyl)chromones according to Liao's research [20]. In conclusion, there were considerable discrepancies in low-weight aromatic compounds between CNA and OA.

4.3. The Phytochemical Characteristics of New-Found CNA Are Consistent with Those of Wild CNA

Considering the chemical composition and application characteristics of agarwood, we comprehensively explored the phytochemical characteristics of CNA induced from new-found Chi-Nan germplasm of *A. sinensis*. Comparatively, CNA and OA showed dramatic differences in chemical composition. Notably, the phytochemical characteristics of CNA were consistent with those of wild CNA reported by Chinese and Japanese researchers in terms of phytochemical characteristics. In addition, we analyzed a wild CNA sample (CNA8) under the same

conditions, and the corresponding results are shown in Fig. S2-S3. Therefore, it is acceptable for us to speculate that CNA in this study is a kind of agarwood that is similar to wild CNA originating from *A. sinensis* or *A. malaccensis*, which is reported by both foreign and domestic researchers [11, 12]. Thus, this is the first study to comprehensively reveal the distinguishing phytochemical characteristics of volatile and semi-volatile components of this newly identified CNA, and the findings indicated that CNA may originate from Chi-Nan germplasm of *Aquilaria* spp. rather than a unique environment, induction method, or species. We also identified the original plant of this CNA via DNA barcoding technology as *A. sinensis*. The relevant conclusions will be published in another paper.

5. Conclusion

We demonstrated the historical phytochemical characteristics of CNA for the first time. Here, we reveal distinguishing differences in terms of 2-(2-phenylethyl)chromones, sesquiterpenes, and low-molecular-weight aromatic compounds between CNA and OA. The main chemical characteristic of CNA is the single composition of 2-(2-phenylethyl)chromones, which is characterized by extremely high contents of 2-(2-phenylethyl)chromone and 2-[2-(4'-methoxybenzene)ethyl]chromone in CNA. Regarding the different types of 2-(2-phenylethyl)chromones, CNA mainly contains FTPECs, while the contents of DEPECs, EPECs, and THPECs, such as agarotetrol, are extremely low. The content of low-molecular-weight aromatic compounds in the smoke of agarwood detected by heating from the pyrolysis of FTPECs was higher than that in OA, especially for 4-phenyl-2-butanone, benzaldehyde, 4-methoxybenzaldehyde, and 2-hydroxybenzaldehyde. Nevertheless, the concentrations of acetic acid, benzaldehyde, and 4-phenyl-2-butanone were much higher in OA than in CNA. Remarkably, the guaiane and eudesmane derivatives with higher content in CNA were likely to be the crucial contributors to the unique fragrance of CNA when used as incense.

Due to the significant differences between CNA and OA, it can be inferred that CNA may originate from Chi-Nan germplasm of *Aquilaria* spp., which can produce CNA in a short period of time without a special environment, place of origin, or method. The unique phytochemical characteristics of CNA may be related to the genetic information of the original plant germplasm or endophytic fungi. However, the biosynthesis mechanism of the abovementioned chemical characteristics needs further research for clarification.

Acknowledgments

The authors deeply appreciate Mr. Lin BZ, Wen ZX, Huang CL, and Qin WY who provided CNA samples. This work was supported by the National Key Research and Development Project of China (2018YFC1706403), Hainan Provincial Natural Science Foundation of China (2019RC340), National Natural Science Foundation of China (81703660), and CAMS Innovation Fund for Medical Sciences (2016-I2M-2-003).

Glossary

Abbreviations

CNA: Chi-Nan agarwood

OA: Ordinary agarwood

SHS-GC-MS: Static headspace sampling gas chromatography mass spectrometry

UPLC: Ultra-performance liquid chromatography

Q-ToF-MS: Quadrupole time-of-flight mass spectrometry

HRMS: High-resolution mass spectrometry

PCA: Principal components analysis

OPLS-DA: Orthogonal partial least squares discriminant analysis

BCD: Burning-chisel-drilling

Agar-Wit: Whole-tree agarwood-inducing technique

PC: Principal component

PECs: 2-(2-phenylethyl)chromones

THPECs: 5,6,7,8-tetrahydro-2-(2-phenylethyl)-chromones

DEPECs: 5,6,7,8-diepoxy-tetrahydro-2-(2-phenylethyl)chromones

EPECs: Epoxy-tetrahydro-2-(2-phenylethyl)chromones

FTPECs: Flindersia type 2-(2-phenethyl)chromones.

References

- [1] H.-Q. Chen, J.-H. Wei, J.-S. Yang, "Chemical constituents of agarwood originating from the endemic genus *Aquilaria* plants," *Chemistry & Biodiversity*, vol. 9 no. 2, pp. 236-250, DOI: 10.1002/cbdv.201100077, 2012.
- [2] R. Naef, "The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: a review," *Flavour and Fragrance Journal*, vol. 26 no. 2, pp. 73-87, DOI: 10.1002/ffj.2034, 2011.
- [3] S. R. M. Ibrahim, G. A. Mohamed, "Natural occurring 2-(2-phenylethyl) chromones, structure elucidation and biological activities," *Natural Product Research*, vol. 29 no. 16, pp. 1489-1520, DOI: 10.1080/14786419.2014.991323, 2015.
- [4] M. Gao, X. Han, Y. Sun, "Overview of sesquiterpenes and chromones of agarwood originating from four main species of the genus *Aquilaria*," *RSC Advances*, vol. 9 no. 8, pp. 4113-4130, DOI: 10.1039/c8ra09409h, 2019.
- [5] Y. Okudera, M. Ito, "Production of agarwood fragrant constituents in *Aquilaria* calli and cell suspension cultures," *Plant Biotechnology*, vol. 26 no. 3, pp. 307-315, DOI: 10.5511/plantbiotechnology.26.307, 2009.
- [6] D. L. Yang, W. L. Mei, J. L. Yang, Y. B. Zeng, H. F. Dai, "GC-MS analysis of the fragrant sesquiterpenes and 2-(2-phenylethyl)Chromone derivatives in four types of agarwood "Qi-Nan"," *Chinese Journal of Tropical Crops*, vol. 35, pp. 1235-1243, 2014.
- [7] E. Yamagata, "Pharmacognostical studies on the crude drug of agarwood (V). Evaluation of agarwood by principal component analysis and factor analysis," *Shoyakugaku Zasshi*, vol. 40, pp. 275-280, 1986.
- [8] M. Ishihara, "The scent of Kyara," *Koryo*, vol. 258, pp. 55-67, 2013.
- [9] K. Hashimoto, S. Nakahara, T. Inoue, Y. Sumida, M. Takahashi, Y. Masada, "A new chromone from agarwood and pyrolysis products of chromone derivatives," *Chemical and Pharmaceutical Bulletin*, vol. 33 no. 11, pp. 5088-5091, DOI: 10.1248/cpb.33.5088, 1985.
- [10] USDA, A. R. S., National Plant Germplasm System. Germplasm Resources Information Network (GRIN-Taxonomy), 2020, <https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysimple.aspx>
- [11] M. Ishihara, T. Tsuneya, K. Uneyama, "Components of the agarwood smoke on heating," *Journal of Essential Oil Research*, vol. 5 no. 4, pp. 419-423, DOI: 10.1080/10412905.1993.9698252, 1993.
- [12] D. L. Yang, "Study on fragrant constituents in agarwood "Qi-Nan" and quality evaluation of agarwood," 2014.
- [13] Z. W. Xie, *Description of Varieties of Chinese Medicinal Materials*, vol. 1, 1964.
- [14] J. Q. Huang, J. H. Wei, Z. Zhang, "Historical records and modern studies on agarwood production method and overall agarwood production method," *Zhongguo Zhong Yao Za Zhi*, vol. 38, pp. 302-306, 2013.
- [15] Y. Chen, T. Yan, Y. Zhang, Q. Wang, G. Li, "Characterization of the incense ingredients of cultivated grafting Kynam by TG-FTIR and HS-GC-MS," *Fitoterapia*, vol. 142, DOI: 10.1016/j.fitote.2020.104493, 2020.
- [16] Y. Liu, H. Chen, Y. Yang, "Whole-tree agarwood-inducing technique: an efficient Novel technique for producing high-quality agarwood in cultivated *Aquilaria sinensis* trees," *Molecules*, vol. 18 no. 3, pp. 3086-3106, DOI: 10.3390/molecules18033086, 2013.
- [17] Q. P. Zhong, Z. J. Zhong, X. D. Chen, "Analysis of aroma components in agarwood by SHS-GC-MS combined with retention index," *Herald of Medicine*, vol. 35, pp. 1243-1249, 2016.
- [18] M. Ishihara, T. Tsuneya, M. Shiga, K. Uneyama, "Three sesquiterpenes from agarwood," *Phytochemistry*, vol. 30 no. 2, pp. 563-566, DOI: 10.1016/0031-9422(91)83727-3, 1991.
- [19] S. Takamatsu, M. Ito, "Agarotetrol in agarwood: its use in evaluation of agarwood quality," *Journal of Natural Medicines*, vol. 74 no. 1, pp. 98-105, DOI: 10.1007/s11418-019-01349-w, 2019.
- [20] G. Liao, W. H. Dong, J. L. Yang, "Monitoring the chemical profile in agarwood formation within one year and speculating on the biosynthesis of 2-(2-Phenylethyl)Chromones," *Molecules*, vol. 23, DOI: 10.3390/molecules23061261, 2018.
- [21] J. Li, D. Chen, Y. Jiang, Q. Zhang, L. Zhang, P.-F. Tu, "Identification and quantification of 5,6,7,8-tetrahydro-2-(2-phenylethyl)chromones in Chinese eaglewood by HPLC with diode array detection and MS," *Journal of Separation Science*, vol. 36 no. 23, pp. 3733-3740, DOI: 10.1002/jssc.201300687, 2013.

[22] M. Ishihara, T. Tsuneya, K. Uneyama, "Guaiane sesquiterpenes from agarwood," *Phytochemistry*, vol. 30 no. 10, pp. 3343-3347, DOI: 10.1016/0031-9422(91)83206-z, 1991.

[23] D.-L. Yang, W. Li, W.-H. Dong, J. Wang, W.-L. Mei, H.-F. Dai, "Five new 5,11-epoxyguaiane sesquiterpenes in agarwood "Qi-Nan" from *Aquilaria sinensis*," *Fitoterapia*, vol. 112, pp. 191-196, DOI: 10.1016/j.fitote.2016.05.014, 2016.

DETAILS

Subject:	Mass spectrometry; Hydroxybenzaldehydes; Phytochemicals; Aromatic compounds; Trees; Alcohol; Scientific imaging; Germplasm; Discriminant analysis; Chromatography; Chemical composition; Weight
Location:	United States--US; China
Editor:	Shaoping Li
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2021-01-06 (Received); 2021-03-18 (Revised); 2021-03-31 (Accepted); 2021-04-10 (Pub)
DOI:	https://doi.org/10.1155/2021/5593730
ProQuest document ID:	2514170702

Document URL: <https://www.proquest.com/scholarly-journals/remarkable-phytochemical-characteristics-chi-nan/docview/2514170702/se-2?accountid=211160>

Copyright: Copyright © 2021 Meng Yu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-04-19

Database: Publicly Available Content Database

Document 40 of 59

Simultaneous Quantification of Aspirin, Its Metabolite Salicylic Acid, and Salvianolic Acid B in Human Plasma Using UPLC-MS/MS

Cao, Weiyi ¹

; Yao, Hezhi ² ; Qian, Zhenzhen ² ; Wu, Yi ¹ ; Wang, Shuge ¹ ; Li, Rui ¹

; Gao, Rui ¹

¹ Institute of Clinical Pharmacology of Xiyuan Hospital, China Academy of Chinese Medical Sciences, No. 1, R. Xiyuangcaochang, Haidian District, Beijing 100091, China; NMPA Key Laboratory for Clinical Research and Evaluation of Traditional Chinese Medicine, National Clinical Research Center for Chinese Medicine Cardiology, Beijing, China ² Institute of Clinical Pharmacology of Xiyuan Hospital, China Academy of Chinese Medical Sciences, No. 1, R. Xiyuangcaochang, Haidian District, Beijing 100091, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Salvianolic acid B is the main active ingredient in salvianolate injection, which is produced by extracting danshen, the most commonly used Chinese herbal medicine for cardiovascular treatment. Clinically, salvianolate injection and aspirin are commonly combined to treat coronary heart diseases in patients with stable angina. To support clinical studies on drug-drug interactions (DDIs) between salvianolate injection and aspirin, a rapid and sensitive UPLC-MS/MS method for the simultaneous determination of aspirin (acetylsalicylic acid), its metabolite salicylic acid, and salvianolic acid B in human plasma was developed. The analytes and internal standard were extracted from the acidified plasma by liquid-liquid extraction with ethyl acetate and then separated by gradient elution with acetonitrile/0.5% formic acid in water on a C18 column. Salvianolic acid B, acetylsalicylic acid, and salicylic acid were quantified in multiple-reaction monitoring mode with negative ion electrospray ionization. The method was fully validated according to the current regulatory guidance for bioanalysis. Calibration curves in the range 5–6000 ng/mL, all with correlation coefficients greater than 0.99, were established using linear regression models for salvianolic acid B and acetylsalicylic acid and a quadratic model for salicylic acid. The validated method was successfully used to

measure salivianolic acid B, acetylsalicylic acid, and salicylic acid concentrations in human plasma samples from 16 patients to observe the pharmacokinetic changes caused by DDIs.

FULL TEXT

DETAILS

Subject:	Formic acid; Plasma; Aspirin; Regression models; Calibration; Quality control; Negative ions; Liquid-liquid extraction; Metabolites; Ethyl acetate; Correlation coefficients; Heart diseases; Acetonitrile; Acetylsalicylic acid; Solvents; Herbal medicine; Salicylic acid; Cardiovascular disease; Blood plasma; Pharmacokinetics; Regression analysis; Angina pectoris; Acidification
Business indexing term:	Subject: Quality control
Location:	United States--US; China
Editor:	David Touboul
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-11-24 (Received); 2021-02-07 (Revised); 2021-03-15 (Accepted); 2021-03-29 (Pub)
DOI:	https://doi.org/10.1155/2021/6620868

ProQuest document ID: 2510954661

Document URL: <https://www.proquest.com/scholarly-journals/simultaneous-quantification-aspirin-metabolite/docview/2510954661/se-2?accountid=211160>

Copyright: Copyright © 2021 Weiyi Cao et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-04-11

Database: Publicly Available Content Database

Document 41 of 59

Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Vardenafil and Its Application of Bioequivalence

Kok Zheng Gan ¹

; Riyanto Teguh Widodo ¹

; Chik, Zamri ²

; Teh, Lay Kek ³

; Mohd Salleh Rofiee ³

; Mohd Izwan Mohamad Yusof ³

¹ Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Malaya, 50603 Kuala Lumpur, Malaysia ² University of Malaya Bioequivalence Testing Centre (UBAT), Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia ³ Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA, Bandar Puncak Alam 42300, Shah Alam, Selangor, Malaysia

[ProQuest document link](#)

ABSTRACT (ENGLISH)

A simple, rapid, and sensitive method of liquid chromatography-tandem mass spectrometry (LC/MS/MS) method was developed and validated for the determination of vardenafil in rabbit plasma. A simple protein precipitation method with ice-cold acetonitrile was used for plasma extraction. The mass transitions m/z 489151 and m/z 390169 were used to measure vardenafil and tadalafil (internal standard), respectively, with a total assay run time of 6 min. The limit of detection was 0.2 ng/mL. The assay was reproducible with intra-assay and interassay precision ranging 1.17%–9.17% and 1.31%–5.86%, respectively. There was also good intra-assay and interassay accuracy between 89.3%–105.3% and 94%–102% of the expected value, respectively. The linearity range was 0.5–60 ng/mL in rabbit plasma ($r^2 \geq 0.99$). The measured AUC from 0 to 24 h AUC_{0–24h} for the test and reference formulations were 174.38

± 95.91 and 176.45 ± 76.88 , respectively. For the test, C_{max} and T_{max} were 75.36 ± 59.53 ng/mL and 1.42 ± 0.19 h, whereas, for the reference, these were 58.22 ± 36.11 ng/mL and 2.04 ± 0.33 h, respectively. The test formulation achieved a slightly lower AUC_{0–24h} value ($p > 0.05$), higher C_{max} values ($p > 0.05$), faster T_{max} ($p < 0.05$), and almost equal bioavailability compared with the reference formulation.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Vardenafil (2-[2-ethoxy-5-(4-ethylpiperazin-1-yl)sulfonylphenyl]-5-methyl-7-propyl-3H-imidazo [5, 1-f] [1, 2, 4] triazin-4-one; trihydrate; hydrochloride) is a highly potent and selective phosphodiesterase 5 (PDE5) inhibitor [1]. This is generally well-tolerated in men with erectile dysfunction (ED) with no reports of abnormal color vision at recommended doses (5–20 mg) [2]. Vardenafil was shown to have 4- to 25-fold selectivity toward PDE5 compared with sildenafil and tadalafil, which only demonstrated 10- and 5-fold selectivity, respectively [1, 2]. High-fat meals were observed to reduce vardenafil absorption if taken orally with an 18%–50% reduction in maximum concentration C_{max} [3]. Vardenafil is highly bound to plasma protein (95%). The mean half-life $t_{1/2}$ of vardenafil ranges from 3.94 to 4.79 h [2]. It is extensively metabolized in the liver mainly by cytochrome P450 (CYP) 3A4 and a smaller portion by CYP3A5 and CYP2C isoforms [3]. Vardenafil film-coated tablets undergo extensive first-pass metabolism, and their absolute bioavailability is approximately 15% [4].

Formulating vardenafil in an orodispersible tablet dosage form can bypass the first-pass metabolism in the liver and can be taken even after a meal, thus improving patient satisfaction [5–7]. An integrated analysis concluded that vardenafil orodispersible tablets significantly improve erectile function in men with ED, regardless of baseline ED severity, age, or underlying conditions [8]. However, the presently marketed vardenafil orodispersible tablet takes a longer time to reach maximum concentration T_{max} [5, 9]. Inappropriate use of excipients in orodispersible formulations causes the drug to mainly be absorbed via the gastrointestinal route rather than the oral transmucosal route [5]. Thus, the current orodispersible formulation should have a faster onset of action by reducing T_{max} . Additionally, the newly optimized orodispersible formulation must also exhibit similar pharmacokinetic parameters and bioequivalence to the marketed orodispersible tablet.

In this study, there were a few challenges in quantifying vardenafil in rabbit plasma. The 10 mg dose of vardenafil ODT administered in the rabbit was low; only 150 μ L of plasma samples were recovered at each time point of blood collection. Most previous studies required a large sample volume to achieve a very low limit of quantification (LLOQ). Carlucci et al. (2009) developed a method to measure vardenafil in human plasma using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [10]. However, this method was tedious and involves double extraction steps of organic solvents, also requiring a large amount of plasma (1 mL) and a high injection volume (20 μ L) with a running time of 15 min [10]. A validated HPLC tandem fluorescence detector was used to quantify vardenafil in rat plasma [11]. This method has a relatively long run time of 18 min and the LLOQ remained high (10 ng/mL) [11]. Abu El-Enin et al. (2015) developed a spectrofluorimetric method to quantify vardenafil in human plasma and pharmaceutical products [12]. Despite the simple protein precipitation method with acetonitrile only, this method requires a large amount of human plasma (1 mL) to achieve an LLOQ of 10 ng/mL [12].

Apart from the HPLC with UV detection, liquid chromatography with tandem mass spectrometry (LC/MS/MS) can also be used to quantify drugs and metabolites in biological samples because of its high sensitivity and selectivity. This requires only a small sample volume and has a short analytical time.

A few validated LC/MS/MS methods that quantify vardenafil in plasma have previously been published [4, 13]. A method developed by Ku et al. (2009) to measure vardenafil in human plasma requires 0.25 mL of plasma to achieve an LLOQ of 0.5 ng/mL [4]. Another method by Lake et al. (2010) requires 0.2 mL of plasma to achieve an LLOQ of 0.2 ng/mL [13]. These two methods require liquid-liquid extraction and solvent evaporation in their sample preparation [4, 13]. By contrast, Bhadoriya et al. (2018) developed a method to extract tadalafil from 0.2 mL of

human plasma via solid-phase extraction [14]. The calibration curve was linear over the concentration range of 0.5–500 ng/mL [14]. Conversely, Kim et al. (2017) developed a method using acetonitrile to precipitate tadalafil from 0.02 mL of human plasma [15]. This method had achieved an LLOQ of 5 ng/mL with a total run time of 1 min [15]. The aforementioned methods require at least 200 μ L of plasma associated with tedious extraction methods to achieve LLOQ of 0.5 ng/mL. Thus, a new method is required to address the problem of limited samples and achieve a very low LLOQ.

2. Materials and Methods

2.1. Materials and Reagents

Vardenafil hydrochloride trihydrate (USP) was purchased from Medigene (M) Sdn Bhd, Malaysia. The purity of vardenafil hydrochloride trihydrate and tadalafil standard was all >99%. LCMS-grade acetonitrile and formic acid were also purchased from Sigma-Aldrich (M) Sdn Bhd. Standard tadalafil was obtained from the Faculty of Pharmacology, University of Malaya. Pooled blank plasma was obtained from the Animal Experimental Unit (AEU) of the University of Malaya.

2.2. Preparation of Standards and Quality Control (QC)

A stock solution of vardenafil (1 mg/mL) and tadalafil as Internal Standard (IS) were prepared in acetonitrile. The stock solution was further diluted with rabbit plasma to make working standard solutions in concentrations of 0.5, 1, 5, 10, 20, 30, 50, and 60 ng/mL. Internal standards with a final concentration of 40 ng/mL were prepared. The same method was used to prepare three QC samples at low (1.5 ng/mL), medium (25 ng/mL), and high (45 ng/mL).

2.3. Sample Preparation

A simple protein precipitation method was used to extract vardenafil and IS from plasma samples. First, 100 μ L of rabbit plasma was combined with 25 μ L tadalafil (IS) and vortex-mixed for 30 s. Then, 200 μ L of ice-cold acetonitrile was added to the mixture, and it was vortex-mixed again for 30 s to precipitate the protein. Subsequently, the mixture was centrifuged at 14000 rpm at 5°C for 10 min. The supernatant was collected, and 2 μ L of aliquot was injected directly into LC/MS/MS.

2.4. Chromatographic Conditions

Chromatographic separation was conducted using Agilent's Zorbax Eclipse Plus (2.1 $\times$ 50 mm i.d; 1.8 μ m) coupled with a guard column. Gradient elution was achieved using mobile phases consisting of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (100%, solvent B). Table 1 describes the gradient conditions used in the method. The flow rate of liquid chromatography (LC) was maintained at 0.4 mL/min, and the total time to run the test was 6 min. The retention time for vardenafil and tadalafil was 1.56 and 1.84 min, respectively.

Table 1

Gradient settings of mobile phase for vardenafil hydrochloride trihydrate assay.

Time (min)	Percentage of mobile phase in eluent	
A	B	0.0
90	10	0.5
10	90	3.0
90	10	6.0

A: 0.1% (v/v) formic acid in water; B: acetonitrile.

2.5. Mass Spectrometric Conditions

Mass spectrometry with electrospray ionization set at positive mode was used. The collision energy was set at 45 eV, cell accelerator voltage at 4 V, and the fragmentor at 135 V. The gas temperature was set at 280°C with the

nebulizer at 50 psi and a 12L/min flow rate. The aforementioned settings were determined when the highest signal was shown. The mass transitions of vardenafil were identified as m/z 489 151 as the quantifier and m/z 489 312 as the qualifier. Conversely, the mass transitions of tadalafil were identified as m/z 390 169 as the quantifier and m/z 390 135 as the qualifier. Figure 1 shows the mass spectrums of both vardenafil and tadalafil.

[figures omitted; refer to PDF]

2.6. Method Validation

The method was successfully validated to show the accuracy, precision, specificity, linearity, stability, and percentage of recovery from the rabbit plasma. Validation was conducted according to the Bioanalytical Method Validation: Guideline for Industry published by the United States Food and Drug Administration (USFDA) [16]. This method was validated within the concentration range of 0.5–60 ng/mL with three levels of quality control (QC) samples: QC low (1.5 ng/mL), QC medium (25 ng/mL), and QC high (45 ng/mL).

2.7. Animal Ethics

Animal ethics was approved by the University of Malaya Institutional Animal Care and Use Committee (UM IACUC) with Ethics Reference No: 2018-190403/PHARM/PS/GKZ. Rabbits were supplied by the AEU, Faculty of Medicine, University of Malaya.

2.8. Preclinical Application

The study was a randomized, single dose, balanced two-period crossover design with a 7-day washout period. Rabbits were fasted for 12 h before the experiment. Rabbits were allowed free access to water only. During the first period, six rabbits received the test formulation (newly optimized vardenafil ODT), while 6 other rabbits received the reference formulation (Staxyn® ODT 10 mg) according to a randomization schedule. After a 1-week washout period, the rabbits were switched to the other formulation accordingly. Before dosing, rabbits were sedated intramuscularly with a low dose of ketamine (10 mg/kg) and xylazine (1 mg/kg) to facilitate drug administration. Then, test or reference formulations were placed under the tongue of the rabbits, as shown in Figure 2. The rabbits were then wrapped with a towel to restrain movement before drawing 0.5 mL blood samples; these were drawn using the direct puncture technique at each time point with a 27 G $\times$ 1/2" needle from the marginal ear vein of each rabbit at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after drug administration. The blood samples were immediately centrifuged at 14000 rpm at 5°C for 10 min, and plasma samples were stored at –20°C until further analysis by LC/MS/MS. At the end of the study, rabbits were euthanized and disposed of according to the laboratory protocols.

[figure omitted; refer to PDF]

2.9. Bioequivalence Evaluation

ANOVA was performed on $AUC_{0-\infty}$, AUC_{0-t} , and C_{max} , both with and without log transformation. Bioequivalence statistics were calculated using the Phoenix WinNolin® 8.1 software to determine the 90% confidence interval range. IBM SPSS Statistics 25 software was used to determine the significance value of T_{max} and $t_{1/2}$.

3. Results

3.1. Specificity and Selectivity

Six different sources ($n=6$) of blank rabbit plasma were used to investigate the specificity and selectivity. No interfering peak was observed at the retention time of the analytes. Figure 3(a) shows the chromatograms of blank plasma. Conversely, blank plasma spiked with LLOQ and upper limit of quantification (ULOQ) concentrations of vardenafil and IS are illustrated in Figures 3(b) and 3(c), respectively. At the retention time of vardenafil, there were no interfering peaks observed in the plasma. Figure 3(d) shows the plasma samples 1.5 h after drug administration. The test proves that the method has high selectivity and specificity.

[figures omitted; refer to PDF]

3.2. Linearity

Eight different concentrations ranging from 0.5 to 60 ng/mL were used to establish linearity via the least squares linear regression (weighted $1/x$). The coefficient of determination R^2 for eight calibrators was 0.99, and the calibration curve was found to be linear. A lack-of-fit test was conducted to confirm the linearity of the method. Analysis of variance (ANOVA) test showed that the F value was less than the tabulated F value at a 95% confidence

level; hence, the linear regression showed no lack of fit.

The limit of detection of vardenafil was 0.2 ng/mL, whereas the signal-to-noise ratio was 3. The lowest concentration on the calibration curve was determined as the LLOQ with the accuracy and precision achieved within $\pm 20\%$. Three replicates at the lowest concentration of the calibration curve over three consecutive days were used to establish the LLOQ. The LLOQ of vardenafil was found to be 0.5 ng/mL, whereas the signal-to-noise ratio was more than 10.

3.3. Imprecision and Accuracy

Six replicates of LLOQ, ULOQ, and three levels of QC samples were used to determine the accuracy and precision of the method. Initially, the LLOQ, ULOQ, and three QC samples were extracted six times for one batch. Afterward, they were extracted six times in two additional batches. Table 2 shows the results of the intraday and interday tests for imprecision and accuracy. The highest relative standard deviation (RSD) observed was 8.89%. The assay was reproducible, with an intra-assay imprecision ranging from 1.17% to 9.17% and interassay imprecision ranging from 1.31% to 5.86%. The assay exhibited good intra-assay accuracy within 89.3%–105.3% of the expected value, whereas interassay accuracy was within 94%–102% of the expected value. The results shown in Table 2 meet the acceptance criteria; hence, the imprecision and accuracy of the method are adequate.

Table 2

Imprecision and accuracy for the determination of vardenafil in plasma.

	LLOQ 0.5ng/mL	LQC 1.5ng/mL	MQC 25ng/mL	HQC 45ng/mL	ULOQ 60ng/mL
Intraday ($n=6$)					
Day 1					
Mean	0.46	1.48	25.77	47.36	60.16
SD	0.04	2.78	9.17	7.64	4.99
RSD %	9.48	2.81	8.89	7.25	8.29
Accuracy %	92.0	98.9	103.1	105.3	100.27
–					
Day 2					
Mean	0.50	1.50	25.14	46.52	61.73
SD	0.01	1.76	2.34	1.76	1.54
RSD %	2.00	1.76	2.33	1.71	2.57
Accuracy %	100.0	100.0	100.6	103.4	100.3
–					
Day 3					

Mean	0.45	1.34	25.59	44.84	60.64
SD	0.03	7.06	1.17	8.65	1.77
RSD %	5.88	7.90	1.14	8.68	3.01
Accuracy %	90.0	89.30	102.4	99.6	100.1
–					
Interday (<i>n</i> = 18)					
Mean	0.47	1.44	25.50	46.24	59.65
SD	0.03	5.86	1.31	2.86	1.93
RSD %	0.06	6.10	1.29	2.78	3.22
Accuracy %	94.0	96.1	102.0	102.8	99.41

SD: standard deviation; RSD: relative standard deviation; %: percentage; LLOQ: lower limit of quantification; LQC: low quality control; MQC: medium quality control; HQC: high quality control; ULOQ: upper limit of quantification; ng indicates nanograms; mL indicates milliliters.

3.4. Stability

Assessment of stability was conducted with three replicates of low (LQC) and high quality control (HQC) for various storage conditions. The stability of vardenafil as shown in Table 3 was tested in plasma at room temperature for 8 h, at 10°C in an autosampler for 24 h, at 20°C for three freeze-thaw cycles, and at –20°C for 2 months. To meet acceptance criteria, the RSD must be ≤15%. In this study, the highest RSD observed was 12.72%, and the mean accuracy was within 88.7%–108.4% with no significant degradation of vardenafil, as presented in Table 3.

Table 3

Stability test of vardenafil in plasma.

		LQC (AUC ratio)	HQC (AUC ratio)
Post 8 h at 25°C	Mean	0.92	29.04
SD	0.09	1.68	RSD %
10.68	5.79	Accuracy %	98.2
100.1	–		
Post 10 h at 10°C	Mean	0.85	29.92
SD	0.04	3.25	RSD %

4.94	10.89	Accuracy %	90.5
103.1	-		
Three times freeze-thaw at -20°C	Mean	0.92	31.45
SD	0.11	0.76	RSD %
12.72	2.44	Accuracy %	98.1
108.4	-		
One-month stability at -20°C	Mean	0.91	29.08
SD	0.02	0.54	RSD %
2.66	1.86	Accuracy %	96.9
100.2	-		
Two-month stability at -20°C	Mean	0.97	24.21
SD	0.08	2.63	RSD %
8.55	10.22	Accuracy %	103.4

SD: standard deviation; RSD: relative standard deviation; %: percentage; LQC: low quality control; HQC: high quality control; $n=3$.

3.5. Recovery

The mean recovery of vardenafil and IS from plasma was determined to be 101.4% and 70.0%, respectively (Table 4). To investigate the matrix effect, the corresponding peak areas of the postextraction spiked samples were compared with the extraction of samples from mobile phase solution at LQC, MQC, and HQC levels. The acceptance criteria for the matrix effect include an RSD of $\leq 15\%$. In this study, the highest RSD recorded in Table 5 was 4.38%. Therefore, no significant matrix effect was observed.

Table 4

Recovery for vardenafil (standard) and internal standard.

	Standard	Internal standard
Recovery %		
LQC	99.81	74.06
MQC	103.84	67.09

HQC	100.39	68.57
Mean	101.4	70.0
SD	2.17	3.67
RSD %	2.14	5.25

SD: standard deviation; RSD: relative standard deviation; %: percentage; LQC: low quality control; MQC: medium quality control; HQC: high quality control; $n=3$.

Table 5

Matrix effect of vardenafil in plasma.

	LQC (AUC ratio)	HQC (AUC ratio)
Mean	1.22	1.30
SD	0.04	1.46
RSD %	3.59	4.38

SD: standard deviation; RSD: relative standard deviation; %: percentage; LQC: low quality control; MQC: medium quality control; $n=6$.

3.6. Dilution Integrity

A dilution integrity test was conducted with six replicates of plasma samples diluted from plasma containing 350 ng/mL of vardenafil with a 1:10 dilution factor. The dilution was done with rabbit plasma. In this study, a concentration of 350 ng/mL was used for dilution because it covers all the detected concentration ranges, and the diluted concentration (35 ng/mL) does not overlap with any of the QCs or the calibration points. An RSD of $\leq 15\%$ is needed to meet acceptance criteria. The mean accuracy and RSD were 94.85% and 8.35%, respectively (Table 6). This indicates that samples with analytes above the validated range can be diluted into the measurable ranges, as shown in Figure 4.

Table 6

Dilution integrity test of vardenafil in plasma.

Expected concentration	35 ng/mL
Mean %	94.85
SD	7.93
RSD %	8.35

SD: standard deviation; RSD: relative standard deviation; %: percentage; $n=6$.

[figure omitted; refer to PDF]

3.7. Pharmacokinetic Parameter

All pharmacokinetic parameters were calculated using noncompartmental analysis using Phoenix Win Nolin ver.8.0;

these are summarized in Table 7. The value of the measured area under the curve from 0 to 24h AUC_{0-24t} and area under the curve from 0 to infinity AUC_{0-∞} for both test and reference formulations were the same, i.e., 174.3 ± 95.91 and 176.45 ± 76.88 , respectively. Conversely, the maximum concentration C_{max} and time to achieve maximum concentration T_{max} were 75.36 ± 59.53 ng/mL and 1.42 ± 0.19 h for the test formulation, whereas these were 58.22 ± 36.11 ng/mL and 2.04 ± 0.33 h for the reference formulation, respectively. The mean t_{1/2} values for the test and reference formulations were 4.83 and 4.75 h, respectively. The mean AUC_{0-24t} and t_{1/2} for the test and reference formulation were very close to each other. The mean value of t_{1/2} was consistent with a previous report [9]. The C_{max} value for the test formulation was higher than the reference formulation. The T_{max} value for the test formulation was lower than that for the reference formulation, indicating that the test formulation achieved the maximum therapeutic effect in a shorter amount of time.

Table 7

Pharmacokinetic parameters of the optimized formulation of vardenafil orodispersible tablet (test) and marketed orodispersible tablet formulation (reference) in New Zealand white rabbit.

	T _{max} (h)	C _{max} (ng/mL)	AUC _{0-24t} (ng·h/mL)	AUC _{0-∞} (ng·h/mL)	t _{1/2} (h)
Formulation <i>T</i>					
<i>N</i>			12		
Mean	1.42 ± 0.19	75.36 ± 59.53	174.38 ± 95.91	174.38 ± 95.91	4.83 ± 1.63
Min	1.00	21.74	63.22	63.22	1.43
Median	1.50	72.79	163.12	163.12	5.12
Max	1.50	240.20	393.13	393.13	6.64
CV%	13.74	78.99	54.99	54.99	33.85
Geometric mean	1.40	59.18	150.88	150.88	4.45
—					
Formulation <i>R</i>					
<i>N</i>			12		
Mean	2.04 ± 0.33	58.21 ± 36.11	176.45 ± 76.88	176.45 ± 76.88	4.75 ± 0.85
Min	1.50	13.15	66.47	66.47	3.42
Median	2.00	46.24	171.18	171.18	4.63
Max	3.00	140.96	295.46	295.46	5.88

CV%	16.37	62.03	43.57	43.57	17.99
Geometric mean	2.02	48.63	159.56	159.56	4.68

R indicates reference; *T* indicates test; *h* indicates time in hours; ng indicates nanogram; mL indicates milliliters; AUC_{0-t} indicates the measured AUC from 0 to 24 h; $AUC_{0-\infty}$ indicates area under the curve until infinite; *N* indicates numbers of rabbits; SD indicates standard deviation; CV indicates coefficient variation; *T*_{max} indicates time to reach the maximum peak; *C*_{max} indicates the maximum concentration that achieved; *t*_{1/2} indicates the half-life of the drug.

4. Discussion

4.1. Advantages of the Method

Liquid-liquid extraction requires a drying process to enhance the resolution of the peak, which is time-consuming, requires a large amount of organic solvent, and has inconsistent recovery [17]. Solid-phase extraction is a better option with cleaner extraction compared with liquid-liquid extraction [14, 17]. However, this method requires conditioning of the column and is expensive. Comparatively, simple protein precipitation with ice-cold acetonitrile has been used in this study to extract vardenafil. This extraction method is simple, less costly, and time-saving compared with previously published methods.

This newly developed LC/MS/MS method also has high sensitivity, as it requires only a small amount (100 μ L) of plasma and a small injection volume (2 μ L) compared with the previous method. Despite the small amount of sample and injection volume, this method still can achieve a low quantification level of drug concentration (LLOQ=0.5 ng/mL). Sample analysis can be conducted rapidly with a total running time of 6 min. Chromatographic separation was found to have better separation with gradient elution compared to isocratic elution. After 1 min of initiation, vardenafil and IS were separated with retention times of 1.56 and 1.84 min, respectively.

In this study, the intra- and interassay imprecision was $RSD \leq 20\%$ for LQC and $RSD \leq 15\%$ for MQC and HQC. The method exhibited good intra-assay accuracy within 89.3%–105.3% of the expected value and an interassay accuracy within 94%–102% of the expected value. Thus, this method exhibited acceptable precision and accuracy. The stability study of vardenafil shows that vardenafil was stable at room temperature for 8 h, at 10°C in an autosampler for 24 h, at 20°C for three freeze-thaw cycles, and at –20°C for 2 months.

4.2. Rabbit Model

The use of rabbits as models for sublingual delivery investigation has been widely established and reported in the literature. Similar to rabbits, other animals such as dogs, pigs, and monkeys are acceptable models and have nonkeratinized oral mucosa with permeability values similar to those of humans [18]. Rabbits and dogs are generally accepted as the most suitable animal models for humans because of the histological similarities in their oral cavities [19], whereas the ease of handling and animal cost makes rabbits more favorable over dogs [20]. Rabbit and human sublingual mucosa are both nonkeratinized; hence, sublingual drug delivery in rabbits can be correlated to intraoral absorption in humans [20].

4.3. Application of the Method

This newly developed and validated LC/MS/MS method was successfully applied in a pharmacokinetics study of the newly optimized formulation of vardenafil orodispersible tablet (test formulation) compared with the marketed orodispersible tablet (reference formulation) in 12 healthy rabbits. Figure 5 plots the mean plasma concentration versus time profile for vardenafil in 12 rabbits. The test formulation was able to reach maximum concentration in the body faster than the reference formulation and has a faster onset of action. The percentages of extrapolation as shown in Figure 5 were 3.14% and 2.58% for the test and reference formulations, respectively. Low percentages of extrapolation indicate a good sampling point. Additionally, the very low LLOQ achieved in this method enabled the graph to be plotted properly.

[figure omitted; refer to PDF]

4.4. ANOVA and Bioequivalence Statistics

4.4.1. Area under the Curve, AUC_{0-24h}

The 90% confidence interval (CI) for the test/reference ratio was 78.11–114.46. However, the test formulation was not bioequivalent to the reference formulation in terms of AUC because the result is not within the acceptable range of 90% CI of 80%–125% for Log₁₀ AUC. The acceptable range was according to the European Community's—The European Agency for the Evaluation of Medicinal Products (EC-EMA), USFDA, and the National Pharmaceutical Regulatory Agency of Malaysia (NPRA). The Anderson Hauck probability of the result falling outside 80%–125%, which indicates the probability of a value outside 80%–125% is 0.059 (5.9 in 100). This value is larger than 0.05 (5 in 100), the level of significance, and hence, the above hypothesis cannot be rejected. Although test formulation is not bioequivalent to the reference formulation, their mean AUC_{0-24h} values were very close (174.38 and 176.45 ng·h/mL, respectively).

4.4.2. Maximum Concentration, C_{max}

The 90% CI for the test/reference ratio was 89.56–165.35. The test formulation was not bioequivalent to the reference formulation in terms of C_{max} because the result did not fall within the acceptable range of 90% CI of 80%–125% for Log₁₀ AUC. The acceptable range was according to the EC-EMA, USFDA, and NPRA. The Anderson Hauck probability of the result falling outside 80%–125%, which indicates the probability of a value outside 80%–125%, is 0.42 (42 in 100). This value is larger than 0.05 (5 in 100), the level of significance, and hence, the above hypothesis cannot be rejected. Although the test formulation is not bioequivalent to the reference formulation, the mean C_{max} for the test formulation was higher than the reference formulation (75.36 and 58.22 ng/mL, respectively).

4.4.3. Time to Reach Maximum Concentration, T_{max}

The mean T_{max} value for vardenafil was 1.42 and 2.04 h for the test and reference formulations, respectively. Wilcoxon signed-rank test showed a statistically significant difference in terms of T_{max} values (p

4.4.4. Half-Life, t_{1/2}

The mean t_{1/2} values for vardenafil were 4.83 and 4.75 h for the test and reference formulations, respectively. Paired *t*-test showed no statistically significant difference in terms of t_{1/2} values between the two ($p > 0.05$). The above results prove that our newly optimized formulation achieved almost equal bioavailability to the test formulation and even achieved higher maximum concentration at a faster rate compared with the reference formulation. No significant differences were observed in t_{1/2} for both test and reference formulations.

5. Conclusion

We report on the development and validation of a simple, rapid, sensitive, and specific LC/MS/MS method for the determination of vardenafil in rabbit plasma. The major advantage of using the MS/MS system is its specificity in targeting ions of interest. This method was applied successfully to the pharmacokinetic study of vardenafil in healthy rabbits after a single dose of 10 mg vardenafil orodispersible tablet. The newly optimized vardenafil orodispersible tablet formulation exhibited similar pharmacokinetic data in terms of AUC_{0-24h} and t_{1/2}. It also achieved almost equal bioavailability, higher maximum concentration, and shorter time to reach the maximum concentration compared to the reference formulation. The objective of developing a new quantification method to solve the challenges in this study was achieved.

Authors' Contributions

The authors declare that the work was done by the authors named in this article and that the authors will be responsible for any liabilities pertaining to claims relating to the content of this article. Conceptualization was carried out by Riyanto Teguh Widodo, Zamri Chik, and Teh Lay Kek. Methodology was performed by Gan Kok Zheng, Zamri Chik, and Teh Lay Kek. Software was provided by Mohd Salleh Rofiee and Mohd Izwan Mohamad Yusof. Validation and analysis were conducted by Gan Kok Zheng and Mohd Izwan Mohamad Yusof. Investigation was performed by Gan Kok Zheng, Mohd Salleh Rofiee, and Mohd Izwan Mohamad Yusof. Data were collected by Gan Kok Zheng. The manuscript was written by Gan Kok Zheng. The manuscript was reviewed by Riyanto Teguh Widodo, Zamri Chik, and Teh Lay Kek. The study was supervised by Riyanto Teguh Widodo, Zamri Chik, and Teh Lay Kek.

Acknowledgments

The authors acknowledge iPROMISE, UiTM, for their assistance and for lending the facilities and equipment to complete the analysis.

- [1] J. Hui, S. He, R. Liu, Q. Zeng, H. Zhang, A. Wei, "Trends in erectile dysfunction research from 2008 to 2018: a bibliometric analysis," *International Journal of Impotence Research*, vol. 32 no. 4, pp. 409-419, DOI: 10.1038/s41443-019-0161-8, 2020.
- [2] G. M. Keating, L. J. Scott, "Vardenafil," *Drugs*, vol. 63 no. 23, pp. 2673-2703, DOI: 10.2165/00003495-200363230-00010, 2003.
- [3] R. A. Kloner, "Cardiovascular effects of the 3 phosphodiesterase-5 inhibitors approved for the treatment of erectile dysfunction," *Circulation*, vol. 110 no. 19, pp. 3149-3155, DOI: 10.1161/01.cir.0000146906.42375.d3, 2004.
- [4] H. Y. Ku, J. H. Shon, K. H. Liu, J. G. Shin, S. K. Bae, "Liquid chromatography/tandem mass spectrometry method for the simultaneous determination of vardenafil and its major metabolite, N-desethylvaridenafil, in human plasma: application to a pharmacokinetic study," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 877 no. 1-2, pp. 95-100, DOI: 10.1016/j.jchromb.2008.11.015, 2009.
- [5] A. Zucchi, E. Costantini, F. I. Scropo, "The first-generation phosphodiesterase 5 inhibitors and their pharmacokinetic issue," *Andrology*, vol. 7 no. 6, pp. 804-817, DOI: 10.1111/andr.12683, 2019.
- [6] P. Hannan, J. Khan, A. Khan, S. Safiullah, "Oral dispersible system: a new approach in drug delivery system," *Indian Journal of Pharmaceutical Sciences*, vol. 78 no. 1, DOI: 10.4103/0250-474x.180244, 2016.
- [7] M. Sanford, "Vardenafil orodispersible tablet," *Drugs*, vol. 72 no. 1, pp. 87-98, DOI: 10.2165/11208270-000000000-00000, 2012.
- [8] H. Sperling, M. Gittelman, C. Norenberg, E. Ulbrich, S. Ewald, "Efficacy and safety of an orodispersible vardenafil formulation for the treatment of erectile dysfunction in elderly men and those with underlying conditions: an integrated analysis of two pivotal trials," *The Journal of Sexual Medicine*, vol. 8 no. 1, pp. 261-271, DOI: 10.1111/j.1743-6109.2010.02005.x, 2011.
- [9] R. Heinig, B. Weimann, H. Dietrich, M.-F. Böttcher, "Pharmacokinetics of a new orodispersible tablet formulation of vardenafil," *Journal of Men's Health*, vol. 7 no. 3, DOI: 10.1016/j.jomh.2010.09.137, 2010.
- [10] G. Carlucci, P. Palumbo, P. Iuliani, G. Palumbo, "Development of a method for the determination of vardenafil in human plasma by high performance liquid chromatography with UV detection," *Biomedical Chromatography*, vol. 23 no. 7, pp. 759-763, DOI: 10.1002/bmc.1181, 2009.
- [11] C. L. Cheng, G. J. Kang, C. H. Chou, "Development and validation of a high-performance liquid chromatographic method using fluorescence detection for the determination of vardenafil in small volumes of rat plasma and bile," *Journal of Chromatography A*, vol. 1154 no. 1-2, pp. 222-229, DOI: 10.1016/j.chroma.2007.03.077, 2007.
- [12] M. A. B. Abu El-Enin, M. E.-S. A. Al-Ghaffar Hammouda, D. T. El-Sherbiny, D. R. El-Wasseef, S. M. El-Ashry, "Validated spectrofluorimetric method for determination of two phosphodiesterase inhibitors tadalafil and vardenafil in pharmaceutical preparations and spiked human plasma," *Luminescence*, vol. 31 no. 1, pp. 173-178, DOI: 10.1002/bio.2941, 2016.
- [13] S. T. Lake, P. M. Altman, J. Vaisman, R. S. Addison, "Validated LC-MS/MS assay for the quantitative determination of vardenafil in human plasma and its application to a pharmacokinetic study," *Biomedical Chromatography: BMC*, vol. 24 no. 8, pp. 846-851, DOI: 10.1002/bmc.1375, 2010.
- [14] A. Bhadoriya, B. Dasandi, D. Parmar, P. A. Shah, P. S. Shrivastav, "Quantitation of tadalafil in human plasma using a sensitive and rapid LC-MS/MS method for a bioequivalence study," *Journal of Pharmaceutical Analysis*, vol. 8 no. 4, pp. 271-276, DOI: 10.1016/j.jpha.2018.01.003, 2018.
- [15] K.-Y. Kim, M. Nam, H.-J. Kwon, "Validated UPLC-MS/MS method for the determination of tadalafil in human plasma and its application to a pharmacokinetic study," *Translational and Clinical Pharmacology*, vol. 25 no. 1, pp. 21-27, DOI: 10.12793/tcp.2017.25.1.21, 2017.
- [16] U.S.FDA, Bioanalytical Method Validation Guidance for Industry, 2018. <https://www.fda.gov/regulatory->

- [17] V. Anjana, P. Ashok, P. Ajay, V. Amit, P. Nilesh, "Sample preparation in bioanalysis a review," International Journal of Scientific and Technology Research, vol. 4 no. 8, 2015.
- [18] I. A. Siegel, K. T. Izutsu, E. Watson, "Mechanisms of non-electrolyte penetration across dog and rabbit oral mucosa in vitro," Archives of Oral Biology, vol. 26 no. 5, pp. 357-361, DOI: 10.1016/0003-9969(81)90030-3, 1981.
- [19] S. S. M. Rathbone, I. Pather, "Oral mucosal drug delivery and therapy," Advances in Delivery Science and Technology, 2015.
- [20] M. M. Dali, P. A. Moench, N. R. Mathias, P. I. Stetsko, C. L. Heran, R. L. Smith, "A rabbit model for sublingual drug delivery: comparison with human pharmacokinetic studies of propranolol, verapamil and captopril," Journal of Pharmaceutical Sciences, vol. 95 no. 1, pp. 37-44, DOI: 10.1002/jps.20312, 2006.

DETAILS

Subject:	Mass spectrometry; Acetonitrile; Plasma; Rabbits; Assaying; Liquid chromatography; Solvents; Quality control; Linearity; Spectroscopy; Bioequivalence; Bioavailability; Scientific imaging; Methods; Metabolism; Chromatography; Ethics; Metabolites; Drug dosages; Proteins
Business indexing term:	Subject: Quality control
Company / organization:	Name: University of Malaya; NAICS: 611310
Editor:	Victoria F Samanidou
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article

Publication history :

Milestone dates: 2021-02-04 (Received); 2021-03-11 (Revised); 2021-03-17 (Accepted); 2021-03-26 (Pub)

DOI: <https://doi.org/10.1155/2021/5590594>

ProQuest document ID: 2508265520

Document URL: <https://www.proquest.com/scholarly-journals/liquid-chromatography-tandem-mass-spectrometry/docview/2508265520/se-2?accountid=211160>

Copyright: Copyright © 2021 Kok Zheng Gan et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-04-04

Database: Publicly Available Content Database

Document 42 of 59

Identification of 24 Unknown Substances (NIAS/IAS) from Food Contact Polycarbonate by LC-Orbitrap Tribrid HRMS-DDMS³ : Safety Assessment

Yusà, Vicent¹

; López, Antonio²; Dualde, Pablo²; Pardo, Olga²; Fochi, Igor³; Miralles, Pablo²; Coscollá, Clara²
¹ Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, Av. Catalunya, 21, Valencia 46020, Spain; Public Health Laboratory of Valencia, 21, Av. Catalunya, Valencia 46020, Spain
² Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, Av. Catalunya, 21, Valencia 46020, Spain
³ Thermo Fisher, Rodano, MI, Italy

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Twenty-four substances, mainly NIAS, have been tentatively identified in food contact polycarbonate through the application a new, fast, and automated analytical strategy for the investigation of unknowns in food contact materials. Most of the identified compounds were plasticizers, slip agents, antioxidants, and ultraviolet stabilizers and fragrances, and the majority of them have not been previously identified in PC food contact materials. The workflow setup includes an intelligent data acquisition applied using LC-Orbitrap Tribrid-HRMS (MS³), with an automated data processing using Compound DiscovererTM. To obtain a high confidence identification of unknown

substances, a very strict criterion has been established, which comprises exact mass, isotopic profile, MS² match, retention time, and MS³ match. To check for the safety of the migration from the food contact polycarbonate, a risk assessment was achieved using the threshold of the toxicological concern (TTC) approach. Except for the slip agent hexadecanamide, the compounds tentatively identified do not represent a risk.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Plastics for food contact applications must be safe. In Europe, plastic articles may only be placed on the market as food contact materials (FCM) if they are manufactured with substances (monomers, additives, and aids) included in the list incorporated into the Regulation 10/2011 [1] (intentionally added substances, IAS). However, during their manufacturing and use, some impurities and reaction or degradation products can be formed (nonintentionally added substances, NIAS). The potential risk of NIAS should be considered for authorization [2, 3] and for the declaration of compliance at the marketing stages [4].

Between the different thermoplastic materials used as FCM, polycarbonate (PC) is commonly employed for tableware and containers exposed to hot filling or heating after filling, such as plates and bowls, that could be used repeatedly. These PC containers can also be used for reheating food in a microwave oven [5]. Many ambient factors such as temperature, humidity, light together with the repeated uses, and washing cycles can damage the stability of the material and increase the migration of substances to the food [6]. The main concern about degradation of PC food containers is the release of bisphenol A (BPA), a component monomer of PC that is currently of toxicological concern [7, 8].

To assess the safety of food contact plastic materials, appropriate analytical methods are required [9]. Apart from the target analysis, mainly for detection of IAS or specific contaminants, the analysis of NIAS is mandatory to avoid any potential health risk to the consumer [2] and should be performed in accordance with the Regulations 1935/2004 [4] and 10/2011 [1]. The identification of NIAS is challenging and requires a non-target analytical approach [10, 11] using LC-HRMS (Q-Orbitrap/Q-TOF), performed using two main modes, data-dependent acquisition (DDA) and data-independent acquisition (DIA) [12].

Bignardi et al. [13] employed a target and untargeted method, using LC-HRMS-Orbitrap working in the data-dependent acquisition mode (DDA), for characterization of PC food contact plastic. In the target approach, several plastic additives were identified. With the untargeted analysis, some colorants and several oligomers derived from the chain breakage were identified, following a laborious and time-consuming process. In a recent study, the same research team [14] applied the target method with UHPLC-HRMS to determine the migration from PC tableware objects, detecting oligomers from the PC chain and traces of colouring agents.

To perform an exhaustive characterization of NIAs, an extensive acquisition (DDA including Full Scan and MSⁿ) and identification of the unknown substances is necessary. This is challenging because of the large number of features present both in the samples and the background (blanks and/or matrix). In order to automate these procedures, new acquisition tools are developed for background removing, exhaustive precursor selection (inclusion list), and creation of dynamic and real-time exclusion list [15]. Likewise, an automated library search has been improved through the data processing tool Compound Discoverer™ [16].

For the identified NIAS, a risk assessment is necessary to ensure the safe usage of the PC containers. One practical option is to use the threshold of the toxicological concern (TTC) approach that estimates the theoretical toxicity of compounds linked to their molecular structure, and a threshold value is provided below which there is a very low probability of adverse health effects [17, 18].

In this study, a new approach for the determination of unknown substances in PC bowls used for food container was investigated. We evaluated the capacity for exhaustive spectral data acquisition of an innovative intelligent acquisition mode (AcquireX™) using DD-MS³. In the second phase, the automatic search on compound libraries and

spectral databases was employed to putative identification of unknown compounds. Finally, we assessed the risk of the detected migrating substances.

2. Materials and Methods

2.1. Chemicals and Samples

Chemical standards of terfenadine and Val-Try-Val were from Sigma Life Science (St Louis, MO, USA), triallyl phosphite was from Alfa Aesar Thermo Fisher (Kandel, Germany), and sulfaguanidine, sulfadimethoxine, reserpine, caffeine, and acetaminophen were obtained from Sigma Aldrich (St Louis, MO, USA). Solutions of 10 ng/mL of these chemicals were prepared in H₂O:MeOH (70:30, v/v) and used as internal standards.

Chemical standards of bis (2-ethylhexyl) adipate, camphor, dibutyl sebacate, dibutyl phthalate, erucamide, 2-(2'-hydroxy-5'-methylphenyl) benzotriazole (benazol P), methyl dihydrojasmonate, oleamide, and tributyl acetyl citrate (Citroflex-A4), all from Sigma Life Science (St Louis, MO, USA), and diethyl phthalate, dibutyl adipate, and tris(2,4-ditert-butylphenyl) phosphite (Irgafos 168), from LGC Standards (Bury, United Kingdom), were used to quantify and confirm the identity of the tentatively identified substances.

We analysed polycarbonate bowls that, according with the declaration of conformity, were used in repeated contact with miscellaneous foods such as soups and broths, during a maximum time contact of 2 hours and a temperature between 85°C and 89°C and prepared to endure until 2000 washing cycles.

2.2. Migration Test and Sample Preparation

Migration tests were achieved in agreement with the Regulation (EU) No. 10/2011 [1]. We used the food simulant C (20% of ethanol in water (v/v)). The migration test was performed during 2 hours at 100°C. We introduced around 200 mL of simulant into the PC rectangular bowl (10 cm × 5.5 cm × 3 cm) and keep it in the incubator. According with Regulation (EU) No. 10/2011, the migration test was repeated three times. Taking into account this Regulation, 6 dm² is equal to 1 Kg of food.

2.3. LC-HRAMS Analysis

We used a Thermo Ultimate 3000 UHPLC system, with a column Hypersil Gold 100 mm × 2.1 mm × 1.9 μm (Thermo Scientific). The chromatographic system was linked with an Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific, USA). A volume of 5 μL was injected, and the flow rate was 300 μL/min. The chromatography started with 90% phase A (water) and 10% B (methanol). The linear gradient used was 0–18 min, 70% B; 18–21.5 min, 98% B; sustained at 98% B from 21.5 min to 25 min; decrease to 10% B, from 25 min to 26 min; and maintained in this initial conditions until 30 min.

Both positive and negative ionizations were employed. The H-ESI parameters were electrospray voltage of 3.5 kV positive (3.2 Kw negative), sheath gas of 25 arbitrary units (a.u), and auxiliary gas of 5 a.u. The ion transfer tube worked at 270°C and the vaporizer temperature at 180°C. A resolving power of 120,000 FWHM was used in full scan (100–900 m/z). The MS² was acquired at 15,000 FWHM, with a precursor mass range of 125–900 m/z. The MS³ precursor was isolated in the quadrupole mass filter (0.4 Da), fragmented in the HCD cell and detected in the ion trap (FWHM ≤ 0.3).

2.4. Acquisition and Data Processing

For unknowns analysis, an intelligent data approach (AcquireX[®], ThermoFisher Scientific) was employed. The details of this approach were described previously [19]. The acquisition workflows for Deep Scan (DS) and Iterative Processing Exclusion (IPE) modes in DD-MS³ are shown in Figures SI-1-2.

For compound identification, the raw files acquired were processed using Compound Discoverer™ 3.1 (CD) (Thermo Fisher Scientific). The CD workflow is shown in Figure SI-3, and each node is defined in Table SI-1. The CD provides a wide list of annotated compounds (several hundreds). However, the majority of them do not present enough analytical evidence for tentative identification. To consider a compound tentatively identified with a high degree of confidence, we used the criteria previously described by [19]. In short, exact mass is with a Δ mass 70%; MS² match (experimental vs. library (mzCloud) spectra) > 70%; retention time consistent with their log P.

2.5. Safety Assessment

For safety assessment of the identified NIAS, we use the threshold of the toxicological concern (TTC) approach [2].

This was performed using the Toxtree software (<http://toxtree.sf.net/predict>) developed by Idea Consult Ltd., commissioned by JRC Computational Toxicology and Modelling (<https://apps.ideaconsult.net/data/ui/toxtree>). The software estimates the TTC following Cramer rules, and for each of the three Cramer classes, a tolerable daily intake (TDI, mg/person/day) is assigned. Class I, the least toxic class, has a TDI=1.8mg/person/day, Cramer class II (intermediate) has a TDI=0.54mg/person/day, and Cramer class III (most toxic) has a TDI=0.09mg/person/day. The estimated daily intake (EDI) was calculated based on the default assumption in Europe that an adult person consumes 1 Kg of food packaged in a dm³, using the following formula [20].

EDI (mg/person x day)=migration (mg/kg) x 1 kg (food intake per person and day).

Quantification was performed using pure standards (Section 2.1). When standards were not available, a semiquantitative estimation of the migration (mg/Kg) was performed with the average response factor of seven internal standards [21] (Table SI-2).

Figure 1 shows the diagram of the overall analytical procedure.

[figure omitted; refer to PDF]

3. Results and Discussion

3.1. Identification of Unknowns

Out of 727 annotated features with CD, only 24 meet the metrics established for tentative identification of compounds (Table 1). In order to achieve a high degree of identification confidence, very demanding criteria were followed (Section 2.5). All of them present a mass error (ΔM) in general lower than 0.5ppm. Equally, the isotopic patterns present, in general, a SFit% > 70%. Likewise, their MS² spectra match (>70%) with the spectra in the mzCloud library (Table 1). As an orthogonal criteria for increasing the identification confidence, the experimental retention time (Rt_{ex}) was compared with the theoretical one (Rt_{th}). To estimate the theoretical retention time, we build a curve that relates the log P with the Rt of 107 substances of a wide range of polarities (Figure SI-4). Using this curve, we estimated the Rt_{th} of the identified substances. In general, the Rt_{ex} (min) fits well with the Rt_{th} (Table 1).

Table 1

Identified compounds.

Compound ^{a,b} (CAS number)	Structure	Δ mass (ppm)	Isotopic pattern (SFit%)			MS ² match ^c	Rt_{exp} (min)	Rt_{theo} (min)
MS ³ match ^d	Description [Reference]	3, 5-Diterbutyl-4-hydroxybenzaldehyde ^a (1620-98-0)		-0.12		88		75

18.54	18.26	(1) CD in silico	Metabolite of antioxidant butylated hydroxytoluene (BHT) [22]	-		
4-tert-Butylcyclohexyl acetate ^{a,b} (32210-23-4)		0.05	52	71.6	20.40	16.72
mzCloud	Fragrance [23, 24]	-				
Benazol P(2-(2'-hydroxy-5'-methylphenyl)benzotriazole) ^{a,b} (2440-22-4)		-0.01	73	89	19.38	16.84
mzCloud	Ultraviolet light absorber [1, 25]	-				
Benzyl octyl adipate ^{a,b} (3089-55-2)		0.34	91	89.4	21.58	23.48
(1) CD in silico	Plasticizer	-				
Bis (2-ethylhexyl) adipate ^{a,b} (103-23-1)		-0.08	73	63.8	22.56	-
mzCloud	Plasticizer [26]	-				

D,L-Camphor ^{a,b} (76-22-2)		-0.1 2	71	75.6	16.14	13.04
—	Fra gra nce [27, 28]	-				
Citroflex A-4 ^{a,b} (tri-n-butyl acetyl citrate) (77-90-7)		-0.0 2	85	91.4	19.34	23.40
(2)	Pla stic izer [29]	-				
Dibutyl hexanodioate ^{a,b} (dibutyl adipate) (105-99-7)		0.11	56	79.3	19.29	16.94
(1) CD in silico	Pla stic ise [30]	-				
Dibutyl phthalate ^{a,b} (84-74-2)		-0.2 6	75	93.9	15.45	18.41
mzCloud	Pla stic izer [29]	-				
Dibutyl sebacate ^{a,b} (109-43-3)		0.03	82	72.7	21.6	21.88
(1) CD in silico	Pla stic izer [29]]	-				
Diethyl phthalate ^a (84-66-2)		-0.0 8	55	81.9	11.55	12.02

(1) CD in silico	Pla stic izer [31]	-				
Dodecyl sulfate ^{a,b} (151-21-3)		-1.45	55	94.6	22.90	20.13
(2)	Sur fact ant [39, 40]	-				
Dipentyl phthalate ^{a,b} (131-18-0)		0.08	93	70.9	21.00	21.64
(1) CD in silico	Pla stic izer [41]	-				
Erucamide ^{a,b} (112-84-5)		0.3	96	93.2	22.93	—
mzCloud	Slip pro mot er [1, 29, 42]	-				
Galaxolidone ^{a,b} (507442-49-1)		0.19	89	72.9	19.96	20.46
(1) CD in silico	Met abo lite of gal axo lide , a pol ycy clie mu sk [43]	-				

Hexadecanamide ^{a,b} (629-54-9)		-0.42	70.5	91.2	21.96	24.50
(1) CD in silico	Slip agent [42]	-				
Irgafos 168 ^{a,b} (tris(2,4-ditert-butylphenyl) phosphite) (Phosphorous acid, tris(2, 4-di-tert-butylphenyl)ester) (31570-04-4)		0.43	74	87.5	25.10	—
(1) CD in silico	Antioxidant stabiliser [1, 29, 44]	-				
Methyl dihydrojasmonate ^{a,b} (24851-98-7)		-0.14	73	77.3	16.14	11.92
(1) CD in silico	Fragrance [45, 46]	-				
N,N'-Dicyclohexylurea ^{a,b} (2387-23-7)		-0.02	96	62	14.79	12.94
mzCloud		-				
DEET ^{a,b} (N,N-diethyl-m-toluamide) (134-62-3)		-0.09	93	77.3	13.14	10.01

(1) CD in silico	Bio cid e (re pell ing or attr acti ng pes ts)	-				
Octyl decyl phthalate ^{a,b} (119-07-3)		-0.24	79	93.6	22.87	—
mzCloud	Pla stic izer [31]	-				
Oleamide ^{a,b} (301-02-0)		-0.06	88	86.7	21.56	25.20
mzCloud	Slip age nt [1, 42]	-				
Palmitoyl ethanolamide ^{a,b} (544-31-0)		0.03	81	71.7	21.32	23.93
mzCloud	Ant ista tic; vis cos ity con troll ing [31]	-				
Stearamide ^{a,b} (octadecanamide) (124-26-5)		0.05	52	96.9	22.44	24.08

^aDetected with the AcqX_DS mode. ^bDetected with the AcqX_IPE mode. ^aAll compounds present a full match for a predicted composition (molecular predicted composition from libraries from the experimental exact) mass; Δ mass (ppm), mass accuracy. ^bAll compounds have acquired MS² and MS³ spectra (except MS3 for L-camphor). ^cMatch

between the experimental and the MS² spectra in the mzCloud library. Isotopic pattern (SFit%), match between the experimental isotopic pattern and the theoretical one; m/z Logic, a data analysis algorithm that combines the millions of available structural databases with the extensive mass spectral fragmentation library of mzCloud to rank order putative structures for unknowns when there is no direct mass spectral match; Rt_{exp}, experimental retention time; Rt_{theo}, theoretical time bases on log P. ^dMatch between the experimental MS³ and the database MS³ spectra (mzCloud) or the in silico fragmentation (Mass Frontier/CD); mzCloud, MS³ experimental spectra match with mzCloud spectra; CD in silico, match between the in-silico fragmented with the CD and the experimental MS³, (1) mzCloud does not contain a MS³ spectra of this compound and (2) the experimental MS³ experimental do not match with that stored in mzCloud or the in silico fragmentation (Mass Frontier/CD).

For identification of those substances that do not meet the established criteria (only the molecular formula was provided by CD), a more in-deep and time-consuming manual examination is required, using in silico fragmentation tools and bibliographic examination [12].

In previous published studies for non-target screening applied to FCM, acquisitions using the data-dependent approach (DDA) with Q-Orbitrap and data-independent approach (DIA) with Q-TOF-MS instruments have been used [12]. The identification strategy is performed manually utilizing lists of probable candidates (only formula available), spectral libraries (MS/MS² spectra), and bibliographical search. The main drawback of this strategy is that it is labour-intensive, and only a few compounds are tentatively identified. Regarding PC food materials, only a few substances migrating to simulants have been described. As mentioned previously, Bignardi et al. [13] and Bignardi et al. [14] performed a targeted and untargeted suspect screening to identify substances migrating from food contact polycarbonate using DDA-MS² acquisition. The identification of compounds was carried out using a list of additives (target) and through the careful observation of the full scan chromatograms, the evaluation of the isotopic pattern, and the investigation in available databases. In the untargeted analysis, they claim the identification of several organic colorants and polycarbonate degradation products, but no MS² spectra were used for structural identification. Another study [6] investigated, using a target method with LC-HRMS, the presence of the PC monomer bisphenol A (BPA) and several additives in polycarbonate tableware. BPA was determined in all samples (in the range of 0.5–6 µg/Kg), and the UV absorbers Cyasorb UV 5411, Tinuvin 327, and Tinuvin 324 and the whitening Uvitex OB were found in some samples (from 7 ng/Kg to 4460 ng/Kg). The target analysis, in general, permits achieve lower LOQ.

3.1.1. MS³ Spectra

The automated identification achieved by the combination of the intelligent data acquisition (AcqX-ddMS³) combined with the data processing using CD does not use the MS³ spectra. A higher level of confidence could provide the MS³ spectra. As can be seen in Table 1 and Table SI-3, all compounds identified have their MS³ acquired spectrum, except for D,L-camphor. However, it is necessary to compare the experimental and the library (mzCloud) MS³ spectra manually. For those compounds that do not have MS³ spectra in mzCloud, we fragmented them in silico using CD and Mass Frontier 8.0. [32].

Ten of the identified compounds had MS³ spectra that matched with that stored in spectrum library. Figure 2 shows, as an example, the spectra of benazol P and stearamide. In both MS³ spectra, the experimental parent ions (m/z 183.0679; m/z 130.1222, respectively) and the main fragments (m/z 155.06 and m/z 88.02, respectively) match with the library spectra. Must be taken into account that MS³ was acquired in low resolution. On the other hand, there are eleven substances in which experimental MS³ spectra match with the in silico fragmentation performed with the CD. As an example, Figure 3 depicts the experimental spectra of the methyl dihydrojasmonate and benzyl octyl adipate and the in silico fragments assigned to each m/z.

[figures omitted; refer to PDF]

[figures omitted; refer to PDF]

Using the criteria proposed by Schymansky et al. [33], the tentatively identified compounds present a high level of identification confidence (level 2a: unambiguous matching with library data). The other annotated compounds (not discussed here), for which only the molecular formula o exact mass is known, only achieve levels 4-5 (low levels of

identification confidence).

We have confirmed the identity of 12 out of 24 tentatively identified substances using standards (Section 2.1). The rest of substances were not available in our lab.

3.1.2. Description of Identified Compounds

In order to explain the presence in the polycarbonate plastic material of the identified compounds, we search for their nature and technological uses. Table 1 shows the functions of these compounds. Many of them are additives, mainly plasticizers and slip agents used in plastics. Additives are introduced into plastic to enhance the quality of the attributes required for their appropriate use. They improve their physical (mechanical, thermal, and durability) and chemical properties and aid with processing of polymers [34].

Plasticizers are additives used to provide flexibility and improve their process ability, and between them, orthophthalates are the most used plasticizers [35]. The typical amount range 10–70% (w/w). Among the identified substances are included four phthalates (DEP, DBP, DPP, and ODP). Both DBP and DPP are included in the “Authorisation List” of Annex XIV of REACH [36] for their toxic effects for reproduction. However, DBP are listed in the Union List of the Regulation 10/2011 to be used as plasticizer in repeated use materials and articles contacting nonfatty foods with a specific migration limit (SML) of 0.3mg/Kg (we have detected 1.34 mg/Kg). Contrarily, neither DEP nor DPP and ODP are in the Union List. Two other plasticizers such as Citroflex A-4 and bis (2-ethylhexyl) adipate are also included Union List of authorized substances (R. 10/2011).

Another group of identified substances is the slip agents, which reduce friction during and after plastic manufacturing and are used in the range of 0.05–3% (w/w) [37]. Oleamide, erucamide, stearamide, and hexadecanamide are authorized substances to be used as additives in plastics (IAS).

Antioxidants and ultraviolet stabilizers are widely used additives in plastics, usually in the range of 0.05–3% to enhance the lifetime of polymeric material, avoiding the oxidation process by oxygen or UV light [34, 37]. Benazol P is a benzotriazole ultraviolet (UV) light absorber that provides UV protection to a wide variety of polymers, including polycarbonates. It is an authorized substance included in the Union List, with a total specific migration limit (SML-T) of 30mg/Kg for the restriction group of the benzotriazole-type UV stabilizers (three substances). Irgafos 168[®] is a phosphite antioxidant that provides polymers protection against thermooxidative degradation and is used in small amounts, usually between 0.004% and 0.5% [29]. It is included in the Union List (IAS).

We have also identified four odorants, which are added into plastics to add desirable fragrance or to mask any undesirable odor. 4-tert-Butylcyclohexyl acetate and methyl dihydrojasmonate are included in the EU cosmetics database [24] as perfumes. D,L-camphor is used as flavouring substance in foods and for perfuming industrial products [38]. On the other hand, galaxolidone is a metabolite of galaxolide, a polycyclic musk widely used as a fragrance in personal care and consumer products.

To our knowledge, these substances have not been previously determined in the polycarbonate food contact material. In a posterior target analysis using a target LC-MS/MS method, we determined BPA with concentrations ranging from 0.002mg/Kg to 0.008mg/Kg (details not provided). These low levels are difficult to detect with the wide scope of the untargeted methods.

3.2. Safety Assessment

For FCM, it is mandatory to guarantee their safety regarding the IAS and NIAS migration. For IAS, the migration concentration should be compared with the specific migration level (SML), established in the Regulation 10/2011. For those authorized substances with no SML, we have used the limit of 60mg/Kg food (limit for overall migration). For NIAS with toxicological reference values (TDI), we use this as a threshold (Table 2). For the rest of identified compounds, we applied the TTC approach described previously (Section 2.5).

Table 2

Risk assessment of the identified compounds.

Substance	Migration concentration (mg/kg food)	SML (mg/kg food)	EDI (mg/person/day)	Cramer class	TDI _{Cramer} (mg/person/day)	TDI (mg/person/day)	HQ (EDI/TDI)
3,5-Ditert-butyl-4-hydroxybenzaldehyde (1620-98-0) ¹	0.13		0.13	2	0.54		0.24
4-tert-Butylcyclohexyl acetate (32210-23-4) ¹	0.13		0.13	2	0.54		0.24
Benazol P (2440-22-4) ²	0.07	30*	0.07				
Benzyl octyl adipate (3089-55-2) ¹	0.04		0.04	1	1.8		0.02
Bis (2-ethylhexyl) adipate (103-23-1) ²	0.56	18	0.56				
D,L-Camphor (76-22-2) ²	0.03	60**	0.03				
Citroflex A-4 (77-90-7) ²	0.03	60***	0.03				
Dibutyl hexanodioate (105-99-7) ²	0.01		0.01	1	1.8		0.006
Dibutyl phthalate (84-74-2) ²	0.21	0.3	0.21			0.6 ^a	0.35
Dibutyl sebacate (109-43-3) ²	0.09	60***	0.09				
Diethyl phthalate (84-66-2) ²	0.01		0.01			49.2 ^b	0.0002
Dodecyl sulfate (151-21-3) ¹	0.20		0.2	3	0.09		2.22
Dipentyl phthalate (131-18-0) ¹	0.05		0.05	1	1.8		0.03
Erucamide (112-84-5) ²	0.34	60**	0.34				
Galaxolidone (507442-49-1) ¹	0.14		0.14	3	0.09		1.56
Hexadecanamide (629-54-9) ¹	1.22	5	1.22				

Irgafos 168 (31570-04-4) ²	0.99	60**	0.99				
Methyl dihydrojasmonate (24851-98-7) ²	0.02		0.02	2	0.54		0.04
N,N'-Dicyclohexylurea (2387-23-7) ¹	0.02		0.02	3	0.09		0.22
DEET (134-62-3) ¹	0.03		0.03	3	0.09		0.33
Octyl decyl phthalate (119-07-3) ¹	2.44		2.44	1	1.8		1.36
Oleamide (301-02-0) ²	0.04	60**	0.04				
Palmitoyl ethanolamide (544-31-0) ¹	0.03		0.03	3	0.09		0.33
Stearamide (124-26-5) ¹	0.33	60**	0.33				

¹Migration (mg/kg) estimated semiquantitatively using the average response factor of a set of internal standards. ²

Migration (mg/kg) calculated quantitatively using the calibration curve of the substance standard. *Group restriction (sum of substances group restriction 12). Regulation 10/2011. **Limit of 60 mg/kg for authorized substances (IAS) with no SML. Regulation 10/2011. ***Group restriction (sum of substances group restriction 32). Regulation 10/2011.

^aEFSA TDI 0.01 (mg/kg bw/day) [47]. Calculation of TDI in mg person⁻¹ day⁻¹ considering a person's weight of 60kg. ^b

US EPA TDI 0.8 (mg/kg bw/day) [48]. Calculation of TDI in mg person⁻¹ day⁻¹ considering a person's weight of 60kg.

The IAS benzazol P, bis (2-ethylhexyl) Citroflex A-4, and hexadecanamide present a migration concentration lower than the established SML; therefore, their exposures are not of concern (Table 2). None of the authorized substances without SML exceeds 60mg/Kg food. The rest of the identified substances show a HQ

More in-deep investigation is needed to uncover the identity of all annotated but not identified substances and to perform a risk assessment.

4. Conclusions

A new and automated analytical strategy for identification of unknown substances in food contact materials was applied to food contact polycarbonate and permitted the identification of 24 substances, mainly plasticizers, slip agents, and antioxidants. The majority of these substances have not been previously identified in PC food contact materials.

The use of the intelligent data acquisition (AcquireX[®]) in both modes (DE and IPE) has permitted a fast and exhaustive data acquisition, and the use of DDA-MS³ provides profuse structural information that increase the identification confidence.

The implementation of an automated workflow for database searching is essential to avoid labour intensive and time-consuming explorations. The use of a specific CD workflow has permitted a very fast search in different databases, which could be improved increasing the database content with more substances.

We have defined very rigorous criteria for identification of the annotated substances, in order to provide to the analytical strategy with a high level of identification confidence. The use of MS³ provides more structural information that increases this confidence. However, some general accepted guidelines for NIAS identification are necessary.

4.1. Study Limitations

For several identified substances, a semiquantitative migration (mg/kg) was estimated using the average response

of seven internal standards not chemically related with the identified substances. This, obviously, is a rough approximation for risk assessment that could be improved using the standards of the identified substances.

Acknowledgments

The study was performed using analytical instruments financed by the European Commission through the European Regional Development Funds (ERDF) Operational Programme of the Valencia Region (2014–2020). Pablo Dualde acknowledges his “Ayudas para la contratación PTA” (PTA2018-016320-I) from “Ministerio de Ciencia e Innovación” (Spain).

- [1] EU, 2011. COMMISSION REGULATION (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food
- [2] EFSA, "Panel on food contact materials, enzymes, flavourings and processing aids," EFSA Journal, vol. 14 no. 1, DOI: 10.2903/j.efsa.2016.4357, 2016.
- [3] ILSI Europe, Guidance on Best Practices on the Risk Assessment of Non Intentionally Added Substances (NIAS) in Food Contact Materials and Articles, 2015. ISBN: 9789078637424
- [4] EU, 2004, Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with Food and Repealing Directives 80/590/EEC and 89/109/EEC
- [5] K. Bhunia, S. S. Sablani, J. Tang, B. Rasco, "Migration of chemical compounds from packaging polymers during microwave, conventional heat treatment, and storage," Comprehensive Reviews in Food Science and Food Safety, vol. 12 no. 5, pp. 523-545, DOI: 10.1111/1541-4337.12028, 2013.
- [6] C. Bignardi, A. Cavazza, C. Laganà, P. Salvadeo, C. Corradini, "UHPLC-high-resolution mass spectrometry determination of bisphenol A and plastic additives released by polycarbonate tableware: influence of ageing and surface damage," Analytical and Bioanalytical Chemistry, vol. 407 no. 26, pp. 7917-7924, DOI: 10.1007/s00216-015-8958-x, 2015.
- [7] E. J. Hoekstra, C. Simoneau, "Release of bisphenol A from polycarbonate-A review," Critical Reviews in Food Science and Nutrition, vol. 53 no. 4, pp. 386-402, DOI: 10.1080/10408398.2010.536919, 2013.
- [8] Y. Zhang, J. Li, Y. Yan, "Molecular dynamics study of the migration of bisphenol A from polycarbonate into food simulants," Chemical Physics Letters, vol. 741, DOI: 10.1016/j.cplett.2020.137125, 2020.
- [9] Y. Sanchis, V. Yusà, C. Coscollà, "Analytical strategies for organic food packaging contaminants," Journal of Chromatography A, vol. 1490, pp. 22-46, DOI: 10.1016/j.chroma.2017.01.076, 2017.
- [10] M. Calow, P. Alfaro, C. Nerín, E. Jones, E. Riches, "Progress in mass spectrometry for the analysis of set-off phenomena in plastic food packaging materials," Journal of Chromatography A, vol. 1453, pp. 124-133, DOI: 10.1016/j.chroma.2016.05.032, 2016.
- [11] C. Nerin, P. Alfaro, M. Aznar, C. Domeño, "The challenge of identifying non-intentionally added substances from food packaging materials: a review," Analytica Chimica Acta, vol. 775, pp. 14-24, DOI: 10.1016/j.aca.2013.02.028, 2013.
- [12] M. J. Martínez-Bueno, M. J. Gómez Ramos, A. Bauer, A. R. Fernández-Alba, "An overview of non-targeted screening strategies based on high resolution accurate mass spectrometry for the identification of migrants coming from plastic food packaging materials," TrAC Trends in Analytical Chemistry, vol. 110, pp. 191-203, DOI: 10.1016/j.trac.2018.10.035, 2019.
- [13] C. Bignardi, A. Cavazza, C. Corradini, P. Salvadeo, "Targeted and untargeted data-dependent experiments for characterization of polycarbonate food-contact plastics by ultra high performance chromatography coupled to quadrupole orbitrap tandem mass spectrometry," Journal of Chromatography a, vol. 1372, pp. 133-144, DOI: 10.1016/j.chroma.2014.10.104, 2014.
- [14] C. Bignardi, A. Cavazza, C. Laganà, P. Salvadeo, C. Corradini, "Release of non-intentionally added substances (NIAS) from food contact polycarbonate: effect of ageing," Food Control, vol. 71, pp. 329-335, DOI: 10.1016/j.foodcont.2016.07.013, 2017.
- [15] Thermo Scientific, "SN65392-EN 0119M. AcquireX," 2019. Available on themofisher.com/AcquireX. Accessed

May 2020

[16] Thermo Scientific, Thermo Scientific, 2019. Available on:

<https://www.thermofisher.com/es/es/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/compound-discoverer-software.html>

[17] E. Canellas, P. Vera, C. Nerín, "Migration assessment and the 'threshold of toxicological concern' applied to the safe design of an acrylic adhesive for food-contact laminates," *Food Additives and Contaminants: Part A*, vol. 34 no. 10, pp. 1721-1729, DOI: 10.1080/19440049.2017.1308017, 2017.

[18] EFSA, "Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC)," *EFSA Journal*, vol. 10 no. 7, 2012.

[19] V. Yusà, A. López, P. Dualde, "Analysis of unknowns in recycled LDPE plastic by LC-Orbitrap Tribrid HRMS using MS3 with an intelligent data acquisition mode," *Microchemical Journal*, vol. 158, DOI: 10.1016/j.microc.2020.105256, 2020.

[20] FCA, FCA Guidelines on Risk Assessment of Non-listed Substances (NLS) and Non-intentionally Added Substances (NIAS) under the Requirements of Article 3 of the Framework Regulation, 2016.

[21] ILE Europe, Guidance on Best Practices on the Risk Assessment of Non Intentionally Added Substances (NIAS) in Food Contact. Report Commissioned by the Packaging Materials Task Force, 2015.

[22] NHI, National Center of Advancing Translational Sciences, 2020. <https://drugs.ncats.io/drug/95VTI93VUL>

[23] S. P. Bhatia, L. Jones, C. S. Letizia, A. M. Api, "Fragrance material review on 4-tert-butylcyclohexyl acetate," *Food and Chemical Toxicology*, vol. 46 no. 12, pp. S36-S41, DOI: 10.1016/j.fct.2008.09.038, 2008.

[24] CosIng, European Commission Database for Information on Cosmetic Substances and Ingredients, 2020. Accessed on 2020, https://ec.europa.eu/growth/sectors/cosmetics/cosing_en

[25] Z. Bodai, C. Kirchkeszner, M. Novák, "Migration of tinuvin P and irganox 3114 into milk and the corresponding authorised food simulant," *Food Additives and Contaminants: Part A*, vol. 32 no. 8, pp. 1358-1366, DOI: 10.1080/19440049.2015.1055523, 2015.

[26] M. T. D. A. Freire, I. A. Santana, F. G. R. Reyes, "Plasticizers in brazilian food-packaging materials acquired on the retail market," *Food Additives and Contaminants*, vol. 23 no. 1, pp. 93-99, DOI: 10.1080/02652030500241686, 2006.

[27] EFSA, "Camphor in flavourings and other food ingredients with flavouring properties opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission," *The EFSA Journal*, vol. 729, 2008.

[28] H. B. Pyeon, J. E. Park, D. H. Suh, "Non-phthalate plasticizer from camphor for flexible pvc with a wide range of available temperature," *Polymer Testing*, vol. 63, pp. 375-381, DOI: 10.1016/j.polymertesting.2017.08.029, 2017.

[29] ECHA, Mapping Exercise. Plastic Additive Initiative, 2020. <https://echa.europa.eu/fr/mapping-exercise-plastic-additives-initiative#table> . Acceded June 2020

[30] M. Biedermann, K. Grob, "GC method for determining polyadipate plasticizers in foods: transesterification to dibutyl adipate, conversion to migrating polyadipate," *Chromatographia*, vol. 64 no. 9-10, pp. 543-552, DOI: 10.1365/s10337-006-0071-z, 2006.

[31] Pubchem, Pubchem, 2020. <https://pubchem.ncbi.nlm.nih.gov/> . Accesed May 2020

[32] Mass Frontier, Mass Frontier, 2020. <https://www.thermofisher.com/es/es/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/mass-frontier-spectral-interpretation-software.html>

[33] E. L. Schymanski, J. Jeon, R. Gulde, "Identifying small molecules via high resolution mass spectrometry: communicating confidence," *Environmental Science & Technology*, vol. 48 no. 4, pp. 2097-2098, DOI: 10.1021/es5002105, 2014.

[34] A. Shrivastava, Introduction to Plastic Engineering. PDL Handbook Series, 2018.

[35] R. Jamarani, H. Erythropel, J. Nicell, R. Leask, M. Marić, "How green is your plasticizer?," *Polymers*, vol. 10 no. 8, DOI: 10.3390/polym10080834, 2018.

- [36] REACH, "Authorisation list," 2020. <https://echa.europa.eu/es/home> . Accessed Jun 2020
- [37] J. N. Hahladakis, C. A. Velis, R. Weber, E. Iacovidou, P. Purnell, "An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling," *Journal of Hazardous Materials*, vol. 344, pp. 179-199, DOI: 10.1016/j.jhazmat.2017.10.014, 2018.
- [38] EU, 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC
- [39] C. A. M. Eke, J. L. Marks, L. B. Wroblewski, H. S. Raatikainen, S. R. Lenox, K. E. Gebhardt, "Human and environmental toxicity of sodium lauryl sulfate (SLS): evidence for safe use in household cleaning products," *Environmental Health Insights*, vol. 9, pp. EHI.S31765-32, DOI: 10.4137/EHI.S31765, 2015.
- [40] M. A. da Silva, V. Calabrese, J. Schmitt, "Impact of wormlike micelles on nano and macroscopic structure of TEMPO-oxidized cellulose nanofibril hydrogels," *Soft Matter*, vol. 16 no. 20, pp. 4887-4896, DOI: 10.1039/d0sm00135j, 2020.
- [41] R. Benson, "Hazard to the developing male reproductive system from cumulative exposure to phthalate esters- dibutyl phthalate, diisobutyl phthalate, butylbenzyl phthalate, diethylhexyl phthalate, dipentyl phthalate, and diisononyl phthalate," *Regulatory Toxicology and Pharmacology*, vol. 53 no. 2, pp. 90-101, DOI: 10.1016/j.yrtph.2008.11.005, 2009.
- [42] V. García Ibarra, A. Rodríguez Bernaldo de Quirós, P. Paseiro Losada, R. Sendón, "Non-target analysis of intentionally and non intentionally added substances from plastic packaging materials and their migration into food simulants," *Food Packaging and Shelf Life*, vol. 21, DOI: 10.1016/j.fpsl.2019.100325, 2019.
- [43] ECCS, "ECCS.Data bank," 2020. Available at: http://eccs.unicartagena.edu.co/data_sheet.php?id=131&varurl=data . Accessed May 2020
- [44] Y. Yan, C.-Y. Hu, Z.-W. Wang, Z.-W. Jiang, "Degradation of irgafos 168 and migration of its degradation products from PP-R composite films," *Packaging Technology and Science*, vol. 31 no. 10, pp. 679-688, DOI: 10.1002/pts.2405, 2018.
- [45] A. M. Api, D. Belsito, S. Bhatia, "fragrance ingredient safety assessment, methyl hexyl oxo cyclopentanone carboxylate, CAS registry number 37172-53-5," *Food and Chemical Toxicology*, vol. 110 no. Suppl 1, pp. S327-S336, DOI: 10.1016/j.fct.2017.06.028, 2017.
- [46] A. Olejnik, A. Sliwowska, I. Nowak, "Jasmonic acid, methyl jasmonate and methyl dihydrojasmonate as active compounds of topical formulations," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 558, pp. 558-569, DOI: 10.1016/j.colsurfa.2018.09.026, 2018.
- [47] EFSA, "Update of the risk assessment of di-butylphthalate (DBP), butyl-benzyl-phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), di-isononylphthalate (DINP) and di-isodecylphthalate (DIDP) for use in food contact materials," *EFSA Journal*, vol. 17 no. 12, 2019.
- [48] EPA, Phthalates TEACH Chemical Summary, 2020. https://archive.epa.gov/region5/teach/web/pdf/phthalates_summary.pdf Accessed May 2020

DETAILS

Subject:	Antioxidants; Standards; Software; Food; Data processing; Regulation; Risk assessment; Identification; UV stabilizers; Slip; Stabilizers (agents); Life sciences; Workflow; Safety; Bisphenol A; Automation; Libraries; Polycarbonate resins

Business indexing term:	Subject: Automation

Location:	United States--US; Europe
Company / organization:	Name: AcquireX; NAICS: 518210
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-11-18 (Received); 2021-02-17 (Revised); 2021-02-25 (Accepted); 2021-03-27 (Pub)
DOI:	https://doi.org/10.1155/2021/6654611
ProQuest document ID:	2508265372
Document URL:	https://www.proquest.com/scholarly-journals/identification-24-unknown-substances-nias-ias/docview/2508265372/se-2?accountid=211160
Copyright:	Copyright © 2021 Vicent Yusà et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/

Document 43 of 59

Study on the Spectrum-Effect Relationship of the Traditional Effect of Saponins in *Glycyrrhiza uralensis* Fisch

Xia, Wenxin¹; Liu, Qiansong¹; Zhou, Hao¹; Shiyao Hua¹; Lin, Dong¹; Han, Xuebo²
; Fu, Xueyan¹

¹ School of Pharmacy, Ningxia Medical University, Yinchuan 750004, China ² Ningxia Medical University, Yinchuan 750004, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Licorice is a traditional Chinese medicine that has been used for a long time in China and still in great use today. The effect of licorice on tonifying spleen and invigorating qi has been proved for thousands of years, but the material basis of its effect is not clear. In this paper, we established the fingerprints of 21 batches of licorice collected from different origins in China with High-Performance Liquid Chromatography (HPLC) to identify the common peaks. Its effect of tonifying spleen and invigorating qi was confirmed through a series of praxiology experiments. The spectrum-effect relationship between HPLC fingerprints and its effect of tonifying spleen and invigorating qi of licorice was examined by gray relational analysis and partial least squares regression analysis. Results showed that the effect of licorice on tonifying spleen and invigorating qi resulted from various compounds and peaks. X_2-X_6 is presumed to be the main pharmacological substance base. This research successfully identified the spectrum-effect relationship between HPLC fingerprints and the effect of licorice on tonifying spleen and invigorating qi. The research method based on the spectrum-effect relationship helps provide new research ideas and strategies for the study of the basis of the medicinal materials and quality control of traditional Chinese medicine.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Glycyrrhiza uralensis Fisch., known as licorice, is one of the most famous traditional Chinese medicine (TCM). Licorice is an "essential herbal medicine" in China; it is not only used to treat diseases. Still, it has been listed as a medicinal and edible homologous plant by the Ministry of Health of China [1]. According to ancient records, licorice has the functions of clearing away heat and detoxification, relieving cough and expectorating phlegm, tonifying spleen and invigorating qi, delaying and relieving pain, and harmonizing all kinds of medicine. These traditional effects have been confirmed by modern research [2]. Triterpenoid saponin is one of the main active components of licorice, which has antiviral, antitumor, immunomodulatory, and other pharmacological effects [3, 4]. In Chinese Pharmacopoeia, the content of Glycyrrhizic Acid and Liquiritin is used as the quality control standard [5]; such a single measurement index is challenging to meet the current quality control requirements of licorice, so the quality

standard of licorice still needs to be improved [6].

Due to the characteristics of “multicomponent, multitarget, and comprehensive regulation” of TCM, it is not easy to find and accurately identify the main efficacy components [7]. Meanwhile, it is one-sided to take the content of individual components in Chinese medicine as the standard of quality control. Because of its microscopic, integral, and fuzzy characteristics, fingerprint can better reflect the types and contents of chemical components. Also, the fingerprint has the attributes of integrity, can comprehensively reflect the quality of TCM, has become one of the hot spots in the quality evaluation and control of TCM [8, 9].

Although the fingerprint confirms the consistency of TCM, it does not reflect its clinical efficacy. It is necessary to study the relationship between spectral effect to elucidate the relationship between chemical composition and curative effect [10, 11]. The study of spectrum-effect is a new scientific research method, including the establishment of fingerprint, efficacy evaluation, and correlation analysis of spectrum-effect [12].

So far, researchers have conducted a lot of experimental exploration on the spectrum-effect relationship. At present, the most commonly used method is still to establish fingerprint by HPLC [13, 14] and then conduct spectrum-effect relationship analysis through PCA, correlation analysis, GRA, regression analysis, and other data processing methods. Using modern analytical technology is an essential means to realize the separation of chemical components and identify structure in the fingerprint of traditional Chinese medicine. It is also a necessary method for the study of spectrum-effect correlation. In addition to HPLC, GC-MS [15] and a variety of liquid-mass spectrometry technology can also be used to study the spectral effect relationship, such as high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [16], high-performance liquid chromatography-evaporative light scattering (HPLC-ELSD) [17], high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) [18], ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) [19], etc.

In our study, we collected licorice from 21 habitats and enriched the total saponin of licorice. We established the fingerprint of licorice saponins from 21 places of origin, identified and determined its common peaks using the similarity evaluation system software of TCM chromatographic fingerprint. After that, we studied the spectrum-effect relationship between the fingerprint and the effect of tonifying spleen and replenishing qi on licorice by the gray relation analysis (GRA) and partial least squares regression (PLSR). This study expects to provide a scientific basis for the basic research of pharmacodynamic substances and quality control standards of licorice.

2. Materials and Methods

2.1. Plant Materials and Reagents

A total of 21 batches (S1–S21) of licorice samples were collected from different areas in 6 provinces of China: Wuzhong, Ningxia (S1, S2, S3, S4), Longde County, Guyuan City, Ningxia (S5, S6), Lanzhou City, Gansu Province (S7, S8), Wuwei City, Gansu Province (S9), Dingxi City, Gansu Province (S10, S11), Qingyang City, Gansu Province (S12), Baiyin City, Gansu Province (S13), Chifeng City, Inner Mongolia (S14, S15), Erdos City, Inner Mongolia (S16), Luliang City, Shanxi Province (S17), Alar City, Xinjiang Province (S18), Yili, Xinjiang Province (S19, S20), Harbin, and Heilongjiang Province (S21). All batches were authenticated as *Glycyrrhiza uralensis* Fisch. by the pharmacognosy department of Ningxia Medical University.

All standards were purchased from Shanghai Yuanye biotechnology Co., LTD. HPLC-grade acetonitrile, anhydrous ethanol, acetic acid, and methanol were obtained from the Tianjin Damao chemical reagent factory. Also, the instrument used: UV-2600 visible - ultraviolet spectrophotometer (Shimadzu instrument Co., LTD), high-performance liquid chromatography-quadrupole in-flight mass spectrometry (Agilent 1290+6545), Agilent 1260 high-performance liquid chromatography (Agilent technology co. LTD), etc.

2.2. Content Determination

2.2.1. Preparation of Glycyrrhizic Acid Standard Curve

Precision measurement of Glycyrrhizic Acid (GA) reference solution 0.5, 1.0, 2.0, 2.5, 3.0 mL in the test tube, and 60°C dry solvents, plus 5% vanillin-ice acetic acid solution 0.4 mL, shake well, add 1.6 mL perchloric acid, shake well, expose it to 55°C water bath reaction for 20 min, take out with cold water flow cooling to room temperature, do

not contain reference substance of the solvent. The determination of absorbance should be at 337 nm wavelength, and the absorbance A should be used as the ordinate and Glycyrrhizic Acid concentration (mg/mL) as the abscissa, drawing a standard curve; the regression equation is as follows: (1) $Y=0.225x+0.0132$ $R^2=0.9995$

The linear relationship of Glycyrrhizic Acid between 0.049 mg/mL and 0.294 mg/mL was good.

The solution of total saponins from 21 samples from different places of origin was collected, and the absorbance of total saponins was determined by the method above. The content was calculated by substituting it into the regression equation.

2.2.2. Solution Preparation of Standard and Test Samples

The standard of Glycyrrhizic Acid was precisely weighed and was dissolved in 10 mL volumetric bottles. The Glycyrrhizic Acid control solution with concentrations of 0.98 mg/mL was prepared.

About 30 mg of the enriched GS from 21 regions was taken and placed in a 10 mL volumetric bottle. 70% ethanol was adjusted to scale and weighed. After 30 min of ultrasound, it was weighed and its quality was supplemented.

2.2.3. Method Validation for Content Determination

(i) Precision: The precision was evaluated by measuring the control solution of Glycyrrhizic Acid five times in a row.

(ii) Repeatability: The repeatability was assessed by determining the absorbance of 5 parts of the total saponin solution enriched from samples of S1.

(iii) Recovery rate: 5.0 mL of total saponins enriched from 9 samples of S1 origin were taken and placed in 25 mL volumetric bottles, respectively. The Glycyrrhizin Acid control solutions 2.0, 4.0, and 6.0 mL were added to form low, medium, and high concentration solutions, respectively. Absorbance was measured according to the method under "2.2.1", and the recovery rate was calculated.

2.3. Establishment of Fingerprint

2.3.1. Apparatus and Chromatographic Conditions

Agilent 1260 liquid chromatography system, Agilent ZORBAX SB-C₁₈ column (4.6 × 250 mm, 5 meters), the mobile phase was 0.2% glacial acetic acid (A) and acetonitrile (B). Elution conditions: 0~15 min: 15% (B); 15~25 min: 15~20% (B); 25~70 min: 20~50% (B); 70~90 min: 50~70% (B); 90~95 min: 70~15% (B). The detection wavelength was 254 nm and 310 nm, the flow rate was 1 mL · min⁻¹, the column temperature was 30 °C, and the injection volume was 20 μL.

2.3.2. Sample Preparation

About 30 mg of the enriched GS was collected from 21 regions, respectively. In 10 mL volumetric bottles, 70% ethanol was adjusted to scale and weighed. After 30 min of ultrasound, it was weighed and its quality was supplemented. Finally, the sample was filtered by 0.22 μm microporous membrane, and the filtrate was set aside.

2.3.3. Method Validation for HPLC Fingerprint Analysis

(i) Precision: The precision was determined by analyzing the total saponins enriched from samples of S1 origin six consecutive times.

(ii) Stability: The sample stability was evaluated by analyzing one sample in 0, 2, 4, 8, 12, 24 h sample determination.

(iii) Repeatability: The repeatability was assessed by analyzing six independently prepared total saponins enriched from samples of S1.

2.4. Qualitative Analysis of Common Peaks in Fingerprints

2.4.1. Apparatus and Chromatographic Conditions

HPLC chromatographic conditions. Agilent 1290 Infinity liquid chromatography system, Agilent RRHD sb-c18 column (2.1 × 100 mm, 1.8 μm), the flow rate of 0.200 mL · min⁻¹, column temperature of 30 °C, amount of injection of 10 μL. The mobile phase was 0.2% formic acid (A) and acetonitrile (B). Elution conditions: 0~2 min: 15~30% (B); 2~10 min: 30~85% (B); 10~15 min: 85~90% (B); 15~20 min: 90~0% (B).

MS conditions. Dual AJS ESI, with positive particle detection, Gas temperature 350 °C; Capillary voltage: 3500 V; Taper hole voltage: 40 V; Extraction voltage: 4.0 V; Ion source temperature: 120 °C; Desolvent temperature and flow rate: 350 °C, 12 L/min; Atomizing gas voltage: 40 psi; Dryer temperature and flow rate: 350 °C and 10 L/min;

Fragmentor 130V, Skimmer 65V; Quality scan range: 50~1000m/z.

2.4.2. Solution Preparation of Standard and Test Samples

Precisely weigh the standard: Liquiritin, Isoliquiritin, Liquiritigenin, isoliquiritigenin, Liquiritin apioside, Isoliquiritin apioside, Neoliquiritin, Neoisoliquiritin, Glabridin.

Licoagrochalcone A, Licoagrochalcone B, Licoagrochalcone C, Formononetin, Glycyrrhizic Acid: add 70% ethanol to dissolve and dilute them to 4.07, 4.07, 3.38, 4.15, 4.00, 3.84, 3.69, 3.08, 3.30, 3.54, 3.08, 1.92, 3.69, 3.69, 3.69 μ g/ml mixed solution, ultrasonically dissolved and set aside.

The total saponin solution enriched by S1 samples prepared by “2.3.2” method was diluted by 70% ethanol by 100 times so that its concentration was about 30 μ g/mL and then it was stored for standby.

2.4.3. Qualitative Analysis of Common Peaks

The mixed standard and sample solution were injected into the HPLC-Q-TOF/MS according to the chromatographic and mass spectrometric conditions of “2.5.1”, and the chromatogram was recorded. The spectra of the mixed standard were compared with the spectra of the sample solution, and the common peak components were inferred according to the retention time and the characteristics of the fragment ion peak.

2.5. Experimental Animals, Groups, and Dosages

Male SPF grade ICR mice, weighing 18~22g, were provided by the laboratory animal center of Ningxia Medical University [Animal certificate NO.: SCXK (Ning) 2015-0001]. The animals were kept at room temperature of 22°C $\pm$ 2°C, at a relative humidity of 50~60%, and natural light. The ICR mice involved in each of the following pharmacodynamics experiments were randomly divided into the model group, the normal group, and the 21 licorice root administration groups (total flavonoids and total saponins), with 10 mice in each group.

2.6. Experiment on Reinforcing the Spleen to Replenish Qi

In this study, based on the “Experimental Methodology of Pharmacology of TCM” and the literature method, the method of excessive fatigue and indiscipline of diet was adopted to cause spleen deficiency in mice. The mice in the spleen-deficiency model group showed symptoms such as weight loss, tiredness, loose stool, haggard, and chills. Building after 14 days, continuous gavage for a week (total saponins of licorice of 150mg/kg, model group and the normal group was given sodium carboxymethyl cellulose aqueous solution) evaluated the effect on fatigue resistance groups of mice, respectively; mice were put into water depth >20cm, with a temperature of 25 $\pm$ 1°C in the bucket; it was left free to swim and we watched the swimming and recorded the time of death.

2.6.1. Tail Suspension Experiment

Continuous dosing week: 150mg/kg of total saponins of licorice was given to the model group and the normal group was given sodium carboxymethyl cellulose aqueous solution. After the final dose, the mice were coated with medical adhesive 1cm from the tail and hung upside down on a pole 15cm off the ground. The animal struggled to overcome abnormal position, but activity after a while will soon appear desperate behavior. The expression of desperate behavior is no longer a struggle, presenting the character of the quiet state. The mice were observed for 6min and the immobility time of the mice was recorded after 1 min of the experiment beginning.

2.6.2. Forced Swimming Experiments

The drug was administered continuously for one week (150mg/kg of total saponins of licorice was given to the model group, and the normal group was given sodium carboxymethyl cellulose aqueous solution). After the last dose, the mice were placed in a cylindrical beaker with a diameter of about 17 cm, a water depth of 10 cm, and a water temperature of 25°C. The observation was performed for 6min, and the accumulated immobility time was recorded within the last 4 min.

2.7. Spectrum-Effect Relationship

Correlation analysis was conducted between the common peak area and pharmacodynamic data of HPLC chromatogram of radix Glycyrrhiza saponin components from 21 various regions. SPSS 24.0 and DPS 7.05 were used for processing, and the common peaks significantly correlated with the pharmacodynamics were found.

2.8. Statistical Analysis

SPSS 24.0 statistical software was used for statistical data analysis; DPS software was used for grayscale

correlation analysis. GraphPad Prism software was used for mapping, experimental data were represented as means $\pm$ SD, and one-way ANOVA LSD and Dunnett's T3 (3) statistical method were used for comparison between groups.

3. Investigations and Results

3.1. Content of the Total Saponins in Licorice

The total saponins obtained from 21 samples of origin were collected and the contents of saponins were calculated by Ultraviolet Spectrophotometry (UV). The results showed that more than half of the samples contained more than 60% of total saponins. The specific results are shown in Figure 1.

[figure omitted; refer to PDF]

3.2. Analysis of HPLC Fingerprints and Similarities

3.2.1. Method Validation for the HPLC Fingerprint Analysis

The precision, repeatability, and stability of the HPLC fingerprint analysis method were verified. The relative standard deviation (RSD) of the average relative retention time (RRT) and relative peak area (RPA) both meet the prescribed requirements. The variations in the RRT of the characteristic peaks did not exceed 3% and the variations in the RPA did not exceed 5%.

3.2.2. Establishment of the Fingerprint of the Saponins in *Glycyrrhiza uralensis* Fisch

Samples of licorice saponins from 21 different regions were collected, injected into HPLC chromatography, recorded chromatogram, and then imported into the Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine to generate HPLC fingerprints of licorice saponin. The results are shown in Figure 2.

[figure omitted; refer to PDF]

3.2.3. HPLC Fingerprints of Licorice Saponins Samples

The HPLC fingerprints of the 21 batches of licorice from different areas of China were obtained and matched by the Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine. S1 was selected as the reference spectrum, and the software was corrected by multiple points to automatically match the chromatographic peaks. The median method was selected to generate the control fingerprint, and 7 common peaks were determined. The results are shown in Figure 3.

[figure omitted; refer to PDF]

3.2.4. Similarity Analysis of the HPLC Fingerprints

The similarity value of the generated control fingerprint (R) was set as 1, and the similarity of the characteristic chromatogram of 21 batches of licorice total saponins was calculated, respectively. The similarity between each batch of licorice saponins and the control fingerprint was as follows: 0.929 (S1), 0.991 (S2), 0.916 (S3), 0.805 (S4), 0.944 (S5), 0.895 (S6), 0.973 (S7), 0.804 (S8), 0.932 (S9), 0.898 (S10), 0.915 (S11), 0.901 (S12), 0.930 (S13), 0.931 (S14), 0.951 (S15), 0.766 (S16), 0.753 (S17), 0.916 (S18), 0.961 (S19), 0.911 (S20), 0.954 (S21), which indicates good consistency among the *G. uralensis* samples.

3.3. Qualitative Analysis of Common Peaks in HPLC-Q-TOF/MS

HPLC-Q-TOF/MS technology was used to classify the common peak chemical components. According to the above HPLC-Q-TOF/MS analysis conditions, the mixed standard solution and the test solution were, respectively, scanned for positive ions. Based on the cleavage law of licorice compounds in databases such as PubChem and Scifinder, a Qualitative Analysis database of licorice was established by combining the cleavage law and literature reports. Agilent Mass Hunter Qualitative Analysis software was used to analyze the match, combined with the reference, mass spectrometry data in the literature, and chromatographic retention behavior, and finally, the information of the compound was determined. After comparison, four common peaks in fingerprint structures were identified, and the compounds are shown in Table 1.

Table 1

Assignment of Common peaks in the fingerprint of saponins in *Glycyrrhiza uralensis* Fisch. based on HPLC-Q-TOF/MS.

Peak number	Name	Formula	RT	<i>m/z</i>	Mass	Theoretical isotopic
4	Glabrolide	C ₃₀ H ₄₄ O ₄	5.990	468.6679	467.6234	468.3240
5	Stearyl Glycyrrhetate	C ₄₈ H ₈₂ O ₄	8.862	724.1758	723.1622	723.6347
6	Glycyrrhizic Acid	C ₄₂ H ₆₂ O ₁₆	11.308	845.4114	822.4116	822.4038
7	18α-Glycyrrhizic Acid	C ₄₂ H ₆₂ O ₁₆	12.315	845.3916	822.4019	823.4071

3.4. Results of Reinforcing the Spleen to Replenish Qi

As can be seen from the experimental results in Figure 4, compared with the normal group, the swimming time of the model group was significantly reduced (###*P*<0.01), indicating successful modeling. Compared with the model group, the Glycyrrhiza saponins from S1~S21 producing area had a significant effect on fatigue-resistant fruit and increased the time of swimming to death (**P*<0.05, ***P*<0.01).

[figure omitted; refer to PDF]

3.4.1. The Results of the Tail Suspension Experiment

As can be seen from the experimental results in Figure 5, in the suspended tail experiment, compared with the model group, there were significant differences among the 21 producing areas (**P*<0.05, ***P*<0.01).

[figure omitted; refer to PDF]

3.4.2. The Results of the Forced Swimming Experiment

The mice in the spleen-deficiency model group showed symptoms such as weight loss, tiredness, loose stool, haggard, and chills. The general observation of mice in the treatment group was significantly better than that in the model group. In addition, the antifatigue ability of mice in the treatment group was significantly improved in the forced swimming test. As can be seen from the experimental results in Figure 6, in the forced swimming experiment, compared with the model group, there were significant differences among the 21 producing areas (**P*<0.05, ***P*<0.01).

[figure omitted; refer to PDF]

3.5. Analysis of the Spectrum-Effect Relationship

Total saponins parts of tonifying spleen replenishing qi experiment, forced swimming test, and tail suspension test have obvious effect. Therefore, correlation analysis was carried out between the common peak area of the licorice saponins in the HPLC chromatogram of each locality and the pharmacodynamic data of the effective invigorating spleen and invigorating qi experiment, forced swimming experiment, and suspended tail experiment.

3.5.1. Gray Relation Analysis (GRA) Results

Gray relational analysis is a method to describe the size, strength, and order of the factors by using gray relational analysis. In this research, the common peak area of the fingerprint of licorice saponins from 21 places of origin was taken as a group of variables, and the data of the pharmacodynamics experiment was taken as another group of variables. The common peak significantly related to the efficacy was found through DPS 7.05 software gray correlation analysis.

It can be seen from Table 2 that there were 6 peaks with the correlation degree of fatigue resistance and swimming time greater than 0.5 in the experiment of invigorating spleen and replenishing qi, namely, common peaks X_5 , X_2 , X_4 , X_1 , X_6 , and X_3 . It indicated that the above peaks were closely related to the efficacy of medicine and might contribute more to the efficacy of invigorating the spleen and replenishing qi. The correlation degree of peak 7 was less than 0.5, indicating that this compound may contribute little to the effect of invigorating the spleen and replenishing qi.

Table 2

Correlation between the common peak of saponins from Glycyrrhiza uralensis Fisch. and the effect of tonifying

spleen and replenishing qi.

No.	Correlation	No.	Correlation
1	0.5970	5	0.5960
2	0.6605	6	0.6670
3	0.5806	7	0.5846
4	0.6475	—	—

It can be seen from Table 3 that there were 6 peaks whose correlation degree with the cumulative fixed time of the suspended tail experiment was greater than 0.5, namely, common peaks X_6 , X_2 , X_4 , X_1 , X_5 , X_7 , and X_3 . It indicated that the above peaks were closely related to the efficacy of drugs.

Table 3

Correlation between common peaks of saponins from *Glycyrrhiza uralensis* Fisch. and tail suspension test.

No.	Correlation	No.	Correlation
1	0.5970	5	0.5960
2	0.6605	6	0.6670
3	0.5806	7	0.5846
4	0.6475	—	—

It can be seen from Table 4 that there were 5 peaks whose correlation degree with the cumulative fixed time of the forced swimming experiment was greater than 0.5, indicating that the 5 common peaks mentioned above were closely related to the drug effect. The correlation degree of peak X_1 and peak X_7 is less than 0.5, indicating that these two compounds might not play the role of fatigue resistance.

Table 4

Correlation between common peaks of saponins from *Glycyrrhiza uralensis* Fisch. and forced Swimming experiment.

No.	Correlation	No.	Correlation
1	0.5281	5	0.5937
2	0.6035	6	0.5600
3	0.5821	7	0.4424
4	0.6090	—	—

3.5.2. PLSR Analysis Results

Partial least squares regression analysis (PLSR) integrates multiple functions to realize regression modeling, data structure simplification, and correlation analysis between two sets of variables at the same time. It can make maximum use of data information and has the characteristics of high prediction accuracy and easy model interpretation. Through DPS software and PLSR analysis, the peak area of fingerprints of licorice saponins from 21 places was taken as an independent variable (X), and the efficacy data obtained from each experiment was taken as dependent variable (Y) for PLS regression analysis to obtain the regression equation fitted by X and Y . The regression coefficient reflects the contribution of each X to Y . The greater the absolute value of the PLS regression coefficient, the greater the contribution of the common chromatographic peak will be.

Experiment on tonifying spleen and replenishing Qi. By PLSR analysis, the relevant regression equation was fitted as follows: (2) $Y = -0.2178X_1 + 0.0607X_2 + 0.1084X_3 - 0.1177X_4 - 0.0018X_5 + 0.1266X_6 + 0.1508X_7$

In this regression equation, the contribution rates of the seven common peaks were: $X_1 > X_7 > X_6 > X_4 > X_3 > X_2 > X_5$ in turn. In the fingerprint, the peak numbers X_1 , X_4 , and X_5 were negatively correlated with the swimming time. That is, the peak strength of this chromatographic peak was increased, and the corresponding swimming time might be reduced, while the remaining chromatographic peaks were positively correlated.

Tail suspension test. By PLSR analysis, the relevant regression equation was fitted as follows: (3) $Y = -0.0715X_1 + 0.0624X_2 - 0.0401X_3 + 0.0943X_4 + 0.1370X_5 + 0.0248X_6 - 0.0977X_7$

In this regression equation, the contribution rates of the seven common peaks were $X_5 > X_7 > X_4 > X_1 > X_2 > X_3 > X_6$ in turn. In fingerprints, peaks X_1 , X_3 , and X_7 were negatively correlated with the accumulative immobility time. That is, the peak strength of this chromatographic peak increased, the corresponding immobility time might decrease, and the remaining chromatographic peaks were positively correlated.

Forced swimming experiment. By PLSR analysis, the relevant regression equation was fitted as follows: (4) $Y = -0.1047X_1 - 0.0980X_2 - 0.0660X_3 - 0.1469X_4 - 0.1101X_5 - 0.0704X_6 - 0.0460X_7$

In this regression equation, the contribution rates of the seven common peaks were $X_4 > X_5 > X_1 > X_2 > X_6 > X_3 > X_7$ in turn. But in the fingerprint, peaks X_1 , X_2 , X_3 , X_4 , X_5 , X_6 , and X_7 were negatively correlated with the accumulated immobility time. That is, the peak strength of this chromatographic peak was increased; the corresponding immobility time might be reduced.

4. Discussion

Traditional Chinese medicine (TCM) has been welcomed worldwide because of its long history and good curative effect. However, up to now, the quality control standard of TCM still needs further discussion and improvement [20, 21]. In recent years, there are various analytical methods for quality control of traditional Chinese medicine, including biological chromatography, traditional chromatography, DNA method, Fourier infrared, near-infrared, nuclear magnetic resonance, and other spectral methods [22]. However, the existing quality control standards that take the characteristics of a single or multiple chemical components as the standard seem unreasonable [23]. Therefore, it is necessary to develop a reasonable method to find markers for quality control of TCM. Thus, the study of the relationship between fingerprint and efficacy emerged [24]. The study of the spectrum-effect relationship is of great significance to elucidate the active components and the quality control of TCM.

In the present study, a simple, accurate, and validated chromatographic fingerprinting method was developed and used to analyze 21 batches of licorice saponins samples collected from different regions in China. Firstly, the total saponins of licorice were prepared. 1000g of licorice herbs from 21 producing areas was weighed and decocted with five times of water, 3 times in total, 1 h each time. The combined filtrate was left standing at room temperature; 60% alcohol was added and left overnight. The supernatant was filtrated, and the combined filtrate was decompressed and recovered until the extracts of licorice herbs were alcohol-free. The extracts of licorice were, respectively, passed into an 80–100 mesh polyamide chromatography column and then eluted with distilled water, 30% ethanol, and 70% ethanol successively. Each component was eluted until the color became lighter and colorless. The eluent was concentrated under pressure and finally concentrated in three parts with different polarities in each producing area, namely water part, 30% alcohol part, and 70% alcohol part. The eluent of the water part was decompressed

and evaporated to get the total saponins of licorice and stored for later use. Second, HPLC fingerprint was established, which is a common method in the quality evaluation of traditional Chinese medicine. The consistency of traditional Chinese medicine was evaluated more comprehensively than the conventional way [25]. HPLC fingerprint and HPLC-QTOF-MS methods used in the present study are important techniques for quality evaluation and identification of natural products [26, 27], and they are more and more widely used in the research of quality evaluation and control of natural products including herbal medicine. Then, 7 common peaks were found by fingerprint similarity evaluation software, and 4 peaks were identified by HPLC-Q-TOF/MS. Second, we conducted a modern pharmacodynamic study of the traditional effects of licorice. It can be seen from the efficacy test results that the total saponins of licorice root have obvious effects on the spleen and qi strengthening test, forced swimming test, and tail suspension test, indicating that the saponins may be the main material basis of these effects. Finally, the relationship between its biological activity and HPLC fingerprint was analyzed by GRA and PLSR. GRA is based on the similarity of geometric curves of various factors to determine the correlation between the factors [28]. If the trend of differences between two factors is consistent, the two factors are considered strongly related. Regression analysis is a kind of multivariate statistical method, which uses regression equation to study the dependence relationship between multiple variables. The contribution of a drug to pharmacodynamics can be determined by establishing a regression equation of fingerprint peak and pharmacodynamics data. PLSR is one of the regression analysis methods [29].

The experimental results identified a total of 7 common peaks (X_1 , X_2 , X_3 , X_4 , X_5 , X_6 , X_7) and inferred 4 common peaks, namely X_4 (Glabrolide), X_5 (Stearyl Glycyrrhetate), X_6 (Glycyrrhizic Acid), and X_7 (18 α -Glycyrrhizic Acid). X_4 , X_5 , and X_6 all had a high contribution in three experiments of tonifying spleen and invigorating qi, suspended tail, and forced swimming. We can speculate that these three compounds may be the active ingredients to exert the above effects.

There are still some deficiencies in the experimental methods of this study. At first, the enriched GS is usually extracted and prepared in ethanol by sonication, and it may not be the most effective method for extracting active components from the *Glycyrrhiza uralensis*. The emerging potential methods of pretreatment and extraction of active components [30] may bring unexpected insights into the material basis of *Glycyrrhiza uralensis*. Then, there is no reverse validation of the predicted active components, which can further determine the scientific nature of the spectral efficiency relationship. The study of the spectrum-effect relationship can not only effectively identify different sources of *Glycyrrhiza uralensis* but also reveal the material basis of its efficacy. Also, it can provide some scientific basis for improving quality control standards.

Authors' Contributions

Wenxin Xia, Qiansong Liu, and Hao Zhou contributed equally to the study.

Acknowledgments

The work was supported by the National Nature Science Foundation of China (Grant Nos. 81860708 and 81560647) and Project Nos. 2018ZWYQ0008 and 2018BEG03016.

References

- [1] L. Que, "Revision of the list of items that are both food and medicine," Chinese Journal of Pharmacy, vol. 52 no. 7, pp. 521-524, 2017.
- [2] X. Jiang, SSYW, "Progress in the pharmacological action of liquorice," Chemical Industry Times, vol. 31 no. 7, pp. 25-28, 2017.
- [3] C. Sun, "Progress in the study of the chemical constituents of Licorice," Science & Technology Information, vol. 18 no. 2, pp. 64-65, 2020.
- [4] M. Li, "Research overview of licorice," Journal of Gansu College of Traditional Chinese Medicine, vol. 17 no. 3, pp. 56-63, 2000.
- [5] Committee NP, Chinese Pharmacopoeia, 2015.
- [6] Y. Zhou, FCSY, "Study on fingerprint of licorice extract and the content determination of its multi-indicator," Chinese Journal of Modern Applied Pharmacy, vol. 37 no. 9, pp. 1046-1051, 2020.

- [7] S. Gao, H. Chen, X. Zhou, "Study on the spectrum-effect relationship of the xanthine oxidase inhibitory activity of *Ligustrum lucidum*," *Journal of Separation Science*, vol. 42 no. 21, pp. 3281-3292, DOI: 10.1002/jssc.201900531, 2019.
- [8] J. Cheng, S. He, Q. Wan, P. Jing, "Multiple fingerprinting analyses in quality control of *Cassiae Semen polysaccharides*," *Journal of Chromatography B*, pp. 1077-1078, DOI: 10.1016/j.jchromb.2018.01.032, 2018.
- [9] D. Ren, "The significance and function of fingerprint quality control technology of Traditional Chinese medicine," *Journal of Chinese Medicinal Materials*, vol. 24 no. 4, pp. 235-239, 2001.
- [10] Y. Chen, H. Yu, H. Wu, "Tracing novel hemostatic compounds from heating products of total flavonoids in *Flos Sophorae* by spectrum-effect relationships and column chromatography," *Journal of Separation Science*, vol. 38 no. 10, pp. 1691-1699, DOI: 10.1002/jssc.201500100, 2015.
- [11] X. Zhang, J. Chen, J. Yang, Y. Shi, "UPLC-MS/MS analysis for antioxidant components of *Lycii Fructus* based on spectrum-effect relationship," *Talanta*, vol. 180, pp. 389-395, DOI: 10.1016/j.talanta.2017.12.078, 2018.
- [12] S. Xiao, *Study on Identification Method of Bacteriostatic Substances of Iron Amaranth Based on Spectral Effect Relationship*, 2013.
- [13] X. Li, "Spectrum-effect relationship of choleric effect of *Gardeniae fructus* extract based on principal component analysis and gray correlation analysis," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 26 no. 14, pp. 135-141, 2020.
- [14] Q. Wang, X. Wu, X. Yang, "Comprehensive quality evaluation of *Lignum caraganae* and rapid discrimination of *Caragana jubata* and *Caragana changduensis* based on characteristic compound fingerprints by HPLC-UV and HPLC-MS/MS coupled with chemometrics analysis," *Phytochem Analysis*, vol. 31 no. 6, pp. 846-860, DOI: 10.1002/pca.2950, 2020.
- [15] X. Ping, S. Dan, W. Renjie, L. Gonghua, W. Nani, "Study on the spectrum-effect relationship between GC-MS fingerprint and antioxidant activity of volatile oil from *atractylodis Macrocephalae rhizoma*," *Chinese Journal of Modern Applied Pharmacy*, vol. 36 no. 11, pp. 1322-1327, 2019.
- [16] Z. Chunsheng, *Study on the Material Basis and Quality Evaluation of Cichorium intybus for Lowering Uric Acid Based on Spectrum-Effect Relationship*, 2016.
- [17] X. Yan, Y. Yongshou, X. Peiyun, "Effect of *Periplaneta americana* anti - pulmonary fibrosis active site on pulmonary fibrosis rats and its spectral - effect relationship," *Journal of Chinese Medicinal Materials*, vol. 42 no. 1, pp. 196-201, 2019.
- [18] Y. Cao, S. Fang, Z. Yin, "Chemical fingerprint and multicomponent quantitative analysis for the quality evaluation of *cyclocarya paliurus* leaves by HPLC-Q-TOF-MS," *Molecules (Basel, Switzerland)*, vol. 22 no. 11, DOI: 10.3390/molecules22111927, 2017.
- [19] W. J. Du, J. Ji, L. Wang, "Relationship between the UPLC-Q-TOF-MS fingerprinted constituents from *Daphne genkwa* and their anti-inflammatory, anti-oxidant activities," *Biomedical Chromatography: BMC*, vol. 31 no. 12, DOI: 10.1002/bmc.4012, 2017.
- [20] F. Cheung, "TCM: made in China," *Nature*, vol. 480 no. 7378, pp. S82-S83, DOI: 10.1038/480s82a, 2011.
- [21] S. P. Li, J. Zhao, B. Yang, "Strategies for quality control of Chinese medicines," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 55 no. 2011, pp. 802-809, 2011.
- [22] Y. Jiang, B. David, P. Tu, Y. Barbin, "Recent analytical approaches in quality control of traditional Chinese medicines-a review," *Analytica Chimica Acta*, vol. 657 no. 1, DOI: 10.1016/j.aca.2009.10.024, 2010.
- [23] R. Länger, E. Stöger, W. Kubelka, K. Helliwell, "Quality standards for herbal drugs and herbal drug preparations - appropriate or improvements necessary?," *Planta Medica*, vol. 84 no. 6-7, pp. 350-360, DOI: 10.1055/s-0043-118534, 2018.
- [24] C. Zhang, X. Zheng, H. Ni, P. Li, H.-J. Li, "Discovery of quality control markers from traditional Chinese medicines by fingerprint-efficacy modeling: current status and future perspectives," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 159, pp. 296-304, DOI: 10.1016/j.jpba.2018.07.006, 2018.
- [25] Z. Zhen, "Study on HPLC fingerprint and index components of *Radix glycyrrhizae*," *Journal of Sichuan of*

Traditional Chinese Medicine, vol. 36 no. 12, pp. 178-180, 2018.

[26] S. Yu, L. Zhu, Z. Xiao, "Rapid fingerprint analysis of flos carthami by ultra-performance liquid chromatography and similarity evaluation," Journal of Chromatographic Science, vol. 54 no. 9, pp. 1619-1624, DOI: 10.1093/chromsci/bmw115, 2016.

[27] L. Zhu, Z. T. Liang, T. Yi, "Comparison of chemical profiles between the root and aerial parts from three Bupleurum species based on a UHPLC-QTOF-MS metabolomics approach," BMC Complementary & Alternative Medicine, vol. 17 no. 1, DOI: 10.1186/s12906-017-1816-y, 2017.

[28] H. Xu, L. Ke, Y. Chen, "Study on the absorbed fingerprint-efficacy of yuanhu zhitong tablet based on chemical analysis, vasorelaxation evaluation and data mining," PLoS One, vol. 8 no. 12, DOI: 10.1371/journal.pone.0081135, 2013.

[29] Q. Zhang, Q.-G. Ru, H. M. Lin, "Correlation between chemical constituents and anti-inflammatory activity of distillates separated from essential oil of Angelica sinensis Radix by molecular distillation based on PLSR," Global Traditional Chinese Medicine, vol. 8 no. 10, pp. 1153-1158, 2015.

[30] C. Zhao, X. Qiao, Q. Shao, M. Hassan, Z. Ma, "Evolution of the lignin chemical structure during the bioethanol production process and its inhibition to enzymatic hydrolysis," Energy & Fuels, vol. 34 no. 5, pp. 5938-5947, DOI: 10.1021/acs.energyfuels.0c00293, 2020.

DETAILS

Subject:	Mass spectrometry; Quality standards; Research methodology; Acids; Quality control; Spleen; Traditional Chinese medicine; Scientific imaging; Fingerprints; Regression analysis; Saponins; Correlation analysis; Chromatography; High performance liquid chromatography; Chinese medicine; Ultrasonic imaging; Ethanol
Business indexing term:	Subject: Quality standards Quality control
Location:	China; Inner Mongolia China
Editor:	David Touboul
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779

Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-12-29 (Received); 2021-03-05 (Revised); 2021-03-09 (Accepted); 2021-03-17 (Pub)
DOI:	https://doi.org/10.1155/2021/6617033
ProQuest document ID:	2506105875
Document URL:	https://www.proquest.com/scholarly-journals/study-on-spectrum-effect-relationship-traditional/docview/2506105875/se-2?accountid=211160
Copyright:	Copyright © 2021 Wenxin Xia et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-04-20
Database:	Publicly Available Content Database

Document 44 of 59

A Novel Nonenzymatic Hydrogen Peroxide Sensor Based on Magnetic Core-Shell Fe_3O_4 @C/Au Nanoparticle Nanocomposite

Ni, Xiao

; Tian, Mingwei; Sun, Jun; Chen, Xiaojun

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Fe_3O_4 @C/Au nanoparticle (AuNP) nanocomposites were prepared through electrostatic adsorption of AuNPs onto PDPA-functionalized core/shell Fe_3O_4 @C magnetic nanospheres, which had been synthesized by a facile solvothermal method. The morphology and composition of the nanocomposites were characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier transform infrared (FT-IR), etc. Moreover,

highly electrocatalytic activity to the reduction of hydrogen peroxide (H_2O_2) was also exhibited on the Fe_3O_4 @C/AuNP-modified indium tin oxide (ITO) electrode. The effect of solution pH and the modification amount of Fe_3O_4 @C/AuNPs on the performance of electrocatalytic H_2O_2 reduction was investigated. Under the optimal conditions, the catalytic current showed a linear relationship with the increase of H_2O_2 concentration in the range of 0.007–15 mM and a detection limit of 5 μM . The H_2O_2 sensor showed high selectivity for H_2O_2 detection, which could effectively resist the interference of ascorbic acid (AA), uric acid (UA), and citric acid (CA). Finally, the H_2O_2 sensor was used in the real fetal bovine serum to detect H_2O_2 and obtained satisfactory results with the recovery values ranging from 95.14 to 103.6%.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Magnetic nanoparticles have been popularly applied in anode material [1], degradation [2], electromagnetic wave absorption [3], separation [4], and catalysis [5]. Among these diverse magnetic nanomaterials reported before, Fe_3O_4 NPs have been studied extensively because of their excellent magnetism, biocompatibility, and especially, highly effective electrocatalytic activity. For example, Kingsley et al. fabricated Fe_3O_4 nanoparticle- (NP-) modified carbon paste electrode to detect ascorbic acid (AA) and folic acid (FA) at the same time, with the detection limits of 2.51×10^{-7} and 2.01×10^{-9} M, respectively [6]. Qu and coworkers established a novel hydrogen peroxide (H_2O_2) sensor using Fe_3O_4 NPs/chitosan composite-modified electrode, which could electrocatalyze the reduction of H_2O_2 with a detection limit of 1.53×10^{-6} M [7]. However, Fe_3O_4 NPs are unstable and prone to be aggregated and settled in aqueous solution because of the magnetic dipolar-dipolar attraction, resulting in the decrease of their catalytic activity [8]. To overcome this problem, considerable efforts have been devoted to introduce stabilizers on the surface of Fe_3O_4 NPs.

In recent years, core/shell structured magnetic nanocomposites have been proposed, with Fe_3O_4 NPs as the core and inorganic materials or polymers as the shell. These specific core/shell Fe_3O_4 materials possess enhanced stability and compatibility with different charges, functions, or reactive moieties. Owing to the enlarged effective surface area and the synergetic interaction between core and shell, these nanocomposites usually combine the advantageous properties of both core and shell [9]. For example, Beitollahi and coworkers synthesized a carbon paste electrode modified with magnetic Fe_3O_4 @ SiO_2 /MWCNT nanocomposite and found it exhibiting a very good resolution between the voltammetric peaks of hydrazine and phenol, which made it suitable for the detection of hydrazine in the presence of phenol in real samples [10]. Wang and coworkers showed that the Fe_3O_4 @ZnO nanoparticles (NPs) have excellent photocatalytic activity and it is convenient to reuse NPs in wastewater treatment owing to the magnetic Fe_3O_4 core [11]. Therefore, magnetic core/shell nanocomposites with multiple properties have been widely used.

Among these shell materials, carbon and gold are the most applied coating materials due to their excellent conductivity and biocompatibility [12, 13]. In addition, on the surface of carbon shells, abundant amounts of hydroxyl (-OH) and carboxyl (-COOH) groups can effectively protect the Fe_3O_4 NPs from aggregation and facilitate further functional modification over the surface. Hence, the applicability of Fe_3O_4 @C has been extensively promoted. For example, Zhang and coworkers synthesized Fe_3O_4 @C NPs, which showed dramatic ability to remove methylene blue, with a maximum adsorption of $141.3 \text{ mg} \cdot \text{g}^{-1}$ [14]. Zhang and coworkers fabricated another Fe_3O_4 @C NPs immobilized upon the GCE which was modified with molecular imprinted TiO_2 velamen. It was then made into the uric acid sensor, which delivered a linear range of 0.3–34 μM with a detection limit of 0.02 μM [15]. Meanwhile, functionalized Fe_3O_4 @Au NPs have also been applied in As (III) [16] and dopamine [17] sensors, respectively. In this work, Fe_3O_4 @C core/shell nanocomposite was synthesized by a facile solvothermal method from ferrocene as the raw material. It was not only easily separated by an external magnetic field but also possessed excellent dispersion in water. To further enhance the conductivity and catalytic activity, Au NPs were adsorbed on the surface

of the obtained $\text{Fe}_3\text{O}_4@\text{C}$ nanospheres by electrostatic attraction. The synthesized $\text{Fe}_3\text{O}_4@\text{C}/\text{Au}$ NPs were first used to fabricate a nonenzymatic H_2O_2 sensor and showed high electrochemical sensitivity.

2. Materials and Methods

2.1. Materials

Ferrocene, acetone, sodium citrate, absolute ethanol, sodium chloride (NaCl), and Tris base were purchased from Shanghai Lingfeng Chemical Reagent (China). Poly(diallyldimethylammonium chloride) (PDDA, 20 wt. %) was purchased from Aldrich. Chloroauric acid hydrated ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) and sodium citrate were purchased from Nanjing Chemical Reagent (China). H_2O_2 (30%) was obtained from Sinopharm Chemical Reagent. Phosphate buffer saline (PBS, 0.1 M) was prepared by mixing the stock solutions of KH_2PO_4 and K_2HPO_4 and was adjusted to the appropriate pH value. Uric acid (UA), AA, and citric acid (CA) were all purchased from Sigma. Nafion (Nf) was obtained from DuPont as 5 wt% solution and was used as 0.5 wt% solution after dilution with water. Other reagents were of analytical grade and used without further purification. Double distilled water (DDW) was used throughout the experiments.

2.2. Characterization

The structures and morphologies of the $\text{Fe}_3\text{O}_4@\text{C}$ and $\text{Fe}_3\text{O}_4@\text{C}/\text{Au}$ NPs were characterized by transmission electron microscopy (TEM, JEOL JEM-200CX) and field emission scanning electron microscopy (FESEM, Hitachi S4800). Fourier transform infrared (FT-IR) spectra of KBr powder-pressed pellets were recorded on a Bruker model VECTOR 22 Fourier transform spectrometer. Elemental composition and chemical valence analyses were performed on X-ray diffraction (XRD, Rigaku SmartLab) and X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha), respectively.

Cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) were performed using a CHI 660D electrochemical workstation (Shanghai Chenhua Instruments, China). A three-compartment electrochemical cell contained a saturated calomel reference electrode (SCE), a platinum wire auxiliary electrode, and a modified ITO working electrode with a 3 mm diameter controlled area. The determination of H_2O_2 was conducted in 0.1 M PBS, and the sensor responses were measured under deductions for background currents from the total ones.

2.3. Preparation of $\text{Fe}_3\text{O}_4@\text{C}/\text{Au}$ NP Magnetic Nanocomposite

Core/shell structured $\text{Fe}_3\text{O}_4@\text{C}$ magnetic nanocomposite was prepared by Wang's method [18]. First, 0.3 g of ferrocene was dissolved in 30 mL of acetone under ultrasonication at room temperature for 30 min. Then, 1 mL of H_2O_2 was added dropwise into the above ferrocene solution. After reacting for 15 min under stirring, the resulting solution was transferred into a Teflon-lined stainless-steel autoclave with a capacity of 50 mL and heated at 210°C for 24 h. After cooling to room temperature, the product was magnetically separated, washed several times with absolute ethanol, and finally dried under vacuum at 50°C for 5 h.

Au colloid solution was prepared by adding 5 mL of 1% (wt. %) sodium citrate into 200 mL of 0.01% (wt. %) HAuCl_4 , which had been heated to boil beforehand. Heating was stopped by the change of solution color from light blue to violet-red and stirring was continued for 15 min. After cooling to room temperature, Au colloid solution was preserved in a refrigerator at 4°C . Generally, an electrostatic attraction method was used for the immobilization of AuNPs onto the surface of the $\text{Fe}_3\text{O}_4@\text{C}$ core/shell nanocomposite. 50 mg of $\text{Fe}_3\text{O}_4@\text{C}$ nanocomposite was dispersed into 30 mL of 0.2% PDDA that contained 20 mM Tris base and 20 mM NaCl, and the mixture was stirred for 20 min. The product was collected with the help of a magnet and rinsed with DDW six times to remove the residual PDDA. The positively charged $\text{Fe}_3\text{O}_4@\text{C}$ nanocomposite was dispersed in 100 mL of Au colloid solution and stirred for 8 h. The suspension was separated with the help of a magnet. After rinsed with water and ethanol three times successively, the $\text{Fe}_3\text{O}_4@\text{C}/\text{Au}$ NPs nanocomposite was dried under vacuum at 50°C for 6 h.

2.4. Fabrication of $\text{Fe}_3\text{O}_4@\text{C}/\text{Au}$ NP-Modified ITO Magnetism Electrode

Before modification, the ITO chips were cleaned with acetone, ethanol, and water, respectively, and then dried under a stream of nitrogen. The active electrode area was confined by bounding a circular opened 3M polyvinyl chloride (PVC) film on the ITO surface, with a 3 mm diameter on the middle of the electrode. On the other side of the

ITO, a piece of 8mm diameter and 5mm height permanent magnet was adhered by silica rubber adhesive [19]. Then, 10 μL of 1 $\text{mg}\cdot\text{mL}^{-1}$ $\text{Fe}_3\text{O}_4\text{@C/AuNP}$ nanocomposite dispersion (in 5mL 0.5% Nf) was dropped onto the surface of the ITO magnetism electrode and dried at room temperature, which was designated as $\text{Fe}_3\text{O}_4\text{@C/AuNPs/Nf/ITO}$. When the sensor was not in use, it was stored at 4°C in a refrigerator.

3. Results and Discussion

3.1. Structure and Composition Characterization of Samples

The structure and morphology of the nanocomposites were investigated by TEM and FESEM. In Figure 1(a), $\text{Fe}_3\text{O}_4\text{@C}$ with obvious core/shell structures were successfully synthesized. The presence of carbon shells prevented agglomeration of the Fe_3O_4 NPs and improved the dispersity of the $\text{Fe}_3\text{O}_4\text{@C}$, with an average diameter of approximately 125nm. As vividly shown in Figure 1(b), the SEM image of $\text{Fe}_3\text{O}_4\text{@C}$ displays a distinct margin without agglomeration. Figure 1(c) shows the TEM image of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ with a diameter of 141nm. It is clear that there are numerous dark spots embedded in the outer shells, indicating that AuNPs were successfully immobilized on the surface of the $\text{Fe}_3\text{O}_4\text{@C}$ nanospheres, with an average diameter of approximately 15nm. Figure 1(d) shows the SEM image of the rather rough surface of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ with a raspberry-shaped morphology compared to that of $\text{Fe}_3\text{O}_4\text{@C}$. Moreover, some of them are overlapped or amalgamated due to external immobilization of AuNPs, which amplify the superficial roughness. And the dispersity of the $\text{Fe}_3\text{O}_4\text{@C}$ nanocomposites is weakened by electrostatic attraction between AuNPs and $\text{Fe}_3\text{O}_4\text{@C}$ as well. The inset in Figure 1(d) shows the elemental composition of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ and the Au atom mass concentration is 3.57%.

[figures omitted; refer to PDF]

FT-IR was used to characterize the chemical structure of the samples. Figure 2(a) shows the FT-IR spectra of Fe_3O_4 (curve a), $\text{Fe}_3\text{O}_4\text{@C}$ (curve b), and $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ (curve c). The characteristic peak appears at 590cm^{-1} which is related to the Fe-O stretching vibration [20]. Compared with curve a, there is a new strong band in curve b at around 3446cm^{-1} , indicating the presence of -OH in the carbon shell. These phenomena further proved that the carbon shell is successfully coated on the surface of the Fe_3O_4 nanosphere. The peak located at 1399cm^{-1} is attributed to the COO- symmetric vibration [21]. Actually, the carboxyl group-functionalized $\text{Fe}_3\text{O}_4\text{@C}$ nanospheres are negatively charged. This is the reason why we use PDDA to make the surface of carbon shell positively charged for the assembly of negatively charged AuNPs via electrostatic interaction. Curve c shows the FT-IR spectrum of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$. Because AuNPs do not have absorption in the IR region, the characteristic peak of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ is almost the same as that of $\text{Fe}_3\text{O}_4\text{@C}$.

[figures omitted; refer to PDF]

To characterize the crystal information of the prepared $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ further, the XRD has been performed. As shown in Figure 2(b), the diffraction peaks appeared at 30° , 35.5° , 43° , 57° , and 62° are originated from Fe_3O_4 , corresponding to (220), (311), (400), (511), and (440) crystal planes [22]. In addition to those peaks, another peak at 38.2° is in good accordance with (111) plane of AuNPs. It is difficult to discriminate Fe_3O_4 phases from $\gamma\text{-Fe}_2\text{O}_3$ by only using XRD patterns due to the similarity of patterns, and XPS is conducted to characterize the electronic structures of Fe element in the nanocomposite, which is known to be sensitive to Fe^{2+} and Fe^{3+} cations. Figure 2(c) presents two peaks at the binding energies of 724.4 and 711.4eV, which correspond to the maximum intensity of Fe 2p_{1/2} and Fe 2p_{3/2}, illustrating the coexistence of Fe^{2+} and Fe^{3+} cations in $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ [23]. Peaks of Au are also shown in Figure 2(d), corresponding to Au 4f_{7/2} and Au 4f_{5/2}, respectively. From the above characterization results, the well-defined core/shell structured $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ is confirmed to be constructed.

3.2. Characterization of Nanocomposite-Modified ITO Electrodes

The influence of potential scan rate (v) on the response of the $\text{Fe}_3\text{O}_4\text{@C/AuNP}$ -modified ITO electrode in PBS was studied, as shown in Figure 3(a). The reduction and oxidation peak currents (i_{pa} and i_{pc}) increased linearly with the square root of scan rate ($v^{1/2}$) accelerating from 40 to $300\text{mV}\cdot\text{s}^{-1}$, indicating a surface-controlled electrochemical process.

[figures omitted; refer to PDF]

As shown in Figure 3(b), we also recorded the CVs of various modified electrodes towards 0.1 $\text{mM H}_2\text{O}_2$ in 0.1M pH

7.4 PBS at a scan rate of $100\text{mV}\cdot\text{s}^{-1}$. At the bare ITO, almost no peak of H_2O_2 is observed (curve a), except for a slight reduction peak. After the electrode was modified with $\text{Fe}_3\text{O}_4@\text{C}$ nanocomposites, a small increase in i_{pa} was observed (curve b), indicating the electrocatalytic reduction activity toward H_2O_2 possessed by $\text{Fe}_3\text{O}_4@\text{C}$ nanocomposites. The reduction peak potential was around -0.35V , similar to another reported nonenzymatic H_2O_2 sensor [24] and the i_{pa} was 1.7 times higher compared with that in curve a. When the $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ were assembled on the electrode, the peak current significantly increased (curve c) and the i_{pa} is 2.4 times that of curve b, demonstrating that the AuNPs significantly improve the catalytic activity of $\text{Fe}_3\text{O}_4@\text{C}$ nanocomposites.

The electron transfer rate and the interfacial resistance, as shown in Figures 4(a) and 4(b), respectively, can reflect the electrochemical properties. In Figure 4(a), obvious oxidation and reduction peaks were observed on both $\text{Fe}_3\text{O}_4@\text{C}$ - and $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNP}$ -modified electrodes, belonging to different valences of Fe element. An increased peak current was found after surface modification with AuNPs, indicating that noble metals could promote electron transfer on the electrode. In addition, the electroactive surface area (ESA) was calculated to be 1.628 cm^2 by Randles–Sevcik equation, which is nearly 23 times the geometric area. It means that the modification of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ increased the roughness of the electrode surface greatly, which could facilitate the absorption, transmission, and catalysis of H_2O_2 .

[figures omitted; refer to PDF]

Similar results were also confirmed by EIS measurements, as shown in Figure 4(b). The diameter of the Nyquist curve revealed charge transfer resistance of the electrode (R_{ct}), and a larger diameter means a greater resistance. Curve a presented a smaller R_{ct} value at $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNP}$ -modified ITO, verifying AuNPs could enhance charge transfer significantly, compared with that in curve b.

3.3. Optimization of Experimental Parameters

The variations of oxidation-reduction potential of H_2O_2 in different pH values indicate diverse oxidation ability of H_2O_2 , resulting in different sensitivities of sensors under different pH circumstances. The effect of solution pH on the electrocatalytic reduction of H_2O_2 was investigated. Figure 5(a) shows the linear plots of the reduction peak current versus the concentration of H_2O_2 from 0.1 to 1 mM over the pH range from 6 to 9. The electrocatalytic activity and sensitivity were much higher at the pH value of 7–8 and lower at 6 and 9. The possible reason was that Fe_3O_4 core was expected to dissolve in acidic circumstances and the catalytic activity faded. Meanwhile, H_2O_2 is prone to lose oxidizing ability with solution alkalinity increasing. Therefore, a nearly neutral condition is suitable for H_2O_2 detection and the PBS with physiologic pH value of human being which is about 7.4 was chosen as the supporting electrolyte.

[figures omitted; refer to PDF]

Figure 5(b) exhibits reduction peak current versus different modification amounts of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$. The response current towards 1 mM H_2O_2 rose along with the modification amount of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ increasing from 0.6 to 1 $\text{mg}\cdot\text{mL}^{-1}$ and reached a plateau when the modification amount exceeded 1 $\text{mg}\cdot\text{mL}^{-1}$. The response speed was supposed to be delayed by thickening modification, which brought hidden troubles of its falling off from the electrode surface. It may lead to a decreased stability of the modified electrode and the smaller response current. In this way, 1 $\text{mg}\cdot\text{mL}^{-1}$ of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ was regarded to be the optimal amount of modification for H_2O_2 detection.

3.4. Amperometric Response of H_2O_2 Sensor

Figure 6 displays CVs in the presence of H_2O_2 at different concentrations under the optimum conditions. The response current shows a good linear relationship with the increase of H_2O_2 concentration in the range of 0.007–15 mM. The linear regression equations is $\Delta i_p (\mu\text{A}) = 0.10 + 2.01 c (\text{H}_2\text{O}_2) (\text{mM})$ ($R^2 = 0.9988$). The detection limit was estimated at $5\mu\text{M}$, and the theoretical sensitivity of this sensor was calculated to be $28.71\mu\text{A}\cdot\text{mM}^{-1}\cdot\text{cm}^{-2}$. The response current became saturated when the concentration of H_2O_2 exceeded 15 mM. Quantitative detection of H_2O_2 concentration can be realized by the calculation with the linear equation. Furthermore, the performances including electrode modification, linear range, and detection limit of the $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}/\text{ITO}$ were compared with those of other H_2O_2 sensors, which are listed in Table 1. It was shown that the proposed H_2O_2 sensor in this work exhibited a wider linear range and a relatively lower detection limit. In comparison with those previous works, the advantages of our work are ascribed to five aspects: (1) the combination of the magnetic core of Fe_3O_4 , thin layer of C, and

uniformly dispersed AuNP modification shows their synergies and thus enhances the catalytic ability towards H_2O_2 ; (2) the C layer prevents Fe_3O_4 catalyst from aggregating and maintains its catalytic activity; (3) the utilization of magnetic electrode increases the modification amount of $\text{Fe}_3\text{O}_4\text{@C/AuNPs}$ and enhances the stability of the electrode modification; (4) the C layer is not compact, which facilitate H_2O_2 and the reaction products transferring between the bulk solution and electrode surface; and (5) the modification of AuNPs enhances the electron transfer and electrochemical activity of the H_2O_2 sensor.

[figures omitted; refer to PDF]

Table 1

The performance comparison of different nonenzymatic H_2O_2 sensors.

Electrode modification	Linear range (mM)	Detection limit (μM)	Ref.
$\text{Co}_3\text{O}_4/\text{MWCNTs}$	0.02–0.43	2.46	[25]
PtAu	0.05–1.15	4.1	[26]
Pd/CNT	0.008–9.5	2.6	[27]
AgCo/MWCNTs	0.05–10	0.74	[28]
NPG@Ni	0.02–9.74	10	[29]
Se/PtNPs	0.01–1.5	3.1	[30]
Ag/NaX	0.02–11.76	9.1	[31]
Ag NW array	0.1–3.1	29.2	[32]
AgNPs	0.05–6.5	27	[33]
$\text{Fe}_3\text{O}_4\text{@C/CuNPs}$	0.08–372	32.6	[34]
$\text{Fe}_3\text{O}_4\text{@C/AuNPs}$	0.007–15	5	This work

3.5. Stability and Repeatability

The application of chemical sensors put forward higher demands on stability and catalytic signal repeatability. DPV and EIS measurements for $\text{Fe}_3\text{O}_4\text{@C/AuNP}$ -modified ITO were conducted in 0.1 M of pH 7.4 PBS containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. As shown in Figure 7, it was found that the DPV peak remained nearly unchanged after 10 cycles of scanning. On the other hand, the Nyquist plots nearly overlapped for 10 times of detection, and the R_{ct} value maintained around 152 Ω . It demonstrated that the immobilized $\text{Fe}_3\text{O}_4\text{@C/AuNP}$ modification layer could keep a good level of stability in a continuous scanning system.

[figures omitted; refer to PDF]

In addition, a repeated measurement towards 1 mM H_2O_2 in 0.1 M pH 7.4 PBS was conducted to verify the repeatability of the sensor. The response signal recorded on 5 parallel electrodes is displayed in Figure 7(c), and the relative standard deviation (RSD) value between them was calculated as 1.8%. The good stability and repeatability of the fabricated H_2O_2 sensor are attributed to the simple modification method, stable property, and good catalytic

ability of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$.

3.6. Interference Study

To evaluate the selectivity of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$, an interference study was carried out. As it is known, AA, UA, and CA are the most common interfering electroactive species during the detection of H_2O_2 . The current responses with the addition of H_2O_2 , AA, UA, and CA at the potential of -0.35V are shown in Figure 8, among which the responses caused by AA, UA, and CA interfering species were no more than 10% response of H_2O_2 . The result implied that the $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ possessed high selectivity for H_2O_2 detection at the potential of -0.35V .

[figure omitted; refer to PDF]

3.7. Determination of H_2O_2 in Fetal Bovine Serum (FBS)

The determination of H_2O_2 in FBS was also investigated. The FBS was diluted with 0.1M of $\text{pH } 7.4$ PBS via the volume ratio of $1:9$. We measured the recovery by adding different concentrations of H_2O_2 into the FBS dilution. The recovery values were calculated ranging from 95.14 to 103.6% , which are presented in Table 2. The relative standard deviations (RSDs) are less than 5% , proving a good application prospect.

Table 2

The recovery of H_2O_2 by standard addition method in FBS samples.

Sample	Added concentration (mM)	Measured concentration (mM)	Recovery (%)
1	0.094	0.09	95.14
2	0.28	0.29	103.6
3	0.47	0.48	102.1
4	0.95	0.94	98.95
5	4.54	4.62	101.8

4. Conclusion

In summary, a novel core-shell structured $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ nanocomposite is successfully synthesized and utilized for fabricating a nonenzymatic H_2O_2 sensor, exhibiting a wider linear range from 0.007 to 15mM and a lower detection limit of $5\mu\text{M}$ with a sensitivity of $28.71\mu\text{A}\text{mM}^{-1}\text{cm}^{-2}$. The advantages of the proposed sensor over the previous works are concluded as four points: (1) the superparamagnetic property of $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ nanocomposite contributes to the construction of a magnetic electrode modification, enhancing the stability of the sensor greatly. (2) The protection of C/AuNPs bilayer ensures the monodispersity of Fe_3O_4 catalyst, maintaining its good catalytic activity for H_2O_2 reduction. (3) The C/AuNPs bilayer possesses hydrophilicity, biocompatibility, electroactivity, and conductivity, facilitating the substance transmission and electron transfer between bulk solution and electrode surface. (4) The detection of H_2O_2 in FBS illustrates high selectivity, displaying good potential for real applications. Of course, the fabricated $\text{Fe}_3\text{O}_4@\text{C}/\text{AuNPs}$ nanocomposite could also be used as substrate or nanolabel in other sensing systems.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (21575064), the Six Talent Peaks Project in Jiangsu Province (2016-SWYY-022), and the Qinglan Project of Jiangsu Education Department (2016).

References

[1] C.-L. Liang, Y. Liu, R.-Y. Bao, "Effects of Fe_3O_4 loading on the cycling performance of $\text{Fe}_3\text{O}_4/\text{rGO}$ composite anode material for lithium ion batteries," *Journal of Alloys and Compounds*, vol. 678, pp. 80-86, DOI:

10.1016/j.jallcom.2016.03.274, 2016.

[2] H. Y. Aziz, S. G. Maryam, "Fe₃O₄/ZnO/Ag₃VO₄/AgI nanocomposites: quaternary magnetic photocatalysts with excellent activity in degradation of water pollutants under visible light," *Separation and Purification Technology*, vol. 166, pp. 63-72, 2016.

[3] Y. Wang, Y. Lai, S. Wang, W. Jiang, "Controlled synthesis and electromagnetic wave absorption properties of core-shell Fe₃O₄@SiO₂ nanospheres decorated graphene," *Ceramics International*, vol. 43 no. 2, pp. 1887-1894, DOI: 10.1016/j.ceramint.2016.10.148, 2017.

[4] A. M. Muliwa, T. Y. Leswif, M. S. Onyango, A. Maity, "Magnetic adsorption separation (MAS) process: an alternative method of extracting Cr(VI) from aqueous solution using polypyrrole coated Fe₃O₄ nanocomposites," *Separation and Purification Technology*, vol. 158, pp. 250-258, DOI: 10.1016/j.seppur.2015.12.021, 2016.

[5] J. Chang, Q. Ma, J. Ma, H. Ma, "Synthesis of Fe₃O₄ nanowire@CeO₂/Ag nanocomposites with enhanced photocatalytic activity under sunlight exposure," *Ceramics International*, vol. 42 no. 10, pp. 11827-11837, DOI: 10.1016/j.ceramint.2016.04.104, 2016.

[6] M. P. Kingsley, P. B. Desai, A. K. Srivastava, "Simultaneous electro-catalytic oxidative determination of ascorbic acid and folic acid using Fe₃O₄ nanoparticles modified carbon paste electrode," *Journal of Electroanalytical Chemistry*, vol. 741, pp. 71-79, DOI: 10.1016/j.jelechem.2014.12.039, 2015.

[7] J. Qu, Y. Dong, T. Lou, X. Du, "Determination of hydrogen peroxide using a novel sensor based on Fe₃O₄ Magnetic nanoparticles," *Analytical Letters*, vol. 47 no. 11, pp. 1797-1807, DOI: 10.1080/00032719.2014.888733, 2014.

[8] J. Xie, H. Cao, H. Jiang, "Co₃O₄-reduced graphene oxide nanocomposite as an effective peroxidase mimetic and its application in visual biosensing of glucose," *Analytica Chimica Acta*, vol. 796, pp. 92-100, DOI: 10.1016/j.aca.2013.08.008, 2013.

[9] R. Tian, X. Chen, X. Xu, C. Yao, "Electrocatalytic activity of core/shell magnetic nanocomposite," *Analytical Biochemistry*, vol. 463, pp. 45-53, DOI: 10.1016/j.ab.2014.06.019, 2014.

[10] H. Beitollahi, S. Tajik, S. Jahani, "Electrocatalytic determination of hydrazine and phenol using a carbon paste electrode modified with Ionic Liquids and Magnetic Core-shell Fe₃O₄@SiO₂/MWCNT Nanocomposite," *Electroanalysis*, vol. 28 no. 5, pp. 1093-1099, DOI: 10.1002/elan.201501020, 2016.

[11] J. Wang, J. Yang, X. Li, "Preparation and photocatalytic properties of magnetically reusable Fe₃O₄@ZnO core/shell nanoparticles," *Physica E: Low-Dimensional Systems and Nanostructures*, vol. 75, pp. 66-71, DOI: 10.1016/j.physe.2015.08.040, 2016.

[12] A. Orhan, C. Aykut, K. Hilal, S. Ozlem, "Dendrimer templated synthesis of carbon nanotube supported PdAu catalyst and its application as hydrogen peroxide sensor," *Electroanalysis*, vol. 31, pp. 1646-1655, 2019.

[13] C. K. Hilal, S. Firat, C. Aykut, K. Hilal, A. Nahit, "Synthesis, characterization, and voltammetric hydrogen peroxide sensing on novel monometallic (Ag, Co/MWCNT) and bimetallic (AgCo/MWCNT) alloy nanoparticles," *Fullerenes, Nanotubes and Carbon Nanostructures*, vol. 26, pp. 145-151, 2018.

[14] W. Zhang, L. Y. Zhang, X. J. Zhao, Z. Zhou, "Citrus pectin derived ultrasmall Fe₃O₄@C nanoparticles as a high-performance adsorbent toward removal of methylene blue," *Journal of Molecular Liquids*, vol. 222, pp. 995-1002, DOI: 10.1016/j.molliq.2016.07.144, 2016.

[15] C. Zhang, S. Si, Z. Yang, "A highly selective photoelectrochemical biosensor for uric acid based on core-shell Fe₃O₄@C nanoparticle and molecularly imprinted TiO₂," *Biosensors and Bioelectronics*, vol. 65, pp. 115-120, DOI: 10.1016/j.bios.2014.10.013, 2015.

[16] J. Wei, S.-S. Li, Z. Guo, X. Chen, J.-H. Liu, X.-J. Huang, "Adsorbent assisted in situ electrocatalysis: an ultra-sensitive detection of As(III) in water at Fe₃O₄ nanosphere densely decorated with Au nanoparticles," *Analytical Chemistry*, vol. 88 no. 2, pp. 1154-1161, DOI: 10.1021/acs.analchem.5b02947, 2016.

[17] J.-D. Qiu, M. Xiong, R.-P. Liang, H.-P. Peng, F. Liu, "Synthesis and characterization of ferrocene modified Fe₃O₄@Au magnetic nanoparticles and its application," *Biosensors and Bioelectronics*, vol. 24 no. 8, pp. 2649-2653, DOI: 10.1016/j.bios.2009.01.022, 2009.

- [18] H. Wang, Q.-W. Chen, Y.-F. Yu, K. Cheng, Y.-B. Sun, "Size- and solvent-dependent magnetically responsive optical diffraction of carbon-encapsulated superparamagnetic colloidal photonic crystals," *The Journal of Physical Chemistry C*, vol. 115 no. 23, pp. 11427-11434, DOI: 10.1021/jp201893z, 2011.
- [19] X. Chen, J. Zhu, Z. Chen, C. Xu, Y. Wang, C. Yao, "A novel bienzyme glucose biosensor based on three-layer Au-Fe₃O₄@SiO₂ magnetic nanocomposite," *Sensors and Actuators B: Chemical*, vol. 159 no. 1, pp. 220-228, DOI: 10.1016/j.snb.2011.06.076, 2011.
- [20] Q. Gao, F. Chen, J. Zhang, "The study of novel Fe₃O₄@ γ -Fe₂O₃ core/shell nanomaterials with improved properties," *Journal of Magnetism and Magnetic Materials*, vol. 321 no. 8, pp. 1052-1057, DOI: 10.1016/j.jmmm.2008.10.022, 2009.
- [21] S. Xuan, Y.-X. J. Wang, J. C. Yu, K. C.-F. Leung, "Preparation, characterization, and catalytic activity of core/shell Fe₃O₄@polyaniline@Au nanocomposites," *Langmuir*, vol. 25 no. 19, pp. 11835-11843, DOI: 10.1021/la901462t, 2009.
- [22] J. Gong, K. Yao, J. Liu, "Striking influence of Fe₂O₃ on the "catalytic carbonization" of chlorinated poly(vinyl chloride) into carbon microspheres with high performance in the photo-degradation of Congo red," *Journal of Materials Chemistry A*, vol. 1 no. 17, pp. 5247-5255, DOI: 10.1039/c3ta10316a, 2013.
- [23] J. Zhang, Y. Ji, P. Wang, Q. Shao, Y. Li, X. Huang, "Adsorbing and activating N₂ on heterogeneous Au-Fe₃O₄ nanoparticles for N₂ fixation," *Advanced Functional Materials*, vol. 30 no. 4, DOI: 10.1002/adfm.201906579, 2020.
- [24] K. Liao, P. Mao, Y. Li, "A promising method for fabricating Ag nanoparticle modified nonenzyme hydrogen peroxide sensors," *Sensors and Actuators B: Chemical*, vol. 181, pp. 125-129, DOI: 10.1016/j.snb.2013.01.038, 2013.
- [25] H. Heli, J. Pishahang, "Cobalt oxide nanoparticles anchored to multiwalled carbon nanotubes: synthesis and application for enhanced electrocatalytic reaction and highly sensitive nonenzymatic detection of hydrogen peroxide," *Electrochimica Acta*, vol. 123, pp. 518-526, DOI: 10.1016/j.electacta.2014.01.032, 2014.
- [26] J. Wang, H. Gao, F. Sun, C. Xu, "Nanoporous PtAu alloy as an electrochemical sensor for glucose and hydrogen peroxide," *Sensors and Actuators B: Chemical*, vol. 191, pp. 612-618, DOI: 10.1016/j.snb.2013.10.034, 2014.
- [27] C. K. Hilal, C. Aykut, A. Tarik, A. Nahit, K. Hilal, "Microstructured prealloyed Titanium-Nickel powder as a novel nonenzymatic hydrogen peroxide sensor," *Journal of Colloid and Interface Science*, vol. 530, pp. 353-360, 2018.
- [28] D. K. Hilal, A. Tarik, C. Aykut, "Electrochemical production of graphene oxide and its application as a novel hydrogen peroxide sensor," *International Journal of Nano Dimension*, vol. 10, pp. 252-259, 2009.
- [29] X. Ke, Z. Li, L. Gan, "Three-dimensional nanoporous Au films as high-efficiency enzyme-free electrochemical sensors," *Electrochimica Acta*, vol. 170, pp. 337-342, DOI: 10.1016/j.electacta.2015.04.144, 2015.
- [30] Y. Li, J.-J. Zhang, J. Xuan, L.-P. Jiang, J.-J. Zhu, "Fabrication of a novel nonenzymatic hydrogen peroxide sensor based on Se/Pt nanocomposites," *Electrochemistry Communications*, vol. 12 no. 6, pp. 777-780, DOI: 10.1016/j.elecom.2010.03.031, 2010.
- [31] S. N. Azizi, S. Ghasemi, S. Kavian, "Synthesis and characterization of NaX nanozeolite using stem sweep as silica source and application of Ag-modified nanozeolite in electrocatalytic reduction of H₂O₂," *Biosensors and Bioelectronics*, vol. 62, DOI: 10.1016/j.bios.2014.05.070, 2014.
- [32] E. Kurowska, A. Brzózka, M. Jarosz, G. D. Jaskuła, M. Jaskuta, "Silver nanowire array sensor for sensitive and rapid detection of H₂O₂," *Electrochimica Acta*, vol. 104, pp. 439-447, DOI: 10.1016/j.electacta.2013.01.077, 2013.
- [33] J. B. Raoof, R. Ojani, E. Rashid-Nadimi, S. Hasheminejad, S. Rashid-Nadimi, "Electrochemical synthesis of Ag nanoparticles supported on glassy carbon electrode by means of p-isopropyl calix[6]arene matrix and its application for electrocatalytic reduction of H₂O₂," *Applied Surface Science*, vol. 258 no. 7, pp. 2788-2795, DOI: 10.1016/j.apsusc.2011.10.133, 2012.
- [34] M. Zhang, Q. Sheng, F. Nie, J. Zheng, "Synthesis of Cu nanoparticles-loaded Fe₃O₄@carbon core-shell nanocomposite and its application for electrochemical sensing of hydrogen peroxide," *Journal of Electroanalytical Chemistry*, vol. 730, pp. 10-15, DOI: 10.1016/j.jelechem.2014.07.020, 2014.

DETAILS

Subject:	Biocompatibility; Reduction; Iron oxides; Uric acid; pH; Reagents; Electrodes; Nanocomposites; Hydrogen peroxide; Morphology; Nanoparticles; Magnetic cores; Voltammetry; Citric acid; Fourier transforms; Scanning electron microscopy; Ethanol; Selectivity; Ascorbic acid; Gold; Electron microscopy; Spectrum analysis; Sodium; Carbon; Sensors; Phenols; Nanospheres; Indium tin oxides
Location:	China
Editor:	Günther K Bonn
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-08-16 (Received); 2021-01-25 (Revised); 2021-02-22 (Accepted); 2021-03-08 (Pub)
DOI:	https://doi.org/10.1155/2021/8839895
ProQuest document ID:	2503352626
Document URL:	https://www.proquest.com/scholarly-journals/novel-nonenzymatic-hydrogen-peroxide-sensor-based/docview/2503352626/se-2?accountid=211160

Copyright:

Copyright © 2021 Xiao Ni et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated:

2023-10-05

Database:

Publicly Available Content Database

Document 45 of 59

Rapid Quantification of Chlorpromazine Residues in Pork Using Nanosphere-Based Time-Resolved Fluorescence Immunoassay Analyzer

Wang, Wei ¹

; Wang, Jingneng ²; Wang, Min ¹; Shen, Juan ¹ ¹ Key Laboratory of Meat Processing and Quality Control, MOE, Key Laboratory of Meat Processing, MARA, Jiangsu Synergetic Innovation Center of Meat Processing and Quality Control, Nanjing Agricultural University, No. 1 Weigang, Nanjing, Jiangsu 210095, China ² Shanghai Xiongtu Biotechnology Co., Ltd., No. 21 Guangfulin Road, Shanghai 201600, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Immunochromatographic assays are good analytical tools for the detection of drug residues. We report a nanosphere-based time-resolved fluorescence immunoassay (nano-TRFIA) based on a monoclonal antibody and a portable TRFIA analyzer for the rapid quantification of chlorpromazine (CPZ) residues in pork. Under optimal conditions, the nano-TRFIA detected CPZ residues within 6 min of sample pretreatment. The results showed good linearity ($R^2=0.991$), with a limit of detection (LOD) of $0.32\mu\text{g/kg}$, a wide dynamic range of $0.46\text{--}10.0\mu\text{g/kg}$, and coefficients of variation (CVs) of the overall intrabatch and interbatch assays of 7.34% and 7.65%, respectively. The nano-TRFIA was also used to detect CPZ at different spiked concentrations in pork, and the results were confirmed via ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The nano-TRFIA was evaluated for the analysis of six commercial pork samples, and the results agreed well with those obtained via UPLC-MS/MS, without significant differences ($P>0.05$). Therefore, the proposed nano-TRFIA is a powerful alternative for the rapid and accurate quantification of CPZ residues in pork to meet the required Chinese maximum residue limits for veterinary drugs in foods.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Chlorpromazine (CPZ) is a typical phenothiazine antipsychotic drug [1–3] and is common in clinical veterinary

practice because of its strong sedative and antiemetic effects [4, 5]. The addition of CPZ into animal feeds causes sedation, hypnosis, weight gain, and fattening and shortens the slaughter time. Chlorpromazine can also attenuate the stress response and maintenance needs of animals and can decrease their weight loss and mortality during long-distance transportation and thus prevent the reduction of meat quality [6, 7]. However, CPZ residues in animal products can adversely affect human health [8–11]. As early as 1997, the European Union issued Commission Regulation (EC) No. 17/97 which banned the addition of CPZ into feeds. Japan has also stipulated that CPZ should not be present in foodstuffs of animal origin. According to the National Food Safety Standard on Maximum Residue Limits for Veterinary Drugs in Foods (GB31650-2019) in China, CPZ is allowed for treatment but should not be present in foods of animal origin. Nevertheless, some vendors still illegally add CPZ into edible animal feeds. Detection methods for CPZ residues are underdeveloped compared with those for other antipsychotic drugs [12]. The CPZ residue detection methods can be classified into two types: the first type is based on chromatography, and it includes liquid microextraction-liquid chromatography [13], liquid chromatography-coulometry [14], high-performance liquid chromatography (HPLC) [15], liquid chromatography-mass spectrometry [16], and gas chromatography-mass spectrometry [17]. These methods require advanced laboratories, tedious sample pretreatment, long detection time, expensive apparatus, and operation by experienced personnel. Moreover, only a small number of samples can be analyzed at one time; therefore, these methods are not suitable for *in situ* detection or batch screening. The other type is based on immunological analyses, including enzyme-linked immunosorbent assay (ELISA), which is the most widely used. ELISA has been widely utilized to detect drug residues because of its high specificity, sensitivity, and simple operation [18–20]. However, this method suffers from low stability during quantification, low-temperature storage of reagents, short action time, long detection cycle, and unsuitability for testing a small number of samples. Thus, there is an urgent need for rapid, simple, convenient, and reliable detection methods.

Time-resolved fluorescence immunoassay (TRFIA) was developed in the early 1980s. This technology uses complexes of lanthanides and fluorescent markers and has been successfully applied to labeled immunoassays and clinical medicine [21, 22]. However, the detection sensitivity needs to be amplified through fluorescence enhancement due to the low fluorescence intensities of lanthanide complexes. Nanosphere-based TRFIA (nano-TRFIA) is a novel TRFIA that combines the long-lived fluorescence of rare-earth elements (Eu^{3+} , Tb^{3+} , etc.) with the signal amplification effect of nanospheres. Rare-earth elements and their complexes are codoped into nanospheres. After surface activation, an antibody is coupled onto the surface of a label to form a complex. During the immunoassay, this complex can elevate the sensitivity and expand the linear dynamic range [23]. In addition to facile operation, stable tracing, wide quantification, application ranges, and no radioactive contamination, nano-TRFIA can also offer higher sensitivity and accuracy than conventional methods; this is because polystyrene nanospheres containing thousands of lanthanide chelates have been developed as attractive labels with remarkable signal amplification potential [24]. Thus, in this study, we established a nano-TRFIA for the rapid quantification of CPZ residues in pork under the optimal experimental conditions described in our previous study [25]. Our goal is to provide references for quality and safety monitoring and the supervision and formulation of detection standards.

2. Materials and Methods

2.1. Materials and Reagents

Chlorpromazine, promethazine (PMZ), thioridazine (TDZ), acepromazine (ACP), prochlorperazine (PCZ), haloperidol (HAL), and fluphenazine (FPZ) were purchased from Dr. Ehrenstorfer Co. (Augsburg, Germany). Horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) and tetramethylbenzidine (TMB) and stop buffer were obtained from Cell Signaling Technology (St. Louis, USA). Chlorpromazine-bovine serum albumin and mouse anti-CPZ monoclonal antibody were prepared in our laboratory [26]. Goat anti-mouse IgG was provided by Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), tert-butyl methyl ether (TBME), and MES buffer (0.05 M, pH 6.0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Europium-chelate-coated nanospheres (PS-COOH) with a size of 300 nm were synthesized and provided by Bangs Laboratories, Inc. (Fishers, IN, USA).

Millipore 135 nitrocellulose (NC) membrane with a flow rate of 135s/4 cm, conjugate pad, sample pad, and absorbent pad were obtained from Millipore (MA, USA). All solutions were prepared in deionized water (18.2MΩ·cm, an Arium® Pro ultrapure water purification system from Sartorius, Göttingen, Germany). Two CPZ-positive pork samples labeled A and B were obtained from the Supervision, Inspection and Testing Center for Quality of Meat Products (Nanjing). Four pork samples labeled C, D, E, and F were acquired from a local supermarket in Nanjing, China. Other analytically pure reagents were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Detection of Cross-Reactivity of Monoclonal Antibody

The specificity of the antibody was determined via indirect competitive ELISA. Chlorpromazine, PMZ, TDZ, ACP, PCZ, HAL, and FPZ solutions at a series of concentrations were prepared and coated on 96-well microplates (Corning Inc., Corning, NY, USA). Mouse anti-CPZ monoclonal antibody was used as the primary antibody, and 0.01M phosphate-buffered saline (PBS, pH 7.2) and CPZ were used as the negative and positive controls, respectively. Horseradish peroxidase-labeled goat anti-mouse IgG was used as the secondary antibody. After the antibody was incubated at room temperature for 1 h, washed, and mixed with TMB for color development, the optical density (OD) at 450nm was measured using a Synergy H1 microplate reader (Bio-Tek, Winooski, VT, USA). The cross-reactivity (CR) was calculated as follows: $(1) CR\% = \frac{IC_{50}CPZ}{IC_{50}CPZ \text{ structural analog}} \times 100\%$, where IC_{50} is the half-maximal inhibitory concentration.

2.3. TRFIA Apparatus

The XT8201A portable TRFIA analyzer was codeveloped with Shanghai Xiongtu Biotechnology Co., Ltd., China, and used for the quantitative determination of CPZ. A light-emitting diode lamp served as the excitation source at 365 nm, and signals were acquired at 610nm using a photomultiplier tube (PMT). The fluorescence signal was recorded using the PMT after a 400 μs delay when the exciting light was irradiated on the test strip area. Meanwhile, the fluorescence with a short relaxation time decayed rapidly, reducing the background noise and achieving a high signal-to-noise ratio. The fluorescence signals on the test line (T line) and the control line (C line) peaks were processed using data processing software for quantitative analysis.

2.4. Labeling of Mouse Anti-CPZ Monoclonal Antibody by Fluorescent Nanospheres

Europium fluorescent nanospheres (100 μL) were added to 500 μL of 0.05M boric acid buffer (pH 8.0). The mixture was vortexed and added to 100 μL of 10M EDC solution (prepared with 0.05M MES buffer, pH 6.0). The obtained mixture was activated by shaking at room temperature for 30min and centrifuged at 8000r/min for 10min to discard the supernatant. The precipitate was then redissolved with 500 μL of 0.05M boric acid buffer (pH 8.0), ultrasonicated for 5min, and added to 100 μL of mouse anti-CPZ monoclonal antibody. After the amount of fluorescent nanosphere-conjugated antibody was adjusted to 30 μg/mL, the reaction was performed at room temperature for 2h. Subsequently, 50 μL of 0.05M boric acid buffer (pH 8.0) containing 10% BSA was added, and the mixture was reacted at room temperature for 2h on a thermomixer and centrifuged at 8000r/min for 10min to discard the supernatant. The precipitate was then redissolved with 500 μL of 0.05M boric acid buffer (pH 8.0) and ultrasonicated for 5min. The labeled fluorescent nanospheres (1:800) were sprayed onto a bonding pad with a T2DDA film-spotting, gold-spraying instrument (Shanghai Hangan Electronic Technology Co., Ltd., Shanghai, China). After drying, the nanospheres were sealed and stored at room temperature prior to use.

2.5. Assembly of Nano-TRFIA Kit

The assembly process of the nano-TRFIA kit is illustrated in Figure 1. First, 1.0mg/mL CPZ-BSA and 1.0mg/mL goat anti-mouse IgG were sprayed onto an NC membrane with a T2DDA film-spotting, gold-spraying instrument (Shanghai Hangan Electronic Technology Co., Ltd., Shanghai, China) as a T line and a C line, respectively (distance: about 1.0cm), and dried overnight at 37°C. Afterward, the sample pad, conjugate pad, NC membrane, and absorbent pad were sequentially pasted on a polyvinyl chloride (PVC) board. The conjugate pad and sample pad were pasted at the T line. The conjugate pad and NC membrane overlapped by about 1-2mm. The absorbent pad was pasted at the C line, and the pad overlapped with the NC membrane by about 1-2mm. The PVC board was then cut into a 3.55mm wide strip with a C6 strip-cutting machine (Shanghai Hangan Electronic Technology Co., Ltd., China) and placed into a plastic card to fabricate a nano-TRFIA kit and stored at room temperature for further

experiments.

[figure omitted; refer to PDF]

2.6. Sample Pretreatment for Nano-TRFIA

Pork tissue was homogenized at 10,000r/min for 1 min, and 2.00g (accurate to 0.01g) was put into a 50mL centrifuge tube with 500 μ L of 5M NaOH solution and vortexed for 30s. The sample was then mixed with 200 μ L of acetonitrile, vortexed for 80s, and then shaken thoroughly for another 30min. Next, 12mL of TBME was added, vortexed for 80s, and centrifuged at 4°C and 13,000r/min for 15min. Subsequently, the supernatant was blow-dried with nitrogen at 40°C, and the residue was redissolved with 2mL of methanol. The solution was diluted with 0.01M PBS (pH 7.2) and used for immediate measurement.

2.7. Nano-TRFIA for CPZ Detection

All operations were performed at room temperature (22°C–28°C). First, 100 μ L of the extracted solutions described above was added into a sample well of the nano-TRFIA kit. After incubation at 37°C for 6min, the nano-TRFIA kit was immediately inserted into the test strip slot of a portable TRFIA analyzer, and the fluorescence signals on the T line and C line were directly recorded under ultraviolet light (Figure 2).

[figure omitted; refer to PDF]

2.8. Nano-TRFIA Standard Curve and Determination of Linearity

A CPZ standard sample was dissolved in methanol, calibrated, and mixed with a 1.0g/kg standard solution. The system was then diluted to 10.0, 5.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, and 0 μ g/kg solutions with 0.01M PBS (pH 7.2). The CPZ standard solution was tested using the nano-TRFIA kit, and a portable TRFIA analyzer (Shanghai Xiongtu Biotechnology Co., Ltd., China) was used to record the fluorescence intensities of T and C lines and their ratios. Each standard was tested five times to plot the standard curve. The curve was drawn with $\ln X$ as the x-axis (where X is the concentration of competitive antigen), and the B/B_0 ratio of the standard solution is seen at each concentration as the y-axis. The linear range was determined, where B is the T/C ratio after the addition of the CPZ standard solution and B_0 is the ratio in the presence of 0 μ g/kg standard solution.

2.9. Determination of Limit of Detection and Limit of Quantification of Nano-TRFIA

Twenty blank samples were randomly taken for the nano-TRFIA strip test to calculate the mass concentration of the blank sample. The average ($\bar{X}$) and standard deviation (SD) were obtained. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to $LOD = \bar{X} + 3SD$ and $LOQ = \bar{X} + 10SD$, respectively.

2.10. Detection of Accuracy and Reproducibility of Nano-TRFIA

The accuracy and reproducibility of this method were represented by intrabatch and interbatch coefficients of variation (CVs). Chlorpromazine standard solutions at three different concentrations were tested. Two batches of the same sample were tested, and each batch was detected 10 times. The average of three CVs for each batch was employed as the intrabatch CV, and the average of two intrabatch CVs was used as the overall CV. The interbatch CV was calculated by measuring the CVs of two batches at each concentration 20 times and then averaging the values.

2.11. Comparison of Nano-TRFIA and Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry Results

To validate the nano-TRFIA results, six negative minced pork samples were treated with CPZ standard solutions at 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 μ g/kg and then subjected to ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Waters Corp., Milford, MA, USA) [16, 27]. The spiked samples were pretreated with acetonitrile to precipitate proteins, followed by extraction with TBME. Separation was performed on a Waters ACQUITY UPLC BEH C18 column (50mm $\times$ 2.1mm, 1.7 μ m). The column temperature was 30°C, and the sample injection volume was 10.0 μ L. Gradient elution was performed with solutions A (acetonitrile) and B (0.1% (v/v) formic acid in water) as the mobile phases, at a flow rate of 0.3mL/min: 0–2min, 10% A and 90% B; 2–4min, 60% A and 40% B; and 4–5min, 10% A and 90% B [25]. Detection was performed via positive-ion electrospray ionization in multiple reaction monitoring mode. The transition of m/z 319.27–85.96 was used to quantify CPZ. The recovery rate and relative standard deviation (RSD) of the samples were calculated.

2.12. Application in Real Pork Sample

To validate our strategy, six pork samples from different products were used to detect CPZ. Chlorpromazine from pork samples was extracted as described, and the extracts were analyzed through both nano-TRFIA and UPLC-MS/MS.

3. Results and Discussion

3.1. CR of Monoclonal Antibody

Indirect competitive ELISA was conducted to detect the CRs between mouse anti-CPZ monoclonal antibody and other six structurally relevant compounds. As presented in Table 1, the antibody had an excellent specificity (CR: <0.77%), with mild reactivity with only PCZ (CR: 5.74%) among the considered chemicals. Therefore, there was either low or no CR during CPZ detection, which ensures the specificity and accuracy of the proposed nano-TRFIA. We postulate that the stronger binding of the antibody to PCZ than to the other chemicals is because the key structure of PCZ is similar to that of CPZ.

Table 1

CR of mouse anti-CPZ monoclonal antibody.

Standard	IC ₅₀ (μg/kg)	CR (%)
CPZ	15.38	100
PCZ	267.90	5.74
ACP	>2000	<0.77
TDZ	>2000	<0.77
PMZ	>2000	<0.77
HAL	>2000	<0.77
FPZ	>2000	<0.77

CR, cross-reactivity; ACP, acepromazine; CPZ, chlorpromazine; FPZ, fluphenazine; HAL, haloperidol; IC₅₀, half-maximal inhibitory concentration; PCZ, prochlorperazine; PMZ, promethazine; TDZ, thioridazine.

3.2. Standard Curve and Linearity of Nano-TRFIA

To establish a standard curve, the fluorescence intensities on the T line and the C line with different CPZ concentrations were recorded using a portable TRFIA analyzer (Figure 3). As shown in Figure 4, the standard curve was plotted with the logarithm of the concentration of the competitive antigen CPZ standard as the x-axis and the B/B_0 ratio as the y-axis. The curve shows good linearity when the logarithm ranges from -2.773 to 2.303, corresponding to concentrations of 0.0625 to 10.0 μg/kg. The fitted linear regression equation is $Y = -0.135X + 0.547$ ($R^2 = 0.991$), meeting the requirement for a linear relationship.

[figure omitted; refer to PDF][figure omitted; refer to PDF]

3.3. LOD and LOQ of Nano-TRFIA

The B/B_0 values measured with 20 blank samples were substituted into the above linear regression equation, giving an average CPZ concentration of 0.26 μg/kg and an SD of 0.02. According to the equations $LOD = \bar{X} + 3SD$ and $LOQ = \bar{X} + 10SD$, the LOD and LOQ were calculated as 0.32 μg/kg and 0.46 μg/kg, respectively. The established nano-TRFIA thus had a much higher sensitivity than those previously reported [7, 17, 26, 28, 29]. This suggests that the nano-TRFIA has practical application value.

3.4. Accuracy and Reproducibility of Nano-TRFIA

The accuracy of the nano-TRFIA was determined by setting three concentrations of CPZ standard solutions (1.0, 5.0, and 10.0 $\mu\text{g/kg}$), dividing the samples into two batches, and repeatedly testing each batch 10 times (Table 2). The intrabatch CVs of the first and second batches were 6.32% and 8.36%, respectively. The overall intrabatch and interbatch CVs were 7.34% and 7.65%, respectively. This method is highly accurate and repeatable since all CVs were lower than 10.0%.

Table 2

CPZ detection by nano-TRFIA.

CPZ concentration ($\mu\text{g/kg}$)	First batch ($n=10$)			Second batch ($n=10$)		
Mean value ($\mu\text{g/kg}$)	SD	CV (%)	Mean value ($\mu\text{g/kg}$)	SD	CV (%)	1.0
0.97	0.02	2.61	0.95	0.06	8.51	5.0
4.86	0.04	7.82	5.04	0.04	8.85	10.0

CPZ, chlorpromazine; CV, coefficient of variation; nano-TRFIA, nanosphere-based time-resolved fluorescence immunoassay; SD, standard deviation.

3.5. Validation by UPLC-MS/MS

To verify the accuracy of this method, the correlation of the measurement results with those of UPLC-MS/MS was analyzed. For UPLC-MS/MS, the standard curve was plotted via CPZ standard solutions at mass concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 $\mu\text{g/kg}$, with a regression equation of $Y=11028.80X-1403.87$ ($R^2=0.994$). The retention time of CPZ was 2.2 min. The LOD was 0.25 $\mu\text{g/kg}$, and the LOQ was 1.0 $\mu\text{g/kg}$.

Samples were tested via nano-TRFIA and UPLC-MS/MS under the same spiked concentration (Table 3). The results were subjected to linear regression analysis, giving an equation of $Y=0.957X+0.147$ ($R^2=0.999$) (Figure 5). The CPZ in the samples could be accurately and reliably detected via the established nano-TRFIA.

Table 3

Comparison of nano-TRFIA and UPLC-MS/MS for CPZ detection in pork ($n=5$).

Spiked concentration ($\mu\text{g/kg}$)	UPLC-MS/MS			Nano-TRFIA		
Detected concentration ($\mu\text{g/kg}$)	Recovery (%)	RSD (%)	Detected concentration ($\mu\text{g/kg}$)	Recovery (%)	RSD (%)	1.0
0.96 $\pm$ 0.03	96.40	3.99	0.93 $\pm$ 0.05	92.60	5.59	2.0
1.92 $\pm$ 0.04	96.20	2.25	1.92 $\pm$ 0.04	95.80	2.11	4.0
3.92 $\pm$ 0.09	98.00	2.63	3.89 $\pm$ 0.12	97.15	3.59	6.0
5.98 $\pm$ 0.10	99.73	1.80	6.04 $\pm$ 0.13	100.63	2.40	8.0

8.03±0.16	100.35	2.23	8.09±0.15	101.15	2.09	10.0

CPZ, chlorpromazine; nano-TRFIA, nanosphere-based time-resolved fluorescence immunoassay; RSD, relative standard deviation; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry.

[figure omitted; refer to PDF]

3.6. Determination and Evaluation of Real Pork Samples

To examine the applicability of the established nano-TRFIA, we selected six different pork samples and compared the results with those of nano-TRFIA and UPLC-MS/MS (Table 4). The results obtained using the nano-TRFIA kits were nearly consistent with the UPLC-MS/MS detection results. There was no significant difference between the results of the two methods ($P>0.05$). The data could be used to evaluate the applicability and reliability of the newly developed method. Thus, this nano-TRFIA is a potential alternative to chromatography for the regulatory analysis of CPZ residues in foodstuffs of animal origin.

Table 4

Comparison of nano-TRFIA and UPLC-MS/MS for CPZ detection in real pork samples ($n=5$).

Sample	CPZ found ($\mu\text{g/kg}$)	
UPLC-MS/MS	Nano-TRFIA	A
1.62±0.07	1.57±0.06	B
1.95±0.09	1.92±0.05	C
Nd	Nd	D
Nd	Nd	E
Nd	Nd	F

CPZ, chlorpromazine; nano-TRFIA, nanosphere-based time-resolved fluorescence immunoassay; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry; nd, not detected.

4. Conclusion

Detecting CPZ residues in meat is important; hence, several countries have formulated standards for CPZ residues. Although China has continuously focused on the monitoring of CPZ during food safety inspections and has enforced a strict ban on the addition of CPZ to animal feed, CPZ residues have still been detected in pork in recent years. The strategy reported in this study combines nano-TRFIA with immunochromatography to realize high specificity, sensitivity, reproducibility, and a wide linear range. The detection results meet the requirements for quantifying actual samples. This method is readily operable and does not require expensive instrumentation. Moreover, the approach can quickly measure CPZ residues in foodstuffs of animal origin *in situ* and may have application value for other drug residues.

Acknowledgments

The authors express their gratitude to Mr. Xudong Zhu for his assistance in the research and development of the nano-TRFIA kit. This research was financially supported by the Natural Science Foundation of Jiangsu Province (no. BK20191307) and the National Key R & D Program of China (no. 2018YFC1602804).

References

- [1] B. Thornley, C. E. Adams, A. G. Awad, "Chlorpromazine versus placebo for the treatment of schizophrenia: a systematic review," *Schizophrenia Research*, vol. 29 no. 1-2, DOI: 10.1016/s0920-9964(97)88697-5, 1998.

- [2] C. A. W. Rijcken, T. B. M. Monster, J. R. B. J. Brouwers, L. T. W. de Jong-van den Berg, "Chlorpromazine equivalents versus defined daily doses: how to compare antipsychotic drug doses?," *Journal of Clinical Psychopharmacology*, vol. 23 no. 6, pp. 657-659, DOI: 10.1097/01.jcp.0000096247.29231.3a, 2003.
- [3] J. Cooper, P. Delahaut, T. L. Fodey, C. T. Elliott, "Development of a rapid screening test for veterinary sedatives and the beta-blocker carazolol in porcine kidney by ELISA," *The Analyst*, vol. 129 no. 2, pp. 169-174, DOI: 10.1039/b311709j, 2004.
- [4] S. Song, X. Shi, R. Li, "Extraction of chlorpromazine with a new molecularly imprinted polymer from pig urine," *Process Biochemistry*, vol. 43 no. 11, pp. 1209-1214, DOI: 10.1016/j.procbio.2008.06.015, 2008.
- [5] J. J. Liu, S. M. Yang, "Development of detect of chlorpromazine residue," *China Animal Husbandry & Veterinary Medicine*, vol. 35 no. 10, pp. 141-144, 2008.
- [6] L. Amaral, M. Viveiros, J. Molnar, "Antimicrobial activity of phenothiazines," *In Vivo*, vol. 18 no. 6, pp. 725-731, 2004.
- [7] P. Lu, Z. N. Qu, W. Q. Tan, R. Zheng, J. Jiang, "Studies on the GC-MS analysis of chlorpromazine residue in pig liver," *Chinese Journal of Animal Quarantine*, vol. 23 no. 7, pp. 30-31, DOI: 10.3969/j.issn.1005-944X.2006.07.016, 2006.
- [8] A. H. Abadi, S. Rafatullah, A. A. Al-Badr, "Chlorpromazine," *Analytical Profiles of Drug Substances and Excipients*, pp. 97-165, DOI: 10.1016/S0099-5428(08)60622-X, 1999.
- [9] Y. Ito, S. Sato, M. Son, "Bioelectric toxicity caused by chlorpromazine in human lung epithelial cells," *Toxicology and Applied Pharmacology*, vol. 183 no. 3, pp. 198-206, DOI: 10.1006/taap.2002.9486, 2002.
- [10] R. Frötschl, S. Weickardt, S. Staszewski, G. Kaufmann, P. Kasper, "Effects of chlorpromazine with and without UV irradiation on gene expression of HepG2 cells," *Mutation Research*, vol. 575 no. 1-2, pp. 47-60, DOI: 10.1016/j.mrfmmm.2005.03.002, 2005.
- [11] T. Li, Q. X. Zhou, "Ecotoxicological effect of chlorpromazine and its influence on human health," *Chinese Journal of Ecology*, vol. 25 no. 12, pp. 1554-1558, 2006.
- [12] R. Feng, Q. Liang, R. T. Zhan, "Optimization of national standard method for determination of chlorpromazine residues in pork," *Medical Information*, vol. 28 no. 9, DOI: 10.3969/j.issn.1006-1959.2015.09.157, 2015.
- [13] M. Cruz-Vera, R. Lucena, S. Cárdenas, M. Valcárcel, "Determination of phenothiazine derivatives in human urine by using ionic liquid-based dynamic liquid-phase microextraction coupled with liquid chromatography," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 877 no. 1-2, pp. 37-42, DOI: 10.1016/j.jchromb.2008.11.017, 2009.
- [14] M. A. Saracino, M. Amore, E. Baioni, C. Petio, M. A. Raggi, "Determination of selected phenothiazines in human plasma by solid-phase extraction and liquid chromatography with coulometric detection," *Analytica Chimica Acta*, vol. 624 no. 2, pp. 308-316, DOI: 10.1016/j.aca.2008.06.040, 2008.
- [15] A. Asghari, E. Fahimi, M. Bazregar, M. Rajabi, L. Boutorabi, "Rapid determination of some psychotropic drugs in complex matrices by tandem dispersive liquid-liquid microextraction followed by high performance liquid chromatography," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 1052, pp. 51-59, DOI: 10.1016/j.jchromb.2017.03.012, 2017.
- [16] L. Zhang, W. Huang, X. H. Jiang, "Determination of chlorpromazine in porcine muscle using high performance liquid chromatography-tandem mass spectrometry," *Journal of Chinese Pharmaceutical Sciences*, vol. 24 no. 10, pp. 690-694, DOI: 10.5246/jcps.2015.10.089, 2015.
- [17] M. N. Shan, X. F. Xu, Y. X. Pu, "Determination of chlorpromazine in pork by gas chromatography-mass spectrometry," *Chinese Journal of Food Hygiene*, vol. 25 no. 5, pp. 438-440, 2013.
- [18] S. X. Lu, Y. L. Zhang, J. T. Liu, "Preparation of anti-pefloxacin antibody and development of an indirect competitive enzyme-linked immunosorbent assay for detection of pefloxacin residue in chicken liver," *Journal of Agricultural and Food Chemistry*, vol. 54 no. 19, pp. 6995-7000, DOI: 10.1021/jf061309q, 2006.
- [19] C. B. Zhao, W. Liu, H. L. Ling, "Preparation of anti-gatifloxacin antibody and development of an indirect competitive enzyme-linked immunosorbent assay for the detection of gatifloxacin residue in milk," *Journal of*

Agricultural and Food Chemistry, vol. 55 no. 17, pp. 6879-6884, DOI: 10.1021/jf070978g, 2007.

[20] Z. Q. Liu, S. X. Lu, C. H. Zhao, "Preparation of anti-danofloxacin antibody and development of an indirect competitive enzyme-linked immunosorbent assay for detection of danofloxacin residue in chicken liver," Journal of the Science of Food and Agriculture, vol. 89 no. 7, pp. 1115-1121, DOI: 10.1002/jsfa.3514, 2009.

[21] J.-C. G. Bünzli, C. Piguet, "Taking advantage of luminescent lanthanide ions," Chemical Society Reviews, vol. 34 no. 12, pp. 1048-1077, DOI: 10.1039/b406082m, 2005.

[22] J. Z. Shen, Z. Zhang, Y. Yao, "A monoclonal antibody-based time-resolved fluoroimmunoassay for chloramphenicol in shrimp and chicken muscle," Analytica Chimica Acta, vol. 575 no. 2, pp. 262-266, DOI: 10.1016/j.aca.2006.05.087, 2006.

[23] D. Wang, Z. Zhang, P. Li, "Europium nanospheres-based time-resolved fluorescence for rapid and ultrasensitive determination of total aflatoxin in feed," Journal of Agricultural and Food Chemistry, vol. 63 no. 47, pp. 10313-10318, DOI: 10.1021/acs.jafc.5b03746, 2015.

[24] Y. Shen, S. Xu, D. He, "A novel europium chelate coated nanosphere for time-resolved fluorescence immunoassay," PLoS One, vol. 10 no. 6, DOI: 10.1371/journal.pone.0129689, 2015.

[25] M. Wang, "Establishment and evaluation of rapid quantitative detection of chlorpromazine in animal-derived foods by time-resolved fluorescence immunoassay," 2018. Master's thesis

[26] M. Wang, W. Wang, Q. Q. Lü, "Rapid detection of chlorpromazine in pork tissue by indirect competitive ELISA," Journal of Nanjing Agricultural University, vol. 43 no. 1, pp. 172-177, DOI: 10.7685/jnau.201812051, 2020.

[27] B. P. Li, J. Hua, H. Li, "Determination of chlorpromazine illegally added in 3 kinds of veterinary preparations by UPLC-MS/MS," Chinese Journal of Veterinary Drug, vol. 49 no. 5, pp. 32-36, 2015.

[28] General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ), Standardization Administration of the People's Republic of China (SAC), National Standard of the People's Republic of China, Method for Determination of Acetopromazine, Chlorpromazine, Haloperidol, Propionylpromazine, Xylazine, Azaperone, Azaperol and Carazolol Residues in Porcine Kidney and Muscle Tissues-LC-MS-MS Method, GB/T 20763-2006, 2006.

[29] Y. L. Hong, X. F. Hao, K. Dong, "HPLC determination of chlorpromazine residue in animal-derived foods," Food Science, vol. 30 no. 14, pp. 269-271, DOI: 10.3321/j.issn:1002-6630.2009.14.059, 2009.

DETAILS

Subject:	Laboratories; Mass spectrometry; Monoclonal antibodies; Polyvinyl chloride; Acids; Feeds; Mathematical analysis; Reagents; Liquid chromatography; Linearity; Scientific imaging; Chromatography; Pretreatment; Immunoassay; Coefficient of variation; Fluorescence; Chlorpromazine; Psychotropic drugs; Residues; Pork; Meat quality; Nanospheres
Location:	Beijing China; United States--US; Germany; China
Editor:	Danilo Corradini
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021

Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-12-09 (Received); 2021-02-25 (Revised); 2021-03-02 (Accepted); 2021-03-09 (Pub)
DOI:	https://doi.org/10.1155/2021/6633016
ProQuest document ID:	2503352570
Document URL:	https://www.proquest.com/scholarly-journals/rapid-quantification-chlorpromazine-residues-pork/docview/2503352570/se-2?accountid=211160
Copyright:	Copyright © 2021 Wei Wang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-03-21
Database:	Publicly Available Content Database

Document 46 of 59

Characterization and Quantification of Luteolin-Metal Complexes in Aqueous Extract of Lonicerae Japonicae Flos and Huangshan Wild Chrysanthemum

ABSTRACT (ENGLISH)

Luteolin is a flavonoid compound widely found in vegetables, fruits, and medicinal plants. In this study, the reaction conditions for luteolin and five metal ions (Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{3+} , and Cu^{2+}) to form complexes in hot water were optimized, which was at a molar ratio of 1:1 for luteolin and metal ions at 90°C in a volume of 20 mL for 2 h, and the ability of luteolin to form complexes with Cu^{2+} was the strongest. The DPPH scavenging test showed that luteolin exerted a dose-dependent effect on the clearance of free radicals; luteolin- Cu^{2+} complexes and luteolin- Fe^{3+} complexes accentuated the clearance of free radicals. Furthermore, we used high performance liquid chromatography (HPLC) to analyze luteolin in samples from two medicinal plants, obtained from the dissolution of aqueous extracts in two different solvents. The results showed that the peak areas for luteolin in the samples dissolved in 20% formic acid-methanol were significantly larger than those from the samples dissolved in methanol alone, with increases in the peak area being 135.6% (*Lonicerae Japonicae Flos*), and 161.16% (*Huangshan wild chrysanthemum*). The aforementioned results indicate that complexes formed from organic compounds and metal ions are present in the decoction of a plant.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Plants are an important source of many biologically active and clinically relevant organic compounds, including phenylpropanoid, flavonoid, anthraquinone, steroids, terpenoids, and alkaloids [1]. They also contain many metal ions such as calcium, magnesium, iron, and copper that form complexes with organic compounds which perform multiple biological activities [2]. Phenolic hydroxyl, carbonyl, carboxyl, sulfhydryl, and amino groups and nitrogen-containing aromatic rings are nearly universally present in organic compounds and may readily form complexes with metals. Rybak and Ruzik [3] found that manganese formed complexes with rutin, alizarin, and asperulosidic acid in noni juice. Using $\mu\text{HPLC-ESI-MS/MS}$, Wojcieszek et al. [4] detected seven types of copper complexes and four types of zinc complexes in ionic liquid and pectinase extracts of *Lycium barbarum*. Weber and Koniecznyński [5] detected manganese and magnesium complexes in *Folium Betulae* (birch leaves), *Folium Menthae* (peppermint leaves), and *Radix Taraxaci* (dandelion roots) by size-exclusion chromatography combined with atomic absorption spectroscopy. Using the membrane filter and macroporous resin, Liu et al. [6] identified by flame atomic absorption spectrometry many complexes between zinc and proteins or polycarbohydrates in the decoction of *Flammulina velutipes*. These studies indicated that multiple complexes of ions and organic compounds are present in plants in nature.

Luteolin is a flavonoid that is widely present in nature and is mainly distributed in medicinal herbs such as *Lonicerae Japonica Flos*, *Origanum vulgare*, chrysanthemum, and rosemary and in foods including peanuts, carrots, celery, cucumber, and buckwheat. [7, 8] Luteolin possesses many biological activities including antioxidant [9, 10], antibacterial [11], antiviral [12], and antitumor activities [13, 14] and also confers cardiocerebrovascular protection [15, 16]. The phenolic hydroxyl and carbonyl groups have intact π conjugation and strong superdelocalizability and proper planar structure, which facilitate complex formation between luteolin and ions.

In the current study, we investigated luteolin ion complex formation by measuring postreaction luteolin content by high performance liquid chromatography (HPLC). We further determined the effects of reaction temperature,

volume, and time on the formation of complexes between luteolin and five types of ion including calcium, magnesium, iron, zinc, and copper in the aqueous extract. We also studied the effects of ions on free radical scavenging activities of luteolin. Finally, we determined the presence of luteolin ion complexes in *Lonicerae Japonica Flos* (*L. japonica*) and Huangshan wild chrysanthemum by HPLC analysis of aqueous extract of *L. japonica* and Huangshan wild chrysanthemum in methanol and 20% formic acid-methanol.

2. Materials and Methods

2.1. Chemicals

Luteolin was purchased from Haochen Biotechnology Co. (Shaanxi, China), and its purity was confirmed to be greater than 98% by HPLC. Luteolin standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade and mass spectrometry grade) was purchased from Merker, Germany; formic acid (HPLC grade) and deuterated methanol were purchased from Aladdin, USA, and 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) was purchased from TCI Chemicals (Shanghai, China). Magnesium chloride hexahydrate, anhydrous calcium chloride, anhydrous zinc chloride, iron chloride hexahydrate, copper chloride dihydrate, ethyl acetate, and DMSO were all analytical grade. Weight of chemicals used was measured using an XS203S electronic scale (METTLER TOLEDO, Swiss).

2.2. Plants

L. japonica and Huangshan wild chrysanthemum were purchased from Yifeng Pharmacy (Nanchang, China). *L. japonica* was grown in Henan Province, China, and Huangshan wild chrysanthemum was grown in Anhui Province, China. They were officially authenticated by the School of Pharmacy, Jiangxi Science and Technology Normal University. *L. japonica* was dried flower buds of *Lonicera japonica* Thunb., and Huangshan wild chrysanthemum was the dried capitulum of *Dendranthema morifolium* (Ramat) Tzvel. cv. Gongju.

2.3. Preparation of Luteolin Standard Curve

HPLC separations were performed on YMC-Pack ODS-A C18 chromatographic column (4.6 mm × 150 mm, 5 μm) with 30°C column temperature. Luteolin was eluted using 2 mmol L⁻¹ ammonium formate solution (0.1% formic acid) (A) and methanol (B) (60:40, V/V) as the mobile phase with 60% B isocratic elution. The flow rate was 1.0 mL·min⁻¹. The injection volume was 1 μL. The wavelength of the VWD detector was set at 350 nm.

Luteolin methanol stock solution (2.5 mmol/L) was prepared and diluted into the following concentrations: 2.25, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75, 0.5, and 0.25 mmol/L. The peak area of HPLC was determined. Luteolin standard curve was plotted with the HPLC peak area as the x-axis and luteolin concentrations as the y-axis. The following regression equation was derived: (1) $Y = 0.001X + 0.0008$ $R^2 = 0.9999$, indicating that luteolin concentration from 0.25 mmol/L to 2.5 mmol/L had a good linear relationship.

2.4. ¹H-NMR

Luteolin (0.1 mmol) was allowed to react with 0.02 mmol anhydrous calcium chloride, anhydrous zinc chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate in 40 mL distilled water at 90°C for 4 h under heat backflow using a Hei-VAP rotary evaporator (Heidolph, Germany) and mixed with a DF-101S heat collection type isothermal magnetic heating stirrer (Yuhua Co., Gongyi, Henan, China). Thereafter, the reactants were spun dry and washed with distilled water several times to remove ions that did not form complexes with luteolin. The remaining solid portion was washed several times with 100 mL ethyl acetate, and after the solid portion was spun dry, it was dissolved in deuterated methanol. Free luteolin and the reactants in deuterated methanol were then studied by nuclear magnetic resonance (NMR) spectroscopy as routinely performed using a Bruker AVANCE 400-MHz spectrometer.

2.5. Determination of Reaction Conditions

To determine the effect of reaction temperature on luteolin ion salt complex formation, we mixed 0.1 mmol luteolin with 0.02 mmol anhydrous calcium chloride, anhydrous zinc chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate in 40 mL distilled water. The reaction was allowed to proceed for 4 h under heat backflow at a temperature of 30°C, 50°C, 70°C, or 90°C. Furthermore, to determine the effect of reaction time on luteolin ion salt complex formation, we carried out the above reaction at 90°C for 0.5, 1, 2, and 4 h, respectively.

In addition, to determine the effect of reaction volume on luteolin ion salt complex formation, we carried out the above reaction in 20, 40, 80, or 160 mL distilled water at 90°C for 2 h. We also investigated the effect of molar ratios of luteolin to ion salts on luteolin ion salt complex formation at a ratio of 10:1, 5:1, 2:1, and 1:1 (0.1 mmol luteolin added to 0.002, 0.004, 0.01, and 0.02 mmol anhydrous calcium chloride, anhydrous zinc chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate, respectively). The reaction was allowed to proceed for 2 h under heat backflow at 90°C. Finally, we examined the ability of luteolin to complex with different ion salts. Luteolin (0.1 mmol) was allowed to react with 0.1 mmol anhydrous calcium chloride, anhydrous zinc chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate in 20 mL distilled water at 90°C under heat backflow for 2 h. Free luteolin without ion salt served as blank control. The reactants were spun dry and washed with distilled water several times to remove ions that did not form complexes with luteolin. The remaining solid portion was washed several times with 100 mL ethyl acetate, and after the ethyl acetate lotion was spun dry, it was dissolved in 20 mL methanol. Luteolin content was analyzed by HPLC as described above.

2.6. DPPH Scavenging Test

Luteolin (0.1 mmol) was allotted to react with 0.1 mmol anhydrous calcium chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate in 20 mL distilled water at 90°C under heat backflow for 2 h. The reactants were spun dry and dissolved in DMSO in 10 mmol/L stock solution (using the molar concentration of luteolin). Luteolin and the 4 ion salts were also prepared in equivalent molar concentration. Radical scavenging capacity was evaluated by a DPPH radical test as previously described [17]. The experiments were performed three times independently in triplicate, and the mean value was used to calculate clearance using the following equation: $\text{Clearance\%} = 1 - \frac{A_s - A_c}{A_0} \times 100\%$, where A_s represents the mean absorbance of the sample and DPPH, A_c represents the mean absorbance value of the sample, and A_0 represents the mean absorbance value of DPPH solution.

2.7. HPLC Analysis

L. japonica and Huangshan wild chrysanthemum (20 g each) were dissolved in 200 mL distilled water and after soaking at room temperature for 30 min, were subjected to heat backflow decoction at 95°C for 2 h. After filtration, the filtrate was spin-dried to obtain dried decoction. The dried decoction was dissolved in 20 mL methanol or 20 mL 20% formic acid-methanol, and after sonication for 1 min, 1 mL solute was filtered using 0.22 μm membrane and then subjected to HPLC.

HPLC separations were performed on an Agilent Eclipse XDB-C18 analytical column (250 mm $\times$ 4.6 mm, 5 μm) with 30°C column temperature. Samples were eluted using 2 mmol L⁻¹ ammonium formate solution (0.2% formic acid or methanol) (A) and acetonitrile (B) (60:40, V/V) as the mobile phase 0–80 min, 20% B 60% B; 80–100 min, 60% B 100% B. The flow rate was 0.2 mL $\cdot$ min⁻¹. The injection amount was 10 μL . The wavelength of the DAD detector was set at 350 nm.

3. Results

3.1. Effect of Reaction Temperature on Luteolin-Metal Complex Formation

¹H-NMR spectroscopy showed that after its reaction with anhydrous calcium chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate, luteolin complexes in ethyl acetate exhibited similar spectra to those of free luteolin (Figure 1), indicating that luteolin did not completely react with the five types of ion salts, and luteolin ion salt complexes were not dissolvable in ethyl acetate. Therefore, in subsequent experiments, HPLC was used to quantify luteolin content in ethyl acetate, with higher luteolin content in ethyl acetate indicating a lower capacity to form complexes with these ions.

[figure omitted; refer to PDF]

We then allowed luteolin to react with anhydrous calcium chloride, magnesium chloride hexahydrate, iron chloride hexahydrate, or copper chloride dihydrate at different temperatures to investigate the effect of reaction temperature on luteolin ion complex formation. We observed a gradual reduction in the peak area of luteolin as the reaction temperature rose from 30°C to 90°C (Figure 2). At 90°C, the peak area of luteolin was 67.2% of that at 30°C. The

reaction temperature was set at 90°C for subsequent experiments.

[figure omitted; refer to PDF]

3.2. Effect of Reaction Time and Volume on Luteolin-Metal Complex Formation

We also delineated the effect of reaction time on luteolin ion complex formation. The amount of luteolin gradually decreased as the reaction time increased (Figure 3(a)). At 4h, the peak area of luteolin decreased by 9.7% versus that at 2h, suggesting that most luteolin ion complexes had already formed by 2h. The reaction time was set at 2h for subsequent experiments. We further studied the effect of solvent volume on luteolin ion complex formation. We observed no apparent difference in the content of luteolin in ethyl acetate among different reaction volumes (Figure 3(b)). The peak area at a volume of 40mL was the smallest and declined only by 1.98% versus that at a volume of 20mL, which fell within the standard error of weighing. The peak area at a volume of 80mL and 160mL increased only by 6.00% and 9.04%, respectively, compared with that at a volume of 20mL. This could be due to lower ion concentrations with increased volume, which does not facilitate complexation. The reaction volume was set at 20mL for subsequent experiments.

[figures omitted; refer to PDF]

3.3. Effect of Molar Ratios of Luteolin to Ions and Different Types of Ions on Luteolin Ion Complex Formation

Luteolin was added at a molar ratio of 10:1, 5:1, 2:1, or 1:1 to ions. As shown in Figure 4(a), when the molar ratio of luteolin to ions was 1:1, the amount of luteolin in ethyl acetate was only 31.8% of the amount of luteolin when the luteolin to ion ratio was 10:1, suggesting that the amount of ions had a marked effect on luteolin ion complex formation: the greater the amount of ions was, the greater the amount of luteolin ion complexes was formed. In subsequent studies, the ratio of luteolin to ions was maintained at 1:1.

[figures omitted; refer to PDF]

We further examined luteolin ion complex formation between luteolin and different types of ions. Under condition for water decoction at 90°C under heat backflow for 2h, the peak area was the smallest following luteolin and Cu^{2+} reaction (Figure 4(b)). The peak area after luteolin and Zn^{2+} reaction increased 1.82% compared to that with no ions, indicating virtually no formation of the luteolin- Zn^{2+} complex. The ability to form luteolin ion complexes was as follows: $\text{Cu}^{2+} > \text{Fe}^{3+} > \text{Ca}^{2+} > \text{Mg}^{2+} > \text{Zn}^{2+}$.

3.4. Effect of Metal Ions on Clearance of Free Radicals by Luteolin

Luteolin exhibited a dose-dependent effect on clearance of free radicals. The clearance rate of free radicals was 18.34% for luteolin at 0.10mmol, which shot up to 80.27% for luteolin at 1.0mmol (Table 1). The clearance rate of free radicals stood at 89.69%, 73.44%, 94.63%, and 79.46% for luteolin- Fe^{3+} complexes, luteolin- Mg^{2+} complexes, luteolin- Cu^{2+} complexes, and luteolin- Ca^{2+} complexes at 1.0mmol/L, respectively. In the absence of luteolin, the clearance rate of free radicals was 29.81%, 7.00%, 39.39%, and 5.70% for Fe^{3+} , Mg^{2+} , Cu^{2+} , and Ca^{2+} salt only at 1.0 mmol/L, respectively. These findings suggested that luteolin- Cu^{2+} complexes and luteolin- Fe^{3+} complexes accentuated the clearance of free radicals.

Table 1

Effect of luteolin, metal salt and luteolin, and metal ion reaction products on clearance of DPPH free radicals.

Concentration, mmol/L	Clearance%								
Luteolin	Fe complex	Mg complex	Cu complex	Ca complex	Fe salt	Mg salt	Cu salt	Ca salt	1.0
80.27	89.69	73.44	94.63	79.46	29.81	7.00	39.39	5.70	0.1
69.66	72.66	73.30	86.54	76.02	16.9	-0.84	7.83	4.79	0.01

3.5. Luteolin-Metal Complexes in *L. japonica* and Huangshan Wild Chrysanthemum Decoction

The chromatographic profiles of the methanol extract and 20% formic acid-methanol extract of *L. japonica* decoction are shown in Figure 5, and the retention time and peak area of ten well-isolated chromatographic peaks are given Table 2. The chromatographic profiles of the methanol extract and 20% formic acid-methanol extract of Huangshan wild chrysanthemum decoction are shown in Figure 6, and the retention time and peak area of ten well-isolated chromatographic peaks are given Table 3.

[figure omitted; refer to PDF]

Table 2

Retention time and peak area of *L. japonica* decoction ($\bar{X} \pm S$, $n=3$).

No.	20% formic acid-methanol sample		Methanol sample		Increase in peak area (%)
Retention time	Peak area	Retention time	Peak area		
18562.99±1078.91	33.898	8072.82±487.77	129.94	1	33.806
55410.47±3220.53	35.055	16619.99±1004.20	233.40	2	34.925
8282.763±481.41	38.186	2587.756±156.35	220.08	3	38.134
37570.57±2183.65	42.982	13548.2±818.59	177.31	4	42.941
486.37±28.27	54.257	88.5658±5.35	449.17	5	54.314
91.60±5.32	Not detected	Not detected	—	6	55.249
73.73±4.29	Not detected	Not detected	—	7	56.273
141.49±8.22	57.637	33.18±2.00	326.48	8	57.655
				9	58.699

195.47± 11.36	58.725	43.25±2.61	351.95	10	60.25 1 (luteolin)

[figure omitted; refer to PDF]

Table 3

Retention time and peak area of Huangshan wild chrysanthemum decoction ($\bar{X} \pm S$, $n=3$).

No.	20% formic acid-methanol sample		Methanol sample		Increase in peak area (%)
Retention time	Peak area	Retention time	Peak area	1	33.77
25286.71± 1699.06	33.908	7805.70±689.08	223.95	2	34.93 7
72512.27± 4872.24	35.089	24210.14± 2137.26	199.51	3	36.44 6
34578.05± 2323.36	36.556	9695.21±855.89	256.65	4	42.80 4
60551.63± 4068.58	42.970	19756.52± 744.10	206.49	5	48.32 9
7949.30± 534.13	48.391	1785.63±157.63	345.18	6	55.57 5
3824.70± 256.99	55.591	936.94±82.71	308.21	7	57.30 5
1077.09± 72.37	57.309	224.54±19.82	379.69	8	58.69 8
3877.06± 260.51	58.714	1091.58±96.36	255.18	9	60.24 0 (luteolin)
1538.59± 103.38	60.258 (luteolin)	664.29±58.64	131.61	10	61.67 7

The retention time of the methanol extract of *L. japonica* decoction was 60.26 ± 0.02 min, and the peak area was 1091.53 ± 13.15 . The retention time of the 20% formic acid-methanol extract of *L. japonica* decoction was 60.24 ± 0.02 min, and the peak area was 1094.37 ± 9.28 . Luteolin contains multiple phenolic hydroxyl groups and is weakly acidic, which, to a certain extent, modifies its chromatographic behavior; therefore, in the 20% formic acid-methanol extract, the peak appeared earlier than that in the methanol extract. However, there was no difference in the peak area between the two extracts.

The chromatographic profiles of the methanol extract and 20% formic acid-methanol extract of *L. japonica* decoction and Huangshan wild chrysanthemum decoction were largely in agreement with one another (Figures 5 and 6). In Huangshan wild chrysanthemum decoction, the number of chromatographic peaks, resolution, and peak form were identical in the two extracts. *L. japonica* decoction had two apparent chromatographic peaks at 55.249 min and 56.273 min in the 20% formic acid-methanol sample. Though no apparent chromatographic peaks were observed around the above two retention times using automatic detection software, two small protuberances, which were similar in shape to the two chromatographic peaks in the 20% formic acid-methanol sample, were present in the baseline by manual examination, suggesting the presence of similar compounds at 55.249 min and 56.273 min. We noticed that luteolin-metal complexes were slightly dissolved in methanol but completely dissolved in 20% formic acid-methanol into ligands and corresponding metal ions (data not shown). The chromatographic peak area of both *L. japonica* and Huangshan wild chrysanthemum decoction in 20% formic acid-methanol samples was higher than that in the methanol samples (Tables 2 and 3). The chromatographic peak of luteolin in *L. japonica* decoction in 20% formic acid-methanol samples increased 1.36 fold, while that of Huangshan wild chrysanthemum decoction increased 1.61.

4. Discussion

Luteolin is one of the important plant metabolites. Due to the presence of carbonyl and hydroxyl groups, luteolin can coordinate metal ions to form complexes, and the metal complexes have shown higher biological effects than free luteolin. Over recent years, many luteolin-metal ion complexes have been synthesized including luteolin complexes with copper [18, 19], cadmium [20], chromium [20, 21], vanadium [22–24], aluminum [24–26], manganese [27], zinc [28, 29], iron (III) [30, 31], and rare earth elements [32]. However, these luteolin-metal ion complexes are synthesized in an organic solvent or alkaline aqueous solution. Anna et al. demonstrated by NMR that the energetically favored Zn chelation sites of the 1:1 zinc-luteolin complexes were 4=O and 5-OH [25]. Rygula et al. [26] showed that, after dissolving in sodium hydroxide, luteolin and Zn^{2+} mainly coordinate at 3'-OH and 4'-OH of luteolin in Tris-HCl (pH 7.4). Hao et al. [27] detected three reaction products, such as $[(\text{Lu}-2\text{H})^{2-} + \text{Fe}^{3+} + \text{NO}_3^- + \text{H}^+]$, $[(\text{Lu}-2\text{H})^{2-} + \text{Fe}^{3+} + 2\text{CH}_3\text{CH}_2\text{OH}]$, and $[(\text{Lu}-2\text{H})^{2-} + \text{Fe}^{3+} + \text{NO}_3^- + 2\text{CH}_3\text{CH}_2\text{OH} + \text{H}^+]$, by ESI-TOF-MS when luteolin reacted with $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in ethanol; moreover, these reaction products enhanced the clearance of DPPH free radicals. As most edible and medicinal plants are decocted in water before use, the current study used distilled water as solvent to investigate the formation of complexes between luteolin and metal ions in plant aqueous extracts. Our study demonstrated that luteolin and Zn^{2+} did not form complexes in distilled water, while the reaction products of luteolin and Fe^{3+} lowered the clearance of DPPH free radicals by luteolin, suggesting that reaction solvent affects the ability of luteolin to complex with metal ions, chelation sites, and activities of complexes. In analyzing methanol and 20% formic acid-methanol samples of *L. japonica* and Huangshan wild chrysanthemum decoction, we took great efforts to minimize variations in weighing and extracting the medicinal herbs and divided a decoction into two parts equivalent in volume which was then spun dry and dissolved in solutions. Furthermore, filtration was performed at 95°C in the waterbath to prevent precipitation of compounds due to decrease in filtrate temperature, leading to great variations in results. HPLC revealed that the area of chromatographic peaks for luteolin and other compounds increased to various extents in *L. japonica* and Huangshan wild chrysanthemum decoction in 20% formic acid-methanol samples. Though *L. japonica* and Huangshan wild chrysanthemum contain, apart from luteolin, glycosides from luteolin complexation with carbohydrates, we used moderate to strong acid, formic acid, which was of relatively low concentration in the solution, and the dissolution condition was relatively mild; consequently, the luteolin peak area in 20% formic acid-methanol solution markedly increased. Increased

luteolin content was unlikely caused by increased luteolin-glycoside complexes but more likely due to dissolution of luteolin-metal complexes in the presence of formic acid. Moreover, the amplitude of increase in the luteolin peak area of the decoctions of the two plants was different in 20% formic acid-methanol, which may be due to the difference in the types and contents of metal ions in the two plants.

5. Conclusion

The current study has demonstrated that, apart from organic compounds and metal ions, complexes formed from organic compounds and metal ions are also present in the decoction of a plant. These findings suggest that investigators should also characterize and study the biological significance of organic compound-metal ion complexes in plants.

Acknowledgments

This work was supported by grants obtained from Jiangxi Youth Science Fund Project (20122BAB215017) and the Jiangxi Science and Technology Normal University Undergraduate Research Fund Project (20181204073).

References

- [1] "Antihyperglycemic effect of *Passiflora glandulosa* cav. fruit rinds flour in streptozotocin-induced diabetic mice," *Asian Pacific Journal of Tropical Medicine*, vol. 11 no. 9, pp. 510-517, 2018.
- [2] M. Chatatikun, T. Yamauchi, K. Yamasaki, A. Chiabchalard, S. Aiba, "Phyllanthus acidus (L.) Skeels and *Rhinacanthus nasutus* (L.) Kurz leaf extracts suppress melanogenesis in normal human epidermal melanocytes and reconstitutive skin culture," *Asian Pacific Journal of Tropical Medicine*, vol. 12 no. 3, pp. 98-105, DOI: 10.4103/1995-7645.254935, 2019.
- [3] J. Rybak, L. Ruzik, "Application of chromatography and mass spectrometry to the characterization of cobalt, copper, manganese and molybdenum in *Morinda citrifolia*," *Journal of Chromatography A*, vol. 1281, pp. 19-25, DOI: 10.1016/j.chroma.2013.01.040, 2013.
- [4] J. Wojcieszek, P. Kwiatkowski, L. Ruzik, "Speciation analysis and bioaccessibility evaluation of trace elements in goji berries (*Lycium Barbarum*, L.)," *Journal of Chromatography A*, vol. 1492, DOI: 10.1016/j.chroma.2017.02.069, 2017.
- [5] G. Weber, P. Koniecznyński, "Speciation of Mg, Mn and Zn in extracts of medicinal plants," *Analytical and Bioanalytical Chemistry*, vol. 375 no. 8, pp. 1067-1073, DOI: 10.1007/s00216-002-1706-z, 2003.
- [6] F. Liu, F. Pei, A. M. Mariga, L. Gao, G. Chen, L. Zhao, "Separation and speciation analysis of zinc from *Flammulina velutipes*," *Journal of Food and Drug Analysis*, vol. 23 no. 4, pp. 630-635, DOI: 10.1016/j.jfda.2014.06.008, 2015.
- [7] M. Lopez-Lazaro, "Distribution and biological activities of the flavonoid luteolin," *Mini Reviews in Medicinal Chemistry*, vol. 9 no. 1, DOI: 10.2174/138955709787001712, 2009.
- [8] K. H. Miean, S. Mohamed, "Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants," *Journal of Agricultural and Food Chemistry*, vol. 49 no. 6, pp. 3106-3112, DOI: 10.1021/jf000892m, 2001.
- [9] G. Seelinger, I. Merfort, C. Schempp, "Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin," *Planta Medica*, vol. 74 no. 14, pp. 1667-1677, DOI: 10.1055/s-0028-1088314, 2008.
- [10] U. zgen, A. Mavi, Z. Terzi, "Relationship between chemical structure and antioxidant activity of luteolin and its glycosides isolated from *T. Sipyleus* subsp. *sipyleus* var. *sipyleus*," *Records of Natural Products*, vol. 13 no. 5, 2010.
- [11] T. Zhang, Y. Qiu, Q. Luo, "The mechanism by which luteolin disrupts the cytoplasmic membrane of methicillin-resistant *Staphylococcus aureus*," *The Journal of Physical Chemistry B*, vol. 122 no. 4, DOI: 10.1021/acs.jpcc.7b05766, 2018.
- [12] X. Lin, S. Weiheng, J. Jun, "Identification of luteolin as enterovirus 71 and coxsackievirus A16 inhibitors through reporter viruses and cell viability-based screening," *Viruses*, vol. 6 no. 7, pp. 2778-2795, 2014.
- [13] X. Lu, Y. Li, X. Li, H. A. Aisa, "Luteolin induces apoptosis in vitro through suppressing the MAPK and PI3K signaling pathways in gastric cancer," *Oncology Letters*, vol. 14 no. 2, pp. 1993-2000, DOI: 10.3892/ol.2017.6380,

2017.

- [14] K. A. Kang, M. J. Piao, Y. S. Ryu, "Luteolin induces apoptotic cell death via antioxidant activity in human colon cancer cells," *International Journal of Oncology*, vol. 51 no. 4, pp. 1169-1178, DOI: 10.3892/ijo.2017.4091, 2017.
- [15] H. Qiao, X. Zhang, C. Zhu, "Luteolin downregulates TLR4, TLR5, NF- κ B and p-p38MAPK expression, upregulates the p-ERK expression, and protects rat brains against focal ischemia," *Brain Research*, vol. 1448, pp. 71-81, DOI: 10.1016/j.brainres.2012.02.003, 2012.
- [16] X. Wu, T. Xu, D. Li, "ERK/PP1a/PLB/SERCA2a and JNK pathways are involved in luteolin-mediated protection of rat hearts and cardiomyocytes following ischemia/reperfusion," *PLoS One*, vol. 8 no. 12, DOI: 10.1371/journal.pone.0082957, 2013.
- [17] M. D. Catarino, A. M. S. Silva, S. C. Saraiva, "Characterization of phenolic constituents and evaluation of antioxidant properties of leaves and stems of *Eriocephalus africanus*," *Arabian Journal of Chemistry*, vol. 196, pp. 62-69, 2015.
- [18] S. Niu, B. Han, W. Cao, S. Zhang, "Sensitive DNA biosensor improved by Luteolin copper(II) as indicator based on silver nanoparticles and carbon nanotubes modified electrode," *Analytica Chimica Acta*, vol. 651 no. 1, pp. 42-47, DOI: 10.1016/j.aca.2009.08.002, 2009.
- [19] S. Shuyun, Z. Yuping, C. Xiaoqin, "Investigation of flavonoids bearing different substituents on ring C and their Cu²⁺ complex binding with bovine serum albumin: structure-affinity relationship aspects," *Journal of Agricultural and Food Chemistry*, vol. 59 no. 19, pp. 10761-10769, 2011.
- [20] L.-G. Gao, H. Wang, X.-L. Song, W. Cao, "Research on the chelation between luteolin and Cr (III) ion through infrared spectroscopy, UV-vis spectrum and theoretical calculations," *Journal of Molecular Structure*, vol. 1034 no. 6, pp. 386-391, DOI: 10.1016/j.molstruc.2012.10.053, 2013.
- [21] W. Cao, "Luteolin and luteolin-Cr (III) complexes: antioxidation and reaction mechanism with hydrogen peroxide radical," *Chinese Journal of Inorganic Chemistry*, vol. 31 no. 11, pp. 2229-2235, 2015.
- [22] S. Roy, S. Mallick, T. Chakraborty, "Synthesis, characterisation and antioxidant activity of luteolin-vanadium (II) complex," *Food Chemistry*, vol. 173, pp. 1172-1178, DOI: 10.1016/j.foodchem.2014.10.141, 2015.
- [23] L. Naso, V. R. Martínez, L. Lezama, "Antioxidant, anticancer activities and mechanistic studies of the flavone glycoside diosmin and its oxidovanadium(IV) complex. Interactions with bovine serum albumin," *Bioorganic & Medicinal Chemistry*, vol. 24 no. 18, pp. 4108-4119, DOI: 10.1016/j.bmc.2016.06.053, 2016.
- [24] L. G. Naso, L. Lezama, M. Valcarcel, "Bovine serum albumin binding, antioxidant and anticancer properties of an oxidovanadium(IV) complex with luteolin," *Journal of Inorganic Biochemistry*, vol. 157 no. 5, pp. 80-93, DOI: 10.1016/j.jinorgbio.2016.01.021, 2016.
- [25] A. Anna, C. Catia, M. Costanza, "Complexation of apigenin and luteolin in weld lake: a DFT/TDDFT investigation," *Physical Chemistry Chemical Physics Pccp*, vol. 12 no. 25, pp. 6672-6684, 2010.
- [26] A. Rygula, T. P. Wrobel, J. Szklarzewicz, M. Baranska, "Raman and UV-vis spectroscopy studies on luteolin-Al (III) complexes," *Vibrational Spectroscopy*, vol. 64 no. 8, pp. 21-26, DOI: 10.1016/j.vibspec.2012.10.005, 2013.
- [27] D. Hao, X. Yang, J. He, "Enhanced antioxidant activity, antibacterial activity and hypoglycemic effect of luteolin by complexation with manganese(II) and its inhibition kinetics on xanthine oxidase," *Rsc Advances*, vol. 7 no. 84, pp. 53385-53395, 2017.
- [28] P. Alexandra, M. Gloria, L. Christina, "Understanding zinc(II) chelation with quercetin and luteolin: a combined NMR and theoretical study," *Journal of Physical Chemistry B*, vol. 119 no. 1, pp. 83-95, 2015.
- [29] B. Xiao, H. Wang, X. Zhao, Y. Li, "Selective recognition of luteolin and quercetin based on the specific interaction of ortho-dihydroxy substituents with a zinc (II) complex," *Analytical Methods*, vol. 6 no. 9, pp. 2894-2899, DOI: 10.1039/c4ay00066h, 2014.
- [30] A.-H. Yang, X.-Y. Shi, X. Li, "Spectroscopic and electrochemical studies on the evaluation of the radical scavenging activities of luteolin by chelating iron," *Rsc Advances*, vol. 4 no. 48, pp. 25227-25233, DOI: 10.1039/c4ra01396d, 2014.
- [31] R. Jun, M. Sheng, C. E. Lekka, "Complexation of flavonoids with iron: structure and optical signatures," *Journal*

of Physical Chemistry B, vol. 112 no. 6, pp. 1845-1850, 2008.

[32] J. Li, L. Wang, H. Bai, B. Yang, H. Yang, "Synthesis, characterization, and anti-inflammatory activities of rare earth metal complexes of luteolin," Medicinal Chemistry Research, vol. 20 no. 1, pp. 88-92, DOI: 10.1007/s00044-009-9289-2, 2011.

DETAILS

Subject:	Formic acid; Acids; Zinc; Nuclear magnetic resonance--NMR; Ferric ions; Biological products; Copper; Scientific imaging; Heat; Chromatography; Metal ions; Plants (botany); Scavenging; Chloride; Methanol; Coordination compounds; Pharmacy; Herbal medicine; Flavonoids; Free radicals; Magnesium; High performance liquid chromatography; Organic compounds; Calcium ions
Location:	Germany; China
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestonedates:	2020-11-03 (Received); 2021-02-08 (Revised); 2021-03-01 (Accepted); 2021-03-12 (Pub)
DOI:	https://doi.org/10.1155/2021/6677437

ProQuest document ID: 2503352481

Document URL: <https://www.proquest.com/scholarly-journals/characterization-quantification-luteolin-metal/docview/2503352481/se-2?accountid=211160>

Copyright: Copyright © 2021 Weilan Cai et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-03-21

Database: Publicly Available Content Database

Document 47 of 59

Development and Validation of a LC-MS/MS Method for the Determination of Nitrofurantoin Metabolites in Soft-Shell Turtle Powder Health Food Supplement

Ryu, EunChae ¹
; Ji Sung Park ² ; Giri, Sib Sankar ¹
; Se Chang Park ¹

¹ Laboratory of Aquatic Biomedicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, Republic of Korea ² Seoul Regional Office, Animal and Plant Quarantine Agency, Seoul 07670, Republic of Korea

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Soft-shell turtle (SST; freshwater terrapin or tortoise) is a popular and important health functional food (HFF) product in many Asian countries. HFFs containing SST must be safe, but several HFFs have been found to be contaminated with dangerous substances, such as nitrofurantoin metabolites (NFM). This finding suggests that the consumption of HFFs results in the regular exposure of vulnerable individuals to hazardous substances. Importantly, nitrofurantoin antibiotics have been banned for use in food-producing animals since the 1990s by the European Union. Thus, in this study, we propose a reliable and quick method to reduce the time required for the detection of four NFMs in SST powder that conventional methods are unable to quantify. Our method involves the derivatization and hydrolysis of SST powder and was validated in accordance with the requirements of European Commission Decision 2002/657/EC. The method achieves an apparent mean recovery of 82.2–108.1%, repeatability of 1.5–3.8%, and reproducibility of 2.2–4.8% for 0.5–10.0 $\mu\text{g kg}^{-1}$ of 1-aminohydantoin, semicarbazide, 3-amino-2-oxazolidinone, and 3-amino-5-morpholinomethyl-2-oxazolidinone. In addition, linearity was achieved with correlation coefficients of 0.999, and the detection capability ($CC\beta$) and decision limit ($CC\alpha$) were found to be reliable, indicating that this is a fast and accurate method for the analysis of SST powder. The validated method was successfully applied to detect

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Currently, health functional foods (HFFs, also known as health-promoting foods or health beneficial foods) represent the fastest growing area in the food industry and health food market [1]. In particular, the production of HFFs containing plant or animal extracts is rapidly gaining importance [2]. In this context, soft-shell turtle (SST; freshwater terrapin or tortoise) products are sold as dietary supplements, as well as for traditional Chinese medicinal purposes. Typically, SST is sold in powdered form in capsules or liquid form and is available from both traditional and online markets. SST is, thus, an important commercial product and is a popular delicacy in East and Southeast Asian countries for its nutritional and medicinal properties [3, 4]. The production of Chinese SST reached 331,424 tons in 2012, and sale as a dietary product was the main commercial use [5]. SST has a high nutritional content and is known for the beneficial effects in preventing osteoporosis and bone loss in old age, promoting childhood development, and improving conditions such as back pain, diabetes, allergy, hypertension, asthma, and thyroid disorders, as reported by practitioners of traditional Chinese medicine, which has resulted in its increased popularity and scale of farming [6–8].

The use of antimicrobial veterinary medicines is necessary to rear animals profitably in an intensive fashion, but the use of these products can result in toxicological hazards in the edible products produced from these animals [9]. Furazolidone (FZD), furaltadone (FTD), nitrofurazone (NFZ), and nitrofurantoin (NFT) are commonly used nitrofurantoin (NF-) based veterinary drugs [10]. They are typically used to treat gastrointestinal infections in cattle, pigs, and poultry [11]. In addition, many antimicrobial drugs containing NFs are used in the farming of shrimp and fish [12]. However, NFs are prohibited (zero-tolerance policy) in many countries, as initiated by the European Union (EU) in 1993, because of the carcinogenic, teratogenic, and mutagenic potential of NFs, as reported by the World Health Organization (WHO) in 1989 and 1993 [12–15]. The hazardous potential of NFs has been reported for many years [10]. For example, the mutagenicity of NFT and FZD in Chinese hamster ovary cell strains has been examined [16]. It was found that FZD can induce oxidative DNA damage by increasing the number of intracellular reactive oxygen species, thus resulting in the abnormal progression or arrest of the cell cycle in human hepatoma G2 cells [17]. NFZ and FTD have been evaluated toxicologically and show severe toxicity to *Raphidocelis subcapitata*, *Daphnia magna*, and *Musca domestica* [18]. NFs are direct-acting mutagens in the sense that they induce mutations in bacteria and cultures of mammalian fibroblasts without the addition of exogenous activating systems [19]. Because of these findings, the EU has set the minimum required performance level (MRPL) of $1 \mu\text{g kg}^{-1}$ for each NF [20]. Thus, analytical laboratories must be able to achieve this detection limit, and sensitive and selective methods are required to detect and quantify the residues of these compounds in animal tissues and related food products.

NFMs have been found in products containing SST, but conventional methods are not capable of sufficient analytical accuracy. Thus, we aimed to develop a reliable method to enable the detection and quantification of NFMs in SST food products to enable the better protection of the thousands of people, including children, pregnant women, and the elderly who use these HHFs regularly. Immunoassay techniques have been used for the detection of NFs; however, those methods are problematic due to the interference by other substances in the matrix and the high affinity of immune antibodies having high-risk false-positive and false-negative responses as a fundamental problem methodologically [21, 22]. Although HPLC/UV and HPLC-DAD have long been used for the screening NFs metabolites due to the acceptable reliability, it was lacking specificity as a confirming method, so it could be a costly and time-consuming way [21]. MS analysis offers more improved specificity than immunoassay, HPLC/UV, and HPLC-DAD. Presently, they could be highly sensitive with the best reliability and repeatability. Further, GC-MS is not suitable for routine use in laboratories for chemical detection of NF metabolites [21]. LC-MS/MS analysis is the best choice to screen, determine, and confirm the NF metabolites than those methods in terms of accuracy, precision,

sensitivity, and sample preparation. In this article, a simplified and more reliable method was used for the determination of the NF metabolites using LC-MS/MS in soft-shell turtle powder.

The physical and chemical properties of SST powder (SSTP) are as follows: the product has a moisture content of <5%, an ash content of 20–40%, and a crude protein content of 50–70%. These characteristics make SSTP very different from most processed foods [23]. Furthermore, these properties make the analysis of NFMs in the SSTP matrix challenging. For example, conventional testing of SSTP resulted [11, 24–26] in no peaks being detected, even when internal standards (ISs) were used for the mass spectrometry (MS) analysis. Thus, standard analytical experimental conditions are not appropriate for this specific sample, and the method based on the characteristics of SSTP must be developed.

The four most important NFs (FZD, FTD, NFZ, and NFT) are rapidly metabolized into 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD), respectively, after administration, and remain as stable protein-bound compounds in animal tissues for long periods [11, 24–26]. Therefore, the majority of methods for analyzing NFs are based on the determination of these highly stable metabolites as markers [11, 27]. However, in some samples, for example, products containing azodicarbonamide treated with chlorine, red seaweed, carrageenan, milk powders, whey powders, egg powder, and wild forest honey from New Zealand, trace amounts of SEM have been detected and are considered environmental contaminants, reaction products formed during processing, or natural substances (for example, in certain seafood) [28, 29]. However, in most samples, detected SEM can only originate from the NFZ used to treat food-producing animals [29]. Thus, we did not exclude SEM from method development. In fact, several analytical methods have been described for the simultaneous detection of NFMs in food products from animals [11, 24–26, 30–33].

In this study, we developed a simultaneous determination method for NFMs in SSTP. The method proposed here is based on liquid chromatography-tandem MS (LC-MS/MS), which has been optimized to achieve the quick, accurate, and sensitive analysis of the complex SSTP matrix. Our method will enable better compliance with HFF food safety policies, which is crucial, considering that the market for these products includes vulnerable groups.

2. Materials and Methods

2.1. Chemicals and Reagents

Reference standards for AOZ, AMOZ, SEM, and AHD were purchased from Sigma-Aldrich (Steinheim, Germany). Isotopically labeled AOZ- d_4 , AMOZ- d_5 , $^{13}C^{15}N_2$ -SEM, and AHD- $^{13}C_3$, which were used as ISs, were also bought from Sigma-Aldrich (Steinheim, Germany). All reference standard purities were over 99.0%. The derivatization reagents 2-nitrobenzaldehyde (2-NBA) and 0.1M potassium phosphate dibasic solution (K_2HPO_4) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide (NaOH, reagent grade) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Analytical reagent grade aqueous (37%) hydrogen chloride (HCl) was purchased from Sigma-Aldrich (Steinheim, Germany).

High-performance LC (HPLC) grade ethyl acetate, methanol, and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). MS analysis grade ammonium formate (AF) and formic acid (FA) solutions were obtained from Sigma-Aldrich (Steinheim, Germany). HPLC grade *n*-hexane and dimethyl sulfoxide (DMSO, analytical grade) were purchased from Merck (Darmstadt, Germany). Deionized ultrapure water was generated using a Milli-Q Plus water purification system (Millipore, Darmstadt, Germany). Water and other mobile-phase solvents were filtered through a 0.2 μm Whatman membrane filter, and a 0.22 μm polytetrafluoroethylene (PTFE) syringe filter was used for samples (Millipore Ltd., Bedford, MA, USA).

2.2. Equipment

An Applied Biosystems triple quadrupole mass spectrometer 4500 (AB Sciex, Framingham, MA, USA) was used for analysis with an electrospray ionization (ESI) source operating in positive ion mode. LC was carried out using an Agilent (Santa Clara, CA, USA) 1290 Infinity System. Analyst[®] (version 1.6.2, AB Sciex Corp., Framingham, MA, USA) was used for method development and data analysis. The data were acquired in multiple reaction monitoring (MRM) mode.

2.3. Chromatographic Conditions

The HPLC separation was performed on an Aegispak C18-L (2.0mm × 100mm, 5 μm particle size) obtained from Young Jin Biochrom Co., Ltd (Sung Nam, Korea). The temperatures of the column oven and autosampler tray were set to 40 and 4°C, respectively. The mobile phase was composed of solvent A (10mM AF, 0.1% FA in 20% MeCN/MeOH (1:1 v/v)) and solvent B (10mM AF, 0.1% FA in MeCN/MeOH (1:1 v/v)). The injection volume was 20 μL. The flow rate was set at 250 μL mL⁻¹, with a linear gradient applied for 10 min to obtain sufficient statistics for each analyte, in the following conditions: 0.0–1.0 min, 100% A; 1.0–2.0 min, 100–0% A; 2.0–7.0 min, 0.0–0.0% A; and 7.1–10.0 min, 0–100% A.

2.4. LC-MS/MS Analysis

Stable and common product ions were selected for target analyte identification using syringe injections of the diluted standard solutions of each target analyte at 100 μg L⁻¹, respectively. The 2-nitrophenyl (2-NP) derivatized NFM (2-NP-NFM) and their ISs were detected in positive-ion mode ESI. The corresponding cone voltage and collision energy under optimal operating conditions, along with the MS/MS transitions used for the analysis, are summarized in Table 1. MS parameter tests were performed for all analytes at an ion source temperature (TEMP) of 600°C and an ionization spray voltage (ISV) of 5500 keV. Nitrogen was used as a nebulizer for the curtain gas (CUR) and collision gas (CG) in accordance with the manufacturer's guidelines. The ion source gas 1 (GS 1, nebulizer gas) and ion source gas 2 (GS 2, turbo gas) flows were set to 30 L h⁻¹, and the CUR flow was set to 25 L h⁻¹.

Table 1

Mass spectrometry parameters for the optimal operational analysis of the derivatized NF metabolites.

Analytes	Retention time (min)	Precursor ion (<i>m/z</i>)	Product 1 ion (<i>m/z</i>)	DP/CE/C XP (eV)	Product 2 ion (<i>m/z</i>)	DP/CE/C XP (eV)	Product 3 ion (<i>m/z</i>)	DP/CE/C XP (eV)
2-NP-AHD	3.37	248.9	134.0*	106/17/10	178.1	106/21/8	104.0	106/27/8
2-NP-AOZ	3.57	236.0	134.1*	116/17/8	149.0	116/19/12	104.0	116/33/12
2-NP-AMAZ	1.95	334.7	291.2*	96/17/8	262.1	96/23/10	128.1	96/27/10
2-NP-SEM	3.23	209.0	166.1*	10/13/8	134.0	66/15/8	192.0	66/17/8
2-NP- ¹³ C ¹⁵ N ₂ -S-EM	3.22	211.6	168.0	56/15/8	—	—	—	—
2-NP- ¹³ C ₃ -AHD	3.37	252.0	179.1	61/21/6	—	—	—	—
2-NP-AOZ-d4	3.55	240.0	134.0	111/17/10	—	—	—	—
2-NP-AMAZ-d5	1.93	340.0	296.2	46/17/14	—	—	—	—

RT: retention time (min); DP: declustering potential (electron volt); CE: collision energy (electron volt); CXP: collision cell exit potential (electron volt). *Transitions for quantifier peaks.

2.5. Standard Solutions

Standard solutions of NFMs and their IS solutions were prepared by diluting individual stock solutions containing 100mg L^{-1} of each standard dissolved in MeCN. These solutions were cooled to -20°C in a freezer immediately after preparation. The working standard solution was mixed and then diluted with MeCN to each concentration, i.e., 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and $100.0\text{ }\mu\text{g L}^{-1}$. The working IS solutions of $200\text{ }\mu\text{g L}^{-1}$ were prepared by mixing and diluting from the stock solution in MeCN. The 2-NBA solution was freshly prepared for use when required at a concentration of 0.05M in DMSO.

2.6. Sample Preparation

Samples were kindly provided by associates in the import trade. The SSTP samples were obtained in two different batches. One batch of SSTP was an uncontaminated clean sample. Another batch of SSTP was contaminated and found to contain 2-NP-AOZ by the screening tests before the study using the conventional analytical method. The sample was an ordinary hot-air-dried powder, and it was stored at room temperature as is typical for this type of foodstuff.

2.7. Optimizing the Preparation of SSTP Samples

2.7.1. Sample Weight

The weight of the SSTP samples can affect the reaction pH value when the hydrolysis and derivatization processes occur. The analyte-free SSTP was used as a blank matrix. To obtain the optimal sample weight, samples weighing 5.0 (conventional sample weight), 2.0, 1.0, 0.5, and 0.2g were tested, and the results are shown in Figure 1. This analysis was based on the conventional extraction method outlined in the Korean Food Code [34]. This conventional method followed the previously reported hydrolysis and derivatization processes, which require 16h (overnight) at 37°C [11, 24, 26, 30, 31, 35, 36].

[figure omitted; refer to PDF]

The samples weighed from 5.0 to 0.2g with a margin of error of 10% of each weight range in 50mL polypropylene centrifuge tubes. The working standards at $10\text{ }\mu\text{g kg}^{-1}$ and working IS solutions were placed in $200\text{ }\mu\text{L}$ volumes into the tubes. The samples were kept for about 15min at room temperature. Then, a 10mL aliquot of a 0.125M HCl solution and a $200\text{ }\mu\text{L}$ aliquot of a 0.05M 2-NBA solution in DMSO were added to each tube. Then, the tube was vortex-mixed for 0.5min and kept in a precision reciprocal shaking water bath (Thermo Scientific, NC, USA) for 16h (overnight) at 37°C in the dark and agitated at 80rpm. After cooling to room temperature, a 1 mL aliquot of 0.1M K_2HPO_4 was added to each centrifuge tube, and the pH was adjusted to 7.0 ± 0.1 . All tubes were centrifuged at 2000 rpm for 10min in an Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA). The upper aqueous layer was transferred to a new tube. Then, 10mL of hexane was added and vortex-mixed for 30s. The hexane layer was discarded after centrifugation. Then, 8mL of EtOAc was added and vigorously vortex-mixed for 30s. After each sample had been centrifuged at 2500rpm for 10min, the supernatant was transferred to a new centrifuge tube. This EtOAc extraction was performed twice.

The extract volume was reduced under a gentle stream of nitrogen at a temperature of less than 40°C using an OA-SYS N-EVAPTM 112 N_2 evaporator (Organization, Berlin, MA, USA). The dried eluate was diluted with 0.5mL of the mobile phase and transferred to a 2mL centrifuge tube and centrifuged at 7500rpm for 10min. The clear supernatant was filtered through a $0.22\text{ }\mu\text{m}$ PTFE syringe filter (Millipore Ltd., Bedford, MA, USA) for subsequent HPLC-MS/MS analysis.

2.8. Optimization of Reaction Conditions through Direct Use of Incurred SSTP Samples

Using the incurred SSTP samples, the most optimal preparation procedure was obtained. The sample ($1.0\text{g}\pm 0.1\text{g}$) was weighed in 50mL polypropylene centrifuge tubes with $200\text{ }\mu\text{L}$ of IS solution ($200\text{ }\mu\text{g L}^{-1}$), on the basis of the optimal sample weight shown in Figure 1. The change in pH with time was determined by measurements from 1 to 6 h in 1h intervals and after 16h using the conditions of the conventional method; the results are shown in Figure 2. The concentration of 2-NP-AOZ in the incurred samples was detected using 10, 20, 30, and 50mL of 0.125M HCl at

37°C. The variation in the volume of HCl solutions in the concentration of 0.125 and 0.5M is shown in Figure 2 and was used to find the optimum volume for each acid concentration. The amount of detected 2-NP-AOZ in the samples is shown in the bar graph in Figure 2.

[figure omitted; refer to PDF]

2.9. Reaction Time

The detection of 2-NP-AOZ was carried out on samples with 10, 12, 13, 14, 15, and 20mL of 0.5M HCl at 45°C. Using the results shown in Figure 2, the best time period was determined based on the amount of detected 2-NP-AOZ in the incurred samples. The optimal time period was obtained using a 1.0g sample with 14 mL of 0.5M HCl at 45°C, as shown in Figure 3. Other experimental conditions are the same as those reported in the "Sample weight" section of the materials and methods.

[figure omitted; refer to PDF]

2.10. Method Validation

The developed method was validated for selectivity, specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), detection limit ($CC\beta$), decision limit ($CC\alpha$), and recovery in accordance with the European Commission (EC) regulations [37]. This method was validated on a multiresidue scale for the simultaneous analysis of AOZ, AMOZ, SEM, and AHD as their derivatized nitrophenyl counterparts.

Selectivity and specificity were assessed by analyzing the samples and by testing spiked NFMs in blank samples. The absence of possible interfering compounds around the retention time of the target compounds was verified and compared to those of the spiked samples with the working standard solutions.

Linearity was measured using matrix-matched calibration curves with NF-free SSTP samples at seven different standard concentrations, as shown in Table 2. The calibration curve was obtained from the area ratio of each target compound to the IS concentration to compensate and correct for matrix effects.

Table 2

Calibration curves, sensitivity, corrected intraday and interday accuracies, and precision for samples spiked with working standard solution ($n=6$).

Target analyte	Linearity range ($\mu\text{g kg}^{-1}$)	Spiked level ($\mu\text{g kg}^{-1}$)	Calibration curve equation (r)	Intraday series (% RSD)	Interday series (% RSD)	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	$CC\alpha$ ($\mu\text{g kg}^{-1}$)	$CC\beta$ ($\mu\text{g kg}^{-1}$)
2-NP-AHD	0.2–20	0.5	$y=0.0124x+0.1991$ (0.99969)	98.8 (3.2)	92.8 (3.6)	0.07	0.23	0.25	0.30
1.0	96.9 (3.2)	101.3 (3.8)	2.0	102.5 (2.1)	98.3 (2.9)	10.0	98.7 (1.7)	99.4 (2.5)	
2-NP-SEM	0.2–20	0.5	$y=0.0477x+0.1280$ (0.99971)	95.5 (3.8)	92.6 (4.5)	0.05	0.17	0.18	0.30
1.0	92.5 (3.1)	98.2 (3.5)	2.0	94.8 (2.1)	92.7 (3.8)	10.0	95.2 (2.2)	93.9 (2.8)	

2-NP-AOZ	0.3–20	0.5	$y=0.0127x+0.2459$ (0.99941)	82.2 (3.7)	85.2 (4.8)	0.09	0.29	0.30	0.34
1.0	84.7 (3.2)	88.5 (3.4)	2.0	87.5 (2.4)	108.1 (2.8)	10.0	90.4 (2.8)	87.6 (3.1)	
2-NP-AMAZ	0.2–20	0.5	$y=0.0235x+0.2192$ (0.99980)	92.6 (2.2)	90.4 (3.1)	0.05	0.17	0.27	0.32

The repeatability and reproducibility were tested in three different days by spiking the working standard solution on the blank SSTP.

LOD is usually reported using the signal-to-noise (S/N) ratio [38], in which the signal must be three times higher than the baseline noise. In this study, we define the LOD from the spiked real samples at the lowest standard solution concentration at which a measurement has been carried out [39]. The LOQ was obtained as 10 times the S/N ratios from the spiked standard solutions of blank samples at the lowest concentration. $CC\alpha$ and $CC\beta$ were acquired from the testing blank samples and calculated by plotting all data obtained as described in the EC 2002/657/EC [37, 39].

3. Results and Discussion

3.1. Optimization of SSTP Sample Preparation

3.1.1. Sample Weight

First, the optimal sample weight for the method was determined because of the peculiar characteristics of SSTP samples. When SSTP samples were analyzed using the conventional method, no chromatographic peaks (including IS peaks) were detected, indicating that derivatization did not occur in the SSTP samples with the conventional method. SSTP is a hot-air-dried product that is nutrient and mineral-dense. SSTP is prepared from the whole body, including bones, of SST. Therefore, the lack of reaction is assumed to be a result of the unique characteristics of SSTP. Thus, finding the optimal sample weight is a reasonable first step. The studied sample weights were 0.2, 0.5, 1.0, 2.0, and 5.0g (conventional sample weight) of SSTP using the fortified standard solution at a concentration of $10\mu\text{gkg}^{-1}$.

Presumably, hydrolysis and derivatization reactions do not occur for samples weighing 2.0 to 5.0g. On the basis of the pH results, a pH around 5.2 or greater cannot induce sufficient hydrolysis and derivatization in the SSTP matrix for detection. At a pH of 5, the density of the mineral content could be the reason for the nonreactiveness, but further research is required to determine this. The peaks could only be observed with efficient recoveries below these weights when using the conventional method. The samples weighing 0.2, 0.5, and 1.0g yielded similar recoveries for the four target analytes. However, the use of very small samples in the field could introduce errors. Thus, we aimed to balance the use of a truly representative sample without reducing the sample weight excessively. Therefore, a sample weight of 1.0g was chosen, as shown in Figure 1.

3.2. Optimization of Reaction Conditions (Temperature and pH) Using Real Incurred SSTP Samples

Efficient analytical methods for NFMs based on Raman spectrometry and enzyme-linked immunosorbent assays have been investigated but have focused on optimizing the reaction temperature following the published HPLC and LC-MS/MS methods [31–34]. In this part of the study, we aimed to develop a practical, convenient, and facile method for SSTP analysis.

The results of the search for the optimal acid volume and concentration and temperature of the incurred SSTP are shown in Figures 2 and 2. The change in pH during the reaction with 2-NBA was measured over time to optimize the reaction time. The temperature is also a key reaction parameter. The derivatization of NFMs with 2-NBA has been reported to be optimal at 37°C [2, 3, 15–18, 21–24, 26, 35]. However, a recent report shows significant differences ($P<0.05$) in peak areas of four derivatized NFMs when the reaction temperature exceeded temperatures of 50, 60,

and 40°C [36]. Considering earlier works and the results of our pilot study prior to this work, temperatures of 37 and 45°C were used in this study.

The detected concentrations of 2-NP-AOZ in the incurred samples using 10, 20, 30, and 50 mL of 0.125 M HCl at 37°C are shown as a bar graph in Figure 2. The experiment using 50 mL of 0.125 M HCl was stopped during preparation because of excess errors arising from the large volume of the solution. For the remaining samples, the pH value increased slightly with time, but the most noticeable change was from 3 to 5 h, as shown by the line plot. When using 10, 20, and 30 mL of 0.125 M HCl, the pH changed from 4.3 to 5.2. Within this pH range, derivatization and hydrolysis are possible, but the reaction rate could be reduced. In muscle tissue samples, protein hydrolysis and NFM detachment from protein occur simultaneously with an increase in the pH value [32, 40–42].

Most published analytical methods target the analysis of tissues such as unprocessed meat and organs [32, 40–42]. These studies have reported the release of NFMs from the bound state under mildly acidic conditions followed by detection by reaction with 2-NBA to produce the derivatized form [24, 33]. Well-ground muscle tissue is slightly viscous, and proteins could be aggregated when the tissue samples are exposed to excessively strong acid solution without continuous vigorous agitation during the hydrolysis process. This can delay the hydrolysis reaction and prevent the protein from unfolding and releasing NFMs from the tissue for detection. To develop a rapid analysis method, these effects must be carefully considered. SSTP samples are well-dried powders with no viscosity or emulsification during sample preparation. Thus, the concentration of the acid can be increased slightly compared to that used for wet tissue samples.

Consequently, to develop our facile method, 0.5 M HCl and small volumes of the acid solution were tested (Figure 2). In these experiments, the pH increased slightly with time, mainly around 3 to 5 h between pH 0.9 and 3.8. The detected concentrations are not significantly different from those shown in Figure 2. However, the pH was slightly higher for the samples treated with 14, 15, and 20 mL compared to those treated with other volumes. Nevertheless, the pH was lower than those of the samples treated with 0.125 M HCl. The pH of the samples treated with 14, 15, and 20 mL volumes are similar, and the reaction occurred below pH 2.5. Thus, the use of 14 mL of 0.5 M HCl allows easy handling while achieving good performance.

3.3. Reaction Time

On the basis of the acquired results, the incurred samples were tested to optimize the reaction period using 14 mL of 0.5 M HCl at 45°C as shown in Figure 3. The amount of 2-NP-AOZ was determined in the samples after 1 to 16 h. At 1, 2, and 3 h, the detected amount increases with an increase in time. However, there was no significant change in the detected amount after 4 h. ANOVA was used for data analysis using OriginPro software (version 8, OriginLab, USA). Significance was set at $P < 0.05$, and the results are expressed as mean $\pm$ SD. Previously published methods have mainly used 16 h (overnight) for hydrolysis and derivatization with 2-NBA [2, 21, 23, 27, 28, 32, 33]. However, in our experiments, 4 h was sufficient reaction time and was selected as the optimum reaction period.

3.4. Sample Treatment Optimization

SSTP samples contain bound fatty acids, minerals, and other nutrients. To remove lipids from the SSTP samples, a single defatting process is not sufficient. However, the addition of hexane washing steps or the use of solid-phase extraction (SPE) will reduce the ease, simplicity, and cost-effectiveness of the method. Consequently, the eluate was reconstituted with 0.5 mL of mobile phase A and transferred to a 2 mL centrifuge tube and centrifuged at 11,500 rpm for 20 min at 0°C to remove lipids by freezing. Then, the clear supernatant was filtered through a 0.22 μ m PTFE syringe filter.

Concerning the abundant mineral content of the samples, centrifugation was used to isolate the aqueous layer after the derivatization and hydrolysis processes. The addition of EtOAc allowed the extraction of the target NFMs from the aqueous layer and accelerated mineral precipitation, which is visible by the naked eye after centrifugation.

3.5. Optimization of LC-MS/MS Conditions

Optimization of the MS parameters was carried out by performing a series of syringe injections with diluted standard solutions of the derivatized NFMs ($100 \mu\text{g L}^{-1}$) with 50% mobile phase B using ESI+ as the ionization source. The analysis was carried out with Analyst (version 1.6.2) to determine the parent ions and the optimal fragment voltage

for syringe injections of the tuning solution. The optimized fragment voltage was then chosen, and the precursor ion was fragmented and collected according to the change in collision energy, where the product ions were selected by the software. The most abundant ion was selected for quantification purposes. Two other transition ions were chosen for identification. MRM was used to evaluate the performance of this method with positive electrospray ionization. Each analyte was examined in ESI+ mode by monitoring the selected reaction. The most abundant and stable product ions were chosen to identify the target analytes. Table 1 lists the MRM transitions, individual declustering potentials, and collision energy voltages used in the analysis. Then, MS parameter tests were performed for all analytes at an ion source temperature (TEMP) of 600°C, an ionization spray voltage (ISV) of 5500 keV, a curtain gas (CUR) pressure of 25 kPa, a collision gas pressure of 8 kPa, an ion source gas 1 (GS 1, nebulizer gas) a pressure of 40 kPa, and an ion source gas 2 (GS 2, turbo gas) pressure of 45 kPa. The MS/MS transitions and transition ratios for derivatives of the NFMs are presented in Table 1.

3.6. Method Validation

Method validation was conducted based on EC regulations 2002/657/EC [37], as summarized in Table 2. The selectivity, specificity, and sensitivity were evaluated and were consistent with EC regulations 2002/657/EC, as shown by the good results (including at a concentration of $1 \mu\text{g kg}^{-1}$), as shown in Table 2. The linearity was evaluated using calibration curves constructed using a blank sample spiked with the target NFMs. All analytes exhibit excellent linearity in the studied concentration range, having correlation coefficients higher than 0.999. The S/N ratio was determined by comparison with the signals with the target analytes of the lowest concentration and the blank samples. The LOD and LOQ were determined from the minimum concentration, i.e., 3 and 10 times larger than the S/N, respectively [39]. The $\text{CC}\alpha$ and $\text{CC}\beta$ values were also acceptable: 0.18–0.30 and 0.30–0.34, respectively. The accuracy and precision were verified for each NFM based on their intraday and interday variabilities. The accuracy and precision were identified with each recovery and percentage root square deviation (% RSD) and determined in terms of the peak areas at four concentration levels (0.5, 1.0, 2.0, and $10.0 \mu\text{g kg}^{-1}$) assuming an acceptability criterion of $\pm 15\%$.

The intraday and interday accuracy were 82.2–102.5% and 85.2–108.1%, respectively, for all analytes. The ranges of interday mean recovery for each analyte were 92.8–101.3% for 2-NP-AHD, 92.6–98.2% for 2-NP-SEM, 85.2–108.1% for 2-NP-AOZ, and 90.4–96.5% for 2-NP-AMAZ. The result values are consistent with the legislated MRPLs for the four NFMs [40]. Chromatograms of the SSTP matrices spiked with NFMs and ISs are shown in Figure 4. Thus, our developed method enables rapid analysis of the SSTP matrix with reliable sensitivity, which has not been previously reported.

[figure omitted; refer to PDF]

3.7. Findings in Samples Obtained from Local Markets

To verify the utility of the method developed in this work, the method was applied for the determination of illegal NFs in real HFF samples. SSTP is typically encapsulated and used as an ingredient in HFFs. SST is believed to increase longevity in Asia and known to be highly nutritious, so, as a healthy food, it is traded at a high price. Primarily, it is sold to promote the health of the elderly and the growth of young children. Thirteen HFF products obtained from Internet sellers were analyzed. SEM and AOZ were detected in three samples, as shown in Table 3. In one sample (R4), SEM was detected below the LOQ. SEM was detected in three samples (C11, C12, and C13) at concentrations of 12.13, 1.02, and $0.54 \mu\text{g kg}^{-1}$, respectively, with reasonable precision. AOZ was detected in one sample (C11) at a concentration of $9.30 \mu\text{g kg}^{-1}$ with excellent precision.

Table 3

Detection of NF metabolites in HFF products purchased online.

	AOZ	AHD	SEM	AMAZ

CON ($\mu\text{g kg}^{-1}$)	STV	% RSD	CON ($\mu\text{g kg}^{-1}$)	STV	% RSD	CON ($\mu\text{g kg}^{-1}$)	STV	% RSD	CON ($\mu\text{g kg}^{-1}$)	STV	% RSD	R1
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R2
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R3
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R4
ND	ND	ND	ND	ND	ND	BL	BL	BL	ND	ND	ND	R5
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R6
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R7
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R8
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R9
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	R10
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	C11
9.30	0.17	1.86	ND	ND	ND	12.13	0.23	1.90	ND	ND	ND	C12
ND	ND	ND	ND	ND	ND	1.02	0.04	3.70	ND	ND	ND	C13

ND: not detected; BL: below the LOQ; CON: concentration; STV: standard deviation.

The results of the analysis of real product samples show that our method is suitable for the simultaneous determination of illegal NFs in HFF by routine analysis. However, as is typical of HFFs, these residues are consumed in capsule form regularly (e.g., three times a day), and there is no information regarding effects on consumers exposed to this regular pattern, which will be different from the irregular consumption of aquatic produces or meat containing residues after cooling. Thus, further research is needed in this area.

Finally, as discussed in the Introduction, proper sample preparation is key to accurate determination because of the wide sample variety. As shown in Table 3, our method can be successfully applied to commercially available HFF samples.

4. Conclusion

By using incurred SSTP samples, a fast and robust LC-MS/MS method has been developed for the simultaneous determination of NFMs in the unique, challenging SSTP matrix. The method reduced the reaction time required for derivatization and hydrolysis from 16 to 4 h using simple demineralization and freezing-defatting steps and achieved desirable reliability, sensitivity, simplicity, and time efficiency in the total test process.

The present method was applied to real SSTP samples, and the result demonstrates the strong detection and quantification of NF marker residues. Furthermore, the method is fully compliant with the recommended guidelines established by the EC regulation 2003/181/EC, as shown by experiments using real contaminated SSTP samples. In summary, this developed method is a simple, efficient, and powerful tool to improve food safety in a current blind spot in food industry regulation.

Acknowledgments

This research was supported by the "Brain Pool Program" through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (KRF: 2016H1D3A1909005) and by the Cooperative Research Program of the Centre for Companion Animal Research of the Rural Development Administration, Republic of Korea (PJ0139852020).

References

- [1] D. Maurice, L. JoAnne, "Functional foods: a conceptual definition," *British Food Journal*, vol. 110 no. 11, pp. 1133-1149, DOI: 10.1108/00070700810918036, 2008.
- [2] P.-G. Rosa, S.-G. Jesus, "A critical review of bioactive food components, and of their functional mechanisms, biological effects and health outcomes," *Current Pharmaceutical Design*, vol. 23 no. 19, pp. 2731-2741, DOI: 10.2174/1381612823666170317122913, 2017.
- [3] Z. Zhang, B. Chen, L. Yuan, C. Niu, "Acute cold stress improved the transcription of pro-inflammatory cytokines of Chinese soft-shelled turtle against *Aeromonas hydrophila*," *Developmental & Comparative Immunology*, vol. 49 no. 1, pp. 127-137, DOI: 10.1016/j.dci.2014.10.010, 2015.
- [4] J. Zhang, D. Kuang, F. Wang, "Turtles as a possible reservoir of nontyphoidal *Salmonella* in Shanghai, China," *Foodborne Pathogens and Disease*, vol. 13 no. 8, pp. 428-433, DOI: 10.1089/fpd.2015.2107, 2016.
- [5] Z. Zhang, R. Song, X. Xing, L. Wang, C. Niu, "Division of Chinese soft-shelled turtle intestine with molecular markers is slightly different from the morphological and histological observation," *Integrative Zoology*, vol. 13 no. 1, pp. 112-121, DOI: 10.1111/1749-4877.12261, 2018.
- [6] J. Yin, Y. Tezuka, L. Subehan, "A combination of soft-shell turtle powder and essential oil of a unicellular chorophyte prevents bone loss and decreased bone strength in ovariectomized rats," *Biological and Pharmaceutical Bulletin*, vol. 28 no. 2, pp. 275-279, DOI: 10.1248/bpb.28.275, 2005.
- [7] P. Sionneau, Pao Zhi: *An Introduction to the Use of Processed Chinese Medicinals*, 1995.
- [8] S. Haitao, J. F. Parham, F. Zhiyong, H. Meiling, Y. Feng, "Evidence for the massive scale of turtle farming in China," *Oryx*, vol. 42 no. 1, DOI: 10.1017/s0030605308000562, 2008.
- [9] T. P. Van Boeckel, C. Brower, M. Gilbert, "Global trends in antimicrobial use in food animals," *Proceedings of the National Academy of Sciences*, vol. 112 no. 18, pp. 5649-5654, DOI: 10.1073/pnas.1503141112, 2015.
- [10] E. Ryu, J.-S. Park, S. S. Giri, S. C. Park, "A simplified modification to rapidly determine the residues of nitrofurans and their metabolites in aquatic animals by HPLC triple quadrupole mass spectrometry," *Environmental Science and Pollution Research*, vol. 28 no. 6, pp. 7551-7563, DOI: 10.1007/s11356-020-11074-7, 2021.
- [11] L. Rodziewicz, "Determination of nitrofurantoin metabolites in milk by liquid chromatography-electrospray ionization tandem mass spectrometry," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 864 no. 1-2, pp. 156-160, DOI: 10.1016/j.jchromb.2008.01.008, 2008.
- [12] C. Douny, J. Widart, E. De Pauw, "Development of an analytical method to detect metabolites of nitrofurans," *Aquaculture*, vol. 376-379, pp. 54-58, DOI: 10.1016/j.aquaculture.2012.11.001, 2013.
- [13] World Health Organisation, "Evaluation of certain veterinary drug residues in food (thirty-fourth report of the joint FAO/WHO expert committee on food additives," 1989. WHO Technical Report Series No. 788
- [14] World Health Organisation, "Evaluation of certain veterinary drug residues in food (fortieth report of the joint FAO/WHO expert committee on food additives," 1993. WHO Technical Report Series No. 832
- [15] Commission Regulation (EC), "No 1442/95 of 26 June 1995 amending Annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin," *Official Journal of the European Communities*, vol. 143, 1995.
- [16] N. Gao, Y.-C. Ni, J. R. Thornton-Manning, P. P. Fu, R. H. Heflich, "Mutagenicity of nitrofurantoin and furazolidone in Chinese hamster ovary cell strains," *Mutation Research Letters*, vol. 225 no. 4, pp. 181-187, DOI: 10.1016/0165-7992(89)90117-6, 1989.
- [17] X. Jin, S. Tang, Q. Chen, "Furazolidone induced oxidative DNA damage via up-regulating ROS that caused cell

- cycle arrest in human hepatoma G2 cells," *Toxicology Letters*, vol. 201 no. 3, pp. 205-212, DOI: 10.1016/j.toxlet.2010.12.021, 2011.
- [18] E. Sbardella, "Toxicological evaluation of nitrofurazone and furaltadone on *Selenastrum capricornutum*, *Daphnia magna*, and *Musca domestica*," *Ecotoxicology and Environmental Safety*, vol. 8 no. 2, pp. 101-105, DOI: 10.1016/0147-6513(84)90053-8, 1984.
- [19] D. R. McCalla, "Mutagenicity of nitrofurans derivatives: review," *Environmental Mutagenesis*, vol. 5 no. 5, pp. 745-765, DOI: 10.1002/em.2860050512, 1983.
- [20] Commission Decision, "2003/181/EC: Commission Decision of 13 March 2003 amending decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin," *Official Journal of the European Union*, vol. 15, 2003.
- [21] L. Hartig, K. v. Czapiewski, "Detecting nitrofurans metabolites in animal products using LC/MS/MS," *Spectroscopy Europe*, vol. 17 no. 3, pp. 21-23, 2005.
- [22] K. Terato, C. T. Do, D. Cutler, T. Waritani, H. Shionoya, "Preventing intense false positive and negative reactions attributed to the principle of ELISA to re-investigate antibody studies in autoimmune diseases," *Journal of Immunological Methods*, vol. 407, pp. 15-25, DOI: 10.1016/j.jim.2014.03.013, 2014.
- [23] Dosic, Hot Air Dried Turtle Powder, 2020.
- [24] S.-P. Khong, E. Gremaud, J. Richoz, "Analysis of matrix-bound nitrofurans residues in worldwide-originated honeys by isotope dilution high-performance liquid Chromatography–Tandem mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 52 no. 17, pp. 5309-5315, DOI: 10.1021/jf0401118, 2004.
- [25] P.-S. Chu, M. I. Lopez, "Determination of nitrofurans residues in milk of dairy cows using liquid chromatography–Tandem mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 55 no. 6, pp. 2129-2135, DOI: 10.1021/jf063391v, 2007.
- [26] Y. Zhang, H. Qiao, C. Chen, Z. Wang, X. Xia, "Determination of nitrofurans metabolites residues in aquatic products by ultra-performance liquid chromatography-tandem mass spectrometry," *Food Chemistry*, vol. 192, pp. 612-617, DOI: 10.1016/j.foodchem.2015.07.035, 2016.
- [27] P.-S. Chu, M. I. Lopez, "Liquid Chromatography–Tandem mass spectrometry for the determination of protein-bound residues in shrimp dosed with nitrofurans," *Journal of Agricultural and Food Chemistry*, vol. 53 no. 23, pp. 8934-8939, DOI: 10.1021/jf051615o, 2005.
- [28] R. H. Stadler, L. Verzeznassi, W. Seefelder, L. Racault, "Why semicarbazide (SEM) is not an appropriate marker for the usage of nitrofurazone on agricultural animals," *Food Additives & Contaminants: Part A*, vol. 32 no. 11, pp. 1842-1850, DOI: 10.1080/19440049.2015.1086028, 2015.
- [29] J.-W. Kwon, "Semicarbazide: natural occurrence and uncertain evidence of its formation from food processing," *Food Control*, vol. 72, pp. 268-275, DOI: 10.1016/j.foodcont.2016.02.022, 2017.
- [30] J. Barbosa, A. Freitas, J. L. Mourão, M. I. Noronha da Silveira, F. Ramos, "Determination of furaltadone and nifursol residues in poultry eggs by liquid chromatography-electrospray ionization tandem mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 60 no. 17, pp. 4227-4234, DOI: 10.1021/jf205186y, 2012.
- [31] C. Bock, P. Gowik, C. Stachel, "Matrix-comprehensive in-house validation and robustness check of a confirmatory method for the determination of four nitrofurans metabolites in poultry muscle and shrimp by LC-MS/MS," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 856 no. 1-2, pp. 178-189, DOI: 10.1016/j.jchromb.2007.05.044, 2007.
- [32] P. Mottier, S.-P. Khong, E. Gremaud, "Quantitative determination of four nitrofurans metabolites in meat by isotope dilution liquid chromatography-electrospray ionisation-tandem mass spectrometry," *Journal of Chromatography A*, vol. 1067 no. 1-2, pp. 85-91, DOI: 10.1016/j.chroma.2004.08.160, 2005.
- [33] H. An, M. Henry, T. Cain, B. Tran, H. C. Paek, D. Farley, "Determination of total nitrofurans metabolites in shrimp muscle using liquid chromatography/tandem mass spectrometry in the atmospheric pressure chemical ionization mode," *Journal of AOAC International*, vol. 95 no. 4, pp. 1222-1233, DOI: 10.5740/jaoacint.11-305, 2012.
- [34] MFDS, Korean Food Code, Chapter 9. Common test method, Article 1. Test method for food constituents.

Available from: <http://www.mfds.go.kr> , Ministry of Food and Drug Safety (2016-154) (2016) page 1106.

[35] L. A. P. Hoogenboom, M. C. J. Berghmans, T. H. G. Polman, R. Parker, I. C. Shaw, "Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs," *Food Additives and Contaminants*, vol. 9 no. 6, pp. 623-630, DOI: 10.1080/02652039209374117, 1992.

[36] J. K. Finzi, J. L. Donato, M. Sucupira, G. De Nucci, "Determination of nitrofurantoin metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 824 no. 1-2, pp. 30-35, DOI: 10.1016/j.jchromb.2005.05.012, 2005.

[37] Commission Decision, Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (2002/657/EC), 2002.

[38] United States Pharmacopeia, Validation of Compendial Methods, 2003.

[39] B. Magnusson, U. Örnemark, The Fitness for Purpose of Analytical Methods—A Laboratory Guide to Method Validation and Related Topics, A Laboratory Guide to Method Validation and Related Topics, 2014.

[40] R. J. McCracken, D. G. Kennedy, "Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography-thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 691 no. 1, pp. 87-94, DOI: 10.1016/S0378-4347(96)00448-3, 1997.

[41] A. Conneely, A. Nugent, M. O'Keeffe, "Isolation of bound residues of nitrofurantoin drugs from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection," *Analytica Chimica Acta*, vol. 483 no. 1-2, pp. 91-98, DOI: 10.1016/S0003-2670(02)01023-1, 2003.

[42] L. A. P. Hoogenboom, M. Van Kammen, M. C. J. Berghmans, J. H. Koeman, H. A. Kuiper, "The use of pig hepatocytes to study the nature of protein-bound metabolites of furazolidone: a new analytical method for their detection," *Food and Chemical Toxicology*, vol. 29 no. 5, pp. 321-328, DOI: 10.1016/0278-6915(91)90203-j, 1991.

DETAILS

Subject:	Laboratories; Farm buildings; Food products; Reproducibility; Animals; Linearity; Turtles; Metabolites; Antibiotics; Methods; Hazardous materials; Immunoassay; Natural & organic foods; Correlation coefficients; Cell cycle; Functional foods & nutraceuticals
Location:	United States--US; Germany
Editor:	Charles L Wilkins
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York

Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-06-22 (Received); 2021-01-14 (Revised); 2021-02-24 (Accepted); 2021-03-09 (Pub)
DOI:	https://doi.org/10.1155/2021/8822448
ProQuest document ID:	2503352241
Document URL:	https://www.proquest.com/scholarly-journals/development-validation-lc-ms-method-determination/docview/2503352241/se-2?accountid=211160
Copyright:	Copyright © 2021 EunChae Ryu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-05-21
Database:	Publicly Available Content Database

Document 48 of 59

Rapid Determination of Pemetrexed Concentration and Distribution in Human Breast Cancer Cells (McF-7) Based on UHPLC-MS/MS

Zhang, Mengwei ¹ ; Wang, Zhipeng ²
; Zhang, Qiqiang ³ ; Ye, Qing ³ ; Xu, Fengjing ² ; Cui, Lili ² ; Liu, Yanping ² ; Gao, Shouhong ²
; Chen, Wansheng ²
; Liu, Yan ³

¹ Department of Pharmacy, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China; Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China; Key Laboratory for Research on Active Ingredients in Natural

ABSTRACT (ENGLISH)

Cell is the basic unit of structure and function of all living bodies. The study of intracellular drug concentration distribution is helpful to understand the drug efficacy of target site. Pemetrexed is a new multitarget folate antagonist with pyrrolidine group as its core structure. An ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was developed for rapid quantification of pemetrexed concentration in human breast cancer cells (MCF-7). Sample pretreatment was completed by protein precipitation using methanol. The optimized chromatographic separation was achieved on a ZORBAX SB-C₁₈ column (2.1 × 150 mm, 3.5 μm). The column was equilibrated with initial mobile phase and eluted under gradient phases containing 0.1% formic acid in water (phase A) and in acetonitrile (phase B). The gradient program started at 5% B, increased to 95% B in 2 min, and then held at 95% B for 1.5 min. The linear range of pemetrexed in cells and nucleus was 2.0–200.0 ng/mL, while in the medium sample it was 50.0–5000.0 ng/mL, and the correlation coefficients were all greater than 0.99. The recovery was 47.50–67.55% (2.0–200.0 ng/mL) and 82.72–97.15% (50.0–5000.0 ng/mL), and the matrix effect was 98.10–100.62% (2.0–200.0 ng/mL) and 89.78–97.65% (50.0–5000.0 ng/mL). Interday precision and intraday precision (RSD%) were less than 15.0% (for LLOQ, less than 20%), and accuracy (RE%) was within ±15%; the deviation of stability was within ±15%, all meeting the requirements of biological sample analysis. The results of intracellular samples showed that the concentration of pemetrexed reached its peak at 3 h after administration. The concentration of pemetrexed in the nucleus continued to increase 2 h after administration and may have reached the maximum concentration at 6 h.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Breast cancer has become a serious disease endangering women's health all over the world. According to the 2020 global cancer report [1] released by the international agency for research on cancer, in 2018, the incidence of breast cancer in females ranked first among malignant tumors (11.6%) and the mortality ranked fifth among malignant tumors (6.6%). The National Cancer Report [2] 2019 shows that, in 2015, the incidence of breast cancer ranked the first among female malignant tumors in China, with about 304,000 new cases, and the mortality ranked the fifth among female malignant tumors (8.16%), with both morbidity and mortality, showing an increasing trend in recent years.

At present, the treatment of breast cancer is still mainly through surgery and chemotherapy [3]. Efficacious chemotherapeutic regimen scan reduces the metastasis and recurrence, thereby increasing the possibility of extending survival time [4]. Nowadays, the chemotherapeutic agents included anthracycline, paclitaxol, platinum, and antimetabolism medicines, etc. [5]. Although impressive improvements have been made in adjuvant therapy with anthracyclines and taxanes in breast cancer, development of drug resistance to these agents in recurrent tumors is common [6–8], and there are still 20%–30% of patients with primary drug resistance in clinical practice. Even if the initial treatment is effective, a vast majority of patients still have disease progression, and eventually develop metastatic disease. As a third-line chemotherapy agent, pemetrexed is a multitarget antimetabolite drug that inhibits folate metabolism and DNA synthesis enzymes. Response rates to pemetrexed as a single agent varied from 8% to 31%, and in combination regimen which was reported between 15.8% and 55.7% [9]. Collective studies

supported that pemetrexed effectively prolongs survival estimation in a proportion of advanced breast cancer patients, which was a better agent, especially followed with anthracycline- and taxane-containing regimens [10–12]. Pemetrexed has good efficacy and compliance in patients with metastatic breast cancer. However, variable treatment response of pemetrexed chemotherapy was observed in different pathological types of tumors [13]. The concentration of pemetrexed in cells is considered to be an important factor affecting the efficacy. There is an urgent need for a dose optimization of pemetrexed for metastatic breast cancer that prolongs survival and improve tolerance. Studying the distribution of intracellular drug concentration is helpful to understand the drug efficacy in target sites, but there still is not a LC-MS/MS method reported until now. Thus, this study will develop an accurate and stable method based on UHPLC-MS/MS technology that can quickly determine the concentration and distribution of pemetrexed in cells, which is helpful for deep understanding of the drug efficacy of pemetrexed.

2. Methods

2.1. Chemicals

The standards of pemetrexed (Lot: D1103AS) and nilotinib (internal standard, IS) (Lot: D1201A) were purchased from Meilun Biotech Co., Ltd. (Dalian City, China). HPLC-grade methanol and acetonitrile were obtained from Merck (Merck Company, Darmstadt, Germany). HPLC-grade dimethyl sulfoxide (DMSO) was purchased from Tedia Company Inc (Tedia, Fairfield, OH, USA). HPLC-grade isopropanol was bought from Shanghai Titan technology Co., Ltd. (Titan, Shanghai, China). HPLC-grade formic acid was from Macklin biochemical Technology Co., Ltd. (Macklin, Shanghai, China). High glucose cell culture medium was supplied by Hyclone company (Logan, Utah, USA). Distilled water was purchased from Watsons Distilled Water Co. Ltd. (Watsons, Shenzhen, China). Human breast cancer McF-7 cell line was provided by Shanghai Branch of China Science Academy (Shanghai, China). Penicillin-Streptomycin solution was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Yuanye, Shanghai, China). RIPA Lysis buffer was supplied from Shanghai Yuanye Biotechnology Co., Ltd. (Yuanye, Shanghai, China). The cell nucleus extraction kit was got from Abbkine Scientific Co., Ltd. (Wuhan, China).

2.2. Instrumentation

All experiments were carried out on an Agilent 1290 series UHPLC system including an online degasser, a quatpump, an autosampler, and a column oven and interfaced to an Agilent 6460A triplequadrupole mass spectrometer with an electrospray ionization source (ESI, Agilent Technologies, USA). All data were collected and analyzed through Agilent Masshunter workstation. Biosafety cabinet was from Thermo Scientific, Germany; inverted microscope was from Olympus, Tokyo, Japan; and temperature controlled magnetic agitator was from Abbott company, Zhengzhou, China.

2.3. Chromatographic Conditions

The ZORBAX SB-C18 column was selected as the analysis column of pemetrexed (2.1 × 150 mm, 3.5 μm, Agilent technology, USA). The mobile phase is acetonitrile and water (containing 0.1% formic acid). The mobile phase consisted of acetonitrile as organic phase (phase A) and water (containing 0.1% formic acid) as aqueous phase (phase B). The mobile phases were degassed automatically using the online degasser system, and the flow rate was 0.3 mL/min. The gradient program started at 5% A, and increased to 95% A in 2 min, and then was held at 95% A for 1.5 min, the pastime was 2 min, and the total run time was 5.5 min. The column temperature was maintained at 35°C, and the injection volume was set at 5 μL.

2.4. Mass Spectrum Parameters

In positive ion mode, ESI ion source was selected for mass spectrometry detection. Data scanning was performed in multiple reaction monitoring (MRM) mode (Figure 1). The dry gas, sheath gas, and nebulizer gas are all high pure nitrogen. The drying temperature was 325°C, and the drying gas flow rate was 10 L/min. The temperature of sheath gas was 350°C, and the flow rate of sheath gas was 12 L/min. The capillary voltage was 4000 V and the nebulizer pressure was 50 psi. The collision gas was high purity nitrogen and was set at 0.2 mPa. The main mass spectral parameters of pemetrexed and internal standard (IS, nilotinib) are shown in Table 1.

[figures omitted; refer to PDF]

Table 1

Main mass spectral parameters of pemetrexed and IS.

Name	Ionization type	Precursor ions (m/z)	Fragmentor (V)	Collision energy (eV)	Product ions (m/z)
Pemetrexed	+	428.3	80	10	281.2
Nilotinib (IS)	+	530.2	130	32	289.2

2.5. Preparation of Standard Solution

Accurately weighted 2.00mg pemetrexed was put into a 2mL volumetric flask, and an appropriate amount of methanol was added to dissolve it with a few drops of DMSO. The stock solution was aliquoted and stored in a refrigerator at -80°C . The above-mentioned stock solution was diluted with 10% methanol to obtain a series of working solutions of the following concentrations: 20.0, 50.0, 100.0, 200.0, 500.0, 1000.0, 2000.0, 5000.0, 10000.0, 20000.0, 50000.0ng/mL for pemetrexed. Two calibration curves were prepared and the working solution was diluted 10 times with blank medium to obtain the calibration sample; the concentration range of pemetrexed was 2.0–200.0 ng/mL and 50.0–5000.0ng/mL for intracellular and extracellular samples, respectively. The quality control (QC) sample of pemetrexed is prepared separately in the same way. Finally, QC samples with concentration of 100.0, 500.0, 2000.0ng/mL and 5.0, 20.0, 100.0ng/mL were obtained. The IS solution was prepared using the same method, and 2mg nilotinib was dissolved in 2mL methanol, and then diluted with methanol to obtain IS solutions with final concentrations of 2 and 20ng/mL, which were stored at -20°C for later use.

2.6. Cell Culture

The human breast cancer cell line McF-7 was cultured with DMEM containing 10% fetal bovine serum and double antibiotics (100 $\mu\text{g}/\text{mL}$ penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, complete medium) in incubator (37°C , 5% CO_2) and the medium was refreshed every 2 or 3 days. The cells were passaged or transferred to prepare stock solutions when they reach an 80–90% confluence.

2.7. Sample Collection and Pretreatment

2.7.1. Experiment Execution and Cell Samples Collection

When the cells grew to 90% confluence, the culture medium was discarded, and the cells were washed twice with 1 $\times$ PBS. Pemetrexed solution in complete medium with a final concentration of 6 $\mu\text{g}/\text{mL}$ was added to incubate the cells for 0, 0.5, 1, 2, 3, and 6h, respectively. The culture medium was harvested as the medium sample (as a reference). After PBS wash, the cells were digested with 0.25% trypsin containing EDTA for cell detachment from the growth surface. The trypsin was neutralized with complete medium, and the mixture was transferred to a 15mL centrifuge tube for centrifugation ($1000 \times g$, 5min, 4°C). The supernatant was discarded and 100 μL of RIPA lysate was added for full cleavage. The supernatant was collected as the intracellular sample after a centrifuge at $13400 \times g$ for 5min. The experiments were carried out three times in triplicate using the same method. For the nuclear sample, a parallel assay was carried out and cells were cultured and treated using the same way, and the nuclear samples were collected after the pemetrexed treatment using an extraction kit.

2.7.2. Sample Pretreatment

Sample pretreatment was performed by ultrafiltration-protein precipitation method. An appropriate amount of culture medium sample was filtered through 0.22 μm microporous filter membrane, and 60 μL filtrate was transferred to a 1.5 mL Eppendorf tube prior to adding 340 μL methanol (containing 20ng/mL of IS solution). For intracellular and nuclear samples, 60 μL filtrate was transferred to a 1.5mL Eppendorf tube before adding 180 μL methanol (containing 2 ng/mL of IS solution). After vortex mixing for 3min, the mixture was centrifuged at room temperature of $13000 \times g$ for 10min. Then, 5 μL supernatant was directly injected for analysis.

2.8. Methodological Study

In order to ensure the accuracy, reliability, and stability of the determination results, the precision and accuracy,

extraction recovery and matrix effect, stability, and specificity of this method should meet the requirements of biological sample analysis. Methodological verification was conducted in accordance with the Chinese pharmacopeia (version 2015) and FDA guidelines, and the experiments were carried out as we reported previously [14, 15].

3. Results and Discussion

3.1. Chromatography Condition Optimization

During the method development, several chromatographic columns had been tested, such as Zorbax SB-C₁₈, Eclipse XDB-C₁₈, Poroshell 120 SB-C₁₈, Eclipse Plus-C₁₈, etc. By comparing their chromatographic characteristics (resolution, peak time, response, peak shape, etc.), the results showed that the pemetrexed and IS had better peak shapes and responses on Zorbax SB-C₁₈ chromatographic column and could achieve complete separation from the internal interferent substances in a short time. Therefore, Zorbax SB-C₁₈ column was selected for the development of methodology. Different mobile phase, acetonitrile and methanol, and additives including formic acid and ammonium acetate were selected to improve the peak shape, response, and retention time, but the methanol brought a longer retention time and trailing peak when ammonium acetate was added to the mobile phase, and the response of the analyte was low and the peak shape was asymmetrical. It was found that 0.1% formic acid (V:V) in the water phase could significantly increase the response of the analyte, and 0.1% formic acid (V:V) in the organic phase has a similar effect on the response of analyte [16], so a 0.1% formic acid was simultaneously added to the water and organic phase to achieve a better response of pemetrexed in the method development. In order to shorten the analysis time and optimize the gradient of the mobile phase, we slightly accelerated the increase of organic phase which could shorten the retention time of the analyte, because a quick increase of organic phase would lead to a worse peak shape of the analyte. In general, improving the ratio of the organic phase in the initial mobile phase could reduce the retention time of most analytes, but in our experiment, a higher ratio of acetonitrile damaged the symmetry of peak, so the total analytical time was 5 min and the ratio of organic phase was set to 5% in the initial mobile phase.

3.2. Sample Pretreatment

Sample pretreatment is an important step in the process of analytes extraction. The process of sample treatment has a significant impact on the experimental results, for example, reducing the matrix effect, which is indispensable to improving the precision and accuracy. Therefore, a further improvement of the resolution and sensitivity of the chromatographic system requires a more suitable sample pretreatment method. When a sample has relatively high purity and concentration of the analyte, a simpler pretreatment could meet the analyte extraction; otherwise, a complex extraction process, for instance, adsorption and enrichment, etc., is needed [17]. The main purpose of sample pretreatment is to remove proteins, some lipids, and soluble salts. At present, protein precipitation, solid-phase extraction, and liquid-liquid extraction are commonly used for sample pretreatment, and every method has its own advantages and disadvantages. The protein precipitation method is simple, fast, and economical, but there are still many impurities in the matrix after treatment, which usually bring a strong matrix effect. The sample processed using liquid-liquid extraction is clean, but the extraction recovery largely depends on the physicochemical property of analytes and the cost is higher than protein precipitation. The solid-phase extraction method often presents a stable extraction rate and clean matrix, but the cost is highest in the three methods and a sophisticated skill was necessary to gain a stable recovery. By comparing the extraction recovery and matrix effect of methanol and acetonitrile in different proportion, the results showed a higher extraction recovery when methanol was applied as precipitant and the ratio of methanol to sample is 3:1. In order to further purify the sample and reduce the contamination of cell debris in the sample, we first filtered the sample with 0.22 μ m microporous membrane and then precipitated the protein in the filtrate with methanol. The ultrafiltration combined with protein precipitation method is more suitable in this experiment.

3.3. Method Validation

3.3.1. Specificity

By comparing the chromatograms of blank samples, IS, LLOQ, and measured samples (Figure 2), there were no

interfering substances in the same retention time of pemetrexed, indicating that this method has good specificity and meets the relevant requirements.

[figures omitted; refer to PDF]

3.3.2. Linearity of Calibration Curves and LLOQ

The linear range of intracellular samples was 2–200 ng/mL and that of extracellular samples was 50–5000 ng/mL. The results of intracellular sample calibration curves are shown in Table 2. The results show that the regression type of correction curve is linear, the weight factor is $1/x^2$, and the linear correlation coefficient R^2 is greater than 0.99, which indicates that the correction curve has a good linear relationship and meets the requirements of correlation analysis. The LLOQ for intracellular samples was 2 ng/mL. The carryover of pemetrexed that needed to be investigated after the method was developed. After the calibration sample with the highest concentration of pemetrexed was detected, the blank sample was then tested, repeating for 3 cycles to observe the response of pemetrexed and IS in the blank sample. Compared with the LLOQ sample, the response of pemetrexed in the blank sample was less than 20% of that in LLOQ sample, and the response of IS in the blank sample was less than 5% of that in LLOQ sample (Figure 3) (the methodological data for the other calibration curve of 50–5000 ng/mL are shown in Supplementary 1).

Table 2

Linearity regression parameters of pemetrexed.

Analyte	Regression type	Linear range	Weighing factor	Regression equations	R^2
Pemetrexed	Linearity	2–200 ng/mL	$1/x^2$	$Y=0.003429 \cdot x+0.001325$	0.998

[figures omitted; refer to PDF]

3.3.3. Interprecision and Intraprecision and Accuracy

QC samples of three concentrations (low, middle, and high) and LLOQ were selected to investigate the precision and accuracy of the method. The results of intraday and interday precision and accuracy of pemetrexed are shown in Table 3. The results show that the intraday precision and interday precision (RSD%) of pemetrexed were below 14.67%; the accuracy (RE%) of pemetrexed in the low, middle, and high QC samples was within $\pm 15\%$, and the accuracy (RE%) of pemetrexed in the LLOQ samples was within $\pm 20\%$, showing this method has good precision and accuracy.

Table 3

Interprecision and intraprecision and accuracy of pemetrexed ($n=5$).

Analyte	Nominal concentration (ng/mL)	Intraday			Interday		
Measured concentration (ng/mL, mean $\pm$ SD)	Precision (RSD%)	Accuracy (RE%)	Measured concentration (ng/mL, mean $\pm$ SD)	Precision (RSD%)	Accuracy (RE%)	Pemetrexed	2

1.74±0.40	14.12	92.49	1.70±0.28	14.67	85.19	5	4.84±0.65
13.43	96.78	4.50±0.60	13.40	90.08	20	19.15±0.70	3.63
95.76	17.34±1.58	9.09	86.71	100	100.51±3.14	3.12	100.51

3.3.4. Extraction Recovery and Matrix Effect

In this method, the extraction recovery and matrix effect results of pemetrexed are shown in Table 4. The results show that the extraction recovery of pemetrexed was between 47.50% and 67.55%, and the matrix effect was between 98.10% and 100.62%. In addition, the RSD of pemetrexed extraction recovery and matrix effect were less than 15%, which indicated that the extraction recovery and matrix effect of pemetrexed were stable.

Table 4

The extraction recovery and matrix effect results of pemetrexed.

Analyte	Spiked concentration (ng/mL)	Recovery		Matrix effect	
Mean (%) ±SD	RSD (%)	Mean (%)±SD	RSD (%)	Pemetrexed	5
63.55±0.12	9.25	98.10±0.05	5.32	100	47.50±0.01

3.3.5. Stability

Stability investigation includes long-term stability, short-term stability, and freeze-thaw stability. Long-term stability refers to the stability of the QC sample after 3 months in a refrigerator at -80°C; short-term stability is the stability of the QC sample after being placed in the automatic sampler at 4°C for 12 hours; freeze-thaw stability was assessed using QC sample after three freeze-thaw cycles. The stability of pemetrexed under different conditions was investigated at low and high concentration and the results are shown in Table 5. The results show that the accuracy (RE%) of pemetrexed ranged from 87.35% to 96.80%, indicating that pemetrexed was stable in the conditions mentioned above.

Table 5

Results of stability investigation of pemetrexed (n=6).

Analyte	Nominal concentration (ng/mL)	Freeze-thaw stability	24 h in autosampler	Long-term stability (3 months)

Measured concentration (ng/mL)	Accuracy (RE%)	Measured concentration (ng/mL)	Accuracy (RE%)	Measured concentration (ng/mL)	Accuracy (RE%)	Pemetrexed	5
4.52	90.40	4.84	96.80	4.47	89.40	100	88.29

3.4. The Concentration of Pemetrexed in McF-7 Cell and Medium

The measured results of culture medium samples, intracellular samples, and nuclear samples are shown in Table 6. The results of cell samples show that the concentration of pemetrexed in cells reached its peak at 3h after administration. The concentration of pemetrexed in the nucleus continued to increase 2h after administration and reached the maximum concentration at 6h. The pemetrexed was usually administrated with 500mg/m² as a third-line chemotherapeutic drug in breast cancer treatment on day 1 in a three-week course. Both the uptake and elimination process in cancer cell had certain influence on the intracellular concentration. Some research reported that the overexpression of TP-binding cassette (ABC) superfamily, including P-glycoproteins (P-gp/ABCB1), breast cancer resistance proteins (BCRP, also named ABCG2), and ABCC subfamily (such as ABCC1-12), significantly decreased the intracellular concentration of pemetrexed based on accelerated drug efflux [18–20], which was an important factor for drug resistance of breast cancer to pemetrexed [21]. Our results presented the concentration variation of pemetrexed in McF-7 cells, which may be a hint to drug combination and dose optimization to achieve a synergistic effect, and finally a better response.

Table 6

The concentration variations of pemetrexed in medium, cytoplasm, and cell nucleus ($n=3$, mean $\pm$ SD).

Time (h)	Intracellular sample concentration (ng/mL)	Nuclear sample concentration (ng/mL)	Medium sample concentration (ng/mL)
0	ND	ND	ND
0.5	4.24 $\pm$ 0.53	3.60 $\pm$ 0.04	1689.69 $\pm$ 83.79
1	7.45 $\pm$ 2.53	4.25 $\pm$ 0.01	1793.39 $\pm$ 18.75
2	19.94 $\pm$ 1.26	6.52 $\pm$ 0.34	1742.55 $\pm$ 24.40
3	41.2 $\pm$ 0.76	4.98 $\pm$ 1.87	1741.00 $\pm$ 42.47
6	35.32 $\pm$ 0.64	29.10 $\pm$ 0.66	1468.20 $\pm$ 43.84

ND: not detected.

4. Conclusion

In this study, a sensitive and reliable UHPLC-MS/MS method was established, which can quickly determine the content of pemetrexed in cells. The pretreatment method, ultrafiltration-protein precipitation, is simple, rapid, and economical for the pemetrexed extraction for from cells, and the results presented a good specificity. The linear range, LLOQ, extraction recovery, matrix effect, interday and intraday precision and accuracy, stability, and so on of this method all met the requirements of biological sample analysis. The results of cell samples showed that the

concentration of pemetrexed reached its peak 3h after administration. The concentration of pemetrexed in the nucleus increased from 2h to 6h and may continue to accumulate inside the nucleus. Further study about the concentration and distribution of pemetrexed in cells will be helpful to understand the therapeutic effect of pemetrexed.

Authors' Contributions

Mengwei Zhang and Zhipeng Wang contributed equally to this work.

References

- [1] C. P. Wild, E. Weiderpass, B. W. Stewart, *Cancer Research for Cancer Prevention*, 2020.
<http://publications.iarc.fr/586>
- [2] R. S. Zheng, K. X. Sun, S. W. Zhang, "An analysis of the prevalence of malignant tumors in China in 2015," *Chinese Journal of Oncology*, vol. 43 no. 1, pp. 19-28, 2019.
- [3] F. Bai, Y. Yin, T. Chen, "Development of liposomal pemetrexed for enhanced therapy against multidrug resistance mediated by ABCB5 in breast cancer," *International Journal of Nanomedicine*, vol. 13, pp. 1327-1339, DOI: 10.2147/ijn.s150237, 2018.
- [4] S. H. A. N. Fei, L. I. U. Yu-Lin, Q. I. A. N. G. Wang, "Thymidylate synthase predicts poor response to pemetrexed chemotherapy in patients with advanced breast cancer," *Oncology Letters*, vol. 16, pp. 3274-3280, 2018.
- [5] Q.-Q. Deng, X.-E. Huang, L.-H. Ye, Y.-Y. Lu, Y. Liang, J. Xiang, "Phase II trial of loubio (lobaplatin) and pemetrexed for patients with metastatic breast cancer not responding to anthracycline or taxanes," *Asian Pacific Journal of Cancer Prevention*, vol. 14 no. 1, pp. 413-417, DOI: 10.7314/apjcp.2013.14.1.413, 2013.
- [6] A. Sheri, S. Johnston, "New developments and future directions in systemic therapy," *Clinical Oncology*, vol. 25 no. 2, pp. 117-126, DOI: 10.1016/j.clon.2012.10.007, 2013.
- [7] V. Kataja, M. Castiglione, "Locally recurrent or metastatic breast cancer: ESMO Clinical Recommendations for diagnosis, treatment and follow-up," *Annals of Oncology*, vol. 19 no. suppl 2, pp. ii11-ii13, DOI: 10.1093/annonc/mdn072, 2008.
- [8] D. M. Parkin, F. Bray, J. Ferlay, P. Pisani, "Global cancer statistics, 2002," *CA: A Cancer Journal for Clinicians*, vol. 55 no. 2, pp. 74-108, DOI: 10.3322/canjclin.55.2.74, 2005.
- [9] L.-Y. Zhou, Y.-H. Shi, Y.-S. Jia, "Potential role of pemetrexed in metastatic breast cancer patients pretreated with anthracycline or taxane," *Chronic Diseases and Translational Medicine*, vol. 23 no. 1, pp. 27-35, DOI: 10.1016/j.cdtm.2015.02.008, 2015.
- [10] A. Garin, A. Manikhas, M. Biakhov, "A phase II study of pemetrexed and carboplatin in patients with locally advanced or metastatic breast cancer," *Breast Cancer Research and Treatment*, vol. 110 no. 2, pp. 309-315, DOI: 10.1007/s10549-007-9722-5, 2008.
- [11] A. Llombart-Cussac, M. Theodoulou, K. Rowland, "Pemetrexed in patients with locally advanced or metastatic breast cancer who had received previous anthracycline and taxane treatment: phase II study," *Clinical Breast Cancer*, vol. 7 no. 5, pp. 380-385, DOI: 10.3816/cbc.2006.n.054, 2006.
- [12] C. Dittich, E. Solska, A. Manikhas, "A phase II multicenter study of two different dosages of pemetrexed given in combination with cyclophosphamide as first-line treatment in patients with locally advanced or metastatic breast cancer," *Cancer Investigation*, vol. 30 no. 4, pp. 309-316, DOI: 10.3109/07357907.2012.658938, 2012.
- [13] N. J. Robert, P. R. Conkling, M. A. O'Rourke, "Results of a phase II study of pemetrexed as first-line chemotherapy in patients with advanced or metastatic breast cancer," *Breast Cancer Research and Treatment*, vol. 126 no. 1, pp. 101-108, DOI: 10.1007/s10549-010-1286-0, 2011.
- [14] Z. Wang, Y. Yang, F. Zhang, "A direct, sensitive and efficient method for determination of alpha-fluoro-beta-alanine in urine: evaluating the influence of magnesium isoglycyrrhizinate on excretion in rat model," *Journal of Chromatography B*, vol. 1102-1103, pp. 17-22, DOI: 10.1016/j.jchromb.2018.10.016, 2018.
- [15] S. Gao, Z. Tao, J. Zhou, "One-step solid extraction for simultaneous determination of eleven commonly used anticancer drugs and one active metabolite in human plasma by HPLC-MS/MS," *Journal of Analytical Methods in Chemistry*, vol. 2018, DOI: 10.1155/2018/7967694, 2018.

- [16] J. Zhou, S. Gao, F. Zhang, "Liquid chromatography-tandem mass spectrometry method for simultaneous determination of seven commonly used anticancer drugs in human plasma," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 1, 2012.
- [17] Y. Liu, Z. Rong, X. Dong, "Detection technologies and metabolic profiling of bile acids: a comprehensive review," *Lipids in Health and Disease*, vol. 17, DOI: 10.1186/s12944-018-0774-9, 2018.
- [18] M. M. Gottesman, T. Fojo, S. E. Bates, "Multidrug resistance in cancer: role of ATP-dependent transporters," *Nature Reviews Cancer*, vol. 2 no. 1, pp. 48-58, DOI: 10.1038/nrc706, 2002.
- [19] A. W. Ravna, I. Sylte, G. Sager, "A molecular model of a putative substrate releasing conformation of multidrug resistance protein 5 (MRP5)," *European Journal of Medicinal Chemistry*, vol. 43 no. 11, pp. 2557-2567, DOI: 10.1016/j.ejmech.2008.01.015, 2008.
- [20] M. Dean, T. Fojo, S. Bates, "Tumour stem cells and drug resistance," *Nature Reviews Cancer*, vol. 5 no. 4, pp. 275-284, DOI: 10.1038/nrc1590, 2005.
- [21] J. Liang, T. Lu, Z. Chen, C. Zhan, Q. Wang, "Mechanisms of resistance to pemetrexed in non-small cell lung cancer," *Translational Lung Cancer Research*, vol. 8 no. 6, pp. 1107-1118, DOI: 10.21037/tlcr.2019.10.14, 2019.

DETAILS

Subject:	Mass spectrometry; Acetonitrile; Formic acid; Womens health; Metastasis; Cancer therapies; Mortality; Nuclei (cytology); Stability analysis; Females; High performance liquid chromatography; Penicillin; Tumors; Pretreatment; Correlation coefficients; Cell culture; Chemotherapy; Breast cancer; Drug resistance
Location:	United States--US; Germany; China
Editor:	Barbara Bojko
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English

Document type: Journal Article

Publication history :

Milestone dates: 2020-12-13 (Received); 2021-02-07 (Revised); 2021-03-02 (Accepted); 2021-03-13 (Pub)

DOI: <https://doi.org/10.1155/2021/6614332>

ProQuest document ID: 2503352218

Document URL: <https://www.proquest.com/scholarly-journals/rapid-determination-pemetrexed-concentration/docview/2503352218/se-2?accountid=211160>

Copyright: Copyright © 2021 Mengwei Zhang et al. This is an open access article distributed under the Creative Commons Attribution License (the “License”), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-04-01

Database: Publicly Available Content Database

Document 49 of 59

Study of Zuojin Pill in Treating Chronic Atrophic Gastritis by UPLC-Q-TOF/MS Based on Serum and Urine Metabolomics Combined with Network Pharmacology

Wu, Shihua ¹

; Chen, Xing ¹ ; Liu, Honghong ² ; Wang, Ruilin ² ; Li, Jianyu ² ; Wen, Jianxia ¹ ; Yang, Tao ¹ ; Wei, Ying ¹ ; Ren, Sichen ¹ ; Wei, Shizhang ¹ ; Manyi Jing ³ ; Li, Haotian ³ ; Wang, Min ³ ; Xia, Houlin ⁴ ; Zhao, Yanling ³

¹ College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China; Department of Pharmacy, The Fifth Medical Center of PLA General Hospital, Beijing, China ² Integrative Medical Center, The Fifth Medical Center of PLA General Hospital, Beijing, China ³ Department of Pharmacy, The Fifth Medical Center of PLA General Hospital, Beijing, China ⁴ College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Zuojin Pill (ZJP) is widely used for the treatment of gastrointestinal diseases, while its specific mechanism has not been systematically investigated. The aim of this study was to explore the therapeutic effects and potential mechanism of ZJP in chronic atrophic gastritis (CAG) through UPLC-Q-TOF/MS-based metabolomics combined with network pharmacology. ZJP and omeprazole significantly reduce contents of IL-1 β , IL-6, IL-10, and iNOS and improve pathological characteristics. Metabolomic results indicated that the therapeutic effects of ZJP were mainly related to ten metabolites, namely, choline, L-threonine, hydroxypyruvic acid, creatine, taurine, succinic acid, cis-aconitic acid, citric acid, succinic acid semialdehyde, and uric acid. Pathway analysis showed that the treatment of CAG by ZJP was associated with taurine and hypotaurine metabolism; glyoxylate and dicarboxylate metabolism; glycine, serine, and threonine metabolism; glycerophospholipid metabolism; citrate cycle (TCA cycle), alanine, aspartate, and glutamate metabolism; butanoate metabolism; and purine metabolism. Validation of metabolic markers and key targets of network pharmacology through RT-PCR analysis showed that ZJP significantly downregulated a series of inflammatory markers, such as MAPK1, PKIA, RB1, SCN5A, RXRA, E2F1, PTGS1, IGF2, ADRB1, ADRA1B, PTGS2, and GABRA1. This study was the first to use a combination of metabolomics and network pharmacology to clarify the therapeutic effects of ZJP on CAG and the regulation of multiple metabolic pathways.

FULL TEXT

DETAILS

Subject:	Cancer; Markers; Uric acid; Discriminant analysis; Disease; Succinic acid; Nuclear magnetic resonance--NMR; Cytokines; Creatine; Pharmacology; Urine; Scientific imaging; Metabolites; Metabolism; Chromatography; Chinese medicine; Citric acid; Stomach; Mass spectrometry; Principal components analysis; Ulcers; Choline; Biomarkers; Glycine; Alanine
Location:	Beijing China; United States--US; China
Company / organization:	Name: Agilent Technologies Inc; NAICS: 334515, 334516, 334519, 339112, 513210
Editor:	Mohamed Abdel-Rehim
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry

ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-11-02 (Received); 2021-02-06 (Revised); 2021-02-19 (Accepted); 2021-03-01 (Pub)
DOI:	https://doi.org/10.1155/2021/6649600
ProQuest document ID:	2501177444
Document URL:	https://www.proquest.com/scholarly-journals/study-zuojin-pill-treating-chronic-atrophic/docview/2501177444/se-2?accountid=211160
Copyright:	Copyright © 2021 Shihua Wu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2022-07-14
Database:	Publicly Available Content Database

Document 50 of 59

HPLC-MS/MS-Mediated Analysis of the Pharmacokinetics, Bioavailability, and Tissue Distribution of Schisandrol B in Rats

Liang, Dahu ¹

; Wu, Zijiang ² ; Liu, Yanhao ³ ; Li, Chao ³ ; Li, Xianghong ¹ ; Yang, Bin ¹ ; Xie, Haitang ¹ ; Sun, Hua ¹

¹ Anhui Provincial Center for Drug Clinical Evaluation, Yijishan Hospital of Wannan Medical College, Wuhu 241001, Anhui, China ² Department of Pharmacy, Bengbu First People's Hospital, Bengbu 233000, China ³ Wannan Medical College, Wuhu 241000, Anhui, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Schisandrol B, a lignan isolated from dried *Schisandra chinensis* fruits, has been shown to exhibit hepatoprotective, cardioprotective, renoprotective, and memory-enhancing properties. This study sought to design a sensitive and efficient HPLC-MS/MS approach to measuring Schisandrol B levels in rat plasma and tissues in order to assess the pharmacokinetics, oral bioavailability, and tissue distributions of this compound *in vivo*. For this analysis, bifendate was chosen as an internal standard (IS). A liquid-liquid extraction (LLE) approach was employed for the preparation of samples that were subsequently separated with an Agilent ZORBAX Eclipse XDB-C₁₈ (4.6 × 150 mm, 5 μm) column with an isocratic mobile phase consisting of methanol and water containing 5 mM ammonium acetate and 0.1% formic acid (90:10, v/v). A linear calibration curve was obtained over the 5–2000 ng/mL and 1–1000 ng/mL ranges for plasma samples and tissue homogenates, respectively. This established method was then successfully applied to investigate the pharmacokinetics, oral bioavailability, and tissue distributions of Schisandrol B in Sprague-Dawley (SD) rats that were intravenously administered 2 mg/kg of Schisandrol B monomer, intragastrically administered Schisandrol B monomer (10 mg/kg), or intragastrically administered 6 mL/kg SCE (equivalent to 15 mg/kg Schisandrol B monomer). The oral absolute bioavailability of Schisandrol B following intragastric Schisandrol B monomer and SCE administration was approximately 18.73% and 68.12%, respectively. Tissue distribution studies revealed that Schisandrol B was distributed throughout several tested tissues, with particular accumulation in the liver and kidneys. Our data represent a valuable foundation for future studies of the pharmacologic and biological characteristics of Schisandrol B.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Schisandra chinensis is a perennial deciduous woody vine that is primarily found in China, Japan, and Korea and that is a popular Traditional Chinese Medicine (TCM) that is recorded in pharmacopeias in China, Japan, and the United States [1, 2]. Recent pharmacological research suggests that *Schisandra chinensis* contains many chemical compounds including lignans, volatile oils, organic acids, vitamins, terpenoids, and polysaccharides [3]. Of these, lignans are the primary bioactive compounds therein, accounting for up to 8% of plant biomass. These lignans exhibit a range of antitumor, anti-inflammatory, antioxidant, antithrombotic, and neuroprotective activities [4–7]. Schisandrol B is a lignan that can be isolated from dried *Schisandra chinensis* fruits and that exhibits hepatoprotective, cardioprotective, renoprotective [8–12], and memory-enhancing properties [13]. There is also prior evidence that Schisandrol B can suppress or prevent vascular fibrotic disorders at least in part via disrupting TGFβ₁-assisted NF-κB signaling [14]. In mice, Schisandrol B can also drive benign liver enlargement consistent with enhanced hepatocyte energy metabolism and energy utilization [15]. Together, these data suggest that Schisandrol B may be a pharmacologically valuable compound worthy of future research. While prior research has elucidated some of the pharmacological activities of this compound, no studies to date have comprehensively evaluated Schisandrol B monomer pharmacokinetics, bioavailability, or tissue distributions *in vivo*. As such, it is vital that a sensitive and efficient approach to measuring tissue and plasma Schisandrol B levels be developed in order to facilitate such analyses in a rat model system.

To date, several methods have been used to measure Schisandrol B levels in different sample types, including gas chromatography-mass spectrometry [16], HPLC with UV detection [17, 18], and HPLC coupled with mass spectrometry [19–24]. These approaches, however, are limited by factors including complexity, a narrow linear range, and prolonged running times. In addition, these studies have primarily evaluated Schisandrol B pharmacokinetics following the administration of complex medicinal herbal mixtures or lignan mixtures. Few studies to date have simultaneously assessed the pharmacokinetics, tissue distributions, and bioavailability of Schisandrol B *in vivo*. Herein, an HPLC-MS/MS approach was established and validated for the evaluation of these

pharmacokinetic parameters in male Sprague-Daley (SD) rats administered Schisandrol B. Together, our data represent a valuable foundation for future studies of the pharmacologic and biological properties of Schisandrol B.

2. Material and Methods

2.1. Chemicals and Reagents

Schisandra chinensis was obtained from the Yijishan Hospital of Wannan Medical College and was positively identified by Professor Lu Jianping of Wannan Medical College (Wuhu, China). Schisandrol B (>98% pure) was obtained from Shanghai Yuanye Biological Technology Co., Ltd. Internal standard (IS) bifendate (>98% pure) was procured from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). HPLC-grade formic acid, methanol, and methyl tert-butyl ether (MTBE; Merck, Germany) were used for the present study, as was analytical grade ammonium acetate. A Milli-Q water system (Millipore, USA) was employed for the preparation of dH_2O .

2.2. Apparatus

A SCIEX Triple Quad 3200 HPLC-MS/MS system (Sciex, Applied Biosystems Inc, USA) and a Shimadzu LC-20AD binary pump equipped with a SIL-HTc autosampler and CTO-10ASvp column oven (Shimadzu, Japan) were used for the present study. An Agilent ZORBAX Eclipse XDB-C₁₈ (4.6×150mm, 5μm) column was used for all chromatographic separation. A Pro 200 Hand-Held or Post-Mounted Laboratory Homogenizer (PRO Scientific Inc, USA) was used to prepare tissue homogenates.

2.3. HPLC-MS/MS Conditions

Schisandrol B chromatographic separation was carried out with a mobile phase composed of methanol with 0.1% formic acid (A) and water containing 5mM ammonium acetate and 0.1% formic acid (90:10, v/v) (B). Separation was conducted at a 0.5mL/min flow rate, while the temperature of the column and autosampler was maintained at 30°C and room temperature, respectively. A 10μL injection volume was used for these analyses. MS detection was conducted using an ESI source in positive ion mode. Ion quantification was conducted in MRM mode, with transitions of m/z 399.4368.2 for Schisandrol B and m/z 387.1328.3 for bifendate (IS). For these MS analyses, settings were as follows: Gas₁ (N₂) 40 psi; Gas₂ (N₂) 40 psi; ion spray voltage 5500V; ion source temperature 550°C; curtain gas (N₂) 25 psi. Compound-specific declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CXP) were 45, 29, 6, and 7V for Schisandrol B, and 76, 28, 9, and 6V for bifendate, respectively. Acquisition and analysis of data were evaluated with the Analyst 1.6.2 software (AB Sciex, USA).

2.4. Standard and Stock Solution Preparation

The preparation of stock solutions of Schisandrol B and IS was carried out by dissolving these standards in methanol at respective concentrations of 450 and 730μg/mL. These stock solutions were diluted as appropriate to prepare working solutions. Quality control (QC) samples and calibration standards were generated by adding appropriate working solution volumes to samples of blank plasma. For analyses of plasma samples, calibration standards were prepared at 5.0, 10.0, 50.0, 100.0, 500.0, 1000.0, and 2000.0ng/mL, while the preparation of QC samples was carried out at 15 (LQC), 150 (MQC), and 1500 (HQC) ng/mL. For analyses of tissue homogenate samples, calibration standards of seven different concentrations (1.0, 5.0, 10.0, 50.0, 100.0, 500.0, and 1000.0 ng/mL) were prepared, and QC samples were prepared at 2 (LQC), 40 (MQC), and 800 (HQC) ng/mL. Additionally, the preparation of a diluted solution (500ng/mL) of IS was carried out in methanol using the stock solution. All working solutions were kept at 4°C before use.

2.5. *Schisandra chinensis* Extract (SCE) Preparation

Schisandra chinensis was dried and crushed, and a 50g sample of the resultant powder was subjected to ultrasonic extraction in 400mL of 95% ethanol for 30 minutes. This extraction procedure was repeated twice, after which these extracts were filtered, pooled, evaporated in a rotary evaporator, and resuspended in 25mL of normal saline. The resultant SCE preparation was equivalent to 2g of crude *Schisandra chinensis* per milliliter and was stored at 4°C before use. Optimized HPLC-MS/MS conditions were used to determine that the Schisandrol B level in this prepared extract was 2.5mg/mL.

2.6. Sample Preparation

Samples of rat plasma (100 μ L) were combined with 10 μ L of the IS working solution, after which samples were shaken for 2 minutes. Next, protein was precipitated via the addition of 1 mL of methyl tert-butyl ether. IS and Schisandra B were then extracted from plasma samples via shaking for 5 minutes. Samples were then spun for 5 minutes at 4,000 rpm, and supernatants were dried at 45°C under nitrogen and were then resuspended in methanol (150 μ L) containing formic acid (0.1%). The final solution was then shaken for 2 minutes, after which it was spun for 5 minutes at 12,000 rpm. A 10 μ L volume of the resultant supernatant was then injected for HPLC-MS/MS analysis. Tissue samples were homogenized in physiological saline (1:3, w/v), after which 10 μ L of an IS solution (250 ng/mL) was combined with 100 μ L of each tissue homogenate sample. These homogenates were then processed and analyzed identically to plasma samples.

2.7. Methodological Validation

This analytical approach was validated as per the Bioanalytical Method Validation Guidance for Industry issued by the US-FDA [25] (Food and Drug Administration 2018). Key metrics used to confirm the validity of this analytical approach were specificity, selectivity, linearity, precision and accuracy, recovery, matrix effects, and stability. These analyses were conducted prior to the assessment of plasma or tissue samples.

2.7.1. Sensitivity and Specificity

For selectivity and specificity analyses, blank rat plasma or tissue homogenates and samples mixed with lower limit of quantification (LLOQ) concentrations of Schisandrol B or IS were analyzed in parallel with samples obtained from rats following oral SCE or Schisandrol B monomer administration in order to exclude the interference of endogenous substance.

2.7.2. Calibration Curves and LLOQ

Calibration curves for Schisandrol B in rat plasma (5–2000 ng/mL) and tissue homogenates (1–1000 ng/mL) were generated by plotting analyte to IS peak area ratios against expected analyte concentrations and were fitted via a weighted least-squares linear regression approach using $1/x^2$ as a weighting factor. LLOQ, described as the concentration of Schisandrol B with a S/N ratio >10, was evaluated based upon six replicate analyses with a percentage relative standard deviation (RSD) <20% and a percentage relative error (RE) not exceeding $\pm 20\%$.

2.7.3. Accuracy and Precision

Intra-day accuracy and precision values were assessed by evaluating six replicate QC samples prepared at HQC, MQC, LQC, and LLOQ concentrations on a given day, whereas inter-day accuracy and precision were calculated via evaluating these same replicate samples on three subsequent days. The overall accuracy of this analytical approach was determined based upon the calculated analyte concentration as a percentage of the expected nominal concentration. Precision was defined by RSD values. The acceptable criteria were an RSD <15% (LLOQ <20%) and an accuracy within 85–115% (LLOQ: 80–120%).

2.7.4. Recovery and Matrix Effects

Schisandrol B extraction recovery was assessed by comparing peak area values for extracted LQC, MQC, and HQC samples to the peak area of extracted plasma or tissue homogenate samples that had been spiked with the corresponding analyte. The matrix effect was determined by comparing the peak areas of Schisandrol B and IS in the spiked samples after extraction and a standard sample directly dissolved with a comparable concentration of solvent.

2.7.5. Stability

Schisandrol B stability was assessed in plasma and tissue homogenate samples by assessing QC samples that had been stored under a range of conditions. To evaluate analyte benchtop stability over the course of handling, QC samples were stored at approximately 25°C for 4 h before analysis. The stability of these samples in storage was evaluated via freezing them at -80°C for 20 days, while freeze-thaw stability was assessed by passing samples through three cycles of freezing and thawing at -80°C and approximately 25°C, respectively. Autosampler stability was assessed by analyzing QC samples that had been stored for 24 h in the autosampler.

2.8. Pharmacokinetics, Bioavailability, and Tissue Distribution Studies

Specific pathogen-free (SPF) male SD rats (180–220 g) were obtained from Qinglong Mountain Animal Breeding

Farm in Jiangning District, Nanjing, Jiangsu Province, with license number: SCXK (Su) 2017–0001. The approval for this study was provided by the Animal Ethics Committee of Wannan Medical College, and these experiments were conducted in a manner consistent with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. Animals were housed in a climate-controlled facility ($22 \pm 2^\circ\text{C}$, $50 \pm 10\%$ relative humidity) with free food and water access, and rats were fasted with free access to water for 12h prior to experimentation.

For pharmacokinetic analyses, SD rats were randomized into three treatment groups. Rats in Group 1 received an intravenous tail vein injection of Schisandrol B monomer (2mg/kg). Rats in Group 2 and Group 3 were intragastrically administered a single dose of Schisandrol B monomer (10mg/kg) or SCE (6mL/kg; equivalent to 15 mg/kg Schisandrol B), respectively. Samples of blood (250 μL) were collected from the ocular fundus vein into heparinized 2mL tubes. For Group 1, samples were collected at 0, 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 12h postinjection, while for Group 2 samples were obtained at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24h postoral administration, and for Group 3 samples were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 36h postoral administration. Blood samples were spun for 5 minutes at 4500rpm, after which plasma was collected and stored at -80°C .

For tissue distribution studies, 18 rats were randomized into six groups ($n=3/\text{group}$). Rats were orally administered Schisandrol B monomer (10mg/kg), after which the samples of heart, lung, kidney, liver, spleen, and brain tissues were obtained at 0.5, 1, 2, 4, 8, and 12h postdosing. Tissue samples were washed to remove blood using normal saline, after which they were dried using filter paper, weighed, and stored at -20°C .

2.9. Data Processing

Plasma and tissue Schisandrol B concentrations were determined using calibration curves. Average plasma pharmacokinetic parameters including the area under the curve (AUC) for concentrations versus time and the elimination half-life ($t_{1/2}$) were obtained using Phoenix WinNonlin (v.8.0, CERTARA, NJ, USA) with a noncompartment model. Following intravenous or oral administration, the maximum drug plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were determined based upon concentration-time curves. Absolute oral bioavailability (BA) was calculated with the equation $F(\%) = (\text{AUC}_{\text{ig}} / \text{AUC}_{\text{iv}}) \times (\text{Dose}_{\text{iv}} / \text{Dose}_{\text{ig}}) \times 100\%$. The concentrations of Schisandrol B in tested rat tissues were expressed in $\mu\text{g/g}$ and were calculated with the equation $C_t = C_s \times V_s / W$, where C_t represented the tissue concentration ($\mu\text{g/g}$), while C_s , V_s , and W were the concentration (ng/mL), the volume (mL), and the weight (g) of the tissue samples, respectively. Data are given as means $\pm$ SD.

3. Results and Discussion

3.1. Mass Spectrometric Detection and Chromatographic Separation

To optimize ESI conditions for this study, Schisandrol B and internal standard (IS) bifendate solutions at appropriate concentrations were injected directly into the mass spectrometer. Negative as well as positive ion modes were tested in this study, and the positive ion mode was found to yield a better signal-to-noise ratio. The primary precursor molecular ion peaks of Schisandrol B and bifendate were observed at m/z 399.4 and 387.1, respectively. The most frequently occurring and stable product ions were observed at m/z 368.2 for Schisandrol B and m/z 328.3 for bifendate in the product ion scan. We therefore selected mass transitions of m/z 399.4/368.2 for Schisandrol B and m/z 387.1/328.3 for bifendate in MRM mode (Figure 1).

[figures omitted; refer to PDF]

We initially tested both methanol-water and acetonitrile-water mobile phases to optimize chromatographic conditions. We determined that the addition of formic acid to the aqueous phase in these analyses was sufficient to improve sensitivity, while ammonium acetate improved peak shape. Methanol was found to yield a superior peak shape relative to acetonitrile and was associated with less background noise. We therefore utilized a mobile phase containing 90% methanol and 0.1% formic acid (A) and water containing 5mmol ammonium acetate and 0.1% formic acid (90:10, v/v) (B). This combination yielded optimal peak shape, lower background noise, higher intensity, and shorter running times for both Schisandrol B and IS.

3.2. Sensitivity and Specificity

The specificity and selectivity of this analytical approach for IS and Schisandrol B were next assessed.

Representative MRM chromatograms of pooled blank rat plasma and liver homogenate samples, as well as chromatograms from plasma and liver homogenate samples spiked with LLOQ Schisandrol B levels, plasma samples obtained at 1 h postdosing, and liver homogenate samples collected at 0.5h postdosing, are shown in Figure 2.

[figures omitted; refer to PDF]

No endogenous substances were found to interfere with Schisandrol B or IS retention time owing to the elevated level of MRM selectivity.

3.3. Linearity and LLOQ

Calibration curves for homogenates (plasma and tissue) were found to exhibit significant linearity in the 5–2000 ng/mL and 1–1000 ng/mL ranges, respectively. The LLOQ for Schisandrol B in rat plasma and tissue homogenates was 5 ng/mL and 1 ng/mL, respectively, as depicted in Table 1. These values were sufficient to facilitate pharmacokinetic analyses and tissue distribution studies.

Table 1

Summary of LLOQ, linear ranges, regression equations, and correlation coefficients of analytes in rat plasma and tissue homogenate samples.

Sample	Calibration curve	R^2	Linear range (ng/mL)
Plasma	$y=0.0081x-0.0137$	0.990	5–2000
Heart	$y=0.00825x-0.00473$	0.997	1–1000
Liver	$y=0.00868x-0.00608$	0.990	1–1000
Spleen	$y=0.0292x+0.00648$	0.995	1–1000
Lung	$y=0.00127x+0.000556$	0.993	1–1000
Kidney	$y=0.00443x+0.00519$	0.990	1–1000
Brain	$y=0.00445x+0.00258$	0.997	1–1000

3.4. Precision and Accuracy

The precision and accuracy of this analytical approach were next tested at four QC levels (LLQC, LQC, MQC, HQC), as shown in Table 2. For plasma and tissue homogenate samples, the intra- and interday precision (RSD) were not >15%, and the accuracy (RE) was within the $\pm 15\%$ range. All of these results were consistent with the acceptance criteria for the present study, indicating that this analytical approach was a reproducible and accurate means of measuring Schisandrol B levels in rat plasma and tissue homogenates.

Table 2

Precision and accuracy of the approach to Schisandrol B measurement in biological samples ($n=6$).

Samples	Spiked conc (ng/mL)	Intra-day ($n=6$)	Inter-day ($n=6 \times 3$)

Measured conc. (ng/mL)	Precision (RSD, %)	Accuracy (RE, %)	Measured conc. (ng/mL)	Precision (RSD, %)	Accuracy (RE, %)	Plasma	5
5.46±0.26	4.73	9.22	5.45±0.27	4.99	8.99	15	13.60±1.40
10.32	-9.31	13.68±1.43	10.46	-8.78	150	145.30±13.05	8.98
-3.14	143.58±14.11	9.82	-4.28	1500	1436.42±93.55	6.51	-1.08
1410.53±112.67	7.99	-5.96	-				
Heart	1	0.99±0.08	7.91	-1.27	0.97±0.03	3.08	-3.41
2	2.00±0.22	10.82	0.17	2.01±0.02	0.96	0.50	40
36.90±0.39	1.06	-7.74	37.11±0.22	0.59	-7.22	800	789.33±48.32
6.12	-1.33	753.10±32.12	4.27	-5.86	.		
Liver	1	0.98±0.02	2.47	-2.16	0.96±0.06	6.50	-4.06
2	1.83±0.06	3.11	-8.49	1.85±0.02	0.90	-7.53	40
38.32±0.96	2.51	-4.20	38.09±0.21	0.55	-4.78	800	713.49±7.44
1.04	-10.81	735.12±18.94	2.58	-8.11	.		
Spleen	1	0.96±0.03	3.24	-4.17	0.95±0.03	2.66	-4.52

2	1.90±0.10	5.02	-5.17	1.85±0.05	2.80	-7.3 1	40
40.18±1.77	4.42	0.45	39.38±0.78	1.97	-1.54	800	761. 79± 53.2 2
6.99	-4.48	762.48±9.57	1.26	-4.69	.		
Lung	1	1.00±0.10	10.02	0.10	1.01±0.02	1.52	0.72
2	2.06±0.10	4.82	2.82	2.03±0.06	3.12	1.41	40
36.27±2.27	6.26	-9.32	37.01±0.88	2.37	-7.47	800	722. 25± 43.0 9
5.97	9.72	723.66±4.49	0.62	-9.54	.		
Kidney	1	0.90±0.05	6.08	-10.33	0.97±0.07	6.96	-2.9 3
2	1.85±0.11	6.06	-7.70	1.88±0.04	2.08	-5.8 6	40
41.92±3.88	9.26	4.81	40.94±0.88	2.15	2.36	800	721. 96± 30.5 7
4.23	9.76	748.65± 25.22	3.37	-6.42	.		
Brain	1	1.03±0.06	6.25	2.73	0.98±0.05	5.10	-2.0 2
2	2.04±0.06	3.07	1.95	1.89±0.14	7.31	-5.4 7	40
43.12±2.37	5.51	7.81	39.06±3.55	9.09	-3.35	800	749. 13± 31.0 1

3.5. Matrix Effect and Extraction Recovery

Extraction recovery values at all three tested concentration levels were >75%, indicating that this extraction approach was reliable and efficient. The matrix effect of Schisandrol B in plasma and tissue homogenate samples ranged within 90.62%–114.08%, suggesting that matrix did not significantly interfere with Schisandrol B analyses (Table 3).

Table 3

Recovery and matrix effects of Schisandrol B in plasma and tissues homogenate samples ($n=6$).

Samples	Conc. (ng/mL)	Extraction recovery (mean±SD)	Matric effect (mean±SD)
Plasma	15	75.55±11.85	103.68±9.77
150	87.72±10.91	110.32±13.18	1500
87.44±11.36	105.71±5.72	-	
Heart	2	78.9±9.10	101.31±8.79
40	76.7±4.40	99.88±3.09	800
75.5±10.70	101.38±14.30	-	
Liver	2	97.89±6.19	106.57±12.41
40	96.03±10.05	114.08±4.92	800
87.79±10.74	107.39±7.02	-	
Spleen	2	83.33±8.40	94.56±12.11
40	91.12±5.47	101.61±9.46	800
79.99±3.04	105.42±2.18	-	
Lung	2	93.19±5.16	107.50±4.81
40	96.52±6.81	95.92±12.51	800
92.41±12.10	99.31±8.43	-	

Kidney	2	86.40±9.95	113.13±3.42
40	91.03±6.55	101.98±3.54	800
86.45±5.00	105.82±1.66	-	
Brain	2	91.30±3.29	90.62±7.57
40	94.26±1.79	93.93±9.74	800

3.6. Stability

Stability was evaluated by assessing analyte levels in six replicate low and high concentration QC samples stored under a range of conditions. No significant differences in Schisandrol B concentrations were observed under any of these tested conditions for either QC sample type (Table 4), suggesting that Schisandrol B is relatively stable in both samples under relevant processing and storage conditions.

Table 4

Stability of Schisandrol B in rat plasma and tissues homogenate samples under various conditions ($n=6$).

Samples	Spiked conc. (ng/mL)	Measured conc.			
Bench-top (for 4h)	Autosampler (at room temperature for 24h)	Three freeze-thaw cycles (at -80°C)	Long term (at -80°C for 20 days)	Plasma	15
13.95±1.21	14.04±1.21	13.70±0.98	13.92±0.96	1500	1345.92±97.42
1433.13±111.16	1333.92±58.09	1387.98±72.00	-		
Heart	2	2.00±0.25	2.03±0.13	1.80±0.09	1.91±0.16
800	728.09±12.10	741.90±66.63	743.67±44.47	732.25±32.14	.
Liver	2	1.86±0.05	1.86±0.04	1.81±0.06	1.82±0.21
800	748.70±10.92	743.18±18.64	761.94±36.57	756.91±42.36	.
Spleen	2	1.80±0.01	1.87±0.07	1.85±0.11	1.84±0.09

800	753.27±8.88	772.37±10.61	781.57±22.05	764.19±25.38	
Lung	2	1.96±0.24	2.07±0.16	1.89±0.07	1.93±1.98
800	728.69±21.07	720.05±15.93	777.29±42.83	748.07±38.92	
Kidney	2	1.92±0.25	1.88±0.11	1.88±0.04	1.86±0.17
800	772.08±88.79	751.92±50.02	773.92±47.36	766.91±50.09	
Brain	2	1.77±0.19	1.87±0.12	1.98±0.04	1.80±0.11

3.7. Pharmacokinetic Study

After successfully validating this method, we assessed the pharmacokinetics of Schisandrol B in rats following intravenous Schisandrol B monomer administration (2mg/kg), intragastric Schisandrol B monomer administration (10mg/kg), or intragastric SCE administration (6mL/kg; equivalent to 15mg/kg Schisandrol B monomer).

Schisandrol B mean plasma concentration-time profiles and key pharmacokinetic parameters were calculated based on these analyses (Figure 3, Table 5). In these three experimental groups, AUC_{0-t} (ng.h/mL) values were 456.68 ± 91.24 , 426.22 ± 32.64 , and 2288.87 ± 276.44 respectively, while $AUC_{0-\infty}$ (ng.h/mL) values were 456.84 ± 91.63 , 427.93 ± 33.15 , and 2334.08 ± 381.07 , respectively. Schisandrol B monomer was found to exhibit an oral bioavailability of 18.73%, whereas the bioavailability of Schisandrol B in SCE was as high as 68.12%. When comparing the pharmacokinetics of Schisandrol B in rats administered SCE to those in rats administered Schisandrol B monomer, we found that Schisandrol B derived from SCE exhibited a longer $T_{1/2}$ (2.55 ± 0.66 vs. 1.59 ± 1.42), a longer $MRT_{0-\infty}$ (9.09 ± 1.51 vs. 3.31 ± 0.46), a lower CL (6.43 ± 1.83 vs. 23.37 ± 3.25), a later T_{max} (2.00 ± 0.87 vs. 0.54 ± 0.40), and a higher dosage exposure and bioavailability (68.12% vs. 18.73%) relative to Schisandrol B monomer at an equivalent dosage. Previous data indicated that Schisandrol B monomer is eliminated within 12h of absorption, whereas this period extends for 24h following SCE administration. As such, the final collection time points in Groups 2 and 3 were 24h and 36h, respectively. This suggests that other components in SCE may enhance the absorption of Schisandrol B, decreasing its elimination and enhancing its bioavailability.

[figures omitted; refer to PDF]

Table 5

Pharmacokinetic parameters of Schisandrol B in rat plasma.

Parameters	Schisandrol B		SCE
Group 1 (2mg/kg; iv)	Group 2 (10mg/kg; ig)	Group 3 (6mL/kg; ig)	$t_{1/2}$ (h)
1.10±0.10	1.59±1.42	2.55±0.66	CL (L/h/kg)
4.38±1.12	23.37±3.25	6.43±1.83	$MRT_{0-\infty}$ (h)

0.67±0.01	3.31±0.46	9.09±1.51	$T_{\max}$ (h)
—	0.54±0.40	2.00±0.87	$C_{\max}$ (ng/mL)
—	133.09±18.71	288.67±67.47	AUC _{0-t} (ng.h/mL)
456.68±91.24	426.22±32.64	2288.87±276.44	AUC _{0-∞} (ng.h/mL)
456.84±91.63	427.93±33.15	2334.08±381.07	F (%)

3.8. Tissue Distribution Study

Having successfully validated this HPLC-MS/MS approach, it was additionally used to assess Schisandrol B concentrations in heart, liver, spleen, lung, kidney, and brain homogenates prepared at 0.5, 1, 2, 4, 8, and 12h after the single dose (10mg/kg) intragastric administration of Schisandrol B. The tissue distribution profiles of Schisandrol B were assessed over time (Figure 4), revealing that this compound was rapidly absorbed and readily diffused throughout all analyzed tissues. Following its oral administration, maximal Schisandrol B concentrations were observed in all analyzed tissues at 1 hour postdosing, after which they declined markedly over the following 12 hours, indicating that Schisandrol B does not accumulate substantially in any of the analyzed tissues. Compared to plasma levels in rat which were intragastrically administered a single dose of Schisandrol B monomer (10mg/kg), the $T_{\max}$ values for these tissues were ~0.5 hours slower, confirming that the absorption of oral Schisandrol B monomer requires processing such that some amount of time is required for Schisandrol B to affect target organs. Maximal Schisandrol B concentrations were detected in the liver, followed by the kidneys, heart, lung, spleen, and brain, respectively. This suggests that Schisandrol B may be best-suited to the treatment of hepatic diseases. Given their high levels of vascularization and blood flow, it is perhaps unsurprising that the liver and kidneys exhibited the highest levels of Schisandrol B, which is likely to be primarily metabolized in the former and excreted via the latter of these organs. Given that we were also able to detect Schisandrol B in the brain following its oral administration, this suggests that it can penetrate the blood-brain barrier and may thus warrant future study as a potentially valuable pharmaceutical agent capable of targeting the central nervous system.

[figure omitted; refer to PDF]

4. Conclusion

Herein, we developed and validated a rapid and sensitive HPLC-MS/MS-based approach to evaluating Schisandrol B levels in rat plasma and tissue homogenates. This study is the first time to our knowledge to have assessed Schisandrol B oral bioavailability and tissue distribution patterns. We determined that the absolute oral bioavailability of Schisandrol B monomer was 18.73%, while for SCE it was 68.12%. We also found that Schisandrol B preferentially localized to the liver, kidneys, heart, and brain, providing a scientific basis for the beneficial effects of this compound on these organs. Together, our data offer a foundation for future studies of the pharmacological activity of Schisandrol B *in vivo*.

Disclosure

Zijing Wu and Yanhao Liu are the co-first authors.

Authors' Contributions

Dahu Liang, Zijing Wu, and Yanhao Liu contributed equally to this work. D. H. L., Z. J. W., C. L., and B. Y. conducted the experiments. X. H. L. and Y. H. L. participated in the data analyses. H. T. X. and S. H. contributed to the design and supervision of the project. D. H. L. wrote the manuscript. All authors have approved the final version of the manuscript.

Acknowledgments

This research study was sponsored via the Youth Foundation of Wannan Medical College (Grant number WK2018F05) and "Climbing Peak" Training Program for Innovative Technology team of Yijishan Hospital of Wannan

References

- [1] J. N. Chun, M. Cho, I. So, J.-H. Jeon, "The protective effects of Schisandra chinensis fruit extract and its lignans against cardiovascular disease: a review of the molecular mechanisms," *Fitoterapia*, vol. 97, pp. 224-233, DOI: 10.1016/j.fitote.2014.06.014, 2014.
- [2] K. Sowndhararajan, P. Deepa, M. Kim, S. J. Park, S. Kim, "An overview of neuroprotective and cognitive enhancement properties of lignans from Schisandra chinensis," *Biomedicine & Pharmacotherapy*, vol. 97, pp. 958-968, DOI: 10.1016/j.biopha.2017.10.145, 2018.
- [3] X. Liu, L. Cong, C. Wang, "Pharmacokinetics and distribution of schisandrol A and its major metabolites in rats," *Xenobiotica*, vol. 49 no. 3, pp. 322-331, DOI: 10.1080/00498254.2017.1418543, 2019.
- [4] M. Hong, Y. Zhang, S. Li, "A network pharmacology-based study on the hepatoprotective effect of fructus schisandrae," *Molecules*, vol. 22 no. 10, DOI: 10.3390/molecules22101617, 2017.
- [5] M.-K. Jang, J. S. Nam, J. H. Kim, "Schisandra chinensis extract ameliorates nonalcoholic fatty liver via inhibition of endoplasmic reticulum stress," *Journal of Ethnopharmacology*, vol. 185, pp. 96-104, DOI: 10.1016/j.jep.2016.03.021, 2016.
- [6] S. Onay, S. Hofer, M. Ganzera, "Rapid analysis of nine lignans in Schisandra chinensis by supercritical fluid chromatography using diode array and mass spectrometric detection," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 185, DOI: 10.1016/j.jpba.2020.113254, 2020.
- [7] A. Szopa, R. Ekiert, H. Ekiert, "Current knowledge of Schisandra chinensis (Turcz.) Baill. (Chinese magnolia vine) as a medicinal plant species: a review on the bioactive components, pharmacological properties, analytical and biotechnological studies," *Phytochemistry Reviews*, vol. 16 no. 2, pp. 195-218, DOI: 10.1007/s11101-016-9470-4, 2017.
- [8] Y.-M. Jiang, Y. Wang, H.-S. Tan, "Schisandrol B protects against acetaminophen-induced acute hepatotoxicity in mice via activation of the NRF2/ARE signaling pathway," *Acta Pharmacologica Sinica*, vol. 37 no. 3, pp. 382-389, DOI: 10.1038/aps.2015.120, 2016.
- [9] Y. Jiang, X. Fan, Y. Wang, "Schisandrol B protects against acetaminophen-induced hepatotoxicity by inhibition of CYP-mediated bioactivation and regulation of liver regeneration," *Toxicological Sciences*, vol. 143 no. 1, pp. 107-115, DOI: 10.1093/toxsci/kfu216, 2015.
- [10] S.-H. Kim, Y. S. Kim, S. S. Kang, K. Bae, T. M. Hung, S.-M. Lee, "Anti-apoptotic and hepatoprotective effects of gomisin A on fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide in mice," *Journal of Pharmacological Sciences*, vol. 106 no. 2, pp. 225-233, DOI: 10.1254/jphs.fp0071738, 2008.
- [11] A. Panossian, G. Wikman, "Pharmacology of Schisandra chinensis baill: an overview of Russian research and uses in medicine," *Journal of Ethnopharmacology*, vol. 118 no. 2, pp. 183-212, DOI: 10.1016/j.jep.2008.04.020, 2008.
- [12] H. Zeng, Y. Jiang, P. Chen, "Schisandrol B protects against cholestatic liver injury through pregnane X receptors," *British Journal of Pharmacology*, vol. 174 no. 8, pp. 672-688, DOI: 10.1111/bph.13729, 2017.
- [13] D. H. Kim, T. M. Hung, K. H. Bae, "Gomisin A improves scopolamine-induced memory impairment in mice," *European Journal of Pharmacology*, vol. 542 no. 1-3, pp. 129-135, DOI: 10.1016/j.ejphar.2006.06.015, 2006.
- [14] J. N. Chun, S. Park, S. Lee, "Schisandrol B and schisandrin B inhibit TGF β 1-mediated NF- κ B activation via a Smad-independent mechanism," *Oncotarget*, vol. 9 no. 3, pp. 3121-3130, DOI: 10.18632/oncotarget.23213, 2017.
- [15] Y. Y. Zhao, X. P. Yao, T. Y. Jiao, "Lipidomics analysis on schisandrol B-induced liver enlargement in mice," *Acta Pharmaceutica Sinica*, vol. 55, pp. 922-929, 2020.
- [16] Y. Matsuzaki, E. Ishibashi, S. Koguchi, "Determination of gomisin A (TJN-101) and its metabolite in rat serum by gas chromatography-mass spectrometry," *Yakugaku Zasshi*, vol. 111 no. 10, pp. 617-620, DOI: 10.1248/yakushi1947.111.10_617, 1991.
- [17] H. Wei, L. Sun, Z. Tai, S. Gao, W. Xu, W. Chen, "A simple and sensitive HPLC method for the simultaneous determination of eight bioactive components and fingerprint analysis of Schisandra sphenanthera," *Analytica*

Chimica Acta, vol. 662 no. 1, pp. 97-104, DOI: 10.1016/j.aca.2009.12.039, 2010.

[18] H. Zhang, G. Zhang, Z. Zhu, "Determination of six lignans in Schisandra chinensis (Turcz.) Baill fruits and related Chinese multiherb remedies by HPLC," Food Chemistry, vol. 115 no. 2, pp. 735-739, DOI: 10.1016/j.foodchem.2008.12.010, 2009.

[19] S.-J. Kim, H. Shin, S.-M. Cheon, "A sensitive UHPLC-MS/MS method for the simultaneous quantification of three lignans in human plasma and its application to a pharmacokinetic study," Journal of Separation Science, vol. 40 no. 17, pp. 3430-3439, DOI: 10.1002/jssc.201700588, 2017.

[20] K. Liu, Y. Song, Y. Liu, "An integrated strategy using UPLC-QTOF-MSE and UPLC-QTOF-MRM (enhanced target) for pharmacokinetics study of wine processed Schisandra Chinensis fructus in rats," Journal of Pharmaceutical and Biomedical Analysis, vol. 139, pp. 165-178, DOI: 10.1016/j.jpba.2017.02.043, 2017.

[21] S. Mao, H. Zhang, L. Lv, "Rapid determination and pharmacokinetics study of lignans in rat plasma after oral administration of Schisandra chinensis extract and pure deoxyschisandrin," Biomedical Chromatography, vol. 25 no. 7, pp. 808-815, DOI: 10.1002/bmc.1520, 2010.

[22] H. Wei, W. Xu, F. Cai, "Simultaneous determination of five lignan constituents of Wuzhi capsule in rat plasma by LC-MS/MS: application to pharmacokinetic study," Journal of Pharmaceutical and Biomedical Analysis, vol. 52 no. 5, pp. 741-746, DOI: 10.1016/j.jpba.2010.02.003, 2010.

[23] S. Zhan, Q. Shao, X. Fan, Z. Li, "Development of a sensitive LC-MS/MS method for simultaneous quantification of eleven constituents in rat serum and its application to a pharmacokinetic study of a Chinese medicine Shengmai injection," Biomedical Chromatography, vol. 29 no. 2, pp. 275-284, DOI: 10.1002/bmc.3273, 2014.

[24] Y. Zhang, Q. H. Zhang, H. Jiang, G. X. Zou, X. M. You, K. S. Bi, "Determination of gomisin A in rat plasma by HPLC-MS and its application," China Pharmaceuticals, vol. 21, 2012.

[25] FAD Administration, Bioanalytical Method Validation Guidance for Industry, 2018. Available online: <https://www.fda.gov/files/drugs/published/BioanalyticalMethod-Validation-Guidance-for-Industry.pdf>

DETAILS

Subject:	Mass spectrometry; Formic acid; Ammonium acetate; Plasma; Accuracy; Lignans; Acids; Monomers; Calibration; Pharmacology; Liquid-liquid extraction; Pharmacokinetics; Bioavailability; Scientific imaging; Chromatography; Bioaccumulation
Location:	United States--US; China; Japan
Company / organization:	Name: Food & Drug Administration--FDA; NAICS: 922190
Editor:	David Touboul
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York

Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-09-20 (Received); 2021-01-27 (Revised); 2021-02-03 (Accepted); 2021-02-24 (Pub)
DOI:	https://doi.org/10.1155/2021/8862291
ProQuest document ID:	2497887 245
Document URL:	https://www.proquest.com/scholarly-journals/hplc-ms-mediated-analysis-pharmacokinetics/docview/2497887245/se-2?accountid=211160
Copyright:	Copyright © 2021 Dahu Liang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-03-07
Database:	Publicly Available Content Database

Document 51 of 59

Assessment of Some Physicochemical Parameters and Heavy Metals in Hand-Dug Well Water Samples of Kafta Humera Woreda, Tigray, Ethiopia

Kiros Gebremichail Gebresilasie ¹ ; Goitom Gebreyohannes Berhe ²
; Tesfay, Amanual Hadera ² ; Gebre, Samuel Estifanos ²

¹ Tsegede Wereda Education Office, Tigray Regional State Education Bureau, P.O. Box 231, Mekelle, Ethiopia ² College of Natural and Computational Sciences, Mekelle University, P.O. Box 823, Mekelle, Ethiopia

ABSTRACT (ENGLISH)

Groundwater is one of the most important sources of drinking water in Kafta Humera Woreda; hence, it is important to assess the quality of these water sources. The aim of this study was to assess the levels of some physicochemical parameters and heavy metals in hand-dug well water sources of Kafta Humera Woreda. The results showed that the physicochemical concentrations of the hand-dug well water samples were given as follows: temperature, 27.67 ± 0.15 to $28.30 \pm 0.25^\circ\text{C}$; pH, 6.90 ± 0.33 to 8.20 ± 0.36 ; dissolved oxygen, 5.60 ± 0.06 to 6.2 ± 0.04 mg/L; turbidity, 1.67 ± 0.02 to 1.89 ± 0.03 NTU; EC, 148.50 ± 0.89 to 932.00 ± 0.98 $\mu\text{S}/\text{cm}$; TDS, 105.80 ± 0.62 to 664.28 ± 0.70 mg/L; total hardness, 71.80 ± 3.05 to 295.30 ± 2.38 mg/L; total alkalinity, 75 ± 5.0 to 215 ± 5.0 mg/L; calcium, 12.02 ± 0.82 to 75.88 ± 0.93 mg/L; magnesium, 9.80 ± 0.80 to 25.70 ± 0.17 mg/L; potassium, 0.130 ± 0.003 to 0.86 ± 0.04 mg/L; sodium, 2.20 ± 0.16 to 12.75 ± 0.87 mg/L; chloride, 12.86 ± 0.02 to 42.72 ± 0.20 mg/L; sulfate, 17.24 ± 0.96 to 118.67 ± 0.46 mg/L; phosphate, 0.018 ± 0.005 to 0.020 ± 0.002 mg/L; and nitrate, 1.86 ± 0.03 to 5.43 ± 0.06 mg/L. Generally, the concentrations of most physicochemical parameters of the hand-dug well water samples of Kafta Humera Woreda were within the permissible limit of World Health Organization and Ethiopian Standard Agency guideline for drinking water. The statistical Pearson's correlation analysis on the water quality parameters revealed that all parameters are more or less correlated with each other. Electrical conductivity and total dissolved solids of the water samples were found to be significantly correlated with total hardness ($r=0.989$), total alkalinity ($r=0.827$), calcium ($r=0.988$), magnesium ($r=0.881$), sodium ($r=0.995$), potassium ($r=0.996$), chloride ($r=0.998$), sulfate ($r=1$), and nitrate ions ($r=0.972$). Out of the selected seven heavy metals, Fe, Cu, Zn, Mn, Cr, Cd, and Pb, only iron was detected in all water samples and its concentration was above the permissible limit of WHO and ESA for drinking water. Therefore, the government should adopt some treatment technologies such as sedimentation and aeration to minimize the concentration of iron for safe drinking the water to the community of Kafta Humera Woreda.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Water is one of the most important compounds of the ecosystem, and all living organisms on Earth need water for their survival and growth. It is also used for drinking, cooking, agricultural activities, and industrial activities; transportation and recreation are among the most uses of water [1]. Water can be obtained from surface water sources such as freshwater, lakes, rivers, streams, and groundwater sources such as hand-dug well and borehole water. Groundwater is a major source of water for domestic use in both urban and rural areas of the world. It contains over 90% of the freshwater resources and it is an important reserve of good quality water [2]. Groundwater quality has become an important water resources issue due to rapid increase of population, rapid industrialization, unplanned urbanization, and too much use of fertilizers and pesticides in agriculture [3]. Good quality of water is essential for life, but the occurrence of physicochemical parameters and heavy metals above the permissible standards make it unsafe for drinking. The quality of drinking water should be checked at regular time interval because due to the use of contaminated drinking water, human population suffers from a variety of waterborne diseases [1]. The majorities of the populations in developing countries are inadequately supplied with potable water and are thus bound to use water from sources like shallow wells and boreholes that have high potential of contamination and provide the unsafe water for domestic and drinking purposes [4]. The availability and amount of freshwater continue to decrease from time to time in Africa. In Sub-Saharan African countries, the requirements of water for domestic and industrial purposes are usually not met. The natural chemical quality of groundwater is generally good, but at higher concentration a number of constituents can cause problems for water use [5]. In Ethiopia, the most common source of drinking water used to supply major urban and rural communities is

obtained from hand-dug wells, boreholes, and shallow wells and covers about 85% of the public supply [6]. Hand-dug well water source is the major source of water for drinking and other domestic purposes for the community of Kafta Humera community (Kunama Adigoshu, Hagere selam, May Woyni, and Habesha Adigoshu. However, the physicochemical parameter and heavy metal concentrations of the hand-dug well water sources have not been tested before in the study area and to compare the results with the minimum requirement of World Health Organization (WHO) and the Ethiopian Standard Agency (ESA). Therefore, the aim of this study is to assess the levels of some physicochemical parameter and heavy metal concentrations of hand-dug well water samples of Kafta Humera Woreda, Tigray, Ethiopia.

1.1. Description of the Study Area

Water samples were collected from Western Zone of Tigray Region, Kafta Humera Woreda specifically Kunama Adigoshu, Hagere Selam, May Woyni, and Habesha Adigoshu Kebeles (Figure 1). It is located 587km Northwest of Mekelle, the capital city of Tigray Region, bordering Sudan on the West and Eritrea on the North. Geographically, the area lies between 13° 40' N and 14° 27' N and between 36° 27' E and 37° 32' E with an elevation varying from 550m to 2800m above sea level. The annual rainfall in the area ranges from 500 to 1500mm. The relative humidity is highest during the months of rain (June-July) and lowest at the end of the dry season in April [7]. Based on the 2007 national census conducted by the central statistical agency of Ethiopia, the Woreda has a total population of 92,167 [8].

[figure omitted; refer to PDF]

2. Materials and Methods

2.1. Sampling and Preservation

Water samples were collected from four sampling sites Kafta Humera Woreda (Kunama Adigoshu, Hagreselam, May Woyni, and Habesha Adigoshu) on May 2019. Simple random sampling method was employed on selecting the four hand-dug wells' water from each site of the study area. Water samples were collected in 2L polyethylene plastic bottle previously washed with detergents, acidified with 5% HNO₃ for 24 h, rinsed twice with distilled water, and then finally rinsed with sample water three times before filling the sample water [9]. Samples from the wells were obtained by direct immersion of plastic containers into the reservoir handled by 10m rope and the fetched water was poured immediately into the polyethylene plastic sample containers. Finally, all the collected samples from the study area were labeled, preserved in ice box at temperature of 4 to 10°C to avoid any contamination, and transported to Mekelle University, College of Natural and Computational Science, School of Geochemistry laboratory and Ezana Mining Analytical Laboratory Development P.L.C for analysis. Standard methods [10] were used for sample collection, handling, and preservation to ensure data quality and consistency.

2.2. Analysis of Physicochemical Parameters in the Water Samples

The water samples were analyzed for physicochemical parameters such as temperature, pH, turbidity, dissolved oxygen (DO), electrical conductivity (EC), total dissolved solids (TDS), total alkalinity, total hardness, calcium, magnesium, sodium, potassium, chlorine, sulfate, phosphate, and nitrate using standard analytical methods [11]. The pH and total dissolved solids were measured using pH meter (HI-99130, Italy). Electrical conductivity was measured using conductivity meter (JENWAY, multi-3410, UK). Turbidity was measured using turbidimeter (AL 250T-IR, Germany). The temperature of the water samples was measured on-site using mercury thermometer. Dissolved oxygen was determined using multi parameter (Multi-3410, Germany). The total alkalinity was determined using titrimetric method. Total hardness, calcium, and magnesium hardness were determined using EDTA titrimetric method. Chloride concentration was determined using argentometric titration method. The cations potassium and sodium were determined using flame photometry (JENWAY, PFP7, UK). The anions sulfate, phosphate, and nitrate were measured using UV-visible spectrophotometer (Lambda, CE1021, and Australia) [11].

2.3. Analysis of Heavy Metal in the Water Samples

Exactly 50mL of filtered aliquot water sample was pipetted into a digestion flask and mixed with 10mL of 68% HNO₃ and then allowed to heat on hot plate for about six hours at a temperature of 80°C until the volume was reduced to one-fifth of its original volume. The solution was cooled, filtered using Whatman number 41 filter paper, and then

diluted to 100mL volumetric flask using distilled water. The concentrations of heavy metals were determined using FAAS (AA240FC, Australia) at the analytical laboratory of Ezana Mining Development P.L.C, Mekelle, Ethiopia [12].

2.4. Data Analysis

The physicochemical parameters and heavy metals were compared with WHO and Ethiopian Standard Agency (ESA) guidelines for drinking water. The relationships between the physicochemical parameters and heavy metals in the water samples were established using Pearson product correlation coefficient (r) at 1% level of significance. Data collected was statistically analyzed using Statistical Package for Social Sciences (SPSS version 23) [13].

3. Results and Discussion

The mean values of different selected physicochemical parameter and heavy metal concentrations are tabulated in Tables 1 and 2.

Table 1

The physicochemical parameter concentrations (mean $\pm$ SD, $N=3$) of hand-dug well samples of Kafta Humera Woreda.

Physicochemical parameters (mean $\pm$ SD)	Water sources				WHO [14]	ESA [15]
Kunama Adigoshu	Hagere Selam	May Woyni	Habesha Adigoshu	Temperature (°C)	27.90 $\pm$ 0.10	27.73 $\pm$ 0.15
28.30 $\pm$ 0.25	27.67 $\pm$ 0.15	25	—	Turbidity (NTU)	1.79 $\pm$ 0.02	1.71 $\pm$ 0.03
1.89 $\pm$ 0.03	1.67 $\pm$ 0.03	5	5	EC (μ S/cm)	932 $\pm$ 0.98	148.5 $\pm$ 0.89
367 $\pm$ 0.36	408.2 $\pm$ 0.96	750	1500	TDS (mg/L)	664.28 $\pm$ 0.70	105.8 $\pm$ 0.62
261.57 $\pm$ 0.26	291.03 $\pm$ 0.60	500	1000	pH	7.4 $\pm$ 0.20	7.6 $\pm$ 0.22
6.9 $\pm$ 0.32	8.2 $\pm$ 0.36	6.5-8.5	6.5-8.5	DO (mg/L)	5.7 $\pm$ 0.12	5.98 $\pm$ 0.05
5.6 $\pm$ 0.06	6.2 $\pm$ 0.04	5-7	—	TA (mg/L)	215 $\pm$ 5.00	75 $\pm$ 5.00
185 $\pm$ 5.00	165 $\pm$ 5.00	120	200	TH (mg/L)	295.3 $\pm$ 2.38	71.8 $\pm$ 3.05
142.3 $\pm$ 3.42	174.57 $\pm$ 3.70	300	300	Calcium	75.88 $\pm$ 0.93	12.02 $\pm$ 0.82
29.13 $\pm$ 1.30	33.45 $\pm$ 1.32	100.00	75.00	Magnesium	25.70 $\pm$ 0.17	9.80 $\pm$ 0.80

16.87±0.09	22.07±0.58	50.00	50.00	Sodium	12.75±0.87	2.20±0.16
4.83±0.07	5.67±0.04	200.00	200.00	Potassium	0.86 ±0.04	0.13±0.03
0.35±0.05	0.36±0.03	10.00	1.50	Chloride	42.72±0.20	12.87±0.02
24.56±0.17	34.95±0.07	250.00	250.00	Sulfate	118.63±0.46	17.24±0.96
47.13±0.33	52.96± 0.27	250.00	250.00	Phosphate	0.018±0.006	0.023±0.002
0.072±0.005	0.073±0.002	2.00	2.00	Nitrate	5.43±0.63	1.86±0.26

WHO: World Health Organization; ESA: Ethiopian Standard Agency; SD: standard deviation; ND: not reported.

Table 2

The heavy metal concentrations (mean±SD, N=3, mg/L) of hand-dug well water sources of Kafta Humera Woreda.

Heavy metals (mean±SD)	Water sources				WHO [14]	ESA [15]
Kunama Adigoshu	Hagere Selam	May Woyni	Habesha Adigoshu	Iron	0.97±0.06	1.86±0.24
0.33±0.02	0.69±0.08	0.3	0.3	Copper	BDL	BDL
BDL	BDL	0.5	2	Zinc	BDL	BDL
BDL	BDL	0.2	5	Manganese	BDL	BDL
BDL	BDL	0.5	0.5	Cadmium	BDL	BDL
BDL	BDL	0.01	0.01	Lead	BDL	BDL
BDL	BDL	0.01	0.01	Chromium	BDL	BDL

BDL: below detection limit; WHO: World Health Organization; ESA: Ethiopian Standard Agency.

3.1. Temperature

Temperature measures the degree of coldness or hotness of a substance [14]. The temperature of the well water samples varied between 27.67°C and 28.30°C (Table 1). Similar studies conducted in selected Hand-Dug Wells for Water Quality in Ilesa Metropolis, Southwest Nigeria, scored temperature value of 28.04±0.12°C [16]. The values of temperature for all well water samples were above the permissible limit of WHO for drinking water, 25°C [14]. These higher water temperatures could be attributed to the environmental temperature as well as other climatic conditions

prevailing in the study area at the time of collecting sample. Higher water temperature increases chemical reactions in the aquifer such as weathering of rocks which leads to the release of the chemical contaminants in water and decreases the level of dissolved in aquatic level [17]. Temperature has a negative correlation with dissolved oxygen ($r=-0.708$) and pH ($r=-0.666$), but positive correlation with turbidity ($r=0.892$), electrical conductivity ($r=0.532$), and TDS ($r=0.532$) (Table 3).

Table 3

Pearson's correlation analysis of the physicochemical parameters and heavy metals of hand-dug well water samples of Kafta Humera Woreda.

	T^0	pH	TU	EC	TDS	TH	TA	Ca^{2+}	Mg^{2+}	K^+	Na^+	Cl^-	SO_4^{2-}	PO_4^{3-}	NO_3^-	DO	Fe
T^0	1	pH	-0.666*	1	TU	0.892*	-0.848	1	EC	0.532*	-0.110	0.422	1	TDS	0.532*	-0.110	0.422
1.00**	1	TH	0.348	-0.022	0.265	0.989**	0.989**	1	TA	0.411	-0.239	0.474	0.827**	0.827**	0.862**	1	Ca^{2+}
0.246	-0.045	0.157	0.988**	0.988**	0.978**	0.780**	1	Mg^{2+}	0.255	0.137	0.055	0.881**	0.881**	0.939**	0.887**	0.871*	1
K^+	0.221	-0.10	0.188	0.995**	0.995**	0.985**	0.813**	0.986**	0.875**	1	Na^+	0.309	-0.126	0.259	0.996**	0.996*	0.984*
0.829*	0.983**	0.872**	0.987**	1	Cl^-	0.154	-0.129	0.223	0.998**	0.998**	0.981**	0.805**	0.987**	0.857**	0.996**	0.994*	1
SO_4^{2-}	0.186	-0.108	0.223	1.00**	1.00**	0.992**	0.838**	0.988**	0.891**	0.995**	0.996**	0.997**	1	PO_4^{3-}	0.252	0.080	0.124
-0.330	-0.330	-0.220	0.213	-0.362	0.081	-0.343	-0.328	-0.374	-0.307	1	NO_3^-	0.104	-0.098	0.152	0.972**	0.972*	0.937*
0.681*	0.973**	0.768**	0.971**	0.968**	0.982**	0.966**	-0.540	1	DO	-0.708*	0.869**	-0.867**	-0.381	-0.380	-0.295	-0.472	-0.299
-0.101	-0.371	-0.403	-0.398	-0.375	0.169	-0.363	1	Fe	-0.565	0.214	-0.492	-0.298	-0.298	-0.374	-0.772**	-0.240	-0.559

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). T^0 : temperature ($^{\circ}\text{C}$); EC: electrical conductivity; Tu: turbidity; TDS: total dissolved solids; TH: total hardness; TA: total alkalinity; Ca^{2+} : calcium ion; Mg^{2+} : magnesium ion; Na^{+} : sodium ion; K^{+} : potassium ion; SO_4^{2-} : sulphate ion; PO_4^{3-} : phosphate ion; NO_3^{-} : nitrate ion; DO: dissolved oxygen; Fe: iron.

3.2. Turbidity

Turbidity is a measure of the degree to which the water loses its transparency due to the presence of suspended particles. The turbidity values of the well water samples varied between 1.67 NTU and 1.89 NTU (Table 1). The recorded turbidity values show that all well water samples had values which were within the permissible limit of WHO and ESA for drinking water below 5 NTU [14, 15]. Similarly, the drinking water quality in the State of Perak, Malaysia, indicates that the turbidity of all the samples studied was below the maximum standard limit of 5 NTU [18]. The occurrence of suspended soil, sediment, carbon-based substance, inorganic material, and other invisible living things enhances turbidity. Highly turbid water reduces light penetration to the wells, therefore affecting the level of photosynthesis aquatic plants. This increasing in water temperature is due to absorption of sunlight [19]. Turbidity has a significant negative correlation with dissolved oxygen ($r = -0.867$) at $p < 0.01$ (Table 3).

3.3. Electrical Conductivity

Conductivity is a measure of the conducting ability of water and it is determined by the presence of ionic species in the water. Electrical conductivity value for all the sampling sites varied from 148.5 to 932 $\mu\text{S}/\text{cm}$ (Table 1). The measured EC value indicates that all examined water samples had values which were within the permissible limit of WHO 750 $\mu\text{S}/\text{cm}$ and ESA 1500 $\mu\text{S}/\text{cm}$ for drinking water except Kunama Adigoshu well water source in WHO [14, 15]. These results are higher in conductivity than the average conductivity value of the drinking water in the State of Perak, Malaysia, which is 102.1 $\mu\text{S}/\text{cm}$ [18]. The presence of a slightly high value of electrical conductivity in the water sample shows that contaminations due to dissolve ions are high, because electrical conductivity is directly proportional to the total dissolved solids. This may be due to the soil type and geology of the study area or the wastes entering from the surrounding water sources [20]. Electrical conductivity shows significant correlation with ten parameters such as sulfate ion and TDS ($r = 1$), total hardness ($r = 0.989$), total alkalinity ($r = 0.827$), calcium ($r = 0.988$), magnesium ($r = 0.881$), potassium ($r = 0.996$), sodium ($r = 0.995$), chloride ($r = 0.998$), and nitrate ions ($r = 0.972$) at $p < 0.01$. This shows that the presence of these ions in dissolved form in water can increase the electrical conductivity of water (Table 3).

3.4. Total Dissolved Solids

Total dissolved solids are the amount of mobile charged ions, including minerals, salts, or dissolved metals in a given volume of water in mg/L. The mean total dissolved solids (TDS) obtained from well water samples were from 105.80 mg/L to 664.30 mg/L (Table 1). All the measured well water samples recorded TDS concentration values within the permissible limit of WHO (500 mg/L) and ESA (1000 mg/L) for drinking water, except Kunama well water source in WHO [14, 15]. These results are in agreement with a study conducted in India; total dissolved solids values ranged from 146 mg/L to 467 mg/L [21]. High level of TDS in groundwater may be due to dissolution of weathered materials from rock formation [22]. The occurrence of high values of TDS in certain well water samples could be unpleasant to consumers and may cause excessive scaling in water heaters, boilers, and household equipment [23]. Total dissolved solids show significant positive correlation with ten parameters such as sulfate ion and EC ($r = 1$), total hardness ($r = 0.989$), total alkalinity ($r = 0.827$), calcium ($r = 0.988$), magnesium ($r = 0.881$), potassium ($r = 0.996$), sodium ($r = 0.995$), chloride ($r = 0.998$), and nitrate ions ($r = 0.972$) at $p < 0.01$. This shows that the presence of these ions in dissolved form in water can increase the amount of total dissolved solids in water (Table 3).

3.5. pH

pH values of the well water samples varied between 6.9 ± 0.32 and 8.2 ± 0.36 (Table 1). All the wells recorded pH values within the acceptable limit of WHO and ESA standards 6.5–8.5 [14, 15]. Similar results were recorded from groundwater sources of Dire Dawa City, Eastern Ethiopia, which were within the permissible pH limits (6.5–8.5) [24]. The pH has a negative significant correlation with turbidity ($r = -0.848$), but strong positive correlation with dissolved

oxygen ($r=0.869$) value at $p<0.01$ (Table 3).

3.6. Dissolved Oxygen

Dissolved oxygen is an important water quality parameter and it is an indicator of water contamination. The small amount of dissolved oxygen in water indicates the microbial contamination or corrosion of chemical substances in the aquifer [25]. The dissolved oxygen values of the well water samples varied between 5.6mg/L and 6.2mg/L (Table 1). From the analyzed result, all well water samples recorded dissolved oxygen values within the acceptable limit of WHO standard for drinking water, 5–7mg/L [14]. These results are similar to a study conducted in groundwater around Singrauli Coalfield Areas, Singrauli district of Madhya Pradesh (India); it varies from 4.98 to 5.72mg/L [21]. The level of dissolved oxygen in natural water highly reduced with increasing of water temperature and high organic concentrations as a result of increased decomposer activities [26].

3.7. Total Alkalinity

Alkalinity is a measure of the ability of water to neutralize acids and it mainly occurs due to the presence of carbonates and bicarbonates in the water. The concentrations of total alkalinity in the well water samples varied between 75mg/L and 215mg/L (Table 1). The measured values of total alkalinity in all well water samples were above the permissible limit of WHO (120mg/L) for drinking water, except Hagere Selam well water sample [14]. However, the total alkalinity of all well water samples was within the permissible limit of ESA 200mg/L [15]. These results are in line with a study conducted in Greece where water alkalinity varied from 23.56 to 267.00mg/L [27]. Total alkalinity has a positive significant correlation with calcium ($r=0.78$), magnesium ($r=0.887$), sodium ($r=0.813$), potassium ($r=0.829$), chloride ($r=0.805$), and sulfate ($r=0.838$) at $p<0.01$. This indicates that the presence of these ions in dissolved form in water can increase the amount of total alkalinity of water (Table 3).

3.8. Total Hardness

Hardness of water is caused by the presence of dissolved polyvalent metallic ions, predominantly calcium ions (Ca^{2+}) and magnesium ions (Mg^{2+}). The values of total hardness concentrations recorded in the well water samples varied between 71.80mg/L and 295.33mg/L (Table 1). These amounts of total hardness concentrations are within the maximum allowable limit of WHO and ESA for drinking water 300mg/L [14, 15]. Higher water hardness concentration in groundwater may be due to the dissolution rocks containing gypsum and dolomite which are responsible for water hardness [27]. Similar results were observed in Guya Village and its surrounding area, Tigray, Northern Ethiopia; hardness values varied from 170.00 to 327.50mg/L [28]. Hardness has a significant positive correlation with calcium ($r=0.978$), magnesium ($r=0.939$), sodium ($r=0.985$), potassium ($r=0.984$), chloride ion ($r=0.981$), sulfate ion ($r=0.992$), and nitrate ion ($r=0.937$) at $p<0.01$ (Table 3). This implies that the presence of these ions in water may have a direct effect on hardness values. The hardness observed in the hand-dug well water samples of the study area may be permanent hardness, because it has a strong positive correlation with permanent hardness causing ions such as chloride, sulfate, and nitrate salts.

3.9. Calcium and Magnesium Ion

Calcium and magnesium ions are the major constituents of various types of rock and they are the most common constituents present in natural waters ranging from zero to several hundred mg/L [29]. The highest calcium amount (75.88mg/L) of the well water was recorded at Kunama Adigoshu whereas the lowest value (12.02mg/L) was found in Hagere Selam well water sample (Table 1). All the wells recorded calcium ions within the acceptable limit of WHO and ESA standard for drinking water, 100mg/L [14, 15]. Calcium ion (Ca^{2+}) can occur naturally in groundwater through the dissolution of carbonate minerals and the decomposition of the sulfate, phosphate, and silicate minerals. High calcium concentrations in water may lead to the formation of solid scales in pipes and kitchen utensils and increased soap consumption [30]. The concentration of magnesium ions in the wells varied between 9.80mg/L and 25.70mg/L (Table 1). From the analyzed result, all the well water samples recorded magnesium ions within the permissible limit of WHO and ESA constants, 50mg/L, for drinking water [14, 15]. The main sources of magnesium in the underground water sampled may be attributed to geological sources such as dolomite and other magnesium containing compounds in sediments and soils of the water samples [18]. Calcium and magnesium have a significant positive correlation with potassium, sodium, chloride, sulfate, and nitrate ions (Table 3).

3.10. Sodium and Potassium Ion

The natural source of sodium and potassium in groundwater was from the weathering of rocks, but the elevated quantities in contaminated water may be attributed to the release of waste water [23]. The amount of sodium ions in well water samples varied between 2.20mg/L and 12.75mg/L (Table 1). The amount of sodium recorded in all well water samples was below the maximum permissible limit of WHO and ESA for drinking water, 200mg/L [14, 15]. The potassium levels recorded in the study areas were very low ranging from 0.13mg/L to 0.86mg/L (Table 1). The concentration of potassium obtained from all well water samples was far below the maximum permissible limit of WHO and ESA constant, 10mg/L, for drinking water [14, 15]. Similar results were recorded in the groundwater of Dire Dawa City, Eastern Ethiopia, concentration of sodium (7.31 mg/L–74.94 mg/L) and potassium (0.55mg/L–3.33 mg/L) [24]. Sodium shows a significant positive relationship with chloride ($r=0.994$), sulfate ($r=0.996$) and nitrate ($r=0.968$) values. Potassium value has a positive strong correlation with sodium ($r=0.987$), chloride ion ($r=0.996$), sulfate ($r=0.995$), and nitrate ion ($r=0.971$) at $p<0.01$.

3.11. Chloride Ion

Chlorides enter into surface and groundwater from both anthropogenic and natural sources such as from run-off wastes from human activities, discharges of wastewaters into water bodies, use of inorganic fertilizers, landfill leachates, and septic tank effluents [31]. The concentration of chloride ion in the well water samples varied between 12.86mg/L and 42.72mg/L (Table 1). The level of chloride ion recorded in all water samples was within permissible limit of WHO and ESA for drinking water, 250mg/L [14, 15]. These slight variations in the concentrations might be due to different levels of chloride salts in the soil and sediments or a result of differences in degree of wastes entering into the hand-dug wells [32]. The findings of this study are similar to hand-dug wells in Hantebet Catchment, Tigray, and Northern Ethiopia, varying from 15.45 to 49.44 mg/L [33]. Chloride value has a positive strong correlation with sulfate ion ($r=0.997$) and nitrate ion ($r=0.982$). This shows that other factors governing the loading of these ions may directly affect chloride distribution at $p<0.01$.

3.12. Sulfate, Phosphate, and Nitrate Ions

The concentration of sulfate ion in the well water samples varied between 17.24mg/L and 118.67 mg/L (Table 1). From the analyzed result, the sulfate level of all well water samples was within the permissible limit of WHO and ESA for drinking water 250mg/L [14, 15]. The high sulfate content of some well water samples of the study area may be due to the presence of sulfate containing minerals such as gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) and anhydrite (CaSO_4) in the sites [34]. Sulfate has a positive strong correlation value with nitrate ($r=0.966$) at $p<0.01$. The phosphate levels obtained in the well water samples of the sampling area were very low, ranging from 0.018 to 0.073mg/L (Table 1). The concentrations of phosphate in all well water samples were within the permissible limit of WHO and ESA for drinking water, 2mg/L [14, 15]. The low concentration of the phosphate in the well water samples might be due to the geology of the area [32]. The nitrate levels in the well water samples ranged from 1.86 to 5.43mg/L (Table 1). All the well water samples recorded nitrate ion concentration within the permissible limit of WHO and ESA for drinking water, 45mg/L and 50mg/L, respectively [14, 15]. Nitrate level in groundwater may be attributed to the introduction of sewages of humans and animals from the surrounding of the well, because it is very close to human residents. Sulfate has a significant positive correlation with nitrate value ($r=0.966$) at $p<0.01$.

3.13. Iron

Iron was the only heavy metal detected in all well water samples of the study area. The concentration of iron measured in all well water samples varied from 0.33mg/L to 1.86mg/L (Table 2). From the analyzed result, all the well water samples had iron concentration above the permissible limit of WHO and ESA for drinking water, 0.3mg/L [14, 15]. The higher value of iron from the study areas could also be from the natural sources. This may be due to the weathering of minerals, soil type, and sediments which are iron-rich materials [32]. Iron may not pose any health hazards but gives a bitter taste to the water when present in large concentrations. People consuming water sources with high concentration of iron were suffering from taste, color, corrosion of plumbing systems, and liver diseases. However, those exposed to low concentration would be highly susceptible to anemia [34]. A similar study conducted in hand-dug wells of Ilaro and Aiyetoro, Ogun State, Southwestern Nigeria, showed ranges from 0.26 to 1.37 mg/L

[35].

3.14. Copper, Zinc, Manganese, Cadmium, Lead, and Chromium

The elements of Cu, Zn, Mn, Cd, Pb, and Cr were below the detection limit in all hand-dug well water samples of the study area. Therefore, these elements may not have an immediate threat to the health of individuals in the study area who drink this water.

4. Concluding remarks

The aim of this study was to assess the level of some physicochemical parameters and heavy metals of water samples from hand-dug well water sources of Kafta Humera Woreda, specifically, Kunama Adigoshu, Hagere Selam, May Woyni, and Habesha Adigoshu Kebeles, Tigray Region, Ethiopia. The results showed that the concentrations of most physicochemical parameters of the water samples, such as turbidity, pH, total hardness, total alkalinity, calcium, magnesium, potassium, sodium, chloride, sulfate, phosphate, and nitrate ions, were within the permissible limit of the World Health Organization and Ethiopian Standard Agency guideline for drinking water with the exceptions of some physicochemical parameters, such as temperature in all hand-dug wells, total dissolved solids, and electrical conductivity in Kunama Adigoshu hand-dug well water sample which was above the WHO permissible limit, but within the permissible limit of ESA for drinking water. The concentrations of total alkalinity were above the WHO permissible limit in all hand-dug well water samples except in Hagere Selam hand-dug well water source. Out of the selected seven heavy metals, Fe, Cu, Zn, Mn, Cr, Cd, and Pb, only iron (Fe) was detected in all water samples and its concentration was above the permissible limit of WHO and ESA for drinking water. Generally, the water quality of the four hand-dug well water samples was good with the exception of some physicochemical parameters and heavy metals. It is recommended to check the quality of the water sources regularly in the study area. This assessment of some physicochemical and heavy metal quality of water is necessary but not sufficient to determine its suitability for drinking purpose. As such, it is recommended that further research should be a study of other trace heavy metals like mercury, arsenic, and biological parameters to confirm the study reliability.

Acknowledgments

The authors are highly indebted to the Mekelle University and Tigray Regional State Education Bureau for sponsoring the study.

References

- [1] B. Simpi, S. M. Hiremath, K. N. S. Murthy, K. N. Chandrashekarappa, A. N. Patel, E. T. Puttiah, "Analysis of water quality using physicochemical parameters Hosahalli Tank in Shimoga District, Karnataka, India," *Global Journal of Science Frontier Research*, vol. 11 no. 3, pp. 31-34, 2011.
- [2] S. Kanmani, R. Gandhimathi, "Investigation of physicochemical characteristics and heavy metal distribution profile in groundwater system around the open dump site," *Applied Water Science*, vol. 3 no. 2, pp. 387-399, DOI: 10.1007/s13201-013-0089-y, 2013.
- [3] M. A. Joarder, F. Raihan, J. B. Alam, S. Hasanuzzaman, "Regression analysis of groundwater quality data of Sunamjang district, Bangladesh," *International Journal of Environmental Research*, vol. 2 no. 3, pp. 291-296, 2008.
- [4] WHO, *Guidelines for Drinking Water Quality*, 2011.
- [5] A. B. Gebrehiwot, N. Tadesse, E. Jigar, "Application of water quality index to assess suitability of groundwater quality for drinking purposes in Hantebet watershed, Tigray, Northern Ethiopia," *ISABB Journal of Food and Agriculture Science*, vol. 1 no. 1, pp. 22-30, 2011.
- [6] H. M. Getchew, "Study on groundwater and requirements for drilling and tapping ground in Ethiopia," *Proceedings of the International Conference and Exhibition on Ground in Ethiopia*, UNECA Conference Center, .
- [7] M. A. Momodu, C. A. Anyakora, "Heavy metal contamination of groundwater: the Surulere case study," *Environmental and Earth Sciences Research Journal*, vol. 2 no. 1, pp. 39-43, 2010.
- [8] G. Hailu, B. Aferu, "Retrospective assessment of black leg in Kafta Humera Woreda," *Momona Ethiopian Journal of Science*, vol. 7 no. 1, pp. 134-140, DOI: 10.4314/mejs.v7i1.117244, 2015.
- [9] S. M. Rao, P. Mamatha, "Water quality in sustainable water management," *Current Science*, vol. 87 no. 7, pp. 942-947, 2004.

- [10] APHA, Standard Methods for Examination of Water and Waste Water, 1998.
- [11] American Public Health Association, Standard Methods for Examination of Water and Waste Water, 2012.
- [12] K. J. Inoti, K. Fanuel, O. George, O. Paul, "Assessment of heavy metal concentrations in urban grown vegetables in Thika Town, Kenya," *African Journal of Food Science*, vol. 6 no. 3, pp. 41-46, 2012.
- [13] B. Lukubye, M. Andama, "Physico-chemical quality of selected drinking water sources in Mbarara municipality, Uganda," *Journal of Water Resource and Protection*, vol. 9 no. 7, pp. 707-722, DOI: 10.4236/jwarp.2017.97047, 2017.
- [14] World Health Organization, Guidelines for Drinking Water Quality, 2008.
- [15] Ethiopia Standard Agency, Compulsory Ethiopian Standard: Drinking Water Specifications, 2013.
- [16] A. J. Omotoso, E. O. Dada, O. Oyedepi, "Physicochemical and microbiological assessment of selected hand-dug wells for water quality in Ilesa Metropolis, southwest Nigeria," *Notulae Scientia Biologicae*, vol. 10 no. 1, pp. 52-59, DOI: 10.15835/nsb10110058, 2018.
- [17] S. B. Saana, S. A. Fosu, G. E. Sebiawu, N. Jackson, T. Karikari, "Assessment of the quality of groundwater for drinking purposes in the upper west and northern regions of Ghana," *Springer Plus*, vol. 5 no. 1, 2016.
- [18] N. Rahmanian, S. H. B. Ali, M. Homayoonfard, "Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia," *Journal of Chemistry*, vol. 2015, DOI: 10.1155/2015/716125, 2015.
- [19] D. E. Hawkins, S. Mann, "The world bank's role in tourism development," *Annals of Tourism Research*, vol. 34 no. 2, pp. 348-363, DOI: 10.1016/j.annals.2006.10.004, 2007.
- [20] A. A. Christine, J. K. Kibet, A. K. Kiprop, M. L. Were, "The assessment of bore-hole water quality of Kakamega County, Kenya," *Applied Water Science*, vol. 8 no. 1, DOI: 10.1007/s13201-018-0688-8, 2018.
- [21] A. K. Sonkar, A. Jama, "Physicochemical characteristics of groundwater around Singrauli Coalfield areas, Singrauli district of Madhya Pradesh (India)," *RASĀYAN Journal of Chemistry*, vol. 12 no. 2, pp. 608-615, 2019.
- [22] Y. M. John, S. A. Kareem, H. M. Kefas, A. Polycarp, "Quality assessment of hand dug well in Hongtown," *International Journal of Scientific Research Engineering & Technology (IJSRET)*, vol. 3 no. 2, pp. 306-311, 2014.
- [23] S. A. Ganiyu, O. T. Olurin, S. B. Otun, "Assessment of physico-chemical properties of hand-dug wells around abandoned dumpsite in Ibadan Metropolis, southwestern Nigeria," *The Journal of Solid Waste Technology and Management*, vol. 43 no. 3, pp. 195-206, DOI: 10.5276/jswt.2017.195, 2017.
- [24] M. Dawit, A. Nagari, H. Hailu, "Ground water quality assessment of the rural administrative, Dire Dawa city, eastern Ethiopia," *Journal of Hydrogeology & Hydrologic Engineering*, vol. 6 no. 3, 2017.
- [25] D. Chapman, V. Kimstach, "Selection of water quality variables," *Water Quality Assessments. A Guide to the Use of Biota, Sediments and Water in Environmental Monitoring*, pp. 243-318, 1996.
- [26] A. Ebenezer, "Assessment and mapping of groundwater quality in the Thiririka sub catchment Kiambu County, Kenya," 2014.
- [27] T. Spanos, A. Ene, C. Xatzixristou, A. Papaioannou, "Assessment of groundwater quality and hydrogeological profile of Kavala area, Northern Greece," *Environmental Physics*, vol. 60, pp. 1139-1150, 2015.
- [28] G. Kassa, "Assessment of groundwater quality for drinking purpose in Guya village its surrounding area, Tigray, northern Ethiopia," *Assessment*, vol. 7 no. 10, 2015.
- [29] M. M. Ndamitso, S. Idris, M. B. Likita, J. O. Tijani, A. I. Ajai, A. A. Bala, "Physico-chemical and Escherichia coli assessment of selected sachet water produced in some areas of Minna, Niger State, Nigeria," *International Journal of Water Resources and Environmental Engineering*, vol. 5 no. 3, pp. 134-140, 2013.
- [30] M. K. Mensah, "Assessment of drinking water quality in Ehi Community in the ketu-north district of the volta region of Ghana," 2011. Doctoral dissertation
- [31] B. K. Gupta, R. R. Gupta, "Physicochemical and Biological study of drinking water in Satna Madhya Pradesh," *Pollution Research*, vol. 18 no. 4, pp. 523-525, 1999.
- [32] R. A. Obasi, A. O. Talabi, "The physiochemical characteristics of water in hand dug wells around Ekiti state university Ado-Ekiti, southwest Nigeria," *British Journal of Earth Sciences Research*, vol. 4 no. 2, 2015.
- [33] N. Tadesse, A. Bairu, K. Bheemalingeswara, "Suitability of groundwater quality for irrigation with reference to

hand dug wells, Hantebet Catchment, Tigray, and Northern Ethiopia," Momona Ethiopian Journal of Science, vol. 3 no. 2, DOI: 10.4314/mejs.v3i2.67711, 2011.

[34] M. I. Ocheri, L. A. Odoma, N. D. Umar, "Groundwater quality in Nigerian urban areas: a review," Global Journal of Science Frontier Research: (H) Environment & Earth, vol. 14, pp. 35-45, 2014.

[35] A. O. Eruola, G. C. Ufoegbune, J. A. Awomeso, S. A. Abhulimen, "Assessment of cadmium, lead and iron in hand dug wells of Ilaro and Aiyetoro, Ogun State, South-Western Nigeria," Research Journal of Chemical Sciences, vol. 1 no. 9, 2011.

DETAILS

Subject:	Laboratories; Nitrates; Magnesium; Aeration; Alkalinity; pH; Iron; Water sampling; Electrical resistivity; Copper; Drinking water; Water quality; Potassium; Turbidity; Freshwater resources; Groundwater; Cadmium; Sedimentation; Calcium; Chlorides; Quality assessment; Polyethylene; Zinc; Heavy metals; Statistical methods; Manganese; Parameters; Chromium; Correlation analysis
Location:	United Kingdom--UK; Australia; Ethiopia; Germany
Company / organization:	Name: World Health Organization; NAICS: 923120
Editor:	Lorenzo Giuntini
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article

Publication history :

Milestone dates:	2020-05-12 (Received); 2020-09-11 (Revised); 2021-02-05 (Accepted); 2021-02-16 (Pub)
DOI:	https://doi.org/10.1155/2021/8867507
ProQuest document ID:	2494043522
Document URL:	https://www.proquest.com/scholarly-journals/assessment-some-physicochemical-parameters-heavy/docview/2494043522/se-2?accountid=211160
Copyright:	Copyright © 2021 Kiros Gebremichail Gebresilasie et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-02-28
Database:	Publicly Available Content Database

Document 52 of 59

Total Oxidation of Isopropanol in Its Liquid Phase, at a Low Temperature in the Presence of Prepared and Characterized Zinc Oxide

Younes Dehmani

; Amhoud, Abdellatif; Abouarnadasse, Sadik

[ProQuest document link](#)

ABSTRACT (ENGLISH)

The complete oxidation of isopropanol in its liquid phase at a low temperature was studied in the presence of zinc oxide (ZnO). This solid was prepared with the precipitation method. Structural analysis (infrared in Fourier transform and diffraction of X-rays) and textured (adsorption/desorption of N₂) were conducted for the wurtzite structure results, an IV type isotherm with a type H3 hysteresis. This solid presents a good catalytic activity against the complete oxidation of isopropanol, a constant of selectivity equal to 1; however, the studied temperatures were 40, 60, and 80°C. In addition, a kinetic study of the oxidation was performed and showed that the reaction follows a successive mechanism isopropanol-acetone-carbon dioxide. The low value of the apparent energy of the activation of this solid confirms the high value of the initial rate of the catalytic oxidation reaction of isopropanol in the temperature range studied.

FULL TEXT

1. Introduction

Volatile organic compounds (VOCs) are a major source of pollution. These compounds bring together various families: alkanes, alkenes, aromatics, ketones, alcohols, acetates, aldehydes, and chlorine [1]. The volatility of these compounds gives them the ability to propagate more or less far from their place of emission, thus leading to direct (toxicity and odor) and indirect impacts [2–5] (“Smog” and warming) on the air. Concern over this observation has prompted many countries to control their emissions of gases into the atmosphere [6].

Several processes have been proposed for their destruction, and among them is the catalytic oxidation of these pollutants into carbon dioxide and water. Catalytic oxidation has many advantages over thermal oxidation, such as low cost [7]. It is, above all, profitable for treating effluents which are less concentrated in pollutants, thermal destruction then being discredited due to excessive energy consumption, in particular when the solvent content is low. However, the choice of catalyst is a very important parameter for obtaining the expected success in this type of process. This will allow the reaction rate to increase and operate at much lower temperatures [8–10].

Oxides of transition metals such as Cr, Fe, Ni, Mn, Cu, Co, and Zn are both typical and cost-effective catalysts for the complete oxidation of VOC. The properties to be attributed to this class of catalyst such as selectivity, durability, nontoxicity, and low cost (commercially accessible (approachable)) make it a considerable area of research under development [7].

The objective of our work is to test the catalytic potential of ZnO, in the reaction of the decomposition of isopropanol in the liquid phase and at low temperature. The choice of isopropanol in this study is based on its classification among VOC. The studied transition metal oxide was prepared by the precipitation method [11]. Characterization of catalyst is carried out by physicochemical methods such as structural, XRD and FTIR, and textural, adsorption/desorption of N₂ gas [12, 13]

2. Experimental Part

2.1. Catalyst Preparation

A mass of zinc nitrate dihydrate (Zn(NO₃)₂·6H₂O) was dissolved in a volume of distilled water. To this mixture, we added (7 ml/min) 20ml molar solution of ammonia (NH₄OH) dropwise. The resulting mixture was heated with constant stirring at 40°C for 1 hour and then filtered in vacuum. The resulting solid was washed several times with distilled water and dried overnight in oven at 100°C. The dried powder was calcined at 500°C for 3 hours [14].

2.2. Catalyst Characterization

(i) XRD: the patterns were recorded using an X'PERT MPD-PRO wide-angle X-ray powder diffractometer equipped with a diffracted beam monochromator and CuKα radiation filtered with Ni (λ=1, 5406 Å) [12, 13].

(ii) FTIR: characterization and structural changes were collected using the JASCO 4100 FTIR spectrometer with a resolution of 4 cm⁻¹ and an accumulation of at least 64 scans [12, 13].

(iii) BET: BET adsorption measurements: N₂ at T=−196°C was carried out using a Micromeritics ASAP 2010. The specific surface area and the mean pore diameter were determined, respectively, according to the standard BET and BJH (Barrett, Joyner, and Halenda) [12, 13].

2.3. Catalytic Test

To a ball to pass lapped 250ml, used as reactor, are added 0.15mL of isopropanol (reactive), 120mg of catalyst, and 200mL of distilled water successively. The temperature (the temperature of the water bath heating (reaction temperature)) is adjusted to the desired value (40°C, 60°C, and 80°C) before you plunge the ball serving reactor. Air (oxidizing reagent) is then admitted in the reactor using a lateral tubing under a flow of the order 40mL/min (more than enough to make the solution constantly saturated in oxygen). The reaction mixture is stirred continuously for the duration of the test in order to facilitate the accessibility of the grains of the reagent catalyst and to ensure work in chemical plan. It should be recalled that each catalytic test was performed with one taken Virgin catalyst test. The chromatographic analysis was conducted using the SHIMADZU CR15A fitted with a column under the following conditions: the column temperature is 80°C and P_{N2}=24.525KPa, P_{H2}=39.24 KPa, and P_{air}=49.05KPa [15].

3. Results and Discussion

3.1. Catalyst Characteristics

3.1.1. Diffraction of X-Rays (XRD)

The sample of the solid ZnO calcined at 500°C for 3 hours was analyzed by diffraction of X-rays. The diffractogram of X-rays obtained is presented in Figure 1.

[figure omitted; refer to PDF]

The diffractogram of zinc oxide shows some peaks of diffraction at $2\theta = 31.77^\circ, 34.25^\circ, 36.25^\circ, 47.54^\circ, 57.77^\circ, 62.85^\circ, 66.46^\circ, 67.93^\circ$, and 69° that can be effected into the crystalline plan of ZnO (100), (002), (101), (102), (110), (103), (200), (112), and (201). All the reflexions can be indicated during ZnO phase hexagonal wurtzite with network constants $a = 0.3249\text{nm}$ and $c = 0.5205\text{nm}$, which is accurate with the standard data (card JCPDS N° 79-2205) [16].

The clearness and the intensity of the pick indicate the good crystalline nature of the prepared sample. No appearance of a line in the ZnO diffractogram indexed to the $\text{Zn}(\text{OH})_2$ structure, which means that $\text{Zn}(\text{OH})_2$ has been completely decomposed into ZnO at 500°C for 3 hours [17, 18].

3.1.2. Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR spectra obtained for ZnO (Figure 2) show bands located at 406, 506, and 806cm^{-1} characterizing ZnO. In addition, bands located at 3420; 1637, and 1385cm^{-1} corresponding to the vibration of the hydroxyl group of water and carbon dioxide are also identified. [18, 19].

[figure omitted; refer to PDF]

The presence of hysteresis sample in this isotherm is associated with the secondary process of capillary concentration, which is translated by filling completely the measured P/P_0 .

Table 1

Textural characteristics of solids.

Solid	Specific surface (m^2/g)	Volume (cm^3/g)	Pore diameter ($\text{\AA}$)
ZnO	80.75	0.573	220

3.2. Catalytic Test: Catalytic Oxidation of Isopropanol

3.2.1. Blind Test

The tests were performed without a catalyst in the same pressure and temperature conditions, which show the absence of every catalytic activity in the homogenous phase, and same results were obtained by Sahroui et al. [21].

3.2.2. Catalyst Mass Effect

We are interested in the evaluation of the impact of the mass of a catalyst on the activity of the oxidation reaction of isopropanol in its liquid phase at a low temperature of 80°C. We tested the zinc oxide with three masses: 120, 100, and 140 mg.

The specific activity is defined as the number of moles of isopropanol disappeared per unit of time and unit of mass of the catalyst [15]. $(1)\text{asmol}\cdot\text{g}^{-1}\cdot\text{s}^{-1} = -1\text{mdndt}$.

The influence of the amount of catalyst on the specific activity is illustrated in Figure 4. The initial specific activity relating to the disappearance of isopropanol keeps a constant value whatever the amount of catalyst is. This means that, under these conditions, the catalyst operates in the chemical regime and is not the diffusion regime.

[figure omitted; refer to PDF]

Zinc oxide allows a total conversion of isopropanol into carbon dioxide, and the water lasts no longer than 100 min, 120 min, and 140 min at a temperature of 80°C, 60°C, and 40°C. It can be concluded from this that the total destruction of isopropanol in the liquid phase is carried out rapidly at slightly moderate temperatures in the presence of this synthetic solid. These results of zinc oxide activity have been noted by many authors [22–26].

The catalyst activity concerning the disappearance of a reactive is defined by the number of the reported moles by the unit of time that disappeared at the moment of the reaction. In addition to the temperature of the reaction and the

composition of the reactor's mixture, the catalytic activity depends on the nature of the catalyst and the structural textural properties. This dependence is expressed by the specific activity defined in the following. This dependence is expressed by the specific activity and defined as the ratio of the catalytic activity and the mass of catalyst as (mole/g·s) follows:

$$r_{\text{cat}} = \frac{1}{W} \left(-\frac{dn_0}{dt} \right)_{t=0}$$

where r_{cat} is the specific activity, W is the mass of catalyst, n_0 is the number of moles of reactant, and t is time.

Table 2

Oxide	T°C	as (mole·g ⁻¹ ·s ⁻¹)	v_0 (mole·s ⁻¹)	$\ln(a_s)$	$\ln(v_0)$
ZnO	40	4.25E-06	5.09E-07	-12.369600	-14.4898641
60	4.28E-06	5.13E-07	-12.362667	-14.4829315	80

3.3. The Kinetics of the Reaction

The curves of Figure 6 present the rate evolution of global transformation TT_G , acetone transformation TT_{act} , and carbon dioxide TT_{CO_2} in terms of TT_G through different temperatures (40, 60, and 80°C).

The analysis of the results shows that the oxidation of isopropanol in carbon dioxide and water is complete at the temperature of 80°C for a reacting duration of 100 min; also, the transformation in carbon dioxide reaches two very important values which are 97% at 60°C and 93% at 40°C. The secondary product formed is acetone, with a trend higher than 72.04% at 80°C. The significant quantity of acetone produced by the decomposition reaction of isopropanol confirms the properties of the zinc oxide surface as a base [33, 34]. The results enable us to conclude that this catalyst presents basic sites and leads a predisposition to form acetone, a result that is confirmed in the bibliography [12]. The power of ZnO catalytic can be explained by the elevated capacity of transporting oxygen, as well as shifting easily between its reduced and oxidized form (the mechanism of Mars–van Krevelen) [2, 34, 35]. These curves behave the same way regardless of the temperature. The curve represents the rate of transformation of acetone in terms of the rate of global conversion pass by a maximum; therefore, the one corresponding to CO₂ grams between 0 and 100 [31]. Therefore, this allows suggesting a mechanistic diagram of the form(3)Isopropanol AcetoneCarbon's dioxide+water

3.3.2. Appeared Energy of Activation

Specific activity can be expressed as $as = A \exp(-Ea/RT)f$, where A is a preexponential Arrhenius factor and f a function of active constituents concentrations and catalyst mass.

The relation of Arrhenius by tracing the line $\ln a_s = \ln A - E_a/R \cdot T$.

Figure 7 presents the variation $\ln(as) = f(1/T)$; from this, we find the value of the appeared activation energy in the order of 3,48 kJ/mol for zinc oxide. It has to be noted that the weakest value of the activation energy is relative to the dispersion of isopropanol and is due to the strongest value of the initial speed in the domain of the studied temperature [21]. The obtained results in this study show weak activation energy in comparison to what Deepak Kulkarni's work has shown [34]. This approves the big catalytic potential of zinc oxide.

[figure omitted; refer to PDF]

4. Conclusions

In conclusion, we can conclude that the structural characteristics show a wurtzite structure, particles of this oxide with a hexagonal shape, that the textural characteristic rises, and that the isotherm is of type IV with a hysteresis loop H3.

Zinc oxide prepared by the precipitation method presents a good catalytic activity against the reaction of decomposition of isopropanol in its liquid phase with a low temperature and acetone as a mediating product. An activity of the order $4.25E-06 \text{ mole}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$ for the temperature of 40°C, $4.28E-06 \text{ mole}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$ for the temperature of 60°C, and $4.96E-06 \text{ mole}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$ for the temperature of 80°C are shown.

Otherwise, the kinetic study of the reaction of the oxidation, in liquid phase of the realized isopropanol, showed that this reaction follows a successive mechanism of isopropanol.

- [1] Y. Dehmani, A. A. Alrashdi, H. Lgaz, T. Lamhasni, S. Abouarnadasse, I.-M. Chung, "Removal of phenol from aqueous solution by adsorption onto hematite ($\alpha\text{-Fe}_2\text{O}_3$): mechanism exploration from both experimental and theoretical studies," *Arabian Journal of Chemistry*, vol. 13 no. 5, pp. 5474-5486, DOI: 10.1016/j.arabjc.2020.03.026, 2020.
- [2] R. Beauchet, J. Mijoin, I. Batonneau-Gener, P. Magnoux, "Catalytic oxidation of VOCs on NaX zeolite: mixture effect with isopropanol and o-xylene," *Applied Catalysis B: Environmental*, vol. 100 no. 1-2, pp. 91-96, DOI: 10.1016/j.apcatb.2010.07.017, 2010.
- [3] X. Li, L. Zhang, Z. Yang, P. Wang, Y. Yan, J. Ran, "Adsorption materials for volatile organic compounds (VOCs) and the key factors for VOCs adsorption process: a review," *Separation and Purification Technology*, vol. 235, DOI: 10.1016/j.seppur.2019.116213, 2020.
- [4] L. Zhu, D. Shen, K. H. Luo, "A critical review on VOCs adsorption by different porous materials: species, mechanisms and modification methods," *Journal of Hazardous Materials*, vol. 389, DOI: 10.1016/j.jhazmat.2020.122102, 2020.
- [5] C. Yang, "Abatement of various types of VOCs by adsorption/catalytic oxidation: a review," *Chemical Engineering Journal*, vol. 370, pp. 1128-1153, 2018.
- [6] Y. Dehmani, S. Abouarnadasse, "Study of the adsorbent properties of nickel oxide for phenol depollution," *Arabian Journal of Chemistry*, vol. 13 no. 5, pp. 5312-5325, DOI: 10.1016/j.arabjc.2020.03.010, 2020.
- [7] Y. Dehmani, A. Ed-Dra, O. Zennouhi, "Chemical characterization and adsorption of oil mill wastewater on Moroccan clay in order to be used in the agricultural field," *Heliyon*, vol. 6 no. 1, DOI: 10.1016/j.heliyon.2020.e03164, 2020.
- [8] M. A. Al-ghouti, M. A. Al-kaabi, M. Y. Ashfaq, D. A. Da'na, "Produced water characteristics, treatment and reuse: a review," *Journal of Water Process Engineering*, vol. 28, pp. 222-239, DOI: 10.1016/j.jwpe.2019.02.001, 2019.
- [9] H. Zhang, C. Luo, Y. Yan, "Adsorption dynamics of isopropanol in structured fixed bed with microfibrillar ZSM-5 zeolite structured composite," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 80, pp. 779-786, DOI: 10.1016/j.jtice.2017.09.020, 2017.
- [10] ", Cristina Giordano "A Neglected World: Transition Metal Nitride and Metal Carbide Based Nanostructures Novel Synthesis and Future Perspectives", . Chapter 2, University of Potsdam, Potsdam, Germany, 2014
- [11] B. B. Mohammed, K. Yamni, N. Tijani, "Adsorptive removal of phenol using faujasite-type Y zeolite: adsorption isotherms, kinetics and grand canonical Monte Carlo simulation studies," *Journal of Molecular Liquids*, vol. 296, DOI: 10.1016/j.molliq.2019.111997, 2019.
- [12] H. Asnaoui, Y. Dehmani, M. Khalis, E.-K. Hachem, "Adsorption of phenol from aqueous solutions by Na-

- bentonite: kinetic, equilibrium and thermodynamic studies," *International Journal of Environmental Analytical Chemistry*, vol. 0 no. 0, DOI: 10.1080/03067319.2020.1763328, 2020.
- [13] Y. Dehmani, L. Sellaoui, Y. Alghamdi, "Kinetic, thermodynamic and mechanism study of the adsorption of phenol on Moroccan clay," *Journal of Molecular Liquids*, vol. 312, DOI: 10.1016/j.molliq.2020.113383, 2020.
- [14] A. Dehbi, Y. Dehmani, H. Omari, "Comparative study of malachite green and phenol adsorption on synthetic hematite iron oxide nanoparticles (α -Fe₂O₃)," *Surfaces and Interfaces*, vol. 21, DOI: 10.1016/j.surfin.2020.100637, 2020.
- [15] Y. Dehmani, S. Abouarnadasse, "Total oxidation of isopropanol in the liquid phase, under atmospheric pressure and low temperature, on transition metal oxides catalysts Cr₂O₃ and Fe₂O₃," *Journal of Chemistry*, vol. 2020, DOI: 10.1155/2020/6129526, 2020.
- [16] M. Yang, G. Pang, L. Jiang, S. Feng, "Hydrothermal synthesis of one-dimensional zinc oxides with different precursors," *Nanotechnology*, vol. 17 no. 1, pp. 206-212, DOI: 10.1088/0957-4484/17/1/034, 2006.
- [17] R. Saravanan, V. K. Gupta, V. Narayanan, A. Stephen, "Comparative study on photocatalytic activity of ZnO prepared by different methods," *Journal of Molecular Liquids*, vol. 181, pp. 133-141, DOI: 10.1016/j.molliq.2013.02.023, 2013.
- [18] J. Kaur, S. Bansal, S. Singhal, "Photocatalytic degradation of methyl orange using ZnO nanopowders synthesized via thermal decomposition of oxalate precursor method," *Physica B: Condensed Matter*, vol. 416, pp. 33-38, DOI: 10.1016/j.physb.2013.02.005, 2013.
- [19] H. Wang, C. Xie, W. Zhang, S. Cai, Z. Yang, Y. Gui, "Comparison of dye degradation efficiency using ZnO powders with various size scales," *Journal of Hazardous Materials*, vol. 141 no. 3, pp. 645-652, DOI: 10.1016/j.jhazmat.2006.07.021, 2007.
- [20] K. T. Alali, T. Liu, J. Liu, "Preparation and characterization of ZnO/CoNiO₂ hollow nanofibers by electrospinning method with enhanced gas sensing properties," *Journal of Alloys and Compounds*, vol. 702, pp. 20-30, DOI: 10.1016/j.jallcom.2017.01.167, 2017.
- [21] H. Sahraoui, S. Abouarnadasse, K. Elkamel, A. Nadiri, A. Yacoubi, "Étude de l'activité catalytique d'une diatomite naturelle marocaine dans la réaction de décomposition de l'isopropanol," *Annales de Chimie Science des Matériaux*, vol. 28 no. 4, pp. 91-105, DOI: 10.1016/s0151-9107(03)00098-9, 2003.
- [22] M. Sadiq, K. Saeed, S. Sadiq, S. Munir, M. Khan, "Liquid phase oxidation of cinnamyl alcohol to cinnamaldehyde using multiwall carbon nanotubes decorated with zinc-manganese oxide nanoparticles," *Applied Catalysis A: General*, vol. 539, pp. 97-103, DOI: 10.1016/j.apcata.2017.04.007, 2017.
- [23] A. Król, P. Pomastowski, K. Rafińska, V. Railean-Plugaru, B. Buszewski, "Zinc oxide nanoparticles: synthesis, antiseptic activity and toxicity mechanism," *Advances in Colloid and Interface Science*, vol. 249, pp. 37-52, DOI: 10.1016/j.cis.2017.07.033, 2017.
- [24] Z. Q. Liu, J. Tu, Q. Wang, "Catalytic ozonation of diethyl phthalate in aqueous solution using graphite supported zinc oxide," *Separation and Purification Technology*, vol. 200, pp. 51-58, DOI: 10.1016/j.seppur.2018.02.026, 2018.
- [25] D. Ali, Y. Dehmani, H. Omari, A. Lamini, K. Elazhari, A. Abdallaoui, "Hematite iron oxide nanoparticles (α -Fe₂O₃): synthesis and modelling adsorption of malachite green », " *Journal of Environmental Chemical Engineering*, vol. 8 no. 1, DOI: 10.1016/j.jece.2019.103394, 2020.
- [26] Y. Dehmani, H. Lgazb, A. A. Alrashdi, T. Lamhasni, S. Abouarnadasse, I.-M. Chung, "Phenol adsorption mechanism on the zinc oxide surface: experimental, cluster DFT calculations, and molecular dynamics simulations," *Journal of Molecular Liquids*, vol. 324, DOI: 10.1016/j.molliq.2020.114993, 2021.
- [27] H. Chen, H. Zhang, Y. Yan, "Gradient porous Co-Cu-Mn mixed oxides modified ZSM-5 membranes as high efficiency catalyst for the catalytic oxidation of isopropanol," *Chemical Engineering Science*, vol. 111, pp. 313-323, DOI: 10.1016/j.ces.2014.02.031, 2014.
- [28] W. Zhou, C. Wang, J. Xu, Y. Du, P. Yang, "Enhanced electrocatalytic performance for isopropanol oxidation on Pd-Au nanoparticles dispersed on poly (p-phenylene) prepared from biphenyl," *Materials Chemistry and Physics*, vol. 123 no. 2-3, pp. 390-395, DOI: 10.1016/j.matchemphys.2010.04.027, 2010.

- [29] A. Ghosh, K. Bhaduri, S. Shah, A. Auroux, J. K. Pandey, B. Chowdhury, "Dehydration of isopropanol to propylene over fullerene[C 60] containing niobium phosphate catalyst: study on catalyst recyclability," *Molecular Catalysis*, vol. 475, DOI: 10.1016/j.mcat.2019.110470, 2019.
- [30] O. N. Fedyaeva, D. O. Artamonov, A. A. Vostrikov, "Effect of H₂O and CO₂ on propane, propene, and isopropanol oxidation at elevated pressures," *Combustion and Flame*, vol. 199, pp. 230-240, DOI: 10.1016/j.combustflame.2018.09.031, 2019.
- [31] J. Medina-Valtierra, C. Frausto-Reyes, G. Camarillo-Martínez, J. A. Ramírez-Ortiz, "Complete oxidation of isopropanol over Cu₄O₃ (paramelaconite) coating deposited on fiberglass by CVD," *Applied Catalysis A: General*, vol. 356 no. 1, pp. 36-42, DOI: 10.1016/j.apcata.2008.12.014, 2009.
- [32] J. Abelleira, J. Sánchez-Oneto, J. R. Portela, E. J. Martínez de la Ossa, "Kinetics of supercritical water oxidation of isopropanol as an auxiliary fuel and co-fuel," *Fuel*, vol. 111, pp. 574-583, DOI: 10.1016/j.fuel.2013.03.033, 2013.
- [33] J. Karuppiyah, L. Sivachandiran, R. Karvembu, C. Subrahmanyam, "Catalytic nonthermal plasma reactor for the abatement of low concentrations of isopropanol," *Chemical Engineering Journal*, vol. 165 no. 1, pp. 194-199, DOI: 10.1016/j.cej.2010.09.017, 2010.
- [34] D. Kulkarni, I. E. Wachs, "Isopropanol oxidation by pure metal oxide catalysts: number of active surface sites and turnover frequencies," *Applied Catalysis A: General*, vol. 237 no. 1-2, pp. 121-137, DOI: 10.1016/s0926-860x(02)00325-3, 2002.
- [35] S. Xing, Z. Liu, Q. Xue, "Rh nanoroses for isopropanol oxidation reaction," *Applied Catalysis B: Environmental*, vol. 259, DOI: 10.1016/j.apcatb.2019.118082, 2019.
- [36] I. Z. Ismagilov, E. M. Michurin, O. B. Sukhova, "Oxidation of organic compounds in a microstructured catalytic reactor," *Chemical Engineering Journal*, vol. 135 no. SUPPL. 1, pp. S57-S65, DOI: 10.1016/j.cej.2007.07.036, 2007.

DETAILS

Subject:	Liquid phases; Nitrates; Catalytic oxidation; Pollutants; Catalytic activity; Chemical precipitation; Spectrum analysis; Fourier transforms; Carbon dioxide; Zinc oxides; Decomposition; Infrared analysis; Zinc oxide; Oxidation; Acetone; Isopropanol; Structural analysis; Low temperature; Selectivity; Wurtzite; X-rays
Editor:	Mohamed Abdel-Rehim
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry

ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-10-25 (Received); 2021-01-21 (Revised); 2021-01-28 (Accepted); 2021-02-09 (Pub)
DOI:	https://doi.org/10.1155/2021/6667551
ProQuest document ID:	2491753216
Document URL:	https://www.proquest.com/scholarly-journals/total-oxidation-isopropanol-liquid-phase-at-low/docview/2491753216/se-2?accountid=211160
Copyright:	Copyright © 2021 Younes Dehmani et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-12-13
Database:	Publicly Available Content Database

Document 53 of 59

Analytical Quality by Design Approach of Reverse-Phase High-Performance Liquid Chromatography of Atorvastatin: Method Development, Optimization, Validation, and the Stability-Indicated Method

Alruwaili, Nabil K

[ProQuest document link](#)

ABSTRACT (ENGLISH)

The use of analytical quality by design (AQbD) approach in the optimization of the high-performance liquid chromatography (RP-HPLC) method is a novel tool. Three factors and three levels of Box–Behnken statistical design (BBD) were used for method optimization and analysis of atorvastatin. The mobile phase (acetonitrile: water), flow rate (Rt), and UV wavelength were used as independent variables. Their effects were observed in the area of the chromatogram (AU), retention time (Rt, min), and tailing factor (%). The optimized HPLC condition was found as acetonitrile:water (50:50), flow rate (0.68 ml/min), and UV wave length (235 nm). It gives the retention time of 2.43 min with the linearity range of 5–30 µg/ml with a high regression value ($r^2=0.999$). The method was found to be precise and accurate with low % RSD (<5%). The refrigeration stability indicated that atorvastatin was stable. The force degradation study showed that the atorvastatin was fully unstable in UV light and stable in 0.1 M basic condition. It concluded that this QbD optimized method is suitable for quantification of the atorvastatin from the formulation as well as pharmacokinetic parameters.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Atorvastatin (ATS) belongs to statin class and is used for lowering the lipid in the body through desensitizing the making of cholesterol in the liver resulting in decreasing the risk of cardiovascular diseases. Mainly, it completely inhibited hydroxymethylglutaryl coenzyme A reductase enzyme [1, 2]. Chemically, it is (3R, 5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propane-2-ylpyrrol-1-yl]-3,5 dihydroxy eptanoic acid [3] (Figure 1). ATS is insoluble in water and soluble in acetonitrile, methanol, ethanol, dimethyl sulfoxide, and other organic solvents [4, 5].

[figure omitted; refer to PDF]

The RP-HPLC is a very sensitive analytical technique for estimation of the drug from the formulation as well as a biological sample [6]. Some HPLC methods for ATS literature have been reported earlier by a researcher, i.e., simultaneous estimation of ATS, amlodipine, and benazepril [7, 8]. Erturk et al. reported the HPLC method for estimation of ATS from the bulk drug and tablet [9, 10]. Martins and associates estimated the ATS from the biological sample [11], but ATS did not well resolve and has longer retention time (Rt).

Several published studies reported the relevance of quality by design approach for optimization of the HPLC method and provide prized information about the interaction effect of the variable over the response as well as give appropriated chromatographic condition in which drug is well separated [12, 13]. The analytical quality by design (AQbD) is a novel tool for optimization of HPLC instrumental conditions such as flow rate, the solvent system, solvent ratio, the effect of temperature, injection volume, the resolution of the drug (Rt) as well as cost and effort. The AQbD gives information on risk associated with the method as well as the influencing factor effect on analytical method performance. Various research publications have been explained by the application of AQbD approaches [14–16]. This approach can create various analytical chromatographic conditions with noticeably improved method presentation. This approach also helps in determining the crucial method variables, which directly impact the performance, enlarge the least resources of time, endeavour, and cost. It helps to understand the risk assessment of the critical factors which are directly influenced in the presentation of the analytical method [17, 18]. It is based on the principles of experimental design that participated in a systematic thoughtful of the reasonable risk and interaction between the experimental variables [19]. The Box–Behnken design has various advantages such as prevent the waste of solvent and time. It gives a lesser number of runs in the various compositions of independent variables. There are many published research works on optimization of RP-HPLC using QbD (Box–Behnken design) reported such as voriconazole [20], methotrexate [21], dapagliflozin [12], lacidipine [13], timolol maleate [22], risperidone [23], and temozolomide [24]. No method has been reported on the RP-HPLC method using a QbD approach for atorvastatin. This research work developed and optimized the analytical condition by using AQbD (Box–Behnken design).

2. Materials and Methods

2.1. Materials

Atorvastatin (ATS, 99.99%) as a gift sample was obtained from Aldrich (St. Louis, US). HPLC grade water, methanol, and acetonitrile were procured from Sigma Aldrich (St. Louis, US). All other chemicals used in this study are analytical grade.

2.2. Methods

2.2.1. Liquid Chromatographic Condition

High-performance liquid chromatography (HPLC, Auto sampler, Shimadzu LC10AD, Kyoto Japan) attached with a UV detector (SPD-20A) was used for the method development of ATS. The ultrafine particle packed 250mm length C₁₈ column (Acclaim120C18, 4.6mm internal diameter, 2.2 μ m particle size) was used for chromatographic separation of ATS. Flow range, i.e., 0.1–1 ml/min, was used for separation of ATS. The different mobile phase compositions of acetonitrile and water (50:50, 60:40, and 70:30) were used as mobile phase. The whole operation was carried out at room temperature. The sample (30 μ L) was applied as injection volume for each sample.

2.2.2. Risk Assessment Study

This study was conducted to understand the various factors affecting the target quality profile (TQP). The critical analytical attributes were applied to visualize the proper relationships between parameters to the target quality profile before risk judgment. This study was used to classify the possible causes of difficulty to obtain the reason for deficiency, discrepancy, and failure (defect). It is also applied to determine the considerable role for the extraction of vital detail required to achieve the target quality profile. The factors which influence the target response are categorized as a low, medium, and high score [25]. The seven factors were used and further divided into low, medium, and high-risk levels for screening and obtaining only a few factors. Finally, three out of seven factors were selected for the optimization of responses using experiment designs (Table 1). Using this study, the target quality profile was recognized in the form of retention time (Rt), area, and tailing factor from the independent seven variables.

Table 1

RP-HPLC analytical method risk assessment for atorvastatin.

Critical attributes response	Various RP-HPLC chromatographic conditions for atorvastatin							
Composition of mobile phase	UV wavelength	Injection volume	Column temperature	Flow rate	Sample temperature	Column dimension	Type of vial	Chromatogram area

+2	+1	-1	-1	+2	-1	+1	-1	Retention time
+2	+1	-1	+1	+2	-1	+1	-1	Tailing factor

+2, high-risk parameter; +1, medium-risk parameter; -1, low-risk parameter.

2.2.3. Optimization

The AQbD method was used to analyze the effect of independent factors onto the responses. Box–Behnken design (BBD, Design Expert 8.0.7.1, Stat-Ease Inc., Write country) is used for the optimization because it gives a few numbers of experimental trials with appropriate composition [21]. The three-factor and three-level BBD were used for the optimization of an analytical method. The mobile phases, i.e., acetonitrile (X_1 , %), flow rate (X_2 , ml/min), and UV wavelength (X_3) and their single and combined effects were examined over the selected responses, i.e., area (Y_1), Rt (min, Y_2), and tailing factor (% , Y_3). In total, 17 experimental runs at various compositions were obtained from software to find out the best composition (Table 2). The experimental data were fitted into different kinetic models such as linear, second-order, quadratic, and cubic models to find the best fit model. The polynomial equation, 3D plot, and contour plot were measured to evaluate the effect of the variable on responses. Statistical analysis ANOVA and regression analysis were performed to determine the significant level for the model and to determine the best fit model.

Table 2

Critical influencing variable and their actual and predicted values of responses.

Standard order	Critical influencing variable				Response				
Acetonitrile (%)	Flow rate (ml/min)	Wavelength (nm)	Area (AU)		Retention time		Tailing factor		Actual value
Predicted value	Actual value	Predicted value	Actual value	Predicted value	1	50	0.35	240	1975990
1975589	2.19	2.18	1.95	1.94	2	90	0.35	240	1501486

1502578	4.18	4.19	2.42	2.41	3	50	1	240	214 967 0
2148578	3.23	3.22	2.12	2.12	4	90	1	240	173 039 5
1730796	5.33	5.34	2.47	2.48	5	50	0.68	235	203 716 1
2038807	2.45	2.47	2.08	2.09	6	90	0.68	235	156 506 9
1565223	4.84	4.84	2.51	2.52	7	50	0.68	245	200 497 0
2004816	2.83	2.83	2.04	2.05	8	90	0.68	245	158 925 3
1587607	4.62	4.6	2.44	2.43	9	70	0.35	235	175 546 5
1754220	2.9	2.89	2.21	2.21	10	70	1	235	198 476 5
1984211	4.02	4.02	2.34	2.33	11	70	0.35	245	177 725 0
1777804	2.98	2.99	2.14	2.15	12	70	1	245	194 777 4
1949019	4.04	4.05	2.26	2.26	13	70	0.68	240	180 478 2
1804782	3.54	3.54	2.01	2.01	14	70	0.68	240	180 478 2

1804782	3.54	3.54	2.02	2.01	15	70	0.68	240	1804782
1804782	3.54	3.54	2.01	2.01	16	70	0.68	240	1804782
1804782	3.54	3.54	2.01	2.01	17	70	0.68	240	1804782

2.2.4. Preparation of Standard Solution and Linearity Curve

The standard solution was prepared by weighing ATS (10mg) and dissolved in HPLC grade methanol (10mL). From this stock solution, further dilution was performed to prepare the concentration range of 5–30 µg/ml using methanol. Each sample was injected (20 µL) in the HPLC instrument by an autosampler, and the different parameters were determined. The linearity curve was plotted between the area of chromatogram vs. concentration (µg/ml), and the linear regression equation was calculated. The analysis was performed in triplicate, and the mean value with standard deviation was used.

2.2.5. Quality Control Sample

For validation of the method, the quality control sample was prepared to form a stock solution at three levels, i.e., low-quality control (1.5 µg/ml), the medium limit of quality control (MQC 12.43 µg/ml), and high-quality control (HQC, 24 µg/ml).

2.2.6. Method Validation

HPLC method was validated according to the approved protocol given in ICH Q2 (R1). The method was validated for linearity, accuracy, sensitivity, precision, recovery, LOD, and LQC of the quality control sample [26, 27].

2.2.7. System Suitability and Specificity

The system suitability of the method was performed, and the area, Rt, and tailing factor were assessed with the same concentration of ATS injection in six replicates. The specificity of the method was assessed to find out any interference on chromatographic separation of the ATS with a blank sample.

2.2.8. Linearity and Sensitivity Analysis

The linearity study of ATS was analyzed at a concentration range of 5–30 µg/ml. The prepared sample was injected into the HPLC instrument, and the peak area was noted. The linearity curve was plotted between AST concentrating vs. the peak area. The slope and regression coefficient were calculated from the curve. The study was carried out in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were analyzed by signal-to-noise ratio of 3:1 and 10:1 level and calculated by the following formula.(1)LOD=3.3×Standard deviation Slope,LOQ=10×Standard deviation Slope.

2.2.9. Precision and Accuracy

Accuracy and precision studies were performed to determine the closeness between several injections of the same concentration. The study was conducted several times on the same day and on different days. The intraday (within a day) and interday (different days) precision and accuracy of the method were analyzed at three different quality samples, i.e., LOC (1.5 µg/ml), MQC (12.43 µg/ml), and HQC (24 µg/ml) for six replicates three times a day and three different days. The regressed standard deviation (% RSD) was calculated for each study.

2.2.10. Recovery Studies

The recovery study of ATS was estimated by spiking the sample after the addition of an extra quantity of standard ATS (50, 100, and 150%). The analysis was performed by HPLC using an optimized mobile phase, flow rate, and UV wavelength in triplicate. The % recovery was calculated by the given formula:(2)% Recovery=Recovered

$\text{concentration}/\text{Initial concentration} \times 100$.

2.2.11. Storage Stability Study

The refrigerated stability of ATS was analyzed at LQC and HQC concentration. The study was conducted at different temperatures for long-term, bench-top, freeze-thaw, and postprocessing stability. The long-term ATS stability was performed at -80°C for 30 days in the deep freezer. The freeze-thaw ATS stability was performed at -20°C to 25°C for three successive days. The bench-top study was carried out at 25°C after 24 h of storage. The postoperation study was carried out at 10°C by storing the sample in an autosampler of HPLC. The study was conducted in six replicates, and drug concentration as well as % RSD was calculated.

2.2.12. Forced Degradation Study

Stress degradation study provides information about the stability of the drug and drug product. It gives information about the stability of substances with acid, base, light, pH, hydrolysis, and oxidation condition. It directly affects the selection of formulation development, packaging, storage, transportation, shelf life, chemical stability of the drug, and drug product [28, 29].

2.2.13. Acid and Base Degradation Analysis

The study was conducted with 1 M hydrochloric acid and 0.1 M sodium hydroxide. An equal volume ratio of the ATS stock solution was taken and added to acid and base solutions and mixed into the round bottom flask. The mixture was refluxed using an air reflux condenser at 90°C for 6 h, and then, the sample was cooled at room temperature. The sample was appropriately diluted with HPLC methanol, filtered through with the membrane filter ($0.45\ \mu\text{m}$), and injected into the HPLC instrument.

2.2.14. Oxidation Degradation Study

The oxidation degradation study was carried out by using the oxidizing agent hydrogen peroxide (30%). The standard stock solution of ATS was mixed with hydrogen peroxide in equal volume. The sample was refluxed with air condenser at 90°C for 6 h and cooled at room temperature, diluted with methanol. The sample ($20\ \mu\text{l}$) was injected into the HPLC instrument and analyzed for drug content.

2.2.15. Photolytic Degradation Study

The photodegradation study was conducted to evaluate the effect of UV light during the processing of API for formulation development. For this study, the standard stock solution of ATS placed into an open quartz glass vial and exposed with UV light (200 watt/square meter) for 24 h. Then, the sample was appropriately diluted with HPLC grade methanol and analyzed by HPLC.

3. Result and Discussion

3.1. Method Development

The RP-HPLC analytical method of ATS was developed and optimized by AQbD. The various mobile phases such as methanol, acetonitrile, water, and phosphate buffer in different compositions were used with different flow rates to obtain a suitable RP-HPLC method. Among different mobile systems with different ratios, a well-resolved peak was not found. The retention time was also found to be high. Finally, acetonitrile and water as the mobile phase was tried at 50:50 v/v and got a well-determined peak at 2.43 min using a flow rate of 0.5 ml/min, at 235 nm. The further mobile phase, flow rate, and UV wavelength were used to optimize BBD software for a robust method.

3.2. Risk Assessment Study

The risk assessment study was carried out for the determination of essential constraint of the method and to get the robust condition. It shows the connection between various parameters and analytical attributes. As per the examination of previously published research work descriptions, various possible input parameters were measured at a different level of risks connected with each experimental parameter of the HPLC method, i.e., the ratio of mobile phase and flow rate. It was found that wavelength and flow rate in the medium risk zone and other constraints have less risk due to the negligible effect on the developed method. There is continuous improvement in the design space that has been found with the use of risk management and screening, experimental design, and response surface methodology to get an optimum analytical condition. The use of the high, medium, and low-risk factors is depicted in Table 1.

3.3. Optimization

The BBD software was used for the optimization of analytical (HPLC) parameters to get the optimum conditions for the estimation of ATS. The independent variables such as acetonitrile (50–90% v/v), flow rate (0.35–1 ml/min), and wavelength (235–245 nm) were used to determine the effect on dependent variables (Rt, area, and tailing factor). Total seventeen experimental runs with five centre points obtained from the software for determination of optimum chromatographic separation of ATS (Table 2). The variables, i.e., mobile phase ratio (water: acetonitrile 30:70% v/v), flow rate (0.7 ml/min), and wavelength (240 nm), are the centre point of composition. The desirable factor was found to be very close to one (0.9653) at centre point, indicating well-fitting of the model, and the responses were within the target value. The effects of variables over the responses were evaluated to select the design space. The data of responses were fitted into different polynomial designing models such as linear, second-order, quadratic, and cubic. The quadratic model was found to be the best fit model because its regression coefficient ($R^2=0.9999$) is significantly higher (pF value, mean square, and p value of each response were also calculated. The 3D and contour plot of each response was constructed from the software and demonstrated the effect of multiple variables (integration effect) on a response at one time (Figures 2(a)–2(c)).

[figures omitted; refer to PDF]

3.4. Effect of Independent Factors on Peak Area

The effect of an independent variable on the peak area was expressed by the 3D response, contour plot, and polynomial equation. The 2nd order quadratic polynomial for response Y_1 (area) is given as follows.

$$\text{Area } (Y_1) = 1804782 - 222698.48X_1 - 100301.57X_2 - 2901.74X_3 + 13807.15X_1X_2 + 14093.63X_1X_3 - 14694.06X_2X_3 - 16298.40X_{12} + 50901.77X_{22} + 10629.64X_{32}.$$

In this polynomial equation, the X_1 , X_2 , X_3 , X_1X_2 , X_1X_3 , X_2X_3 , X_{12} , X_{22} , and X_{32} are the significant model terms because all the model terms have pF value of the quadratic model was found to be 32195.28, and it implies that the model is significant. The predicted R^2 (0.9996) is closer to the adjusted R^2 (0.9999). The adequate precession is greater than four (646.328), and it indicated that the model has sufficient signal. The polynomial equation showed that acetonitrile (X_1 , ACN) has shown a positive effect, whereas flow rate (X_2) and wavelength (X_3) have a negative effect on the peak area. The peak area linearly decreases with an increase in the acetonitrile percentage with respect to water, flow rate, and wavelength (Figure 2(a)). But the percentage of acetonitrile and flow rate has a dominant effect on the peak area of chromatogram as compared to detector wavelength.

3.5. Effect of Independent Factors on Retention Time

The effect of an independent variable on the retention time was expressed by the 3D response, contour plot, and polynomial equation. The 2nd order quadratic polynomial equation for the response Y_2 , retention time, is given as follows.

$$\text{Retention time } (Y_2) = +3.54 + 1.02X_1 + 0.55X_2 + 0.024X_3 + 0.027X_1X_2 - 0.1X_1X_3 - 0.015X_2X_3 + 0.21X_{12} - 0.018X_{12} - 0.037X_{12}.$$

The effects of the different variables on flow rate were expressed by the polynomial equation, 3D plot, and contour plots. The factors X_1 , X_2 , and X_3 showed the synergistic effect on the response. On increasing the % of acetonitrile (X_1), the Rt of ATS increased. The high increment of Rt is not good for analysis because of the wastage of the solvent as well as time. So, it selected the medium percentage of solvent for analysis. The flow rate (X_2) increases as the Rt value increases slightly as compared to acetonitrile. The design showed the quadratic model as the best fit model because the regression coefficient is closer to one ($R^2=0.9999$) than other analytical experimental design models. The model F value (5910.01) implies that the model is significant (pX_1 , X_2 , X_3 , X_1X_2 , X_1X_3 , X_2X_3 , X_{12} , X_{22} , and X_{32}) have pR^2 (0.9997) is closer to the adjusted R^2 (0.9979). The adequate precession value from the software represented is greater than four (1923.940) and indicates that the model has sufficient signal. The 3D and contour plot showed that the Rt increases with increase in the flow rate (Figure 2(b)). In this method, the medium level of flow rate (ml/min) was selected as an optimized condition for the method development. The third-factor wavelength showed increases in the UV wavelength; the Rt value of ATS slightly increases as compared to other factors, i.e., acetonitrile and flow rate. The interaction effect (combined) of acetonitrile, wavelength (X_1X_3) and flow rate,

wavelength (X_2X_3) showed an antagonistic effect on the Rt of ATS.

3.6. Effect of Independent Factors on Tailing Factor

The effect of an independent variable on the retention time was expressed by the 3D response, contour plot, and polynomial equation. The 2nd order quadratic polynomial equation for the response Y_3 (tailing factor) is given as follows.

$$\text{Tailing factor } (Y_3) = +2.012 + 0.206X_1 + 0.059X_2 - 0.032X_3 - 0.03X_1X_2 - 0.008X_1X_3 - 0.002X_2X_3 + 0.129X_{12} + 0.099X_{13} + 0.127X_{23}$$

The quadratic model was found to be the best model rather than other analytical experimental designing models. The regression coefficient is found close to one ($R^2=0.9993$) (Table 3). The model terms X_1 , X_2 , X_3 , X_1X_2 , X_{12} , X_{22} , and X_{32} are the significant effects on response (pX_1X_3 and X_2X_3 are insignificant ($p>0.1$) on response. The model F value was found too high (1113.03) and implies that the model is significant. The F value of lack fit was found to be less (5.42), and it indicates that the model is nonsignificant ($p>0.1$); it is good for the fitted quadratic model. The variable effect on the tailing factor was evaluated by the 3D surface and contour plot. It showed the mobile phase system and flow rate favoured the response, whereas detector UV wavelength has a negative response. The 3D and contour plot showed that increasing acetonitrile percentage (X_1) increases the tailing factor (Figure 2(c)). The high and lesser values of the tailing factor are not suggested for the analytical method (HPLC). For this selected medium, acetonitrile concentration was found to be within the standard limit [30]. The 2nd factor solvent flow rate (X_2) increases; the tailing factor also increases but has a less prominent effect than factor A (Figure 2(c)). Moreover, the third-factor UV wavelength (X_3) has a negative effect on the tailing factor, which means UV wavelength increases from 235nm to 245nm and the tailing factor decrease. The interaction effect of acetonitrile (A) and flow rate showed the negative effect on the tailing factor, and other interaction factors (X_2X_3 and X_1X_3) showed no significant effect ($p>0.1$) on the tailing factor.

Table 3

Statistical ANOVA results for the responses (Y_1 =area; Y_2 =retention time; Y_3 =tailing factor).

Result (ANOVA)	Area	Retention time	Tailing factor
Regression			
Sum of squares	23701247441	1.56	2.58
Degrees of freedom (df)	9	9	9
Mean squares	2633471938	0.17	0.28
F value	253.39	25.40	199.28
P	<0.0001	0.0002	<0.0001
Lack of fit tests			
Sum of squares	55661902.75	0.01	0.004
Degrees of freedom (df)	3	3	3

Mean squares	18553967.58	0.002	0.002
<i>F</i> value	4.34	0.16	0.91
<i>P</i>	0.10	0.92	0.51
Residual			
Sum of squares	72750482.75	0.05	0.01
Degrees of freedom (df)	7	7	7
Mean squares	10392926.11	0.01	0.001

3.7. Optimization by Point Prediction

The further optimization of the chromatographic condition was carried out by point prediction. The changes in the mobile phase composition, flow rate, and wavelength were carried out in the software. The optimized chromatographic condition, i.e., mobile phase (50:50%), wavelength 235nm, and flow rate 0.7 ml/min, shows the responses for area as 2036620 AU, retention time 2.43min, and tailing 2.08%. It showed less error (%) from the predicted value (software value).

4. Validation

4.1. System Suitability and Specificity

The system suitability of ATS was determined at different responses or critical analytical attributes, i.e., the area of the chromatogram, *R_t*, and tailing effect. It was found that there are no significant ($p > 0.05$) differences in responses after the evaluation of six replicates of analysis. The RSD (%) system suitability of the method found to be <1 (0.73) represented the high scale of precision of instrument [31]. The specificity of the developed analytical chromatographic method was performed in the blank and spiked quality control samples, and it was found that there is no interference with blank and standard quality control samples (Figures 3(a) and 3(b)). It confirmed that the method was suitable and specific for the quantification of ATS from the formulation.

[figures omitted; refer to PDF]

4.2. Linearity and Sensitivity Analysis

The standard curve of ATS was conducted at 5–30 µg/ml standard solution using optimized chromatographic conditions (Table 4). The regression coefficient (R^2) was found to be 0.999, and the regression equation was $98881x + 71.42$. The RSD of slope and intercept was calculated and found as 0.89% and 0.76%, respectively. It was found as less than 5%, and it indicates that the method was good and accurate. The limit of detection and limit of quantification was found to be 0.25 µg/ml and 0.83 µg/ml, respectively.

Table 4

Linearity data of ATS.

Parameter	Observed result
Linearity range	5–30 µg/ml
Calibration equation	$Y = 98881x + 71.42$

Slop	98881
Intercept	71.42
Limit of detection	0.254 $\mu\text{g/ml}$
Limit of quantification	0.838 $\mu\text{g/ml}$

4.3. Precision and Accuracy

The precision and accuracy of the developed method at an optimized chromatographic condition in different quality control samples were analyzed in six replicates, and the data are expressed in Table 5. The percentage RSD was determined and found between 1.70 and 2.79 for intraday and 1.25 and 2.71 for interday at LQC, MQC, and HQC quality control sample. RSD was <5%, and as per the guidelines, it indicates that the method was accurate and suitable [32, 33]. The percentage accuracy of the quality control sample (LQC, MQC, and HQC) was found to be 98.66 ± 2.86 – 99.83 ± 1.79 for intraday and 98.33 ± 2.46 – 99.91 ± 2.33 for interday, respectively. The accuracy of the method was found to be within the acceptable limit of the guideline.

Table 5

Precision and accuracy of different quality control samples of ATS.

Quality control sample	Concentration ($\mu\text{g/ml}$)	Intraday precision and accuracy			Interday precision and accuracy		
Experimental concentration	% RSD	Accuracy	Experimental concentration	% RSD	Accuracy	LQ C	1.5
1.48 ± 0.04	2.70	98.66 ± 2.9	1.475 ± 0.04	2.71	98.33 ± 2.46	MQ C	12.44
12.42 ± 0.3	2.41	99.84 ± 2.1	12.43 ± 0.3	2.41	98.91 ± 2.33	HQ C	24

4.4. Recovery Study

The recovery study of ATS was determined by adding an extra amount of drug into analytes. The recovery of ATS was found to be 98.66–100.26, respectively, with the low RSD (0.98–1.91%). The low RSD (<5%) indicates that the method was accurate.

4.5. Storage Stability Study

The refrigerated storage stability of ATS was analyzed at two levels of quality samples, i.e., LQC and HQC (1.5 $\mu\text{g/mL}$ and 24 $\mu\text{g/mL}$), and the result is given in Table 6. The percentage recovery at the LQC level was 99.33–100% and at the HQC level was 99.58–100% in different storage conditions, i.e., short-term stability (25°C, 24 h), long-term stability (–80°C, 30 days), freeze-thaw stability (three cycles, –20°C to 25°C), postpreparative stability (10°C, 24 h), and short-term stability (25°C, 24 h). There was no significant ($p > 0.05$) change found with a low value of % RSD (<5%). The low value of RSD indicated that ATS was stable at different freezing storage conditions.

Table 6

Stability study of ATS at different quality controls.

Quality control sample	Refrigerated condition and time	Experimental concentration	% experimental drug	% RSD
LOC (1.5 µg/ml)	Initial (0h)	1.5±0.02	100	1.33
Short-term storage stability (25°C/24 h)	1.5±0.03	100	1.33	Long-term stability (-80°C, 30 days)
1.49±0.03	99.33	2.01	Three cycles freeze-thaw stability (three cycles, -20°C to 25°C)	1.48±0.02
98.66	1.35	Postpreparative stability (10°C, 24 h), initial (0h)	1.49±0.03	99.33
2.01	-			
HQC (24 µg/ml)	Initial (0h)	24±0.2	100	0.83
Short-term stability (25°C, 24h)	23.99±0.3	99.95	1.25	Long-term stability (-80°C, 30 days)

23.90±0.3	99.58	1.26	Three cycles freeze-thaw stability (three cycles, -20°C to 25°C)	23.92±0.3
99.66	1.25	Postpreparative stability (10°C, 24 h)	23.98±0.3	99.91

5. Forced Degradation Study

5.1. Acid Degradation Study

The chromatogram of ATS after the degradation study showed one additional peak at 3.8min except than the standard ATS peak (2.43min) (Figure 4(a)). The additional peak indicated that ATS was unstable at a high molar concentration of HCl as well as at high temperature. As per previous work' report, the most prominent acid degradation product is lacton. The degradation product form by lactonization of 3,5-dihydroxyheptanoate side chain under mild acidic condition and further may degrade upon exposure with strong acid for long exposure time [34, 35]. [figures omitted; refer to PDF]

5.2. Basic Degradation Study

The base degradation study was conducted in 0.1M sodium hydroxide, and no additional peaks were found in the chromatogram of AST (Figure 4(b)). There was 100% concentration retained, and it indicates that the drug was stable in basic condition.

5.3. Oxidative Degradation Study

An oxidation degradation study was conducted using hydrogen peroxide solution (30% v/v) under reflux condition. One additional well-resolved peak was observed at Rt 3.54min in addition to the standard ATS peak (Rt=2.43min) (Figure 5(a)). The mechanism of hydrogen peroxide oxidation takes place by oxidation of pyrrole ring of ATS and may be the formation of unstable endoperoxide that takes place [36]. [figures omitted; refer to PDF]

5.4. UV Photolytic Degradation Study

The study was carried out by placing the standard sample under UV light (254nm) for 24h, and chromatogram is depicted in Figure 5(b). There are many ATS peaks observed in the degradation chromatogram of ATS. The degradation peaks were observed at different Rts (2.71 min, 3.16 min, 3.38 min, and 3.7 min). It indicates that ATS was completely degraded under UV light after long time exposure due to absorption of energy from UV light by molecules. The molecule is absorbed and indulgences the energy in presence of UV light. ATS molecule activated from the ground to excited state because it contains the carbonyl and c= c group as well as the formation of free radical takes place [37].

6. Conclusion

An analytical quality by design (Box–Behnken) approach was successfully employed for the optimization of the RP-HPLC method. The solvent system (acetonitrile:water, 50:50), flow rate (0.7 ml/min), and UV wavelength (235nm) were found to be the optimized chromatographic condition for atorvastatin. The developed method was evaluated for the validation, quantification of the quality control sample, and stability study. The method showed a well-resolved chromatographic peak (Rt=2.43min) and found to be accurate and precise. The force degradation study showed well-resolved atorvastatin and degradation product peaks. Our finding suggests that QbD approaches can be used for the method development and also for the determination of atorvastatin from formulation and pharmacokinetic parameter in vivo.

Acknowledgments

The author is very thankful to Jouf University, Aljouf, KSA, for providing facility to perform this research.

- [1] M. H. Moghadasian, "Clinical pharmacology of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors," *Life Sciences*, vol. 65 no. 13, pp. 1329-1337, DOI: 10.1016/s0024-3205(99)00199-x, 1999.
- [2] K. D. Tripathi, *Essentials of Medical Pharmacology*, 2010.
- [3] M. Korhonova, A. Doricakova, Z. Dvorak, "Optical isomers of atorvastatin, rosuvastatin and fluvastatin enantiospecifically activate pregnane X receptor PXR and induce CYP2A6, CYP2B6 and CYP3A4 in human hepatocytes," *PLoS One*, vol. 10 no. 9, DOI: 10.1371/journal.pone.0137720, 2015.
- [4] M. S. Rodde, G. T. Divase, T. B. Devkar, A. R. Tekade, "Solubility and bioavailability enhancement of poorly aqueous soluble atorvastatin: in vitro, ex vivo, and in vivo studies," *BioMed Research International*, vol. 2014, DOI: 10.1155/2014/463895, 2014.
- [5] *Indian Pharmacoeia, Solubility*, 2010
- [6] T. Dongala, N. K. Katari, A. K. Palakurthi, S. B. Jonnalagadda, "Development and validation of a generic RP-HPLC PDA method for the simultaneous separation and quantification of active ingredients in cold and cough medicines," *Biomedical Chromatography*, vol. 33 no. 11, DOI: 10.1002/bmc.4608, 2019.
- [7] A. Mohammadi, N. Rezanour, D. M. Ansari, B. F. Ghorbani, M. Hashem, R. B. Walker, "A stability-indicating high performance liquid chromatographic (HPLC) assay for the simultaneous determination of atorvastatin and amlodipine in commercial tablets," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 846 no. 1-2, pp. 215-221, DOI: 10.1016/j.jchromb.2006.09.007, 2007.
- [8] K. R. Naidu, U. N. Kale, M. S. Shingare, "Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 39 no. 1-2, pp. 147-155, DOI: 10.1016/j.jpba.2005.04.001, 2005.
- [9] S. Ertürk, E. Sevinç Aktaş, L. Ersoy, S. Fıçıcıoğlu, "An HPLC method for the determination of atorvastatin and its impurities in bulk drug and tablets," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 33 no. 5, pp. 1017-1023, DOI: 10.1016/s0731-7085(03)00408-4, 2003.
- [10] L. Nováková, D. Šatínský, P. Solich, "HPLC methods for the determination of simvastatin and Atorvastatin," *TrAC Trends in Analytical Chemistry*, vol. 27 no. 4, pp. 352-367, DOI: 10.1016/j.trac.2008.01.013, 2008.
- [11] A. F. Martins, C. d. S. Frank, J. Altissimo, "Determination of statin drugs in hospital effluent with dispersive liquid-liquid microextraction and quantification by liquid chromatography," *Journal of Environmental Science and Health, Part A*, vol. 52 no. 10, pp. 939-945, DOI: 10.1080/10934529.2017.1324706, 2017.
- [12] A. Zafar, I. El-Bagory, N. K. Alruwaili, "Quality by design (QbD) based development and validation of bioanalytical RP-HPLC method for dapagliflozin: forced degradation and preclinical pharmacokinetic study," *Journal of Liquid Chromatography & Related Technologies*, vol. 43 no. 1-2, pp. 53-65, DOI: 10.1080/10826076.2019.1667820, 2020.
- [13] M. Qumbar, A. Zafar, S. S. Imam, M. Fazil, A. Ali, "DOE-based stability indicating RP-HPLC method for determination of lacidipine in niosomal gel in rat: pharmacokinetic determination," *Pharmaceutica Analytica Acta*, vol. 5, 2014.
- [14] T. Moolakkadath, M. Aqil, S. S. Imam, "Analytical quality by design (AQbD) approach based HPTLC method for quantification of fisetin with superior recovery in formulations," *Current Analytical Chemistry*, vol. 16 no. 2, pp. 149-157, DOI: 10.2174/1573411014666180627145031, 2020.
- [15] M. V. Krishna, R. N. Dash, B. Jalachandra Reddy, P. Venugopal, P. Sandeep, G. Madhavi, "Quality by Design (QbD) approach to develop HPLC method for eberconazole nitrate: application oxidative and photolytic degradation kinetics," *Journal of Saudi Chemical Society*, vol. 20 no. 1, pp. S313-S322, DOI: 10.1016/j.jscs.2012.12.001, 2016.
- [16] S. Karmarkar, X. Yang, R. Garber, A. Szajkovichs, M. Koberda, "Quality by design (QbD) based development and validation of an HPLC method for amiodarone hydrochloride and its impurities in the drug substance," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 100, pp. 167-174, DOI: 10.1016/j.jpba.2014.07.002, 2014.
- [17] E. Rozet, E. Ziemons, R. D. Marini, B. Boulanger, P. Hubert, "Quality by design compliant analytical method validation," *Analytical Chemistry*, vol. 84 no. 1, pp. 106-112, DOI: 10.1021/ac202664s, 2012.
- [18] N. Khorshidi, M. Rahimi, I. Salimikia, "Application of aeration-assisted homogeneous liquid-liquid

microextraction procedure using Box-Behnken design for determination of curcumin by HPLC," *Journal of Separation Science*, vol. 43 no. 13, DOI: 10.1002/jssc.202000001, 2020.

[19] P. Nethercote, J. Ermer, "Quality by design for analytical methods: implications for method validation and transfer," *Pharmaceutical Technology*, vol. 36, pp. 74-79, 2012.

[20] G. Srinubabu, C. A. I. Raju, N. Sarath, P. K. Kumar, J. V. L. N. S. Rao, "Development and validation of a HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design," *Talanta*, vol. 71 no. 3, pp. 1424-1429, DOI: 10.1016/j.talanta.2006.04.042, 2007.

[21] A. Jain, S. Beg, S. Saini, T. Sharma, O. P. Katore, B. Singh, "Application of chemometric approach for QbD-enabled development and validation of an RP-HPLC method for estimation of methotrexate," *Journal of Liquid Chromatography & Related Technologies*, vol. 42 no. 15-16, pp. 502-512, DOI: 10.1080/10826076.2019.1626742, 2019.

[22] M. Mitrovic, A. Protic, A. Malenovic, B. Otasevic, M. Zecevic, "Analytical quality by design development of an ecologically acceptable enantioselective HPLC method for timolol maleate enantiomeric purity testing on ovomucoid chiral stationary phase," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 180, DOI: 10.1016/j.jpba.2019.113034, 2020.

[23] S. S. Imam, M. Aqil, M. Akhtar, Y. Sultana, A. Ali, "Optimization of mobile phase by 32-mixture design for the validation and quantification of risperidone in bulk and pharmaceutical formulations using RP-HPLC," *Analytical Methods*, vol. 6 no. 1, pp. 282-288, DOI: 10.1039/c3ay41562g, 2014.

[24] A. Khan, S. S. Imam, M. Aqil, Y. Sultana, A. Ali, K. Khan, "Design of experiment based validated stability indicating RP-HPLC method of temozolomide in bulk and pharmaceutical dosage forms," *Beni-Suef University Journal of Basic and Applied Sciences*, vol. 5 no. 4, pp. 402-408, DOI: 10.1016/j.bjbas.2015.11.011, 2016.

[25] S. S. Panda, V. V. Ravi Kumar Bera, S. Beg, O. Mandal, "Analytical quality by design (AQbD)-Oriented RP-UFLC method for quantification of lansoprazole with superior method robustness," *Journal of Liquid Chromatography & Related Technologies*, vol. 40 no. 9, pp. 479-485, DOI: 10.1080/10826076.2017.1327442, 2017.

[26] ICH Topic Q2 (R1), *Validation of Analytical Procedures*, 1995.

[27] N. Batrawi, S. Wahdan, M. Abualhasan, "Analytical method validation of high-performance liquid chromatography and stability-indicating study of medroxyprogesterone acetate intravaginal sponges," *Analytical Chemistry Insights*, vol. 12, DOI: 10.1177/1177390117690152, 2017.

[28] N. Khan, Ameenuzzafar, A. Ali, F. J. Ahmad, "A novel validated stability-indicating HPTLC method to quantitate forskolin as a bulk drug and in a nanosuspension," *Indian Journal of Pharmaceutical Sciences*, vol. 80 no. 5, pp. 820-826, DOI: 10.4172/pharmaceutical-sciences.1000427, 2018.

[29] S. Sonawane, S. Jadhav, P. Rahade, S. Chhajed, S. Kshirsagar, "Development and validation of stability-indicating method for estimation of chlorthalidone in bulk and tablets with the use of experimental design in forced degradation experiments," *Scientifica (Cairo)*, vol. 2016, DOI: 10.1155/2016/4286482, 2016.

[30] S. Beg, V. Chaudhary, G. Sharma, B. Garg, S. S. Panda, B. Singh, "QbD-oriented development and validation of a bioanalytical method for nevirapine with enhanced liquid-liquid extraction and chromatographic separation," *Biomedical Chromatography*, vol. 30 no. 6, pp. 818-828, DOI: 10.1002/bmc.3613, 2016.

[31] U.S. FDA, *Guidance for Industry: Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation*, 2000.

[32] N. Çankaya, İ. Bulduk, A. M. Çolak, "Extraction, development and validation of HPLC-UV method for rapid and sensitive determination of colchicine from *Colchicum autumnale* L. Bulbs," *Saudi Journal of Biological Sciences*, vol. 26 no. 2, pp. 345-351, DOI: 10.1016/j.sjbs.2018.10.003, 2019.

[33] B. T. Alquadeib, "Development and validation of a new HPLC analytical method for the determination of diclofenac in tablets," *Saudi Pharmaceutical Journal*, vol. 27 no. 1, pp. 66-70, DOI: 10.1016/j.jsps.2018.07.020, 2019.

[34] R. K. Seshadri, M. M. Desai, T. V. Raghavaraju, D. Krishnan, D. V. Rao, I. E. Chakravarthy, "Simultaneous quantitative determination of metoprolol, atorvastatin and ramipril in capsules by a validated stability-indicating RP-

UPLC method," Scientia Pharmaceutica, vol. 78 no. 4, pp. 821-834, DOI: 10.3797/scipharm.1004-14, 2010.

[35] P. Vukkum, J. M. Babu, R. Muralikrishna, "Stress degradation behavior of atorvastatin calcium and development of suitable stability-indicating LC method for the determination of atorvastatin, its related impurities and its degradation products," Scientia Pharmaceutica, vol. 81 no. 1, pp. 93-114, DOI: 10.3797/scipharm.1208-06, 2013.

[36] S. Navaratnum, "Photochemical and photophysical methods used in the study of drug photoreactivity," The Photostability of Drugs and Drug Formulations, pp. 255-284, 2004.

[37] M. Kracun, A. Kocijan, A. Bastarda, R. Grahek, J. Plavec, D. Kocjan, "Isolation and structure determination of oxidative degradation products of atorvastatin," Journal of Pharmaceutical and Biomedical Analysis, vol. 50 no. 5, pp. 729-736, 2009.

DETAILS

Subject:	Ultraviolet radiation; Design analysis; Acetonitrile; Mathematical analysis; Solvents; Optimization; Quality control; Linearity; Variables; Retention; Stability analysis; Regression analysis; Independent variables; Chromatography; High performance liquid chromatography; Design optimization; Flow velocity; Statistical analysis; Risk assessment
Business indexing term:	Subject: Quality control Risk assessment
Location:	United States--US
Editor:	Günther K Bonn
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article

Publication history :

Milestone dates: 2020-09-12 (Received); 2020-12-29 (Revised); 2021-01-27 (Accepted); 2021-02-13 (Pub)

DOI: <https://doi.org/10.1155/2021/8833900>

ProQuest document ID: 2491753158

Document URL: <https://www.proquest.com/scholarly-journals/analytical-quality-design-approach-reverse-phase/docview/2491753158/se-2?accountid=211160>

Copyright: Copyright © 2021 Nabil K. Alruwaili. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License.
<https://creativecommons.org/licenses/by/4.0/>

Last updated: 2021-02-21

Database: Publicly Available Content Database

Document 54 of 59

Rapid Identification and Systematic Mechanism of Flavonoids from *Potentilla freyniana* Bornm. Based on UHPLC-Q-Exactive Orbitrap Mass Spectrometry and Network Pharmacology

Cai, Wei ¹
; Li, Kailin ²
; Qin, Shihan ³ ; Xiong, Pei ²
; Peng, Jie ²
; Shi, Silin ²
; Zhang, Zaiqi ⁴

¹ Hunan Provincial Key Laboratory of Dong Medicine, Hunan University of Medicine, Huaihua 41800, China; School of Pharmaceutical Sciences, Hunan University of Medicine, Huaihua 41800, China ² School of Pharmaceutical Sciences, Hunan University of Medicine, Huaihua 41800, China ³ School of Pharmacy, Weifang Medical University, Weifang 261053, China ⁴ Hunan Provincial Key Laboratory of Dong Medicine, Hunan University of Medicine, Huaihua 41800, China

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Potentilla freyniana Bornm. (*P. freyniana*), belonging to the family Rosaceae, has been used as a folk medicine in China. However, as we know, the constituents and the systematic elucidation of the mechanism were not fully investigated. Therefore, it is necessary to develop a rapid method using LC-MS and network pharmacology for the detection and identification of constituents and the systematic mechanism of *P. freyniana*. Firstly, the flavonoids were detected and identified based on ultra-high-performance liquid chromatography coupled with Quadrupole-Exactive Focus Orbitrap MS (UHPLC-Q-Exactive Orbitrap MS). After that, the potential targets of those constituents were obtained by database mining. Then, the core targets were predicted by protein-protein interaction network and network analysis. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were carried out via DAVID. This finding revealed that *P. freyniana* possessed 43 flavonoids (40 of them were first reported) with 23 core target genes, which are associated with PI3K-Akt, MAPK, TNF signaling pathway, and pathway in cancer. This study demonstrated the multicomponent, multitarget, and multimechanism of *P. freyniana*, which are very beneficial to develop the further study and utilization of this plant including the material basis and quality control research.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Potentilla freyniana Bornm. (*P. freyniana*), a genus *Potentilla* of the family Rosaceae, named Difengzi, is a perennial plant with branched and tufted roots widely distributed and cultivated all-around of China, especially in Hunan, Hubei, Jiangxi. Their roots have been used as a folk medicine in clearing away heat and toxic materials for treating canker, bone tuberculosis, external bleeding [1–3]. Previous investigations on *P. freyniana* showed the presence of different compounds including terpenes and flavonoids [4–7] and possessed a variety of activities such as anti-inflammatory, analgesic effects [8, 9]. However, as we know, the constituents and systematic pharmacological mechanism were not fully investigated. For instance, 14 compounds, including eriodictyol, phlorizin, were separated from the roots of *P. freyniana* [7]. Therefore, it is worthwhile to establish a highly sensitive method for characterizing their chemical constituents and elucidating systematic pharmacological mechanism of *P. freyniana*.

The complexity of chemicals in Traditional Chinese Medicine (TCM) including *P. freyniana* has presented a significant challenge in the rapid identification and characterization of components. During the past decades, HPLC-MS, as a new technique has been used to profile and identify the chemical in TCM due to its validity, sensitivity, and specialness [10, 11]. Especially, UHPLC-HRMS such as UHPLC-Q-Exactive Orbitrap MS, UHPLC-Q-TOF MS, and UHPLC-LTQ-Orbitrap-MS, affording a higher and faster separation and higher resolution of mass, was a much more powerful equipment in the identification of TCM compared to traditional HPLC-MS [12–14].

Network pharmacology is an impressive methodology for investigating the systematic pharmacological mechanism through the constructing and analyzing biological networks such as protein-protein interaction, chemical-target-pathway network, which could provide direction for the further discovery of new drug without enormous time, money, and effort [15–17].

Therefore, this current study was designed to develop a fast and effective method for the chemical characterization and systematic pharmacological mechanism of *P. freyniana* using UHPLC-Q-Exactive Orbitrap MS and network pharmacology. *P. freyniana* possessed 43 flavonoids (40 of them was first reported) with 23 core target genes, which are associated with PI3K-Akt, MAPK, TNF signaling pathway, and pathway in cancer. This study demonstrated the multicomponent, multitarget, and multimechanism of *P. freyniana*, which are very beneficial for the further study and utilization of this plant including the material basis and quality control research.

2. Materials and Methods

2.1. Chemicals and Materials

The chemical reference standards isoquercitrin, luteolin, naringenin, and kaempferol were provided by Chengdu

Herbpurify biotechnology CO., LTD (Chengdu, China); Phlorizin, Phloretin, and Trilobatin were purchased from Sichuan Wei Keqi Biotechnology Co., Ltd (Sichuan, China); eriodictyol and hyperoside were provided by Chengdu Push biotechnology CO., LTD (Chengdu, China); quercetin and apigenin were provided by Chengdu Alfa biotechnology CO., LTD (Chengdu, China); baicalein and wogonin were obtained from Chengdu Desite biotechnology CO., LTD (Chengdu, China). The purities of all chemical reference standards were above 98% by HPLC-DAD.

Acetonitrile and methanol of chromatography grade were provided by MERCK (Darmstadt, Germany); The ultrapure water was produced by a milli-Q water purification system (Millipore, Milford, MA, United States); formic acid of LC-MS grade and all other reagents of analytical grade were purchased from Aladdin Industrial Corporation.

P. freyniana was collected from Tong-Dao country of Huaihua (109.86 longitude, 26.03 latitude), Hunan province and were identified by Professor Wei Cai (Hunan Provincial Key Laboratory of Dong Medicine, Hunan University of Medicine). The voucher specimen was deposited at School of Pharmaceutical Sciences, Hunan University of Medicine.

2.2. Sample Preparation

A total of 10g powdered root of *P. freyniana* was ultrasonically extracted with 50mL of 70% aqueous methanol for 1 h, and then the extracted solution was filtered for further UHPLC Q-Exactive Focus Orbitrap MS analysis.

The reference standards including hyperoside, isoquercitrin, phlorizin, eriodictyol, trilobatin, quercetin, luteolin, naringenin, apigenin, phloretin, kaempferol, baicalein, and wogonin were weighed and dissolved in methanol to obtain the reference solution with the final concentrations of 10.2, 9.8, 10.0, 9.8, 9.9, 10.5, 10.2, 10.8, 9.3, 10.1, 10.6, 9, 4, and 10.5ug/mL, respectively, and then these solutions were stored in -4°C before analysis.

2.3. Instruments and Conditions

Chromatography analysis was performed on an Ultimate 3000 focused system (Dionex, Sunnyvale, CA, USA) consisting of an online vacuum degasser, a binary pump, and an autosampler. The sample separation was carried out on the Hypersil GOLD C18 column (100×2.1 mm, 1.9 μm) at 40°C. The mobile phase consisted of acetonitrile as solvent A and water with 0.1% formic acid as solvent B using the following gradient elution of 5–15 % A at 0–2min; isocratic 15 % A at 2–5min; 15–18 % A at 5–10min; 18–50 % A at 10–15min; 50–100 % A at 15–16min; 100–5 % A at 16–17min; isocratic 5 % A at 17–20min. The flow rate was 0.3mL/min.

All LC-MSⁿ analyses were performed on the Q-Exactive Focus Orbitrap MS connected to the UHPLC system via a heated electrospray ionization source (Thermo Electron, Bremen, Germany). The optimized tune operating parameters in negative ion mode were listed as follows: sheath gas and auxiliary gas flow rate of 30 and 10 arbitrary, respectively; the capillary and auxiliary gas heater temperatures of 320°C and 350°C, respectively; spray voltage of 3.0kV; RF lens of 50; High-resolution MS analysis was performed at full scan MS¹ with the mass range of m/z 100–1000 at a resolution of 35000 and targeted MS² at a resolution of 17500 triggered by parallel reaction monitoring mode; nitrogen was set as sheath, auxiliary, and collision gas; the isolation window was 2amu, and the normalized collision energy (NCE) was 30%.

2.4. Data Processing and Analysis

All high-resolution MS data were acquired and processed using the Xcalibur version (2.0 software, Thermo Fisher Scientific, San Jose, CA, USA). The compounds were detected by the Compound Discover version 3 using the metabolism workflow templates by the expected compounds predicted method [18]. The detailed parameters of the workflow template were set as follows: the minimum peak intensity was set as 10000; the maximum element counts were C30 H60 O20; the mass tolerance of MS and MS² was within 5 and 10ppm, respectively; baicalein and phloretin were set as the carbon skeleton; reduction, oxidation was set as Phase I transformation; glucoside conjugation, glucuronide conjugation, pentoside conjugation, methylation was set as Phase II transformation.

2.5. Target Identification of Flavonoids

TCMSP database is a free and online database for potential target identification of small molecules, especially TCM. The target genes were converted to the official gene symbol by STRING (<https://string-db.org>) or Uniprot (<https://www.uniprot.org>).

2.6. Protein-Protein Interaction Network

STRING was a free tool, which can construct the PPI network by uploading the potential targets. The species was set as "*Homo sapiens*" with a confidence score >0.4. The network analysis was performed at Cytoscape to obtain the core targets.

2.7. GeneMANIA Analysis

GeneMANIA (<http://genemania.org>) is an online free and friendly tool for investigating gene function and gene interaction. The species was set as "*Homo sapiens*."

2.8. GO and Pathway Analysis

The GO and KEGG pathway analysis was performed on the DAVID (<https://david.ncicrf.gov>, Version 6.8). The specific species in the list and background was set as "*Homo sapiens*." The entire compounds, targets, and pathway network were visualized by Cytoscape.

3. Results and Discussion

3.1. Analytical Strategy

In order to identify flavonoids fully, an analytical strategy based on UHPLC Q-Exactive Focus Orbitrap MS was established in this study. First, the sample was prepared and injected into the UHPLC Q-Exactive Focus Orbitrap MS to gain the full scan high-resolution MS data. Then, those data were processed using Compound Discover software with metabolism workflow to predict and detect the molecule of flavonoids. Third, the MS² of the predicted molecule were acquired using UHPLC Q-Exactive Focus Orbitrap MS by parallel reaction monitoring mode. Finally, the compounds were identified based on the full scan MS, MS² data, retention time, and bibliography.

3.2. Identification of Flavonoids

The total content of flavonoids was measured by NaNO₂-Al(NO₃)₃-NaOH spectrophotometric colorimetry [19]. The calibration curve obtained by the rutin standard of absorbance concentrations(mg/mL) using five dilutions was $y = 7.15x - 0.001$, with the corresponding determination coefficient at 0.9999. Finally, the content of flavonoids is $32.17 \pm 0.26\%$. A total of 43 constituents were unanimously and tentatively characterized based on UHPLC Q-Exactive Focus Orbitrap MS combined with the expected compounds predicted method. 40 excluded eriodictyol, phloretin, and hyperoside were reported from *P. freyniana* for the first time. The detailed information of those compounds is listed in Table 1. The high-resolution extracted ion chromatography is shown in Figure 1.

Table 1

The retention time and mass spectrometric data of flavone in *Potentilla freyniana* Bornm.

Peak	t_R	Theoretical mass, m/z	Experimental mass, m/z	Error (ppm)	Formula	MS/MS fragment	Identification
1	3.59	465.1038	465.1039	0.21	$C_{21}H_{22}O_{12}$	MS ² [465]: 285.0406(100), 125.0233(72), 275.0563(45), 177.0185(29), 303.0512(20), 151.0032(17)	Taxifolin-glucoside
2	3.72	465.1038	465.1033	-1.08	$C_{21}H_{22}O_{12}$	MS ² [465]: 285.0405(100), 125.0233(38), 273.0043(28), 177.0185(19), 303.0511(15)	Taxifolin-glucoside
3	3.88	465.1038	465.1042	0.86	$C_{21}H_{22}O_{12}$	MS ² [465]: 285.0405(100), 125.0233(36), 177.0184(18), 275.0566(12), 303.0512(10)	Taxifolin-glucoside
4	4.13	463.0882	463.0881	-0.22	$C_{21}H_{20}O_{12}$	MS ² [463]: 287.0562(100), 259.0612(78), 125.0232(32)	Eriodictyol-glucuronide

5	4. 19	449.1089	449.1093	0.89	$\text{C}_{22}^{21}\text{H}_{11}\text{O}$	MS ² [449]: 259.0611(100), 287.0561(33), 178.9979(15), 125.0231(13)	Eriodictyol-glucoside
6	4. 41	593.1512	593.1535	3.88	$\text{C}_{30}^{27}\text{H}_{15}\text{O}$	MS ² [593]: 353.0667(100), 383.0774(54), 473.1092(35), 125.0234(25), 413.0874(6)	Vicenin II
7	4. 56	449.1089	449.1092	0.67	$\text{C}_{22}^{21}\text{H}_{11}\text{O}$	MS ² [449]: 259.0612(100), 269.0455(76), 287.0563(44), 125.0233(38), 178.9977(22)	Eriodictyol-glucoside
8	5. 61	465.1038	465.1042	0.86	$\text{C}_{22}^{21}\text{H}_{12}\text{O}$	MS ² [465]: 125.0233(100), 285.0405(62), 259.0610(48), 275.0566(32), 303.0512(18)	Taxifolin-glucoside
9	5. 75	479.0831	479.0834	0.63	$\text{C}_{20}^{21}\text{H}_{13}\text{O}$	MS ² [479]: 285.0405(100), 125.0233(41), 303.0512(18), 177.0186(17), 169.0133(15), 259.0613(14)	Taxifolin-glucuronide
10	5. 88	449.1089	449.1092	0.67	$\text{C}_{22}^{21}\text{H}_{11}\text{O}$	MS ² [449]: 269.0456(100), 151.0026(69), 178.9979(39), 125.0231(13), 259.0612(12), 287.0562(10)	Eriodictyol-glucoside
11	6. 12	433.1140	433.1142	0.46	$\text{C}_{22}^{21}\text{H}_{10}\text{O}$	MS ² [433]: 271.0614(100), 151.0027(73), 119.0491(18), 125.0229(8)	Naringenin-glucoside
12	6. 22	625.1408	625.1408	0.00	$\text{C}_{30}^{27}\text{H}_{17}\text{O}$	MS ² [625]: 287.0558(100), 113.0231(40), 151.0030(38)	Eriodictyol-glucoside-glucuronide
13	6. 57	303.0510	303.0507	-0.99	$\text{C}_{12}^{15}\text{H}_7\text{O}$	MS ² [303]: 125.0234(100), 285.0407(38)	Taxifolin
14	6. 59	433.1140	433.1141	0.23	$\text{C}_{22}^{21}\text{H}_{10}\text{O}$	MS ² [433]: 271.0613(100), 151.0027(72), 119.0491(16)	Naringenin-glucoside
15 *	6. 85	463.0882	463.0886	0.86	$\text{C}_{20}^{21}\text{H}_{12}\text{O}$	MS ² [463]: 300.0272(100), 301.0350(70)	Hyperoside
16	6. 90	449.1089	449.1094	1.11	$\text{C}_{22}^{21}\text{H}_{11}\text{O}$	MS ² [449]: 151.0028(100), 287.0562(73), 135.0442(32)	Eriodictyol-glucoside
17 *	7. 18	463.0882	463.0886	0.86	$\text{C}_{20}^{21}\text{H}_{12}\text{O}$	MS ² [463]: 300.0279(100), 301.0358(50), 151.0025(8), 178.9976(7)	Isoquercitrin
18	7. 75	597.1825	597.1832	1.17	$\text{C}_{34}^{27}\text{H}_{15}\text{O}$	MS ² [597]: 357.0983(100), 387.1087(85), 315.0882(22), 417.1165(21), 358.1008(15)	Phloretin-C-diglucoside

19	7. 99	449.1089	449.1085	-0.89	$C_{22}H_{11}O$	MS ² [449]: 167.0340(69), 137.0233(50), 123.0441(12)	Phloretin- glucuronide
20	8. 15	431.0984	431.0992	1.86	$C_{21}H_{10}O$	MS ² [431]: 269.0445(100)	Baicalein- glucoside
21	9. 60	431.0984	431.0988	0.93	$C_{21}H_{10}O$	MS ² [431]: 269.0443(100)	Baicalein- glucoside
22	9. 61	449.1089	449.1086	-0.67	$C_{22}H_{11}O$	MS ² [449]: 151.0026(100), 287.0563(65), 135.0442(22)	Eriodictyol- glucoside
23	9. 68	565.1563	565.1568	0.88	$C_{30}H_{14}O$	MS ² [565]: 271.0614(100), 151.0026(48)	Naringenin- pentoside- glucoside
24	9. 82	433.1140	433.1142	0.46	$C_{21}H_{10}O$	MS ² [433]: 271.0615(100), 151.0028(41), 119.0491(14)	Naringenin- glucoside
25	10 .3 8	431.0984	431.0992	1.86	$C_{21}H_{10}O$	MS ² [431]: 268.0369(100), 269.0444(47)	Apigenin-7- glucoside
26	11 .1 8	463.0882	463.0886	0.86	$C_{21}H_{12}O$	MS ² [463]: 151.0027(100), 113.0231(66), 287.0562(52), 161.0234(40), 337.0569(39), 135.0439(23)	Eriodictyol- glucuronide
27 *	12 .1 6	435.1297	435.1300	0.69	$C_{24}H_{10}O$	MS ² [435]: 273.0769(100), 167.0340(69), 125.00232(9), 179.0341(6)	Phlorizin
28	12 .3 6	477.0674	477.0678	0.84	$C_{21}H_{13}O$	MS ² [477]: 301.0353(100), 151.0029(11), 178.9983(9)	Quercetin- glucuronide
29	12 .5 1	431.0984	431.0986	0.46	$C_{21}H_{10}O$	MS ² [431]: 268.0371(100), 269.0441(18), 239.0337(9)	Apigenin-4'- glucoside
30	12 .6 2	477.0674	477.0678	0.84	$C_{21}H_{13}O$	MS ² [477]: 301.0354(100), 178.9983(12), 151.0029(8)	Quercetin- glucuronide
31	13 .0 0	433.1140	433.1144	0.92	$C_{21}H_{10}O$	MS ² [433]: 271.0612(100), 151.0027(46)	Naringenin- glucoside

32	13 .3 9	567.1719	567.1730	1.94	$\text{C}_{26}\text{H}_{32}\text{O}_{14}$	MS ² [567]: 273.0771(100), 167.0340(42), 125.0020(22)	Phloretin-pentoside-glucoside
33 *	13 .4 5	287.0561	287.0564	1.05	$\text{C}_{15}\text{H}_{12}\text{O}_6$	MS ² [287]: 151.0027(100), 135.0441(69), 107.0125(10)	Eriodictyol
34	13 .5 7	449.1089	449.1085	-0.89	$\text{C}_{21}\text{H}_{22}\text{O}_{11}$	MS ² [449]: 137.0233(100), 167.0340(89), 123.0440(8)	Phloretin-glucuronide
35 *	13 .6 7	435.1297	435.1303	1.38	$\text{C}_{21}\text{H}_{24}\text{O}_{10}$	MS ² [435]: 273.0767(100), 167.0339(52)	Trilobatin
36 *	13 .8 6	301.0354	301.0356	0.66	$\text{C}_{15}\text{H}_{10}\text{O}_7$	MS ² [301]: 151.0027(100), 178.9978(66), 121.0284(21), 107.0125(8)	Quercetin
37 *	13 .9 5	285.0405	285.0407	0.70	$\text{C}_{15}\text{H}_{10}\text{O}_6$	MS ² [285]: 285.0404(100), 151.0033(12), 133.0290(11), 175.0395(8)	Luteolin
38 *	14 .7 9	271.0611	271.0614	1.11	$\text{C}_{15}\text{H}_{12}\text{O}_5$	MS ² [271]: 151.0027(100), 119.0499(36), 177.0184(14), 93.0333(14), 107.0126(9)	Naringenin
39 *	14 .9 5	269.0455	269.0454	-0.37	$\text{C}_{15}\text{H}_{10}\text{O}_5$	MS ² [269]: 269.0455(100), 117.0344(8), 149.0239(7), 151.0035(7)	Apigenin
40 *	14 .9 9	273.0768	273.0771	1.10	$\text{C}_{15}\text{H}_{14}\text{O}_5$	MS ² [273]: 167.0341(100), 123.0438(18), 119.0491(12), 125.0231(8)	Phloretin
41 *	15 .0 7	285.0405	285.0405	0.00	$\text{C}_{15}\text{H}_{10}\text{O}_6$	MS ² [285]: 285.0403(100), 151.0030(5)	Kaempferol
42 *	15 .4 9	269.0455	269.0453	-0.74	$\text{C}_{15}\text{H}_{10}\text{O}_5$	MS ² [269]: 269.0453(100), 241.0504(9), 251.0348(8), 223.0399(7)	Baicalein
43 *	16 .7 1	283.0612	283.0613	0.35	$\text{C}_{16}\text{H}_{12}\text{O}_5$	MS ² [283]: 268.0376(100), 163.0035(7)	Wogonin

*Identified by comparing with reference standards.

[figures omitted; refer to PDF]

Peaks 15, 17, 27, 33, 35–43 were unanimously identified as hyperoside, isoquercitrin, phlorizin, eriodictyol, trilobatin, quercetin, luteolin, naringenin, apigenin, phloretin, kaempferol, baicalein, and wogonin, respectively, by comparing the retention time, high-resolution mass measurement, and MS² spectrum with those reference standards.

Peak 13 was eluted at 6.57 min and possessed the deprotonated ion [M–H][–] at m/z 303.0507 (–0.99 ppm, C₁₅H₁₁O₇). The fragment ions at m/z 125.0234 (–8.14 ppm, C₆H₅O₃) and 285.0407 (0.84 ppm, C₁₅H₉O₆) were detected in the MS² spectrum, which is consistent with the MS data of taxifolin in bibliography [20]. Thus, peak 13 was tentatively identified as taxifolin. Peaks 1–3, and 8 possessed the deprotonated ion [M–H][–] at m/z 465.1039 (0.21 ppm, C₂₁H₂₁O₁₂), m/z 465.1033 (–1.08 ppm, C₂₁H₂₁O₁₂), m/z 465.1042 (0.86 ppm, C₂₁H₂₁O₁₂), and m/z 465.1042 (0.86 ppm, C₂₁H₂₁O₁₂), respectively, 162 Da(C₆H₁₀O₅, glucose moiety) more than that of taxifolin (peak 13). The fragmentation ions at m/z 285.041(C₁₅H₉O₆), 125.023(C₆H₅O₃), 303.051(C₁₅H₁₁O₇) in the MS² spectrum were matched to those attributed to taxifolin. Therefore, Peaks 1–3, and 8 were tentatively characterized as taxifolin-glucoside.

Peaks 4 and 26 were eluted at 4.13 and 11.18 min, respectively. All of them showed the same deprotonated ion [M–H][–] at m/z 463.088 (C₂₁H₁₉O₁₂), 176.032 Da(C₆H₈O₆, glucuronide moiety) more than that of eriodictyol, suggesting they are eriodictyol-glucuronide, which were further identified by the presence of fragmentation ion at m/z 287.056 (C₁₅H₁₁O₆). In a similar way, peaks 9, 28, and 30 were tentatively identified as taxifolin-glucuronide, quercetin-glucuronide, and quercetin-glucuronide, respectively.

Peaks 5, 7, 10, 16, 19, 22, and 34 were eluted at 4.19, 4.56, 5.88, 6.90, 7.99, 9.61, and 13.57 min, with the same deprotonated ion [M–H][–] at m/z 449.109 (C₂₁H₂₁O₁₁). Peaks 19 and 34 possessed the fragment ions at m/z 167.034 (C₈H₇O₄) and m/z 123.044 (C₇H₇O₂), which are the diagnosis fragmentation ions of phloretin, suggesting they were phloretin derivatives. Thus, Peaks 19 and 34 were tentatively inferred as phloretin-glucuronide. Peaks 5, 7, 10, 16, and 22 yielded the same fragmentation ion at m/z 287.056 (C₁₅H₁₁O₆), suggesting they were eriodictyol derivatives. The ion at m/z 287.056 was yielded by the neutral loss of 162.053 (C₆H₁₀O₅, glucose moiety), suggesting the presence of glucose moiety. Therefore, they were tentatively characterized as eriodictyol-glucoside.

Peak 6 with the deprotonated ion [M–H][–] at m/z 593.1535 (3.88 ppm, C₂₇H₂₉O₁₅) was eluted at 4.41 min. It yielded fragment ions at m/z 353.0667 (0.07 ppm, C₁₉H₁₃O₇), 383.0774 (0.42 ppm, C₂₀H₁₅O₈), 473.1092 (0.56 ppm, C₂₃H₂₁O₁₁), and 413.0874 (–0.98 ppm, C₂₁H₁₇O₉), resulting from the loss of C₄H₈O₄ + C₄H₈O₄, C₄H₈O₄ + C₃H₆O₃, C₄H₈O₄, and C₃H₆O₃ + C₃H₆O₃, respectively, suggesting the presence of two carbon-glucoside. According to the published paper [21, 22], peak 6 was tentatively identified as Vicenin II. In a similar way, peak 18 was tentatively identified as Phloretin-C-diglucoside.

Peaks 11, 14, 24, and 31 generated the same quasimolecular ion [M–H][–] at m/z 433.114 (C₂₁H₂₁O₁₀), 162 Da(C₆H₁₀O₅, glucose moiety) more than that of naringenin (peak 38), suggesting they were naringenin-glucoside, which were further confirmed by the presence of m/z 271.061 and 151.003 in MS² spectrum.

Peak 12 eluted at 6.22 min and showed a pseudomolecular ion at m/z 625.1408 (0.00 ppm, C₂₇H₂₉O₁₇), 176.032 Da(C₆H₈O₆, glucuronide moiety) more than that of eriodictyol-glucoside, suggesting it is eriodictyol-glucoside-glucuronide, which was confirmed by the presence of the base peak at m/z 287.0558 (eriodictyol).

Peaks 20, 21, 25, and 29 presented the same deprotonated ion [M–H][–] at m/z 431.099 (C₂₁H₁₉O₁₀) and generated the same fragment ions at m/z 269.044 (C₁₅H₉O₅) by loss of the glucose moiety (C₆H₁₀O₅), which suggested the presence of glucose moiety. The base peak at m/z 268.037 [Y₀–H] ions in the MS² spectrum of peaks 25 and 29 was a characteristic of apigenin aglycone. According to the published paper [23, 24], they were tentatively inferred as apigenin-7-glucoside and apigenin-4'-glucoside, respectively. Meantime, peaks 20 and 21 were tentatively characterized as baicalein-glucoside.

Peak 32 was detected at 13.39 min. It presented a pseudomolecular ion at m/z 567.1730 (1.94 ppm, C₂₆H₃₂O₁₄) and exhibited the MS² fragmentation ions at m/z 273.0771 (1.10 ppm, C₁₅H₁₃O₅), resulting from the loss of glucose moiety and pentoside moiety (294.096). Thus, peak 32 was tentatively characterized as phloretin-pentoside-glucoside. In a similar way, peak 23 was tentatively identified as naringenin-pentoside-glucoside.

3.3. Target Identification of Flavonoids

212 putative targets of flavonoids were obtained from the TCMSP database. A visual compounds-targets network with 224 nodes and 440 edges was built by Cytoscape Version 3.7.2 (Figure S1). Compounds quercetin, apigenin, kaempferol, luteolin, and wogonin are the top 5 compounds with a maximum degree and betweenness in the compound-targets network. The detailed information of putative targets linked to compounds was provided in Supplementary Table S1.

3.4. Protein-Protein Interaction Network

In order to find the key targets of flavonoids, a total of 212 putative targets were imported into the STRING to obtain the protein-protein interaction (PPI) data. The PPI network with 206 nodes and 3980 edges was established by Cytoscape (Figure S2). A total of 23 targets, including AKT1, INS, TP53, IL6, HSP90AA1, EGFR, VEGFA, JUN, EGF, CASP3, MAPK1, ESR1, ERBB2, PTGS2, MYC, MAPK8, MMP9, FN1, FOS, PPARG, CXCL8, CYCS, and CCND1, were selected as the core targets for GO and KEGG pathway analysis by setting the parameters as follows: the degree ≥ 50 ; betweenness centrality ≥ 0.01 ; closeness centrality ≥ 0.6 .

3.5. GeneMANIA Analysis

Among the 23 key target genes and their interacting genes, it was found that 42.75 % had coexpression characteristics, 41.10 % displayed physical interactions characteristic. Other characteristics, including pathway, genetic interactions, colocalization, and shared protein domains, are displayed in Figure 2.

[figure omitted; refer to PDF]

3.6. GO and Pathway Analysis

In order to further study the 23 core target genes, GO and KEGG pathway analysis were performed by DAVID. GO term enrichment analysis results were divided into the biological process (BP, 23/23), cell compound (CC, 23/23), and molecular function (MF, 23/23). A total of 158 BP, 16 CC, and 33MF has a p-value less than 0.05 (Table S2). In GO term enrichment analysis, the BP might be related to positive regulation of transcription from RNA polymerase II promoter (10/23), response to drug (9/23), negative regulation of apoptotic process (9/23), positive regulation of transcription, DNA-templated (9/23), signal transduction (9/23), positive regulation of gene expression (8/23), and positive regulation of cell proliferation (8/23), and so on. The top 4 of CC are nucleus (16/23), nucleoplasm (12/23), cytosol (12/23), and cytoplasm (12/23). The MF are protein binding on 100%, identical protein binding on 56.5%, enzyme binding on 39.1%, and transcription factor binding on 34.8%. The top 10 enriched terms in BP, CC, and MF are displayed in Figure 3. In addition, 83 KEGG pathways (Table S3) were enriched as p-value less than 0.05. The result showed that the pathway was mainly related to the signaling pathway including PI3K-Akt (12/23), MAPK (10/23), TNF (9/23), ErbB (8/23), HIF-1 (8/23), Estrogen (8/23), FoxO (8/23), and cancer in the pathway. The top 20 KEGG pathways are shown in Figure 4.

[figure omitted; refer to PDF][figure omitted; refer to PDF]

3.7. Network Analysis

Based on the target and KEGG pathway analysis, the entire compounds, targets, and pathway network were established by Cytoscape. The network with 122 nodes and 595 edges is shown in Figure 5. The red diamond, green ellipse, and blue triangle represent compounds, genes, and pathways, respectively.

[figure omitted; refer to PDF]

4. Conclusion

In the present investigation, this finding revealed that *P. freyniana* possessed 43 flavonoids (40 of them was first reported) with 23 core target genes, which were associated with PI3K-Akt, MAPK, TNF signaling pathway, and pathway in cancer. This study demonstrated the multicomponent, multitarget, and multimechanism of *P. freyniana*, which are very beneficial for the further study and utilization of this plant including the material basis and quality control research.

Authors' Contributions

Zaiqi Zhang and Wei Cai designed the study. Kailin Li, Wei Cai, Shihan Qin, Pei Xiong, Jie Peng, and Silin Shi performed the experiments. Kailin Li, Wei Cai and Shihan Qin performed data analyses. Kailin Li and Wei Cai wrote

the experiments; Zaiqi Zhang, Shihan Qin, Pei Xiong, Jie Peng, and Silin Shi critically reviewed the manuscript. All authors read and approved the final manuscript. Wei Cai and Kailin Li contributed equally to this work.

References

- [1] Editorial Board, Flora of China, 2003.
- [2] Y. Zhong, X. Y. Qu, Y. D. Zhang, "Medicinal Plants of potentilla genus in Xiangxi," Chinese Journal of Ethnomedicine and Ethnopharmacy, vol. 6 no. 72, pp. 344-345, 2014.
- [3] K. M. Lu, Medicine of Dong Nationality, 1992.
- [4] Y. H. Cai, L. Liu, D. X. Han, C. L. Liu, "Study on chemical components of potentilla freyniana bornm," Journal Wuhan University Technology, vol. 27 no. 9, pp. 32-33, 2014.
- [5] X. H. Wu, C. Mi, Y. He, "Polyphenols from the rhizomes of potentilla freyniana," Medicinal Chemistry, vol. 5, pp. 21-22, 2015.
- [6] X.-H. Wu, J.-L. Ruan, Y.-L. Cai, "Triterpenes from the rhizomes of potentilla freyniana," Biochemical Systematics and Ecology, vol. 7 no. 4, pp. 509-511, DOI: 10.1016/j.bse.2009.04.009, 2009.
- [7] H. Z. Chen, "Study on the chemical constituents of ethy acetate extract from Potentilla freyniana," Hubei University Chinese Medicine Dissertation, vol. 36 no. 5, pp. 744-746, 2015.
- [8] S. H. Wang, G. D. Chen, "Studies on anti-inflammatory and immune effect Potentilla freyniana Bornm.," Journal Hubei University Chinese Medicine, vol. 11, pp. 26-28, 2009.
- [9] B. J. Xiao, G. D. Chen, Y. M. Zhu, "Study on anti-inflammatory and analgesic effect of Potentilla freyniana Bornm.," Tianjin Medicine Journal, vol. 37, pp. 482-484, 2006.
- [10] N. Satheeshkumar, S. Shantikumar, M. Komali, "Identification and quantification of aldose reductase inhibitory flavonoids in herbal formulation and extract of gymnema sylvestre using HPLC-PDA and LC-MS/MS," Chromatography Research International, vol. 2014, DOI: 10.1155/2014/518175, 2014.
- [11] Y. S. Fan, Y. M. Li, Y. Y. Wu, "Identification of the chemical constituents in simiao wan and rat plasma after oral administration by GC-MS and LC-MS," Evidence-Based Complementary and Alternative Medicine, vol. 2017, DOI: 10.1155/2017/6781593, 2017.
- [12] L. Liu, J. Zhang, B. Zheng, "Rapid characterization of chlorogenic acids in duhaldea nervosa based on ultra-high-performance liquid chromatography-linear trap quadrupole-orbitrap-mass spectrometry and mass spectral trees similarity filter technique," Journal of Separation Science, vol. 41 no. 8, pp. 1764-1774, DOI: 10.1002/jssc.201701047, 2018.
- [13] M. Chandradevan, S. Simoh, A. Mediani, "UHPLC-ESI-Orbitrap-MS analysis of biologically active extracts from gynura procumbens (lour.) merr. and Cleome gynandra L. Leaves," Evidence-Based Complementary and Alternative Medicine, vol. 2020, DOI: 10.1155/2020/3238561, 2020.
- [14] X. D. Ye, Y. H. Wang, J. P. Zhao, "Identification and characterization of key chemical constituents in processed gastrodia elata using UHPLC-MS/MS and chemometric methods," Journal of Analytical Methods in Chemistry, vol. 2019, DOI: 10.1155/2019/4396201, 2019.
- [15] Y. Chen, D. Chen, S. Liu, "Systematic elucidation of the mechanism of genistein against pulmonary hypertension via network pharmacology approach," International Journal of Molecular Sciences, vol. 20 no. 22, DOI: 10.3390/ijms20225569, 2019.
- [16] Y.-F. Zhang, Y. Huang, Y.-H. Ni, Z.-M. Xu, "Systematic elucidation of the mechanism of geraniol via network pharmacology," Drug Design, Development and Therapy, vol. 13, pp. 1069-1075, DOI: 10.2147/dddt.s189088, 2019.
- [17] J. Chen, J. Chen, J. Lu, "Systematic elucidation of the mechanism of oroxylum indicum via network pharmacology," Evidence-Based Complementary and Alternative Medicine, vol. 2020, DOI: 10.1155/2020/5354215, 2020.
- [18] W. Cai, K. L. Li, P. Xiong, "A systematic strategy for rapid identification of chlorogenic acids derivatives in duhaldea nervosa using UHPLC-Q-exactive orbitrap mass spectrometry," Arabian Journal of Chemistry, vol. 13, pp. 3751-3761, DOI: 10.1016/j.arabjc.2020.01.007, 2020.

- [19] Y. J. Min, Y. P. Zhang, Q. Gao, "Study on the technology of extraction and content of flavonoids from potentilla chinensis series," Food and Fermentation Industries, vol. 6, pp. 176-179, 2008.
- [20] M. A. Alvarez-Fernández, A. B. Cerezo, A. M. Cañete-Rodríguez, "Composition of nonanthocyanin polyphenols in alcoholic-fermented strawberry products using LC-MS (QTRAP), high-resolution MS (UHPLC-Orbitrap-MS), LC-DAD, and antioxidant activity," Journal of Agricultural and Food Chemistry, vol. 63, pp. 2041-2051, DOI: 10.1021/jf506076n, 2015.
- [21] C. Terreaux, Q. Wang, J. R. Ioset, "Complete LC/MS analysis of a Tinneveli senna pod extract and subsequent isolation and identification of two new benzophenone glucosides," Planta Medica, vol. 68, pp. 349-354, DOI: 10.1055/s-2002-26738, 2002.
- [22] A. Taamalli, D. Arráez-Román, L. Abaza, "LC-MS-based metabolite profiling of methanolic extracts from the medicinal and aromatic species mentha pulegium and origanum majorana," Phytochemical Analysis, vol. 26, pp. 320-330, DOI: 10.1002/pca.2566, 2015.
- [23] M. Yasir, B. Sultana, F. Anwar, "LC-ESI-MS/MS based characterization of phenolic components in fruits of two species of solanaceae," Journal of Food Science and Technology-Mysore, vol. 55, pp. 2370-2376, DOI: 10.1007/s13197-017-2702-9, 2018.
- [24] L. Bevilacqua, F. Buiarelli, F. Coccioli, "Identification of compounds in wine by HPLC-tandem mass spectrometry," Annali di Chimica, vol. 94 no. 9-10, pp. 679-690, DOI: 10.1002/adic.200490085, 2004.

DETAILS

Subject:	Mass spectrometry; Software; Encyclopedias; Constituents; Quality control; Investigations; Genes; Identification; Pharmacology; Metabolism; Chromatography; Network analysis; Chinese medicine; Proteins; Quadrupoles; Flavonoids; High performance liquid chromatography; Biotechnology; Genomes
Location:	United States--US; Germany; China
Editor:	Richard G Brereton
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779

Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-10-11 (Received); 2020-11-27 (Revised); 2021-01-16 (Accepted); 2021-01-27 (Pub)
DOI:	https://doi.org/10.1155/2021/6619959
ProQuest document ID:	2487051257
Document URL:	https://www.proquest.com/scholarly-journals/rapid-identification-systematic-mechanism/docview/2487051257/se-2?accountid=211160
Copyright:	Copyright © 2021 Wei Cai et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-02-14
Database:	Publicly Available Content Database

Document 55 of 59

Optimization of 25% Sulfosalicylic Acid Protein-to-Creatinine Ratio for Screening of Low-Grade Proteinuria

Ambulugala Gamage Rajika Greshamali Jinadasa ¹

; Lanka Acharige Shalka Madushan Srimantha ¹

; Siriwardhana, Indika Deepani ²

; Gunawardana, Kalani Buddika ¹

; Attanayake, Anoja Priyadarshani ³

¹ Department of Medical Laboratory Science, Faculty of Allied Health Sciences, University of Ruhuna, Galle, Sri Lanka ² Department of Pathology, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka ³ Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Proteinuria is an important prognostic marker in the diagnosis and management of kidney diseases. Sulfosalicylic acid method (SSA) is a simple, low cost, qualitative test, widely used to assess proteinuria. The aim of this study was to optimize SSA test as a quantitative screening tool to assess proteinuria at lower excretory levels which would facilitate the screening and early diagnosis of renal impairment using protein-to-creatinine ratio (PCR). The study was conducted in two phases. In phase I, optimum SSA percentage to detect low-grade proteinuria was selected by comparing the performance of 3%, 6%, and 25% SSA methods in manual spectrophotometric analysis. In phase II, clinical applicability of the optimized method was evaluated using retained urine samples of patients with chronic kidney disease (CKD) assessed for urine protein by the pyrogallol red (PGR) method in a tertiary care hospital in Sri Lanka. Optimized 25% SSA protein-to-creatinine ratio (PCR) was compared with PGR PCR and albumin-to-creatinine ratio (ACR). Sensitivity, specificity, degree of agreement, correlation, and diagnostic accuracy were evaluated. Turbidimetric analysis using 25% SSA was linear in the range 3–50 mg/dL giving the highest analytical sensitivity. The test yielded a sensitivity of 86.5% and specificity of 96.5% and a degree of agreement of 5 mg/dL with the PGR method. Optimal cut-off for 25% SSA PCR in receiver operating characteristic analysis was 166 mg/g. Spearman's correlation coefficient for 25% SSA PCR versus ACR was $r=0.823$, $p<0.0001$, and for 25% SSA PCR versus PGR PCR was $r=0.913$, $p<0.0001$. The 25% SSA PCR has a sensitivity of 92% against ACR, the current prognostic marker for proteinuria in patients with CKD. The 25% SSA test is a simple method, and it performs satisfactorily as a screening test with a cut-off for PCR optimized at 166 mg/g. The test merits further evaluation due to its low cost.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Chronic kidney disease (CKD) has become a major healthcare problem all over the world with a global mean prevalence of 13.14% in 2016 [1]. With respect to this global prevalence, a remarkable increase of the disease has emerged in Sri Lanka within the past two decades [2]. Major etiologies for CKD are diabetes mellitus and hypertension. Meanwhile, a new form of the disease, with no identifiable etiology termed as chronic kidney disease of unknown etiology (CKDu) has emerged in several countries, including Sri Lanka [3]. International Society of Nephrology defines CKD as abnormalities of kidney structure or function persisting for more than three months with implications for health leading to dialysis and kidney transplant in the final stage [4]. Diagnosis of renal damage in the early course of the disease would prevent its development to the chronic irreversible stage and facilitate better prognosis. Thus, sensitivity in screening and specificity in diagnostic methods of CKD play a major role in disease management. Among the available screening techniques, persistent proteinuria has become a key evaluation target in assessing kidney function and progressive kidney damage. It refers to urinary protein excretion ≥ 150 mg/24 hours. Albuminuria (albumin-to-creatinine ratio (ACR) ≥ 3 mg/mmol or 30 mg/g) is a common finding in CKD due to diabetic nephropathy. Assessment of protein concentration in 24-hour urine collection is considered as the gold standard for proteinuria estimation [5]. However, sample collection for 24-hour test has limitations. Kidney Disease Improving Global Outcomes (KDIGO) guidelines states estimated glomerular filtration rate (eGFR) and ACR as the recommended evaluation criteria for the classification of CKD [4]. Furthermore, it is mentioned that eGFR begins to decline following a greater damage (nearly 50% damage) to renal tissues [6]. Thus, ACR is a better marker over eGFR for early diagnosis of renal damage. Dipsticks are currently being used in population screening programmes for CKD, but dipsticks are more sensitive to urine albumin. Dye binding methods, like pyrogallol red (PGR) and coomassie brilliant blue, and immunoturbidimetric methods are being used at clinical laboratories for quantitative analysis of urine protein. Cystatin-C, kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and alpha 1 microglobulin (A1M) are more specific urine biomarkers which are being evaluated in research

[7]. However, the requirement of expensive chemicals and analyzers for some of these have limited their use in hospital laboratory set up and these are not cost-effective for community based screening [2, 8]. Thus, cost-effective, convenient screening methods could immensely contribute to disease management and early diagnosis of CKD and CKDu patients.

Sulfosalicylic acid (SSA) method is a noninvasive, cost-effective turbidimetric test routinely used for semiquantitative evaluation of proteinuria up to 500 mg/dL in clinical laboratories. Addition of SSA to an acidified protein solution results in denaturation of proteins followed by precipitation. The content of protein can be estimated semiquantitatively using a grading scale or quantitatively by measuring the turbidity. It has long been used for quantitative evaluation of total protein in urine and cerebrospinal fluid before dye-binding methods, such as PGR, were introduced [9].

This study was aimed to investigate the feasibility of using SSA turbidimetric method for quantitative evaluation of proteinuria in the lower range and to assess its clinical applicability by assessing protein-to-creatinine ratio (PCR) which minimizes the effects of factors, like pyrexia, status of hydration, and strenuous exercises. We were interested to optimize it for the lower range (0–50 mg/dL) since it would facilitate the detection and the follow-up of the progression of disease at early stages using a low-cost screening method. It is important to evaluate protein-to-creatinine ratio (PCR) and compare against standard ACR since PCR and ACR provide better clinical information of kidney disease rather than assessing total urine protein concentration in a random urine sample [10–12]. The current gold standard for assessing urine protein is PGR dye binding method, which requires expensive reagents and automated analyzers, which constrain its usability as a screening test. Therefore, development of SSA test to assess PCR as a reliable quantitative low-cost screening method could be useful in early diagnosis of renal impairment.

2. Materials and Methods

SSA test method is used in the clinical setup with various modifications. Different concentrations of SSA (3%, 6%, and 25%) have been used in the current laboratory setup for semiquantitative estimation of urine protein.

Therefore, in this study, we had to address two basic research questions. First, can we use SSA for quantitative estimation of urine protein in the lower range, and if so, what is the optimum percentage? Second, is it clinically applicable? In order to address these two questions, we conducted the study in two phases using convenient sample method. In phase I, SSA method was optimized to quantitate urine protein in the lower range (0–50 mg/dL), and the clinical validity of the optimized method was assessed using samples received from the Nephrology Clinic at Teaching Hospital, Karapitiya, Sri Lanka, for analysis of urine protein by PGR method. In phase II, the clinical applicability of the optimized method was evaluated by comparing urine PCR with gold standard urine ACR on samples from CKD patients [4]. Ethical approval for the study was granted by the Ethics Review Committee of Faculty of Allied Health Sciences, University of Ruhuna, Galle, Sri Lanka (14.02.2018.005).

Retained urine samples of patients aged 18–65 years, admitted to wards and/or attending the Nephrology Clinic at Teaching Hospital, Karapitiya, Sri Lanka, were used for this study. They were already analyzed by PGR dye binding method using Thermo Scientific Indiko Plus Fully Automated Biochemistry Analyzer (Finland) for urine protein concentration. Samples with urine total protein ≤ 20 mg/dL and ACR ≤ 30 mg/g were considered as normal, in phase I and II, respectively. In identification of CKD patients, serum creatinine value measured within the past three months was collected from laboratory information system, and eGFR was calculated using CKD/EPI formula according to 2012 KDIGO guidelines [4].

2.1. Phase I Method Development

This phase was conducted to find out the most suitable concentration of SSA to quantitatively evaluate urine protein in the lower range. Therefore, standard curves were prepared using 3%, 6%, and 25% SSA in a dilution series of bovine serum albumin factor V using manual spectrophotometric analysis. Absorbance was used to generate standard curves. The optimum SSA percentage was selected depending on analytical sensitivity (gradient of the standard curve), lowest detection limit, and linearity range.

2.2. Preparation of Standard Curves

A dependent dilution series of albumin standard (2.125–200 mg/dL) was prepared. This was used for the generation

of albumin standard curve [13]. Albumin standard of 2.0mL was acidified with 50 μ L of trichloroacetic acid followed by 100 μ L of SSA at 25 $\pm$ 2°C. Proteins were allowed to precipitate for three minutes, and the absorbance was measured at 600nm against reagent blank in a double beam spectrophotometer (SHIMADZU 1800 UV-VIS, Japan) using manual procedures. Standard curves were generated for 3%, 6%, and 25% SSA assays by plotting absorbance verses concentration of albumin standards.

2.3. Clinical Validity of Standard Curve

A comparative cross-sectional study was performed to assess the performance of the selected SSA percentage on clinical samples. Protein concentrations of 81 urine samples were tested on the same day of sample collection with modified SSA method. Two (2) mL of urine sample was initially acidified with 50 μ L of trichloroacetic acid followed by addition of 100 μ L of SSA, and the turbidity was measured as described above. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were evaluated considering PGR method as the gold standard. Since patient samples were used for the study, samples with proteinuria less than 20mg/dL measured by the PGR method were considered as normal population for the study. Intra-assay precision was assessed using albumin spiked normal urine sample at the lower range (20mg/dL) and a pooled patient sample at the upper range (40.6mg/dL). Bland-Altman plot and linear regression were used to assess the degree of agreement and correlation between the two methods, respectively (Figure 1).

[figure omitted; refer to PDF]

To validate the optimized 25% SSA curve, a clinical study was performed using 81 patient samples (Table 3). The assessment of clinical validity at 95% confidence interval (CI) showed a sensitivity of 86.5% (95% CI 74.4–93.63) and specificity of 96.5% (95% CI 81.3–99.9), PPV of 97.8% (95% CI 87.6–99.9), NPV of 80.0% (95% CI 63.8–90.2), and accuracy of 90.1% (95% CI 81.4–95.1) when compared with the PGR method at a cut-off of 20mg/dL for proteinuria.

Table 3

Patient data for the calculation of performance characteristics of the 25% sulfosalicylic acid protein concentration versus pyrogallol red protein concentration.

	Diseased (PGR protein value $\geq$ 20 mg/dL)	Nondiseased (PGR protein value <20 mg/dL)
25% SSA (protein value $\geq$ 20 mg/dL)	45	1
25% SSA (protein value <20 mg/dL)	7	28

SSA: sulfosalicylic acid. PGR: pyrogallol red.

The patient data deviated from normal distribution with skewness 0.968 and kurtosis 0.200; outliers were removed and statistical analysis was performed. In linear regression, a positive correlation (r) of 0.9023 ($R^2=0.8143$) was observed between 25% SSA method and PGR method. The equation of [PGR]=1.4395 [SSA] was derived through linear regression.

The degree of agreement between the SSA method and PGR method was evaluated in Bland-Altman analysis. Data range used was 3–50mg/dL. The plot showed a bias of –5.5mg/dL and a SD of 5.3mg/dL. The lower limit and the upper limit of agreement were –15.8 and 4.9mg/dL, respectively (Figure 3).

[figure omitted; refer to PDF]

AUC observed for SSA and PGR was 0.904 (95% CI, 0. 803–1.00) and 0.962 (95% CI, 0.908–1.00), respectively. AUC being above 0.5 indicates that both SSA and PGR methods are effective in identifying the presence of a disease. The optimal cut-off derived for 25% SSA method from ROC analysis is 166mg/g at 95% CI of sensitivity

and specificity. This is closer to the currently accepted cut-off for PCR of 150mg/g in detecting proteinuria [4].

4. Discussion

In this study, conventional qualitative 25% SSA test for urine protein measurement was optimized as a low cost clinically applicable sensitive test by evaluating protein-to-creatinine ratio for the quantitative determination of urine proteins at lower excretory levels. The cost evaluation studies show that cost per test of 25% SSA method is 0.27 LKR, whereas, for PGR method and for microalbumin method, it is 25.00 LKR and 168.00 LKR, respectively, in Sri Lankan rupees. An optimal cut-off of 166mg/g was derived for PCR in 25% SSA method which is closely associated with the current cut-off for PCR of 150mg/g in diagnosing proteinuria [10–12].

Urine protein and creatinine were assayed using the 25% SSA and Jaffe methods, respectively, using spectrophotometric analysis. Albumin was measured in immunoturbidimetric method in BTS 350 semiautomatic system. The 25% SSA test was performed on the same day of sample collection. Manually measured creatinine values were used to calculate 25% SSA PCR and ACR. The PGR PCR were derived from Indiko Plus Fully Automated Biochemistry Analyzer at the Pathology Laboratory, Teaching Hospital, Karapitiya, Sri Lanka. A previous study has derived an optimum cut-off of 160mg/g for PCR, which is closer to 166mg/g when SSA was used to assess urine protein and Jaffe method for creatinine [11]. Therefore, the current study has been able to regenerate similar figures in a different laboratory setting. Limited sample numbers were used in the project depending on the feasibility within the limited time frame. However, similar sample numbers have been used in previous method comparison studies for urine protein measurement [8, 15]. KDIGO guidelines recommend eGFR together with ACR for diagnosis and classification of CKD [4]. Nevertheless, ACR targets only a fraction of total protein amount excreted in urine. The 25% SSA test could be clinically applicable as a screening test in early diagnosis of proteinuria (both albumin and nonalbumin) because of its improved sensitivity at lower levels of protein excretion. Tubulointerstitial diseases of kidney predominantly give rise to nonalbumin proteinuria, which is not detected by ACR. CKDu is an example where tubulointerstitial damage predominates [16].

CKDu has shown an alarming rise in prevalence among the rural agricultural communities in Sri Lanka over the last three decades [17]. The disease has been reported among similar socioeconomic communities around the globe in the developing world [3]. Early diagnosis of the disease is important to ensure better patient outcomes. Therefore, a sensitive and cost-effective test for early identification of tubular proteinuria is invaluable in the early diagnosis of CKDu. Although newer biomarker assays, molecular markers, and histopathological tests have been found to be more sensitive and specific for accurate diagnosis of CKDu, sophisticated technology, expensive reagents, competent human resources, and invasive sampling methods have limited their use in population screening [7, 18–21]. A large-population-based study conducted in Sri Lanka in the year 2015 has recommended the use of combined eGFR and urine ACR or urine PCR (UPCR) as screening tests for early identification of CKDu [22]. The performance of eGFR and urine based screening tests have been studied among the population at risk. Dipstick proteinuria, urine ACR, UPCR using PGR method, and semiquantitative screening test for urine protein detection using SSA had been used in the aforementioned study. The aforementioned large-population-based study has revealed that the sensitivity of UPCR and semiquantitative SSA test were superior to that of ACR and dipstick protein test, in screening for CKDu [22]. Thus, combined evaluation of PCR using quantitative SSA test, as observed in our study (25% SSA PCR), would be effective in screening programmes for CKDu.

The modified 25% SSA PCR test is a simple and cost-effective test for identification of low-grade proteinuria which appears early in tubulointerstitial diseases. The test is easily applicable in population screening programmes for CKD/CKDu to identify low-grade proteinuria. However, measurement of urine protein concentration alone is not clinically significant. Therefore, evaluation of PCR in a random sample is essential in clinical diagnosis. In addition, PCR is highly correlated with 24-hour protein excretion, which is the recommended sample of urine protein estimation [10–12]. The 25% SSA test in our study has been validated and its clinical applicability in PCR has been assessed.

Limitations of this method include the requirement of fresh urine samples; the test is nonresponsive in refrigerated urine samples. The current study was conducted in manual spectrophotometric analysis. Applicability of the test on

semiautomated or automated systems with minimum sample volume should be evaluated for it to be applicable for population screening. The test needs further evaluation as a screening test among CKDu endemic and nonendemic regions to further determine its clinical applicability.

5. Conclusion

In conclusion, the 25% SSA PCR method has a sensitivity of 92% against ACR, the current prognostic marker for proteinuria in patients with CKD. The test, with its ability to identify patients with low-grade proteinuria optimized at a cut-off 166mg/g, merits further evaluation as a screening test due to its cost-effectiveness and high sensitivity.

Acknowledgments

The authors are grateful to Prof. Bilesha Perera, Department of Community Medicine, Faculty of Medicine, University of Ruhuna, for the statistical consultation in the project. The authors greatly acknowledge consultant chemical pathologist Dr. Manjula Dissanayake and laboratory staff, Teaching Hospital, Karapitiya, and the Department of Medical Laboratory Science, Faculty of Allied Health Sciences, University of Ruhuna, for their kind collaboration to make the present study a success. Furthermore, the authors are grateful to Triviron Nawakrama Medical Technologies (Pvt) Ltd, Peliyagoda, Sri Lanka, for providing BTS 350 semiautomatic biochemistry analyzer, Spain, to perform the urine albumin assay. This study was funded by the Faculty of Medicine and Faculty of Allied Health Sciences, University of Ruhuna, Sri Lanka.

References

- [1] N. R. Hill, S. T. Fatoba, J. Oke, "Global prevalence of chronic kidney disease-a systematic review and meta-analysis," *PloS One*, vol. 11 no. 7, DOI: 10.1371/journal.pone.0158765, 2016.
- [2] A. V. Ranasinghe, G. W. G. P. Kumara, R. H. Karunarathna, "The incidence, prevalence and trends of chronic kidney disease and chronic kidney disease of uncertain aetiology (CKDu) in the North Central Province of Sri Lanka: an analysis of 30,566 patients," *BMC Nephrology*, vol. 20 no. 1, DOI: 10.1186/s12882-019-1501-0, 2019.
- [3] M. Almaguer, R. Herrera, C. M. Orantes, "Chronic kidney disease of unknown etiology in agricultural communities," *MEDICC Review*, vol. 16 no. 2, 2014.
- [4] K. M. Andrassy, "Comments on KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease," *Kidney International*, vol. 84 no. 3, pp. 622-623, DOI: 10.1038/ki.2013.243, 2013.
- [5] I. Saxena, S. Kapoor, R. C. Gupta, "Detection of proteinuria in pregnancy: comparison of qualitative tests for proteins and dipsticks with urinary protein creatinine index," *Journal of Clinical and Diagnostic Research*, vol. 7 no. 9, pp. 1846-1848, DOI: 10.7860/JCDR/2013/6656.3330, 2013.
- [6] D. Steubl, M. Block, V. Herbst, "Plasma uromodulin correlates with kidney function and identifies early stages in chronic kidney disease patients," *Medicine (Baltimore)*, vol. 95 no. 10, DOI: 10.1097/md.0000000000003011, 2016.
- [7] B. N. T. W. Fernando, A. Alli-shaik, R. K. D. Hemage, "Pilot study of renal urinary biomarkers for diagnosis of CKD of uncertain etiology," *Kidney Int Reports*, vol. 4 no. 10, pp. 1401-1411, DOI: 10.1016/j.ekir.2019.07.009, 2019.
- [8] Q.-E. Cao, Z. Ding, R. Fang, X. Zhao, "A sensitive and rapid method for the determination of protein by the resonance Rayleigh light-scattering technique with Pyrogallol Red," *The Analyst*, vol. 126 no. 8, pp. 1444-1448, DOI: 10.1039/b101827m, 2001.
- [9] C. K. Cheung, Y. T. Mak, R. Swaminathan, "Automated trichloroacetic acid precipitation method for urine total protein," *Annals of Clinical Biochemistry: International Journal of Laboratory Medicine*, vol. 24 no. 2, pp. 140-144, DOI: 10.1177/000456328702400203, 1987.
- [10] C. P. Price, R. G. Newall, J. C. Boyd, "Use of protein: creatinine ratio measurements on random urine samples for prediction of significant proteinuria: a systematic review," *Clinical Chemistry*, vol. 51 no. 9, pp. 1577-1586, DOI: 10.1373/clinchem.2005.049742, 2005.
- [11] P. Patil, V. Shah, B. Shah, "Comparison of spot urine protein creatinine ratio with 24 hour urine protein for estimation of proteinuria," *The Journal of the Association of Physicians of India*, vol. 62 no. 5, pp. 406-410, 2014.
- [12] J. Kamińska, V. Dymicka-Piekarska, J. Tomaszewska, J. Matowicka-Karna, O. M. Koper-Lenkiewicz, "Diagnostic utility of protein to creatinine ratio (P/C ratio) in spot urine sample within routine clinical practice," *Critical*

- reviews in clinical laboratory sciences, vol. 57 no. 5, pp. 345-364, DOI: 10.1080/10408363.2020.1723487, 2020.
- [13] M. M. Gunathilaka, K. D. K. Silva, M. M. Hunais, Manual on Standard Operating Procedures, Sample Collection and Reference Ranges for Clinical Chemistry, 2005.
- [14] J. Peat, B. Barton, Medical Statistics: A Guide to Analysis and Critical Appraisal, 2008.
- [15] P. Yalamati, M. L. Karra, A. V. Bhongir, "Comparison of urinary total proteins by four different methods," Indian Journal of Clinical Biochemistry, vol. 31 no. 4, pp. 463-467, DOI: 10.1007/s12291-016-0551-3, 2016.
- [16] J. Wijkstrom, C. Jayasumana, R. Dassanayake, "Morphological and clinical findings in Sri Lankan patients with chronic kidney disease of unknown cause (CKDu): similarities and differences with Mesoamerican nephropathy," PLoS One, vol. 13 no. 3, DOI: 10.1371/journal.pone.0193056, 2018.
- [17] T. Ruwanpathirana, S. Senanayake, N. Gunawardana, "Prevalence and risk factors for impaired kidney function in the district of Anuradhapura, Sri Lanka: a cross-sectional population-representative survey in those at risk of chronic kidney disease of unknown aetiology," BMC Public Health, vol. 19 no. 1, DOI: 10.1186/s12889-019-7117-2, 2019.
- [18] P. M. C. S. D. Silva, K. S. M. Abdul, E. M. D. V. Eakanayake, "Urinary biomarkers KIM-1 and NGAL for detection of chronic kidney disease of uncertain etiology (CKDu) among agricultural communities in Sri Lanka," PLoS Neglected Tropical Diseases, vol. 10 no. 9, 2016.
- [19] K. Wanigasuriya, I. Jayawardene, C. Amarasiriwardena, R. Wickremasinghe, "Novel urinary biomarkers and their association with urinary heavy metals in chronic kidney disease of unknown aetiology in Sri Lanka: a pilot study," Ceylon Medical Journal, vol. 62 no. 4, pp. 210-217, DOI: 10.4038/cmj.v62i4.8568, 2017.
- [20] S. Ratnayake, Z. Badurdeen, N. Nanayakkara, T. Abeysekera, N. Ratnatunga, R. Kumarasiri, "Screening for chronic kidney disease of uncertain aetiology in Sri Lanka: usability of surrogate biomarkers over dipstick proteinuria," BMC Nephrology, vol. 18 no. 199, DOI: 10.1186/s12882-017-0610-x, 2017.
- [21] S. Sayanthooran, D. N. Magana-arachchi, L. Gunerathne, T. Abeysekera, "Potential diagnostic biomarkers for chronic kidney disease of unknown etiology (CKDu) in Sri Lanka: a pilot study," BMC Nephrology, vol. 18 no. 31, DOI: 10.1186/s12882-017-0440-x, 2017.
- [22] N. Herath, R. Dassanayake, M. Dissanayake, "Normality data of eGFR and validity of commonly used screening tests for CKD in an area with endemic CKD of unknown etiology; need for age and sex based precise cutoff values," BMC Nephrology, vol. 20 no. 298, DOI: 10.1186/s12882-019-1477-9, 2019.

DETAILS

Subject:	Markers; Acids; Regression analysis; Sensitivity analysis; Kidney diseases; Urine; Medical laboratories; Diagnosis; Automation; Correlation coefficients; Proteins; Patients; Disease management; Validity; Low cost; Albumins; Proteinuria; Spectrophotometry; Creatinine; Optimization; Diagnostic systems; Methods; Teaching hospitals; Nephrology
Business indexing term:	Subject: Automation
Location:	Sri Lanka
Editor:	David M Lubman
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021

Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-10-20 (Received); 2021-01-07 (Revised); 2021-01-12 (Accepted); 2021-01-28 (Pub)
DOI:	https://doi.org/10.1155/2021/6688941
ProQuest document ID:	2487051251
Document URL:	https://www.proquest.com/scholarly-journals/optimization-25-sulfosalicylic-acid-protein/docview/2487051251/se-2?accountid=211160
Copyright:	Copyright © 2021 Ambulugala Gamage Rajika Greshamali Jinadasa et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-02-14
Database:	Publicly Available Content Database

Determination and Pharmacokinetics of Omeprazole Enantiomers in Human Plasma and Oral Fluid

Utilizing Microextraction by Packed Sorbent and Liquid Chromatography-Tandem Mass Spectrometry

Ahmed, Hytham ¹ ; Abdel-Aziz, Wahbi ² ; Elmongy, Hatem ² ; Amini, Ahmad ³ ; Hirsh Koyi ⁴ ; Branden, Eva ⁴ ; Abdel-Rehim, Mohamed ⁵

¹ Department of Pharmaceutical Analysis, Menoufia University, Menofia Governorate, Egypt ² Department of Pharmaceutical Analytical Chemistry, University of Alexandria, Alexandria 21521, Egypt ³ Swedish Drug Agency, Uppsala, Sweden ⁴ Department of Respiratory Medicine Gävle Hospital and Centre for Research at Uppsala University/County Council of Gävleborg, Gävle, Sweden ⁵ Karolinska Institute, Solna SE17176, Sweden

[ProQuest document link](#)

ABSTRACT (ENGLISH)

In the present work, the determination of omeprazole (OME) enantiomers in oral fluid and plasma samples was carried out utilizing microextraction by packed sorbent (MEPS) and liquid chromatography-tandem mass spectrometry. A chiral column with cellulose-SB phase was used for the first time for enantiomeric separation of OME with an isocratic elution system using 0.2% ammonium hydroxide in hexane-ethanol mixture (70:30, v/v) as the mobile phase. OME enantiomers were determined utilizing a triple quadrupole tandem mass spectrometer in positive ion mode (ESI+) monitoring mass transitions: m/z 346.3 198.0 for OME and m/z 369.98 252.0 for internal standard. The limits of detection and quantification of the present method for both enantiomers were 0.1 and 0.4 ng/mL, respectively. The method validation provided good accuracy and precision. The matrix effect factor was less than 5%, and no interfering peaks were observed. The interday precision values ranged from 2.2 to 7.5 (%RSD), and the accuracy of determinations varied from -9.9% to 8.3%. In addition, the pharmacokinetics (PK) of omeprazole enantiomers in healthy subjects after a single oral dose was investigated. (S)-Enantiomers showed higher levels than (R)-enantiomers throughout 24 h. It was found that the mean maximum concentrations of (R)- and (S)-omeprazole in plasma samples were about two times higher than in oral fluid.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Omeprazole (OME) is the first proton pump inhibitor (PPI) that acts by blocking the H⁺- K⁺ATPase enzymes (proton pumps) irreversibly. OME is indicated in treatment of all acid-related diseases, where the target of treatment is to reduce gastric acid secretion, such as dyspepsia, peptic ulcer, gastric ulcer, and gastroesophageal reflux disease [1]. It is a chiral drug but used as a racemic mixture and pure enantiomer for medicinal purposes [2]. Commonly, the pharmacodynamics and pharmacokinetics (PK) of a chiral drug are potentially different between the optical isomers. Therefore, it is important to study the individual enantiomers to get clear knowledge about pharmacokinetics and pharmacodynamics of any chiral drug. In OME drug, the (S)-enantiomer (esomeprazole) showed better effect, and it was formulated as an enantio-pure drug by AstraZeneca under the commercial name of Nexium® [3–5]. Solid-phase extraction (SPE) is the mainly employed sample preparation technique for extracting OME from plasma [6–9]. In addition, liquid-liquid extraction (LLE) [10] and 96-well LLE [11] have been applied for OME measurement in biological samples. However, SPE provides some advantages, such as selectivity and efficiency, over other techniques, especially LLE [12, 13]. Moreover, achiral-chiral column-switching technique has been used for online

precolumn extraction of OME [14]. However, there is an increasing demand for miniaturized and automated sample preparation techniques which are simple, cheap, and provide acceptable drug recovery and selectivity [15].

Microextraction by packed sorbent (MEPS) is a miniaturized SPE technique, in which the volumes of sample and solvents is highly reduced. MEPS is efficient for the removal of contaminants from samples. In MEPS, the packed sorbent is integrated directly into the syringe via sampling needle which could be used several times for biological samples instead of single-use SPE cartridges [16], can be fully automated, and be used for small-volume samples [16–21].

As oral fluid is easier to handle and is less complex than the plasma matrix, it has been a promising matrix for drug analysis and pharmacokinetic studies [22–27]. Oral fluid samples can be easily collected from infants, disabled, or anxious patients [28]. In comparison with plasma, oral fluid can provide shorter sampling time, more simple and noninvasive sampling strategy. Furthermore, daily secretion of human oral fluid is too high (up to 1.5L) which enables sample collection at short intervals [29, 30]. Therefore, oral fluid can be considered as an ideal biological fluid for drug monitoring.

OME enantiomers have been separated by using chiral columns with polysaccharide-based stationary phases formed of carbamate derivatives of cellulose (Chiralpak AD column, Chiralcel OJ column, Chiralpak AS column, and Chiralcel OD column) [7, 8, 13] or amylose [14, 31] as well as protein-based chiral stationary phase (bovine serum albumin) [6].

Several studies have been conducted for investigating the pharmacokinetic parameters of OME and its enantiomers in human plasma after single oral dose [32] or repeated oral doses [33]. Oral fluid samples provide a safe solution for sample collection procedures. The unbound drug molecules, being responsible for the therapeutic activity of the administered drug, are distributed to the oral fluid [34].

The present work describes an enantioselective chromatographic method with mass spectrometric detection for the separation and quantification of OME enantiomers in plasma and oral fluid samples using MEPS for sample preparation and chiral column cellulose-SB tris(3,5dimethylphenylcarbamate) for the chiral separation. The developed method aims to provide a reliable determination of OME enantiomers in oral fluid as an alternative specimen to plasma to monitor the therapeutic concentrations of the drug. To our knowledge, the present study is the first study for the analysis of OME enantiomers in human oral fluid samples, using a cellulose-SB-based stationary phase.

2. Materials and Methods

2.1. Chemicals

OME racemate, (R)-OME, (S)-OME, and (S)-lansoprazole (l.S) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade hexane, ethanol, formic acid, and ammonium hydroxide were purchased from Merck (Darmstadt, Germany). A Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA) was used for water purification.

2.2. Preparation of Standards and Quality Control Samples

Two stock solutions ($5\text{ }\mu\text{g}\cdot\text{mL}^{-1}$, each) were prepared in ethanol (one to prepare calibration standards and one for the quality control (QC) samples), and the standards and QC samples were prepared in blank pooled human plasma (heparin) and oral fluid samples (from six different subjects). The stock solutions showed stable for at least one month at 4°C. The concentration of the standard samples in plasma was in the range of 25–600 ng/mL and in oral fluid was 25–300 ng/mL. QC samples were prepared in plasma and oral fluid in three concentration levels: low (QCL), medium (QCM), and high (QCH) as reported in Table 1.

Table 1

Accuracy and precision of QC samples in human plasma and oral fluid.

Sample matrix drug	QC (ng/mL)	Intraday ($n=6$)	Interday ($n=18$)

RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	Plasma	(R)	75
5.7	-8.8	4.9	-5.7	250	2.4	-5.9
2.2	-6.1	500	5.1	-10.3	6.6	-2.4
(S)	75	2.3	-3.5	4.4	-4.4	250
5.0	-7.0	3.2	-1.9	500	5.8	-7.1
5.6	-7.0	-				
Oral fluid	(R)	75	5.3	-5.5	5.3	-3.5
150	4.3	-3.7	4.5	8.3	250	4.6
-4.0	3.5	-5.4	(S)	75	5.6	5.0
7.5	4.8	150	4.5	-4.7	5.3	-9.9

2.3. Instrumentation

The liquid chromatography system (LC) consists of two pumps, Shimadzu LC-10ADvp, (Kyoto, Japan), an autosampler (CTC-Pal, Analytics AG, Zwingen, Switzerland), and a 50 μL sample loop. The used chromatographic column was a CHIRAL ART cellulose-SB (150 $\times$ 4.6 mm, 5 μm particle size) and was obtained from YMC Europe GmbH (Dinslaken, Germany). Different mobile phase composition utilizing ethanol and hexane with different additives (formic acid and ammonium hydroxide) was investigated. The best chromatographic conditions consisted of 0.2% ammonium hydroxide in n-hexane/ethanol (70:30, v/v). The mobile phase flow rate was at 0.8 mL/min with split of 1:1 before the MS interface. A 50 μL sample volume was injected. eVol sampling device with MEPS syringe and C8 sorbent pins were supplied by SGE Analytical Science (Melbourne, Australia).

Electrospray ionization in positive ion mode (ESI+) was performed using a triple quadrupole mass spectrometer detector (Quattro-micro, Waters, Manchester, UK). 150 and 400 $^{\circ}\text{C}$ were the MS source block and desolvation temperatures, respectively. Nitrogen gas was utilized not only for drying (950 Lh^{-1}) but also for nebulization (60 Lh^{-1}). Argon gas was utilized as collision gas (collision energy 10 eV for OME and 20 eV for lansoprazole). OME enantiomers analysis in plasma and oral fluid samples was performed by using LCMS/MS with multiple reaction monitoring (MRM) of the transitions: m/z 346.30 $>$ 198.05 for OME and m/z 369.98 $>$ 252.02 for I. S. utilizing a dwell time of 0.2 sec/transition. Peak-area ratios (OME/I. S.) were used for all calculations. Data analysis was done by using MassLynx software (version 4.1).

2.4. Sample Preparation

The QC samples in plasma and oral fluid were prepared and stored at -20°C until the analysis, while the standard calibrations were prepared freshly for each validation assay. A 100 μL of each sample was mixed with a 100 μL of the I. S. (20 $\mu\text{g mL}^{-1}$ in methanol), and then the samples were diluted with water (1:4) and centrifuged for few minutes (1–3 min). Thereafter, extraction utilizing a programmed eVol device with MEPS syringe contains C8 sorbent. The sorbent was conditioned using 100 μL ethanol followed by 100 μL of water. The sample was drawn 6 times for preconcentration of the analytes. Then, the sorbent was washed by 5% methanol in water (2 $\times$ 100 μL) two times to take away the interfering materials in the samples. A 500 μL ethanol (2 $\times$ 250 μL) was utilized for the analyte and I.S. elution. The samples were then subjected to evaporation until dryness under nitrogen, and the residues

were reconstituted with 150 μ L ethanol. The MEPS sorbent was reused after washing 3–4 times with water and 4–5 with ethanol to overcome carry over.

2.5. Method Validation

The validation of the proposed method was carried out according to FDA guidelines [35] including accuracy, precision, matrix effect, selectivity, linearity, limits of detection and quantitation, recovery, and robustness. Quality control samples at three different levels: low (QCL), medium (QCM), and high (QCH) ($n=6$) were used for the determination of precision and accuracy of the method. For intraday precision and accuracy, a single analytical batch was analyzed. On the other hand, three different batches with three triplicates were analyzed for the evaluation of the interday precision and accuracy. The accepted data for the precision with relative standard deviation (RSD) should be within $\pm 15\%$, and the accuracy with relative error (RE) from the nominal values should not exceed 15% and 20% for lower limit of quantitation (LLOQ).

The calibration curve for plasma ranged from 25 ng/mL to 600 ng/mL (25, 50, 100, 150, 200, 250, 300, and 600 ng/mL), while for oral fluid, it was in the range of 25–300 ng/mL (25, 50, 100, 150, 200, 250, and 300 ng/mL). Each back calculated standard concentration should not be more than two-third of the points to be accepted with $\%RSD \leq 15\%$ except lowest concentration ($\leq 20\%$).

The stability of OME in plasma and oral fluid was performed using triplicates of the QC solutions. Four types of stability studies were done for QC samples. First type was short-term stability by defrost the samples at room temperature for eight hours before analysis time; the second type was long-term stability via keeping the samples at -20°C for fifteen days before analysis time; the third type was three freeze-thaw stability cycles by leaving the iced samples at -20°C to melt at room temperature. After that, samples were restored at -20°C , and this procedure was repeated three times before analysis time, and finally, the last type was postpreparative stability of the processed samples by keeping them for 24 h at 4°C before analysis time. If assay results were within the acceptable limits of accuracy ($\pm 15\%$) and precision (15%), then samples could be considered to be stable.

2.6. PK Analysis

The PK parameters were calculated from both oral fluid and plasma samples using the concentration-time profile data for each enantiomer. C_{max} and T_{max} were calculated directly from the concentration-time plot of the individual enantiomer. The elimination half time ($t_{1/2}$) and the elimination rate constant (k_e) were estimated from the terminal slope of the plasma/fluid concentration-time profile based on first-order kinetics.

3. Result and Discussion

3.1. Method Development

The extraction of OME enantiomers from plasma and oral fluid samples was achieved using MEPS eVol sampling device. Different factors affecting extraction efficiency such as sorbents (C8, C18, and polystyrene), washing, and eluting solutions (methanol, ethanol, and isopropanol) were investigated. Best results were obtained with C8 as sorbent (Figure 1), water as washing solution, and ethanol as eluting solvent (Figure 2).

[figure omitted; refer to PDF]

The chromatographic separation of OME enantiomers was achieved using chiral-SB column packed with cellulose tris-(3,5-dimethylphenylcarbamate) immobilized on silica gel as the chiral selector. Hexane containing different volumes of ethanol or isopropanol was investigated as a mobile phase. Different mobile phase composition consisting of ethanol and hexane with different additives (formic acid and ammonium hydroxide) was investigated. It is known that the addition of formic acid in the mobile phase results in good ionization in ESI interface, but in our case, it did not work well and it resulted in poor peak shape and poor enantio-separation. The addition of ammonium hydroxide on the other hand generated Gaussian peak shape and good MS signal for both the OME enantiomers and internal standard. Thus, the best result was obtained using a mobile phase consisting of n-hexane/ethanol (70:30, v/v) and ammonium hydroxide (0.2% v/v). The presence of ammonium hydroxide as a basic modifier in the mobile phase improved the enantiomeric selectivity and peak symmetry (Figure 3). The mobile phase flow rate was 0.8 mL·min⁻¹, and a baseline separation between all analytes was achieved within 10 min of the run.

[figures omitted; refer to PDF]

The triple quadrupole mass spectrometry was employed for the identification of OME enantiomers. For both enantiomers the precursor and product ions were appeared at 346.30 and 198.05 m/z values, while the corresponding ions for the internal standard were detected at 369.98 and 252.02 m/z , respectively.

3.2. Method Validation

The validation of the method was run as described previously including linearity, limit of detection (LOD), lower limit of quantification (LLOQ), accuracy, precision, recovery, matrix effects, selectivity, and carry over for determination of OME in human plasma and oral fluid samples in line with FDA guidelines. The calibration curves were prepared in the range of 25–600 $\text{ng}\cdot\text{mL}^{-1}$ and 25–300 $\text{ng}\cdot\text{mL}^{-1}$ in human plasma and oral fluid samples, respectively, with LLOQ of 25 ng/mL and a quadratic equation weighted with $1/x$ was used for the calculations. The calculated limits of detection ($S/N \geq 3$) and quantitation ($S/N \geq 10$) for both enantiomers in plasma and oral fluid were 0.1 and 0.4 ng/mL , respectively. The accuracy and precision were determined by the analysis of QC samples (QC low, QC medium, and QC high) as mentioned in Table 1.

3.2.1. Calibration

The peak area ratio of each enantiomer to I. S. was plotted against the corresponding concentration for construction of calibration curves for plasma and oral fluid samples. The quality-control samples were treated in the same way as the standards. Quadratic equation was used for the standard curve due to the complexity of the plasma and oral fluid matrices. Using oral fluid and plasma, a close relationship between concentration and peak area ratio (OME/I. S.) was observed in the concentration range 25–600 ng/mL and 25–300 ng/mL in plasma and oral fluid, respectively. The correlation coefficient (R^2) values obtained for plasma and oral fluid matrices were ≥ 0.999 .

(S)-Lansoprazole was used as internal standard, and the LLOQ was set to 25 ng/mL . A good correlation between peak area ratios and analyte concentrations was obtained which demonstrated reliability of the method. Figure 4 shows LC-MS/MS chromatograms of OME in plasma and oral fluid.

[figures omitted; refer to PDF]

3.2.2. Accuracy and Precision

Table 1 reports the accuracy and precision data from the QC samples in plasma and oral fluid samples. Precision was calculated as the percentage of RSD, and the accuracy was calculated as the percent deviation of the obtained value from nominal value. The method accuracy and precision were within the accepted range, i.e., the accuracy and the precision values for plasma samples ranged from –1.9% to –10.3% and between 2.2 and 5.8%, respectively, for both intra- and interday assays. For oral fluid samples, the accuracy values varied between –9.9% and 8.3%, and the precision ranged from 4.3 to 7.5% for both intra- and interday assays.

3.2.3. Extraction Recovery

The average absolute recovery for each enantiomer in both plasma and oral fluid samples using QC samples was 95%.

3.2.4. Matrix Effect and Selectivity

Matrix effects of the tested biological fluids samples of OME enantiomers were shown to be within 95% and 103%, and thus no significant ion suppression was observed.

For the method selectivity, blank plasma and oral fluid samples from different subjects ($n=6$) were analyzed. The results showed that no interfering peak was detected at the elution positions of at the enantiomer's and I. S.

3.2.5. Stability

The stability studies were conducted analyzing each QC sample in triplicate. No significant change in the enantiomers concentration in short- and long-term being indicated by the mean recovery values was observed. Also, there was no effect on the quantification of target analyte in the three freeze-thaw cycles as well. In addition, the samples were assayed after 24 h at 4°C (postpreparative stability). The obtained data indicated that both used biological samples could be analyzed under such laboratory conditions and after storage at –20°C for up to two weeks without significant change in the drug chemistry or quantity (Table S1).

3.3. Comparison of Developed Method for OME Analysis

Several methods for extraction and analyzing of OME have been developed. In this part, ability of the developed

method was compared with other methods, and the results of this part are presented in Table 2.

Table 2

Determination of OME with different methods.

Method	Linear range (ng/mL)	Matrix (OM form)	Sample preparation method	Extraction efficiency (%)	Ref.
RP-HPLC	2–2000	Human plasma (racemate)	LLE	84	[10]
HILIC-MS/MS	2–1000	Human plasma (racemate)	LLE	87–98	[11]
UHPLC-MS/MS	10–500	Rat plasma (racemate)	Protein precipitation and LLE	87	[36]
LC-UV	50–1000	Human plasma (enantiomers)	SPE	93–94	[37]
LC-MS/MS	1.25–2500	Human plasma (enantiomers)	Protein precipitation (PP)	105–106	[38]
LC-MS/MS	25–500	Human plasma, oral fluid (enantiomers)	MEPS	94–98	Present study

3.4. Method Application to PK Study of OME in Healthy Individuals

The PK study of OME enantiomers was performed in a group of 4 healthy subjects. The patient's samples were provided by the center of clinical research, Gavle hospital, Sweden. The subjects were 3 females and one male within an age range of 32–63 years old and a weight varying from 60 to 95kg (Table 3). The study addresses the PK parameters based on a single oral administration of 20mg OME tablets (racemate) to each volunteer. Blood and saliva samples were collected from each individual throughout 24 h after administration of the drug.

Table 3

The physical data of selected volunteers.

Subject no.	Sex	Weight	Age
1	Male	80	56
2	Female	95	59
3	Female	60	63
4	Female	70	32
	Average	76.25	52.5

The OME enantiomers in human plasma and saliva samples were analyzed by the present method. The PK characteristics being expressed by its parameters, such as elimination rate constant (k_e) and half-time ($t_{1/2}$), were then estimated using the concentration-time profile data for each enantiomer. $C_{\max}$ and $T_{\max}$ were estimated directly from the concentration-time curves.

The PK parameters were evaluated for each enantiomer in the four healthy persons by calculating the mean values (Table 4). The results showed that the (S)-enantiomer has higher $C_{\max}$ and exposure as expressed as the area under the concentration-time curve (AUC) PK values compared to that found for the (R)-enantiomer ((R)-OME) by approximately 1.5 and 1.3-fold, respectively. $C_{\max}$ for both enantiomers reached 2.5h after drug administration ($T_{\max}$) (Figure S1). The elimination rate of the (R)-enantiomer was slightly higher than that of the (S)-enantiomer. The half-time ($t_{1/2}$) of the S-enantiomer was approximately 0.7h compared to that obtained for the R-enantiomer. The higher oral clearance rate (CL/f) of the (R)-enantiomer provides a proof of the stereoselective metabolism. The (Vd/f) values of both enantiomers were approximately similar indicating high tissue distribution of both forms. The (S)-enantiomer showed higher $C_{\max}$ than that of (R)-OME with similar AUC₀₋₂₄ values (Table 4). Both enantiomers showed faster $T_{\max}$ values in saliva compared to that seen in plasma, while both enantiomers attained higher AUC and $C_{\max}$ values in plasma than that in saliva.

Table 4

Main pharmacokinetic parameters of OME enantiomers in plasma and oral fluid following single oral dose of 20 mg to healthy volunteers ($n=4$).

Parameter	Plasma		Oral fluid	
(R)-OME	(S)-OME	(R)-OME	(S)-OME	$C_{\max}$ (ng·mL ⁻¹)
62.44	97.24	40.01	43.61	$T_{\max}$ (h)
2.50	2.50	2.00	2.00	k_{el} (h ⁻¹)
0.267	0.207	1.811	1.151	$t_{1/2}$ (h)
2.59	3.35	0.58	0.60	AUC ₀₋₂₄
384.86	510.80	125.99	130.66	AUC _{0-∞}
411.72	544.25	128.59	134.79	CL/f (L·kg ⁻¹ ·h ⁻¹)
0.631	0.477	1.944	1.855	VD/f (L·kg ⁻¹)

4. Conclusion

A new method based on LC-MS/MS has been developed and validated for the assay of the enantiomers of OME in human plasma and oral fluid samples. Both plasma and oral fluid samples were treated with MEPS technique before analysis. Quantitative analysis of OME enantiomers in both matrices were achieved successfully with good accuracy and precision. The method demonstrated to be an excellent method for the chiral separation of OME enantiomers as well as for the monitoring of OME in plasma. Therefore, the method was used for the PK study of the omeprazole enantiomers.

The evaluation of the PK parameters in oral fluid for omeprazole showed poor correlation to those in plasma. However, the use of oral fluid serves as a fast and easy sampling medium for screening of the drug's therapeutic levels. On the other hand, possible enzymatic actions in the oral fluid and the intersubject variations in the enzymatic

composition of oral fluid s can contribute to the variation in the drug levels that requires further investigation.

References

- [1] L. S. Goodman, L. L. Brunton, A. Gilman, B. Chabner, B. C. Knollmann, Goodman and Gilman's: The Pharmacological Basis of Therapeutics, 2011. 12th edition
- [2] W. Asghar, E. Pittman, F. Jamali, "Comparative efficacy of esomeprazole and omeprazole: racemate to single enantiomer switch," DARU Journal of Pharmaceutical Sciences, vol. 23, pp. 50-57, DOI: 10.1186/s40199-015-0133-6, 2015.
- [3] A. Rahman, M. Rashedul Haque, Z. Sultan, M. M. Rahman, M. A. Rashid, "Enantiomeric determination of omeprazole and esomeprazole by a developed and validated chiral HPLC method and stability studies by microthermal analysis," Journal of Pharmaceutical Sciences, vol. 16 no. 2, pp. 221-233, 2017.
- [4] T. Lind, L. Rydberg, A. Kylebäck, "Esomeprazole provides improved acid control vs. omeprazole in patients with symptoms of gastro-oesophageal reflux disease," Alimentary Pharmacology & Therapeutics, vol. 14 no. 7, pp. 861-867, DOI: 10.1046/j.1365-2036.2000.00813.x, 2000.
- [5] A. Choudhury, S. Das, S. Bahadur, S. Saha, A. Roy, "Formulation and evaluation of omeprazole tablets for duodenal ulcer," Indian Journal of Pharmaceutical Sciences, vol. 72 no. 4, pp. 491-494, DOI: 10.4103/0250-474x.73922, 2010.
- [6] A. M. Cairns, R. H.-Y. Chiou, J. D. Rogers, J. L. Demetriades, "Enantioselective high-performance liquid chromatographic determination of omeprazole in human plasma," Journal of Chromatography B: Biomedical Sciences and Applications, vol. 666 no. 2, pp. 323-328, DOI: 10.1016/0378-4347(94)00587-u, 1995.
- [7] H. Elmogy, M. Abdel-Rehim, "Oral fluid as an alternative specimen to plasma for drug bioanalysis: a review," TrAC Trends in Analytical Chemistry, vol. 83, pp. 70-79, DOI: 10.1016/j.trac.2016.07.010, 2016.
- [8] H. Kanazawa, A. Okada, M. Higaki, H. Yokota, F. Mashige, K. Nakahara, "Stereospecific analysis of omeprazole in human plasma as a probe for CYP2C19 phenotype," Journal of Pharmaceutical and Biomedical Analysis, vol. 30 no. 6, pp. 1817-1824, DOI: 10.1016/s0731-7085(02)00524-1, 2003.
- [9] M. Ishii, M. Sato, M. Ogawa, T. Takubo, K. i. Hara, Y. Ishii, "Simultaneous determination of omeprazole and its metabolites (5'-hydroxyomeprazole and omeprazole sulfone) in human plasma by liquid chromatography-tandem mass spectrometry," Journal of Liquid Chromatography & Related Technologies, vol. 30 no. 12, pp. 1797-1810, DOI: 10.1080/10826070701360590, 2007.
- [10] N. L. Rezk, K. C. Brown, A. D. M. Kashuba, "A simple and sensitive bioanalytical assay for simultaneous determination of omeprazole and its three major metabolites in human blood plasma using RP-HPLC after a simple liquid-liquid extraction procedure," Journal of Chromatography B, vol. 844 no. 2, pp. 314-321, DOI: 10.1016/j.jchromb.2006.07.047, 2006.
- [11] Q. Song, W. Naidong, "Analysis of omeprazole and 5-OH omeprazole in human plasma using hydrophilic interaction chromatography with tandem mass spectrometry (HILIC-MS/MS)-eliminating evaporation and reconstitution steps in 96-well liquid/liquid extraction," Journal of Chromatography B, vol. 830 no. 1, pp. 135-142, DOI: 10.1016/j.jchromb.2005.10.042, 2006.
- [12] A. Abdel-Rehim, M. Abdel-Rehim, "Dried saliva spot as a sampling technique for saliva samples," Biomedical Chromatography, vol. 28 no. 6, pp. 875-877, DOI: 10.1002/bmc.3193, 2014.
- [13] H. Stenhoff, Å. Blomqvist, P.-O. Lagerström, "Determination of the enantiomers of omeprazole in blood plasma by normal-phase liquid chromatography and detection by atmospheric pressure ionization tandem mass spectrometry," Journal of Chromatography B: Biomedical Sciences and Applications, vol. 734 no. 2, pp. 191-201, DOI: 10.1016/s0378-4347(99)00324-2, 1999.
- [14] Q. B. Cass, V. V. Lima, R. V. Oliveira, N. M. Cassiano, A. L. G. Degani, J. Pedrazzoli, "Enantiomeric determination of the plasma levels of omeprazole by direct plasma injection using high-performance liquid chromatography with achiral-chiral column-switching," Journal of Chromatography B, vol. 798 no. 2, pp. 275-281, DOI: 10.1016/j.jchromb.2003.09.053, 2003.
- [15] R. Said, M. Kamel, A. El-Beqqali, M. Abdel-Rehim, "Microextraction by packed sorbent for LC-MS/MS

- determination of drugs in whole blood samples," *Bioanalysis*, vol. 2 no. 2, pp. 197-205, DOI: 10.4155/bio.09.187, 2010.
- [16] M. Abdel-Rehim, "Recent advances in microextraction by packed sorbent for bioanalysis," *Journal of Chromatography A*, vol. 1217 no. 16, pp. 2569-2580, DOI: 10.1016/j.chroma.2009.09.053, 2010.
- [17] M. M. Moein, A. Abdel-Rehim, M. Abdel-Rehim, "Microextraction by packed sorbent (MEPS)," *TrAC Trends in Analytical Chemistry*, vol. 67, pp. 34-44, DOI: 10.1016/j.trac.2014.12.003, 2015.
- [18] M. Abdel-Rehim, "Microextraction by packed sorbent (MEPS): a tutorial," *Analytica Chimica Acta*, vol. 701 no. 2, pp. 119-128, DOI: 10.1016/j.aca.2011.05.037, 2011.
- [19] M. M. Moein, A. Abdel-Rehim, M. Abdel-Rehim, "On-line determination of sarcosine in biological fluids utilizing dummy molecularly imprinted polymers in microextraction by packed sorbent," *Journal of Separation Science*, vol. 38 no. 5, pp. 788-795, DOI: 10.1002/jssc.201401116, 2015.
- [20] Z. Altun, A. Hjelmström, M. Abdel-Rehim, L. G. Blomberg, "Surface modified polypropylene pipette tips packed with a monolithic plug of adsorbent for high-throughput sample preparation," *Journal of Separation Science*, vol. 30 no. 12, pp. 1964-1972, DOI: 10.1002/jssc.200600523, 2007.
- [21] M. Abdel-Rehim, "New trend in sample preparation: on-line microextraction in packed syringe for liquid and gas chromatography applications," *Journal of Chromatography B*, vol. 801 no. 2, pp. 317-321, DOI: 10.1016/j.jchromb.2003.11.042, 2004.
- [22] L. A. Tabak, "A revolution in biomedical assessment: the development of salivary diagnostics," *Journal of Dental Education*, vol. 65 no. 12, pp. 1335-1339, DOI: 10.1002/j.0022-0337.2001.65.12.tb03492.x, 2002.
- [23] S. Chiappin, G. Antonelli, R. Gatti, E. F. De Palo, "Saliva specimen: a new laboratory tool for diagnostic and basic investigation," *Clinica Chimica Acta*, vol. 383 no. 1-2, pp. 30-40, DOI: 10.1016/j.cca.2007.04.011, 2007.
- [24] Y.-H. Lee, D. T. Wong, "Oral fluid: an emerging biofluid for early detection of diseases," *American Journal of Dentistry*, vol. 22 no. 4, pp. 241-248, 2009.
- [25] D. Malamud, "Saliva as a diagnostic fluid," *Dental Clinics of North America*, vol. 55 no. 1, pp. 159-178, DOI: 10.1016/j.cden.2010.08.004, 2011.
- [26] N. Spielmann, D. Wong, "Saliva: diagnostics and therapeutic perspectives," *Oral Diseases*, vol. 17 no. 4, pp. 345-354, DOI: 10.1111/j.1601-0825.2010.01773.x, 2011.
- [27] L. F. Hofman, "Human saliva as a diagnostic specimen," *The Journal of Nutrition*, vol. 131 no. 5, pp. 1621s-1625 s, DOI: 10.1093/jn/131.5.1621s, 2001.
- [28] J. Liu, Y. Duan, "Saliva: a potential media for disease diagnostics and monitoring," *Oral Oncology*, vol. 48 no. 7, pp. 569-577, DOI: 10.1016/j.oraloncology.2012.01.021, 2012.
- [29] C. Streckfus, L. Bigler, "Saliva as a diagnostic fluid," *Oral Diseases*, vol. 8 no. 2, pp. 69-76, DOI: 10.1034/j.1601-0825.2002.10834.x, 2002.
- [30] G. Iorgulescu, "Oral fluid between normal and pathological. Important factors in determining systemic and oral health," *Journal of Medicine and Life*, vol. 2 no. 3, pp. 303-307, 2009.
- [31] Q. B. Cass, A. L. G. Degani, N. Cassiano, "The use of a polysaccharide-based column on multimodal elution," *Journal of Liquid Chromatography & Related Technologies*, vol. 23 no. 7, pp. 1029-1038, DOI: 10.1081/jlc-100101505, 2000.
- [32] T. Andersson, L. Weidolf, "Stereoselective disposition of proton?? pump inhibitors," *Clinical Drug Investigation*, vol. 28 no. 5, pp. 263-279, DOI: 10.2165/00044011-200828050-00001, 2008.
- [33] T. Andersson, K. Röhss, E. Bredberg, M. Hassan-Alin, "Pharmacokinetics and pharmacodynamics of esomeprazole, the S-isomer of omeprazole," *Alimentary Pharmacology & Therapeutics*, vol. 15 no. 10, pp. 1563-1569, DOI: 10.1046/j.1365-2036.2001.01087.x, 2001.
- [34] H. Inoue, K. Ono, W. Masuda, "Gender difference in unstimulated whole saliva flow rate and salivary gland sizes," *Archives of Oral Biology*, vol. 51 no. 12, pp. 1055-1060, DOI: 10.1016/j.archoralbio.2006.06.010, 2006.
- [35] Food and Drug Administration, "FDA policy statement for the development of new stereoisomeric drugs," *Chirality*, vol. 4 no. 5, pp. 338-340, 1992.

[36] Z. Wang, W. Sun, Z.-F. Lin, "A UHPLC-MS/MS method coupled with liquid-liquid extraction for the quantitation of phenacetin, omeprazole, metoprolol, midazolam and their metabolites in rat plasma and its application to the study of four CYP450 activities," Journal of Pharmaceutical and Biomedical Analysis, vol. 163, pp. 204-210, DOI: 10.1016/j.jpba.2018.10.012, 2019.

[37] R. M. Orlando, P. S. Bonato, "Simple and efficient method for enantioselective determination of omeprazole in human plasma," Journal of Chromatography B, vol. 795 no. 2, pp. 227-235, DOI: 10.1016/s1570-0232(03)00570-1, 2003.

[38] L. N. Sun, Y. Zhang, Y. Q. Yang, "Simultaneous enantioselective determination of omeprazole, rabeprazole, lansoprazole, and pantoprazole enantiomers in human plasma by chiral liquid chromatography-tandem mass spectrometry," Journal of Separation Science, vol. 43 no. 16, pp. 3183-3196, DOI: 10.1002/jssc.202000226, 2020.

DETAILS

Subject:	Mass spectrometry; Plasma; Accuracy; Acids; Ethanol; Ions; Liquid chromatography; Pharmacology; Quality control; Positive ions; Chromatography; Ammonium hydroxide; Automation; Quality standards; Quadrupoles; Pharmacodynamics; Cellulose; Ulcers; Spectroscopy; Blood plasma; Pharmacokinetics; Enantiomers; Hexanes; Sorbents; Column chromatography; Water purification
Business indexing term:	Subject: Quality control Automation Quality standards
Location:	Germany
Editor:	Eladia M Pena Mendez
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English

Document type:	Journal Article
Publication history :	
Milestone dates:	2020-08-30 (Received); 2020-12-05 (Revised); 2021-01-07 (Accepted); 2021-01-19 (Pub)
DOI:	https://doi.org/10.1155/2021/8845139
ProQuest document ID:	2484173676
Document URL:	https://www.proquest.com/scholarly-journals/determination-pharmacokinetic-s-omeprazole/docview/2484173676/se-2?accountid=211160
Copyright:	Copyright © 2021 Hytham Ahmed et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2022-08-04
Database:	Publicly Available Content Database

Document 57 of 59

Point-of-Care Diagnoses and Assays Based on Lateral Flow Test

Pohanka, Miroslav

[ProQuest document link](#)

ABSTRACT (ENGLISH)

Analytical devices for point-of-care diagnoses are highly desired and would improve quality of life when first diagnoses are made early and pathologies are recognized soon. Lateral flow tests (LFTs) are such tools that can be easily performed without specific equipment, skills, or experiences. This review is focused on the use of LFT in point-of-care diagnoses. The principle of the assay is explained, and new materials like nanoparticles for labeling, new recognition molecules for interaction with an analyte, and new additional instrumentation like signal scaling by a smartphone camera are described and discussed. Advantages of the LFT devices as well as their limitations are described and discussed here considering actual papers that are properly cited.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

There are standard laboratory methods (chromatographic, mass, immunochemical, genetical, etc.) suitable for the analysis of various compounds and substances. Although the aforementioned laboratory methods exert superior features, they are quite expensive for both purchasing and cost per one assay. Moreover, education related to the type of analysis or experiences at least are required for staff controlling the instruments.

In the bioanalytical approaches, there is a little different situation to the standard analytical methods in the central laboratories. While the dominant part of analyses is expected to be made in clinical laboratories, it is also necessary to perform some analyses outside. The term point-of-care testing has emerged in current medicine. It can be explained as a simple test suitable to be finished including data evaluation in the home conditions by a patient or by a caregiver with no education in the bioanalyses or similar disciplines. Disposable urine test strips for multiple biochemical parameters and glucose biosensors for a fast glycemia assay can be mentioned as the standard commercial devices. Research on diagnostical biosensors is ongoing, and a number of new biosensor devices suitable for point-of-care testing have been investigated [1–5]. Other types of point-of-care tests like the colorimetric one based on a digital camera are developed [6–9].

The current review is focused on lateral flow immunochromatographic assays also known as lateral flow tests (LFTs) and their use in point-of-care. The LFT has started quite a long ago, and many analytical and diagnostical methods have been developed on the platform. In recent time, further improvement was achieved due to the use of advanced materials like colored nanoparticles. The recent progress in the field of LFT is surveyed here, and the progress is analyzed and discussed. The actual literature on LFT is summarized here.

2. LFT Development as a Standard Platform

Various paper tests for measuring a wide scale of parameters like pH or thin-layer chromatography assay have been extensively researched since the beginning of modern chemistry. Chromatography as a general method is connected with the work of Russian scientist Mikhail Tsvet in the early 1900s, and thin-layer chromatography was first reported by Russian scientists Izmailov and Shreiber in 1938 [10]. Further research on immunoassays including the latex fixation test provided a simple analytical tool significantly improving and simplifying the previous methods [11, 12]. The first LFT devices were developed as an outcome of knowledge from previous methods and a series of patents applied in the 1980s. LFTs for proving pregnancy by the assay of human chorionic gonadotropin in urine were the first commercial tests working on the lateral flow principle and using specific antibodies against the hormone [13].

The original types of LFT were based on the recognition capacity of antibodies that served as a recognition part of the assay. The general principle is shown in Figure 1 and can be described as follows: the assay is performed on a sheet-shaped matrix from paper, cellulose, etc., that contains freely adsorbed antibodies labeled by color or fluorescent mark and specific against the analyte on the first end. The matrix also contains two zones with immobilized antibodies on the second end: the first zone contains antibodies specific to the analyte, and the second zone immobilized antibodies specific against free-labeled antibodies. A liquid sample is applied on the first end and the analyte presented in the sample interacting with the labeled antibodies. The complex analyte-labeled antibody and the unreacted antibodies are carried by the lateral flow on and in the hydrophilic matrix. The complex analyte-labeled antibody is captured on the first zone forming a colored spot visible by a naked eye. The unreacted labeled antibodies interact with the second zone and form a visible spot as well. The coloration caused by an analyte is frequently called the test line, while the spot by unreacted antibodies is frequently called the control line.

[figures omitted; refer to PDF]

As seen from the principle of the LFT, it is a simple method suitable for simple assay relying on a naked eye, no specific instrumentation is necessary, and even liquid sample can be measured directly without further treatment. It is typically suitable for field applications [14]. Though LFT can be called by a synonym lateral flow immunochromatographic assay and antibodies are relevant and are also the most traditional recognition part in

them, other recognition molecules fully replacing the antibodies can be also embedded. Aptamers can be exemplified [15].

Gravidity tests for semiquantitative determination of human chorionic gonadotropin in urine are well known and are mass-produced types of LFT [16]. However, other types of LFT are currently available in the market, and many of them serve for the purpose of point-of-care diagnosis. The LFT kits for the detection of lipoarabinomannan in urine as a marker of *Mycobacterium tuberculosis* and diagnosis of tuberculosis disease [17, 18], detection of antibodies responsible for allergic reactions like the antibodies causing allergic bronchopulmonary aspergillosis [19, 20], detection of allergenic substances like peanut and hazelnut in food products [21], diagnosis of coronavirus disease 2019 (COVID-19) including antigens and specific antibodies [22–25], detection of antibodies against *Brucella* sp. to diagnose brucellosis [26], peste des petits ruminants virus disease diagnosis by antigen detection in fecal and nasal swab samples [27], measuring of C reactive protein in the blood, blood plasma, and serum [28], and assay of biological warfare agents and toxins like *Bacillus anthracis*, *Escherichia coli* O157:H7, staphylococcal enterotoxin B, ricin, botulinum toxin, *Francisella tularensis*, and *Yersinia pestis* [29–32] can be mentioned as examples of commercially available devices. The appearance of a commercially available LFT is depicted in Figure 2.

[figure omitted; refer to PDF]

The standard methods have a lack in the inability to measure the exact concentration of the analyte, and the assays can be performed as a semiquantitative test only. The spots formed on the LFT matrix are typically narrow which is optimal for coloration scaling by a naked eye but not an ideal solution for colorimetry. Digital cameras including the cameras integrated into smartphones are considered as the future tool for coloration scaling in various analytical protocols [8, 33–36]. The assays can be also improved by spot design or by manufacturing more positive spots with an unequal affinity toward analyte placing in the LFT strip, so the concentration of analyte would be better estimated by a naked eye. The next evolution of LFT should be made with regard to the measuring platforms making the formerly qualitative or semiquantitative tests to be the quantitative ones.

2.1. Current Trends in LFT Construction in Point-of-Care Diagnosis

Though the original LFT devices from the 1980s are the functional ones, further improvement is desired to improve their analytical specifications and reduce costs. Comparing to the original devices from the 1980s, the currently researched and developed LFT contains typically alterations in selected recognition molecule and substance responsible for the visualization of the interaction with the analyte. Evolution of materials for matrix manufacturing, overall design resolving problems with manipulation by an unskilled worker, and improving LFT package to make it have long-term stability can be mentioned as the other areas of improvement. The evolution of LFTs is not of course related to devices for diagnosis only because the platform gained overall popularity in analytical chemistry and various applications are known for this moment.

The immunoassay-based point-of-care diagnostic tool was, for instance, described for COVID-19. The researchers investigated seroprevalence for COVID-19 using standard enzyme-linked immunosorbent assay (ELISA) and compared it with a standard LFT based on antibodies labeled by colloidal gold [37]. The LFT and ELISA mutually correlated and the authors concluded their work by a recommendation that LFT is suitable for point-of-care in the healthcare setting and COVID-19 monitoring. In another study on COVID-19, an LFT device for immunoglobulins (Ig) M and G in blood was constructed using selenium nanoparticles labeled SARS-CoV-2 nucleoproteins causing interaction with IgM and IgG antibodies [38]. The assay exerted a limit of detection of 20 ng/ml for IgM and 5 ng/ml for IgG in a 10-minute lasting assay. Other types of nanoparticles can be also used for an LFT immunoassay. For instance, LFT based on carbon nanoparticles conjugated with p48 protein was developed for the diagnosis of mycoplasma caused by *Mycoplasma bovis* [39]. The assay exerted 100% specificity and no cross-reactivity with antibodies to other bovine pathogens. A full correlation with ELISA was also reached. An LFT test using monoclonal antibodies labeled by gold nanoparticles was developed by Liu and coworkers for assay of dinitolmide in chicken tissue [40]. The researchers reported a limit of detection of 2.5 µg/kg for chicken tissue containing dinitolmide, and the assay was fully comparable to liquid chromatography and ELISA. Gold nanoparticles (gold spheres with size 30 and 100 nm or gold-silica shells with size 150 nm) and antibody-based detection were also used in the development

of an LFT for the human immunodeficiency virus [41]. The gold nanoparticles were covered by a monoclonal antibody against protein p24 of the human immunodeficiency virus, and the whole assay was made in a standard manner. Signal was recorded by thermal contrast reading using an IR camera and laser. The assay was highly sensitive as a limit of detection of 8 pg/ml of p24 was achieved.

The relevance of LFT can be largely perceived in the recent events when a fast test for the diagnosis of COVID-19 was demanded. The standard diagnosis of COVID-19 was based on the polymerase chain reaction (presence of pathogen) and ELISA (prove of antibodies), but the tests have to be performed in specialized laboratories, and they require a quite long time to be finished. LFTs were successfully introduced as an alternative to the polymerase chain reaction and ELISA, and they were proved to be suitable for routine diagnosis based on the detection of COVID-19 antigen. Though they are not a replacement of polymerase chain reaction and ELISA, they were proved to be a suitable tool to be developed in a short period and used wherever necessary.

Concurrently, the recognition capability of antibodies suitable for antigens measuring respective antigens when antibodies are assayed is frequently replaced by the use of aptamers by many assays. Extensive progress in aptamer preparation has been reached in recent years, and the aptamers typically comprise ribonucleic acid (RNA), deoxyribonucleic acid (DNA), peptides, or proteins [42–48]. Their potency for LFT development was recognized as well [49]. Aptamers found their way to the construction of LFT, and they have become a relevant recognition molecule in LFT devices' development. In a work by Tripathi and coworkers, the LFT test was constructed for the point-of-care cancer diagnosis by the measurement of marker CA125 level [50]. The researchers used aptamer linked with gold nanoparticles serving as peroxidase mimetic and optimized the assay for the detection of CA125 in human serum. The assay had a limit of detection of 3.71 U/ml and correlated with ELISA. A DNA aptamer for residual penicillin antibiotic ampicillin was prepared by Lin and coworkers [51]. The researchers used hexachlorofluorescein for oligonucleotide labeling and were able to detect ampicillin in a range of 10 to 200 ng/l and a limit of quantification of 2.71 ng/l when water sample was analyzed. DNA aptamer was also used for the detection of dopamine in urine [52]. In this study, dopamine duplex DNA aptamers were conjugated to 40 nm gold nanoparticles, and LFT was performed in a standard manner resulting in a limit of detection of 50 ng/ml. Protein osteopontin representing a new marker for cancer was detected by a biotinylated aptamer and then streptavidin modified gold nanoparticles served for the visualization purpose [53]. The assay had a limit of detection of 0.1 ng/ml with dynamic detection of osteopontin in a range from 10 to 500 ng/ml for a measuring time of 5 minutes. Aptamer-based LFT was chosen by Ali and coworkers for the early diagnosis of type-2 diabetes by assay of protein vaspin using fluorescent upconverting nanoparticles [54]. Vaspin was recognized in a range of 0.1–55 ng/ml with a limit of detection of 39 pg/ml. LFT does not have to be targeted on defined molecules only. In a work by Yu and coworkers, LFT was developed for tumor-derived exosomes for rapid diagnosis of lung cell cancer [55]. Aptamer specific to CD63 on exosome surface and gold nanoparticles giving good visualization and observability of the assay by a naked eye were used for the LFT construction. The assay was proved on exosomes isolated from human lung carcinoma cells in a dilution of 6.4×10^9 particles/ml. Quantum dots are another material that can serve for the purpose of reaction visualization and macromolecules labeling [56–59]. An LFT method based on CdTe quantum dots was, for instance, constructed by Lu and coworkers in order to detect Shiga toxin type II [60]. The authors also worked with gold nanoparticles as a material for labeling antibodies. While labeling by quantum dots provided a limit of detection of 25 ng/ml, the LFT based on CdTe quantum dots had a five times lower limit of detection: 5 ng/ml. The LFT tests can be improved by connecting with other techniques improving samples and sensitivity increased. For instance, detection of *Escherichia coli* O157:H7 was performed in two steps: the first step was based on bacterium captured by immunomagnetic nanoparticles and monoclonal antibody conjugate with beta-lactamase and gold nanoparticles; the second step contained penicillin solution application and hydrolysis by the beta-lactamase [61]. The final test containing LFT consisted of an ultrasensitive penicillin immunochromatographic test strip. The assay had a limit of detection of 137 CFU/ml. Further investigation is also focused on other types of recognition parts and labels like molecularly imprinted polymers [62] and the use of liposome enhanced signal amplification [63]. Highly fluorescent europium chelate loaded silica nanoparticles can be mentioned as another type of label [64]. Research

on matrix composition is also demanded, and it can improve analytical properties. Nitrocellulose or nitrocellulose coated with nanocolloids appears to be promising [40, 65]. An overview of the aforementioned LFTs is given in Table 1.

Table 1

Overview of LFT in point-of-care diagnosis.

Assay/diagnosis of a pathology	Type of recognition part	Type of label attached to the recognition part	Analytical specifications	Reference
COVID-19	Antibody	Colloidal gold	Full correlation with ELISA for clinical samples testing	[37]
–				
COVID-19-specific antibodies recognition	SARS-CoV-2 nucleoproteins	Selenium nanoparticles	20 ng/ml for IgM and 5 ng/ml for IgG in 10 minutes	[38]
–				
Mycoplasma	p48 protein	Carbon nanoparticles	100% specificity, no cross-reactivity, full correlation with ELISA	[39]
–				
Dinitolmide in tissue	Monoclonal antibody	Gold nanoparticles	Limit of detection of 2.5 µg/kg for chicken tissue containing dinitolmide	[40]
–				
Protein p24 of human immunodeficiency virus	Monoclonal antibody	Gold nanoparticles	Limit of detection of 8 pg/ml	[41]
–				
Diagnosis of cancer by CA125 assay	Aptamer	Gold nanoparticles	Limit of detection of 3.71 U/ml	[50]
–				
Ampicillin in water	DNA aptamer	Hexachlorofluorescein	Limit of quantification of 2.71 ng/l	[51]
–				
Dopamine in urine	DNA aptamer	Gold nanoparticles	Limit of detection of 50 ng/ml	[52]
–				

Cancer marker osteopontin	Biotinylated aptamer	Conjugate streptavidin-gold nanoparticles	Limit of detection 0.1 ng/ml, with a dynamic range of 10 to 500 ng/ml, time per assay 5 minutes	[53]
–				
Assay of vaspin as an early marker of type-2 diabetes	Aptamer	Fluorescent upconverting nanoparticles	Limit of detection for vaspin of 39 pg/ml	[54]
–				
Exosomes for rapid diagnosis of lung cell cancer	Aptamer specific to CD63 on exosomes surface	Gold nanoparticles	Detection of 6.4×10^9 exosomes/ml	[55]
–				
Shiga toxin type II	Antibodies	Gold nanoparticles and CdSe quantum dots	25 ng/ml (labeling by gold nanoparticles), 5 ng/ml (labeling by quantum dots)	[60]

Considering the current trends, it is clear that the new directions in LFT research are focused on two major areas. Firstly, new recognition elements are researched. Secondly, new types of nanoparticles are used for LFT construction. All the new materials can improve the final analytical parameters of a final LFT, but the suitability of the particular materials will depend on the type of assay and other conditions. There probably will never be an ideal recognition element or a label for any assay scenarios. Molecularly imprinted polymers and aptamers can be prospective recognition elements, but antibodies will probably remain an irreplaceable part of many commercial LFTs. Standard chemical labels like fluorescein will also remain a part of standard LFTs though nanoparticles like the gold one or quantum dots will probably represent a better alternative gaining higher popularity in the future praxis.

Considering the current options in analytical chemistry, LFT remains probably the major tool for point-of-care specific diagnosis of various pathologies besides the devices like Clark glucose biosensor and simple urine colorimetric test strips. Compared to the other tests, LFT has quite high versatility for the diagnosis of good presumptions to be used under point-of-care conditions; on the other hand, LFT has a limitation on the molecular weight of analyte because the assay is on an affinity principle. Analytes with low molecular weight are not suitable for a standard immunoassay, and a competitive format is the only possibility of how to use an immunoassay for the analysis of a small compound. However, the new types of recognition elements like aptamers bring improvement and even LFT for small molecules like dinitolmide, ampicillin, and dopamine can be seen in the examples of new research on LFT.

2.2. Instrumentation of LFT for Point-of-Care Diagnosis

LFT methods are typically intended to be either qualitative or semiquantitative, and the coloration is determined by a naked eye. If the assay is performed as a semiquantitative, the found range of value is highly inaccurate. The overall simplicity of the method and no necessity to use an analytical device, electricity, or elaborative sample manipulation are the major advantages of LFT. On the other hand, there are disadvantages as well. The scaling of coloration by a naked eye is highly subjective and also depends on ambient light conditions. The subjective perception of color may be a problem when the point-of-care diagnosis is performed by elderly or disabled people. Development of coloration readers suitable for standard LFT is a way of how to improve the assay. The reader devices are especially desired in point-of-care testing [66]. The improved LFT assays are quantitative or at least semiquantitative with acceptable accuracy of concentration range determination.

In the current time, broad attention is given to digital photography because of the good availability of cameras and their integration into smartphones. Standard cameras integrated into smartphones are able to provide at least 8-bit digital photography in a format like jpg and have information about color depth for the 8-bit photography equal to 256 variables for each channel. Better cameras giving figures in 12, 14, 16, and more bits and providing raw data from the digital sensor are also widely available in the market. Digital photography is highly suitable for colorimetry by color depth analysis and colorimetric tests performed on a thin-layer-like paper-based assays and detector strips like the pH and others can be recorded this way, and the digital photography-assisted assay is well suitable for point-of-care testing [7, 36, 67–69]. Digital photography has also its limitations making the assay inaccurate under some conditions. In the first point, the light source has to be mentioned. The light conditions are crucial when a sensor is photographed; integrated light-emitting diodes can have problems with the light temperature setting. There can be also problems with the setting of white balance and color temperature in the camera. Problems with lens quality can also play a role when a cheap camera is used for point-of-care testing. Nevertheless, the use of digital cameras in personal diagnosis is considered as the next direction of research and application into praxis [70–75].

Instrumental analysis of spots formed in LFT was introduced in several applications. In the aforementioned study by Zhan and coworkers, detection of p24 protein of the human immunodeficiency virus was made using thermal contrast reading [41]. The spot on LFT was formed by a complex of gold nanoparticle conjugate with p24 from a sample and capturing zone on the LFT pad. The spot was focused by laser with wavelength 532 or 800 nm with power adjusted in the range from 10 to 500 mW. The alighted spot was recorded, and temperature measured by an IR camera and digital data for further processing and signal scaling were recorded. Digital camera containing complementary metal-oxide-semiconductor (CMOS) chip served for the spot color recording in the study by Jahanpeyma and coworkers [76]. The researchers tested their LFT device for the hybridization of DNA, and visualization was made by the application of a biotinylated detector probe in the presence of peroxidase-streptavidin conjugate. Just the peroxidase was responsible for the chemiluminescence reaction recorded by a camera. The assay was tested for proving the 16S rRNA gene from *Escherichia coli*, and the lowest reached limit of detection was equal to 1.5 pmol/l. The use of peroxidase-catalyzed reaction in an LFT was also described in a paper by Mirasoli and coworkers [77]. They adopted their method for the detection of fumonisin in food samples, and the mycotoxin was detected in a range of 2.5–500 µg/l with a limit of detection of 2.5 µg/l for an assay lasting for 25 minutes. The detection signal was evaluated by a charge-couple device (CCD) camera. The authors stated that the peroxidase reaction generating chemiluminescence products is more suitable for quantitative LFT assay than an assay where colloidal gold is used instead of peroxidase. The digital scaling of coloration can be even made by simpler devices than cameras. Digital scanner was selected as an analytical tool in the work by Posthuma-Trumple and coworkers [78]. The authors successfully performed a standard LFT test for progesterone assay using antibodies and carbon colloid as a label and the LFT strips scanned and analyzed digitally. Spots on an LFT test can be evaluated, and coloration was determined by a smartphone camera which makes the assays more available to most people. A smartphone camera assay based on an LFT was investigated for the detection of mercury [79]. The assay comprised of the use of streptavidin-biotinylated DNA probes modified with gold nanoparticles and adsorbing mercury was proved with a limit of detection of 2.53 nmol/l. In another smartphone application, uricemia (uric acid content in the blood) was measured by a combination of an LFT where coloration was initiated Prussian blue nanoparticles as artificial nanozymes and standard smartphone for spots characterization [80]. The assay exerted a linear range of 1.5–8.5 mg/dl for uric acid. An overview of the types of instrumentation applicable for an LFT assay is shown in Table 2.

Table 2

LFT signal recording by an analytical instrument.

Type of assay	Type of instrumentation	Physical principle of the instrumental assay	Reference
LFT for p24 protein from human immunodeficiency virus, gold nanoparticles conjugated with a monoclonal antibody against p24 were used	Laser alighting specific spots, IR camera visually recording temperature	Thermal contrast reading	[41]
-			
Detection of 16S rRNA gene from <i>Escherichia coli</i> by hybridization and use of biotinylated probe and peroxidase conjugated with streptavidin, peroxidase was responsible for the chemiluminescent reaction	Digital camera with CMOS chip	Digital camera recorded chemiluminescence provided by peroxidase	[76]
-			
Detection of fumonisin, labeling of antibodies by peroxidase allows performing chemiluminescent chemical reaction that is instrumentally measured	Digital camera with CCD chip	Digital camera recorded chemiluminescence provided by peroxidase	[77]
-			
LFT immunoassay of progesterone based on labeling by carbon colloid	Digital scanner	Strips were scanned and digital data analyzed	[78]
-			
The LFT assay comprised of the use of streptavidin-biotinylated DNA probes modified with gold nanoparticles	Smartphone camera	Detected spots were photographed by a smartphone camera, and coloration was measured	[79]
-			
LFT where coloration was initiated Prussian blue nanoparticles as artificial nanozymes	Smartphone camera	Detected spots were photographed by a smartphone camera, and coloration was measured	[80]

3. Conclusions

LFT devices and kits can be found in the current market as standard devices, and new improved types are researched. Three major directions of improvement can be observed when the current research is compared with the traditional LFT devices on immunoassay principle: (1) new labels and materials including nanoparticles providing

contrast coloration, (2) new recognition molecules selectively interacting with analytes, and (3) types of instrumentation making the LFT-based assays quantitative from the originally qualitative one. All the facts make the LFT a significant tool in point-of-care diagnostic where it can be performed for multiple diagnoses or analyses of harmful substances or microorganisms. Overall simplicity and growing sensitivity allow making LFT a tool for a wide number of markers. Though the traditional analytes in an LFT assay were molecules with higher molecular weight, it is expected that the LFT will become a universal tool even for analytes with lower molecular weight and make the assay more universal. The relevance of LFT was also evident during the COVID-19 crisis when the tests COVID antibodies and antigens were urgently developed and marketed in a quite short time.

Acknowledgments

This work was supported by a long-term organization development plan "Medical Aspects of Weapons of Mass Destruction" (Faculty of Military Health Sciences, University of Defense, Czech Republic) and Technological Agency of Czech Republic (TACR) (TH03030336).

References

- [1] P. Babaie, A. Saadati, M. Hasanzadeh, "Recent progress and challenges on the bioassay of pathogenic bacteria," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 14, DOI: 10.1002/jbm.b.34723, 2020.
- [2] S. Menon, M. R. Mathew, S. Sam, K. Keerthi, K. G. Kumar, "Recent advances and challenges in electrochemical biosensors for emerging and re-emerging infectious diseases," *Journal of Electroanalytical Chemistry*, vol. 878, DOI: 10.1016/j.jelechem.2020.114596, 2020.
- [3] Q. H. Nguyen, M. I. Kim, "Nanomaterial-mediated paper-based biosensors for colorimetric pathogen detection," *TrAC Trends in Analytical Chemistry*, vol. 132, DOI: 10.1016/j.trac.2020.116038, 2020.
- [4] M. Pohanka, "The piezoelectric biosensors: principles and applications, a review," *International Journal of Electrochemical Science*, vol. 12, pp. 496-506, DOI: 10.20964/2017.01.44, 2017.
- [5] B. Senf, W.-H. Yeo, J.-H. Kim, "Recent advances in portable biosensors for biomarker detection in body fluids," *Biosensors*, vol. 10 no. 9, DOI: 10.3390/bios10090127, 2020.
- [6] S.-u. Hassan, A. Tariq, Z. Noreen, "Capillary-driven flow microfluidics combined with smartphone detection: an emerging tool for point-of-care diagnostics," *Diagnostics*, vol. 10 no. 8, DOI: 10.3390/diagnostics10080509, 2020.
- [7] D. S. Y. Ong, M. Poljak, "Smartphones as mobile microbiological laboratories," *Clinical Microbiology and Infection*, vol. 26 no. 4, pp. 421-424, DOI: 10.1016/j.cmi.2019.09.026, 2020.
- [8] M. Pohanka, "Small camera as a handheld colorimetric tool in the analytical chemistry," *Chemical Papers*, vol. 71 no. 9, pp. 1553-1561, DOI: 10.1007/s11696-017-0166-z, 2017c.
- [9] H. A. Watson, R. M. Tribe, A. H. Shennan, "The role of medical smartphone apps in clinical decision-support: a literature review," *Artificial Intelligence in Medicine*, vol. 100, DOI: 10.1016/j.artmed.2019.101707, 2019.
- [10] N. A. Izmailov, M. S. Schraiber, "Spot chromatographic adsorption analysis and its application in pharmacy," *Communication I Farmatsiya*, vol. 3, 1936.
- [11] F. Klein, M. B. Janssens, "Standardisation of serological tests for rheumatoid factor measurement," *Annals of the Rheumatic Diseases*, vol. 46 no. 9, pp. 674-680, DOI: 10.1136/ard.46.9.674, 1987.
- [12] J. M. Singer, S. C. Edberg, R. L. Markowitz, J. D. Glickman, L. Miller, R. Marchitelli, "Performance of latex-fixation kits used for serologic diagnosis of rheumatoid factor in rheumatoid arthritis sera," *American Journal of Clinical Pathology*, vol. 72 no. 4, pp. 597-603, DOI: 10.1093/ajcp/72.4.597, 1979.
- [13] A. E. Urusov, A. V. Zherdev, B. B. Dzantiev, "Towards lateral flow quantitative assays: detection approaches," *Biosensors*, vol. 9 no. 3, DOI: 10.3390/bios9030089, 2019.
- [14] B. O'Farrell, "Lateral flow technology for field-based applications-basics and advanced developments," *Topics in Companion Animal Medicine*, vol. 30 no. 4, pp. 139-147, DOI: 10.1053/j.tcam.2015.12.003, 2015.
- [15] A. Chen, S. Yang, "Replacing antibodies with aptamers in lateral flow immunoassay," *Biosensors and Bioelectronics*, vol. 71, pp. 230-242, DOI: 10.1016/j.bios.2015.04.041, 2015.

- [16] Y. Zhang, X. Liu, L. Wang, "Improvement in detection limit for lateral flow assay of biomacromolecules by test-zone pre-enrichment," *Scientific Reports*, vol. 10 no. 1, DOI: 10.1038/s41598-020-66456-1, 2020.
- [17] S. D. Lawn, "Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: a state of the art review," *BMC Infectious Diseases*, vol. 12 no. 1, pp. 1471-2334, DOI: 10.1186/1471-2334-12-103, 2012.
- [18] M. Shah, C. Hanrahan, Z. Y. Wang, "Lateral flow urine lipoarabinomannan assay for detecting active tuberculosis in HIV-positive adults," *Cochrane Database of Systematic Reviews*, vol. 10, DOI: 10.1002/14651858.CD011420.pub2, 2016.
- [19] E. S. Hunter, I. D. Page, M. D. Richardson, D. W. Denning, "Evaluation of the LDBio Aspergillus ICT lateral flow assay for serodiagnosis of allergic bronchopulmonary aspergillosis," *PLoS One*, vol. 15, DOI: 10.1371/journal.pone.0238855, 2020.
- [20] P. Schubert-Ullrich, J. Rudolf, P. Ansari, "Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview," *Analytical and Bioanalytical Chemistry*, vol. 395 no. 1, pp. 69-81, DOI: 10.1007/s00216-009-2715-y, 2009.
- [21] M. Röder, S. Vieths, T. Holzhauser, "Commercial lateral flow devices for rapid detection of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) cross-contamination in the industrial production of cookies," *Analytical and Bioanalytical Chemistry*, vol. 395 no. 1, pp. 103-109, DOI: 10.1007/s00216-009-2716-x, 2009.
- [22] F. Cui, H. S. Zhou, "Diagnostic methods and potential portable biosensors for coronavirus disease 2019," *Biosensors and Bioelectronics*, vol. 165, DOI: 10.1016/j.bios.2020.112349, 2020.
- [23] H. A. Hussein, R. Y. A. Hassan, M. Chino, F. Febbraio, "Point-of-Care diagnostics of COVID-19: from current work to future perspectives," *Sensors*, vol. 20 no. 15, DOI: 10.3390/s20154289, 2020.
- [24] Z. Lyu, M. Harada Sassa, T. Fujitani, K. H. Harada, "Serological tests for SARS-CoV-2 coronavirus by commercially available point-of-care and laboratory diagnostics in pre-COVID-19 samples in Japan," *Diseases*, vol. 8 no. 4, DOI: 10.3390/diseases8040036, 2020.
- [25] L. Weidner, S. Gäsndorfer, S. Unterweger, "Quantification of SARS-CoV-2 antibodies with eight commercially available immunoassays," *Journal of Clinical Virology*, vol. 129, DOI: 10.1016/j.jcv.2020.104540, 2020.
- [26] D. M. Pfukenyi, E. Meletis, B. Modise, "Evaluation of the sensitivity and specificity of the lateral flow assay, Rose Bengal test and the complement fixation test for the diagnosis of brucellosis in cattle using Bayesian latent class analysis," *Preventive Veterinary Medicine*, vol. 181, DOI: 10.1016/j.prevetmed.2020.105075, 2020.
- [27] S. Halecker, S. Joseph, R. Mohammed, "Comparative evaluation of different antigen detection methods for the detection of peste des petits ruminants virus," *Transboundary and Emerging Diseases*, vol. 5, DOI: 10.1111/tbed.1366, 2020.
- [28] J. C. Wei, Y. M. Gao, G. Q. Li, "A lateral flow immunochromatographic assay (LFIA) strip reader based on scheduler and 8051 IP core," *Automatika*, vol. 57 no. 4, pp. 1079-1088, DOI: 10.7305/automatika.2017.12.2009, 2016.
- [29] F. Gessler, S. Pagel-Wieder, M.-A. Avondet, H. Böhnel, "Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism," *Diagnostic Microbiology and Infectious Disease*, vol. 57 no. 3, pp. 243-249, DOI: 10.1016/j.diagmicrobio.2006.07.017, 2007.
- [30] H.-L. Hsu, C.-C. Chuang, C.-C. Liang, "Rapid and sensitive detection of *Yersinia pestis* by lateral-flow assay in simulated clinical samples," *BMC Infectious Diseases*, vol. 18 no. 1, DOI: 10.1186/s12879-018-3315-2, 2018.
- [31] M. Pohanka, "Current trends in the biosensors for biological warfare agents assay," *Materials*, vol. 12 no. 14, DOI: 10.3390/ma12142303, 2019.
- [32] I. Ziegler, P. Vollmar, M. Knüpfer, P. Braun, K. Stoecker, "Reevaluating limits of detection of 12 lateral flow immunoassays for the detection of *Yersinia pestis*, *Francisella tularensis*, and *Bacillus anthracis* spores using viable risk group-3 strains," *Journal of Applied Microbiology*, DOI: 10.1111/jam.14863, 2020.
- [33] E. Frantz, H. Li, A. J. Steckl, "Quantitative hematocrit measurement of whole blood in a point-of-care lateral flow device using a smartphone flow tracking app," *Biosensors and Bioelectronics*, vol. 163, DOI:

10.1016/j.bios.2020.112300, 2020.

[34] Y. Jung, Y. Heo, J. J. Lee, A. Deering, E. Bae, "Smartphone-based lateral flow imaging system for detection of food-borne bacteria *E. coli* O157:H7," *Journal of Microbiological Methods*, vol. 168, DOI: 10.1016/j.mimet.2019.105800, 2020.

10.1016/j.mimet.2019.105800, 2020.

[35] M. Pohanka, "Photography by cameras integrated in smartphones as a tool for analytical chemistry represented by an butyrylcholinesterase activity assay," *Sensors*, vol. 15 no. 6, pp. 13752-13762, DOI: 10.3390/s150613752, 2015.

[36] M. Pohanka, "Colorimetric hand-held sensors and biosensors with a small digital camera as signal recorder, a review," *Reviews in Analytical Chemistry*, vol. 39 no. 1, DOI: 10.1515/revac-2020-0111, 2020.

[37] S. Pickering, G. Betancor, R. P. Galão, "Comparative assessment of multiple COVID-19 serological technologies supports continued evaluation of point-of-care lateral flow assays in hospital and community healthcare settings," *PLOS Pathogens*, vol. 16 no. 9, DOI: 10.1371/journal.ppat.1008817, 2020.

[38] Z. Wang, Z. Zheng, H. Hu, "A point-of-care selenium nanoparticle-based test for the combined detection of anti-SARS-CoV-2 IgM and IgG in human serum and blood," *Lab on a Chip*, vol. 20 no. 22, DOI: 10.1039/d0lc00828a, 2020.

[39] F. Shi, Y. Zhao, Y. Sun, C. Chen, "Development and application of a colloidal carbon test strip for the detection of antibodies against *Mycoplasma bovis*," *World Journal of Microbiology and Biotechnology*, vol. 36 no. 10, DOI: 10.1007/s11274-020-02930-2, 2020.

[40] C. Liu, S. Fang, Y. Tian, "Rapid detection of *Escherichia coli* O157:H7 in milk, bread, and jelly by lac dye coloration-based bidirectional lateral flow immunoassay strip," *Journal of Food Safety*, DOI: 10.1111/jfs.12862, 2020.

[41] L. Zhan, T. Granade, Y. Liu, "Development and optimization of thermal contrast amplification lateral flow immunoassays for ultrasensitive HIV p24 protein detection," *Microsystems & Nanoengineering*, vol. 6 no. 1, DOI: 10.1038/s41378-020-0168-9, 2020.

[42] F. Farshchi, M. Hasanzadeh, "Nanomaterial based aptasensing of prostate specific antigen (PSA): recent progress and challenges in efficient diagnosis of prostate cancer using biomedicine," *Biomedicine & Pharmacotherapy*, vol. 132, DOI: 10.1016/j.biopha.2020.110878, 2020.

[43] Y. Guo, F. Yang, Y. Yao, "Novel Au-tetrahedral aptamer nanostructure for the electrochemiluminescence detection of acetamiprid," *Journal of Hazardous Materials*, vol. 401, DOI: 10.1016/j.jhazmat.2020.123794, 2021.

[44] N. Cheng, Y. Liu, O. Mukama, "A signal-enhanced and sensitive lateral flow aptasensor for the rapid detection of PDGF-BB," *RSC Advances*, vol. 10 no. 32, pp. 18601-18607, DOI: 10.1039/d0ra02662j, 2020.

[45] T. N. Navien, R. Thevendran, H. Y. Hamdani, T.-H. Tang, M. Citartan, "In silico molecular docking in DNA aptamer development," *Biochimie*, vol. 180, pp. 54-67, DOI: 10.1016/j.biochi.2020.10.005, 2021.

[46] Y. Ning, J. Hu, F. Lu, "Aptamers used for biosensors and targeted therapy," *Biomedicine & Pharmacotherapy*, vol. 132, DOI: 10.1016/j.biopha.2020.110902, 2020.

[47] F. Xue, Y. Chen, Y. Wen, "Isolation of extracellular vesicles with multivalent aptamers," *The Analyst*, vol. 146 no. 1, DOI: 10.1039/d0an01420f, 2020.

[48] T. Yang, X. Yang, X. Guo, "A novel fluorometric aptasensor based on carbon nanocomposite for sensitive detection of *Escherichia coli* O157:H7 in milk," *Journal of Dairy Science*, vol. 103 no. 9, pp. 7879-7889, DOI: 10.3168/jds.2020-18344, 2020.

[49] R. Reid, B. Chatterjee, S. J. Das, S. Ghosh, T. K. Sharma, "Application of aptamers as molecular recognition elements in lateral flow assays," *Analytical Biochemistry*, vol. 593, DOI: 10.1016/j.ab.2020.113574, 2020.

[50] P. Tripathi, A. Kumar, M. Sachan, S. Gupta, S. Nara, "Aptamer-gold nanozyme based competitive lateral flow assay for rapid detection of CA125 in human serum," *Biosensors and Bioelectronics*, vol. 165, DOI: 10.1016/j.bios.2020.112368, 2020.

[51] H. Lin, F. Fang, J. Zang, "A fluorescent sensor-assisted paper-based competitive lateral flow immunoassay for the rapid and sensitive detection of ampicillin in hospital wastewater," *Micromachines*, vol. 11 no. 4, DOI:

10.3390/mi11040431, 2020.

[52] S. Dalirirad, A. J. Steckl, "Lateral flow assay using aptamer-based sensing for on-site detection of dopamine in urine," *Analytical Biochemistry*, vol. 596, DOI: 10.1016/j.ab.2020.113637, 2020.

[53] O. Mukama, W. Wu, J. Wu, "A highly sensitive and specific lateral flow aptasensor for the detection of human osteopontin," *Talanta*, vol. 210, DOI: 10.1016/j.talanta.2019.120624, 2020.

[54] M. Ali, M. Sajid, M. A. U. Khalid, "A fluorescent lateral flow biosensor for the quantitative detection of Vaspine using upconverting nanoparticles," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 226, DOI: 10.1016/j.saa.2019.117610, 2020.

[55] Q. Yu, Q. Zhao, S. Wang, "Development of a lateral flow aptamer assay strip for facile identification of therapeutic exosomes isolated from human lung carcinoma cells," *Analytical Biochemistry*, vol. 594, DOI: 10.1016/j.ab.2020.113591, 2020.

[56] J. Cao, B. Zhu, K. Zheng, "Recent progress in NIR-II contrast agent for biological imaging," *Frontiers in Bioengineering and Biotechnology*, vol. 7, DOI: 10.3389/fbioe.2019.00487, 2020.

[57] M. Ganesan, P. Nagaraaj, "Quantum dots as nanosensors for detection of toxins: a literature review," *Analytical Methods*, vol. 12 no. 35, pp. 4254-4275, DOI: 10.1039/d0ay01293a, 2020.

[58] M. Pan, X. Xie, K. Liu, J. Yang, L. Hong, S. Wang, "Fluorescent carbon quantum dots-synthesis, functionalization and sensing application in food analysis," *Nanomaterials*, vol. 10 no. 5, DOI: 10.3390/nano10050930, 2020.

[59] M. Pohanka, "Quantum dots in the therapy: current trends and perspectives," *Mini Rev Med Chem*, vol. 20, 2017.

[60] T. Lu, K.-D. Zhu, C. Huang, "Rapid detection of Shiga toxin type II using lateral flow immunochromatography test strips of colorimetry and fluorimetry," *The Analyst*, vol. 145 no. 1, pp. 76-82, DOI: 10.1039/c9an01996k, 2020.

[61] W. Chen, S. Shan, J. Peng, "Sensitive and hook effect-free lateral flow assay integrated with cascade signal transduction system," *Sensors and Actuators B: Chemical*, vol. 321, DOI: 10.1016/j.snb.2020.128465, 2020.

[62] Y. He, S. Hong, M. Wang, "Development of fluorescent lateral flow test strips based on an electrospun molecularly imprinted membrane for detection of triazophos residues in tap water," *New Journal of Chemistry*, vol. 44 no. 15, pp. 6026-6036, DOI: 10.1039/d0nj00269k, 2020.

[63] S. Kim, H. D. Sikes, "Liposome-enhanced polymerization-based signal amplification for highly sensitive naked-eye biodetection in paper-based sensors," *ACS Applied Materials & Interfaces*, vol. 11 no. 31, pp. 28469-28477, DOI: 10.1021/acsami.9b08125, 2019.

[64] X. Xia, Y. Xu, X. Zhao, Q. Li, "Lateral flow immunoassay using europium chelate-loaded silica nanoparticles as labels," *Clinical Chemistry*, vol. 55 no. 1, pp. 179-182, DOI: 10.1373/clinchem.2008.114561, 2009.

[65] P. Manta, R. Nagraik, A. Sharma, "Optical density optimization of malaria Pan rapid diagnostic test strips for improved test zone band intensity," *Diagnostics*, vol. 10 no. 11, DOI: 10.3390/diagnostics10110880, 2020.

[66] R. Xiao, L. Lu, Z. Rong, "Portable and multiplexed lateral flow immunoassay reader based on SERS for highly sensitive point-of-care testing," *Biosensors and Bioelectronics*, vol. 168, DOI: 10.1016/j.bios.2020.112524, 2020.

[67] G. M. Fernandes, W. R. Silva, D. N. Barreto, "Novel approaches for colorimetric measurements in analytical chemistry - a review," *Analytica Chimica Acta*, vol. 1135, pp. 187-203, DOI: 10.1016/j.aca.2020.07.030, 2020.

[68] H. Chen, Z. Li, L. Zhang, P. Sawaya, J. Shi, P. Wang, "Quantitation of femtomolar-level protein biomarkers using a simple microbubbling digital assay and bright-field smartphone imaging," *Angewandte Chemie International Edition*, vol. 58 no. 39, pp. 13922-13928, DOI: 10.1002/anie.201906856, 2019.

[69] M. Pohanka, J. Zakova, I. Sedlacek, "Digital camera-based lipase biosensor for the determination of paraoxon," *Sensors and Actuators B: Chemical*, vol. 273, pp. 610-615, DOI: 10.1016/j.snb.2018.06.084, 2018.

[70] L. Harendarcikova, J. Petr, "Smartphones & microfluidics: marriage for the future," *Electrophoresis*, vol. 39, pp. 1319-1328, DOI: 10.1002/elps.201700389, 2018.

[71] I. Nishidate, M. Minakawa, D. McDuff, "Simple and affordable imaging of multiple physiological parameters with RGB camera-based diffuse reflectance spectroscopy," *Biomedical Optics Express*, vol. 11 no. 2, pp. 1073-1091,

DOI: 10.1364/boe.382270, 2020.

[72] D. Quesada-González, A. Merkoçi, "Mobile phone-based biosensing: an emerging "diagnostic and communication" technology," *Biosensors and Bioelectronics*, vol. 92, pp. 549-562, DOI: 10.1016/j.bios.2016.10.062, 2017.

[73] T. Sergeyeva, D. Yarynka, L. Dubey, "Sensor based on molecularly imprinted polymer membranes and smartphone for detection of Fusarium contamination in cereals," *Sensors*, vol. 20 no. 15, DOI: 10.3390/s20154304, 2020.

[74] K. Tomimuro, K. Tenda, Y. Ni, Y. Hiruta, M. Merckx, D. Citterio, "Thread-based bioluminescent sensor for detecting multiple antibodies in a single drop of whole blood," *ACS Sensors*, vol. 5 no. 6, pp. 1786-1794, DOI: 10.1021/acssensors.0c00564, 2020.

[75] A. K. Yetisen, M. S. Akram, C. R. Lowe, "Paper-based microfluidic point-of-care diagnostic devices," *Lab on a Chip*, vol. 13 no. 12, pp. 2210-2251, DOI: 10.1039/c3lc50169h, 2013.

[76] F. Jahanpeyma, M. Forouzandeh, M. J. Rasaei, N. Shoaie, "An enzymatic paper-based biosensor for ultrasensitive detection of DNA," *Frontiers in Bioscience (Scholar Edition)*, vol. 11, pp. 122-135, 2019.

[77] M. Mirasoli, A. Buragina, L. S. Dolci, "Chemiluminescence-based biosensor for fumonisins quantitative detection in maize samples," *Biosensors and Bioelectronics*, vol. 32 no. 1, pp. 283-287, DOI: 10.1016/j.bios.2011.11.039, 2012.

[78] G. A. Posthuma-Trumpie, J. Korf, A. van Amerongen, "Development of a competitive lateral flow immunoassay for progesterone: influence of coating conjugates and buffer components," *Analytical and Bioanalytical Chemistry*, vol. 392 no. 6, pp. 1215-1223, DOI: 10.1007/s00216-008-2362-8, 2008.

[79] Z. Guo, Y. Kang, S. Liang, J. Zhang, "Detection of Hg(II) in adsorption experiment by a lateral flow biosensor based on streptavidin-biotinylated DNA probes modified gold nanoparticles and smartphone reader," *Environmental Pollution*, vol. 266, DOI: 10.1016/j.envpol.2020.115389, 2020.

[80] N.-S. Li, Y.-T. Chen, Y.-P. Hsu, "Mobile healthcare system based on the combination of a lateral flow pad and smartphone for rapid detection of uric acid in whole blood," *Biosensors and Bioelectronics*, vol. 164, DOI: 10.1016/j.bios.2020.112309, 2020.

DETAILS

Subject:	Laboratories; Nanoparticles; Coronaviruses; Point of care testing; COVID-19; Medical research
Editor:	Adil Denizli
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York

Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-11-26 (Received); 2021-01-05 (Revised); 2021-01-11 (Accepted); 2021-01-20 (Pub)
DOI:	https://doi.org/10.1155/2021/6685619
ProQuest document ID:	2484134040
Document URL:	https://www.proquest.com/scholarly-journals/point-care-diagnoses-assays-based-on-lateral-flow/docview/2484134040/se-2?accountid=211160
Copyright:	Copyright © 2021 Miroslav Pohanka. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2021-02-01
Database:	Publicly Available Content Database

Document 58 of 59

Computer-Aided Rapid Establishment of Fingerprint of Xiaojin Capsule by HPLC

Jiang, Hui ¹

; Xiao, Yuansheng ² ; Xue, Xingya ² ; Jin, Hongli ² ; Yang, Xiang ³ ; Liu, Yanfang ² ; Jin, Gaowa ²

¹ Key Lab of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China; University of Chinese Academy of Sciences, Beijing 100049, China ² Key Lab of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China ³ Jianmin Pharmaceutical Group Co., Ltd., Wuhan, Hubei 430000, China

ABSTRACT (ENGLISH)

Traditional Chinese medicine (TCM) formulas have a significant clinical efficacy, and the fingerprint technology has been widely accepted to fully reveal the quality of TCM. Whereas, it is a great challenge to establish the fingerprint chromatogram which can fully reflect every single herb material in a short time. In this study, we used Xiaojin capsule (XJC) as a case and developed a rapid fingerprint method based on increasing the column temperature and flow rate simultaneously combined with computer-aided. First, the elution gradient was optimized based on the retention parameters and peak shape parameters of the four linear gradients, and then, the column temperature and flow rate were increased simultaneously to shorten the analysis time. Next, the standard fingerprint chromatogram of XJC, which can reflect every herb material, was generated. Finally, quality markers were screened through unsupervised cluster analysis and supervised orthogonal partial least squares discrimination analysis. Combining computer-aided with increasing column temperature and flow rate simultaneously can develop the rapid method for establishing HPLC fingerprint of XJC, which can fully reflect every single herb material and provide comprehensive quality control. The strategy for establishing HPLC fingerprint of TCM formula could be applied to other traditional Chinese medicine formulas and herbal medicine.

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Traditional Chinese medicines (TCMs) have been used around the world for centuries in the prevention and treatment of human diseases. However, due to the complex material basis and the complex structure type of compounds of the TCMs, the uncontrollable quality is a bottleneck for its modernization and globalization all the time [1]. TCM formulas, containing two or more herbs, have a very significant clinic effect [2]. However, it is a great challenge to control the quality of TCM formulas, particularly those who contain ten or more herb materials. The fingerprint, a spectrum, or chromatogram of the overall chemical composition can qualitatively and quantitatively reveal the changes in the pharmacodynamics of TCM. It can be obtained by modern separation and analysis methods such as chromatography or spectroscopy. Nowadays, fingerprint technology has been widely accepted to fully reveal the quality characteristics of TCMs all over the world, including the World Health Organization (WHO), the Chinese State Food and Drug Administration (SFDA), the Food and Drug Administration (FDA), and the European Medical Organization (EMA). Many methods have been proposed, including HPTLC, HPLC, GC, and CE for establishing characteristic fingerprints to identify TCM formula [3–5]. Among them, high-performance liquid chromatography (HPLC) has been widely used for establishing fingerprints of TCM owing to its high separation efficiency, good reproducibility, high precision, and easy operation. It has been reported that the chromatographic fingerprint can provide a comprehensive evaluation for TCM formulas [6, 7].

The complex components in TCM formula have a wide range of retention time and different retention behaviors in HPLC, so that it is a difficult task to establish the fingerprint of TCM formula in a short time. Several studies have established the HPLC fingerprints of TCM formula with a particularly long analysis time [8–10]. Longer analysis time means more solvent consumption and lower analysis efficiency. Gradient elution has been widely used in the analysis of TCM formula to improve separation efficiency [11]. Trial-and-error optimizations are frequently used to find out the appropriate gradients, and they result in a long experimental cycle and low efficiency to establish the analytical method in yet another way. Optimization of the gradient elution conditions coupled with computer-aided allows for satisfactory separation without unnecessary experimentation. Optimization time will be saved, and optimization quality will be improved. To simplify and accelerate the optimization process, several computer simulation software packages, such as DryLab, Preopt-W, Osiris, Chrom, Sword, and PIOTR, have been widely

used in reversed-phase high-performance liquid chromatography (RP-HPLC) [12–19]. Our laboratory developed a complex sample analysis software system (CSASS) to resolve the separation of complex samples [11]. The retention parameters and peak shape parameters can be calculated based on 3–5 linear gradient mobile phase conditions. Then, the simulated chromatogram under other gradient conditions can be exported, and the moved overlapping separation range mapping (OSRM) was drawn, so that the satisfactory separation condition of target peaks can be selected [20]. However, most reports referred to CSASS focus on the separation and analysis of herb materials or target compounds, such as Li et al. proposed a HPLC-DAD-ESI-MS/MS method for the analysis of iridoid glycosides (IGs) from *Hedyotis diffusa* Willd. through CSASS [21] and few studies referred to establishing fingerprints of TCM formula via CSASS.

Xiaojin capsule (XJC), a TCM formula originating from Xiaojin Dan, which was first recorded in Waike Zhengzhi Quansheng Ji by Hongxu Wang in the Qing Dynasty, has been recorded by China pharmacopoeia (version 2015). It is composed of artificial *Moschus* (Shexiang, SX), *Faeces Troglodyteris* (Wulingzhi, WLZ), *Pheretima* (Dilong, DL), *Momordicae Semen* (Mubiezi, MBZ), *Aconiti Kusnezoffii Radix* Cocta (Caowu, CW), *Angelicae Sinensis Radix* (Danggui, DG), *Liquidambaris Resina* (Feng Xiang Zhi, FXZ), *Olibanum* (Ru Xiang, RX), *Myrrha* (Mo Yao, MY), and fragrant ink (Xiangmo, XM). The sources of these herb materials are complicated, including botanic, resinous, mineral, and creatural medicine. Xiaojin capsule and Xiaojin tablet are modified dosage forms of Xiaojin pill, and the following are collectively referred to as XJC. The three dosage forms were extensively used to treat multiple diseases, such as anti-inflammatory [22], hyperplasia of mammary gland [23], and prostate disease [24]. To develop a method for establishing HPLC fingerprints of TCM formula rapidly, we proposed to shorten analysis time through increasing the column temperature and flow rate simultaneously combined with computer-aided. Taking XJC as a case, the complex sample analysis software system (CSASS) was used to optimize the elution gradient. Then, the developed method was applied to analyze several batches of XJC samples and establish the standard fingerprint chromatogram. As a result, the standard fingerprint chromatogram can reflect every herb material in XJC and the differences of XJC samples from different manufacturers.

2. Experimental

2.1. Crude Drugs, Reference Standards, and Reagents

HPLC grade methanol was obtained from Meryer (Shanghai) Chemical Technology Co., Ltd., HPLC grade acetonitrile was purchased from Merck, the water was purified using a Milli-Q water purification system (Bedford, MA, USA), and formic acid and phosphoric acid were purchased from J and K Scientific Ltd. (Beijing, China). A total of 36 batches of XJC samples were obtained from seven manufacturers (named A, B, C, D, E, F, and G), and their related messages are given in Table 1. The 10 herb materials were provided and authenticated by the manufacturer A.

Table 1

Source and serial number of each passel sample.

Serial no.	Item no.	Batch no.	Serial no.	Item no.	Batch no.
S1	A1	181173	S19	C3	1706002Z
S2	A2	181174	S20	D1	170401
S3	A3	181175	S21	D2	170602
S4	A4	181176	S22	E1	17010002
S5	A5	181177	S23	E2	17080011

S6	A6	181178	S24	E3	17110013
S7	A7	181179	S25	E4	17120015
S8	A8	181280	S26	E5	17120016
S9	A9	181281	S27	F1	170804
S10	A10	181282	S28	F2	171002
S11	A11	181283	S29	F3	171004
S12	A12	181284	S30	F4	171005
S13	A13	181285	S31	F5	171104
S14	A14	181286	S32	F6	171204
S16	A15	181287	S33	F7	171206
S16	B1	171106	S34	F8	171208
S17	C1	1703002Z	S35	G1	17040016
S18	C2	1703003Z	S36	G2	17040018

2.2. Apparatus and Chromatographic Conditions

HPLC analysis was carried out by a Waters Alliance HPLC instrument (Waters Corporation, MA, USA), including the HPLC quaternary pump, an autosampler, a column oven, a binary solvent manager, and a PDA detector, connected to a Waters Empower 3 software. Separation was performed on a Waters-ACCHROM Tnature C18 column (250 mm × 4.6 mm i.d., 5 μm, Waters, USA), and the column temperature was set at 40°C. On the basis of the eluent system with optimized ionization efficiency, the linear gradient consisted of A (acetonitrile) and B (0.2% (v/v) phosphoric acid in aqueous phase) with a flow rate of 1.5 mL·min⁻¹. Four linear gradient program conditions were investigated in (i)–(iv) to obtain the optimized separation with the CSASS software. The optimized elution gradient was 0–40 min, 12%–98% A and 40–55 min, 98%–98% A, and the injection volume was 10 μL.

(i) 0–120 min, 6%–98% A and 120–145 min, 98% A

(ii) 0–90 min, 8%–98% A and 90–115 min, 98% A

(iii) 0–60 min, 10%–98% A and 60–85 min, 98% A

(iv) 0–30 min, 14%–98% A and 30–55 min, 98% A

2.3. Preparation of XJC Sample

In order to extract the effective components from XJC, the pretreatment of samples is necessary. In several of the studies, XJC samples were extracted by ultrasonic extraction with methanol as extraction solvent. Their research results show that this extraction method was effective [22, 25]. Therefore, in our research, the powders of XJC samples and ten herb materials were precisely weighed (2.0 g) and then ultrasonically extracted with 25 mL of methanol for 35 minutes below 30°C. After the extracting solution was agitated and cooled down, it was filtered through a 0.22 μm microfiltration membrane. Then, the test solutions were obtained.

2.4. Methodology

2.4.1. Measurement of Determination of Dead Time and Gradient Delay in the System

Dead time was measured with uracil on the Tnature C18 column. The mobile phase was isocratic elution with a composition of 50% methanol and 50% 0.1% (v/v) formic acid in aqueous phase. The absorbance wavelength was 254 nm.

Using A (water) and B (0.1% (v/v) acetone in aqueous phase) as mobile phase, a specific step gradient was designed to measure the gradient delay in the system. The C18 column was replaced by a capillary tube (25 cm, 0.001 in i.d), and the absorption spectrum at 265 nm was recorded. The elution gradient was 0–5 min, 95%–95% A; 5–25 min, 95%–5% A; and 25–30 min, 5%–5% A.

2.4.2. Optimization of Elution Gradient by CSASS

The XJC sample was analyzed according to the four linear gradients in Section 2.2, and the absorption spectrum at 240 nm was recorded. The dead time, delay time, and four linear gradient analysis results were input into the CSASS software to optimize the elution gradient. The optimization process is shown in Figure 1.

[figure omitted; refer to PDF]

2.4.3. Precision, Repeatability, and Stability

The precision test was determined by the successive analyses of six injections of the same sample solution, respectively. Repeatability was determined by analyzing eight independent sample solutions extracted from the same batch of XJC samples. The stability was estimated by testing the same batch of sample solution stored at the same temperature for 0, 2, 4, 6, 8, 12, 24, 36, and 48 h, respectively. The XJC samples were analyzed, and the chromatograms were recorded.

2.4.4. Generation of the Standard Fingerprint Chromatogram

The professional software Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version 2004A) was used to generate the standard fingerprint chromatogram based on thirty-six batches of XJC samples. The similarities between different batches of XJC samples and the standard fingerprint chromatogram were evaluated.

2.4.5. Identification of the Origin of Common Peaks

The XJC samples and ten herb materials solutions were analyzed by the method described in Section 2.3. The origin of common peaks could be identified based on the chromatogram and spectral absorption results acquired at 240 nm.

2.5. Data Analysis

Taking the common peak area in the standard fingerprint chromatogram as variables, the cluster analysis was performed based on the relative peak areas of those common characteristic peaks calculated by IBM SPSS Statistics 23. In this study, SIMCA-14.1 (Umetrics, Umea, Sweden) was used to carry out OPLS-DA for the standardized concentrations of all common peaks. Finally, the quality markers causing the differences between the XJC samples of different groups could be screened.

3. Results and Discussion

3.1. Optimization of Column Temperature and Mobile Phase

The effect of the column temperature (25, 30, 35, and 40°C) on the chromatographic peak separation was investigated, and the HPLC chromatograms with different column temperature were very similar, and 25°C or 30°C will be selected in general. It was worth mentioning that according to the van der Waals equation, the optimum flow rate of the column with an inner diameter of 4.6 mm is $1.0 \text{ mL} \cdot \text{min}^{-1}$ with room temperature as column temperature [26–29]. In this research, the analysis time was up to 90 minutes when the flow rate was $1.0 \text{ mL} \cdot \text{min}^{-1}$, and the result is shown in Figure 2(a). To shorten the analysis time, considering the higher column temperature can enhance the velocity of convection mass transfer, so that the decline in column efficiency of column could be reduced; we propose to increase the flow rate from $1.0 \text{ mL} \cdot \text{min}^{-1}$ to $1.5 \text{ mL} \cdot \text{min}^{-1}$, and 40°C was used as column temperature. The results under the higher flow rate are shown in Figure 2(b). In terms of peak shape and retention time of chromatographic peaks, the HPLC chromatograms under different column temperature and flow rate were

consistent. The analysis time was reduced from 90min to 60min by increasing flow rate and column temperature. These results indicated that the method improves the separation analysis efficiency significantly.

[figures omitted; refer to PDF]

Different mobile phase compositions were investigated, including acetonitrile-water, acetonitrile and 0.1% (v/v) phosphoric acid aqueous solution, and acetonitrile and 0.2% (v/v) phosphoric acid aqueous solution. The results demonstrated that the acetonitrile and 0.2% (v/v) phosphoric acid aqueous solution exhibited a satisfactory resolution, moderate retention time, and smooth baseline, which was adopted as the mobile phase in this study.

3.2. Optimization of Elution Gradient

It is possible to predict retention behavior and optimize chromatographic separation [11] with CSASS. Based on the retention parameters and peak shape parameters obtained under four linear gradients, CSASS could give a simulated chromatogram for any elution conditions. The dead time (1.485 minutes), delay time (0.542 minutes), and retention parameters and peak shape parameters (Figure S1 and Tables S1) under four linear gradients were input into the CSASS software to predict the appropriate separation conditions, and then, the simulation conditions were experimentally performed and verified. The predicted optimal elution gradient was 0–40 min, 12%–98% A and 40–55 min, 98%–98% A; the simulated chromatogram is shown in Figure 3(a), and the experimental verification chromatogram is shown in Figure 3(b). Both the main and trace components in validated chromatogram have the same retention behavior with simulated chromatogram. Thus, the elution gradient was selected for analysis of the XJC samples.

[figures omitted; refer to PDF]

3.3. Validation of the HPLC Fingerprint Method

The developed HPLC analytical approach was validated by estimating the precision, repeatability, and stability. The validation of precision, repeatability, and stability was performed on the relative standard deviation (RSD, %) of relative retention times (RRTs) and relative peak areas (RPAs) of the common peaks, and the peak with RT=30.79 min was selected as the reference peak. The results for the precision, repeatability, and stability tests are shown in Table S3, respectively. In the precision test, the RSD values of the RRTs and RPAs were less than 0.20% and 4.14%. In the repeatability test, the RSD values of the RRTs and RPAs were less than 0.09% and 5.98%. Moreover, in the stability test, the RSD values of RRTs and RPAs were less than 0.21% and 6.30%. These results showed that the developed method is precise, accurate, and stable, and it is suitable to identify the fingerprint and twenty-five common peaks.

3.4. Generation of the Standard Fingerprint Chromatogram and Similarity Calculation

The HPLC fingerprint profiles of thirty-six batches of XJC samples acquired from seven manufacturers were obtained (Figure S2). According to the similarity calculation results of samples from the same manufacturer, the samples from A had a higher stability and consistency than other manufacturers. Therefore, XJC samples from A were fitted to generate the standard fingerprint chromatogram. The standard fingerprint chromatogram generated by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version 2004A). And then, the similarities between different batches of XJC samples' fingerprints and the standard fingerprint chromatogram were calculated (Table S2). The result showed that the similarities of XJC samples from both different and same manufacturers had a big difference. Therefore, it is necessary to control the source and quality of single herb material and the process to maintain the stability and consistency of XJC.

3.5. Identification of the Origin of Common Peaks

In this research, twenty-five common peaks were assigned based on the comparison of their retention time and absorption spectra with the herb material solutions (Figures S3 and S4). The standard fingerprint chromatogram contained twenty-five common peaks as shown in Figure 4. Peaks 4 was the characteristic peak from WLZ, and the extracts of WLZ can inhibit the proliferation of lymphocyte U937 in vitro [30]. Peaks 7, 11, 13, 14, 17, 20, 21, 22, 23, 24, and 25 were the characteristic peaks from FXZ, and FXZ could activate blood and relieve pain in traditional Chinese medicine theory. Peak 9 was the characteristic peak from SX, which had antithrombin activity [31]; moreover, SX for tumor growth in nude mice has a certain antitumor effect [32]. Peak 10 was the characteristic peak

from RX. According to modern pharmacology research, RX extracts possess anti-inflammatory [33], antiasthma [34], antioxidant bioactivities [35], and antiulcer [36]. Peak 12 was a characteristic peak from MY. Prior to the discovery of morphine, MY was a commonly used analgesic, which had many activities such as anti-inflammatory [37], antibacterial [38], anthelmintic [39], and anticancer [40]. Peaks 1, 2, 3, 5, 6, 8, 15, 16, 18, and 19 were from two or more herb materials. Finally, the origins of twenty-five common peaks were confirmed, and ten herb materials were all reflected in the standard fingerprint chromatography.

[figure omitted; refer to PDF]

3.6. Chemical Pattern Recognition

3.6.1. Clustering Analysis (CA)

The cluster analysis showed that thirty-six batches of XJC were sorted into two clusters by selecting 15 as distance degree (Figure 5). The samples in cluster I were from manufactures A, B, C, D, E, and G, and the cluster II come from F. It indicated that the samples from F had a difference with the other manufactures. The differences may be caused by both the source of the herb materials and the processing of XJC samples.

[figure omitted; refer to PDF]

3.6.2. Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA)

Orthogonal partial least squares discrimination analysis (OPLS-DA) was carried out based on the CA, and the results are shown in Figure 6(a). The model principal component regression coefficients were $R^2_x=0.911$, $R^2_y=0.983$, and $Q^2=0.881>0.5$. In the prediction model, Q^2 was greater than 0.5. This value indicated that the OPLS-DA prediction model was effective, and two groups of XJC samples were distinguished well. The results are consistent with the CA. Both the CA result and the OPLS-DA result indicated that the samples from F had a big difference with other manufacturers.

[figures omitted; refer to PDF]

The VIP values of variables in the OPLS-DA model represent the roles they played in classification, and the bigger the value means more important. Thus, the VIP values of twenty-five common peaks were extracted and arranged from large to small in the OPLS-DA model (Figure 6(b)). The VIP values of the peaks 9, 8, 15, 18, 3, 19, 20, 13, 12, and 4 were greater than 1; it means that these common peaks played more important roles in distinguishing the two groups of XJC samples. Among them, peak 9 originated from SX, peak 8 originated from FXZ and XM, peak 15 originated from DG and FXZ, peak 18 originated from MY, DL, and RX, peak 3 originated from WLZ, SX, and MBZ, peak 19 originated from FXZ and RX, peak 20 and peak 13 originated from FXZ, peak 12 originated from MY, and peak 4 originated from WLZ. Overall, the above 10 peaks originated from nine herb materials making up XJC except CW. The content of diester-type alkaloids from CW was controlled strictly in the Chinese pharmacopoeia of the 2015 edition. Above all, the 10 peaks with VIP value >1 could be selected as the markers to evaluate the quality consistency of XJC.

4. Conclusions

In this study, we developed a fast method for the establishment of the HPLC fingerprint of TCM formula by increasing the flow rate and column temperature simultaneously combined with computer-aided. Taking XJC as an example, the established method can complete the chromatographic analysis of XJC in 55 minutes. After that, the fast-developing HPLC method was used to analyze 36 batches of XJC samples, and the standard fingerprint chromatogram was generated. In the standard fingerprint chromatogram, there were twenty-five common peaks derived from the ten herb materials. What is more, thirty-six batches of XJC samples were divided into two groups, and a total of ten quality markers were screened out through CA and OPLS-DA. Combining simultaneous increase of column temperature and flow rate with computer-aided can quickly establish HPLC fingerprints of TCM formula, and it can improve the efficiency of separation and analysis significantly. At the same time, the established fingerprints can provide comprehensive quality control for TCM formula. The proposed method for establishing HPLC fingerprint could be applied to other traditional Chinese medicines and herbal medicine.

Acknowledgments

This work was supported by “National Science and Technology Major Project (2018ZX09735-002),” LiaoNing

Revitalization Talents Program (XLYC1908035), and Guangxi Innovation-Driven Development Project (GuiKeAA18242040).

Glossary

Abbreviations

TCM:Traditional Chinese medicine

XJC:Xiaojin capsule

CSASS:Complex sample analysis software system

CA:Cluster analysis

OPLS-DA:Orthogonal partial least squares discrimination analysis

OSRM:Overlapping separation ranging mapping

SX:Moschus (She xiang)

WLZ:Faeces Troglodyter (Wu ling zhi)

DL:Pheretima (Di long)

MBZ:Momordicae Semen (Mu bie zi)

CW:Aconiti Kusnezoffii Radix Cocta (Cao wu)

DG:Angelicae Sinensis Radix (Dang gui)

FXZ:Liquidambaris Resina (Feng Xiang Zhi)

RX:Olibanum (Ru Xiang)

MY:Myrrha (Mo Yao)

XM:Fragrant ink (Xiang mo).

References

- [1] Y. Jiang, B. David, P. Tu, Y. Barbin, "Recent analytical approaches in quality control of traditional Chinese medicines-A review," *Analytica Chimica Acta*, vol. 657 no. 1, DOI: 10.1016/j.aca.2009.10.024, 2010.
- [2] Q. Liu, J. Wen, Z. Peng, F. Liu, X Tong, "Review of the powder and decoction formulae in Traditional Chinese Medicine based on pharmacologically active substances and clinical evidence," *Journal of Traditional Chinese Medicine = Chung I Tsa Chih Ying Wen pan*, vol. 35 no. 3, pp. 355-360, DOI: 10.1016/s0254-6272(15)30110-2, 2015.
- [3] H. Zhang, M. Meng, L. Chen, "Fingerprint analysis and the application of high-performance thin-layer chromatography for the quality assessment of QiYi capsules with the reference of *Tripterygium wilfordii*," *Journal of Planar Chromatography - Modern TLC*, vol. 28 no. 3, pp. 218-222, DOI: 10.1556/1006.2015.28.3.4, 2015.
- [4] Y. Zhang, C. Wang, F. Yang, Z. Yang, F. Wang, G. Sun, "UHPLC-ESI-Q-TOF-MS/MS analysis, antioxidant activity combined fingerprints for quality consistency evaluation of compound liquorice tablets," *Rsc Advances*, vol. 8 no. 49, pp. 27661-27673, DOI: 10.1039/c8ra02431f, 2018.
- [5] J. Xing, H.-M. Sun, Z.-Y. Li, X.-M. Qin, "Comparison of volatile components between raw and vinegar baked radix bupleuri by GC-MS based metabolic fingerprinting approach," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, DOI: 10.1155/2015/653791, 2015.
- [6] J. Zhang, G. Sun, "Assessment of quality consistency in traditional Chinese medicine using multi-wavelength fusion profiling by integrated quantitative fingerprint method: niuhuang Jiedu pill as an example," *Journal of Separation Science*, vol. 42 no. 2, pp. 509-521, DOI: 10.1002/jssc.201800988, 2019.
- [7] C. M. Luo, F. L. Yi, Y. L. Xia, Z. F. Huang, "Comprehensive quality evaluation of the lateral root of *Aconitum carmichaelii* Debx. (Fuzi): simultaneous determination of nine alkaloids and chemical fingerprinting coupled with chemometric analysis," *Journal of Separation Science*, vol. 42 no. 5, pp. 980-990, 2019.
- [8] J. Liu, S. Y. Dai, L. M. Ye, C. M. Fu, "Study on the chemical profiling of fangfeng tongsheng pills by HPLC," *Indian Journal of Pharmaceutical Sciences*, vol. 78 no. 3, pp. 344-U142, DOI: 10.4172/pharmaceutical-sciences.1000124, 2016.
- [9] S. Chen, G. Sun, D. Ma, L. Yang, J. Zhang, "Quantitative fingerprinting based on the limited-ratio quantified fingerprint method for an overall quality consistency assessment and antioxidant activity determination of Lianqiao

- Baidu pills using HPLC with a diode array detector combined with chemomet," *Journal of Separation Science*, vol. 41 no. 2, pp. 548-559, DOI: 10.1002/jssc.201700566, 2018.
- [10] K. Wang, H. Zeng, Y. Zhang, "A hierarchical screening method for detection of illegal adulterants in Fur seal ginseng pills by profiling analysis of HPLC multi-dimensional fingerprints," *Journal of Separation Science*, vol. 42 no. 8, pp. 1509-1519, DOI: 10.1002/jssc.201900003, 2019.
- [11] Y. Jin, X.-y. Xue, Y.-f. Liu, Y.-s. Xiao, "A novel method of prediction and optimization for preparative high-performance liquid chromatography separation," *Journal of Chromatography A*, vol. 1183 no. 1-2, pp. 76-86, DOI: 10.1016/j.chroma.2008.01.008, 2008.
- [12] N. Lundell, "Implementation and use of gradient predictions for optimization of reversed-phase liquid chromatography of peptides," *Journal of Chromatography A*, vol. 639 no. 2, pp. 97-115, DOI: 10.1016/0021-9673(93)80245-4, 1993.
- [13] P. Jandera, "Simultaneous optimisation of gradient time, gradient shape and initial composition of the mobile phase in the high-performance liquid chromatography of homologous and oligomeric series," *Journal of Chromatography A*, vol. 845 no. 1-2, pp. 133-144, DOI: 10.1016/s0021-9673(99)00331-3, 1999.
- [14] R. G. Wolcott, J. W. Dolan, L. R. Snyder, "Computer simulation for the convenient optimization of isocratic reversed-phase liquid chromatographic separations by varying temperature and mobile phase strength," *Journal of Chromatography A*, vol. 869 no. 1-2, DOI: 10.1016/s0021-9673(99)00876-6, 2000.
- [15] V. Morris, J. Hughes, P. Marriott, "Spherical coordinate representations of solvent composition for liquid chromatography method development using experimental design," *Journal of Chromatography A*, vol. 1008 no. 1, pp. 43-56, DOI: 10.1016/s0021-9673(03)01021-5, 2003.
- [16] G. Vivó-Truyols, J. R. Torres-Lapasió, M. C. García-Alvarez-Coque, "Enhanced calculation of optimal gradient programs in reversed-phase liquid chromatography," *Journal of Chromatography A*, vol. 1018 no. 2, pp. 183-196, DOI: 10.1016/j.chroma.2003.08.043, 2003.
- [17] P. Nikitas, A. Pappa-Louisi, K. Papachristos, "Optimisation technique for stepwise gradient elution in reversed-phase liquid chromatography," *Journal of Chromatography A*, vol. 1033 no. 2, pp. 283-289, DOI: 10.1016/j.chroma.2004.01.048, 2004.
- [18] T. Baczek, R. Kaliszan, H. A. Claessens, M. A. V. Straten, "Computer-assisted optimization of reversed-phase HPLC isocratic separations of neutral compounds," *LC-GC Europe*, vol. 14, pp. 99-106, 2001.
- [19] B. W. J. Pirok, S. Pous-Torres, C. Ortiz-Bolsico, G. Vivó-Truyols, P. J. Schoenmakers, "Program for the interpretive optimization of two-dimensional resolution," *Journal of Chromatography A*, vol. 1450, pp. 29-37, DOI: 10.1016/j.chroma.2016.04.061, 2016.
- [20] G. Jin, X. Xue, F. Zhang, Y. Jin, X. Liang, "Computer-aided target optimization for traditional Chinese medicine by ultra-performance liquid chromatography," *Talanta*, vol. 78 no. 1, pp. 278-283, DOI: 10.1016/j.talanta.2008.11.009, 2009.
- [21] C. Li, X. Xue, D. Zhou, "Analysis of iridoid glucosides in *Hedyotis diffusa* by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48 no. 1, pp. 205-211, DOI: 10.1016/j.jpba.2008.05.013, 2008.
- [22] X. Xiong, Y. N. He, B. Feng, Y. Pan, "Screening for the anti-inflammation quality markers of Xiaojin Pills based on HPLC-MS/MS method, COX-2 inhibition test and protein interaction network," *Scientific Reports*, vol. 8, DOI: 10.1038/s41598-018-25582-7, 2018.
- [23] L.-k. Sun, F. Liu, W.-q. Chen, P.-m. Li, "Systematic evaluation on xiao jin pill treatment of hyperplasia of mammary glands," *Chinese Journal of Drug Evaluation*, vol. 33 no. 5, pp. 448-451+456, 2016.
- [24] X.-W. Qu, S.-W. Zhang, P.-H. Zhang, J. Yin, "Xiaojin Wan inhibits the expression of COX-2 in prostate tissues of prostatitis pain rats," *Zhonghua Nan Ke Xue = National Journal of Andrology*, vol. 14 no. 8, pp. 759-762, 2008.
- [25] X. Xiong, Y.-n. He, B. Feng, "Identification of quality markers of Xiaojin Pills using a combination of high-performance liquid chromatography tandem mass spectrometry and multivariate analysis," *Tropical Journal of Pharmaceutical Research*, vol. 17 no. 8, pp. 1663-1672, DOI: 10.4314/tjpr.v17i8.28, 2018.

- [26] X.-y. Zhao, J.-m. Zhou, X. Qin, "Study on the determination of column efficiency of high performance liquid chromatography silica gel column (in Chinese)," *Mechanical and Electrical Information*, vol. 2, pp. 28-30, 2012.
- [27] Y.-h. Ji, H. Gao, Y.-f. Chang, X.-l. Li, "Correlation analysis of HPLC fingerprint chromatograms of dictamni cortex decoction pieces-standard decoction-dispensing granules," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 24 no. 18, pp. 81-87, 2018.
- [28] X.-j. Yang, S. Li, Z.-j. Yang, Z.-y. Pan, "HPLC fingerprint spectrum and chemical pattern recognition of Radix Hedysari from different habitats of Gansu," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 33 no. 7, pp. 3070-3073, 2018.
- [29] H.-y. Liu, J. Wu, L.-n. Liu, Z. Jia, "Research on HPLC fingerprint of jinsangqi kangdu dropping pills," *Chinese Journal of Information on Traditional Chinese Medicine*, vol. 25 no. 6, pp. 90-94, 2018.
- [30] J. Zhao, L.-x. Dai, T. Lei, Q. Lu, "Chemical constituents of trogopterus xanthipes (in Chinese)," *Natural Product Research and Development*, vol. 22 no. 4, pp. 541-543, 2010.
- [31] Y. Luo, T. Tan, X.-L. Liang, H.-P. Zhao, "Quality evaluation of musk based on the biological potency of its anti-thrombin activity," *Zhongguo Zhongyao Zazhi = China Journal of Chinese Materia Medica*, vol. 43 no. 10, pp. 2112-2117, 2018.
- [32] C.-x. Cao, H.-b. Wang, R.-y. Yang, Z. Sun, "The effects of musk on HER 2 , VEGF and tumor markers in human gastric cancer cell lines," *Henan Medical Research*, vol. 27 no. 23, pp. 4225-4228, 2018.
- [33] H. P. T. Ammon, T. Mack, G. Singh, H. Safayhi, "Inhibition of leukotriene B₄ Formation in rat peritoneal neutrophils by an ethanolic extract of the gum resin exudate of *Boswellia serrata*," *Planta Medica*, vol. 57 no. 3, pp. 203-207, DOI: 10.1055/s-2006-960074, 1991.
- [34] P. Calzavara-Pinton, C. Zane, E. Facchinetti, R. Capezzera, A. Pedretti, "Topical Boswellic acids for treatment of photoaged skin," *Dermatologic Therapy*, vol. 23, pp. S28-S32, DOI: 10.1111/j.1529-8019.2009.01284.x, 2010.
- [35] A. Assimopoulou, S. Zlatanov, V. Papageorgiou, "Antioxidant activity of natural resins and bioactive triterpenes in oil substrates," *Food Chemistry*, vol. 92 no. 4, pp. 721-727, DOI: 10.1016/j.foodchem.2004.08.033, 2005.
- [36] W.-x. Mei, C.-c. Zeng, "Study of the effect of Frankincense extraction on ulcer healing quality in gastric ulcerated rats," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 21 no. 5, pp. 478-479+486, 2012.
- [37] I. Kimura, M. Yoshikawa, S. Kobayashi, "New triterpenes, myrrhanol A and myrrhanone A, from guggul-gum resins, and their potent anti-inflammatory effect on adjuvant-induced air-pouch granuloma of mice," *Bioorganic & Medicinal Chemistry Letters*, vol. 11 no. 8, pp. 985-989, DOI: 10.1016/s0960-894x(01)00111-1, 2001.
- [38] K. Asres, A. Tei, G. Moges, F. Sporer, M. Wink, "Terpenoid composition of the wound-induced bark exudate of *Commiphora tenuis* from Ethiopia," *Planta Medica*, vol. 64 no. 5, pp. 473-475, DOI: 10.1055/s-2006-957489, 1998.
- [39] M. A. Birkett, S. A. Abassi, T. Kröber, "Antiectoparasitic activity of the gum resin, gum hagggar, from the East African plant, *Commiphora holtziana*," *Phytochemistry*, vol. 69 no. 8, pp. 1710-1715, DOI: 10.1016/j.phytochem.2008.02.017, 2008.
- [40] T. Shen, W. Wan, H. Yuan, "Secondary metabolites from *Commiphora opobalsamum* and their antiproliferative effect on human prostate cancer cells," *Phytochemistry*, vol. 68 no. 9, pp. 1331-1337, DOI: 10.1016/j.phytochem.2007.01.013, 2007.

DETAILS

Subject:

Software; Acids; Quality control; Cluster analysis; Spectrum analysis; Ions; Solvents; Herbal medicine; Optimization; Traditional Chinese medicine; Computer simulation; Herbs; Fingerprints; Chromatography; Flow velocity; Parameters; Drug dosages; Efficiency

Location:	United States--US; China
Company / organization:	Name: Food & Drug Administration--FDA; NAICS: 922190
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Journal Article
Publication history :	
Milestone dates:	2020-09-21 (Received); 2020-12-14 (Revised); 2021-01-03 (Accepted); 2021-01-16 (Pub)
DOI:	https://doi.org/10.1155/2021/8858501
ProQuest document ID:	2480125509
Document URL:	https://www.proquest.com/scholarly-journals/computer-aided-rapid-establishment-fingerprint/docview/2480125509/se-2?accountid=211160
Copyright:	Copyright © 2021 Hui Jiang et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/

Document 59 of 59

Corrigendum #2 to “Quantitative Detection of miRNA-21 Expression in Tumor Cells and Tissues Based on Molecular Beacon”

Liu, Qingxin ¹ ; Fan, Jialong ² ; Zhou, Chuang ³ ; Wang, Liqun ³ ; Zhao, Bin ⁴ ; Zhang, Haibin ⁴ ; Liu, Bin ² ; Tong, Chunyi ²

¹ College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China; Jiangsu Vocational College of Agriculture and Forestry, Jurong, Jiangsu 212400, China ² College of Biology, Hunan University, Changsha, Hunan 410082, China ³ Jiangsu Vocational College of Agriculture and Forestry, Jurong, Jiangsu 212400, China ⁴ College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

[ProQuest document link](#)

FULL TEXT

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. In the article titled “Quantitative Detection of miRNA-21 Expression in Tumor Cells and Tissues Based on Molecular Beacon” [1], the authors’ previous similar work on pre-miRNA-21 was not cited and discussed [2]. The authors said the two studies are similar in strategy, using a fluorescent probe to detect RNA, but with different probes and targets. With the structural differences of pre-miRNA and miRNA, they used a chimeric molecular beacon (the stem contains RNA bases) to detect pre-miRNA-21 in the earlier study [2], while they used a molecular beacon (entirely DNA bases) to directly detect miRNA-21 in the second article [1]. The studies were conducted at the same time and have similar outcomes, in that both LOD are 0.5nM of the targets and there is a similar linear range (1–100nM vs. 1–50 nM). The original version of Figure 3 that was replaced in an earlier corrigendum [3] was reproduced in error from Figure 2 in that earlier article.

References

- [1] Q. Liu, J. Fan, C. Zhou, "Quantitative detection of miRNA-21 expression in tumor Cells and Tissues based on molecular beacon," International Journal of Analytical Chemistry, vol. 2018, DOI: 10.1155/2018/3625823, 2018.
- [2] H.-L. Xie, T.-T. Wu, Z.-H. Xie, J.-L. Fan, C.-Y. Tong, "Quantitative detection of PremicroRNA-21 based on chimeric molecular beacon," Chinese Journal of Analytical Chemistry, vol. 46 no. 7, pp. e1847-e1853, DOI: 10.1016/s1872-2040(18)61099-0, 2018.
- [3] Q. Liu, J. Fan, C. Zhou, "Corrigendum to “quantitative detection of miRNA-21 expression in tumor Cells and Tissues based on molecular beacon,” International Journal of Analytical Chemistry, vol. 2020, DOI: 10.1155/2020/1515794, 2020.

DETAILS

Subject:	Analytical chemistry
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2021
Publication year:	2021
Publication date:	2021
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New York
Publication subject:	Chemistry--Analytical Chemistry
ISSN:	16878760
e-ISSN:	16878779
Source type:	Scholarly Journal
Language of publication:	English
Document type:	Corrections/Retraction
Publication history :	
Milestone dates:	2020-11-05 (Received); 2020-11-05 (Accepted); 2021-01-15 (Pub)
DOI:	https://doi.org/10.1155/2021/8406935
ProQuest document ID:	2480125499
Document URL:	https://www.proquest.com/scholarly-journals/corrigendum-2-quantitative-detection-mirna-21/docview/2480125499/se-2?accountid=211160
Copyright:	Copyright © 2021 Qingxin Liu et al. This is an open access article distributed under the Creative Commons Attribution License (the "License"), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Notwithstanding the ProQuest Terms and Conditions, you may use this content in accordance with the terms of the License. https://creativecommons.org/licenses/by/4.0/
Last updated:	2022-01-26
Database:	Publicly Available Content Database

Bibliography

Citation style: APA 6th - Annotated with Abstracts - American Psychological Association, 6th Edition

Atinafu G/Mariam, Diro, A., Tsegaye, G. A., Jado, D., & Melak, F. (2021). Spectroscopic determination of fluoride using eriochrome black T (EBT) as a spectrophotometric reagent from groundwater. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/2045491>

Fluoride health problem is a great concern worldwide, most often as a result of groundwater intake. Thus, determination of fluoride is vital to take appropriate measures upon fluoride contamination of water. Potentiometric method of analysis is reliable for the determination of fluoride in various samples. In addition, spectroscopic methods are found important to quantify fluoride levels from water; however, several factors hinder its easier determination. Among the bottlenecks, the use of toxic chemicals and tedious steps in preparing chemicals (e.g., SPADNS method) are to mention a few. In this study, a spectrophotometric method was developed for the determination of fluoride from groundwater using Eriochrome Black T (EBT) as a spectroscopic reagent. Experimental parameters that influence the determination of fluoride including ligand type, kinetics, pH, and ligand-to-metal ratio were assayed. Evaluation of fluoride levels showed that Beer–Lambert's law is obeyed in the range of 0.3–5.0 mg/L at 544 nm. The calibration curve, resulting in good linearity ($R^2=0.9997$), was considered during quantitative analysis of the samples and in the spiking analysis. The limits of detection (LOD) and quantification (LOQ) of the method were found to be 0.19 and 0.64 mg/L, respectively. The precision studied in terms of intraday and interday at three concentration levels showed less than 5.4% RSD. Applicability of the method was investigated by analyzing groundwater samples spiked with fluoride standards, and satisfactory recoveries in the range of 98.18–111.4 were demonstrated. The developed spectrophotometric method has been successfully applied for fluoride determinations in groundwater samples. Thus, it could be used as an attractive alternative for the determination of fluoride from groundwater.

Houssaini, J., Mohammed, N. B., Ziyat, H., Arhzaf, S., Qabaqous, O., & Amhoud, A. (2021). Study of the catalytic activity of the compounds hydrotalcite type treated by microwave in the self-condensation of acetone. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/1551586>

The self-condensation reaction of acetone, producing diacetone alcohol (DAA), is of great industrial importance. It was used to study the catalytic activity of Mg–Al catalysts synthesized by the coprecipitation method. For this purpose, we synthesized Mg–Al based hydrotalcite with a molar ratio of 3, obtained either after conventional heating or after microwave irradiation with of 100W for three minutes. Structural and chemical properties of the obtained catalysts were characterized, using different techniques: X-ray diffraction (XRD), Fourier transform infrared spectra (FTIR), scanning electron microscope (SEM), equipped with energy dispersive X-ray (EDX), and specific surface area of the catalysts were determined by the methylene blue (MB) adsorption method. Also, these catalysts were tested in the self-condensation reaction of acetone at 273K in the liquid phase without solvent, a reaction which requires very high catalytic activity. The microwave treatment improves the catalyst activity, and the conversion of acetone to diacetone alcohol increases from 13.2 to 18.3% after 8 h of reaction. Moreover, the microwave-treated hydrotalcite catalyst, calcined at 723K and rehydrated under a flow of N₂, is the most active and gives conversion of acetone of 52% under the same reaction conditions.

Doering, C., Strassner, J., & Fouckhardt, H. (2021). Microdroplet actuation via light line optoelectrowetting (LL-OEW). *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/3402411>

Meanwhile, electrowetting-on-dielectric (EWOD) is a well-known phenomenon, even often exploited in active micro-optics to change the curvature of microdroplet lenses or in analytical chemistry with digital microfluidics (DMF, lab on a chip 2.0) to move/actuate microdroplets. Optoelectrowetting (OEW) can bring more flexibility to DMF because in OEW, the operating point of the lab chip is locally controlled by a beam of light, usually impinging onto the chip perpendicularly. As opposed to pure EWOD, for OEW, none of the electrodes has to be structured, which makes the chip design and production technology simpler; the path of any actuated droplet is determined by the movement of the light spot. However, for applications in analytical chemistry, it would be helpful if the space both below as well as that above the lab chip were not obstructed by any optical components and light sources. Here, we report on the

possibility to actuate droplets by laser light beams, which traverse the setup parallel to the chip surface and inside the OEW layer sequence. Since microdroplets are grabbed by this surface-parallel, nondiverging, and nonexpanded light beam, we call this principle “light line OEW” (LL-OEW).

Guo-wei, Z., Yong-mei, L., Chun-ni, L., Hong-min, R., & Hai-ying, L. (2021). Rapid simultaneous determination of 43 pesticide residues in *schizonepeta tenuifolia* by gas chromatography mass spectrometry. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/8934998>

A simple, fast, and reliable method was established for simultaneous determination of 43 pesticides in *Schizonepeta tenuifolia*. The samples were prepared using solid-phase extraction (SPE) method. Pesticides were extracted from *Schizonepeta tenuifolia* using acetonitrile, cleaned with Pesticarb/NH₂, and eluted by mixed solvents of acetonitrile and toluene (3:1, v/v). Selected pesticides were identified using DB-35MS capillary column and detected by gas chromatography mass spectrometry. Samples were quantified by external standard method. Recoveries for the majority of pesticides at spike levels of 0.2, 0.5, and 1 mg kg⁻¹ ranged between 70 and 120% (except for Chlorothalonil, Thiamethoxam, and Dicofof), and the relative standard deviations (RSDs n=6) were 1.32%–13.91%. Limits of detection (LODs) were 0.0011–0.0135 mg kg⁻¹, whereas limits of quantification (LOQs) were 0.0038–0.0451 mg kg⁻¹. The satisfactory accuracy and precision, in combination with a good separation and few interferences, have demonstrated the strong potential of this technique for its application in *Schizonepeta tenuifolia* analysis.

Alzahrani, E., & Alkhudidy, A. T. (2021). Synthesis, optimization, and characterization of ecofriendly production of gold nanoparticles using lemon peel extract. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/7192868>

This study examines the importance of utilizing green synthesis using lemon peel for gold nanoparticles over other chemicals since it is environmentally friendly, available, and cheap. Several parameters were optimized to ensure the extraction of the GNPs concentration of lemon peels using HAuCl₄ and lemon peel extract having a ratio of 2:1. For the optimum result, the ratio used was 2:1. The gold nanoparticles fabrication happened in 10 minutes. The initial observation was the color change of the solution. The UV-visibility spectroscopic studies are performed to confirm the result. The experiments are done concurrently to ensure the solution is mixed on the proper ratio. The GNP is also characterized by the different techniques in their sizes and electronic transmission microscopy, essential in extracting gold nanoparticles. Other elements of the composition are removed by the EDAX methods, the FTIR method, and the TEM methods, all of which reveal the real reason behind the required extraction capacity. Most gold nanoparticles show a maximum absorption rate at the peak of 535 to 579 nm. The result obtained from the TEM and the SEM analysis revealed that the grain size is analogized to the average size of 6.67 nm. With a simple synthesis of the price, some processes show that the medically available nanoparticles are necessary. The used method in this paper to fabricate GNPs is cheap, easy, fast, and sustainable and it can be done with ease in any laboratory.

Kumatia, E. K., Ofosu-Koranteng, F., Appiah, A. A., & Barimah, K. B. (2021). Standardization and quality control of the herbal medicine mist nibima, employed to treat malaria and COVID-19, using physicochemical and organoleptic parameters and quantification of chemical markers via UHPLC-MS/MS. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/6390481>

Mist Nibima is an essential herbal medicine used to treat malaria, bacterial, yeast, and COVID-19 infections. However, the drug has not been standardized and its active chemical ingredients are also not known. This study employed physicochemical, organoleptic, qualitative, and quantitative phytochemical analysis to establish standards for Mist Nibima. Additionally, UHPLC was used to quantify the alkaloid cryptolepine in the drug using calibration curve. The chemical ingredients in Mist Nibima were thereafter characterized using UHPLC-MS. Organoleptic evaluation shows that Mist Nibima is a very bitter, cloudy, broom yellow decoction with the following physicochemical parameters: pH=6.10±0.08 (at 28.3°C), total solid residue=5.34±0.27%w/v, and specific gravity=1.0099±0.0000. The total alkaloid (23.71±1.311%) content of the drug is 3 times its total saponins (7.993±0.067%)

content. Methyl cryptolepinoate (37.10%), cryptolepine (33.56%), quindoline (20.78%), 11-isopropylcryptolepine (5.16%), and hydroxycryptolepine (3.14%) were the active chemical ingredients in the drug with the concentrations of 18.64 ± 0.255 , 16.85 ± 0.231 , 10.42 ± 0.143 , 2.56 ± 0.034 , and 1.70 ± 0.023 $\mu\text{g/mL}$, respectively. Administration of a single oral therapeutic dose (30 mL) of Mist Nibima corresponds to ingestion of 559.2 ± 7.662 , 505.5 ± 6.930 , 312.6 ± 4.285 , 76.8 ± 1.028 , and 51.0 ± 0.699 μg of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine, respectively. This translates into a corresponding daily dose of 1677.6 ± 22.986 , 1516.5 ± 20.790 , 937.8 ± 12.855 , 230.4 ± 3.084 , and 153.0 ± 2.097 μg of methyl cryptolepinoate, cryptolepine, quindoline, 11-isopropylcryptolepine, and hydroxycryptolepine. These results could now serve as tools for authentication, standardization, and quality control of Mist Nibima to ensure its chemical and pharmacological consistency and safety.

Ding, L., Zhang, X., & Zhang, J. (2021). Antioxidant activity in vitro guided screening and identification of flavonoids antioxidants in the extract from *tetragium hemsleyanum diels et gilg*. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/7195125>

This study aimed to investigate the extract with high antioxidant activity of *Tetragium hemsleyanum Diels et Gilg* and identify the antioxidant components in vitro. α , α -Diphenyl- β -picrylhydrazyl (DPPH) radical assay, Trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing antioxidant power (FRAP), and hydroxyl radical scavenging method were used to screen the extract with high antioxidant activity. The antioxidant capacity of the extracts was evaluated by the free radical scavenging ability of DPPH. The ability of extracts to scavenge 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical was evaluated by TEAC assay. The FRAP method was used to evaluate the ability of extracts to reduce Fe^{3+} . The ability to scavenge hydroxyl radicals produced by the interaction of hydrogen peroxide and Fe^{2+} was measured by monitoring the change in the absorbance of the reaction mixture at 536 nm. Then, high-performance liquid chromatography-DPPH (HPLC-DPPH) and HPLC-hydroxyl radical scavenging methods were used to screen the antioxidant components in the extract. The molecular weight of the above antioxidant components was investigated using the qualitative analytical method of high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-Q-TOF LC/MS). Based on the concentrations of the samples (0.2–4 mg/mL), the DPPH free radical scavenging ability, ABTS+ free radical scavenging ability, hydroxyl free radical scavenging ability, and Fe^{3+} reducing ability of the ethyl acetate extract (EAE) were stronger than that of the crude extract (CE), petroleum ether extract (PEE), and n-butanol extract (BE). The EAE has higher antioxidant activity than CE, PEE, and BE. Six antioxidant components, rutin, quercetin, isoquercetin, astragalin, kaempferol, and kaempferol-3-o-rutside, were identified in the EAE.

Wu, X., Shen, X., Cao, X., Nie, R., Zhang, H., Tang, C., & Wang, W. (2021). Simultaneous determination of amphenicols and metabolites in animal-derived foods using ultrahigh-performance liquid chromatography-tandem mass spectrometry. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/3613670>

Amphenicols are widely used to prevent and treat animal diseases. However, amphenicol residues accumulate in livestock and poultry and harm consumers. We hypothesized that one can combine solid-phase extraction (SPE) with ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to simultaneously determine amphenicols and metabolites in pork, beef, lamb, chicken, and their products and meet government regulations for maximum residue limits. We extracted crude samples with ethyl acetate and ammonia water (98:2, v/v), purified the samples with a CNW Si SPE column, defatted the samples with acetonitrile-saturated n-hexane, and then determined the resulting analytes by UHPLC-MS/MS. The limit of detection of the analytes in livestock and poultry meat was 0.03–1.50 $\mu\text{g/kg}$, and the limit of quantification was 0.05–5.00 $\mu\text{g/kg}$. Measured chloramphenicol, thiamphenicol, and florfenicol concentrations were linear over the range 0.50–50 $\mu\text{g/kg}$; and the florfenicol amine concentration was linear over the range 5.00–200 $\mu\text{g/kg}$ (all with correlation coefficients >0.9990). The recovery of the spiked samples was between 72% and 120%. The intraday relative standard deviation (RSD) ranged from 1% to 9%, and the interday RSD ranged from 1% to 12%. Based on the above results, the current method is sensitive, accurate, and reproducible with the detection limits being well below the maximum residue limits as per Chinese standard GB 31650-2019, and thus, our research hypothesis could be confirmed.

Azrul, H. S., May, Y. A., & Ng, S. Y. (2021). Optimization of residual hexane in edible oils analysis using static headspace gas chromatography. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/1941336>

This study aims to determine the residual hexane in four edible oils in Malaysia using a simple, rapid, and automated method in order to improve the efficiency and productivity of the analysis. Gas chromatography (GC/FID) equipped with a headspace autosampler (HS-20) was used to perform the analysis. Incubation time for each injection was successfully optimized from one hour to 30 minutes (50% reduction) compared to the official AOCS method Ca 3b-87. Out of the four tested edible oils, only the hexane residues detected in sunflower oil exceeded the maximum residue limit (MRL) set by the European Union regulation. Significant difference of the results obtained between large calibration range (0–938 mg kg⁻¹) and small calibration range (0–68 mg kg⁻¹) suggests that there is a need to use a lower standard calibration concentration to avoid misinterpretation of analysis results. Method validation applies to the technical hexane; 2-methylpentane, 3-methylpentane, cyclohexane, and methylcyclopentane, the signal-to-noise (S/N), as well as the limit of quantification (LoQ) values was found to be 218.20, 221.45, 746.37, 97.37 and 0.85, 0.84, 0.25, 1.93 mg kg⁻¹, respectively. Good linearity, repeatability, and low carryover of this method have provided an alternative way to analyze the content of the residual hexane in edible oils in a more efficient manner. Current study might provide a fundamental reference for the improvement of the AOCS official Ca 3b-87 method for determination of hexane residues in fats and oils analysis in the future.

Basheer, C. (2021). Electrospun membrane for the extraction of acrylamide in pet food samples. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/1285501>

A simple microextraction procedure was developed using an electrospun nanostructured membrane to determine acrylamide in pet food samples. Polyvinyl chloride, polyvinyl alcohol, and polyvinyl alcohol/hydroxyethyl cellulose electrospun membranes were prepared and investigated as a sorbent to extract acrylamide. The characterization of the synthesized electrospun membrane was accomplished using field-emission scanning electron microscopy (FESEM). FESEM images showed uniform morphology and beadless nanofibers. Quantification was done by high-performance liquid chromatography with ultraviolet detection. A series of microextraction parameters were optimized before quantitative analysis of dry pet food samples. The calibration curve exhibited good linearity with a correlation coefficient of 0.996 across a 1–100 µg/kg concentration range. The recovery of acrylamide for pet food samples spiked with 5 and 10 µg/kg was in the range of 79.6–113.9 (n=3). The intraday precision of the method was less than 12% for three replicated real spiked samples at the 5 µg/kg level. The results demonstrated that the electrospun nanostructured membrane has good extraction selectivity and minimal matrix effect with an enrichment factor of 180-fold.

Panthi, V. K., Jha, S. K., Chaubey, R., & Pangeni, R. (2021). Formulation and development of serratiopeptidase enteric coated tablets and analytical method validation by UV spectroscopy. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9749474>

Serratiopeptidase (SRP) is a proteolytic enzyme that emerged as one of the most potent anti-inflammatory and analgesic drugs. The purpose of the present study was to formulate and evaluate enteric-coated tablets for SRP and investigate their stability using a simple and validated analytical method by ultraviolet (UV) spectroscopy. The colloidal silicon dioxide (2.50%), sodium starch glycolate (3.44%), and croscopovidone (2.50%) were used as appropriate excipients for the development of core part of tablets. To protect the prepared tablets from acidic environment in the stomach, white shellac, castor oil, HPMC phthalate 40, and ethyl cellulose were used. The seal coating and enteric coating attained were 2.75% and 6.74%, respectively. SRP was found to be linear at 265 nm in the concentration range of 25–150 µg/mL. The results revealed that our developed method was linear (R²=0.999), precise (RSD % = 0.133), and accurate (% recovery = 99.96–103.34). The formulated SRP tablets were found to be stable under accelerated conditions as well as under room temperature for 6 months (assay %: >97.5%). The in vitro drug release study demonstrated that enteric-coated tablets were able to restrict SRP release in both acidic environments: 0.1 N HCl and simulated gastric fluid (pH 1.2). Moreover, at 60 minutes, the formulated SRP tablets revealed 13.0% and 8.98% higher drug release in phosphate buffer (pH 6.8) and simulated intestinal fluid (pH 6.8),

respectively, compared to the marketed tablet formulation. This study concludes that enteric-coated tablets of SRP with higher drug release in the intestine can be prepared and examined for their stability using validated analytical technique of UV spectroscopy.

Sidgwick, F., Porter, A., Ayuk, P., Kamali, F., & Truemann, A. (2021). Determination of dabigatran concentration in human plasma and breast milk. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5949385>

Venous thromboembolism (VTE) is an important cause of death following childbirth. Dabigatran etexilate can be a useful prophylaxis in susceptible women during the postpartum period. However, it is not clear whether dabigatran is excreted into breast milk in amounts which can be harmful to the suckling baby. We have developed an accurate, sensitive, and specific assay for the quantitation of dabigatran in both human plasma and breast milk. This is particularly useful for the determination of the extent by which dabigatran is secreted into breast milk in relation to its systemic availability. Dabigatran was enriched from both matrices using solid-phase extraction prior to separation on a C8-RPLC column and detection using SRM on a QqTrap mass spectrometer. The assay was validated for specificity, sensitivity, linearity, precision, accuracy, and stability of the analyte in human plasma and breast milk. The lower limit of detection for dabigatran was 20 pg/ml in plasma and 75 pg/ml in breast milk. This assay will aid future studies for the measurement of dabigatran concentrations in human breast milk to help determine if dabigatran etexilate can safely be administered to breast-feeding women.

Yuan, M., Liu, X., Sun, Y., Wang, L., Jin, P., Zhuang, X., . . . Nie, L. (2021). Chromatographic fingerprinting based on column switching technology for quality evaluation of tianmeng oral liquid. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/2514762>

Separation power was limited when the conventional high-performance liquid chromatography (HPLC) fingerprinting method based on a single column was used to analyze very complex traditional Chinese medicine (TCM) preparations. In this research, a novel HPLC fingerprinting method based on column switching technology by using a single pump was established for evaluating the quality of Tianmeng oral liquid (TMOL). Twelve batches of TMOL samples were used for constructing HPLC fingerprints. Compared with the 16 common peaks in fingerprinting with a single column, 25 common peaks were achieved with two columns connected through a six-way valve. The similarity analysis combined with bootstrap method was applied to determine the similarity threshold, which was 0.992 to distinguish expired samples and unexpired samples. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were also applied to classify the TMOL samples, and results revealed that expired and unexpired samples are classified into two categories. The HPLC fingerprinting based on column switching technology with better separation power and higher peak capacity could characterize chemical composition information more comprehensively, providing an effective and alternative method to control and evaluate the quality of TMOL, which would offer a valuable reference for other TCM preparations.

Solagbade, S. A., Oyeyode, J. O., Shafik, W., Sunusi, Z. A., & Adegoke, A. A. (2021). Proximate analysis of poultry-mix formed feed using maize bran as a base. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/8894567>

The purpose of this research was to demonstrate the proximate analysis of poultry-mix made using maize bran as a basis. Red beans, soya beans, and benny beans were the three samples utilised in this study. This work investigates the appropriate poultry mix for birds breed for meat and egg. Thirty grammes of proteinous feedstock were weighed and homogeneously combined with 70 grammes of maize bran. The following was revealed in a proximate analysis of the feeds: moisture ranged from 1.18% to 1.54%, unrefined lipids 0.99–3.08%, total carbohydrate 57% to 72%, ash content 38.48% to 38.92%, unrefined protein 18.38% to 22.53% and unrefined fiber 2.0% to 4.65% respectively for broilers and layers. In terms of nutritional concentrations, all feed samples showed a substantial variation. Based on the findings of the study, it can be stated that Soya bean-maize bran is an excellent poultry-mix formulation that has deep well-disposed benefits and meets nearly all nutritional needs for meat and egg-producing birds.

Sun, Y., Zhu, X., Shen, X., & Wang, W. (2021). Determination of carrageenan in livestock and poultry meat by ultrahigh-performance liquid chromatography-tandem mass spectrometry. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5277453>

Ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has become the main method for the detection and analysis of food additives because of its good separation, high selectivity, and high sensitivity. The aim of this study was to establish an UHPLC-MS/MS method that can quickly and accurately measure the content of carrageenan in livestock and poultry meat. Chromatographic separation was performed on an ACQUITY UPLC BEH HILIC C18 column (2.1 mm×50 mm, 1.7 μm) using a gradient elution with methanol and 0.1% (v/v) formic acid in water as a mobile phase. The quantitative analysis was executed using a triple quadrupole mass spectrometer in which electrospray ionization, multiple reaction monitoring, and negative mode were operated. The retention time was about 1.3 min for carrageenan. The carrageenan content showed a good linear relationship from 0.05 to 1.00 g/kg. The limit of detection (LOD) was 0.06 g/kg, and the limit of quantification (LOQ) was 0.18 g/kg. The standards were spiked at three levels (low, medium, and high) and were analyzed in six replicates. The recovery values of carrageenan in pork, beef, lamb, chicken, and duck meat were 82.06–111.55%, 85.43–112.50%, 89.55–116.00%, 83.80–102.15%, and 82.41–110.90%, respectively. The relative standard deviations (RSDs) were all lower than 7.51%. The developed method shows a high recovery rate and good precision and can be used for the rapid detection of carrageenan in livestock and poultry meat.

Liu, Y., Liu, H., Xia, Z., Wang, Z., Yun, Y., Zhang, G., . . . Chen, W. (2021). Simultaneous and rapid determination of six tyrosine kinase inhibitors in patients with non-small cell lung cancer using HPLC-MS/MS. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5524361>

Objective. To develop a new method for quantitatively analyzing six tyrosine kinase inhibitors (gefitinib, erlotinib, icotinib, afatinib, osimertinib, and crizotinib) used in the treatment of non-small cell lung cancer (NSCLC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Methods.** The analytes were detected in the selected reaction monitoring mode on a triple quadrupole mass spectrometer with the positive ionization mode. Carbamazepine was utilized as the internal standard. The pretreatment of the plasma sample was completed based on protein precipitation with acetonitrile, and the analytes were separated on an Agilent Zorbax SB-C18 reversed-phase column (2.1 mm×100 mm, 3.5 μm, Agilent, USA) using gradient elution. The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The flow rate was 0.3 mL/min, and the injection volume was 5 μL. The column temperature was set and maintained at 35°C. **Results.** The calibration curves were linear over the range from 5.0 to 1000.0 ng/mL for gefitinib, crizotinib, and osimertinib; from 50.0 to 4000.0 ng/mL for icotinib and erlotinib; and from 5.0 to 400.0 ng/mL for afatinib. Linear correlation coefficients were >0.990 for all regression curves. The intra- and interday accuracy and precision of the method were within ±15.0% and not more than 15.0%, respectively. The mean recovery of all the analytes ranged from 70.18% to 110.76%, the matrix effect was from 88.85% to 127.58%, and stability was within ±15.0%. **Conclusion.** This newly developed method was sensitive, simple, and robust and could be used in therapeutic drug monitoring of six tyrosine kinase inhibitors in NSCLC patients.

Yang, M., Tan, D., Lu, A., Lin, Q., Wang, C., Ling, H., . . . He, Y. (2021). Identification and simultaneous determination of the main toxic pyrrolizidine alkaloids in a compound prescription of traditional Chinese medicine: Qianbai biyan tablet. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5209618>

Qianbai biyan tablet (QT) is a compound prescription of traditional Chinese medicine which is used to treat nasal congestion, rhinitis, and nasosinusitis, with *Senecio scandens* as its main plant material. Several pyrrolizidine alkaloids (PAs) were reported in *Senecio scandens* and others of *Senecio* species. Although *Senecio scandens* is assigned as the legal plant material of QT, whether replaced use of it by other *Senecio* plants can bring toxicity is unknown because of the lack of quantitative data about toxic PAs between different *Senecio* species. In the present study, adonifoline, senkirkine, and another PA presumed as emiline have been identified in QT; however, there was no senecionine detected in all tablets. PA contents in QTs varied in different companies and different batches. Adonifoline existed only in *Senecio scandens*, and senecionine was detected in all eight *Senecio* plants investigated

in the present study. Data showed that replaced use of *Senecio scandens* with a low level of senecionine by other *Senecio* plants such as *Senecio vulgaris* containing a high level of senecionine is advertised to be forbidden. Data of the present study may be used as a reference to make new drug quality regularity and recommendation guideline for the safety of QT.

Valkova, P., & Pohanka, M. (2021). Novel trends in electrochemical biosensors for early diagnosis of Alzheimer's disease. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9984876>

Background. Alzheimer's disease (AD) is a multifactorial progressive and irreversible neurodegenerative disorder affecting mainly the population over 65 years of age. It is becoming a global health and socioeconomic problem, and the current number of patients reaching 30–50 million people will be three times higher over the next thirty years. **Objective.** Late diagnosis caused by decades of the asymptomatic phase and invasive and cost-demanding diagnosis are problems that make the whole situation worse. Electrochemical biosensors could be the right tool for less invasive and inexpensive early diagnosis helping to reduce spend sources— both money and time. **Method.** This review is a survey of the latest advances in the design of electrochemical biosensors for the early diagnosis of Alzheimer's disease. Biosensors are divided according to target biomarkers. **Conclusion.** Standard laboratory methodology could be improved by analyzing a combination of currently estimated markers along with neurotransmitters and genetic markers from blood samples, which make the test for AD diagnosis available to the wide public.

Khamis, A. S., & Chai, L. C. (2021). Chemical and antimicrobial analyses of *juniperus chinensis* and *juniperus seravschanica* essential oils and comparison with their methanolic crude extracts. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9937522>

Juniperus chinensis and *Juniperus seravschanica* are commonly used in the traditional folk medicine to treat microbial infection. In this study, the essential oils obtained from the leaves of *J. chinensis* growing in Malaysia and *J. seravschanica* growing in Oman were analysed by head space-solid phase microextraction-gas chromatography mass spectrometry (HS-SPME-GC-MS) and screened for antimicrobial activities against *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC 10662), *Bacillus subtilis* ATCC6059, *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (NCTC 6571), and methicillin-resistant *S. aureus* (MRSA; ATCC 33591). To compare the antimicrobial activities of extracts using different extraction methods, methanol extraction was performed to obtain crude extracts from the leaves of *J. chinensis* and *J. seravschanica* for antimicrobial analysis. The HS-SPME-GS-MS analysis of the essential oils from the leaves of *J. chinensis* and *J. seravschanica* identified 37 and 36 components, respectively. Essential oils from these two species had distinctive chemical component profiles, with α -pinene (27.2%) as the major component of *J. chinensis* essential oil, while dl-limonene (45.2%) constitutes the major component of *J. seravschanica* essential oil. Essential oils of these two species shared only six similar terpenoids compounds: α -pinene, β -pinene, γ -elemene, sabinene, elemol, and 3-cyclohexen-1-ol. Overall, the essential oils showed antimicrobial activities against all the six bacterial strains tested, with the highest antagonistic activity against *M. luteus* and *B. cereus*; while, methanolic crude extracts showed the highest activities against *S. aureus* and MRSA strains. The methanolic crude extracts demonstrated significantly higher antibacterial activity against the Gram-positive bacteria ($p < 0.005$); while, the essential oils of *Juniperus* did not show significant differences between Gram-positive and Gram-negative bacteria. Future studies are needed to investigate the active compounds present in the essential oils and methanolic crude extracts that confer the selectivity in the antimicrobial activity.

Liang, Q., Zhuang, Y., Ma, J., Wang, J., Feng, R., He, R., . . . Zhan, R. (2021). A rapid screening method for sibutramine hydrochloride in natural herbal medicines and dietary supplements. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/8889423>

Herbal weight loss drugs are becoming more widely used in the fight against obesity, but ineffective regulation of these products have resulted in harmful additives. These products may contain adulterants such as sibutramine hydrochloride that may result in serious adverse health events including death. This work established a color precipitation reaction-based rapid screening method for illegal adulteration of sibutramine hydrochloride in natural

herbal medicines (NHM) and dietary supplements (DS). While a variety of chromatography- and electrophoresis-based systems have been reported to measure this analyte, they generally suffer from high costs, complicated sample preparation, and a costly analytical infrastructure. In contrast, we present a simple, handheld kit to assay for sibutramine. The performance metrics of this tool include an average detection time of approximately 3 minutes, which is markedly shorter than conventional methods (HPLC or HPLC-MS, etc.), a detection limit of 0.1 mg per aliquot, and an accuracy of 99.02% (n=820). More strikingly, the sensitivity is 100% (n=278), and the specificity is 98.52% (n=542). The rapid test kit developed from this screening method was evaluated by FDA. In summary, this screening method is a rapid, simple, and low-cost tool for the detection of sibutramine in NHM and DS with superior selectivity and sensitivity. For these reasons, this method is especially suitable for underdeveloped settings because it can be employed onsite without any instrumentation. In addition, this approach could rapidly exclude most of the negative samples to boost efficiency in large-scale samples assay. If necessary, positive samples can undergo further alternate testing methods to confirm the positive results of sibutramine hydrochloride content.

Yu, G., & Fang, D. (2021). Evaluation of neuraminidase inhibitory activity of compounds and extracts from traditional medicines by HPLC-FLD. *International Journal of Analytical Chemistry*, 2021
doi:<https://doi.org/10.1155/2021/6694771>

A simple and effective method was established and validated to determine 4-methylumbelliferone (4-MU) for screening the natural neuraminidase inhibitors (NAIs) from traditional medicines (TMs) by high performance liquid chromatography combined with fluorescence detection (HPLC-FLD). 4-MU and TMs compounds were separated on a Heder TM ODS column (5 μ m, 4.6 $\times$ 250mm) using an isocratic elution of 55% methanol at 35°C. The flow rate was 1 mL min⁻¹. The excitation and emission wavelength were performed at 320nm and 480nm. Some extracts of TMs and compounds were selected as examples to demonstrate the feasibility of the new HPLC-FLD method. It was found that the results of most compounds except for the auto fluorescence substances determined by HPLC-FLD were in good agreement with NA enzyme-based inhibitory assays. Comparing to traditional NA enzyme-based inhibitory assays, the HPLC-FLD method could prevent interference from fluorescence pigments of compounds. It was considered a simple, effective, and economical technique for the screening the natural neuraminidase inhibitors from traditional medicines.

Wang, Y., Wang, X., Zhang, H., Jiang, J., & Fotina, H. (2021). Synthesis of zearalenone immunogen and comparative analysis of antibody characteristics. *International Journal of Analytical Chemistry*, 2021
doi:<https://doi.org/10.1155/2021/7109383>

Background. This study aimed to explore the zearalenone (ZEN) immunogen synthesis method, immunogenicity, and antibody characteristics and to lay a foundation for the establishment of immunoassay methods for ZEN single residue and ZEN and its analogs total residue. **Methods.** Based on the molecular structure and active sites of ZEN, oxime active ester (OAE), condensation mixed anhydride (CMA), formaldehyde (FA), and 1,4-butanediol diglycidyl ether method (BDE) were designed and used for immunogen (ZEN-BSA) synthesis. The immunogens were identified by infrared (IR) and ultraviolet (UV) spectra and gel electrophoresis (SDS-PAGE) and were then used to immunize Balb/c mice to prepare ZEN polyclonal antibody (ZEN pAb). The titers and sensitivity of the ZEN pAb were determined by indirect noncompetitive ELISA (inELISA) and indirect competitive ELISA (icELISA), respectively, and its specificity was assessed by the cross-reaction test (CR). **Results.** ZEN-BSA was successfully synthesized, and the molecular binding ratios of ZEN to BSA were 17.2:1 (OAE), 14.6:1 (CMA), 9.7:1 (FA), and 8.3:1 (BDE), respectively. The highest inELISA titers of ZEN pAb of each group were 1:(6.4 $\times$ 10³) (OAE), 1:(3.2 $\times$ 10³) (CMA), 1:(1.6 $\times$ 10³) (FA), and 1:(1.6 $\times$ 10³) (BDE), respectively. The 50% inhibition concentrations (IC₅₀) for ZEN by icELISA of each group were 11.67 μ g/L (OAE), 16.29 μ g/L (CMA), 20.92 μ g/L (FA) and 24.36 μ g/L (BDE), respectively. ZEN pAb from the mice immunized with ZEN-BSA (OAE) and ZEN-BSA (CMA) had class broad specificity to ZEN and its analogs. The CRs of ZEN pAb with α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZON were 36.53%, 16.98%, 64.33%, 20.16%, and 10.66%, respectively. ZEN pAb from the mice immunized with ZEN-BSA (FA) and ZEN-BSA (BDE) had high specificity for ZEN. The CRs of ZEN pAb with its analogs were all less than 1.0%. **Conclusion.** This study demonstrated that the preparation of the class broad-specificity antibodies of ZEN and its analogs can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the OAE method, while the preparation of highly

specific antibodies can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the FA method. These findings lay the material and technical foundation for immunoassay of ZEN single residue and ZEN and its analogs total residue.

Pan, X., Li, H., Chen, D., Zheng, J., Yin, L., Zou, J., . . . He, F. (2021). Comparison of essential oils of *Houttuynia cordata* Thunb. from different processing methods and harvest seasons based on GC-MS and chemometric analysis. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/8324169>

Houttuyniae Herba (HH) refers to the dried aerial part of *Houttuynia cordata* Thunb. (DHC) or the fresh whole grass of *Houttuynia cordata* Thunb. (FHC), where DHC are harvested in summer and FHC around the year. However, harvest seasons and processing methods (i.e., medicinal parts and drying process) might affect the quality of HH. To compare the essential oils (EOs) of DHC and FHC and their two harvest seasons, GC-MS analysis combined with chemometric analysis was applied. The results showed that the oil yield of FHC ($0.076 \pm 0.030\%$) was higher than that of DHC ($0.038 \pm 0.029\%$), and oil yield was higher in summer than in autumn ($0.044 \pm 0.029\%$ for DHC1, $0.036 \pm 0.028\%$ for DHC2, $0.084 \pm 0.026\%$ for FHC1, and $0.067 \pm 0.033\%$ for FHC2, respectively). Moreover, hierarchical cluster analysis (HCA) and principal component analysis (PCA) successfully distinguished the chemical constituents of DHC and FHC oils. Additionally, according to orthogonal partial least squares discriminant analysis (OPLS-DA), eleven components were selected as chemical markers for discriminating DHC and FHC, and two and four chemical markers for discriminating two harvest seasons of DHC and FHC, respectively. Among these markers, the average contents of α -pinene, limonene, β -phellandrene, α -terpineol, 4-tridecanone, and ethyl decanoate were higher in FHC oils. In contrast, the average contents of nonanal, 1-nonanol, β -cyclocitral, n-hexadecanoic acid, and octadecanol were higher in DHC oils. Additionally, the contents of 4-tridecanone and ethyl decanoate were both higher in DHC1 oils than in DHC2 oils. Moreover, the contents of β -myrcene and β -phellandrene were higher in FHC1 oils, while the contents of 2,6-octadien-1-ol, 3,7-dimethyl-, acetate, and (z)-phytol were higher in FHC2 oils. For these reasons, this study provides a scientific basis for quality control and clinical medication.

Jia, X., Hu, C., Zhu, X., Yuan, Y., & Zhou, Y. (2021). Simultaneous determination of 25 ginsenosides by UPLC-HRMS via quantitative analysis of multicomponents by single marker. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9986793>

A method using UPLC-HRMS has been developed for a rapid, simultaneous qualitative and quantitative analysis of twenty-five ginsenosides. Chromatographic separation was achieved on a C18 analytical column with an elution gradient comprising 0.1% aqueous formate/acetonitrile as the mobile phase. HRMS detection acquired full mass data for quantification and fullms-ddms2 (i.e., data-dependent scan mode) yielded product ion spectra for identification. Furthermore, quantitative analysis of multiginsenosides by single marker (QAMS) was developed and validated using a relative correction factor. Under optimal conditions, we could simultaneously separate eight groups of isomers of the 25 ginsenosides. Good linearity was observed over the validated concentration range for each analyte ($r^2 > 0.9924$), showing excellent sensitivity (LODs, 0.003–0.349 ng/mL) and lower limit quantification (LOQs, 0.015–1.163 ng/mL). The LC-MS external standard method (ESM) and QAMS were compared and successfully applied to analyze the ginsenoside content from *Panax ginseng* roots. Overall, our UPLC-HRMS/QAMS approach provides high precision, stability, and reproducibility and can be used for high-throughput analysis of complex ginsenosides and quantitative analysis of multiple components and quality control of traditional Chinese medicines (TCM).

Lu, Q., Yuan, N., Han, G., Cheng, B., Zhang, D., Song, J., & Mu, Y. (2021). Determination of pendimethalin dynamic residual distribution in crucian carp tissues and associated risk assessment. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9984230>

Pendimethalin has been considered a moderately to extremely toxic compound for fish and aquatic organism. This study developed the determination of dynamic residual distribution for pendimethalin in crucian carp tissues (muscle, liver, kidney, gill, and blood) under semistatic exposure system by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method. The pendimethalin residues in various fish tissues increased initially

and then decreased, and the residue amount of pendimethalin varied from tissue to tissue of crucian carp. Particularly, the pendimethalin accumulation in most fish tissues made significant decreases at two-time points. Pendimethalin was initially absorbed and enriched by fish body, and then partial pendimethalin was discharged into the outside environment through the metabolism function of crucian carp. The residue levels of pendimethalin distributed in crucian carp were ranked in the following decreasing order: liver>kidney>gill or muscle>blood, attributed to the fact that pendimethalin tends to accumulate in lipid-rich tissues of fish. Risk assessment results indicated that the chronic risk from dietary exposure to pendimethalin through crucian carp consumption for Chinese residents was acceptable, along with a lower estimated exposure dose (EED) than acceptable daily intake (ADI) and risk quotient (RQ) < 1. This study performed the first analysis for pendimethalin residual distribution in crucian carp tissues under semistatic exposure condition and provided a reference for pollution control and risk assessment of pendimethalin aimed at aquatic products.

Saoudi, M. M., Bouajila, J., Rahmani, R., & Alouani, K. (2021). Phytochemical composition, antioxidant, antiacetylcholinesterase, and cytotoxic activities of *Rumex crispus* L. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/6675436>

Rumex crispus L. (*R. crispus*) is regarded as an aromatic plant. It was used for its excellent biological properties in traditional medicine. The aerial part was extracted successively by maceration with three solvents increasing polarity (cyclohexane (CYH), dichloromethane (DCM), and methanol (MeOH)) to evaluate their chemical compositions and biological activities. The extracts were rich in phenolic compounds (13.0 to 249.8 mg GAE/g of dry weight (dw)). The MeOH extract has presented remarkable IC₅₀=6.2 µg/mL for anti-DPPH and 31.6 µg/mL for anti-AChE. However, the DCM extract has the highest cytotoxic activity against the two cancer cells (HCT-116 and MCF-7) (69.2 and 77.2% inhibition at 50 µg/mL, respectively). Interestingly, GC-MS analysis enabled to identify three new compounds in *R. crispus* extracts, such as L-(-)-arabitol (5), D-(-) fructopyranose (7) detected only in MeOH extract, and 2, 5-dihydroxyacetophenone (3) detected in all extracts. For HPLC chromatograms, cardamomin (8), 5-hydroxy-3'-methoxyflavone (17), and 3'-hydroxy-b-naphthoflavone (18) showed the highest concentrations of 74.0, 55.5, and 50.4 mg/g of dw, respectively, among others who are identified. Some phenolic compounds were identified and quantified by HPLC in more than one organic extract, such as 4', 5-dihydroxy-7-methoxyflavone (13), 4', 5-dihydroxy-7-methoxyflavone (14), 5-hydroxy-3'-methoxyflavone (17), and 3'-hydroxy-b-naphthoflavone (18), were found for the first time in the *R. crispus* extracts. Our results showed that the biological activities of this plant might be linked to their phenolic compounds and that the polar extracts could be considered as new natural supplements to be used in food and pharmaceuticals.

Kerrou, M., Bouslamti, N., Raada, A., Elanssari, A., Mrani, D., & My, S. S. (2021). The use of sugarcane bagasse to remove the organic dyes from wastewater. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5570806>

In the present study, the potential of sugarcane bagasse (SCB) was evaluated by methylene blue (MB) retention. The selected low-cost adsorbent was characterized by scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), BET method, and determination of the point of zero charge (pH_{zpc}). Batch kinetic and isothermal studies were performed to examine the effects of contact time, initial dye concentration, adsorbent dose, pH, and temperature. The results show that the kinetic study of MB adsorption on sugarcane bagasse is very fast; the equilibrium is reached after only 20 minutes. The kinetic model of pseudo-second-order and the Langmuir isotherm model perfectly explain the adsorption process of MB with a monolayer adsorption capacity equal to 49.261 mg·g⁻¹ activation parameters' values such as free energy (ΔG°), enthalpy (ΔH°), and entropy (ΔS°) also determined as -4.35 kJ·mol⁻¹, -31.062 kJ·mol⁻¹, and -0.084 J·mol⁻¹·K⁻¹, respectively. Besides, the thermodynamic parameters of the methylene blue sugarcane bagasse system indicate that the exothermic adsorption process is spontaneous.

Chen, F., Fang, B., Li, P., & Wang, S. (2021). A fast and validated HPLC method for the simultaneous analysis of five 5-HT₃ receptor antagonists via the quantitative analysis of multicomponents by a single marker. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5533646>

In this study, a new strategy for the simultaneous quantization of five serotonin 5-hydroxytryptamine receptor antagonists—ondansetron, azasetron, ramosetron, granisetron, and tropisetron—either in infusion samples or in injection dosage form was first established based on high-performance liquid chromatography combined with a quantitative analysis of multiple components by a single marker. The quantitative analysis of multicomponents by a single marker method was conducted with ondansetron as an internal reference substance and performed using relative retention time and ultraviolet spectral similarity as the double indicator. The quantitative analysis of the 5-HT₃ receptor antagonists was calculated and investigated based on the relative correction factors. Chromatographic separation was achieved using a C18 column (150mm×4.6mm, 5.0μm), and the mobile phase was composed of acetonitrile-0.05mol·L⁻¹ potassium dihydrogen phosphate (pH 4.0) (25:75) at a flow rate of 1.0mL·min⁻¹ and detection wavelengths of 307nm (ondansetron, azasetron, ramosetron), 302nm (granisetron), and 285nm (tropisetron). In addition, the accuracy of the quantitative analysis of multicomponents by a single marker method was compared with an external standard method, and no significant difference was observed between the two methods. The established method is rapid, is easy, and does not require many reference substances, and it can be successfully applied as part of the quality control of the five 5-HT₃ receptor antagonists in their injection dosage form and infusion sample drugs in hospitals.

Mensah, J. N., Brobbey, A. A., Addotey, J. N., Ayensu, I., Asare-Nkansah, S., Opuni, K. F. M., & Adutwum, L. A. (2021). Ultraviolet-visible spectroscopy and chemometric strategy enable the classification and detection of expired antimalarial herbal medicinal product in ghana. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/5592217>

To meet the growing demand for complementary and alternative treatment for malaria, manufacturers produce several antimalarial herbal medicinal products. Herbal medicinal products regulation is difficult due to their complex chemical nature, requiring cumbersome, expensive, and time-consuming methods of analysis. The aim of this study was to develop a simple spectroscopic method together with a chemometric model for the classification and the identification of expired liquid antimalarial herbal medicinal products. Principal component analysis model was successfully used to distinguish between different herbal medicinal products and identify expired products. Principal component analysis showed a clear class separation between all five herbal medicinal products (HMP) studied, with explained variance for first and second principal components as 37.51% and 26.38%, respectively, while the third principal component had 18.74%. Support vector machine classification gave specificity and accuracy of 1.00 (100%) for training set data for all the products. The validation set HMP1, HMP2, and HMP3 had sensitivity, specificity, and accuracy of 1.00. HMP4 and HMP5 had sensitivity and specificity of 0.90 and 1.00, respectively, and an accuracy of 0.98. The support vector machine classification and principal component analysis models were successfully used to identify expired herbal medicinal products. This strategy can be used for rapid field detection of expired liquid antimalarial herbal medicinal products.

Ho, J. K., Mi, J. L., Hur, S. H., & Jeong, M. H. (2021). Development of a gas chromatography-time-of-flight method for detecting glucosinolate metabolites and volatile organic compounds in kimchi. *International Journal of Analytical Chemistry*, 2021 doi:<https://doi.org/10.1155/2021/9978251>

Database copyright © 2023 ProQuest LLC. All rights reserved.

[Terms and Conditions](#) [Contact ProQuest](#)