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Quantification of Rhodojaponin II and Rhodojaponin III in Rat Plasma by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT (ENGLISH)

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was developed to determine the concentrations of Rhodojaponin II and Rhodojaponin III in rat plasma, and their pharmacokinetic profiles were investigated. A UPLC HSS T3 (2.1 mm × 50 mm, 1.8 μm) chromatographic column was employed at a temperature of 40°C. The mobile phase consisted of acetonitrile-0.1% formic acid in water, and a gradient elution method with an elution time of 6 min and flow rate of 0.4 mL/min was utilized for analysis purposes. Methodological investigations were conducted accordingly. The plasma concentrations of Rhodojaponin II and Rhodojaponin III exhibited excellent linearity within the range of 2 ng/mL–1250 ng/mL. Moreover, both intraday and interday precision were below 15%, while accuracy ranged from 88% to 115%. Additionally, matrix effect fell within the range of 90%–110%, and recoveries ranged from 78% to 87%. These results comply with relevant regulations for drug analysis in biological samples. Therefore, this method is deemed suitable for quantifying Rhodojaponin II and Rhodojaponin III levels in rats.

FULL TEXT

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1. Introduction

Rhododendron molle (Blum) G. Don, a species of shrub, has gained popularity as an ornamental plant in recent years and is widely recognized by the public [1]. However, its medicinal properties derived from the roots, flowers, and fruits remain relatively unknown. Extensive research has demonstrated that each medicinal component of this plant exhibits diverse effects such as anti-inflammatory and analgesic properties, antipyretic activity, and hypotensive effects [2]. Despite its therapeutic potential, it should be noted that *Rhododendron molle* possesses significant toxicity with no specific treatment available; hence, clinical management primarily focuses on symptomatic relief [3]. Toxic symptoms include nausea, vomiting, diarrhea, bradycardia, hypotension, dyskinesia, and dyspnea which can lead to fatal outcomes in severe cases [1]. Consequently, the flowers are categorized as toxic herbs requiring careful handling. Studies have identified diterpenoids like Rhodojaponin II and Rhodojaponin III as the primary active and toxic constituents of *Rhododendron molle* (Blum) G. Don [4–6]. Diterpenoids exhibit potent insecticidal properties while also reducing blood pressure and heart rate along with inhibiting inflammatory responses. Additionally, it demonstrates remarkable analgesic efficacy. Notably, Rhodojaponin III surpasses morphine in terms of acute pain models as well as inflammatory pain models. Its potency for diabetic neuropathic pain models is 100 times greater than gabapentin's effectiveness [2, 7]. However, it was discovered that both

intraperitoneal administration and oral consumption of Rhodojaponin III resulted in severe acute toxicity in mice. Moreover, it was implicated as one of the components associated with cardiotoxicity observed in rats treated with orally administered *Rhododendri Mollis* Flos extract. To date, research on Rhodojaponin II and Rhodojaponin III remains limited.

The present study establishes an analytical method based on ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and successfully applies it to determine the concentrations of Rhodojaponin II and Rhodojaponin III in rat plasma. This approach aims to elucidate the metabolic characteristics of these compounds in rats, providing valuable insights for their rational use in clinical practice.

2. Experimental

2.1. Reagents

Chromatographically pure acetonitrile and methanol were procured from Merck GmbH (Darmstadt, Germany). Chromatographically pure formic acid was obtained from Tedia Ltd. (Ohio, USA). Rhodojaponin II and Rhodojaponin III (purity >98%, Figure 1) were sourced from Chengdu Manster Biotechnology Co. (Chengdu, China). Ultrapure water was generated using the Millipore Milli-Q purification system (Bedford, MA, USA).

[figure(s) omitted; refer to PDF]

2.2. Animals

The healthy male Sprague–Dawley (SD) rats weighing 200 g ± 20 g was procured from the Experimental Animal Center of Wenzhou Medical University.

2.3. Equipment

Waters Xevo TQ-S Micro mass with ACQUITY H-Class UPLC (Waters Corp., Milford, MA, USA) was utilized in this study. Data acquisition and instrument control were performed using Masslynx 4.1 software (Waters Corp., Milford, MA, USA). Additionally, a Multifuge XIR low-temperature high-speed centrifuge (Hermos, Germany) was employed.

2.4. Chromatographic Conditions

The UPLC analysis was performed using a HSS T3 column (2.1 mm × 50 mm, 1.8 μm) at a controlled temperature of 40°C. The mobile phase consisted of acetonitrile-0.1% formic acid in water, and the flow rate was maintained at 0.4 mL/min throughout the experiment. A total run time of 6.0 min was employed, following the gradient elution process outlined in Table 1.

Table 1

Structure of gradient elution mobile phases.

Runtime (min)	Acetonitrile (v/v%) (%)	0.1% formic acid in water (v/v%) (%)
0–0.2	10	90
0.2–2.4	10–75	90–25
2.4–5.0	75–90	25–10
5.0–5.1	90–10	10–90
5.1–6.0	10	90

2.5. Mass Spectrometry Conditions

Nitrogen was used as the desolventization gas (800 L/h) and cone gas (50 L/h). The capillary voltage was set at 2.2 kV, while the ion source temperature was maintained at 150°C. Desolventization was carried out at a temperature of 400°C using electrospray ionization (ESI) as the ion source.

The daughter ions of Rhodojaponin II and Rhodojaponin III were not obvious (Figure 2), and then the mother ions

were selected. Positive ion detection and multiple reaction monitoring (MRM) were employed for analysis, with Rhodojaponin II m/z 455.2 455.2 and Rhodojaponin III m/z 413.2 413.2 being the selected ions for quantitative analysis, Figure 3.

[figure(s) omitted; refer to PDF]

2.6. Sample Pretreatment

The plasma sample (50 μL) was transferred into a 1.5 mL Eppendorf tube, followed by the addition of acetonitrile (200 μL). After vortexing for 1.0 min, the mixture was centrifuged at 13,000 rpm for 10 min at 4°C. A volume of 2 μL from the resulting supernatant was injected for subsequent analysis.

2.7. Preparation of Standard Curve

The Rhodojaponin II and Rhodojaponin III standards were accurately weighed, and a stock solution of Rhodojaponin II (0.5 mg/mL) and Rhodojaponin III (0.5 mg/mL) was prepared using methanol. Subsequently, the stock solution of Rhodojaponin II and Rhodojaponin III was diluted with acetonitrile to generate a series of standard working solutions. The plasma standard curves for Rhodojaponin II and Rhodojaponin III were established by combining blank rat plasma with an appropriate amount of standard working solution within the concentration range of 2–1250 ng/mL.

2.8. Method Validation

The UPLC-MS/MS method was validated according to the international regulatory guidelines (selectivity, lower limit of quantitation, accuracy, intraday/interday precision, matrix effect, recovery, and stability) [8, 9].

2.9. Pharmacokinetics

The pharmacokinetic study protocol was approved by the Laboratory Animal Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (WYYY-AEC-2023-144). Due to the inherent variability of SD rats, twelve rats were divided into two groups. The disparity in toxicity between Rhodojaponin II and Rhodojaponin III necessitates the need for refinement. Then, one group received intravenous injections of Rhodojaponin II at a dose of 0.5 mg/kg, while the other group received intravenous injections of Rhodojaponin III at a dose of 0.25 mg/kg. Blood samples (0.300 mL/rat/at one time point) were collected from the caudal vein at ten time points (0.0833 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h). These samples were centrifuged at a rate of 3,000 revolutions per minute for ten minutes at −4°C; the obtained plasma samples were then stored at −20°C.

3. Results and Discussion

3.1. Selectivity

The blank plasma of rats was utilized and supplemented with Rhodojaponin II and Rhodojaponin III separately. Following the plasma treatment protocol outlined in Section 2.6, a specificity examination was conducted to obtain the corresponding chromatography, as depicted in Figure 4. No endogenous and cross-talk interferences was found in the retention time of Rhodojaponin II (tR = 2.11 min) and Rhodojaponin III (tR = 1.80 min).

[figure(s) omitted; refer to PDF]

3.2. Standard Curve and Lower Limit of Quantification

The solutions at concentrations of 2, 5, 12, 50, 125, 250, 500, and 1250 ng/mL were prepared by adding Rhodojaponin II and Rhodojaponin III to blank rat plasma (100 μL) following the “sample handling” procedure. Subsequently, the samples were treated and their spectra recorded. Based on the calibration curves, the lowest concentration was identified as the lower limit of quantification (LLOQ). For Rhodojaponin II, the equation $y = 4.8558x + 70.633$ ($r = 0.9991$) was obtained, whereas for Rhodojaponin III it was $y = 2.968x + 112.06$ ($r = 0.9991$), where x represents the analyzed concentration and y denotes peak area. LLOQ of Rhodojaponin II and Rhodojaponin III was 2 ng/mL.

3.3. Accuracy, Precision, Matrix Effect, and Recovery

Four quality control (QC) samples with plasma concentrations of 2, 4, 100, and 1000 ng/mL were prepared for UPLC-MS/MS analysis following the same procedure as the standard curve preparation. Accuracy, precision, matrix effect, and recovery were assessed through six simultaneous preparations at each concentration. The precision for both intraday and interday measurements was below 15%, while the accuracy ranged from 88% to 115%.

Rat blank plasma was extracted and supplemented with Rhodojaponin II and Rhodojaponin III at concentrations of 2, 4, 100, and 1000 ng/mL (n=6) in order to evaluate the matrix effect. The matching peak areas were then contrasted with those derived from pure standard solutions at comparable concentrations [10]. By comparing the peak area of extracted quality control samples with that of reference quality control solutions reconstituted in blank plasma extracts (n=6), the recovery of both Rhodojaponin II and Rhodojaponin III was assessed. The matrix effect fell within the range of 90%–110%, and the recovery exceeded 78%. These values meet the criteria for drug analysis in biological samples outlined in Table 2.

Table 2

Accuracy, precision, matrix effect, and recovery of Rhodojaponin II and Rhodojaponin III in rat plasma (n=6).

Compound	Concentration (ng/mL)	Accuracy (%)		Precision (RSD%)		Matrix effect (%)	Recovery (%)
Intraday	Interday	Intraday	Interday	Rhodojaponin II	2	114.8	88.4
13.1	13.4	102.0	83.0	4	92.6	98.7	11.2
12.4	101.1	84.5	100	106.6	96.7	9.4	4.5
106.2	86.6	1000	97.2	101.3	6.5	9.0	109.2
85.4	–						
Rhodojaponin III	2	95.5	97.2	12.7	14.7	90.5	81.7
4	101.5	107.2	9.3	8.4	100.1	83.6	100
100.1	109.2	7.8	10.3	106.8	78.8	1000	103.1

3.4. Stability

The plasma samples (4, 100, and 1000 ng/mL) were subjected to three cycles of freezing and thawing after pretreatment, followed by incubation at room temperature for 24 hours. A stability test was conducted at −20°C for a duration of 30 days to assess their stability. Rhodojaponin II and Rhodojaponin III exhibited an accuracy range of 86–112%, with a within-group standard deviation of 13%. Based on these findings, both Rhodojaponin III and Rhodojaponin II demonstrated excellent stability.

The stability of the Rhodojaponin II (1000 ng/mL) and Rhodojaponin III (1000 ng/mL) standard solution tests was conducted at room temperature for 24 hours, and at −20°C for a duration of 30 days. It exhibited an accuracy range of 90–108%, with a standard deviation of 10%.

3.5. Pharmacokinetic Study

Six rats were administered 0.5 mg/kg of Rhodojaponin II via the sublingual route, while another six rats received 0.25 mg/kg of Rhodojaponin III using the same administration method. The pharmacokinetic parameters were determined utilizing UPLC-MS/MS and analyzed employing Drug and Statistics (DAS) 2.0 software (Wenzhou Medical University, Wenzhou, China), as presented in Table 3, and concentration-time curve of rats is shown in Figure 5.

Table 3

Main pharmacokinetic parameters after intravenous administration of Rhodojaponin II (iv, 0.5 mg/kg) and Rhodojaponin III (iv, 0.25 mg/kg) in rats (n=6).

Compound	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	t1/2z	CL_z	Vz	Cmax
ng/mL*h	ng/mL*h	h	L/h/kg	L/kg	ng/mL	Rhodojaponin II
550.2±97.6	631.0±153.2	7.6±4.3	0.8±0.2	8.5±4.1	133.9±29.9	Rhodojaponin III

AUC: area under the plasma concentration-time curve; t1/2: half-life; CL: plasma clearance; *V*: apparent volume of distribution; Cmax: maximum plasma concentration.

[figure(s) omitted; refer to PDF]

The concentration of Rhodojaponin III was determined using an UPLC-MS/MS method by Zhang et al., which was developed and validated over a range of 1–200 ng/mL. This method was then applied to investigate the pharmacokinetics of Rhodojaponin III in mice following intravenous (0.06 mg/kg) or oral (0.24 mg/kg) administration [3]. The results demonstrated rapid oral absorption of Rhodojaponin III with a time to peak concentration of 0.08 h, as well as favorable oral bioavailability (73.6%). Furthermore, both intravenous and oral administration resulted in quick elimination of Rhodojaponin III, with half-life values of 0.19 h and 0.76 h, respectively [3]. In contrast, the half-life (t1/2) of Rhodojaponin III after intravenous administration at a dose of 0.25 mg/kg was found to be 2.6±1.3 h in this study.

Dong et al. developed a LC-MS method to detect the rat plasma concentrations of three major Rhodojaponins (Rhodojaponin II, and III) [11]. Notably, the pharmacokinetic parameters [(area under the plasma concentration-time curve (AUC), half-life (t1/2), time to reach maximum plasma concentration (tmax), maximum plasma concentration (Cmax))] differed significantly among Rhodojaponin II, and III. After oral administration of *Rhododendri Mollis* Flos extract at doses of 21.44 mg/kg and 112.56 mg/kg respectively, the t1/2 values for Rhodojaponin II they were 133.74 ±66.05 min and 215.96±163.68 min; while for Rhodojaponin III they were 83.69±39.57 min and 219.63±91.11 min. While in this study, after intravenous administration, the t1/2 of Rhodojaponin II was determined to be 7.6±4.3 h following intravenous administration, and the t1/2 of Rhodojaponin III was found to be 2.6±1.3 h after intravenous administration, Table 3.

4. Conclusion

The UPLC-MS/MS method established for the determination of Rhodojaponin II and Rhodojaponin III in rats demonstrates desirable attributes, including specificity, accuracy, precision, matrix effect, and stability. These methods fulfill the methodological requirements and are suitable for high-throughput detection. This study showcases the potential of integrated pharmacokinetics and provides a valuable reference for a more comprehensive understanding of the pharmacokinetic behavior of *Rhododendron molle* (Blum) G. Don and its efficacy.

Authors' Contributions

Cheng Sun and Wanhang Wang conceptualized the study, investigated the study, and proposed the methodology. Xi Bao, Dizhong Chen, and Shenshen Mei performed formal analysis. Xi Bao and Xiajuan Jiang collected resources. Jianshe Ma and Xiajuan Jiang wrote the original draft of the manuscript and supervised the study. Xi Bao and Jianshe Ma reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript. Cheng Sun and Wanhang Wang contributed equally to this work and are the co-first authors.

Acknowledgments

This study was supported by grants from the Wenzhou Science and Technology Bureau (Y2020881).

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DETAILS

Subject:	Mass spectrometry; Acetonitrile; Formic acid; Plasma; Accuracy; Toxicity; Elution; Liquid chromatography; Quality control; Biological properties; Scientific imaging; Chromatography; Laboratory animals; Analgesics
Business indexing term:	Subject: Quality control
Location:	United States--US; Germany
Company / organization:	Name: Waters Corp; NAICS: 334516

Editor:	Danilo Corradini
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-12-22 (Received); 2024-04-20 (Revised); 2024-06-06 (Accepted); 2024-06-18 (Pub)
DOI:	https://doi.org/10.1155/2024/6386570
ProQuest document ID:	3073789064
Document URL:	https://www.proquest.com/scholarly-journals/quantification-rhodojaponin-ii-iii-rat-plasma/docview/3073789064/se-2?accountid=211160
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Last updated:	2024-07-01
Database:	Publicly Available Content Database

Residents' Knowledge regarding Recreational Drug Screening Immunoassays at a Swiss Hospital Group

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ABSTRACT (ENGLISH)

Introduction. In case of suspected acute recreational drug toxicity, immunoassays are commonly used as diagnostic tools. Although easy to handle, understanding of their limitations is necessary for a correct interpretation of the results. The aim of this project was to investigate residents' knowledge regarding drug screening immunoassays at a Swiss hospital group. *Methods*. All residents of a large hospital group in Switzerland were invited by e-mail to participate in an anonymous survey. Following ten multiple choice questions on drug screening tests, the participants were also asked about their demographics, whether they used drug screening tests on a regular basis, and how confident they felt in their ability to interpret test results. *Results*. The ten knowledge questions were answered by 110 of the 1026 residents (11%). Among the 108 participants with available demographics, 90% were 25–35 years old, 63% were female, and 70% were at least in their 4th year of residency. The median score of correct answers was 4 out of 10 (range 0–7) and in 50% of the questions, the correct answer was the most frequently selected response. No significant differences in the knowledge scores were found based on the training, confidence level, or the frequency of drug tests used in daily work. *Conclusion*. This survey revealed widespread knowledge gaps among residents regarding the interpretation of immunoassay-based drug test results. These findings can be used to implement educational measures on this topic and might provide a basis for targeted information on common pitfalls to be included in laboratory reports.

FULL TEXT

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1. Introduction

In patients presenting with suspected acute recreational drug toxicity, drug screening tests can provide a helpful diagnostic tool and are commonly used in emergency departments. Although more specific analytical methods such as liquid or gas chromatography coupled with tandem mass spectrometry [1–3] can detect a large number of substances with high sensitivity and specificity, such methods are more expensive, need specialized personnel, and the results are commonly not available during the acute patient management. Therefore, other analytical methods, i.e., immunoassays, are the tests that are commonly used in most emergency departments. These tests are easy to perform and the results are quickly available. However, these immunoassays use antibodies to qualitatively determine the presence of a specific substance or substance group and have several limitations. For example,

especially for the group of amphetamines, false-positive results due to cross-reactivity are very common [4], while a positive result for substances such as tetrahydrocannabinol (THC), whose main metabolite THC-COOH can be detected in urine for weeks after chronic use in some cases, does not necessarily represent acute use [5]. Furthermore, each commercially available immunoassay can detect a specific number of substances or substance groups, and if the suspected substance is not of the test panel, the test will be negative even in case of acute intoxication. Commonly included substances besides THC are cocaine, opiates, and amphetamines, but depending on the assay, other substances or substance groups such as benzodiazepines or phencyclidine (PCP) can also be included. Importantly, novel psychoactive substances such as cathinones and synthetic cannabinoid receptor agonists that have emerged in the last years [6] can typically not be detected with the commonly used drug screen immunoassays.

Due to these aspects, the notion that these immunoassays are easy to use might also be misleading. Although easy to handle (e.g., no special instruments are needed), knowledge of the characteristics of the test and the properties of the suspected substances is necessary for a correct interpretation of the results. Depending on the clinical situation, a wrong interpretation might remain without consequences or go unnoticed (e.g., if the test result does not affect the clinical management), or it could lead to a misdiagnosis and jeopardize patient management (e.g., in the case of a false-negative result) or strain the patient-physician relationship (e.g., in the case of a false-positive result). Prior surveys from other countries among family and primary care physicians [7, 8] and internal medicine residents [9] have demonstrated that a considerable proportion of the participants were not adequately trained regarding the interpretation of such tests thus highlighting the importance of educational measures focussing on this topic.

The aim of this project was to investigate residents' knowledge regarding the interpretation of drug screening immunoassays at the Insel Hospital Group including the University Hospital Bern, a tertiary hospital and one of the biggest healthcare providers in Switzerland, in order to provide a basis for strategies to optimize residents' training regarding drug screening tests.

2. Materials and Methods

The survey was conducted electronically using the SurveyMonkey tool which is supported by the University Hospital Bern. The Ethics Committee of the canton Bern reviewed the study and exempted it from approval (Req-2022-00979). Potential participants (all resident physicians employed by the Insel Hospital Group irrespective of discipline) were invited by e-mail to voluntarily and anonymously participate. No power analysis was performed for this descriptive, cross-sectional study, and the final number of included participants depended on the return rate.

The final questionnaire consisted of questions modified from previous similar surveys [7, 9] and questions created by two of the authors based on experiences from clinical practice. Before distribution, the questionnaire was sent to physicians active in the fields of clinical pharmacology and toxicology ($n=8$), emergency ($n=1$), internal medicine ($n=1$), and anesthesiology ($n=1$) for pilot testing regarding clarity. The final questionnaire was distributed by e-mail with a link to the electronic survey, followed by a reminder e-mail after two weeks. The survey was open for a total of one month (from January 30 to February 28, 2023).

The participants could choose between a German and a French version of the questionnaire. A short description of the drug screening test used at the University Hospital Bern ("Triage® TOX Drug Screen," manufactured by Quidel Cardiovascular Inc., San Diego, USA), urine immunoassay for amphetamines, methamphetamines, barbiturates, benzodiazepines, cocaine, methadone metabolite (EDDP), opiates, tetrahydrocannabinol (THC), and tricyclic antidepressants [10], which represents a typical panel included in clinically used urine drug tests [11]) was included in the preface. Participants were then asked to answer ten multiple choice questions, which referred to typical urine drug tests such as the one used at our institution, unless otherwise specified. For each question, five possible answers were provided and to continue, one answer had to be chosen. As a result of this requirement, there were no missing data in the survey answers to the knowledge questions. Following the 10 questions on drug test interpretation, a series of general and demographic questions was asked, including participant's sex, age group, current hospital department and position (resident or senior resident), current year of training, whether they use or order drug screening tests on a regular basis, how confident they feel in their ability to interpret the results of urine

drug tests (5-point Likert scale, 1 = not at all confident and 5 = very confident), and how many standard urine drug screening tests they already have ordered, performed, or interpreted during their medical training (0, 1–100, or >100).

The primary outcome was the knowledge score, calculated by giving one point for each correct answer (maximum possible score: 10). Numerical data are presented as median and range if not normally distributed and categorical data as number of cases and percentages. Differences between the two groups were explored using the Mann–Whitney test for nonnormally distributed variables and a p value of <0.05 was considered statistically significant. The association between sex, training level, confidence, and prior experience with drug tests as independent variables and the primary outcome (knowledge score) as the dependent variable was investigated using multiple regression models. We used both a linear regression model with the primary outcome (the 10-item knowledge score) expressed as a continuous variable and an ordered logistic regression model with the knowledge score expressed as an ordinal variable. A test for internal consistency was performed a posteriori for the 10-item questionnaire using McDonald's omega. Analyses were conducted using the R statistical package (version 4.1.2), R package "psych" and RStudio (2021.09.02). Data visualization was performed with GraphPad Prism version 8.0.1 (GraphPad Software, La Jolla California, CA).

3. Results

The questionnaire was sent to 1026 residents working at the Insel Hospital Group. Among these, 110 (10.7%) completed the questionnaire on drug test knowledge and 108 also provided data on training and demographics (Table 1).

Table 1

Participants' demographics and training characteristics (*N* = 108).

	n (%)
Sex	
Female	68 (63.0)
Male	38 (35.2)
Diverse/other nonbinary/no answer	2 (1.9)
–	
Age	
<25 years	1 (0.9)
25–30 years	39 (36.1)
31–35 years	58 (53.7)
>35 years	10 (9.3)
–	

Year of residency	
1 year	13 (12.0)
2 year	4 (3.7)
3 year	15 (13.9)
4 year	20 (18.5)
5 year	23 (21.3)
6 year	15 (13.9)
>6 year	18 (16.7)
–	
Position	
Resident	97 (89.8)
Senior resident	11 (10.2)
–	
Use or order of drug screening tests on a regular basis	
Yes	20 (18.5)
No	88 (81.5)
–	
Number of standard urine drug screening tests ordered, conducted, or evaluated	
0	27 (25.0)
1–100	80 (74.1)
>100	1 (0.9)

Thirty-four participants (31.5%) were from the General Internal Medicine department, followed by residents in paediatrics (*n*=12, 11.1%), neurology (*n*=8, 7.4%), and anesthesiology (*n*=7, 6.5%). The departments of angiology, diabetology and endocrinology, intensive care medicine, emergency medicine (adults), orthopaedic and traumatology, and pneumology had four participants each, while departments with fewer participants were gynecology (three participants, 2.8%), surgery, neurosurgery, gastroenterology, emergency medicine (children),

visceral surgery, and “other” (two participants (1.9%) each), otorhinolaryngology, hematology, infectiology, oral and maxillofacial surgery, nephrology, osteoporosis, and radiology (one participant (0.9%) each).
The participants’ own perception regarding their ability to interpret the results of urine drug tests is shown in Figure 1; the results of the ten multiple choice knowledge questions are presented in Figure 2. The median of correct answers to the ten knowledge questions was 4 (range: 0–7), and the score distribution is shown in Figure 3. The McDonald’s omega (total) was 0.39, indicating a low internal consistency.
[figure(s) omitted; refer to PDF]
On bivariate analysis, there were no significant differences in the knowledge score between participants with 1–3 and ≥4 years of residency (p=0.59), confidence level 1-2 and ≥3 (p=0.72), or based on the use or order of drug tests on a regular basis (p=0.51). There were also no significant differences in the knowledge score when stratifying the analysis by sex or when accounting for sex, training, confidence, and drug test use in multivariable models.

4. Discussion

In this anonymous, electronic survey, resident physicians at the Insel Hospital Group most frequently selected the correct answer in 5 of the ten questions. More than one-third of the participants did not feel confident at all in their ability to interpret drug screening results and the majority of the participants had a knowledge score of <5/10 points. These results are broadly in line with prior research on drug knowledge in general practitioners, internal medicine residents, psychiatry residents and fellows, as well as pediatricians [7–9, 12]. As these studies were conducted several years ago and had a more narrow focus (e.g., on specific drugs such as opioids or specific medical disciplines), the findings of this survey expand prior observations and suggest that the poor proficiency in drug test interpretation remains an ongoing issue for several substances included in a typical urine drug test panel and is generalizable to the setting of a hospital group (including a large tertiary care university center as well as community hospitals), encompassing various disciplines and levels of training.
Similar to the studies of Reisfeld et al. [7], Starrels et al. [9], and Suzuki et al. [12], knowledge was not associated with prior experience in drug test use. However, in contrast to Starrels et al. who saw significant differences in knowledge between interns and more experienced residents and divergent correlations between knowledge and confidence when stratifying according to sex (negative correlation in male participants and positive correlation in female participants), neither sex nor training level were associated with the knowledge score in our study. The lack of effect of both professional experience and prior drug test use on knowledge likely reflects in part the insufficient emphasis placed on drug test interpretation during medical education and training.
One aspect that seems to cause confusion is the ability of the usual immunoassays to detect and differentiate between various opiates (naturally occurring substances such as morphine) and synthetic opioids, such as methadone and fentanyl. When using an immunoassay drug test that can detect “opiates” as a substance group, a positive result is expected after morphine use, but not following the use of synthetic substances such as oxycodone or fentanyl [9, 12]. Immunoassay tests for synthetic opioids such as methadone and its metabolite EDDP are available, but these results are provided separately from the “opiates” group, which would be expected to be negative in the case of the use of methadone only. Immunoassays for both methadone and EDDP are commonly used in the context of checking for compliance with substitution therapy. A positive result for methadone and EDDP indicates therapy adherence, a negative result for both would represent no consumption, a positive result for EDDP only indicates fast metabolism (genetically or due to interaction with other substances), and a positive result for methadone only indicates sample adulteration by the addition of methadone directly into the urine [13]. When using immunoassays for detecting opiates, it is important to consider that both heroin and codeine are metabolized to morphine [13]. Depending on the time since use, this could lead to a positive result for “opiates” when using these tests, due to the detection of morphine and not of heroin or codeine directly. Since heroin is first metabolized to 6-monoacetylmorphine (6-MAM), some immunoassays available on the market can additionally detect 6-MAM as a specific marker for heroin use, but due to the short detection time window for 6-MAM (elimination half-life: 6–25 minutes), morphine might be the only detectable substance following heroin use [13]. Codeine is metabolized to morphine by cytochrome P450 2D6 (CYP2D6). The rate of this step is, among others, influenced by CYP2D6

genetic polymorphisms, leading to some people being “ultrarapid” and other people being “poor” metabolizers [13, 14]. Besides codeine-containing drugs, poppy seeds also contain traces of opiates and can thus lead to a positive result [13, 15].

Similar to naturally occurring opiates vs. synthetic opioids, structural differences are also relevant for the detection of THC metabolites vs. synthetic cannabinoids. In contrast to the naturally occurring psychoactive cannabinoid THC, synthetic cannabinoids (or more accurately described as synthetic cannabinoid receptor agonists) are synthetic substances that also act as agonists at the cannabinoid receptors but are not detected in most immunoassays, due to their markedly different structure compared to THC [16, 17]. This aspect seemed to be unknown to most of the participants in our study. Conversely, while pure formulations of the naturally occurring cannabinoid cannabidiol (CBD) usually do not show up on drug screening tests [18], oral ingestion of hemp products can lead to a positive cannabis test result [19], due to varying THC content reflecting jurisdiction-dependent legal limits (usually ranging from 0.2 to 1% THC content for hemp) [20]. THC metabolites can be detected for days or weeks after regular cannabis consumption [13, 21], without representing recent use or acute intoxication. This seemed to be better known among participants compared to the aspects mentioned above. On the other hand, except in the case of massive exposure in unventilated areas, second-hand smoke is very unlikely to produce false-positive results if a cutoff of 50 ng/ml THC-COOH is used [22], as is the case for the immunoassay used at our institution.

Knowledge about cross-reactivities in drug tests was not widespread based on the results of the survey, even though false-positive results due to interacting substances are well described, mainly for the amphetamine component of drug screening immunoassays [23]. The antidepressants trazodone and bupropion, the H2-antihistamine ranitidine, and stimulants such as ephedrine or methylphenidate are among the substances that can cross-react with amphetamines in immunoassays [24]. Information on the product-specific cross-reactivities, as well as the cutoff values for the specific substances, is available in the package insert of the commercially available immunoassays (commonly also available online). Confirmatory testing with more specific methods (e.g., chromatography coupled with mass spectrometry) should be considered in unclear situations, especially when confronted with a positive amphetamine immunoassay result. In contrast, the test for the cocaine metabolite benzoylecgonine displays a more favorable specificity for recent cocaine exposure, although intriguingly false-positive results have been reported in patients undergoing evaluation for organ transplantation [25].

Most survey participants thought that either specific gravity or osmolality rather than creatinine was the most commonly used marker to detect purposefully diluted samples, a scenario which might be encountered when testing for drugs of abuse. If the urine creatinine is below 20 mg/dL, the sample is usually considered too diluted to adequately reflect a patient’s drug exposure and hence does not allow reliable interpretation [26]. Quantification of creatinine concentration is usually the preferred method to screen for dilution in clinical practice due to the ease of measurement [27]. In guidelines for workplace drug testing, specific gravity is recommended as a secondary measurement to confirm dilution if urine creatinine is less than 20 mg/dL [28]. While a low urine osmolality can also indicate dilution, it is rarely used to assess the validity of drug screening tests because the method is time consuming [27]. Table 2 summarizes some important aspects to consider when interpreting drug test immunoassays.

Table 2

Limitations and pitfalls to consider when interpreting drug test immunoassay results (selection) [9, 12, 13, 17, 18, 22, 25–29].

(i) Cross-reactivity with other compounds can cause false-positive results (common issue within the amphetamine group and very unlikely in the case of cocaine metabolite testing).

(ii) Possible reasons for a false-negative result include the test's inability to identify a specific compound (e.g., novel psychoactive substances), concentrations below the assay's cutoff, and a short detection window of the substance of interest (e.g., GHB and 6-MAM).
(iii) Immunoassays for "opiates" typically do not detect synthetic opioids (e.g., oxycodone and fentanyl); similarly, immunoassays for THC metabolites do not adequately detect synthetic cannabinoids and Z-drugs (e.g., zolpidem) give a negative result for "benzodiazepines".
(iv) 6-MAM is a specific marker for heroin use that can be detected with some immunoassays shortly after use; otherwise, a positive result for "opiates" does not allow differentiation between heroin, codeine, and morphine use.
(v) Following regular/chronic cannabis use, THC metabolites can be detected in urine for several weeks without necessarily representing acute intoxication.
(vi) Some commonly abused substances such as ketamine, GHB, LSD, and novel psychoactive substances (e.g., synthetic cathinones and synthetic cannabinoids) are not commonly included in most drug test immunoassays currently in clinical use, which might lead to a negative result despite acute intoxication.
(vii) Additional analytical methods (e.g., chromatography coupled with mass spectrometry) can provide more information in unclear cases and are thus recommended especially in cases with potential legal consequences.
(viii) Validity of urine samples should be assessed by measuring creatinine concentrations to detect dilution; when urine creatinine is below 20 mg/dL, interpretation is not recommended.

6-MAM, 6-monoacetylmorphine; GHB, *γ*-hydroxybutyrate; LSD, lysergic acid diethylamide; THC, tetrahydrocannabinol.

To remedy this lack of knowledge and improve urine drug test interpretation by physicians, several options could be considered. This study's findings of knowledge gaps irrespective of training status may indicate that this topic should be emphasized more prominently at multiple levels of medical training and continuous education, e.g., in pregraduate medical education, during postgraduate training, and as information provided to the practicing physician. In general, education on laboratory medicine topics is not preeminently featured in medical school curricula [29]. Its teaching is often limited to theoretical aspects and eschews intricacies that might be encountered clinically later on. Discussions on the strengths and limitations of laboratory-based diagnostic methods such as drug tests should be considered in future attempts to standardize medical education in laboratory sciences and analytical chemistry [30]. During residency, emphasis could be placed on challenges in drug test interpretation that are likely to be of high clinical relevance. Fields making frequent use of these tests (e.g., emergency and addiction medicine) should consider integrating education measures on this topic as integral parts of their curricula to be explored on grand rounds or as case-based learning. Most useful would probably be to provide the information just in time (i.e., when and how it is needed) to the ordering physician. This can be achieved by including short comments on the main pitfalls in drug test interpretation (Table 2) next to the test results and by adding a reference to the laboratory guide for a more in-depth discussion of general and specific test-related limitations. Finally, encouraging clinicians to contact the laboratory to assist in the interpretation of unclear cases by explicitly mentioning this possibility in the report might lead to quality improvements [31].

This study had several limitations. The relatively low return rate (10.7% of the invited participants) compared to previous studies (40–75%, corresponding to 60–359 participants [7–9]) and associated small sample sizes limited statistical power to detect significant differences in drug test knowledge in groups of interest. A test of internal consistency performed a posteriori showed poor reliability, which is likely attributable to the fact that the test items

measure very different aspects of the drug test interpretation proficiency, assessing knowledge of different substances and test characteristics and using questions of varying levels of difficulty. However, our aim was not to design a standardized testing instrument but to increase awareness among residents about common pitfalls of drug test interpretation in clinical practice. This resulted in a small number of markedly different questions, which negatively affected reliability in retrospect. As participation in this study was voluntary, a significant sampling bias is likely, e.g., participation of mostly motivated residents, knowledge of nonparticipants could thus have been even lower. Reporting of prior drug test use was based on self-assessment which is subject to recall bias. It cannot be excluded that some participants consulted the internet for the correct answer. The survey questionnaire was newly developed for this study and was not a previously validated assessment tool. While it was designed based on prior research and examples taken from clinical practice and also was pilot-tested by experienced physicians, it is unclear whether it represents an optimal instrument for the evaluation of medical trainees. For instance, some questions were very specific and probably very difficult for most physicians who do not regularly use drug tests in their daily routine. This could have also contributed to the very low return rate.

In conclusion, this project identified widespread knowledge gaps regarding drug test immunoassays among medical residents of a hospital group, across several disciplines and levels of training. These findings can guide the implementation of specific educational and quality improvement measures such as teaching sessions and targeted information in laboratory reports. The correct interpretation of a drug test immunoassay may be challenging in clinical practice since having an understanding of the specific immunoassay used, its limitations (e.g., cutoff values and cross-reactivities), and the pharmacology of the substances of interest (e.g., metabolic pathways and detection windows) is necessary to avoid misinterpretations that might endanger patient management or negatively affect the patient-physician relationship. In unclear cases, consultation with specialists (e.g., the hospital’s laboratory or specialized toxicology or forensics units) is recommended.

Acknowledgments

The authors would like to thank all the colleagues who participated in the pilot testing and provided helpful feedback regarding the questions and their clarity.

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DETAILS

Subject:	Emergency medical care; Tetrahydrocannabinol--THC; Drug testing; Questionnaires; Internal medicine; Urine; Confidence; Immunoassay; Physicians; Narcotics; Recreational drugs; Questions; Anesthesiology; Otolaryngology; Medicine; Surgery; Confidence intervals; Hospitals; Knowledge; Cocaine; Departments; Screening; Benzodiazepines; Amphetamines
Business indexing term:	Subject: Physicians Hospitals
Editor:	Charles Wilkins
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-07-16 (Received); 2024-03-28 (Revised); 2024-05-29 (Accepted); 2024-06-10 (Pub)
DOI:	https://doi.org/10.1155/2024/4400606
ProQuest document ID:	3071320964
Document URL:	https://www.proquest.com/scholarly-journals/residents-knowledge-regarding-recreational-drug/docview/3071320964/se-2?accountid=211160
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Last updated:	2024-06-24
Database:	Publicly Available Content Database

Structure Elucidation of a Novel Polysaccharide Isolated from Euonymus fortunei and Establishing Its Antioxidant and Anticancer Properties

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ABSTRACT (ENGLISH)

Euonymusfortunei polysaccharides (EFPs) have not been extensively investigated yet in terms of their extraction and biological activity. The orthogonal experimental design was employed in this study to evaluate the optimum yield of EFPs. A maximum yield of 2.63 ± 0.23% was attained using material-liquid ratios of 60 mL/g, extraction temperature of 80°C, ultrasonic power of 144 W, and extraction time of 75 mins. The polysaccharide content reached 53.47 ± 0.31% when deproteinized thrice. An analysis of monosaccharide composition revealed that these polysaccharides consist of Gal, Glc, Man, Fuc, and Rha with a molar ratio of 7.14 : 23.99 : 6.29 : 6.55 : 1.00, respectively, in EFPs. Subsequently, the *in vitro* scavenging capacities of 2,2-diphenylpicrylhydrazyl (DPPH) and ·OH and superoxide anion radicals, along with the reducing power of EFPs, were studied. Results revealed that EFPs have higher antioxidant activity, particularly ·OH scavenging, as well as reducing power, as compared to *Astragalus* polysaccharides (ASPs) and *Lycium barbarum* polysaccharides (LBPs). The Cell Counting Kit-8 (CCK-8) method was used to evaluate the effects of different concentrations of polysaccharides on SKOV3 cell proliferation, and the results revealed their inhibition at concentrations in the range of 200–800 μg/mL. In addition, findings from flow cytometry further confirmed that EFPs blocked the cell cycle at G0/G1 and S phases and induced SKOV3 cell apoptosis. In a word, EFPs could be exploited and used further based on the experimental results from this study.

FULL TEXT

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1. Introduction

Euonymus fortunei (*E. fortune*) has been frequently used in traditional Chinese medicine for maintaining health and treating diseases since ancient times. Modern medical research also shows that it contains various active ingredients such as polysaccharides, dulcitol, flavonoids, catechins, and triterpenoids [1–3]. Polysaccharides are among the main bioactive substances with various effects such as improving immunity, antitumor, antifatigue, liver protection, blood glucose lowering, antioxidation, and antiaging [4, 5]. However, reports on the study of polysaccharides from *E. fortunei* are very limited. It is necessary to clarify its pharmacological action, monosaccharide composition, and antioxidant effects and further study the relevant mechanisms.

In females, ovarian cancer is one of the three prevalent malignant tumors of the reproductive system, which has the highest mortality rate among gynecological malignant tumors [6]. Treatment strategies for ovarian cancer involve surgical resections and systemic pharmacological treatment. Cisplatin and paclitaxel are routinely used drugs, but their application has difficulties because the tumor patient would suffer a series of adverse reactions. Additionally, natural plant-based drugs play a crucial role in the chemotherapy of malignant tumors [7–12]. Hence, an exploration of low-toxicity and high-efficiency antitumor drugs of plant origin is gaining huge interest in tumor treatment. Currently, a growing number of researchers have realized that cell apoptosis induction, as well as affecting cell cycle, can be a new method to treat tumors, which could be achieved via the mechanism of inhibiting tumor cell proliferation and promoting apoptosis. Likewise, for ovarian cancer, Zhou et al. [13] have reported that a traditional Chinese medicine from *Salvia miltiorrhiza*, *tanshinone IA*, could considerably inhibit *in vitro* cell proliferation and *in vivo* tumor growth via apoptosis induction and autophagy promotion through inactivating the PI3K/AKT/mTOR pathway. Based on these findings, our current research focused on using ultrasonic extraction and alcohol-based precipitation for extracting crude polysaccharides from *E. fortunei*, where an extraction procedure has been optimized via orthogonal experiments, followed by column chromatography-based EFP purification. In the initial

identification of *E. fortunei*, polysaccharide content was determined, and we also analyzed the monosaccharide composition of EFPs. The antioxidant activities of EFPs were evaluated, and antiovarian adenocarcinoma activity was studied *in vitro*. It is anticipated that this study will serve as a basis to evaluate the clinical application values of EFPs. Thus, suppose it could be proven that EFPs could treat ovarian cancer by regulating the body's immunity, cytotoxicity, and cell proliferation. In that case, it is expected to overcome the limitations of high side effects, easy drug resistance, and low survival rate of patients caused by chemotherapy drugs such as cisplatin or paclitaxel in the treatment of tumors.

2. Materials and Methods

2.1. Materials

E. fortunei was provided by the Pharmaceutical Factory of Guangxi University of Traditional Chinese Medicine (shown in Figure 1) and identified as genuine *E. fortunei* by Prof. Wei Songji from the Pharmaceutical Factory of Guangxi University of traditional Chinese medicine. The whole herb was crushed through an 80-mesh sieve using a grinder to obtain a fine powder, dried to a constant weight, and placed in a dryer till further use. The AB-8 macroporous adsorption resin, monosaccharide standards, and *Astragalus* and *Lycium barbarum* polysaccharides were all purchased from Beijing Solarbio Science &Technology Co., Ltd. (Beijing, China). Both the superoxide anion free radical detection kit and the hydroxyl radical detection kit were provided by Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China). The rest of the chemicals used for the tests were of analytical and chromatographic grade and were procured from regional chemical vendors in China. The SKOV3 cells were procured from the Chinese Academy of Sciences (Shanghai, China). The RPMI 1640 and fetal bovine serum were purchased from Gibco (Grand Island Biological Company, NY, USA). Other agents such as penicillin-streptomycin liquid and trypsin were brought from Solarbio Science &Technology Co., Ltd. (Beijing, China). The CCK-8 and fluorescein isothiocyanate-conjugated Annexin V (Annexin V-FITC)/propidium iodide (PI) apoptosis assay kits were procured from DOJINDO (Shanghai, China) and BD (Becton, Dickinson and Company, NJ, USA), respectively.

[figure(s) omitted; refer to PDF]

2.2. Sample Preparation of EFPs

2.2.1. Single-Factor Experiment (SFE)

Fifteen grams of finely dry powder was defatted with 300 mL of anhydrous ethanol at 95°C for 5 h using a Soxhlet extractor (shown in Figure 2) and then placed in the dryer until a constant weight was achieved. The constant weight powder was mixed with sterilized water before being extracted using the ultrasonic extractor. After filtration by vacuum pumping, the water extract was collected and then concentrated to 1/5th of the initial volume using a rotary evaporator at 55°C, followed by centrifugation at 5,000 rpm for 10 mins. The chloroform (1 : 5 diluted) was added to the collected supernatant, which was placed into the separating funnel to remove the protein. Absolute ethanol was slowly added while stirring to the collected supernatant, making the final ethanol concentration of the mixture reach 80%. Afterward, the solution was cooled at 4°C for 24 h, and then, centrifugation was carried out at 5,000 rpm for 10 mins for precipitate collection. Next, the residue was washed with ethanol and acetone. The crude polysaccharide sample was obtained by dryer drying (50°C) and then kept in a desiccator till further use. For further improving the yield of crude polysaccharide extraction from *E. fortunei*, the following five parameters were considered: material-liquid ratio, extraction temperature, ultrasonic power, and extraction times. The different values of these factors used for optimization involved the material-liquid ratios (20, 30, 40, 50, 60, and 70 mL/g); extraction temperatures (40, 50, 60, 70, 80, and 90°C); ultrasonic power (144, 198, 252, 306, and 360 W); and extraction time at 15, 30, 45, 60, and 90 mins. By using the measured EFP content, the extraction rate (ER) has been computed.

[figure(s) omitted; refer to PDF]

2.2.2. Orthogonal Test Design for Optimization

Based on the outcomes of SFE, the following four parameters were used: extraction temperatures, ultrasonic power, material-liquid ratios, and extraction time, which were selected in an orthogonal test to optimize the optimal extraction process. Then, the orthogonal experiment was carried out following Table 1. All experiments were conducted in triplicates.

Table 1

Factors and levels used in the orthogonal experimental design.

Variable	Level		
1	2	3	(A) Material-liquid ratios (mL/g)
40	50	60	(B) Extraction temperature (°C)
60	70	80	(C) Ultrasonic power (W)
144	198	252	(D) Extraction time (min)

2.2.3. EFP Purification

The crude polysaccharides were resuspended in the deionized water to a suitable volume and then injected into an AB-8 macroporous adsorption resin chromatography column (2.5 × 100 cm). Elution was carried out using a gradient of water and 5% and 15% ethanol solutions with a flow rate of 1 BV/h, whereas eluent collection was performed at 10 mL per tube with the aid of an automated collector. To determine the polysaccharide content in each tube, an elution curve was generated, using the phenol-sulfuric acid method. Fractions containing peak components were subjected to concentration, freezing, and drying.

2.3. Estimation of Polysaccharide Content

First, for the glucose standard curve, 0, 0.04, 0.06, 0.08, 0.1, 0.2, and 0.4 mL from glucose stock (1 mg/mL) were added to seven dry grinding test tubes with plugs, respectively, and the final volume of each tube was 2 mL with the deionized water. Afterward, tubes were added with 1 mL of phenol (5%) and 5 mL of concentrated H_2SO_4. The samples were gently mixed and kept for 15 mins at room temperature (RT). The absorbance was recorded at 485 nm using a spectrophotometer (Shanghai Mapada Co., Ltd., China). In general, the glucose standard curve was drawn with the standard glucose concentration (mg/mL) as the abscissa and the absorbance value as the ordinate. Finally, 5 mg of EFPs was thoroughly dissolved in 100 mL of deionized water and subjected to the same reaction as with the glucose standard solution. The absorbance was recorded at 485 nm and substituted into a glucose standard curve for estimating the polysaccharide concentration.

2.4. Monosaccharide Composition Assays

A combination of high-performance liquid chromatography (HPLC) and 1-phenyl-3-methyl-5-pyrazolone (PMP) was used to detect the monosaccharide compositions of EFPs as described previously [14]. In detail, 20 mg of polysaccharide was infiltrated using 2.0 mL of trifluoroacetic acid (2.0 mol/L) in a nitrogen-sealed tube at 110°C for two hours. In the next step, following cooling the reaction mixture to RT, centrifugation was performed at 1000 rpm for 5 mins. Afterward, the supernatant was transferred to the evaporation pan, and the trifluoroacetic acid was removed with 1.5 mL of methyl alcohol. After drying at atmospheric pressure, the residue was resuspended in 2 mL of deionized water and kept at 4°C till used.

Herein, 100 μL of the sample solution, including hydrolyzed polysaccharide or monosaccharide, was added with 100 μL each of aq. NaOH (0.3 mol/L) and PMP (0.5 mol/L) solutions. In the following step, the solutions were mixed, sealed, reacted at 70°C for 30 mins, and then cooled to ambient temperature. Next, a neutralization operation was

performed via the addition of 100 μL of HCl (0.3 mol/L) solution. Finally, the neutralization solution was extracted by using 3 mL of chloroform and the aqueous phase was leached with a 0.45 μm membrane filter to obtain the filtrate, which was subjected to HPLC analysis. In individual test tubes, 10 different standard monosaccharide solutions were added at concentrations of 0.1, 0.5, 1, 2, 5, and 10 μmol/L to obtain the calibration curve.

For conducting the HPLC analysis, separation was carried out on an InertSustain C18 column (4.6 mm × 250 mm, 5 microns), run at 42°C, using a flow rate (1.0 mL/min). The mobile phase contained potassium phosphate buffer (A, 0.05 mol/L, pH 6.9) and acetonitrile (B), and the elution concentration was 92% (A)-8% (B). UV peak detection was carried out at 254 nm. All the chromatography solvents/solutions were filtered through membrane filters (0.45 μm, Millipore) and sonicated for degassing in an ultrasonic bath before use.

2.5. Antioxidant Activity Assays

2.5.1. Scavenging Rate of DPPH Radical

Emanuel's method was applied to determine the scavenging rate of DPPH radical [15]. Herein, 2 mL of the polysaccharide solution at varying concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) was absorbed in the test tubes and added with 2 mL of the anhydrous ethanol and 2 mL of the DPPH ethanol (0.1 mmol/L) solution. After homogeneous mixing, the solution was placed in the dark for 30 mins at RT, and then, the absorbance was recorded at a wavelength of 517 nm. The experiment was carried out in parallel three times, where butylated hydroxytoluene (BHT), ASPs, and LBPs were chosen as the positive (+ve) controls. The rate of DPPH radical scavenging was evaluated by applying the following formula:(1)DPPH scavenging rate %=1−Ai−AjA0×100,where Ai = absorbance of the mixture, Aj = absorbance of the sample solution, and A0 = absorbance of the DPPH solution.

2.5.2. Inhibition Capacity of Hydroxyl Radical

The hydroxyl radical assay kit was applied to evaluate the inhibition potential of hydroxyl radical as per the Fenton reaction [16]. For this, individual test tubes were added with the sample solution at varying concentrations (0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 mg/mL) and subsequently mixed with the different working solutions from the kit as per the provided instructions. The solutions were placed at RT for 20 mins, and then, their absorbance was recorded at a wavelength of 550 nm using the deionized water as the blank. The experiment was carried out in parallel three times, with vitamin C (VC), ASPs, and LBPs serving as +ve controls. The rate of hydroxyl radical inhibition was evaluated by applying the following formula:(2)Hydroxyl radical inhibition rate%=ODcontrol−ODdeterminedODcontrol×100.

2.5.3. Inhibition Capacity of Superoxide Anion Radical

The superoxide anion radical assay kit was applied to test the inhibition capacity of the superoxide anion radical [17]. For this, individual test tubes were added with the sample solution at varying concentrations (0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 mg/mL) and subsequently mixed with different working solutions from the kit as per instructions. The solutions were placed at RT for 20 mins, and then, the absorbance was recorded at a wavelength of 550 nm using the deionized water as the blank. The experiment was carried out in parallel three times, with VC, ASPs, and LBPs serving as +ve controls. The rate of superoxide anion radical inhibition was evaluated by applying the following formula:(3)Superoxide anion radical inhibition rate%=ODcontrol−ODdeterminedODcontrol×100.

2.5.4. Determination of Reducing Capacity

The reducing capacity of EFPs was determined by implementing Prussian Blue's method [18]. For this, the individual test tubes were added with the sample solution at varying concentrations (0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 mg/mL) and subsequently added with 2.5 mL each of the phosphate buffer (0.2 mol/L) and 1% of the potassium ferricyanide solution. After homogeneous mixing, the solutions were kept for 20 mins in a 50°C water bath. Afterward, the solutions were brought to RT and immediately added with 2.5 mL of trichloroacetic acid (10%). The blending solutions were subjected to centrifugation at 3,000 rpm for 10 mins, and the individual supernatants were separated. Finally, the absorbance of the mixture, including the supernatant, deionized water, and 0.1% of the trichlorinated iron solution, was measured at 700 nm by setting the deionized water as the blank. The experiment was measured in parallel three times, and BHT, ASPs, and LBPs were chosen as +ve controls.

2.6. Cell Proliferation and Cycle Assays

The proliferation of EFP-treated SKOV3 cells was assessed by means of the CCK-8 method as described earlier

[19]. For this, the SKOV3 cell resuspension was carried out in the RPMI 1640 medium containing 10% of the fetal bovine serum. Then, the cells were diluted to 1.0×10^5 cells/mL using the serum-free medium and inoculated into 96-well tissue culture plates. Following incubation with the EFPs at different concentrations (200, 400, and 800 μg/mL) at 37°C for 24 h, 48 h, 72 h, and 96 h, respectively, in a 5% CO_2 humidified atmosphere, cell proliferation was assessed by measuring absorbance at 450 nm using a microplate reader (Bio-Rad, USA). Each experiment was carried out independently at least three times. The inhibition rate of cell proliferation on polysaccharide treatment was calculated according to the following formula: (4) $\text{The inhibition rate}\% = B - AB - C \times 100$, where A is the absorbance of the experimental group, B is the absorbance of the control group, and C is the absorbance of the blank group.
A cell cycle distribution for EFP-treated SKOV3 cells was examined using the Annexin V-FITC/PI staining method. Briefly, the cells were plated using a density of 5.0×10^5 cells/well and subjected to EFP treatment procedures at concentrations (200, 600, and 1000 μg/mL) for 72 h. Following centrifugation, the cell precipitates were collected and resuspended in the precooled absolute ethanol added slowly. After being fixed away from light at 4°C for more than 2.5 h, the cell precipitates were collected by centrifugation and resuspended in the precooled phosphate buffer saline. This cell resuspension solution was again centrifuged to collect the cell precipitates, to which PI staining solution (PI 50 mg/L and RNase A 1 g/L) was added and placed in the dark at RT for 15 mins. Lastly, the cell cycle was evaluated using a Guava easyCyte flow cytometry with a Guavasoft 3.1.1 (BD, USA).

2.7. Statistical Analysis

All statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, version v23.0 (IBM Corp., Armonk, NY, USA)) and plotted with GraphPad Prism (v7.0) software. When following normal distributions, the data were analyzed using Student's *t*-test, or else, the nonparametric Mann–Whitney test was applied. A $p<0.05$ (or less) was taken to indicate a statistically significant difference. Measurement data were expressed as the mean ± standard deviation (SD).

3. Results

3.1. SFE for EFP Extraction Parameters

The effects of various extraction parameters on the ER of EFPs are illustrated in Figures 3(a)–3(d). As shown in Figure 3(a), when the material-liquid ratio was 50 mL/g, the polysaccharide extraction rate (PER) reached a maximum, but this phenomenon was reversed on the material-liquid ratio exceeding 50 mL/g, indicating 50 mL/g as an optimum level for the next experiments. In Figure 3(b), when the temperature was 60°C, the PER increased obviously and it increased with the increase in temperature. According to the heat that could destroy the structure of polysaccharides, 70°C has been selected as an alternative temperature for EFP extraction. Furthermore, Figure 3(c) depicts a decrease in PER on increasing ultrasonic power. With a view to the influence of other components in EFPs and the excessive ultrasonic power that could destroy the structure of polysaccharides, 144 W was selected as an alternative ultrasonic power for EFP extraction. Figure 3(d) illustrates that with an increase in extraction time from 15 to 105 mins, the PER increased, but the onward PER slowed down with the increase in extraction time from 75 to 105 mins. Thus, considering time and benefit, 75 mins was chosen for future experiments.
[figure(s) omitted; refer to PDF]

3.2. Orthogonal Test Analysis

The outcomes from extreme differences reveal the influence of four factors on the EFP yield in order as follows: water to extraction temperature ($R = 1.607$) > ultrasonic power ($R = 0.590$) > extraction time ($R = 0.484$) > material-liquid ratio ($R = 0.210$) (Table 2). The results were in line with F values from the analysis of variance (ANOVA) (Table 3). According to ANOVA, the influence of four factors ($p<0.01$) on the EFP yield was highly significant. Based on extreme differences and ANOVA, the optimum parameters influencing EFP extraction involved the water-to-material-liquid ratio of 60 mL/g, the extraction temperature of 80°C, the ultrasonic power of 144 W, and the extraction time of 75 mins. A verification test was performed three times under these conditions to determine the highest yield, and this EFP yield reached 2.63%. After deproteinization, the polysaccharide content was 53.47%. The powdered polysaccharides were then used for further monosaccharide compositions and antioxidant activity testing, thus evaluating the effect on the proliferation and the cycle of SKOV3 cells.

Table 2

Orthogonal test design and results for the ER of EFPs (n=3).

No.	Factors				ER (%)
A	B	C	D	1	1
1	1	1	1.860±0.001	2	2
1	2	2	1.840±0.017	3	3
1	3	3	1.810±0.023	4	2
2	1	3	2.117±0.017	5	3
2	2	1	1.927±0.043	6	1
2	3	2	2.047±0.021	7	3
3	1	2	2.627±0.041	8	1
3	2	3	2.247±0.021	9	2
3	3	1	2.243±0.021	$k1$	6.154
5.510	6.604	6.030		$k2$	6.200
6.091	6.014	6.514		$k3$	6.364
7.117	6.100	6.174		R	0.210

A: material-liquid ratio; B: extraction temperature; C: ultrasonic power; D: extraction time.

Table 3

Variance analysis of the orthogonal test.

Factors	SS	df	MS	F value	P value	Significant
A	0.024	2	0.012	18.293	≤0.001	***
B	1.324	2	0.662	994.000	≤0.001	***
C	0.203	2	0.102	152.505	≤0.001	***
D	0.124	2	0.062	92.725	≤0.001	***

Deviation	0.012	18	0.001			

SS: sum of square; *df*: degree of freedom; MS: mean of square; ***: p≤0.001; A: material-liquid ratio; B: extraction temperature; C: ultrasonic power; D: extraction time.

3.3. Monosaccharide Composition

The existence of monosaccharides was validated by comparing the outcomes of the PMP derivatives of standard monosaccharides (Figure 4(a)). As shown in Figure 4(b) and Table 4, the EFPs were composed of Gal, Glc, Man, Fuc, and Rha. The monosaccharide contents and the molar ratios of EFPs are listed in Table 4, which shows the highest Glc and lowest Rha contents.

[figure(s) omitted; refer to PDF]

Table 4

Monosaccharide composition of EFPs.

Number	Monosaccharide	Contents (mg/g)	Molar ratio
1	Gal	12.675	7.14
2	Glc	42.560	23.99
3	Man	11.162	6.29
4	Fuc	11.622	6.55
5	Rha	1.774	1.00

Gal: galactose; Glc: glucose; Man: mannose; Fuc: fucose; Rha: rhamnose.

3.4. The DPPH Radical Scavenging Capacity

Figure 5(a) illustrates the DPPH scavenging activity of EFPs at varying concentrations. The results showed that EFPs scavenged DPPH in a concentration-dependent manner, whereas the inhibition rate increased from 24.00 to 49.00% in the range of 0.1 mg/mL to 1.0 mg/mL. The DPPH scavenging activity of EFPs was weaker than that of ASPs and LBPs at the concentration of 0.4 mg/mL to 1.0 mg/mL ($p<0.05$), but it was similar to that of BHT, a commonly used antioxidant, which was consistent with the result of the IC_{50} values shown in Table 5.

[figure(s) omitted; refer to PDF]

Table 5

The IC_{50} values of various polysaccharide samples from antioxidant properties.

Sample	IC_{50} value (mg/mL)		
DPPH scavenging activity	·OH scavenging activity	$O_2^{\cdot-1}$ scavenging activity	EFPs
1.751 ± 0.03^{b}	0.481 ± 0.01^{b}	3.441 ± 0.22^{a}	ASPs
0.379 ± 0.02^{a}	3.708 ± 0.15^{a}	4.985 ± 0.50^{a}	LBPs
0.373 ± 0.01^{a}	2.680 ± 0.14^{a}	4.305 ± 0.29^{a}	BHT

1.231 ± 0.03			VC

The IC_{50} value is half the maximal inhibitory concentration. Data are presented as mean ± standard deviation (n = 3). The superscript letters a and b indicate a significant difference at the 0.05 significance level.

3.5. The ·OH Radical Scavenging Capacity

The ·OH scavenging activity of EFPs at various concentrations is illustrated in Figure 5(b). The EFPs demonstrated scavenging activity on ·OH in a concentration-dependent manner, whereas the inhibition rate was found to increase from 11.30 to 64.20% at a concentration range of 0.05–0.4 mg/mL. The ·OH scavenging activity of EFPs was weaker than that of VC, which is a commonly used antioxidant, but it was higher than that of ASPs and LBPs at the concentration range of 0.2 mg/mL to 0.4 mg/mL ($p<0.05$), which was also consistent with the IC_{50} values shown in Table 5.

3.6. The Superoxide Anion Radical Scavenging Capacity

As illustrated in Figure 5(c), the EFPs demonstrated superoxide anion scavenging activity in a concentration-dependent manner, whereas the inhibition rate was found to increase from 11.60% to 32.89% at a concentration range of 0.1–1.0 mg/mL. Also, the ability of EFPs to scavenge superoxide anion was lower than that of VC but higher than that of ASPs and LBPs at the concentration range of 0.8–1.0 mg/mL ($p<0.05$), which was consistent with the result of the IC_{50} values shown in Table 5.

3.7. Reducing Power Assay

As shown in Figure 5(e), linear growth of reducing power was observed with increasing sample concentration at a concentration range of 0.1 to 1.0 mg/mL (r = 0.996 5). The dose-dependent effect of EFPs on reducing power is illustrated in Figure 5(d). At 0.2 mg/mL to 1.0 mg/mL, EFPs exhibited a lower reducing power than BHT, but a higher power than ASPs and LBPs at the same concentration ($p<0.05$).

3.8. *In Vitro* Analysis of Anti-Ovarian Adenocarcinoma Activity for EFPs via CCK-8 Assay

To identify the anti-ovarian adenocarcinoma activities of EFPs *in vitro*, the viability of SKOV3 cells on treatment with various concentrations of EFPs for varied periods (24 h, 48 h, 72 h, and 96 h) was assessed using the CCK-8 method. Figure 6(a) illustrates an increase in the growth inhibition of SKOV3 cells with increasing EFP concentration and time. Compared with other time intervals, the EFPs have a considerable inhibitory effect on the inhibition rate of SKOV3 cell growth in the dose range of 200–800 μg/mL at 48 and 72 hours, where the differences were statistically significant ($p<0.05$). Therefore, EFPs in the concentration range (200, 400, and 800 μg/mL) with a treatment duration of 72 h were selected for further anti-ovarian adenocarcinoma activity analyses. As shown in Figure 6(b), the SKOV3 cells possessing normal morphologies showed irregular spindle forms, large numbers, complete shapes, and clustered adherent growth. In comparison with the blank group (0 μg/mL), the SKOV3 cells experienced a gradual loss of their original cellular morphology with an increase in EFP concentrations. This indicated the cell number declined considerably, becoming stripped with gradually increased floating.

[figure(s) omitted; refer to PDF]

3.9. EFPs Suppressed the Cell Cycle and Promoted Apoptosis of SKOV3 Cells

The EFP effect on the cell cycle of SKOV3 cells was examined by analyzing DNA amount (%) at the G0/G1, S, and G2/M phases of the cell cycle. In comparison with the blank group (0 μg/mL), the proportion of cells in G1-phase cells increased from 81.49% ± 2.01 to 85.93% ± 1.01 ($p < 0.05$) in the concentration range of EFPs (200, 400, and 800 μg/mL) with a treatment duration of 72 h, whereas the value in S-phase cells declined from 18.82% ± 1.22 to 6.90 ± 1.50% ($p < 0.05$) demonstrating the concentration-dependent effect, whereas the effect in G2/M phase cells was considerably insignificant (Figure 7(a) and Table 6). Following treatment with EFPs for 72 h, the total apoptosis rates of the blank group (0 μg/mL) and the treatment group (400 μg/mL and 800 μg/mL) were 4.46% and 4.71%, respectively (Figure 7(b) and Table 7), with statistically significant differences ($p<0.05$).

[figure(s) omitted; refer to PDF]

Table 6

The EFP effect on the cell cycle distribution of SKOV3 cells treated for 72 h (n = 3).

Cell cycle (%)	Concentration (μg/mL)			
0	200	400	800	G0/G1
81.49±2.01	81.53±1.17*	83.17±0.36*	85.93±1.01*	S
18.82±1.22	13.01±0.12*	8.57±1.18*	6.90±1.50*	G2/M

Note. The SKOV3 cells were treated with EFPs (200 μg/mL, 400 μg/mL, and 800 μg/mL) for 72 h (mean±SD, *$p<0.05$ vs. non-EFP-treated group).

Table 7

The EFP effect on the cell apoptosis of SKOV3 cells treated for 72 h (n=3).

Concentration (μg/mL)	Apoptosis (%)
0	2.83±0.22
200	3.28±0.57
400	4.46±0.90*
800	4.71±0.92*

Note. SKOV3 cells were treated with EFPs (200 μg/mL, 400 μg/mL, and 800 μg/mL) for 72 h (mean±SD, *$p<0.05$ vs. non-EFP-treated group).

4. Discussion

In this research, multiple experiments were performed to understand the monosaccharide composition and antioxidant and anticancer effects of EFPs. As we know, polysaccharides are among the most versatile natural active substances due to their wide variety of pharmacological activities such as antiaging [20–22], liver injury protection [23–25], blood glucose lowering [26–29], antifatigue [30, 31], immunoregulatory activity [32–35], and antitumor activity [36–39]. Presently, frequently used extraction methods for polysaccharides mainly include hot water extraction [40], alcohol extraction [40], ultrasonic-assisted extraction [41], microwave-assisted extraction [42], and infrared-assisted extraction [43]. Compared with the other extraction methods, ultrasonic extraction was chosen to carry out a single-factor test as this method could break up the plant cell walls and accelerate the diffusion and dissolution of polysaccharides. Importantly, this method had the advantages of simple operation, low cost, and stable extraction. The optimum extraction conditions were obtained by SFE (material-liquid ratio: 50 mL/g, temperature: 70°C, ultrasonic power: 144 W, and extraction time: 75 mins). However, the single-factor experiments just considered the influence of each factor on the extraction yield and did not involve the interactions among them, as well as the influence of each factor on the extraction yield. Therefore, the optimal experimental range of material-liquid ratios, extraction temperature, ultrasonic power, and extraction time was determined by the orthogonal test [44, 45]. In the orthogonal test, only nine experiments were conducted for four factors at three levels each, whereas 81 experiments would be necessary for the full-factorial design. At last, $A_2B_2C_1$ was reasonably confirmed to be the best optimum combination of different parameters. After deproteinization, the polysaccharide content was 53.47%, which was no less than that of mushroom polysaccharides [46], indicating that it may have a similar function to mushroom polysaccharides.

To analyze the monosaccharide compositions, we chose the HPLC method, which has the advantages of fast separation speed, high resolution, good precision and reproducibility, and high stability [47–49]. It was shown that

EFPs have five main monosaccharide components, including Gal, Glc, Man, Fuc, and Rha. It has been extensively reported that ASPs and LBPs are commonly used natural antioxidants [50, 51]. ASPs are composed of five monosaccharides, which are Rha, Gal acid, Glc, Gal, and Ara, whereas LBPs are composed of four monosaccharides, viz., Rha, Man, Glc, and Gal. Moreover, Deng [52] and Zhang [53], respectively, found that both ASPs and LBPs have antioxidant and antitumor activities. Numerous research studies revealed that mushroom polysaccharides also have antioxidant and antitumor activities [46, 54, 55]. Therefore, it was speculated that EFPs could also have antioxidant and antitumor activities. In this study, no unreported monosaccharides have been found in EFPs, but we initiatively extracted EFPs for antiovarian cancer research, which could provide a new direction on more anticancer effects of *E. fortunei*.

To prove the antioxidant activity of polysaccharides, DPPH, ·OH, superoxide anion, and reducing power analysis were performed in the following experiments. DPPH as a stable free radical in organic solvents is commonly used to evaluate the ability of natural compounds to scavenge free radicals [56, 57]. Usually, the DPPH free radical inhibition rate is measured to evaluate their free radical scavenging ability and antioxidant capacity. In reactive oxygen species, hydroxyl radicals are among the most harmful radicals, causing severe oxidative damage to nearby biomolecules (such as carbohydrates, proteins, lipids, and DNA) [58, 59]. Superoxide radicals are highly toxic substances produced during various biological and photochemical reactions and also play an important role in the formation of other reactive oxygen species [58, 59]. Reducing power is a key indicator for evaluating the antioxidant activity of substances. Antioxidant substances give electrons by their reduction and scavenge free radicals to interrupt the chain reaction of lipid peroxidation. Antioxidant strength can be evaluated by measuring the reducing power [58, 59]. In this study, the DPPH scavenging activity of EFPs was weaker than that of ASPs and LBPs, but it was similar to that of BHT. In addition, the ·OH and superoxide anion scavenging activity of EFPs was weaker than that of VC, but it was higher than that of ASPs and LBPs. Also, EFPs exhibited a lower reducing power than BHT, but a higher power than ASPs and LBPs at the same concentration. In short, it suggested that EFPs and other polysaccharides had potential antioxidant activity to be developed as antioxidants *in vitro*.

It has been widely reported that plant polysaccharides possessing antioxidant properties may prevent tumor cells from proliferating. Based on the published literature, the antitumor activities of polysaccharides are associated with their antioxidant and free radical scavenging activities. ASPs could inhibit the proliferation of both lung cancer cells (A549) and liver cancer cells (HepG2) [60, 61]. LBPs could also result in the inhibition of MCF-7 and HepG2 cell proliferation, thus inducing apoptosis of both types of cells [62, 63]. Polysaccharides from *Pleurotus ostreatus* may reduce the viability of A549, SKOV3, HepG2, and MCF-7 cells in a concentration-dependent manner while inducing apoptosis of A549 cells [64]. *Duchesnea indica (Andr.) Focke* polysaccharides significantly inhibited the proliferation of SKOV3 and HepG2 cancer cell lines [65]. Polysaccharides from *Cordyceps gunnii* could significantly induce apoptosis in SKOV3 cells through the p53-Bax-caspase pathway [66]. In the above research, we found that EFPs have antioxidant effects. Therefore, we speculated that the antioxidant EFPs also have certain antitumor effects and took it as the next research direction. In the present research, the changes in cell viability and morphology illustrated an increase in the growth inhibition of SKOV3 cells with increasing EFP concentration and time. Moreover, the SKOV3 cells were arrested by EFPs at the G0/G1 and S phases of the cell cycle and the total apoptosis rate increased in the treatment group (400 μg/mL and 800 μg/mL) with statistically significant differences ($p<0.05$). These outcomes illustrated that EFPs could induce apoptosis and inhibit the proliferation of SKOV3 cells *in vitro*. We speculated that EFPs could block the division of SKOV3 cells in the G0/G1 phase, affect DNA replication and transformation into S and M phases, and ultimately slow down the growth of SKOV3 cells to reduce their proliferative activity and lead to cell apoptosis.

Notably, Sudha Govindan [54] found that *Hypsizygus ulmarius* polysaccharides exhibited antioxidant, liver protective, and lipid-lowering activities in ethanol-induced rats. Nataraj et al. [46] found that the antioxidant properties of crude polysaccharides from *Calocybe indica* (CICP) were assessed using a variety of in vitro tests and also inhibited the growth of HeLa, PC3, HT29, HepG2, and Jurkat cells in a concentration-dependent manner. The results of Shen et al. [63] revealed that LBPs caused growth inhibition of MCF-7 cells with the arrest of the cell cycle

in the S phase and induced apoptosis through the ERK pathway. The results of Li et al. [35] revealed that anticancer activity in vivo of ASPs may be achieved by restoring immune organs, regulating cellular immune responses, and increasing cytokine levels. Compared with the above traditional Chinese medicine or medicinal plant polysaccharides, EFPs have similar polysaccharide composition and in vitro antioxidant and antitumor effects, indicating that they could be used as the potential natural antioxidant and antitumor agents. However, the molecular mechanism needs further study. Of course, we need to find out the genes or proteins related to the effect of EFPs on the proliferation and apoptosis of ovarian cancer cells, discover their signaling pathway, and reveal the specific mechanism.

5. Conclusions

This study, for the first time, used a single factor combined with an orthogonal experiment to detect the factors influencing EFP extraction to obtain optimal extraction conditions. The results showed that the optimal parameter combination of EFPs was material-liquid ratios of 60 mL/g, extraction temperature of 80°C, ultrasonic power of 144 W, and extraction time of 75 mins. In this condition, the highest yield of 2.63 ± 0.23% was obtained. After deproteinization 3 times, the polysaccharide content increased and the protein content decreased, respectively. Subsequently, we comprehended the monosaccharide composition of the polysaccharides, including Gal, Glc, Man, Fuc, and Rha. Furthermore, the antioxidant capacity of EFPs *in vitro* was analyzed using five different evaluation methods and the results showed that EFPs have a certain antioxidant capacity, which was also superior to that of ASPs and LBPs to a certain extent. Finally, the effects of various polysaccharide concentrations on the proliferation of SKOV3 cells, cell cycle, and apoptosis were evaluated. The cell proliferation was inhibited following treatment with different concentrations of EFPs (200, 400, and 800 μg/mL). Also, there was a cell arrest by EFPs at the G0/G1 and S phases of the cell cycle, which illustrates that EFPs can induce apoptosis. These results demonstrate that polysaccharides isolated and purified from *E. fortunei* exert *in vitro* antitumor activity, which deserves further examination to investigate the antitumor mechanisms of EFPs. However, we did not detect the expression of apoptosis-related genes and proteins, reveal the signaling pathway, and clarify the anticancer mechanism. Therefore, further works are focused on the expressions of caspase-3, caspase-9, PARP, p53, Bax, and Bcl-2 in the SKOV3 cells, which were studied via Western blotting, and animal experiments revealed whether EFPs inhibit tumors within an ovarian tumor model rat, modeled with the SKOV3 cells.

Authors' Contributions

Luo Yu, Cai Danzhao, and Wen Qilong contributed to the conception and design of the evaluation, acquisition of the data, and analysis and interpretation of the data. Luo Yu and Wen Qilong drafted the article and revised it critically for important intellectual content. Luo Yu, Cai Danzhao, and Chen Hongtao contributed to the acquisition and analysis of the data. Huang Chunxi contributed to the statistical analysis of the data. Luo Yu, Cai Danzhao, and He Shujia contributed to the conception and design of the evaluation and revised the article critically for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The work was supported by funds from the National Natural Science Foundation of China (grant no. 81360688) and the Innovation Project of Guangxi Graduate Education (grant no. YCBZ2020053).

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DETAILS

Subject:	Polysaccharides; Monosaccharides; Ratios; Cancer therapies; Drugs; Antioxidants; Chromatography; Biological activity; Chinese medicine; Apoptosis; Cell cycle; Ethanol; Ovarian cancer; Scavenging; Pharmaceuticals; Temperature; Medical research; Anticancer properties; Glucose; Design of experiments; Phenols; Tumors; Cell growth; Chemotherapy; Flow cytometry
Business indexing term:	Subject: Ratios
Location:	Beijing China; United States--US; China
Editor:	Adil Denizli
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-02-07 (Received); 2024-04-13 (Revised); 2024-04-23 (Accepted); 2024-05-24 (Pub)
DOI:	https://doi.org/10.1155/2024/8871600
ProQuest document ID:	3063164061
DocumentURL:	https://www.proquest.com/scholarly-journals/structure-elucidation-novel-polysaccharide/docview/3063164061/se-2?accountid=211160

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Last updated:	2024-06-03
Database:	Publicly Available Content Database

Label-Free Ratiometric Homogeneous Electrochemical Strategy Based on Exonuclease III-Aided Signal Amplification for Facile and Rapid Detection of miR-378

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ABSTRACT (ENGLISH)

MiR-378 is abnormally expressed in various cancers, such as hepatocellular carcinoma, renal cell carcinoma, and nonsmall cell lung cancer. Here, we developed a label- and immobilization-free ratiometric homogeneous electrochemical strategy based on exonuclease III (Exo III) for the facile and rapid determination of miR-378. Two 3′-protruding hairpin DNA probes (HPs) are designed in this strategy. Doxorubicin (DOX) and potassium ferrocyanide (Fe^{2+}) were used as label-free probes to produce a response signal (I_{DOX}) and a reference signal (I_{Fe}^{2+}) in the solution phase. When no target was present in the solution, the HP was stable, most of the DOX was intercalated in the stem of the HP, and the diffusion rate of DOX was significantly reduced, resulting in reduced electrochemical signal response. When miR-378 was present, double-cycle signal amplification triggered by Exo III cleavage was initiated, ultimately disrupting the hairpin structures of HP1 and HP2 and releasing a large amount of DOX into the solution, yielding a stronger electrochemical signal, which was low to 50 pM. This detection possesses excellent selectivity, demonstrating high application potential in biological systems, and offers simple and low-cost electrochemical detection for miR-378.

FULL TEXT

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1. Introduction

With the continuous development of medical technology in recent years, several deadly diseases have become curable. Cancer continues to pose a serious threat to human health. In its early stages, cancer often presents with no notable symptoms. Upon diagnosis, the treatment typically includes surgery [1], radiotherapy [2], chemotherapy [3], targeted therapy [4], and immunotherapy [5]. Regardless of the treatment approach, early diagnosis is crucial for its treatment [6]. With the advancements in cancer research, new diagnostic methods are constantly being proposed, and the detection of microRNAs (miRNAs) is a highly promising avenue of research [7–9].

MiRNAs are a class of small, endogenous, noncoding RNAs that regulate target gene expression at the posttranscriptional level by binding to the 3′ untranslated region (3′ UTR). Several cancers exhibit an abnormal expression of specific miRNAs during development. Abnormal miR-378 expression has been reported to be associated with several types of cancers, including hepatocellular carcinoma [10], renal cell carcinoma [11], and nonsmall-cell lung cancer [12]. However, owing to the low expression levels of miRNAs, using highly sensitive detection methods is necessary.

Electrochemical detection, which is characterized by high sensitivity, rapid response, good selectivity [13], and simplicity of operation, has been employed by researchers for microRNA detection [14–20]. However, conventional heterogeneous electrochemical biosensors typically involve the immobilization of a probe on a working electrode, which requires laborious and time-consuming electrode pretreatment and probe immobilization steps [21–25]. Moreover, the efficiency of probe immobilization may vary after electrode pretreatment, hindering the reproducibility of the test results. In addition, probes situated in the same plane may face limitations due to steric hindrance, which subsequently decreases the effectiveness of recognition and binding. By contrast, homogeneous electrochemical biosensors, whose recognition and response processes occur in the solution phase instead of on the electrode surface, avoid complicated immobilization processing [26–29].

Currently, most homogenous electrochemical sensors use DNA probes labeled with methylene blue (MB) as signal-responsive substance [30–32]. However, these labeled probes are time consuming and costly. Moreover, methylene blue, a dye molecule, exhibits significant adsorption properties that could affect the accuracy of the test results. DOX can specifically intercalate into dsDNA without strong nonspecific adsorption [33].

Ratio electrochemical sensors utilize two independent response signals to compare and detect targets, effectively eliminating the interference caused by external factors and improving the accuracy and reliability of the results [34, 35]. Chen et al. developed an electrochemical aptamer-based biosensor for a highly sensitive thrombin assay using MB and ferrocene (Fc) as electrochemically active markers, which exhibited different signal changes in the presence of thrombin. Through amplification of the ratio signal, high-sensitivity detection of thrombin was achieved with a detection limit as low as 56 fM [36]. Zhu et al. utilized aptamer and hybrid chain reactions using Fc as an internal standard to achieve the highly sensitive detection of aflatoxin B1 based on the ratio of methylene blue to Fc signals [37].

In this study, we developed a label-free homogeneous electrochemical sensor for miR-378 determination based on exonuclease III (Exo III)-assisted recycling amplification. HP1 and HP2 were designed as 3′-protruding structures. In the absence of the target, DOX in the solution was embedded in the stem-loop structures of HP1 and HP2, thus reducing its diffusion rate in the solution and exhibiting a lower electrochemical signal. When miR-378 is present, it triggers the cleavage process of Exo III. Ultimately, the stem-loop structures of HP1 and HP2 were disrupted, releasing a large amount of DOX into the solution, resulting in a higher electrochemical signal. Throughout this process, the electrochemical signal of Fe^{2+} remained relatively constant. miR-378 was achieved by calculating the ratio of DOX to Fe^{2+} current. This method allows for the sensitive, rapid, and accurate determination of miR-378.

2. Experiment

2.1. Reagents

Magnesium chloride, potassium chloride, sodium hydroxide, and sodium chloride were purchased from Sinopharm

Chemical Reagents (China). DOX and potassium ferrocyanide were purchased from Aladdin Chemical (Shanghai, China). Agarose, 50× TAE buffer, Tris(hydroxymethyl)aminomethane (Tris), Exo III, Fetal bovine serum, and all oligonucleotides are from Sangon Biotech (China). The sequence is listed in Table S1.

Exo III reaction buffer (10 mM Tris HCl, 10 mM $MgCl_2$, 10 mM KCl, 50 mM NaCl, and pH 7.6). Milli-Q water was used for all of the solutions.

2.2. Instrumentation

All of the electrochemical measurements were carried out on a CHI 660C workstation (Chenhua, Shanghai) with a three electrode system of an indium tin oxide (ITO) working electrode, a platinum wire counter electrode and a reference electrode of Ag/AgCl (sat. KCl). Differential pulse voltammetry (DPV) was performed at a scanning potential from −0.7 to 0.6 V with an increment potential of 4 mV, the amplitude, the pulse width, and the pulse period were set to values of 0.05 V, 0.05 s, and 0.5 s. Before measurement, the ITO electrode was treated with water ethanol and acetone for 10 min repeated. Then, the ITO electrode was immersed in 1 mM NaOH solution for 5 h and sonicated in ultrapure water for 10 min. Finally, a negatively charged working electrode surface was obtained [38].

2.3. Construction and Detection Process of This Assay

HP was preheated to 95°C for 5 min and cooled to room temperature before use. Then, a mixture of 100 μL containing 0.7 μM HP1, 3.5 μM HP2, 0.16 unit/μL Exo III, and varying concentrations of target DNA in a 10 mM Tris-HCl buffer was incubated at 37°C for 75 min; subsequently, add 100 μL of the sample to 1 mL of 10 mM Tris HCl buffer containing 0.4 μM DOX and 10 μM Fe^{2+}. The solution was mixed at room temperature in the dark for 20 min before electrochemical testing.

2.4. Gel Electrophoresis

Gel electrophoresis was conducted on a 5% agarose gel at room temperature in TAE buffer at 120 mA for 60 min. The red-stained gel was imaged using a Tanno 1600+ Imaging System (Tanon, China).

2.5. Cell Culturing and Extraction of miRNA-378

Human normal liver cells (L02) and human cervical cancer cell lines (HeLa) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. During the exponential phase of growth, cells were collected and washed twice with phosphate buffered saline (pH 7.4), thereafter suspended in 1× CHAPS lysis buffer, shaken at 4°C for 30 min, and centrifuged at 10,000 rpm for 30 min at 4°C. The resulting supernatant was collected and stored at −80°C until used for further analysis.

3. Results and Discussion

3.1. Design Principle of the Sensor

Here, we report a label-free homogeneous electrochemical sensing platform for an ultrasensitive miR-378 assay based on Exo III-aided recycling amplification.

The nucleic acids in this detection system typically contained hairpin probes HP1 and HP2. The HP could form a stable stem-loop structure, including a 3′-protruding DNA fragment. As shown in Scheme 1, in the absence of miR-378, the HP was stable, most of the DOX was intercalated into the stem of the HP, and the diffusion rate of DOX was significantly reduced. Furthermore, owing to the electrostatic repulsion of the HP towards the indium tin oxide (ITO) electrode, the intercalated DOX molecules could not easily reach the electrode surface, resulting in a reduced electrochemical signal response.

[figure(s) omitted; refer to PDF]

When miR-378 is present, HP1 binds to miR-378, forming a 3′ terminus recognized by the Exo III. Exo III is then triggered to cleave HP1, releasing both miR-378 and the DNA fragment of HP1. The released miR-378 then binds to the next HP1, whereas the remaining DNA fragment of HP1 binds to HP2, triggering endonuclease III. Thus, a single miRNA, miR-378, can repeatedly and consecutively trigger a signaling response.

Ultimately, the stem-loop structures of HP1 and HP2 are disrupted, releasing a large amount of DOX into the solution, resulting in a higher electrochemical signal. Fe^{2+} maintained its free state in solution throughout the reaction, and a constant I_{Fe}^{2+} was used as an internal reference signal to enhance the reliability of this sensing system.

Consequently, the Exo III-based label-free ratiometric homogeneous electrochemical biosensor for detecting the miR-378 was developed because of two signal variations.

3.2. Feasibility of Homogeneous Electrochemical-Based miR-378 Assay

As shown in Figure 1(a), the signal changes in DOX under different conditions were analyzed. Upon adding HP to the system, DOX was intercalated into the HP, exhibiting a lower peak current. Furthermore, upon the addition of both HP and the target, the peak current remained largely unchanged compared to that when only HP was present. In the absence of the target, the addition of HP and Exo III resulted in a slight increase in the DOX peak current, possibly due to nonspecific cleavage by Exo III. Only when HP, the target, and Exo III were simultaneously present in the system, a significant increase in the peak current occurred.

[figure(s) omitted; refer to PDF]

Gel electrophoresis experiments were also performed to verify Exo III-aided target recognition in this homogeneous system. As shown in Figure 1(b), the migration of HP1 cells (lane a) was slower than that of HP2 cells (lane b). No new bands appeared in the presence of HP1 + HP2 (lane c), indicating the stable coexistence of HP1 and HP2 in the absence of the target. With the addition of miR-378 to HP1 + HP2 (lane d), a band with a molecular weight greater than that of HP1 emerged, indicating successful binding between HP1 and miR-378. In the presence of HP1 + HP2 + Exo III (lane e), no significant change in band intensity was observed, suggesting that Exo III was not triggered without a target. However, the band nearly disappeared when miR-378 and Exo III were added to the HP1 + HP2 (lane f), indicating the successful initiation of the cleavage process for Exo III.

3.3. Optimization of Experimental Conditions

This experiment involved two hairpin probes, with HP1 primarily responsible for target recognition and HP2 amplifying the signal. The concentration ratio of the two probes influences the sensitivity of the experiment [39, 40]. Under the conditions of HP1-HP2 total concentration 2.1 μM, target concentration 5 nM, DOX 1.0 μM, and Fe^{2+} 10 μM, electrochemical tests were conducted at 37°C for 30 min with the addition and without the addition of 0.2 U/μL Exo III. Subsequently, the Δ (I_{DOX}/I_{Fe}^{2+}) for both cases was calculated. As shown in Figure 2(a), results demonstrated that no noticeable change was observed beyond a 1 : 5 ratio of HP1-HP2. Therefore, a concentration ratio of 1 : 5 was used in the subsequent experiments.

[figure(s) omitted; refer to PDF]

Optimization was performed under specific experimental conditions to achieve the optimal performance. As shown in Figure S1, the initial signal of DOX at different concentrations is denoted as I0, whereas the signal after the addition of HP at different concentrations is denoted as I1. Conditions with the greatest variation relative to the blank were obtained. The ratio of I0–I1 was investigated when different concentrations of DOX were added to varying concentrations of the probes.

At a constant HP concentration, as the concentration of DOX increased, the magnitude of the signal initially increased and then decreased. This phenomenon can be attributed to excessive DOX reducing the proportion of DOX signals embedded in the double-stranded structure, whereas insufficient DOX fails to respond promptly after HP cleavage, thereby lowering the sensitivity of the detection system. At a constant DOX concentration, as the concentration of hairpin probes increased, the magnitude of the signal change increased. However, excess hairpin probes can affect the cleavage time of Exo III. Considering the probe dosage and subsequent enzyme cleavage reaction, the experimental conditions were ultimately selected as DOX 0.4 μM, HP1 0.7 μM, and HP2 3.5 μM. The effect of HP on the Fe^{2+} signals was tested under these conditions, as shown in Figure S3. No significant differences were observed in the signals before and after the addition of HP.

After determining the probe concentration (Figure 2(b)), the intercalation times for HP and DOX were optimized. After 20 min, no significant signal changes were observed, indicating complete binding between DOX and HP.

After determining the concentration of the HP, the enzyme digestion conditions for Exo III were optimized. Under the aforementioned experimental conditions, 5 nM miR-378 and varying concentrations of Exo III were added, followed by incubation at 37°C for 30 min. Figure 3(a) shows that the electrochemical signal no longer increased significantly at 0.16 U/μL [41–43]. The digestion time was further optimized. As shown in Figure 3(b), the electrochemical signal

ceased to increase after 75 min. In addition, further optimizations were made for digestion temperature (Figure S4) and pH (Figure S5).

[figure(s) omitted; refer to PDF]

3.4. Analytical Performance of the Assay

Under optimized experimental conditions, the DPV responses were tested with a series of concentrations of miR-378. As shown in Figure 4(a), the DPV signals increased successively with increasing miR-378 concentration, indicating a strong dependence of the electrochemical signal of DOX on the concentration of the target DNA. This confirms the working principle of triggering Exo III digestion by hybridizing the target DNA with the hairpin probe. Figure 4(b) demonstrates an excellent linear correlation (correlation coefficient of 0.997) between I_{DOX}/I_{Fe}^{2+} and target concentrations ranging from 0.05 to 5 nM. Direct measurement of the target DNA achieved a detection limit as low as 50 pM.

[figure(s) omitted; refer to PDF]

3.5. Selectivity, Reproducibility, and Stability of the Assay

To assess the selectivity of the proposed method, interference tests using other miRNAs and mismatched sequences with miR-378 were performed at a concentration of 5 nM for both the interfering agents and the target [44]. Figure 5(a) illustrates that the signals from the interfering agents showed little to no discernible difference from the blank, whereas the presence of miR-378 elicited a notable response, indicating the excellent selectivity of the assay. Furthermore, as shown in Figure S2, the reproducibility of the measurements was evaluated by performing parallel measurements of miR-378 at 5 nM nine consecutive times [45], which yielded a relative standard deviation estimate of 1.18%. In addition, to assess stability, the samples were stored in the dark at 4°C for 7 days and tested daily. As shown in Figure 5(b), even after 7 days, the DPV response retained 96.0% of the initial signal after storage at 4°C for 7 days indicating good stability [46].

[figure(s) omitted; refer to PDF]

3.6. miRNA Detection in the Sample

To evaluate the applicability of the assay, varied concentrations of miRNA-378 were introduced into bovine serum diluted at a ratio of 1 : 20. Preprocessing involves thawing the frozen plasma to room temperature and diluting it with a buffer before spiking [47]. As shown in Table 1, the recovery rates ranged from 99.65% to 102.56%. These results demonstrate promising prospects for detecting miRNAs in authentic biological samples.

Table 1

Determination of miRNA-378 in bovine serum sample with suggested methodology.

Sample	Added (nM)	Founded (nM)	Recovery (%)
1	2.5	2.49	99.65
2	10	10.26	102.56
3	25	25.46	101.84

Moreover, the expression level of miRNA-378 in cancer cell was further studied with this electrochemical biosensor. The cell lysates were extracted from HeLa cells and L02 cells and used after 50-fold dilution. The content of miR-378 is higher in HeLa cells than in normal cells (Figure S6). This results consistent with the earlier reported [48] and indicate the potential application in complex sample detection.

4. Conclusion

In summary, a label-free and immobilization-free ratiometric homogeneous electrochemical biosensor based on Exo III was developed. The Exo III-aided method achieved target recycling signal amplification, enabling the rapid and simple detection of miR-378. Compared to other miRNA detection methods, this method avoids probe modification.

DOX and Fe^{2+} can simultaneously generate response signal (I_{DOX}) and reference signal (I_{Fe}^{2+}) in solution. The miR-378 can be quantified by the ratio of I_{DOX}/I_{Fe}^{2+}, reducing the influence of the testing environment on the results and improving the accuracy of the test results. The detection process of this method was conducted in solution, avoiding the intricate modification and probe immobilization steps of heterogeneous electrochemical sensors. Therefore, this strategy holds great promise as a simple and highly sensitive method for miR-378 detection and demonstrates significant practical application prospects for early cancer diagnosis based on miR-378.

Authors' Contributions

Bingyuan Fan and Qian Wang contributed equally to this work.

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DETAILS

Subject:	Labels; Electrochemical analysis; Electrodes; Reference signals; Cancer therapies; Sensors; Amplification; Medical research; MicroRNAs; Diffusion rate; Biosensors; Potassium; Doxorubicin; Iron cyanides; Indium tin oxides
Location:	China
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Number of pages:	8
Publication year:	2024
Publication date:	2024
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:	2024-01-24 (Received); 2024-02-24 (Revised); 2024-05-06 (Accepted); 2024-05-21 (Pub)
DOI:	https://doi.org/10.1155/2024/8368987
ProQuest document ID:	3063163984
Document URL:	https://www.proquest.com/scholarly-journals/label-free-ratiometric-homogeneous/docview/3063163984/se-2?accountid=211160
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Last updated:	2024-06-03
Database:	Publicly Available Content Database

Sorption Characteristics and Chromatographic Separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ from Aqueous Media by Chelex-100 (Anion Ion Exchange) Packed Column

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ProQuest document link

ABSTRACT (ENGLISH)

There is growing demand for separation of ^{90}Y carrier free from ^{90}Sr coexisting to produce high purity ^{90}Y essential for radiopharmaceutical uses. Thus, in this context the sorption profiles of Y^{3+} and Sr^{2+} from aqueous solutions containing diethylenetriaminepenta acetic acid (DTPA), ethylenediaminetetra-acetic acid (EDTA), acetic acid, citric acid, or NaCl onto Chelex-100 (anion ion exchange) solid sorbent were critically studied for developing an efficient and low-cost methodology for selective separation of Y^{3+} from Sr^{2+} ions (1.0×10^{-5}M). Batch experiments displayed relative chemical extraction percentage (98±5.4%) of Y^{3+} from aqueous acetic acid solution onto Chelex-100 (anion ion exchanger), whereas Sr^{2+} species showed no sorption. Hence, a selective separation of Y^{3+} from its parent $^{90}Sr^{2+}$

has been established based upon percolation of the aqueous solution of Y^{3+} and Sr^{2+} ions containing acetic acid at pH 1-2 through Chelex-100 sorbent packed column at a 2 mL min^{-1} flow rate. Y^{3+} species were retained quantitatively while Sr^{2+} ions were not sorbed and passed through the sorbent packed column without extraction. The sorbed Y^{3+} species were then recovered from the sorbent packed column with HNO_3 (1.0 M) at a 1.0 mL min^{-1} flow rate. A dual extraction mechanism comprising absorption associated to “weak-base anion exchanger” and “solvent extraction” of Y^{3+} as $(YCl_6)^{3-}$ and an extra part for “surface adsorption” of Y^{3+} by the sorbent is proposed. The established method was validated by measuring the radiochemical (99.2 ± 2 1%), radionuclide purity and retardation factor (Rf = 10.0 ± 0.1 cm) of $^{90}Y^{3+}$ recovered in the eluate. Ultimately, the sorbent packed column also presented high stability for reusing 2-3 cycles without drop in its efficiency (±5%) towards Y^{3+} uptake and relative chemical recovery. A proposed flow sheet describing the analytical procedures for the separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ using chelating Chelex 100 (anion exchange) packed column is also included.

FULL TEXT

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1. Introduction

In nuclear medicine, ^{90}Y is an important radioisotope due to its satisfactory physical characteristics that include β− emissions that allow tissue diffusion to a moderately extensive range, a suitable half-life (t1/2 = 64.4 h) and a nonradioactive daughter [1–6]. The United States-food and drug administration (US-FDA) has accepted Zevalin drug that incorporates ^{90}Y [7, 8]. So, it is essential to the ^{90}Sr impurity in ^{90}Y samples owing to the radiotoxicity of ^{90}Sr [9, 10]. ^{90}Y decays to the stable ^{90}Zr daughter, thus it is used as a pure *β*-emitter. High complex formation constants of Y^{3+} with complexing ligands make $^{90}Y^{3+}$ valued in preparing radiopharmaceutical reagents [11, 12]. In light of the perceived need to abolish the radiotoxicity risk poses by ^{90} Sr, accurate separation, accurate, and reliable removal of ^{90}Sr impurities from Y samples is of great importance. Therefore, great attention has been oriented towards establishing selective and low cost methods for chromatographic separation of $^{90}Sr^{2+}$ from $^{90}Y^{3+}$ samples with good radiochemical and radionuclide purity.

Recently, huge volumes of radioactive wastes and a diversity of radionuclides are generated from nuclear reaction process are certainly released into the natural environment [13, 14]. Thus, many approaches have been described in the last two decades for complete separation of $^{90}Y^{3+}$ from various solutions via 2-ethylhexyl phosphonic acid mono-2-ethylhexyl ester (PC88A) in kerosene, [15], solvent extraction [16–20], organic resin and chelating agent and TBP-treated resins such as amberlite XAD-4 [21–24], cation ion exchanger [25, 26], pyridyl azo napthol (PAN)/zeolite composite [27], Teflon grain support di (2-ethyl hexyl) phosphoric acid-dodecane [28], Chelex-100 [29], paper chromatography [30], nafion-117, and dowex 50W X8 [31]. A ^{90}Y generator system contained two extraction columns and sec-octylphenoxy acetic acid and tri-n-butyl phosphate as modifier have been used for separation of ^{90}Y from ^{90} Sr [32–34]. $^{90}Sr/^{90}Y$ generator elutes ^{90}Y free from ^{90}Sr with relative chemical recovery over 90% [35, 36]. Inorganic ion exchangers, e.g., cerium (IV) iodotungstate [37], zirconium-vanadte gel [38, 39] and chelating Chelex-100 ion exchange [10] in several steps have been used for complete separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ [31–34]. However, some of these methods have many drawbacks and limitations such as complexity, high cost, the necessity for suitable operation, and preconcentration due to their low capacity. Therefore, searching for establishing low cost, simple systems with short analytical time and ruggedness for chromatographic separation of Y^{3+} from Sr^{2+} species in aqueous solutions with good radiochemical and radionuclide purity are much sought after [37, 38].

With this background in mind, taking into account the importance of highly pure $^{90}Y^{3+}$, the characteristics of the chelating group iminodiacetate moieties of the Chelex-100 anion ion exchanger and some of the synthesized inorganic sorbents as ideal and ecofriendly solid sorbents and in continuation to our previous study [10, 38–43], the current study is aimed to (i) revising the sorption profiles of Y^{3+} and Sr^{2+} onto the anion ion exchanger as a new candidate solid sorbent, (ii) establishing a simple and low cost and selective chromatographic separation of ^{90}Y from its parent ^{90}Sr by chelating Chelex-100 (anion ion exchanger) sorbent packed column and finally, and (iii) testing the reusability of the established chelating Chelex-100 sorbent packed column towards separation of Y^{3+} from its parent

Sr^{2+} species in aqueous solutions. These results are supportive to recognize the analytical utility of the Chelex-100 in nuclear medicine, waste management and to properly assign the physicochemical behavior of radionuclide $^{90}Y^{3+}$ and $^{90}Sr^{2+}$.

2. Materials and Methods

2.1. Chemicals

Analytical reagents chemicals were used as received. High-density polyethylene (HDPE) bottles and all glassware's were immersed in hot detergent for 24 h and soaked in the acid mixture of HCl (50% v/v)-conc. HNO_3 (3.0 M) at 1 : 1 v/v ratio, washed with double distilled water and finally dried in oven at 80–90°C. Stock solutions (1.0 M) of Sigma-Aldrich diethylenetriaminepenta acetic acid (DTPA), ethylenediaminetetra acetic acid (EDTA), HCl, HNO_3, acetic acid, citric acid, and NaCl were prepared in deionized water. The chelating Chelex-100 (anion exchanger) (100–200 mesh) was purchased from BDH (Poole, England) and was used after washing 3-4 times with deionized water before use. ^{89}Sr tracer was used as a substitute for ^{90}Sr and the radio tracers $^{90}Y^{3+}$ and $^{90}Sr^{2+}$ were acquired by exposing the target Y_2O_3 and $SrCO_3$ (99.9% purity) in Al container at an average thermal neutron flux density of 1.3×10^{13} neutrons/cm^2/s at the ERR-1 research reactor (Atomic Energy Authority, Inshas, Egypt). Beta counting (liquid scintillation) was used to check $^{90}Sr^{2+}$ efficiency. Millipore water (resistivity 18.2 MΩcm) was used in all experiments. The test solutions were prepared by spiking 100 mL of the water sample with a certain amount of YCl_3 (1.771×10^{-2} M) and $SrCl_2$ (1.323×10^{-2} M) individually.

2.2. Apparatus

A Geiger–Muller (β− Counter) and window detector and a Scaler Ratementer SR7 (*γ*-Scintillation Counter) were used for ordinary gamma ray counter and it is fixed with well NaI (Tl) crystal. A high purity germanium (HPGe) coupled to a calibrated multichannel gamma analyzer (Silena, Milan, Italy) was employed to test impurities in the irradiated Y_2O_3 and $SrCO_3$ as reported earlier [10]. The activity of ^{90}Sr activity was monitored as reported [10]. Standard radionuclides were prepared from a mixed source of the radioisotopes ^{155}Eu (86.5 and 105.3 keV), ^{57}Co (122.1 and 136.5 keV), and ^{137}Cs (661.6 keV). A mechanical shaker (Corporation Precision Scientific, Chicago, USA) with a shaking rate of 10–250 rpm was used for performing batch experiments. A centrifuge Chermle Z 230 A of 5500 rpm speed was also used. A close-fitting glass Jar chromatography (40 cm L and 5 cm id) of Whatmann paper No 1 (3 cm × 30 cm), Milli-Q Plus water system (Millipore, Bedford, MA, USA), and glass columns of 10.0 cm length (8, 15, and 20.0 mm internal diameter) were used in flow experiments. A digital micropipette (5.0–100 *μ*L) and a Jenway pH meter (model 3510) were used for the preparation and measuring the pH of more diluted working solutions, respectively.

2.3. Preparation of the Radioactive ^{90}Y and ^{09}Sr Tracers

An accurate mass of $SrCO_3$ (200 mg, 99.9% purity, MW = 147.63) or Y_2O_3 (200 mg, 99.9% purity, MW = 225.8) was enfolded in pure Al foil precleaned with acetone and air dried before use to reserve the solid from possible contamination during cooling in the reactor. Al foil was then presented into another outer leak proof of Al and the target compound $SrCO_3$ and Y_2O_3 were exposed for two days in the perpendicular channel of 2 MW water-cooled research reactor ERR-1 at average thermal neutron flux density of 1.3×10^{13} n/cm^2s (Atomic Energy, Inshas, Egypt). The exposed target was left-hand to cool for 24 h before use. Accurate masses of each exposed $SrCO_3$ (200 mg) and Y_2O_3 (200 mg) were dissolved in HCl (50.0 mL, 2.0 M). The test solution of $^{89}SrCl_2$ and $^{90}YCl_3$ was heated to dryness and redissolved in deionized water (100 mL) to achieve the final concentrations of $^{89}SrCl_2$ (1.323×10^{-2} M) and $^{90}YCl_3$ (1.771×10^{-2} M), respectively. In the irradiated product, the impurities were then checked as reported earlier [10]. The specific activity (*S*) was then computed as reported earlier [40–42]. The purity of irradiated $^{90}YCl_3$ was also confirmed from the decay shape over 3 half-lives (t1/2) period at neutron flux density of 1.3×10^{13} neutrons/cm^2/s as reported [44, 45].

2.4. General Batch Extraction Procedures

In a series of precleaned penicillin bottle, accurate masses (0.100 ± 0.002 g) of the precleaned Chelex-100 (Anion ion exchanger) were transferred and equilibrated with 20.0 mL solutions containing known concentrations (1.0×10^{-5} M) of YCl_3 or $SrCl_2$ in acetic acid, DTPA, EDTA, citric acid, or NaCl (1.0×10^{-3} M). The test solutions were then shaken

for 60 min at various pH at 25°C. The solid phase extractor in each solution was allowed to settle down and an accurate volume (1.0 mL) of the aqueous phase of each solution was separated out. The radioactivity of ^{90}Y and ^{90}Sr, the relative extraction percentage (%*E*) and the amount (qe) of Y^{3+} and/or Sr^{2+} between the sorbent phase and the aqueous solution were then computed from their activities before and after extraction as reported [38, 39]. The distribution ratio (D, mL/g) of Y^{3+} and/or Sr^{2+} were also calculated using the following equation [43]:(1)Kd=%E100−% E×VmLWg mL/g,where *V* is the volume of solution (mL) and W is the mass of the dry ion exchanger (g). The quantity (qe) of Y^{3+} extracted per unit mass of the sorbent (mol g^{-1}) was then calculated as reported earlier [39].

2.5. Separation of Y^{3+} from Sr^{2+} by Chelex-100 (Anion Exchanger) Packed Column

An accurate mass (1.0 ± 0.002 g) of the Chelex-100 (anion exchanger) sorbent was homogeneously packed in glass column (10.0 cm length × 0.8 cm i.d). An aqueous solution of acetic acid (1.0×10^{-3} M) of pH 1-2 was introduced into the sorbent packed column and quartz wool was then placed at the top of the resin after the sorbent had established down. This step helps in avoiding the disturbance of the resin particles during percolation of the test solution. Column was then washed with water 2-3 times at a 2.0 mL/min flow rate. The test solution (25 mL) containing Y^{3+} and Sr^{2+} and DTPA (1.0×10^{-3} M) was permeated to pass through the column at a 2.0 mL min^{-1} flow rate. Y^{3+} was only sorbed quantitatively, whereas Sr^{2+} species were passed through the column without sorption as specified from the radioactivity measurement of $^{90}Y^{3+}$ and $^{90}Sr^{2+}$ in the effluent. The sorbed Y^{3+} species were then recovered from the sorbent packed column with HNO_3 (10 mL, 1.0×10^{-1} M) at a 2.0 mL min^{-1} flow rate. The recovered Y^{3+} solutions were heated to dryness, redissolved in ultra-pure water, and the Y^{3+} purity was finally determined via computation of the half life (t1/2) as reported [39, 44]. Moreover, the influence of other parameter such as flow rates (1.0–5 mL min^{-1}) and the internal diameter (0.8, 1.5, and 2.0 cm) on the analytical performance of Chelex-100 packed column for separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ was also examined.

2.6. Determination of Radiochemical and Radionuclidic Purity of ^{90}Y

^{90}Y purity was critically checked as follows: On a strip of Whatmann No. 1 paper, a drop of 5.0 μL was put on the lower end of the chromatographic paper. After the spot has dried, the strip was immersed at its lower end in TRIS buffer (0.1 M) of pH 7 as a developer using ascending chromatograph technique without reaching the spot. The paper was left for 5-6 min to develop; the solution was then reserved out and the paper was allowed to dry. The paper was divided into equal parts (1.0 cm sections) and GM was used for counting β activity and the sorption factor (Rf) was then computed. The radionuclidic purity of $^{90}Y^{3+}$ in the eluate was determined from the purification factor (Pf)=*A*/Ao, where *A* and Ao are the activity of ^{89}Sr in the eluate and solution, respectively. The radionuclidic purity was also computed from the decay curve over a period of at least 3 half-lives. The decay curve of $^{90}Y^{3+}$ was planned by detecting β− activity at one day intervals for 10 days after elution [46, 47].

3. Results and Discussion

3.1. Preliminary Study on the Sorption Profile of Y^{3+} and $^{89}Sr^{2+}$ onto Chelex-100

The majority of ion exchangers using organic resin and chelator/complexing agent are simple and fast for separation of elements. However, they do not offer a ready-to-use eluate [3, 5]. Introductory study on Y^{3+} and Sr^{2+} uptake from the aqueous solution by chelating Chelex-100 (anionic form) displayed significant Y^{3+} sorption in a short time. Thus, a detailed study on the sorption profile of Y^{3+} and Sr^{2+} from the aqueous solution onto the established Chelex-100 sorbent was critically studied.

3.2. Programming of the Analytical Parameters

3.2.1. Impact of pH of the Extraction Media

The pH has a significant influence on the retention capacity for the ion exchange materials since the electrostatic interactions are the driving forces. Thus, in batch experiment, the uptake of Y^{3+} and Sr^{2+} (1.0×10^{-5} M) as YCl_3 or $SrCl_2$ from the test aqueous solutions containing DTPA, EDTA, acetic acid, citric acid, or NaCl (1.0×10^{-3} M) by Chelex-100 sorbent was studied over a wide range of solution pH (pH 1–11) after a shaking period of 60 min. In the aqueous phase, the amount of $Y^{3+}$and Sr^{2+} was then determined. The sorption data of Y^{3+} and Sr^{2+} from the various extraction media into Chelex-100 (anionic form) are summarized in Table 1. In acetic acid and DTPA media, the sorption profiles of Y^{3+} and Sr^{2+} are also illustrated in Figures 1 and 2, respectively. In acetic acid media, the K_d of Y^{3+}

sorption onto Chelex-100 sorbent reached a maximum value at pH 1–6 (Kd = 9930.6 ± 12.4), whereas the Kd gradually decreased on increasing the solution pH and reached minimum value (Kd close to zero negligible value) at pH11 as shown in Figure 1. On the other hand, Sr^{2+} uptake was insignificant in the pH range pH 1–5 (Kd = 0.0) and it gradually increased on growing the pH and reached maximum value at pH 9 (Kd = 9910.6 ± 10.6) and levelled off at higher pH up to pH 11 (Kd = 6000.7 ± 9.3) as shown in Figure 1. The sorption selectivity of Y^{3+} at pH 1–3 onto chelating Chelex-100 sorbent in the various extraction media followed the order: NaCl (Kd = 12278.5 ± 13.8 > acetic acid (Kd = 9930.6 ± 12.4) > EDTA (Kd = 9905.3 ± 5.4) > citric acid (Kd = 9886.4 ± 5.8) > DTPA (Kd = 7004.7 ± 3.3). At pH 1–3, Sr^{2+} species did not retained except in NaCl, where Kd = 66654.6 ± 5.7 (Table 1).

Table 1

Influence of the extraction media (acetic acid, citric acid, DTPA, EDTA and NaCl), and the solution pH on the distribution ratio (Kd) of Y^{3+} and Sr^{2+} towards retention onto Chelex 100 (anion form)†.

pH	Compound									
Acetic acid		Citric acid		DTPA		EDTA		NaCl		Y^{3+}
Sr^{2+}	Y^{3+}	Sr^{2+}	Y^{3+}	Sr^{2+}	Y^{3+}	Sr^{2+}	Y^{3+}	Sr^{2+}	pH 1–pH 3	9930 ± 12.4
0.0	9886 ± 5.8	0.0	7004 ± 3.3	0.0	9905 ± 5.4	0.0	12278 ± 13.8	66654 ± 5.7	pH 4-pH5	9927.7 ± 9.3
0.0	123.5 ± 4.7	0.0	20.4 ± 2.9	0.0	7.3 ± 0.21	4.3 ± 2.5	12273 ± 10.4	4734 ± 12	pH 7	9924 ± 11.3
3924.4 ± 11.3	125.8 ± 5.7	2012.6 ± 3.7	20.4 ± 2.9	2000 ± 2.2	0.0	4.3 ± 0.2	12275 ± 15.1	1154 ± 5.1	pH 9	4027 ± 7.5
9120 ± 12.4	0.0	9654 ± 5.7	20.4 ± 2.1	1734 ± 2.2	0.0	4123 ± 2.3	1027 ± 9.5	12276 ± 5.7	pH 11	100.7 ± 3.7

†The concentration of acetic acid, citric acid, DTPA, EDTA, or NaCl is equal to 1.0×10^{-3} M.

[figure(s) omitted; refer to PDF]

In DTPA or acetic acid media of pH ranging from pH 1 to pH 4, Y^{3+} species were retained quantitatively onto the chelating Chelex-100 ion exchanger sorbent and the values of Kd were reproducible compared to EDTA, citric acid, or NaCl. Representative plot of Kd *versus* of pH of Y^{3+} and Sr^{2+} sorption onto Chelex-100 (anion ion exchanger) from aqueous DTPA solution (1.0×10^{-3} M) after 60 min shaking time at 25 ± 0.1°C is shown in Figure 2. The observed behavior in Figure 2 is most likely attributed to the possible formation of nonpolar complex species of Y^{3+} species $(YCl_6)^{3-}$ with the available iminodiacetate moieties of the chelating Chelex 100 sorbent at pH ≤ 3 (pK_{a1} = 3.2) [40, 42, 43]. At low pH (pH ≤ 3), the possible interaction between the formed complex anion of yttrium $(YCl_6)^{3-}$ and the protonated iminodiacteate moieties of the chelating Chelex−100 anion ion exchanger by forming ternary complex ion associate may also contributed in the observed trend at low pH ≤ 3 [42, 43]. On the other hand, at pH above pH 3, one of the two carboxylic acids of the iminodiacetate moiety is deprotonated carrying a negative charge which attracts other positive cation present in extraction media, e.g., Na^{+} (introduced from pH adjustment by diluted NaOH which compete effectively with Y^{3+} because of their considerably higher concentration in solution [42]. The fact that, in acidic solutions, the N atom of the iminodiactic group retaining free electron pair is protonated, hence the resin is most likely can acts as weakly basic anion exchanger [43]. In addition, deprotonation of the second carboxylic group

of the iminodiacetic moieties could also be proceeded at pH > 7, resulting in destabilization of the “guest-host” complexes between Y^{3+} and aminocarboxylic moieties [43]. This exchanger is also commonly regarded as an amphoteric ion exchanger and its ion exchange function depends on the solution pH that in contact with the resin as presented in Scheme 1.

[figure(s) omitted; refer to PDF]

In DTPA, EDTA, acetic acid, citric acid, or NaCl medium at pH > 7, Chelex-100 sorbent displayed good retention towards Sr^{2+} and the extraction profile of Sr^{2+} followed the order: NaCl (Kd = 12276.9 ± 5.7> citric acid (Kd = 9759.6 ± 5.7) > acetic acid (Kd = 7212.2 ± 5.8) > EDTA (Kd = 3623.4 ± 3.7) > DTPA (Kd = 1614.5 ± 5.2) was achieved. On the other hand, at pH > 7, the chelating Chelex-100 sorbent displayed no affinity towards Y^{3+} from citric acid, EDTA or DTPA. The fact that the chelating agents EDTA and DTPA act as competitors having similar groups with the Chelex-100 sorbent and both are able to form complexes in solution with Y^{3+} and Sr^{2+} preventing their adsorption while acetic acid is the weakest medium [42, 48]. This behavior is most likely attributed to the strong and weak ion-association interaction of the accessible specific active sites of the Chelex-100 solid extractor towards Sr^{2+} and Y^{3+}, respectively, as reported the authors in [42, 48]. On the other hand, it may be thought that for smaller molecules a more pronounced difference between the adsorption sites on the surface of Chelex-100 and inside the sorbent pores as reported [48]. In acetic acid, EDTA or NaCl at pH > 7, separation of Y^{3+} from Sr^{2+} was not complete. Thus, in Y^{3+} separation from Sr^{2+}, acetic acid, or DTPA (1.0×10^{-3} M) was implemented as a preferred extraction medium at lower pH in the subsequent study.

3.2.2. Impact of Shaking Time

The influence of shaking time over 0.0–2.0 h on Y^{3+} uptake from aqueous acetic acid or DTPA solution (1.0×10^{-3} M) onto Chelex-100 was studied. Y^{3+} sorption onto the ion exchanger was fast at the initial stage and attained maximum sorption percentage after 60 min shaking time and remained constant at extra time. This trend was supported from the value of half life (t1/2) of Y^{3+} retention via the plots of log Ct/C0 of Y^{3+} versus shaking time. The value of the half-life (t1/2) of Y^{3+} retention from the aqueous acetic acid or DTPA solution as computed from the plot of log Ct/C0 of Y^{3+} versus shaking time was in the range 2.42 ± 0.05 min. Representative plot is given in the Supplementary Description (SD. 1). Thus, the rate-controlling step for Y^{3+} sorption by the sorbent is not only gel diffusion control as in the ion exchangers [49, 50]. At the initial stage of shaking time, the plot of %*E* of Y^{3+} versus log time was fast and linear approving the occurrence of intraparticle diffusion [10, 50]. Thus, a 60 min shaking time was adopted in the following study.

3.2.3. Influence of Media Polarity

The extraction medium in solid phase extraction procedures has a pronounced effect on the performance of Y^{3+} separation. Thus, Y^{3+} and Sr^{2+} ions uptake from the test aqueous solutions (20.0 mL) containing various known concentrations (1×10^{-5}–1.0 M) of HCl or HNO_3 at standard concentration of Y^{3+} and Sr^{2+} (1.0×10^{-5} M) was studied over a shaking time of 60 min at room temperature. At equilibrium, the remained Y^{3+} and Sr^{2+} ions in the aqueous phase was measured and the extraction percentage (*E*, %) and the *D* were then computed as reported [10]. In HCl media, the data are presented in Figure 3, where the *E*% and *D* of Y^{3+} and Sr^{2+} by the sorbent decreased on rising HCl concentration from 1.0×10^{-5} to 1.0 M. At HCl concentration ≥1.0×10^{-1} M, Sr^{2+} species were not retained, while 75.0 ± 2.1% of Y^{3+} was retained. The strong interaction of the active sites of the sorbent with Y^{3+} may account for this trend [51, 52]. The strong binding of Y^{3+} to form $[YCl_6]^{3-}$ complex species [53] compared to Sr^{2+} in HCl media may also account for the observed trend. In HNO_3 (1.0×10^{-5}–1.0 M), Y^{3+} species did not sorbed onto chelating Chelex-100 sorbent whereas significant sorption of Sr^{2+} (Kd = 650.4 ± 3.64 mL g^{-1}) was noticed at 1.0×10^{-5} M and decreased on rising HNO_3 concentration up to 1.0 M (Kd = 210.2 ± 3.64 mL g^{-1}). The average chemical extraction percentage of ^{89}Sr and ^{90}Y from acetic acid (1.0×10^{-3} M) at different solution pH onto Chelex-100 was also studied. The results are illustrated in Figure 1 where in acetic acid media at pH ≤ 5, Y^{3+} species were retained quantitatively while Sr^{2+} ions did not get sorbed. However, in the subsequent study, HNO_3 (1.0×10^{-1} M) was nominated as a prober reagent for Y^{3+}recovery from Chelex-100 sorbent packed column since it is easily evaporated by gentle heating.

[figure(s) omitted; refer to PDF]

3.3. Possible sorption Mechanism for Y^{3+} Retention

The affinity of the sorbent towards Y^{3+} played an important role on its uptake. The nature and number of the specific sorbent sites are involved instantaneously in Y^{3+} uptake from the solution [16]. The chelating Chelex-100 sorbent acts as an active “weak anion-exchanger” towards complex species of Y^{3+} such as $(YCl_6)^{3-}$ in HCl media [53] and “liquid-liquid extraction” with the salt performing as salting-out reagent in Y^{3+} uptake. The salt added decreases the water molecules available to solvate Y^{3+} ions which would be required out of the solvent onto the sorbent phase. Thus, water structure enforced ion pairing is somewhat the driving force for Y^{3+} uptake and “surface adsorption” effectively take part in the Y^{3+} extraction [53, 54]. Based on the obtainable results and the data reported earlier [54, 55], a dual sorption mechanism involving absorption related to “weak-base anion exchange” and “solvent extraction” in addition to “surface adsorption” of Y^{3+} is proposed. Thus, retention mechanism of Y^{3+} can be stated by the following equation [54, 55]:(2)Cr=Cabs+Cads=DCaq+SKLCaq1+KLCaq,where Cr and Caq are the equilibrium concentrations of Y^{3+} ions onto the sorbent and in solution, respectively. Cabs and Cads are the equilibrium concentrations of Y^{3+} absorbed and adsorbed onto the sorbent while *S* and KL are the parameters of the Langmuir adsorption model [54, 55].

3.4. Separation of $^{90}Y^{3+}$ from Sr^{2+} by Sorbent Chelex-100 (Anion Form) Packed Column

An aqueous solution (25.0 mL) composed of acetic acid (1.0×10^{-3} M), Y^{3+} and Sr^{2+} ions (1.0×10^{-5} M) was permeated through Chelex-100(Anion exchanger) packed column at a reasonable flow rate (2.0 mL min^{-1}). Y^{3+} species were retained quantitatively whereas Sr^{2+} ions were passed without uptake as revealed from ICP-OES determination of $^{90}Y^{3+}$ and $^{90}Sr^{2+}$ ions in the effluent *versus* reagent blank. Selection of proper eluting agent prior to use of $^{90}Y^{3+}$ for labeling and radiolysis of organic support materials is crucial and is identified as the main limitations of current 90Sr/90Y [35, 56]. Thus, the established methodology offered a facile, better selectivity and simple approach compared to the published work [12–21, 57]. Numerous eluting agents such as HNO_3, $HClO_4$, H_2SO_4, and acetic acid (1.0×10^{-1}M) were checked for recovery of Y^{3+} from chelated Chelex-1000 packed column. Among these reagents, good percentage recovery (99.5±2.9%) of $^{90}Y^{3+}$ was only achieved with HNO_3 (10 mL, 1.0×10^{-1}M) as a prober agent for Y^{3+} recovery at a 1.0 mL/min flow rate using Chelex-100 sorbent packed glass column of 8 mm internal diameter. On the other hand, HNO_3 can easily remove from the recovered $^{90}Y^{3+}$ solution by gentle evaporation. The solid residue was redissolved in deionized water and analyzed as reported [56].

Moreover, the impact of the internal column diameter (0.8, 1.5, and 2.0 cm) on the performance of chelated Chelex-100 packed column on the separation of Y^{3+} from Sr^{2+} ions was examined at a 1.0 mL min^{-1} flow rate. Acceptable separation and relative chemical recovery of Y^{3+} from Sr^{2+} was only achieved at 8 mm internal diameter of the column, whereas at internal diameter greater than 8 mm, Y^{3+} recovery was not complete (<90%) The influence of the flow rate (1.0–5 mL min^{-1}) on Y^{3+} separation from $^{9}Sr^{2+}$ ions was critically tested. Good separation with acceptable relative chemical recovery (over 99%) of Y^{3+} from Sr^{2+} ions was achieved at a flow rate of 1.0 mL min^{-1}. Thus, in the subsequent study, the flow rate and the internal diameter of the Chelex −100 sorbent packed column were adopted at a 1.0 mL min^{-1} flow rate and 8 mm internal diameter.

3.5. Radiochemical Purity of $^{90}Y^{3+}$

Validation of Chelex-100 (anion form)-packed column for chromatographic separation of ^{90}Y from ^{90}Sr was critically tested by calculating the retardation factor (Rf) from the radio chromatogram of ^{90}Y on the original spot constructed by plotting radioactivity (cpm) versus travelled distance, cm. The data are presented in Figure 4 and the Rf value was 10.0±0.1 cm of total activity on the original spot in agreement with the data published earlier [31–36]. These data also signify that over 99.2±2.1% of $^{90}Y^{3+}$ species are present in the eluate as $^{90}YCl_3$ as reported by the authors in [46, 56].

[figure(s) omitted; refer to PDF]

3.6. Radionuclides Purity of $^{90}Y^{3+}$

The proposed protocol was tested by measuring the radionuclidic purity using the purification factor (Pf=A/Ao), where *A* and Ao are the $^{90}Sr^{2+}$ activity in the recovered and loaded solution, respectively. The Pf value was lower than 1.1×10^{-6}, demonstrating negligible impurity of $^{90}Sr^{2+}$ in $^{90}Y^{3+}$ solution [45, 46]. The radionuclidic purity of $^{90}Y^{3+}$

was also computed from radioactivity (cpm) plot of $^{90}Y^{3+}$ in solution versus time (day) (Figure 5). The value of half life ($t_{1/2}$) of ^{90}Y as computed from the decay curve (Figure 5) was found equal 64.4 h in good agreement with the data reported earlier [38, 39], revealing high purity of $^{90}Y^{3+}$ with good performance of Chelex-100-packed column towards $^{90}Y^{3+}$ separation from $^{90}Sr^{2+}$. The whole analytical procedures for $^{90}Y^{3+}$ separation from $^{90}Sr^{2+}$ by Chelex−100 sorbent is presented in the proposed flowsheet (Figure 6).

[figure(s) omitted; refer to PDF]

4. Conclusion, Drawbacks, and Future Outlooks

In summary, the current study presented an optimized protocol for selective separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ with good purity using chelating Chelex-100 (anion exchanger) packed column. The membrane-like structures and the available active sites of the Chelex-100 solid extractor permit good separation of Y^{3+} from Sr^{2+} compared to other sorbents [12–22, 57]. Compared to previous methods for separation of $^{90}Y^{3+}$ from ^{90}Sr, Chelex-100 requires slight sample operation to reduce the analysis time, and it does not require solvent evaporations and reconstruction step. This method displays high selectivity for separation of $^{90}Y^{3+}$ from $^{90}Sr^{2+}$ at the low level. The purity of ^{90}Y can be tested by quality control procedures. The established extractor looks low cost and valuable alternative sorbent over the common rigid or granular solid extractors. A dual sorption mechanism of Y^{3+} comprising both “surface adsorption” and an added component of “ion exchanger and/or solvent extraction” is anticipated. In addition, the results revealed the possible use of Chelex-100 sorbent packed column for complete enrichment and recovery of Y^{3+} for 2-3 times without significant decrease in its performance. Work is ongoing for studying the impact of memory effect, various organic materials in water samples and online enrichment of ultratrace levels of Y^{3+} from great volume of water samples followed by subsequent determination. The study also shows that the established extractor can be used as cheap, efficient and ecofriendly solid sorbent for Y^{3+} separation from Sr^{2+}, whereas other methodologies have high operational costs and sometimes yield undesirable by-products when linked to physical and chemical methods. The fact that the use of one factor at a time has many drawbacks and shortcomings and the cooperating results of numerous features might advance the signal and the utility of the proposed methodology. Therefore, design experiment for separation of Y^{3+} from Sr^{2+} is suggested in the forthcoming study. The developed strategy provides new sorbents for establishing a method for radiochemical separation.

Ethical Approval

This article does not require IRB/IACUC approval because there were no human or animal participants.

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DETAILS

Subject:	Aqueous solutions; Radiochemical analysis; Ion exchangers; Acids; pH; Neutrons; Separation; Acetic acid; Anion exchanging; Strontium 90; Chromatography; Percolation; Citric acid; Nuclear medicine; Chelation; Ion exchange; Radioisotopes; Nuclear reactors; Sorption; Packed columns; High density polyethylenes; Ethylenediaminetetraacetic acids; Solvent extraction; Flow velocity; Sorbents; Purity; Yttrium isotopes
Location:	Egypt; United States--US
Editor:	Mahmood Ahmed

Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-12-13 (Received); 2024-02-29 (Revised); 2024-04-09 (Accepted); 2024-05-13 (Pub)
DOI:	https://doi.org/10.1155/2024/6232381
ProQuest document ID:	3060197524
Document URL:	https://www.proquest.com/scholarly-journals/sorption-characteristics-chromatographic/docview/3060197524/se-2?accountid=211160
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Last updated:	2024-05-27
Database:	Publicly Available Content Database

The Analysis of Leontopodium leontopodioides (Willd.) Beauv. Chemical Composition by GC/MS and UPLC-Q-Orbitrap MS

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ABSTRACT (ENGLISH)

Leontopodium leontopodioides (Willd.) Beauv. (*L. leontopodioides*.) has been used to treat lung diseases in traditional Chinese medicine (TCM). However, a systematic analysis of its chemical components has not been reported so far. In this study, UPLC-Q-Orbitrap MS and GC-MS were applied to investigate the chemical composition of the water extracts and essential oils of *L. leontopodioides*. UPLC-Q-Orbitrap MS adopts a heating electrospray ionization source, collecting primary and secondary mass spectrometry data in positive and negative ions, respectively, and uses Compound Discoverer 3.2 software to analyze the collected raw data. As a result, a total of 39 compounds were identified from their high-resolution mass spectra in both positive and negative ionization modes, including 13 flavonoids and their glycosides, 15 phenolic acids, 4 oligosaccharides and glycosides, 4 pentacyclic triterpenoids, and 3 other compounds. Among them, 18 chemical components have not been reported in *L. leontopodioides*. In the GC-MS section, two common organic solvents (n-hexane and diethyl ether) were used to extract essential oils, and the mass spectra were recorded at 70 eV (electron impact) and scanned in the range of 35~450 m/z. Compounds were identified using NIST (version 2017), and the peak area normalization method was used to calculate their relative amounts. Finally, 17 components were identified in the volatile oil extracted with n-hexane, accounting for 80.38% of the total volatile oil, including monoterpenoids, phenylpropene, fatty acids, and aliphatic hydrocarbons. In the volatile oil extracted with diethyl ether, 16 components were identified, accounting for 73.50% of the total volatile oil, including phenylpropene, aliphatic hydrocarbons, monoterpenoids, fatty acids, and esters. This study was the first to conduct a comprehensive analysis of the chemical composition of the *L. leontopodioides* water extract and its essential oil, and a comprehensive chemical composition spectrum was constructed, to lay a foundation for its further pharmacodynamic material basis and quality evaluation.

FULL TEXT

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1. Introduction

As a traditional Chinese medicine, *Leontopodium leontopodioides* (Willd.) Beauv. has the functions of clearing away the pulmonary heat, relieving cough, and expectorating phlegm and is generally used to treat lung diseases in TCM [1]. *L. leontopodioides* belongs to the Asteraceae family and is a perennial herb with a height of 5~45 cm, and it is widely distributed in northeast, north, and northwest China and grows in arid grasslands, loess slopes, gravel, and mountain grasslands at an altitude of 100~3200 m [2], as shown in Figure 1. Previous studies of *L. leontopodioides* were isolated by chromatographic methods, such as silica gel, ODS, Sephadex LH-20, and HPLC, and identified by chemical and physical methods, especially spectral analysis [3–5]. Modern pharmacological studies have shown that *L. leontopodioides* has anti-inflammatory, antibacterial, antioxidant, hypoglycemic, diuretic, and other effects [6–9]. Chen [10] et al. used chemical and spectroscopic methods to study a 70% EtOH extract of the whole plants of

Leontopodium leontopodioides (Wild.) Beauv obtained leontoaerialosides A (1), B (2), C (3), D (4), and E (5). Zhao et al. [11] found that chlorogenic acid and ferulic acid are components with obvious antioxidant effects in *L. leontopodioides*, and most of the chemical components related to antioxidant activity are phenolic acids. Wu et al. [12] obtained an abundant higher monomer compound by silica gel column chromatography and preparative thin-layer chromatography in ethyl acetate parts of the alcohol extract; through analysis of ultraviolet, infrared, hydrogen, and carbon spectrum, it was presumed as para-hydroxyl-acetophenone. Gao et al. [13] reported the essential oil from the aerial parts of *L. leontopodioides* and found that it not only has low antioxidant activity but also possesses a potent antibacterial activity against *S. aureus* and *B. subtilis*. Although some chemical components, such as flavonoids, phenylpropanoids, phenolics [14], and essential oils, have been isolated from *L. leontopodioides*, systematic analysis of its chemical components has not been investigated. More importantly, *L. leontopodioides* has not been recorded in the Chinese Pharmacopoeia. Therefore, it is necessary to carry out a systematic and comprehensive study of the chemical composition in order to elucidate its pharmacodynamic material basis. At present, there is no research on the chemical composition of the water extract and essential oil of *L. leontopodioides* at the same time.

[figure(s) omitted; refer to PDF]

2. Experimental

2.1. Sample Preparation

The whole plant of *L. leontopodioides* was powdered. Pulverized samples of *L. leontopodioides* (50g) were accurately weighed, add 15 times of distilled water, decocting 3 times for 1 hour each time, combining the three filtrates, evaporating, drying and weighing to prepare the *L. leontopodioides* water extrac, and its yield was 27.63%. Fifty grams of crushed *L. leontopodioides* was precisely weighed, and the supercritical CO_2 extraction method was used for 5 h at a temperature of 45°C and a pressure of 18 MPa to obtain 1.20 mL of dark green essential oil of *L. leontopodioides.*

An appropriate amount of *L. leontopodioides* water extract was weighed, and 1 mL of 80% methanol was added to prepare a solution with a concentration of 10 $mg \cdot mL^{-1}$, vortexed, ultrasonicated for 10 min, and centrifuged at 14000 rpm for 10 min. Then, 0.8 mL of the supernatant was placed in a centrifuge tube and centrifuged again, and the supernatant was placed into a sample bottle for analysis by UHPLC-MS.

2.2. Methods

In this study, water extracts and essential oils of *L. leontopodioides* were analyzed using UPLC-Q-Orbitrap MS and GC-MS techniques, respectively. Featuring high resolution, high sensitivity, and high speed, ultra-performance liquid chromatography quadrupole-Orbitrap mass spectrometry (UPLC-Q-Orbitrap MS), a cutting-edge molecular separation and determination technique, has been applied to the analysis of various complex samples [15, 16]. Based on UPLC-Q-Orbitrap MS technology, a rapid identification method was established for the chemical composition of the water extract of *L. leontopodioides*. According to the precise molecular mass and fragmentation information of the compounds, the main compounds were identified by means of databases and references, and their cracking laws were discussed, which provided a data basis for further elucidating their pharmacodynamic material basis. GC-MS a highly effective and versatile analytical technique is widely used in pharmaceutical industries for analytical research and development, quality control, and quality assurance [17]. The essential oil was extracted by supercritical carbon dioxide extraction and then dissolved in n-hexane and diethyl ether. The resulting fractions were analyzed by GC-MS, using NIST (version 2017) for similarity search, enabling identification of the components, while calculating their relative amounts using peak area normalization. The instruments and materials used in the experiment are listed in Table 1.

Table 1

Instruments and materials.

Instruments/materials	Type	Company

Ultra-high-performance liquid chromatography	Ultimate 3000	Dionex, USA
High-resolution mass spectrometer	Thermo Q Exactive Plus	Thermo Fisher Scientific, USA
Compound analysis and identification software	Compound Discoverer, 3.2	Thermo Fisher Scientific, USA
Gas/liquid chromatography-triple quadrupole-tandem mass spectrometer	EXPEC 5250	Hangzhou Puyu Technology Development Co., Ltd
Supercritical extraction equipment	GKSFE220-50-6L	Jiangsu Hi-Tech Pharmaceutical Equipment Co., Ltd
n-Hexane	Analytical grade	Tianjin Fuyu Fine Chemical Co., Ltd
Diethyl ether	Analytical grade	Tianjin Fuyu Fine Chemical Co., Ltd
Methanol	Mass spectrometry grade	Fisher Scientific (Pittsburg, PA, USA)
Acetonitrile	Mass spectrometry grade	Fisher Scientific (Pittsburg, PA, USA)
Formic acid	Mass spectrometry grade	Thermo Fisher Scientific, CN
Ultrapure water	—	—
L. leontopodioides	Whole plant (flowering period) of compositae *Leontopodium leontopodioides*	Xiaojinggou, Daqingshan, Inner Mongolia

2.2.1. Chromatography and Mass Spectrometry Conditions

The UPLC-Q-Orbitrap LC/MS system used a Waters ACQUITY UPLC HSS T3 C_{18} column (2.1 mm × 100 mm, 1.8 μm; Waters Corporation, USA); the column temperature was 35°C, and the flow rate was 0.2 mL·min^{-1}. The injection volume was 5 μL; mobile phase was 0.1% formic acid acetonitrile (A)-0.1% formic acid water (B), gradient elution: 0~10 min, 100% B; 10~20 min, 100%~70% B; 20~25 min, 70%~60% B; 25~30 min, 60%~50% B; 30~40 min, 50%~30% B; 40~45 min, 30%~0% B; 45~60 min, 0% B; 60~60.1 min, 0%~100% B; and 60.1~70 min, 100% B. The wavelength of DAD was set as a full scan in the range of 190~400 nm.

The heating electrospray ionization source (HESI) was used as the ion source to detect the positive and negative ion modes, the positive spray voltage was 3.2 kV, the negative spray voltage was 3.0 kV, the detection method was full MS/dd-MS2, the sheath gas flow was 40 arb, the auxiliary gas flow rate was 15 arb, the capillary temperature was 320°C, and the auxiliary gas heater temperature was 350°C. The resolution of MS was 70000, the resolution of

MS/MS was 17500, and the mass spectrum was recorded with a positive ion spectrum scan range of m/z 100~500. Unknown compounds were identified using Compound Discoverer 3.2 software, and mzCloud (https://www.mzcloud.org/) and mzVault (self-built database) were used to identify compounds.

2.2.2. GC-MS Conditions

Inject 1.0 μL essential oils dissolved in n-hexane/diethyl ether each in splitless mode. An HP-5 ms fused silica capillary column (30 m, inner diameter 0.25 mm, and film thickness 0.25 μm) was used with helium as the carrier gas, and the oven temperature was increased from 80°C to 90°C at a rate of 3°C/min (maintained for 2 min), 95°C to 140°C at a rate of 3°C/min, 155°C to 185°C at a rate of 2°C/min, and finally 195°C at a rate of 5°C/min (hold for 8 min). The total run time was 54.5 min, and the ion source temperature was set at 250°C. The GC interface temperature was 270°C. The mass spectra were recorded at 70 eV (EI) and were scanned in the range 35~450 m/z. Compounds were identified using the NIST Chemistry WebBook (https://webbook.nist.gov).

3. Results

3.1. Compounds Confirmation of *L. leontopodioides* Water Extract by UPLC-Q-Orbitrap MS

First, samples were injected according to the chromatographic and mass spectrometry conditions, and Compound Discoverer 3.2 software was used to search for the target compound peaks on the collected raw data and screen for compounds with a score greater than 80, and after matching, compounds were obtained, and the secondary fragmentation fragment ion information was analyzed to further accurately identify chemical components. As a result, a total of 39 chemical components were identified from the water extract of *L. leontopodioides*, mainly including flavonoids, phenolic acids, pentacyclic triterpenes, oligosaccharides, and glycosides. The total ion chromatogram is shown in Figure 2, and the chemical composition identification results are shown in Table 2.

[figure(s) omitted; refer to PDF]

Table 2

Compounds identified in *L. leontopodioides* water extract by UPLC-Q-Orbitrap MS.

No.	RT (min)	Formula	Ion type	MS	MS/MS fragment ions	PPM	Identification	Classification
1	1.773	$C_5H_{11}NO_2$	$[M+H]^+$	118.0861	103.0627 69.4371 59.0733	−1.20	Betaine*	Alkaloids
2	25.258	$C_{16}H_{18}O_9$	$[M+H]^+$	355.1018	177.0533 163.0389 145.0286 135.0441	−1.72	Chlorogenic acid	Phenolic acids
3	26.935	$C_9H_8O_2$	$[M+H]^+$	149.0597	135.0443 121.0284 106.0414 89.0386	−0.40	Cinnamic acid*	Phenolic acids
4	28.192	$C_{15}H_{10}O_6$	$[M+H]^+$	287.0544	269.0443 245.0444 223.0385 185.0599	−1.57	Scutellarein*	Flavonoids
5	28.209	$C_{21}H_{20}O_{12}$	$[M+H]^+$	465.1023	303.0498 137.0235	−0.99	Isoquercitrin	Flavonoid glycosides
6	28.209	$C_{15}H_{10}O_7$	$[M+H]^+$	303.0495	285.0392 257.0443 229.0495 153.0183 137.0235	−1.62	Morin*	Flavonoids

7	29.127	$C_{15}H_{10}O_6$	$[M+H]^+$	287.0545	259.0600 231.0648 153.0182	−1.57	Kaempferol	Flavonoids
8	29.448	$C_{21}H_{20}O_{11}$	$[M+H]^+$	449.1072	287.0548 153.0183 135.0441	−1.47	Kaempferol-7-O-β-D-glucopyranoside	Flavonoid glycosides
9	29.949	$C_{21}H_{20}O_{11}$	$[M+H]^+$	449.1077	287.0549 153.0183 135.0441	−1.47	Cynaroside	Flavonoid glycosides
10	36.069	$C_{42}H_{60}O_{16}$	$[M+H]^+$	823.4106	471.3467 453.3362 435.3276 285.2217 189.1639	−0.21	Dipotassium glycyrrhizinate*	Pentacyclic triterpenoids
11	40.018	$C_{17}H_{19}NO_3$	$[M+H]^+$	286.1435	201.0545 171.0439 143.0492 115.0542	−1.50	Piperine*	Alkaloids
12	45.320	$C_{30}H_{46}O_3$	$[M+H]^+$	455.3516	437.3575 409.3459 247.1696 201.0545 159.1167	−0.63	Oleanonic acid*	Pentacyclic triterpenoids
13	48.962	$C_{30}H_{48}O_3$	$[M+H]^+$	457.3673	439.3575 411.3622 393.3508	−0.82	Ursolic acid*	Pentacyclic triterpenoids
14	2.169	$C_7H_{12}O_6$	$[M-H]^-$	191.0560	147.0299 127.0400 109.0293 93.0344	−0.58	Quinic acid	Phenolic acids
15	2.189	$C_{12}H_{22}O_{11}$	$[M-H]^-$	341.1085	179.0560 161.0454	−0.86	Sucrose*	Oligosaccharides
16	2.291	$C_{18}H_{32}O_{16}$	$[M-H]^-$	503.1619	341.1079 179.0556 161.0453 89.0243	−1.75	Raffinose*	Oligosaccharides
17	2.421	$C_6H_8O_7$	$[M-H]^-$	191.0197	173.0085 154.9983 129.0194 111.0087 87.0086	0.02	Citric acid*	Phenolic acids
18	4.794	$C_4H_4O_4$	$[M-H]^-$	115.0035	99.0087 73.0294	−1.75	Fumaric acid*	Phenolic acids
19	5.439	$C_{24}H_{42}O_{21}$	$[M-H]^-$	665.2143	485.1498 179.0560 161.0452	−1.75	Nystose*	Oligosaccharides
20	7.307	$C_{12}H_{16}O_7$	$[M-H]^-$	271.0823	161.0452 108.0216 71.0138	−0.21	Arbutin*	Glycosides
21	19.948	$C_7H_6O_4$	$[M-H]^-$	153.0192	109.0293	−0.79	Protocatechuic acid	Phenolic acids

22	24.923	$C_7H_6O_3$	$[M-H]^-$	137.0243	119.0138 109.0294 91.0187	−1.24	Protocatechualdehyde	Phenolic acids
23	25.735	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0874	191.0561 179.0349 135.0451	−1.14	Cryptochlorogenic acid	Phenolic acids
24	25.764	$C_7H_{10}O_5$	$[M-H]^-$	173.0452	155.0348 137.0240 93.0344	−2.54	Shikimic acid*	Phenolic acids
25	26.388	$C_9H_6O_4$	$[M-H]^-$	177.0192	149.0243 133.0294 105.0345	−0.85	Esculetin*	Coumarin
26	26.444	$C_9H_8O_4$	$[M-H]^-$	179.0349	135.0451	−0.59	Caffeic acid	Phenolic acids
27	28.156	$C_9H_8O_3$	$[M-H]^-$	163.0400	119.0501 93.0344	−0.73	p-coumaric acid	Phenolic acids
28	28.482	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.0880	300.0273 271.0247 243.0296	−0.34	Hyperoside	Flavonoid glycosides
29	28.912	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1190	354.0908 336.0805 191.0560 179.0348 173.0453	−1.14	Isochlorogenic acid B	Phenolic acids
30	29.374	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1190	353.0877 191.0561 179.0349 135.0452	−1.17	3,5-dicaffeoylquinic acid	Phenolic acids
31	29.437	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.0922	285.0393 284.0324 255.0298 227.0349	−0.53	Astragalin*	Flavonoid glycosides
32	29.709	$C_{21}H_{20}O_{10}$	$[M-H]^-$	431.0981	269.0439 268.0375 153.0191	−0.73	Apigenin-7-O-β-D-glucoside	Flavonoid glycosides
33	29.852	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1190	353.0876 335.0768 191.0560 179.0349 173.0453	−1.15	Isochlorogenic acid C	Phenolic acids
34	30.625	$C_7H_6O_3$	$[M-H]^-$	137.0243	94.0378 93.0344	−1.24	Salicylic acid	Phenolic acids
35	32.827	$C_{15}H_{10}O_6$	$[M-H]^-$	285.0403	241.0501 151.0035 133.0294	−0.83	Luteolin	Flavonoids
36	32.888	$C_{15}H_{10}O_7$	$[M-H]^-$	301.0351	273.0403 178.9985 151.0035 121.0293	−1.03	Quercetin	Flavonoids

37	34.706	$C_{15}H_{10}O_5$	$[M-H]^-$	269.0452	227.0351 225.0556 151.0035 117.0345	−1.30	Apigenin	Flavonoids
38	35.102	$C_{16}H_{12}O_6$	$[M-H]^-$	299.0559	284.0325 256.0376 227.0349 151.0034	−0.68	Diosmetin*	Flavonoids
39	36.597	$C_{42}H_{62}O_{16}$	$[M-H]^-$	821.3963	351.0562 193.0346 113.0243 85.0294	−0.25	Diammonium glycyrrhizinate	Pentacyclic triterpenoids

*Components that have not been reported in *L. leontopodioides*.

3.1.1. Identification of Flavonoids and Their Glycosides

In this study, a total of 13 flavonoids and their glycosides (peaks 4, 5, 6, 7, 8, 9, 28, 31, 32, 35, 36, 37, and 38) were identified from the extract of *L. leontopodioides*. The excimer ion of peak 35 with a retention time of 32.827 min and a molecular formula of $C_{15}H_{10}O_6$ given in negative ion mode was m/z 285.0403 $[M-H]^-$, and it loses a molecule of carbon dioxide forming m/z 241.0501 $[M-H-CO_2]^-$. At the same time, 1, 3 cracking could occur to generate fragment ions m/z 151.0035 $[M-H]^-$ and m/z 133.0294 $[M-H]^-$. Among them, m/z 133.0294 consisted of residues on the B ring and C ring, and its intensity was larger than that of m/z 151.0035. Combined with the databases, peak 35 was identified as luteolin. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 3.

[figure(s) omitted; refer to PDF]

Peak 9, with a retention time of 29.949 min and a molecular formula of $C_{21}H_{20}O_{11}$, combined with the databases was identified as cynaroside. It responds well in positive ion mode, and the excimer ion given in positive ion mode was m/z 449.1077 $[M+H]^+$. In secondary mass spectrometry, luteoloside lost a glucose to form aglycone ion m/z 287.0549 $[M+H-Glc]^+$, and the aglycone was further cleaved by RDA to form fragment ions m/z 153.0183 $[^{1,3}A]^-$ and m/z 135.0441 $[^{1,3}B]^-$. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 4.

[figure(s) omitted; refer to PDF]

The excimer ion of peak 31 in negative ion mode was m/z 447.0922 $[M-H]^-$. The excimer ion peaks were cracked and lost the fragment groups of $C_6H_{10}O_5$ and $C_6H_{11}O_5$, respectively, and fragment ions of m/z 285.0393 $[M-H-C_6H_{10}O_5]^-$ and m/z 284.0324 $[M-H-C_6H_{11}O_5]^-$ were obtained, respectively. Subsequently, the fragment ion of m/z 284.0324 continued to fragment, losing 1 neutral CO molecule, and producing a fragment ion of m/z 257.0424 $[M-H-C_6H_{11}O_5-CO]^-$. At the same time, the fragment ion of m/z 285.0393 can continue to be fragmented, and after losing one neutral CO molecule, it rearranges and removes 2 H atoms, and a fragment ion of m/z 255.0298 $[M-H-C_6H_{11}O_5-CO-2H]^-$ was produced. Finally, the fragment ion continues to fragment and loses the CO molecule, producing a fragment ion of m/z 227.0349 $[M-H-C_6H_{11}O_5-CO-2H-CO]^-$. Compared with the databases, peak 31 was identified as astragalin. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 5.

[figure(s) omitted; refer to PDF]

3.1.2. Identification of Phenolic Acids

A total of 15 phenolic acid compounds (peaks 2, 3, 14, 17, 18, 21, 22, 23, 24, 26, 27, 29, 30, 33, and 34) were detected in the extract of *L. leontopodioides*, and these compounds responded better in negative ion mode. The excimer ion of peak 21 with a retention time of 19.948 min and a molecular formula of $C_7H_6O_4$ in negative ion mode was m/z 153.0192 $[M-H]^-$. Through the loss of CO_2, the secondary spectrum generates fragment ion peaks at m/z 109.0293 $[M-H-CO_2]^-$. Compared with the databases, it was identified as protocatechuic acid. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 6.

[figure(s) omitted; refer to PDF]

The excimer ion of peak 24 with a retention time of 25.764 min and a molecular formula of $C_7H_{10}O_5$ in negative ion mode was m/z 173.0452 $[M-H]^-$. The excimer ion peak lost one molecule of H_2O to generate m/z 155.0348 $[M-H-H_2O]^-$ and also lost one molecule of H_2O to generate m/z 137.0240 $[M-H-2H_2O]^-$. Finally, this fragment ion continues to fragment and loses the COOH molecule, producing a fragment ion of m/z 93.0344 $[M-H-2H_2O-$

COOH]$^-$. Compared with the databases, peak 24 was identified as shikimic acid. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 7.

[figure(s) omitted; refer to PDF]

3.1.3. Identification of Pentacyclic Triterpenoids

A total of four pentacyclic triterpenoids (peaks 10, 12, 13, and 39) were identified in this study. Ursolic acid is a pentacyclic triterpenoid with a retention time of 48.962 min and a molecular formula of $C_{30}H_{48}O_3$. The excimer ion given in the positive ion mode was m/z 457.3670 $[M+H]^+$. After it lost the neutral molecule H_2O, a fragment ion of m/z 439.3575 was generated, and at the same time, the COOH molecule was lost to obtain a fragment ion of m/z 411.3622, and this fragment further lost H_2O to produce the fragment ion of m/z 393.3508. Based on a comprehensive database, this structure was speculated as ursolic acid, and its MS/MS spectrum and the fragmentation pathway are shown in Figure 8.

[figure(s) omitted; refer to PDF]

3.1.4. Identification of Oligosaccharides and Glycosides

In the negative ion mode, the primary mass spectrometry mainly exists in the form of quasimolecular ion peak $[M-H]^-$. Under the high-energy collision of mass spectrometry, the cleavage of the glycosidic bond mainly occurs and loses the glycosyl group. A total of four oligosaccharide and glycoside compounds (oligosaccharides: peaks 15, 16, and 19; glycosides: peak 20) were identified in this experiment. The excimer ion of peak 15 with a retention time of 2.189 min and a molecular formula of $C_{12}H_{22}O_{11}$ in negative ion mode was m/z 341.1085 $[M-H]^-$. Its negative ion mode of MS^2 spectra revealed 179.0560 $[M-H-Glc]$ and 161.0454 $[M-H-Glc-H_2O]$. Based on a comprehensive database, it was tentatively identified as sucrose, and its MS/MS spectrum and the fragmentation pathway are shown in Figure 9.

[figure(s) omitted; refer to PDF]

3.1.5. Other Compounds

In addition, two alkaloids (peaks 1 and 11) and one coumarin (peak 25) were identified in positive ion mode from the water extract of *L. leontopodioides*. The excimer ion of peak 11 with a retention time of 40.018 min and a molecular formula of $C_{17}H_{19}NO_3$ in positive ion mode was m/z 286.1435 $[M+H]^+$. The most abundant fragment of m/z 201.0545 was formed by the cleavage of the amide bond, and loss of piperidine ring ($-C_5H_{11}N$), in the process of further cracking, will be obtained the characteristic ion m/z 171.0439 $[M+H-C_5H_{11}N-CH_2O]^+$ with molecular formula $C_{11}H_7O_2$. This ion continues to lose CO to obtain the ion m/z 143.0492 $[M+H-C_5H_{11}N-CH_2O-CO]^+$ and m/z 115.0542 $[M+H-C_5H_{11}N-CH_2O-CO-CO]^+$. Compared with the database, this compound was identified as piperine. Its MS/MS spectrum and the fragmentation pathway are shown in Figure 10.

[figure(s) omitted; refer to PDF]

3.2. Compound Confirmation of *L. leontopodioides* Essential Oil by GC-MS

Samples were injected according to the GC-MS conditions and obtained a total ion chromatogram of volatile components in *L. leontopodioides*, and the obtained data were searched and matched by the mass spectrometry database of the National Institute of Standards and Technology (NIST 2017), and compounds with similarity scores above 80% were taken into account. After comparing the chemical composition of essential oils extracted with n-hexane and diethyl ether, a total of 33 volatile compounds (there were seven identical components) were identified, as shown in Figure 11 and Tables 3 and 4. The relative content of each component was estimated by the peak area normalization method. It can be seen from Table 3 that the main components of the volatiles of *L. leontopodioides* extracted with n-hexane mainly included phenylpropene (64.52%), monoterpenes (10.96%), fatty acids (10.03%), and contained some aliphatic hydrocarbons. Among them, the components with higher content were methylconiferylaldehyde (14.77%), (E)-2,6-dimethoxy-4-(prop-1-en-1-yl) phenol (12.04%), and eugenol (11.51%). As shown in Table 4, the main components included fatty acids (28.99%), phenylpropene (28.37%), aliphatic hydrocarbons, and some esters. Among them, the components with higher content were methylconiferylaldehyde (9.61%), pentadecanoic acid (9.25%), and 8-methylnonanoic acid (8.63%). Furthermore, terpinolene, terpinen-4-ol, *γ*-terpinene, methyleugenol, methylconiferylaldehyde, tetradecanoic acid, and n-hexadecanoic acid were identical

components.

[figure(s) omitted; refer to PDF]

Table 3

Chemical composition of *L. leontopodioides* essential oil extracted with *n*-hexane.

No.	tR (min)	Content (%)	Components	Formula	Relative molecular mass	Classification
1	5.91	2.61	Terpinolene*	$C_{10}H_{16}$	136	Monoterpenoids
2	7.03	3.85	*γ*-Terpinene	$C_{10}H_{16}$	136	Monoterpenoids
3	6.23	2.56	Isoterpinolene	$C_{10}H_{16}$	136	Monoterpenoids
4	7.91	2.55	2-Carene*	$C_{10}H_{16}$	136	Aliphatic hydrocarbons
5	10.94	1.94	Terpinen-4-ol	$C_{10}H_{18}O$	154	Monoterpenoids
6	15.14	3.24	3-Methoxycinnamaldehyde*	$C_{10}H_{10}O_2$	162	Phenylpropene
7	19.83	11.51	Eugenol	$C_{10}H_{12}O_2$	164	Phenylpropene
8	21.56	4.19	Methyleugenol*	$C_{11}H_{14}O_2$	178	Phenylpropene
9	23.47	5.27	4-Hydroxy-2-methoxycinnamaldehyde*	$C_{10}H_{10}O_3$	178	Phenylpropene
10	24.55	14.77	Methylconiferylaldehyde*	$C_{11}H_{12}O_3$	192	Phenylpropene
11	35.91	7.68	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	Fatty acids
12	26.01	12.04	(E)-2,6-dimethoxy-4-(prop-1-en-1-yl) phenol*	$C_{11}H_{14}O_3$	194	Phenylpropene
13	27.97	2.98	Methoxyeugenol*	$C_{11}H_{14}O_3$	194	Phenylpropene
14	36.45	1.31	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	Fatty acids

15	30.03	1.07	Isoelemicin*	$C_{12}H_{16}O_3$	208	Phenylpropene
16	34.65	1.77	2-Propenoic acid, 3-(3-methoxyphenyl)-, ethyl ester*	$C_{12}H_{14}O_3$	206	Phenylpropene
17	44.52	1.04	Eicosanoic acid*	$C_{20}H_{40}O_2$	312	Fatty acids

Nos. 1, 2, 5, 8, 10, 11, and 14 are the same in Tables 3 and 4. *Components that have not been reported in *L. leontopodioides*.

Table 4

Chemical composition of *L. leontopodioides* essential oil extracted with diethyl ether.

No.	tR (min)	Content (%)	Components	Formula	Relative molecular mass	Classification
1	6.23	5.63	Terpinolene*	$C_{10}H_{16}$	136	Monoterpenoids
2	7.11	3.83	γ-Terpinene	$C_{10}H_{16}$	136	Monoterpenoids
3	8.00	1.79	Allo-ocimene*	$C_{10}H_{16}$	136	Aliphatic hydrocarbons
4	15.14	2.70	Methyl cinnamate*	$C_{10}H_{10}O_2$	162	Phenylpropene
5	10.94	2.43	Terpinen-4-ol	$C_{10}H_{18}O$	154	Monoterpenoids
6	19.77	8.63	8-Methylnonanoic acid*	$C_{10}H_{20}O_2$	172	Fatty acids
7	23.42	3.98	cis-Methyl isoeugenol*	$C_{11}H_{14}O_2$	178	Phenylpropene
8	21.53	1.48	Methyleugenol*	$C_{10}H_{10}O_3$	178	Phenylpropene
9	25.93	7.29	Elemicin*	$C_{12}H_{16}O_3$	208	Phenylpropene
10	24.48	9.61	Methylconiferylaldehyde*	$C_{11}H_{12}O_3$	192	Phenylpropene
11	27.92	3.31	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	Fatty acids
12	35.73	9.25	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	Fatty acids
13	36.40	1.71	Ethyl tridecanoate*	$C_{15}H_{30}O_2$	242	Esters

14	44.45	2.12	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	Fatty acids
15	51.71	5.68	Linoleic acid	$C_{18}H_{32}O_2$	280	Fatty acids
16	51.97	4.06	17-Octadecynoic acid*	$C_{18}H_{32}O_2$	280	Fatty acids

Nos. 1, 2, 5, 8, 10, 11, and 14 are the same in Tables 3 and 4. *Components that have not been reported in *L. leontopodioides.*

4. Discussion

4.1. UPLC-Q-Orbitrap MS Section

Flavonoids mainly exist in natural plants in the form of free or combined with sugar to form glycosides or in the form of carbon sugars, and they have anti-inflammatory, antioxidant, antibacterial, antidiabetic, antihypertensive, and other pharmacological activities [18]. The mass spectrometry fragmentation characteristics of flavonoid aglycones were mainly the loss of CO, COO, and CH_3 groups, or the loss of neutral molecules such as H_2O and the occurrence of reverse Diels–Alder reaction (RDA) fragmentation to form a series of characteristic ion peaks. Flavonoid glycosides first lose the glycosyl group to form the corresponding aglycone and then further cleave [19, 20]. In this study, taking luteolin, cynaroside, and astragalin as examples, the cracking rules of flavonoids and their glycosides were described, and it was found that the cracking rules of the three were consistent with those reported in the literature [21–23]. Among the 13 flavonoids and their glycosides obtained from the analysis, isoquercitrin, kaempferol, kaempferol-7-O-*β*-D-glucopyranoside, cymaroside, hyperoside, apigenin-7-O-*β*-D-glucoside, luteolin, quercetin, and apigenin compounds with previous reports [24–28] on the chemical composition of *L. leontopodioides* , scutellarein, morin, diosmetin, and astragalin have not been reported.

Phenolic acids mainly contain carbonyl, carboxyl, and hydroxyl groups, so neutral fragments of CO, H_2O, and CO_2 were easily lost in mass spectrometry collisions. The cracking rules of protocatechuic acid and shikimic acid obtained by database analysis are consistent with those reported in the literature [29, 30]. Chlorogenic acid, quinic acid, protocatechuic acid, protocatechualdehyde, cryptochlorogenic acid, caffeic acid, p-coumaric acid, isochlorogenic acid B, isochlorogenic acid C, and salicylic acid have been reported [31, 32]. Moreover, basic research on pharmacodynamics found that protocatechuic acid, protocatechuic aldehyde, chlorogenic acid, and caffeic acid in *L. leontopodioides* can resist acute inflammation [33]. Cinnamic acid, citric acid, fumaric acid, and shikimic acid have not been reported in *L. leontopodioides* in previous research.

The mass spectrometry fragmentation of pentacyclic triterpenoids was mainly loss of neutral molecules, such as H_2O and CO, and the occurrence of Diels–Alder reaction, and the oligosaccharide and glycoside mass spectrometry was relatively simple. The cracking rules of ursolic acid and sucrose were consistent with literature reports [34, 35]. In previous research, pentacyclic triterpenoids, oligosaccharides, and glycosides have not been reported in *L. leontopodioides.* Newly discovered pentacyclic triterpenoids have a wide range of pharmacological effects and important biological activities, including anti-inflammatory, antibacterial, antiviral, immunomodulatory, blood sugar regulation, blood pressure lowering, and antitumor activities [36]. In particular, ursolic acid has the same inhibitory effect on glycosidase in vivo and in vitro and has an obvious hypoglycemic effect [37]. Oligosaccharides possess various bio-activities, including immune regulation, antitumor, antioxidation, and anti-infection, and modulate the gut microflora [38].

In addition, there has been no research on the chemical composition of *L. leontopodioides* using UPLC-Q-Orbitrap MS technology at present. UPLC-Q-Orbitrap MS technology adopts full MS/dd-MS2 mode, which greatly shortens the analysis time and can quickly detect multiple chemical components, with its advantages of high separation, high resolution, and high sensitivity, and it can provide accurate mass, elemental composition, mass spectrometry fragments, and other information required for the structural characterization of compounds without the need for reference substances. Then, the possible structure of the compound can be speculated for rapid qualitative analysis. This study collects data in both positive and negative ion modes to obtain more complete mass spectrometry data.

Therefore, this method was used to analyze the water extract of *L. leontopodioides* in this study. Compared with previous studies, not only flavonoids and phenolic acids, but also pentacyclic triterpenes, oligosaccharides, and glycosides, which have never been reported before, were obtained using UPLC-Q-Orbitrap MS. The above research results show that the anti-inflammatory, antibacterial, antioxidant, and hypoglycemic effects of *L. leontopodioides* may be derived from the presence of chemical components, such as flavonoids, phenolic acids, pentacyclic triterpenes, oligosaccharides, and glycosides.

4.2. GC-MS Section

The essential oil of *L. leontopodioides* was extracted by supercritical carbon dioxide (SC-CO_2) extraction technology. As a new advanced "green" separation technology, SC-CO_2 is easy to operate and can not only extract and separate the desired substances quickly and efficiently but also the yield and purity of the obtained substances are higher than those of traditional methods [39].

The composition of *L. leontopodioides* essential oil was analyzed by GC-MS, and the chemical composition of essential oils extracted with different organic solvents was compared, and it was found that extracting essential oils with n-hexane can obtain a large amount of phenylpropene compounds, such as eugenol. The pharmacological effects of eugenol include antibacterial, anticancer, antioxidant, and other effects [40]; using diethyl ether to extract essential oils can obtain a large amount of fatty acids. Aparna et al. [41] through research suggested that the *n*-hexadecanoic acid might function as an anti-inflammatory agent. Analysis of *L. leontopodioides* essential oil by GC-MS found that the high content of fatty acid components and phenylpropene components may be an essential ingredient for its medicinal effect.

Gao et al. [13] extracted essential oil from aerial parts of *Leontopodium leontopodioides* (Willd.) Beauv. by water distillation and analyzed it by GC-FID and GC-MS. The main components in the essential oil were identified as palmitic acid (11.6%), *n*-pentadecanal (5.7%), linalool (3.8%), *β*-ionone (3.3%), hexahydrofarnesyl acetone (3.2%), bisabolone (3.2%), and *β*-caryophyllene (3.2%).

Compared with this, the results of this study are quite different, but the composition types are roughly the same, which may be related to the origin of *L. leontopodioides*, the extraction methods of volatile oil, and the extraction of volatile oil with different solvents.

5. Conclusions

In this study, UPLC-Q-Orbitrap MS and GC-MS analytical methods were established to comprehensively characterize the chemical composition of *L. leontopodioides* and provide a good research basis for the formulation compatibility and pharmacological mechanism of *L. leontopodioides*. However, the analysis of this study mainly focused on the identification and analysis of chemical components and did not carry out basic research on blood components and pharmacodynamic substances. Therefore, in the future, this analytical technique should be used to further improve the pharmacodynamic material basis of *L. leontopodioides*. At the same time, the mechanism of action of *L. leontopodioides* should be further elucidated by combining serum medicinal chemistry, network pharmacology, metabolomics, and other technologies.

Additional Points

Highlights. (1) In this study, we divided the water extract and volatile oil of *L. leontopodioides* to reveal its chemical constituents by UPLC-Q-Orbitrap MS and GC-MS for the first time. In addition, the constituents of volatile oil dissolved in two different solvents were investigated. (2) This is not only a comprehensive and systematic composition analysis of *L. leontopodioides* but also provides a basis for its development and utilization.

Authors' Contributions

All authors contributed to the study's conception and design. Chula Sa, Yu Dong, and Changxi Bai designed project development and protocol. Yuanyuan Chen, Lin Song, and Buhechaolu Wang performed material preparation, data collection, and analysis. Yuanyuan Chen wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Inner Mongolia Medical University Mongolian Pharmacy "First-Class Discipline"

Young Teachers Innovation Ability Improvement Project (No. myxylxkky2019-01), the Inner Mongolia Medical University Mongolian Pharmacy “First-class Discipline” scientific research project (No. myxylxk2021-11), Scientific and Technologically Innovative Research Team for Inner Mongolia Medical University of Bioanalysis of Mongolian medicine (YKD2022TD037), the General Program of Inner Mongolia Medical University (YKD2021MS040), and University Youth Science and Technology Talent Program (No. NJYT23135).

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DETAILS

Subject:	Mass spectrometry; Software; Acids; Fatty acids; Ionization; Negative ions; Traditional Chinese medicine; Oligosaccharides; Scientific imaging; Essential oils; Antioxidants; Chromatography; Chemical composition; Research &development--R &D; Aliphatic hydrocarbons; Gas flow; Oils &fats; Quality assessment; Diethyl ether; Glycosides; Pharmacodynamics; Phenolic acids; Flavonoids; Esters; Mass spectroscopy; Mass spectra; Electron impact; Hexanes
Location:	United States--US
Company / organization:	Name: National Institute of Standards &Technology; NAICS: 541380, 541714, 926150
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-02-08 (Received); 2024-04-06 (Revised); 2024-04-15 (Accepted); 2024-05-07 (Pub)
DOI:	https://doi.org/10.1155/2024/3525212
ProQuest document ID:	3056517260
Document URL:	https://www.proquest.com/scholarly-journals/analysis-i-leontopodium-leontopodioides-willd/docview/3056517260/se-2?accountid=211160

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Last updated:	2024-05-20
Database:	Publicly Available Content Database

Pharmacokinetic Properties of Baitouweng Decoction in Bama Miniature Pigs: Implications for Clinical Application in Humans

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ABSTRACT (ENGLISH)

Traditional Chinese medicine (TCM) serves as a significant adjunct to chemical treatment for chronic diseases. For instance, the administration of Baitouweng decoction (BTWD) has proven effective in the treatment of ulcerative colitis. However, the limited understanding of its pharmacokinetics (PK) has impeded its widespread use. Chinese Bama miniature pigs possess anatomical and physiological similarities to the human body, making them a valuable model for investigating PK properties. Consequently, the identification of PK properties in Bama miniature pigs can provide valuable insights for guiding the clinical application of BTWD in humans. To facilitate this research, a rapid and sensitive UPLC-MS/MS method has been developed for the simultaneous quantification of eleven active ingredients of BTWD in plasma. Chromatographic separation was conducted using an Acquity UPLC HSS T3 C_{18} column and a gradient mobile phase comprising acetonitrile and water (containing 0.1% acetic acid). The methodology was validated in accordance with the FDA Bioanalytical Method Validation Guidance for Industry. The lower limit of quantitation fell within the range of 0.60–2.01 ng/mL. Pharmacokinetic studies indicated that coptisine chloride, berberine, columbamine, phellodendrine, and obacunone exhibited low Cmax, while fraxetin, esculin, fraxin, and pulchinenoside B4 were rapidly absorbed and eliminated from the plasma. These findings have implications for the development of effective components in BTWD and the adjustment of clinical dosage regimens.

FULL TEXT

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1. Introduction

The Baitouweng decoction (BTWD) is derived from the Treatise on Febrile Diseases authored by Zhongjing Zhang during the Eastern Han Dynasty. It consists of *Pulsatillae radix* (Bai Tou Weng), *Coptidis rhizoma* (Huang Lian), *Phellodendri chinensis cortex* (Huang Bai), and *Fraxini Cortex* (Qin Pi). According to traditional Chinese medicine theory, this decoction exhibits properties such as heat evil clearance, superficial evil expulsion, and blood cooling for diarrhea cessation. In the *Treatise on Febrile Diseases-Bianjueyinbingmaizhengbingzhi*, BTWD has historically been employed primarily for the treatment of dysentery accompanied by symptoms of heat, anal prolapse, and swelling. It has long been regarded as the preferred prescription for hygropyretic dysentery, with a history of usage spanning centuries. In contemporary medicine, BTWD is used for the management of digestive system disorders [1, 2]. The chemical constituents present in BTWD encompass alkaloids, coumarins, saponins, limonins, sterols, and lignanoids. Among these constituents, alkaloids and coumarins are believed to constitute the principal material foundation of BTWD. Coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4 have been recognized as the primary active constituents, exhibiting a broad range of pharmacological effects including antioxidant [3], anti-inflammatory [4], antigastrointestinal cancer [5, 6], hepatic fibrosis amelioration [7], gastroprotective [8], and intestinal epithelial barrier protective activity [9].

The pharmacokinetic properties of these constituents in the human body are of utmost importance for clinical investigations. Several studies have reported on the pharmacokinetic properties of these aforementioned constituents using high-performance liquid chromatography (HPLC) [10] or mass spectrometry (MS) techniques [11–16]. Currently, the majority of pharmacokinetic (PK) studies on BTWD primarily focus on a limited number of its components, with the maximum number of chemicals analyzed being seven [14]. These seven components include anemoside B4, phellodendrine, berberine, palmatine, obacunone, esculin, and esculetin. However, the analysis did not include coptisine chloride, fraxin, and fraxetin, which are important components of BTWD. Therefore, the current studies are insufficient in providing a comprehensive description of the PK properties of BTWD. Consequently, it is

crucial to develop novel analytical methods that can systematically evaluate the pharmacokinetic properties of BTWD.

Currently, the majority of PK studies conducted on BTWD have primarily focused on rats, thereby differing from those conducted on humans. However, Chinese Bama miniature pigs exhibit notable anatomical and physiological resemblances to the human body, rendering them exceptional models for investigating cardiovascular, gastrointestinal, and renal system research [17]. Researchers reported that Bama miniature pigs are suitable for use in drug evaluation studies [18]. Consequently, the PK characteristics identified in Bama miniature pigs hold significant value in informing the clinical application of BTWD in humans.

The objective of this study was to establish a UPLC-MS/MS method that is both rapid and sensitive for the simultaneous quantification of various compounds (coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4) in plasma samples of BTWD. Additionally, this method was used to conduct pharmacokinetic studies on Bama miniature pigs.

2. Materials and Methods

2.1. Chemicals and Reagents

The reference standards, namely coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4, were procured from the National Institute for Food and Drug Control in Beijing, China, with a minimum purity of 98%. Methanol and acetonitrile of HPLC grade were obtained from Merck in Germany, while formic acid with a minimum purity of 99% was sourced from Anaqua Chemicals Supply in America. Pure water with a resistivity of at least 18.2 MΩ•cm was generated using a Milli-Q system manufactured by Millipore in Bedford, USA. All other chemicals used in the study were of analytical grade. *Pulsatillae radix* (originating from Liaoning, China, with batch no. 20181020 and voucher specimen number BTW008) and *Fraxini Cortex* (originating from Liaoning, China, with batch no. 20180126 and voucher specimen number QP012) were procured from Hebei Renxin Pharmaceutical Co., Ltd. *Coptidis rhizoma* (originating from Sichuan, China, with batch no. 20181124 and voucher specimen number HL136) was obtained from Anguo Shenghui Chinese Medicine Yinpian Co., Ltd. *Phellodendri chinensis cortex* (originating from Sichuan, China, with batch no. 20180728 and voucher specimen number HB014) was acquired from Hebei Qiyitang Pharmaceutical Co., Ltd. These samples were subsequently stored in the sample storage room of the Shandong Binzhou Animal Science and Veterinary Medicine Academy. The authenticity and quality of all traditional Chinese medicines used in this study were verified according to the methods outlined in People's Republic of China Veterinary Pharmacopoeia (2020 Edition).

The preparation of BTWD involved combining air-dried *Pulsatillae radix* (30.0 g), *Fraxini Cortex* (24.0 g), *Coptidis rhizoma* (12.0 g), and *Phellodendri chinensis cortex* (24.0 g), followed by extraction with 900 mL of water at 100°C for 1.0 h using a condensing reflux device. This process was repeated twice with 700 mL of water for each extraction, also for 1 h. The resulting extracts were combined and concentrated under reduced pressure using a rotary evaporator at 60°C, resulting in a solution with a concentration of 0.5 g crude herb per 1.0 mL decoction. The solution was then subjected to centrifugation at 3400×g for 10 mins, and the supernatant was further concentrated to achieve a concentration of 1.0 g crude herb per 1.0 mL decoction. The final solution was stored at −20°C until needed.

2.2. Instruments and Analytical Conditions

The LC-MS analysis was conducted using a Waters Acquity UPLC I-Class system (Waters, USA) coupled with a Xevo TQ-XS mass spectrometer equipped with a heated electrospray ionization source. Chromatographic separation was carried out on an Acquity UPLC HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 μm) from Waters, USA, with a flow rate of 0.4 mL/min and a column oven temperature of 40°C. The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B). The gradient elution program was as follows: 0–2.0 min, 10%–60% B; 2.0–2.2 min, 60%–95% B; 2.2–3.2 min, 95% B; 3.2–3.5 min, 95%–10% B; and 3.5–5.5 min, 10% B. Mass spectrometric detection was conducted using both positive and negative ionization modes. The source parameters used were as follows: a spray voltage of 1.00 KV, capillary temperature set at 500°C, desolvation flow maintained at 1000 L/h, cone gas (nitrogen) flow at 150 L/h, and a cone voltage of 5 V. The collision energy and precursor to production transition

m/*z* for each analyte are found in Table 1. The data acquisition was performed in multiple reaction monitoring (MRM) mode.

Table 1

Mass spectrometry parameters of 11 analytes.

Analytes	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Collision energy (eV)	Retention time (min)
Coptisine chloride	320.0 (+)	292.1*, 262, 318	27, 33, 30	1.49
Berberine	336.0 (+)	292*, 306, 321	26, 28, 26	1.65
Columbamine	338.3 (+)	322*, 279	26, 35	1.44
Phellodendrine	342.2 (+)	192.1*, 301	20, 12	0.96
Palmatine	352.1 (+)	336*, 308.1, 337	28, 25, 23	1.62
Obacunone	455.2 (+)	161*, 95	27, 32	2.53
Esculetin	177.0 (−)	89*, 149	22, 22	1.02
Fraxetin	207.0 (−)	191.9*, 163	16, 17	1.11
Esculin	339.0 (−)	177*, 133	25, 28	0.79
Fraxin	369.0 (−)	207*, 192	19, 30	0.93
Pulchinenoside B4	1220 (−)	750*	46	1.52

Note. *Means for quantification.

2.3. Standard Solutions and Quality Control Sample Preparation

Stock solutions of the eleven reference standards were prepared in methanol, with final concentrations of 2.92 mg/mL for coptisine chloride, 2.42 mg/mL for berberine, 2.65 mg/mL for columbamine, 1.50 mg/mL for phellodendrine, 1.91 mg/mL for palmatine, 5.01 mg/mL for obacunone, 4.56 mg/mL for esculetin, 4.92 mg/mL for fraxetin, 4.88 mg/mL for esculin, 5.03 mg/mL for fraxin, and 5.00 mg/mL for pulchinenoside B4. Each reference standard stock solution (1.0 mL) was combined and diluted with methanol to create a 100.0 mL standard mixture stock solution. Subsequently, a series of standard working solutions were generated by sequentially diluting the mixed stock solution with methanol. All working solutions were stored at 4°C in the dark.

Calibration standards were generated by introducing the standard working solutions into the blank plasma, resulting in final concentrations of 1.17 to 292.00 ng/mL for coptisine chloride, 0.97 to 242.00 ng/mL for berberine, 1.06 to 265.00 ng/mL for columbamine, 0.60 to 150.00 ng/mL for phellodendrine, 0.76 to 191.00 ng/mL for palmatine, 2.00 to 501.00 ng/mL for obacunone, 1.82 to 456.00 ng/mL for esculetin, 1.97 to 492.00 ng/mL for fraxetin, 1.95 to 488.00 ng/mL for esculin, 2.01 to 503.00 ng/mL for fraxin, and 2.00 to 500.00 ng/mL for pulchinenoside B4. Three levels of quality control (QC) samples (low, medium, and high) were prepared using the same methodology. All samples were stored at a temperature of −20°C.

2.4. Plasma Sample Preparation

Methanol and acetonitrile were compared in terms of their efficacy in protein precipitation, and acetonitrile was selected due to its superior extraction recovery. Subsequently, a volume of 400 μL of acetonitrile was added to a 100 μL plasma sample. The resulting mixture was subjected to vortexing for 1 minute and centrifuged at a force of 21367 g for 10 minutes at a temperature of 4°C. The resulting supernatant was subjected to evaporation, followed by reconstitution in a volume of 100 μL of 10% acetonitrile. This reconstituted solution was then centrifuged at a force of 21367 g, and subsequently, a volume of 4 μL of the resulting supernatant was used for UPLC-ESI-MS/MS analysis.

2.5. Method Validation

The evaluation of specificity involved the comparison of six separate blank plasma samples, blank plasma samples spiked with analytes, and plasma samples obtained after oral administration of BTWD.

Calibration curves were generated using weighed (1/x2) least-squares regression analysis, plotting the analyte peak areas (y) against the analyte concentrations in blank plasma (x). It was required that each calibration curve exhibit a correlation coefficient (r2) greater than 0.99. The determination of the lower limit of quantification (LLOQ) was based on the lowest concentration in the calibration curve that could be measured with acceptable precision and accuracy, within a range of ±15% for both parameters.

The precision and accuracy of the QC samples were assessed by analyzing eleven analytes in six replicates on the same day and on three separate days. The relative error (RE) and relative standard deviation (RSD) were computed.

Extraction recoveries of three QC levels were analyzed by comparing the peak area of analytes added to blank plasma before and after extraction. The matrix effect was evaluated by analyzing the peak area of the extracted blank plasma added to three QC concentration analytes and the corresponding analyte solutions.

The stability of both short-term and long-term conditions was assessed at room temperature for 24 hours and at −80°C for 10 days, respectively. Freeze-thaw cycle stability was evaluated by subjecting the samples to three cycles of freezing at −80°C and thawing at room temperature. Each test included the analysis of three quality control levels, with each level consisting of six samples. [14].

2.6. Pharmacokinetic Study

The study used six Bama miniature pigs (70 days old, weighing 20 ± 2 kg) obtained from the Experimental Animal Center, Shandong Lvdu Bio-Sciences and Technology Co., Ltd. (Binzhou, China). The pigs were housed under controlled conditions with a temperature of 25 ± 1°C, relative humidity of 65% ± 10%, and a 12/12 h light/dark cycle. The pigs were provided with standard pig feed and water ad libitum, in accordance with the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Following a 12-hour fasting period, the pigs received BTWD intragastrically at a dose of 1.0 mL/kg, with approximate concentrations of coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4 at 457.69, 3084.97, 542.65, 910.00, 736.02, 54.24, 751.43, 1537.92, 2810.50, 1943.49, and 3499.45 μg/kg, respectively. Blood samples (0.5 mL each) were collected from the jugular vein of each pig into heparinized tubes at specific time intervals (0.5 h, 1.0 h, 4.0 h, 8.0 h, 12.0 h, 15.0 h, and 24.0 h) following intragastric administration. The blood sample was promptly subjected to centrifugation at a force of 3400×g for 10 minutes. The resulting supernatant plasma was collected and preserved at a temperature of −80°C until subsequent preparation for LC-MS analysis. The concentration-time data of the analytes were evaluated using either noncompartmental or compartmental methods with the aid of the PKSolver 2.0 software package, and subsequently, pharmacokinetic parameters were computed. The outcomes are presented as the arithmetic mean accompanied by the standard deviation (SD).

3. Results and Discussion

3.1. Method Development

Multiple reaction monitoring (MRM) was employed for the quantification of eleven analytes in pig plasma, thereby ensuring optimal peak shape and anticipated resolution. The optimized mass transition ion pairs (m/z) are delineated in Figure 1 and Table 1. To enhance peak responses and expedite analysis, a gradient elution of acetonitrile-water (0.1% formic acid) was selected. The findings demonstrated that all identified constituents were detected within a

time frame of six minutes.

[figure(s) omitted; refer to PDF]

3.2. Method Validation

Figure 2 displays chromatograms depicting blank plasma, plasma spiked with the analytes, and plasma obtained from a pig following oral administration of the BTWD extract. No discernible interferences were observed for the eleven analytes, indicating a high level of selectivity of the method for BTWD in plasma. The linearity and LLOQ are presented in Table 2. The calibration curves exhibited strong linearity, as evidenced by correlation coefficients ranging from 0.999 to 1. The LLOQs for coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4 were determined to be 1.17, 0.97, 1.06, 0.60, 0.76, 2.00, 1.82, 1.97, 1.95, 2.01, and 2.00 ng/mL, respectively, which were deemed sufficient for the PK studies. In Table 3, the intraday and interday precisions ranged from 1.00% to 13.33% and 0.52% to 9.19%, respectively, while the accuracy ranged from −6.96% to 7.90% and −6.63% to 5.73%. These results conform to the acceptance criteria outlined in the bioanalytical method validation guidelines, indicating that the method employed was reproducible and accurate in detecting all analytes in pig plasma. As indicated in Table 3, the accuracy exhibited a range of −13.38% to 0.67%, −11.92% to −0.98%, and −13.67% to −2.48%, respectively, which provide evidence of satisfactory room temperature stability, long-term stability, and freeze-thaw stability. The extraction recoveries fell within the range of 83.62% to 98.76%, while the matrix effect ranged from 82.93% to 110.91%. These results demonstrate the effectiveness and efficiency of protein precipitation, as well as the negligible influence of the matrix on the detection of analytes in pig plasma. Table 4 presents the detailed results.

[figure(s) omitted; refer to PDF]

Table 2

The linear ranges, regression equations, and LLOQs for the determination of 11 components in pig plasma.

Analytes	Calibration curves	R2	Ranges (ng/mL)	LLOQ (ng/mL)
Coptisine chloride	$y=1E-05x-0.5584$	0.9997	1.17–292.00	1.17
Berberine	$y=8E-06x-2.5152$	1	0.97–242.00	0.97
Columbamine	$y=1E-05x-0.4069$	0.9998	1.06–265.00	1.06
Phellodendrine	$y=4E-06x-0.7192$	0.9998	0.60–150.00	0.60
Palmatine	$y=6E-06x-0.7511$	0.9996	0.76–191.00	0.76
Obacunone	$y=0.0004x-2.0664$	0.9996	2.00–501.00	2.00
Esculetin	$y=0.0025x-1.1961$	0.9991	1.82–456.00	1.82
Fraxetin	$y=0.0013x-0.0661$	0.9997	1.97–492.00	1.97
Esculin	$y=0.0003x-0.6909$	0.9995	1.95–488.00	1.95
Fraxin	$y=0.0006x-2.6841$	0.9996	2.01–503.00	2.01

Pulchinenoside B4	y=0.1017x–7.9946	0.999	2.00–500.00	2.00

Table 3

The accuracy, precision, and stability of eleven ingredients of BTWD in pig plasma (n=6).

Analytes	Spiked (ng/mL)	Intraday		Interday		Stability					
Short term		Long term		Freeze-thaw cycles		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
RSD (%)	RE (%)	RSD (%)	RE (%)	Coptisine chloride	4.67	3.83	−3.73	4.93	−1.30	1.53	−1.21
2.86	−4.11	2.67	−4.83	11.68	3.93	−6.12	5.51	−2.83	2.67	−1.79	3.01
−6.17	3.10	−8.64	116.80	3.71	5.53	5.31	1.81	0.53	−1.59	0.99	−2.31
3.98	−3.67	-									
Berberine	3.87	5.43	−6.96	4.61	−5.06	1.11	−2.95	1.75	−4.82	1.33	−5.52
9.68	3.57	5.10	0.52	5.00	1.47	−2.59	5.88	−6.33	4.72	−3.03	96.80
1.65	−3.52	2.13	−2.55	5.00	−8.48	1.53	−6.64	2.78	−4.33		
Columbamine	4.24	3.93	−0.99	3.64	0.33	2.26	−3.05	1.61	−1.83	3.61	−7.69
10.60	2.64	−1.64	1.50	−0.85	2.08	−8.33	2.19	−2.94	2.36	−4.90	106.00
4.82	−2.85	1.85	−2.81	2.44	−2.75	1.14	−3.68	3.22	−3.94		
Phellodendrine	2.40	3.85	−1.78	8.92	4.62	2.70	−7.50	4.09	−6.67	4.17	−8.33
6.00	3.63	−2.03	3.31	5.29	2.62	−5.71	1.04	−2.44	0.74	−3.57	60.00

6.05	6.60	1.26	5.73	0.65	−1.85	1.03	−3.00	0.66	−2.96		
Palmatine	3.06	13.33	7.90	4.87	1.55	5.42	−6.11	5.77	−7.22	4.81	−8.33
7.64	3.85	−1.42	4.64	0.09	3.20	−4.67	1.19	−3.33	0.69	−4.00	76.40
1.63	−5.88	2.03	−4.45	1.02	−4.61	2.88	−3.91	0.94	−5.00		
Obacunone	8.02	3.66	−1.33	2.65	3.52	2.56	−10.50	2.33	−10.83	1.37	−7.83
20.04	9.20	1.36	9.19	4.92	0.75	−1.15	0.64	−1.79	0.81	−2.48	200.40
4.04	0.45	1.92	−1.90	2.86	−5.52	1.52	−7.53	1.75	−5.21		
Esculetin	7.30	1.24	3.90	2.77	4.98	2.14	−2.67	2.84	−11.33	1.77	−13.67
18.24	1.00	2.02	0.81	−0.31	1.21	−1.67	1.68	−3.73	2.86	−4.31	182.40
3.01	1.69	7.13	3.48	0.59	−3.55	4.82	−7.52	2.80	−4.41		
Fraxetin	7.87	6.54	−4.34	1.21	−2.19	0.87	−5.05	2.66	−7.14	2.30	−5.24
19.68	3.11	6.98	4.12	3.05	1.28	−8.22	1.19	−8.50	1.21	−10.25	196.80
3.32	−0.65	2.33	2.16	5.93	−8.10	1.10	−5.99	1.38	−6.82		
Esculin	7.81	5.12	−1.75	3.06	−0.30	0.74	−11.22	1.39	−9.22	5.33	−11.82
19.52	3.92	4.75	3.64	1.01	0.40	−1.83	3.27	−3.50	0.65	−4.29	195.20

2.35	3.16	2.50	3.64	0.49	−3.38	2.94	−5.45	0.79	−5.52		
Fraxin	8.05	3.69	1.49	3.92	2.94	6.68	−1.82	3.08	−3.58	1.94	−5.12
20.12	4.96	1.71	4.11	−0.31	1.42	−0.90	2.30	−5.24	3.07	−6.75	201.20
2.82	2.75	6.67	3.19	6.37	0.67	3.31	−0.98	0.84	−2.87		
Pulchinenoside B4	8.00	5.54	3.29	5.85	4.62	0.80	−13.38	6.43	−11.92	5.49	−10.00
20.00	1.91	−4.55	2.03	−6.63	1.27	−7.77	0.42	−8.53	0.93	−8.78	200.00

Table 4

Extraction recovery and matrix effect of eleven ingredients in pig plasma (*n*=6).

Analytes	Extraction recovery (%)			Matrix effect (%)		
Low	Medium	High	Low	Medium	High	Coptisine chloride
93.00±1.40	86.70±5.87	92.42±2.32	105.00±6.24	95.50±1.25	93.48±1.65	Berberine
86.65±2.48	85.54±1.26	92.43±0.95	87.94±4.46	85.74±2.27	95.32±10.69	Columbamine
94.50±2.84	83.62±0.94	91.29±2.76	100.24±2.27	91.79±3.02	103.94±1.78	Phellodendrine
91.04±1.39	90.91±7.93	88.22±4.02	103.74±5.16	110.91±1.82	89.33±4.37	Palmatine
92.78±4.19	90.89±1.39	95.39±0.97	101.67±4.41	87.78±2.69	93.67±0.69	Obacunone
87.17±2.84	91.56±3.83	92.72±2.51	104.33±1.53	98.21±0.89	95.88±0.59	Esculetin
92.72±0.66	93.43±1.86	89.42±1.63	101.77±0.38	97.71±2.32	94.55±0.91	Fraxetin

95.55±0.46	98.76±0.24	95.39±1.75	100.76±0.13	99.29±0.22	95.61±0.30	Esculin
88.31±0.27	92.83±0.10	93.60±0.75	103.84±0.22	95.13±0.05	98.29±0.30	Fraxin
92.63±6.10	91.48±5.00	96.24±6.37	93.04±6.51	87.64±5.37	92.91±0.89	Pulchinenoside B4

3.3. Plasma Pharmacokinetics

The validated method was used to assess the pharmacokinetics (PK) of coptisine chloride, berberine, columbamine, phellodendrine, palmatine, obacunone, esculetin, fraxetin, esculin, fraxin, and pulchinenoside B4 in pig plasma subsequent to a single oral administration of BTWD extract (1.0 mL/kg). Figure 3 displays the plasma concentration-time profiles of the eleven analytes in pig plasma following oral administration. Noncompartmental methods were employed to analyze the concentration-time data of esculetin, esculin, coptisine chloride, phellodendrine, pulchinenoside B4, and berberine, whereas compartmental methods were used to analyze fraxetin, fraxin, columbamine, and obacunone in order to calculate the PK parameters, which are presented in Table 5.

[figure(s) omitted; refer to PDF]

Table 5

Pharmacokinetic parameters of 10 components of BTWD after oral administration in pigs (n=6).

Analytes	Cmax (ng/mL)	Tmax (h)	t1/2 (h)	CL/F (mL/h)	AUC0−t (ng/mL·h)	AUC0−inf (ng/mL·h)	MRT (h)
Esculetin	78.17±1.68	4.00	2.91±0.08	0.76±0.02	980.37±26.44	994.38±27.79	8.97±0.08
Esculin	66.43±1.91	1.00	16.63±1.48	3.25±0.43	531.20±52.83	875.86±124.12	25.06±1.91
Fraxetin	118.75±6.14	0.49±0.01	0.33	9.72±0.56	158.57±9.33	158.57±9.33	0.98±0.01
Fraxin	36.83±0.48	0.53±0.02	0.36±0.01	36.38±1.84	53.25±2.70	53.51±2.78	1.07±0.04
Columbamine	3.80±0.08	12.00	6.74±5.08	17.54±0.76	28.35±1.31	30.98±1.36	12.50±2.80
Coptisine chloride	5.90±0.15	12.00	5.70±0.33	6.56±0.14	59.88±0.15	69.80±1.42	14.34±0.31
Berberine	28.36±0.83	12.00	3.14±0.13	14.82±0.93	202.44±12.74	208.64±13.59	11.07±0.03

Phellodendrine	8.18 ± 0.21	12.00	3.81 ± 0.10	12.41 ± 0.47	69.72 ± 2.62	73.39 ± 2.80	11.39 ± 0.14
Obacunone	7.39 ± 0.71	3.49 ± 0.07	0.88 ± 0.15	0.37 ± 0.02	110.70 ± 3.90	1146.51 ± 7.38	17.39 ± 3.00
Pulchinenoside B4	276.70 ± 10.54	1.00	3.27 ± 0.17	1.36 ± 0.05	2525.63 ± 87.16	2580.65 ± 89.09	9.57 ± 0.14

The findings of the present study indicate that the alkaloid compounds berberine, columbamine, phellodendrine, and coptisine chloride exhibited peak concentrations in plasma at 12 hours. These compounds were found to have low plasma concentrations, with berberine demonstrating the highest maximum plasma concentration (Cmax) of 28.36 ± 0.83 ng/mL. On the contrary, columbamine, coptisine chloride, and phellodendrine demonstrated plasma concentrations below 10.0 ng/mL, indicating restricted absorption via the gastrointestinal tract. Typically, molecules must possess lipophilic properties to facilitate efficient absorption in the gastrointestinal tract. Conversely, polar molecules exhibit reduced lipophilicity. The polar nature and presence of ionic charges in the structures of these three molecules hinder their absorption in the gastrointestinal tract. Additionally, the plasma concentration of palmatine fell below the requisite threshold for the analysis of pharmacokinetic behavior, potentially attributable to its polar structure [12]. The plasma concentration of palmatine was found to be below the threshold required for the analysis of PK behavior. However, the concentration of palmatine at the 12-hour timepoint was determined to be 5.79 ng/mL, exhibiting variance from the documented profile of palmatine in rat and dog plasma. In particular, in rats, the Cmax of palmatine was recorded as 2.14 ± 0.68 ng/mL and 2.50 ± 0.43 ng/mL, with the time to reach maximum concentration (Tmax) values of 0.36 ± 0.074 hours and 3.22 ± 0.81 hours following oral administration of Coptis root granules and Shuanghua Baihe tablets, respectively [19]. In beagle dogs, a Cmax of 8 ng/mL and Tmax of 5 hours were observed after oral administration of 300 mg of palmatine [20], indicating that the pharmacokinetic parameters of palmatine are influenced by coexisting compounds.

The Cmax of esculetin, esculin, fraxetin, and fraxin was determined to be 78.13 ± 1.68, 66.43 ± 1.91, 118.75 ± 6.14, and 36.83 ± 0.48 ng/mL, respectively. The original concentrations of these compounds were measured to be 751.43, 2810.50, 1537.92, and 1943.49 ng/kg, respectively. These findings indicate that the Cmax of fraxetin was higher than that of fraxin, and the Cmax of esculetin was higher than that of esculin. This observation suggests that the conversion of esculin and fraxin into esculetin and fraxetin, respectively, may contribute to these differences in Cmax values.

The Tmax and t1/2 values of pulchinenoside B4 were determined to be 1.00 h and 3.27 ± 0.17 h, respectively, indicating rapid absorption and elimination from the plasma. In comparison with other compounds, pulchinenoside B 4 exhibited the highest Cmax of 276.70 ± 10.54 ng/mL and the largest AUC0−t of 2525.63 ± 87.16 ng/mL·h, suggesting a high level of bioavailability.

On the contrary, the obacunone exhibited a low Cmax of 7.39 ± 0.71 ng/mL, attributed to its low initial concentration. However, it demonstrated a prolonged Cmax of 3.49 ± 0.07 h and MRT of 17.39 ± 3.00 h, contrasting with the previously reported Tmax of 1–2 h and MRT of 4.30 ± 0.16 h in rats following oral administration of 10 mg/kg obacunone [21]. Additionally, compared to the Tmax of 1.67 ± 0.29 h and MRT of 4.90 ± 2.60 h in rats administered with the fruit of *Tetradium ruticarpum* and licorice extracts together [22], the extended Tmax and MRT of obacunone suggest that its pharmacokinetic behavior can be altered when used in combination with other drugs.

The concentration-time profiles of esculetin, fraxetin, esculin, columbamine, coptisine chloride, phellodendrine, pulchinenoside B4, and berberine displayed biphasic patterns, indicating the potential involvement of enterohepatic circulation, distribution re-absorption, or biotransformation [11]. The absorption of drugs is a multifaceted process influenced by interactions with various physicochemical and physiological factors. Factors such as the absorption window along the gastrointestinal tract, enterohepatic recirculation, variable gastric emptying, and drug-drug

interactions can impact the absorption kinetics. Distribution re-absorption occurs when the drug concentration in tissue exceeds that in plasma, leading to the transfer of the drug from tissue to plasma and resulting in a secondary peak in plasma levels. For example, berberine, with its high concentration in bile during distribution, may facilitate enterohepatic circulation and distribution re-absorption [23]. The second peaks of esculetin and fraxetin may be caused by the esculin and fraxin biotransformation of their respective precursors, esculin and fraxin. The dual peak phenomena observed in these constituents may play a role in the sustained elevation of their blood concentrations *in vivo*, thereby enhancing the pharmacodynamic effects of BTWD [24].

In this study, the PK behaviors of pulchinenoside B4, phellodendrine, berberine, obacunone, esculin, and esculetin exhibited variations compared with the findings reported by Yang et al. [14, 16]. Similarly, fraxin demonstrated dissimilarities from the observations made by Wang et al. [13]. These disparities may be attributed to drug-drug interactions within the multiherbal mixture, leading to alterations in the PK parameters of the individual components [15]. Additionally, the use of different experimental animals could have contributed to these discrepancies. We assert that our results are more reliable as the animal model employed closely resembles that of humans.

4. Conclusion

This study presents the development and validation of a novel UHPLC-MS/MS method for the simultaneous quantification of eleven analytes in Bama miniature pig plasma. The method incorporates a straightforward plasma sample preparation technique. Rigorous validation procedures were conducted to assess the method's specificity, sensitivity, accuracy, and reproducibility. All validation parameters were found to meet the necessary bioanalysis criteria. Furthermore, the method was effectively used in pharmacokinetic studies of pigs following a single oral administration of 1.0 mL/kg BTWD. BTWD is commonly used in the management of digestive system disorders, with variations in its pharmacokinetic characteristics observed between normal and ulcerative colitis rats. Therefore, further investigation is warranted to assess the pharmacokinetic properties of BTWD compounds following administration to an ulcerative colitis model of Bama miniature pigs. Given the alkaloid compounds' low Cmax and their significant therapeutic roles in digestive system diseases, they may be extracted separately and these compounds were administered through nonoral routes.

Disclosure

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Authors' Contributions

Qianqian Xu conceptualized the study, proposed the methodology, provided resources, wrote the original draft, visualized the data, administered the project, and acquired funding. Huilan Gao provided resources and designed the methodology. Fuqiang Zhu involved in the formal analysis and validated the data. Wenliang Xu wrote the original draft. Yubo Wang reviewed and edited the manuscript. Jinwen Xie and Guangjun Guo investigated the data. Limei Yang and Li Ma curated the data. Zhiqiang Shen and Jichang Li supervised the data.

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DETAILS

Subject:	Acetonitrile; Mass spectrometry; Quality standards; Chloride; Plasma; Acetic acid; Quality control; Traditional Chinese medicine; Pharmacokinetics; Veterinary medicine; Scientific imaging; Health services; Chinese medicine; Chemical treatment
Business indexing term:	Subject: Quality standards Quality control
Location:	United States--US; China
Editor:	Bishnu Regmi
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-12-08 (Received); 2024-04-08 (Revised); 2024-04-23 (Accepted); 2024-05-10 (Pub)
DOI:	https://doi.org/10.1155/2024/5535752
ProQuest document ID:	3056517254
Document URL:	https://www.proquest.com/scholarly-journals/pharmacokinetic-properties-baitouweng-decoction/docview/3056517254/se-2?accountid=211160

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Last updated:	2024-05-20
Database:	Publicly Available Content Database

Exploiting of Green Synthesized Metal Oxide Nanoparticles in the Potentiometric Determination of Metformin Hydrochloride in Pharmaceutical Products

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ProQuest document link

ABSTRACT (ENGLISH)

The advanced and highly functional properties of Al_2O_3 and NiO nanoparticles promote the widespread use of metal oxides as remarkable electroactive materials for sensing and electrochemical applications. The proposed study describes a comparison of the sensitivity and selectivity of two modified wire membrane sensors enriched with Al_2O_3 and NiO nanoparticles with conventional wire membranes for the quantification of the antidiabetic drug metformin hydrochloride (MTF). The results show linear relationships of the enriched Al_2O_3 and NiO nanosensors over the concentration ranges 1.0×10^{-10}–1.0×10^{-2} mol L^{-1} and 1.0×10^{-6}–1.0×10^{-2} M for both the modified sensors and the conventional coated wire membrane sensors. The regression equations were EmV = (52.1 ± 0.5) log (MTF) + 729 for enriched nanometallic oxides, EmV = (57.04 ± 0.4) log (MTF) + 890.66, and EmV = (58.27 ± 0.7) log (MTF) + 843.27 with correlation coefficients of 0.9991, 0.9997, and 0.9998 for the aforementioned sensors, respectively. The proposed method was fully validated with respect to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC). The newly functionalized sensors have been successfully used for the determination of MTF in its commercial products.

FULL TEXT

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1. Introduction

Various nanostructured metal oxides have already found wide applications. They are being researched for their promising applications in almost all scientific fields, including optics, catalysis, energy, electronics, sensors, environment, information technology, medicine, materials chemistry, biomedicine, and agriculture. These diverse applications have led scientists to develop different variants for the production of metal oxide nanoparticles with desired properties [1].

Nanomaterials play an important role in the development of chemosensors and biosensors, especially due to their distinct physical and chemical properties, such as good conductivity, surface-to-volume ratio, high mechanical strength, and excellent electrocatalytic activity [2]. Green nanotechnology is a topic of great interest in research studies around the world as it is the best way to reduce the negative impacts of nanomaterial production and use while reducing the risks of nanotechnology [3]. The principles of green synthesis can therefore be explained by a number of factors, including minimizing waste, reducing pollution, and using safer solvents. The use of plant extracts is one of the currently available green synthesis techniques for metal oxide nanoparticles (MONPs) [4].

Nowadays, the main focus is on the use of metal oxide-modified sensors for the detection and quantification of pharmaceutical compounds. The chemical properties and advanced physical characteristics of nickel oxide nanoparticles (NiONPs) and aluminum oxide nanoparticles (Al_2O_3NPs) promote their use in various applications [5]. The selectivity and sensitivity of ion-selective sensors are known to depend on the type of ionophore and the properties of the plasticizers and additives used. They also depend on the composition of the membrane during the manufacturing process. Nanoparticles are an excellent addition for enhancing electrode performance and reducing electrical resistance [6].

Pennisetum glaucum (millet) is considered to be one of the most nutritious cereals of all. It is unique among cereals in having a higher mineral content and micronutrient density than rice, wheat, barley, etc., and has outstanding nutritional properties. The richness in phytochemicals (polyphenols and fiber) increases the nutraceutical potential of millet and makes it a powerhouse of health-promoting nutrients [7]. The bioactive compounds present in millet, such as flavonoids, alkaloids, saponins, tannins, phenols, terpenoids, proteins, carbohydrates, and amino acids, have a large number of functional groups (O-H, N-H, S-H, -COOH, C=O, and C-halide) [8]. These functional groups can serve as reducing, capping, and stabilizing agents in the production of nanomaterials. The literature search revealed various reports on the use of millet extract in the synthesis of nanoparticles [9–11].

Metformin, 1,1-dimethylbiguanide hydrochloride (Figure 1) is an oral hypoglycemic agent used in medicine for the treatment of diabetic patients [12–15]. Metformin is the most commonly prescribed drug for the treatment of type 2 diabetes (T2D) [16]. In contrast to other diabetes medications, metformin has a positive effect on body weight and has no hypoglycemic side effects. The main target of metformin is presumably the liver. The drug reaches the liver

cells and suppresses the production of glucose in the liver, which leads to a reduction in blood glucose levels [17]. [figure(s) omitted; refer to PDF]

The literature search revealed several analytical methods for the detection of MTF. These methods are spectrophotometry [18, 19] and separation chromatography [20, 21]. A number of sensors have been developed for the determination of MTF [22], but they still have some limitations.

Potentiometry is one of the most important electrochemical techniques, and researchers have long been interested in the applications of potentiometric-based sensors [23]. Hundreds of different sensors have been developed in this field and published in the literature to date [24]. Today, potentiometric sensors are the focus of interest due to the successful development of sensors in many applications [25–27]. Due to the high demand and constant technological progress, the world of sensors is diverse and rapidly evolving. Electrochemical sensors are widely used in the food, oil, and agricultural industries as well as in environmental and biomedical applications. They offer a convenient and affordable solution for the detection of variable analytes [28–32].

The use of metal oxides as electroamplifiers in electrochemical sensors has been reported, e.g., the use of aluminum oxide and nickel oxide nanoparticles in various sensor systems. Due to their strong electrocatalytic activity, low cost, high ability to bind organics, small size, high degree of crystallinity, and large surface-to-volume ratio, metal oxide nanoparticles are widely used and have been established as an active electrocatalyst for the detection of a variety of compounds [33]. The active sites, electrochemically active surface area, surface energy, and other factors are generally closely related to the electrocatalytic properties of metal oxide nanoparticles [34]. The catalytic materials were fabricated as small as possible to increase the number of available active sites and the available surface area so that the metal oxide-based nanoparticles exhibit high sensing performance [35–38]. Undoubtedly, a formula for the development and improvement of ion-selective sensors with low detection limits, repeatability, and good chemical stability is needed. Currently, there are no known modified potentiometric sensors for the detection of metformin hydrochloride based on fabricated metal oxide nanoparticles.

The aim of the present study is to use an aqueous millet extract and a natural reduction source to synthesize Al_2O_3 NPs and NiONPs from their precursors (aluminum nitrate and nickel sulfate) under certain optimized conditions. The synthesized metal oxide nanostructures were subsequently confirmed by various spectroscopic and microscopic investigations. Two new and sensitive potentiometric sensors modified with pre-synthesized nanoparticles were fabricated, and their efficiency in the determination of metformin hydrochloride was investigated. In addition, a comparative study between the conventional sensor and the metal oxide nanoparticle-enriched sensors was performed.

2. Experimental

2.1. Chemicals and Reagents

Pure MTF and its pharmaceutical preparation (Metfor® 500 mg/tablet) were provided by Tabuk Pharmaceutical MFG.CO. (Saudi Arabia). Tetrahydrofuran (THF) 97%, methanol 99.9%, acetone 99.9%, phosphotungstic acid (PTA), sodium hydroxide (NaOH), hydrochloric acid (HCl), polyvinyl chloride (PVC) of high molecular weight, ortho-nitrophenyl octyl ether (*o*-NPOE), aluminum nitrate ($Al(NO_3)_3$), and nickel sulfate ($NiSO_4$) were acquired from Sigma-Aldrich, Hamburg, Germany.

2.2. Instrumentation

Potentiometric measurements were performed with a digital pH-mV (HANNA, model-211) with an Ag/AgCl reference electrode in conjunction with an indicator electrode. A pH meter (Metrohm model 744) was also used for pH measurements. UV-vis spectral analysis was performed using a UV 2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A scanning electron microscope (SEM) and a transmission electron microscope (TEM) (JEM-2100F, JEOL Ltd, USA) were used to study the particle size and surface morphology of Al_2O_3NPs and NiONPs. To determine the functional groups that might be present in the Al_2O_3NPs and NiONPs after fabrication, Fourier transform infrared spectroscopy (FT-IR) was performed using a Spectrum spectrometer BX (PerkinElmer, Waltham, USA).

2.3. Preparation of Extract *Pennisetum glaucum* (Millet)

The *Pennisetum glaucum* (millet) seeds were obtained from a local source (Riyadh, Saudi Arabia). 25 g of cleaned seeds were boiled with 400 mL of deionized water for 30 min. The content was cooled and filtered using Whatman filter paper, No. 4. The resulting extract was kept in a refrigerator for storage at 4°C (Scheme 1).
[figure(s) omitted; refer to PDF]

2.4. Optimization Conditions of Green Synthesis of Nanoparticles

The synthesis process of Al_2O_3 and NiO nanoparticles was performed under certain optimized conditions including the use of different volumes of the plant extract (5–30 mL), the effect of reaction time intervals (10–60 min), the suitable pH of the solution (pH = 2–12), and the effect of temperature using three different degrees (25, 35, and 55°C). The synthesis process was performed under constant stirring at 3000 rpm for 30 min. The selected conditions were 20 mL of extract and 30 min reaction time. pH was adjusted using sodium hydroxide to 11.

2.5. Green Synthesis of Al_2O_3 and NiO Nanoparticles

The synthesis of Al_2O_3NPs was carried out under constant stirring by 100 mL of aluminum nitrate (0.2 M) with 20 mL of millet extract subsequently. A dropwise addition of 5 mL of sodium hydroxide solution 0.1 M was made to adjust the pH to 11. Al_2O_3NPs started to form after the mixture had been held under magnetic stirring for 30 minutes at room temperature. To eliminate any excess sodium hydroxide, the produced nanoparticles were filtered through filter paper and then washed three times with distilled water and one time with methanol. The produced Al_2O_3NPs were dried for 24 hours at room temperature [39].

The synthesis of NiONPs was carried out under constant stirring by 100 mL of nickel sulfate (0.2 M) with 20 mL of millet extract subsequently. A dropwise addition of 5 ml of sodium hydroxide solution 0.1 M was made to adjust the pH to 11. NiONPs started to form after the mixture had been held under magnetic stirring for 30 minutes at room temperature. To eliminate any excess sodium hydroxide, the produced nanoparticles were filtered through filter paper and then washed three times with distilled water and one time with methanol. The produced NiONPs were dried for 24 hours at room temperature [40]. The synthesis process was previously illustrated in Scheme 1.

2.6. Preparation of Stock Drug Solution

A stock solution of MTF (1.0×10^{-2} M) was prepared by dissolving 0.13 g in 100 mL of distilled water. Serial dilutions were prepared with distilled water.

2.7. Preparation of Ion Pair

The ion pair (metformin-phosphotungstate) MTF-PT was prepared by mixing 50 mL of 1.0×10^{-2} M of MTF solution and 50 mL of 1.0×10^{-2} M precipitating agent (phosphotungstic acid) PTA. The obtained precipitate was filtered, properly washed with distilled water, and then fully dried overnight at room temperature [41].

2.8. Membrane Composition

Three different coated membranes were prepared using electroactive materials MTF-PT, MTF-PT-Al_2O_3, and MTF-PT-NiO nanoparticles. The conventional coated wire membrane was prepared by mixing 190 mg of PVC and 0.35 mL plasticizer *o*-NPOE and 10 mg of ion pair (MTF-PT) in 5 mL of THF. The prepared mixture was placed in a petri dish with a diameter of 3 cm and allowed to gradually evaporate there at room temperature. 5 mg of the previously synthesized nanoparticles Al_2O_3 and NiO was added separately to the aforementioned composition of the membrane to create the modified membranes [42].

2.9. Coated Wire Membrane Composition

The Al wire's tip was cleaned with distilled water and acetone and then dried. The wire was coated by swiftly dipping the wire into the coating solution multiple times and letting it dry at room temperature. The manufactured sensor was preconditioned by soaking for 24 h in a 1.0×10^{-3} M of MTF solution.

2.10. Electrode Calibration Graph

About 10 mL aliquots of 1.0×10^{-10}–1.0×10^{-2} M standard MTF solution were pipetted into a 50-mL beaker, and the prepared sensors in conjunction with the Ag/AgCl reference electrode were submerged in this solution. The potential was measured and recorded in mV. The slope of the calibration curves was calculated after the electrode potential was plotted against −log concentration of the examined drug.

2.11. Optimizing the Condition of Potential Reading

The impact of pH on the potential of the prepared sensors was measured. The reference electrode Ag/AgCl was connected to the coated wire sensor. About 50 mL aliquots of the drug solution (1.0×10^{-5} M or 1.0×10^{-4} M) were added to a 100 mL beaker and the two electrodes were immersed in it. Potential measurements corresponding to various pH values were then recorded. The pH-mV was measured and plotted after small amounts of 0.1 M HCl were added to the solution in order to first acidify it. Small amounts of 0.1 M NaOH were then added to progressively raise the pH [43].

The separation solution approach was used to determine the selectivity coefficient, and the following equation (1) was applied:(1)Log KMTF.Jz+pot=E2–E1S+logMTF-logJz+1/z,where E1 is the electrode potential of drug solution in 1.0×10^{-3} M, E2 is the potential of the electrode of interfering species in 1.0×10^{-3} M of the interferent ion J^{+}, and S is the slope of the calibration graph. However, the proposed sensors' selectivity toward interfering ingredients, such as some common cations, amino acid, sugars, related organic analytes (guanidine, cycloguanil, synthalin, and galegine), and formulated additives, was studied [44].

2.12. Analysis of MET in Metfor® Tablets

To prepare 1.0×10^{-2} M standard solution, ten tablets of Metfor® (500 mg/tablet) were ground to a fine powder. An exact quantity equivalent to 0.13 g was then dissolved in distilled water. Serial dilutions were done to prepare various concentrations of MET within the range of 1.0×10^{-10}–1.0×10^{-2} M.

3. Results and Discussion

The synthesis process of Al_2O_3 and NiO nanoparticles was carried out under certain optimized conditions. The number of nanoparticles produced was significantly influenced by the amount of plant extract. The absorption pattern changed significantly when the amount of plant extract was increased. The use of 20 mL of plant extract showed the highest absorbance at 290 nm and 400 nm for the Al_2O_3 and NiONPs, respectively. In addition, a pH of 11 and a reaction time of 30 minutes at room temperature were chosen.

3.1. Characterization of Al_2O_3 and NiO Nanoparticles

The obtained Al_2O_3NPs and NiONPs were examined using a variety of spectroscopic and microscopic techniques. Using UV-vis spectroscopy, the optical properties of each metal oxide nanoparticle were studied. The absorption spectra in Figures 2(a) and 2(b) showed absorption peaks at 290 nm for Al_2O_3NPs and 400 nm for NiONPs.

[figure(s) omitted; refer to PDF]

The band gap energy of the prepared Al_2O_3NPs and NiONPs was calculated from the following formula:(2)αhυ=Ahυ-Egn,where α is the absorption coefficient, Eg is the band gap energy, h is the Planck constant (6.626×10^{-34} J·s), n is 1/2 or 2 for direct or indirect transition, and (hυ) is the photon energy (eV). The predicted band gaps for the Al_2O_3 NPs and NiONPs were 4.28 eV and 3.1 eV, respectively.

To validate the role of bioactive compounds in the green synthesis of metal oxide nanostructures, the absence or presence of phytochemicals in the millet extract has been tested using preliminary chemical tests. According to the previous report [45], various reagents were used including, for flavonoids (sodium hydroxide and hydrochloric acid), alkaloids (Dragendroff reagent, HCL), saponin (Foam test), tannins (ferric chloride 1% solution), terpenoids (Salkowski's test), phenols (bromine water, white ppt), proteins (Biuret test, copper sulfate solution), and carbohydrates (Tollen's test). The obtained findings are summarized in Table 1.

Table 1

Qualitative determination for preliminary phytochemicals in the millet extract.

Phytochemicals	Chemical test	Positive indication sign
Flavonoids	Sodium hydroxide few drops and few drops of hydrochloric acid	Deep yellow color was removed by few drops of hydrochloric acid

Alkaloids	Dragendorff reagent	Orange or red precipitate
Tannins	Ferric chloride (1%)	Blackish blue color
Saponin	Foam test	Foam more than 1 cm
Terpenoids	Salkowski's test	Reddish-brown precipitate
Phenols	Bromine water	White precipitate
Proteins	Biuret test, copper sulfate solution	
Carbohydrates	Tollen's reagent	Silver mirror

Due to the presence of phenolic compounds, such as ferulic acid, which is the main bound phenolic compound, the FT-IR analysis of the millet seed extract (Figure 3(a)) revealed different functional groups at 3441, 3356, and 3240 cm^{-1} for O-H stretching vibration. In addition, 2924 and 2854 cm^{-1} are related to asymmetric and symmetric C-H stretching of alkane. The absorption band at 1743 cm−1 is for C=O stretching of esters while the absorption bands at 1658 and 1550 cm−1 are for asymmetric C=O stretching vibration of aqueous carboxylate. The appeared bands at 1458 and 1373 cm^{-1} are related to NO_2 compounds. However, the noticed bands in the range from 1157 to 1041 cm^{-1} are associated with C-O stretching of polysaccharide skeleton in millet carbohydrates. The bands of 717−509 cm^{-1} are for stretching vibration of alkyl-halide compounds [46].

[figure(s) omitted; refer to PDF]

The spectra of Al_2O_3NPs are shown in Figure 3(b). The broad peaks at 3752.52 cm^{-1} and 3471.56 cm^{-1} are for OH stretching, peaks at 1635.93 cm^{-1} and 1544.23 cm^{-1} are for O-H stretching and bending vibration of absorbed water, peaks at 1383.93 cm^{-1} and 961.87 cm^{-1} could be attributed to O-H bending and C=C bending, the peak at 832.95 is for Al-O vibration, and the broad absorption peak at 608 cm^{-1} is assigned to Al-O-AL stretching vibration mode [47] in the FT-IR. The spectra of NiO are shown in Figure 3(c). The broad peaks at 3489.44 cm^{-1} and 3419.07 cm^{-1} are for -OH stretching, the peaks at 1637.17 cm^{-1} and 1401.39 cm^{-1} are for C=C and C=O stretching, the peak at 1116 cm^{-1} could be attributed to C-O stretching, and the broad absorption peak at 616 cm^{-1} is assigned to the Ni-O vibration mode [48].

The XRD analysis was used to study the crystalline shape and determine the crystallite size of the synthesized metal oxide nanostructures. The XRD pattern of Al_2O_3NPs (Figure 4(a)) shows remarkable peaks at $2\theta = 22.9°$, 29.4°, 31.9°, 39.0°, 55.6°, and 64.9° assigned to planes (1 1 4), (0 1 2), (2 2 0), (1 1 0), and (4 2 2), respectively. The obtained findings are matched to JCPDS Card No. 79–1558 [49]. The XRD pattern of NiO (Figure 4(b)) shows different peaks at (2θ) of NiONPs at 37.04°, 43.04°, 62.49°, 74.93°, 78.89, and 94.38 assigned to planes (1 1 1), (2 0 0), (2 2 0), (3 1 1), and (2 2 2), respectively. The obtained findings are matched to JCPDS Card No. 78–0643 [50]. The grain size of the Al_2O_3 and NiO crystallites was determined using the Debye–Scherrer formula:(3)$D = 0.94\lambda\beta\cos\theta$,where D is the crystal size, $\lambda = 1.54060$ is the wavelength of the radiation, β is the line broadening at half the maximum intensity, and θ is the Bragg angle of the X-ray diffraction peak. The average crystallite size of Al_2O_3 and NiO was 17.7 nm and 20.2 nm, respectively.

[figure(s) omitted; refer to PDF]

TEM is a technique that provides much higher resolution than light-based imaging methods by imaging a nanoparticle sample with an electron beam. The TEM examination revealed details about the surface morphology, agglomeration, and particle size. The TEM images of Al_2O_3NPs and NiONPs showed that the particles are fairly uniformly distributed, polydisperse, and spherical (Figures 5(a) and 5(b)) with an average particle size of 43.01 ± 6

and 38.9 ± 10 nm (Figures 5(c) and 5(d)).

[figure(s) omitted; refer to PDF]

The primary application of the versatile and advanced field emission scanning electron microscope (FISEM) is the study of material surface phenomena. Numerous qualitative details about a material, including its composition, topography, morphology, and crystallographic properties, can be obtained using a FISEM. Stated differently, it furnishes data regarding the size, form, and distribution of the particles on the sample's surface, in addition to the surface properties and texture [51]. The characteristics of adsorbents that have the largest effects on their capacity to absorb biomolecules are their surface area, morphology, charge, and state of aggregation. The morphology of the synthesized metal oxide nanoparticles was investigated using the FESEM. The micrographs showed that each Al_2O_3 NP and NiONP had a spherical morphology with particle sizes ranging from 50 to 100 nm (Figures 6(a) and 6(b)). Large cavities and pores along with an uneven, porous, and heterogeneous surface morphology are visible in the nanocomposite's 30,000x magnification FESEM image. There are some visible micropores in addition to the mesopores. Because of its greater surface area, NCS's heterogeneity, pores, and voids greatly enhance the material's adsorption capacity. The size and three-dimensional profile of the synthesized nanomaterials were verified by AFM analysis of their surface characteristics. The AFM images (Figures 6(c) and 6(d)) demonstrate a well-defined spherical shape with a solid, dense structure and an average size of 100 nm for the particles.

[figure(s) omitted; refer to PDF]

3.2. The Fabricated Sensor Behavior

MTF reacts with PTA to form a stable MTF-PT ion pair complex, which was soluble in an organic solvent such as THF but insoluble in water. The addition of the active components with (*o*-NPOE) acting as a solvent mediator with the presence of PVC was used in conventional and modified sensors [52]. Critical response characteristics of the fabricated electrodes over the concentration range 1.0×10^{-6}–1.0×10^{-2} and 1.0×10^{-10}–1.0×10^{-2} M for conventional and modified sensors were studied, and the results are summarized in Table 2.

Table 2

Electrochemical response characteristics of MTF-PT conventional and MTF-PT-Al_2O_3-modified and MET-PT-NiO-modified sensors.

Parameter	MTF-PT conventional sensor	MTF-PT-Al_2O_3-modified sensor	MTF-PT-NiO-modified sensor
Slope (mV decade^{-1})	52.1 ± 0.5	57.01 ± 0.4	58.27 ± 0.7
Intercept (a)	729	890.66	843.27
Correlation coefficient (r)	0.9992	0.9998	0.9999
Linear range (M)	1.0×10^{-6}-1.0×10^{-2}	1.0×10^{-10}-1.0×10^{-2}	1.0×10^{-10}-1.0×10^{-2}
LOD (M)	5.0×10^{-7}	5.0×10^{-11}	5.0×10^{-11}
Response time, (s)	45	20	30
Working pH range	4-9	4-9	4-9
Lifetime (day)	27	45	35

Temperature (°C)	25	25	25
Accuracy (%)	98.98 ± 0.57	99.69 ± 0.38	99.55 ± 0.53

The fabricated sensors gave Nernstian responses with slopes of 52.1 ± 0.5, 57.01 ± 0.4, and 58.27 ± 0.7 for MTF-PT, MTF-PT-Al_2O_3, and MET-PT-NiO, respectively (Figures 7(a)–7(c)). The suggested sensors have fast dynamic response times of 45, 20, and 30 s and are used for a period of 27, 45, and 35 days for MTF-PT, MTF-PT-Al_2O_3, and MTF-PT-NiO, respectively, without any significant change in parameters. The results showed that when compared to the conventional and the modified sensors, those enhanced with metal oxide nanoparticles had quick response times and good stability. This could be caused by the addition of nanoparticles to sensors. They have physicochemical properties not found in the bulk material. These nanoparticles improved interactions with targets in test solutions due to their higher surface-to-volume ratio [53].

[figure(s) omitted; refer to PDF]

The pH effect of sensors on the potential was investigated to determine the appropriate pH range for determining MTF. The result of conventional and modified sensors concluded that they were practically independent in the range of 4–9 and could be safely used for MTF determination. Potential-pH curves for MTF concentration were created, as shown in Figures 8(a)–8(c). Below pH 4, the potential dropped as the acidity of the analyte increased, which could be attributed to the membrane extraction of H^+ ions. The decrease in the electrode response at pH levels greater than 9 may be attributed to the rise in OH^- concentrations.

[figure(s) omitted; refer to PDF]

One of the most important aspects of an ion-selective electrode is undoubtedly its selectivity behavior, which determines the feasibility of a trustworthy measurement in the target sample. For a number of inorganic cations, sugars, amino acids, and related compounds, the separated solution method [54] and the matched potential method [55] were used to determine the selectivity coefficients for MTF cations. How different substances affect the response of MTF-PT, MTF-PT-Al_2O_3, and MTF-PT-NiO-coated wire membrane sensors was investigated. The selectivity of the prefabricated sensors was tested by measuring the potentiometric interference of inorganic cations such as Na^+, Fe^{3+}, Cr^{3+}, Ag^+, Ca^{2+}, K^+, Mg^{2+}, and Co^{2+}; sugars; and amino acids. The physicochemical properties of the ion exchange process at the membrane determine the selectivity of a membrane sensor based on ion pairs. This selectivity could result from the free energy transfer of the MTF^+ ions between the membrane and the surrounding medium. The data show that the proposed electrodes exhibit a high degree of selectivity for MTFs. Due to the different permeability and mobility of the inorganic cations with respect to the MTFs, they do not interfere, with the so-called Hofmeister selectivity sequence [56]. The degree of correspondence between the locations of lipophilicity sites in two competing species on the bath solution side and those in the receptor of the ion exchanger determines the mechanism of selectivity, which is primarily based on stereospecificity and the electrostatic environment [57]. The results (Table 3) showed that there was no interference between amino acids and sugars.

Table 3

Selectivity coefficient (KPot MTF^+) of conventional MTF-PT and MTF-PT-Al_2O_3-modified and MTF-PT-NiO-modified sensors using 1.0×10^{-3} M of MTF solution.

Interferences	Kpot MTF-PT	Kpot MTF-PT-Al_2O_3	Kpot MTF-PT-NiO
Na^+	1.3×10^{-3}	1.4×10^{-4}	9.3×10^{-3}
Fe^{3+}	2.5×10^{-3}	1.6×10^{-3}	5.1×10^{-3}
Cr^{3+}	5.0×10^{-3}	5.1×10^{-3}	1.9×10^{-3}

Ag^{+}	3.2×10^{-3}	2.2×10^{-4}	1.7×10^{-3}
Ca^{2+}	3.8×10^{-3}	5.7×10^{-3}	3.9×10^{-3}
K^{+}	7.9×10^{-3}	1.9×10^{-4}	1.6×10^{-4}
Mg^{2+}	1.1×10^{-3}	4.5×10^{-4}	3.2×10^{-4}
Co^{2+}	1.4×10^{-3}	2.7×10^{-3}	1.0×10^{-3}
Serine	1.3×10^{-3}	5.5×10^{-3}	5.0×10^{-4}
Glycine	2.1×10^{-3}	1.3×10^{-4}	1.6×10^{-3}
Starch	1.9×10^{-3}	2.3×10^{-4}	1.0×10^{-3}
Guanidine	1.1×10^{-3}	9.2×10^{-4}	8.2×10^{-4}
Cycloguanil	2.3×10^{-3}	1.8×10^{-4}	9.3×10^{-4}
Synthalin	5.6×10^{-3}	5.9×10^{-4}	6.5×10^{-4}
Galegine	4.2×10^{-3}	3.6×10^{-4}	7.4×10^{-4}

The selectivity of the produced sensors was investigated with guanidine and other related compounds. No interferences were observed, which can be attributed to the electroactive sites in the membrane (MTF-PT). However, the addition of metal oxide nanoparticles with a large surface area and high dielectric constant increases the conductivity of the sensor and thus improves the selectivity and sensitivity.

3.3. Quantification of Metformin Hydrochloride

The created sensors were used to detect MTF in its bulk powder. The results were obtained using the direct calibration method and were expressed as % recoveries. The analysis's results, which used the suggested electrodes, revealed mean percentage recoveries of 98.87 ± 0.72, 99.60 ± 0.34, and 99.45 ± 0.40 for MTF-PT, MTF-PT-Al_2O_3NPs, and MTF-PT-NiONPs, respectively (Table 4).

Table 4

The results of the MTF determination in bulk powder using MTF-PT, MTF-PT-Al_2O_3NP-modified, and MTF-PT-NiONP-modified sensors.

	MTF-PT conventional sensor			MTF-PT-Al_2O_3NP-modified sensor			MTF-PT-NiONP-modified sensor		
Test* solution	Found*	% recovery	Test* solution	Found*	%Recovery	Test* solution	Found*	% recovery	Statistical analysis

6	5.99	99.83	10	9.96	99.60	10	9.95	99.50	5
4.96	99.20	8	7.99	99.87	8	7.96	99.50	4.3	4.20
97.76	6	5.98	99.66	6	5.94	99.00	4	3.97	99.25
4	4.00	100.00	4	3.99	99.75	3	2.96	98.67	3
2.97	99.00	3	2.97	99.00	2	1.97	98.50	2	1.99
99.50	2	2.00	100.00	-					
Mean ± SD	98.87 ± 0.72			99.60 ± 0.34			99.45 ± 0.40		
–									
n	6			6			6		
–									
Variance	0.52			0.12			0.16		
–									
% SE**	0.29			0.14			0.16		
–									
% RSD	0.73			0.34			0.40		

*Test and found solutions −log conc. (mol L^{-1}) **SE (% Error) = %RSD/√n.

These results showed the ultra-sensitivity of the MTF-PT-Al_2O_3NP- and MTF-PT-NiONP-modified sensors. The special physical and chemical properties of the metal oxide nanoparticles in use improved the conductivity and sensitivity of the modified electrodes for the detection of the selected drug.

3.4. Method Validation

The proposed method was validated according to IUPAC recommendations [58]. Wide linear relationships were displayed by the designed sensors over 1.0×10^{-6}–1.0×10^{-2} M for the conventional sensor, in comparison with 1.0×10^{-10}–1.0×10^{-2} M for the modified sensors. The regression equations were Emv = (52.1 ± 0.5) log [MTF] + 729 for the MTF-PT conventional sensor and Emv = (57.01 ± 0.4) log [MTF] + 890.66 and Emv = (58.27 ± 0.7) log [MTF] + 843.27 for MTF-PT-Al_2O_3NP- and MTF-PT-NiONP-modified sensors, with correlation coefficients r = 0.998, 0.999, and 0.999 for the respective sensors stated, respectively. All sensors' lower limits (LOD) of detection were recorded after the slope's potential reading dropped by 17.9 mV. The obtained results were found to be 5.0×10^{-7}, 5.0×10^{-11}, and 5.0×10^{-11} M. The dielectric constant of NiO is ε = 9.1, and the dielectric constant of aluminum oxide is ε = 9-10; the two metal oxides have almost the same conductivity constant, which is why both sensors modified with Al_2O_3 and NiO

nanoparticles have the same lower detection limit for MTF analysis.

Nine concentrations were used to test the method's accuracy, and the mean percentage recoveries were calculated as 98.98±0.57, 99.69±0.38, and 99.55±0.53 for the above-mentioned sensors, respectively. Additionally, the intermediate precision was evaluated via the inter-day and intra-day assay, and the percentage relative standard deviation (% RSD) was calculated. The % RSD for the MTF-PT-Al_2O_3NP-modified sensor was 0.27% and 0.41%, and for the MTF-PT-NiONP-modified sensor, it was 0.26% and 0.21%. All results are less than 2%, showing a highly precise technique (Table 5).

Table 5

Intraday and interday assays of MTF solution using MTF-PT-Al_2O_3NP-modified and MTF-PT-NiONP-modified sensors.

	MTF-PT-Al_2O_3NP-modified sensor			MTF-PT-NiONP-modified sensor		
Test sample	Found	% recovery	Test sample	Found	% recovery	Intraday
10	10.00	100.00	10	9.99	99.90	8
7.96	99.50	8	7.95	99.38	6	5.99
99.93	6	5.98	99.67	·		
Mean±SD	99.81±0.27			99.65±0.26		
–						
n	3			3		
–						
SE%**	0.16			0.15		
–						
RSD%	0.27			0.26		
–						
Interday	10	9.99	99.90	10	9.95	99.50
8	7.94	99.25	8	7.94	99.25	6
6.00	100.00	6	5.98	99.67	·	
Mean±SD	99.72±0.41			99.47±0.21		
–						

n	3	3
–		
SE%**	0.24	0.12
–		
RSD%	0.41	0.21

**SE (% Error) = %RSD/√n.

Borate buffer with a pH of 9 ± 0.5 was used to ensure the method's robustness. The percentage recovery for the conventional sensor was 99.44 ± 0.4%, the modified MTF-PT-Al_2O_3NPs sensor was 99.65 ± 0.2%, and the modified MTF-PT-NiONPs sensor was 99.77 ± 0.3%. Another test was performed to ensure the ruggedness of the suggested method by using a different model of pH meter (Jenway-3510). The measured mean percentage recoveries were 99.77 ± 0.2%, 99.85 ± 0.1%, and 99.50 ± 0.1% for the previously mentioned sensors. The results showed good agreement with those obtained using the proposed technique with no significant changes observed.

3.5. Determination of MTF Hydrochloride in Tablets

To quantify the metformin hydrochloride in its pharmaceutical form Metfor® (500 mg/tablet), the fabricated MET-PT, MET-PT-Al_2O_3, and MET-PT-NiO sensors were used. The potential readings were recorded for different concentrations of MTF samples, and the recovery percentage was calculated. The results were 99.12 ± 0.62, 99.57 ± 0.46, and 99.35 ± 0.60 for the above-mentioned sensors, respectively (Table 6).

Table 6

The results of the MTF determination in its dosage forms using MTF-PT, MTF-PT-Al_2O_3NP-modified, and MTF-PT-NiONP-modified sensors with respect to the previously reported results.

	MTF-PT conventional electrode			MTF-PT-Al_2O_3NP-modified electrode			MTF-PT-NiONP-modified electrode			Reference method [59]
Test sample	Found	% recovery	Test sample	Found	% recovery	Test sample	Found	%Recovery	Statistical analysis	6

5.98	99.66	10	10.00	100.00	10	9.96	99.60		5	4.97
99.40	8	7.96	99.75	8	7.94	99.25	4.3	4.25	98.83	6
5.94	99.00	6	5.96	99.33	4	3.98	99.50	4	4.00	100.00
4	3.93	98.25	3	2.98	99.33	3	2.99	99.67	3	2.99
99.67	2	1.96	98.00	2	1.98	99.00	2	2.00	100.00	
Mean ± SD	99.12 ± 0.62			99.57 ± 0.46			99.35 ± 0.60			99.25 ± 0.72
–										
n	6			6			6			6
–										
Variance	0.38			0.21			0.36			0.52
–										
%SE	0.25			0.19			0.24			0.29
–										
%RSD	0.63			0.46			0.60			0.74
–										
t-test	0.337 (2.228)*			0.922 (2.228)*			0.266 (2.228)*			

–				
F-test	1.37 (5.05)*	2.48 (5.05)*	1.44 (5.05)*	

*The tabulated values of "*t*-test" and "*F*-test" at confidence level $p = 0.05$.

4. Conclusion

The proposed potentiometric study was conducted by fabricating coated wire sensors enriched with aluminum oxide and nickel oxide nanoparticles. The potential readings of MTF-PT-Al_2O_3NP- and MTF-PT-NiONP-modified sensors were compared with the MTF-PT conventional sensor. For the quantification of diabetic metformin hydrochloride due to their sensitivity and selectivity, the created sensors proved to be effective and superior to the conventional sensors. In addition, the use of Al_2O_3NPs and NiONPs as electro-improved materials increased the sensitivity of the sensors and made it easier to identify the drug under investigation with a low limit and over a wide concentration range. The fabricated electrodes can be applied for the metformin routine analysis in pharmaceutical industries, research laboratories, and hospitals.

Acknowledgments

The authors extend their appreciation to the Researchers Supporting Project, King Saud University, for funding this work through Grant No. RSP2024R272.

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DETAILS

Subject:	Nitrates; Nickel oxides; Wire; Diabetes; Metal oxides; Electrodes; Nanoparticles; Enrichment; Aluminum; Nanomaterials; Nanotechnology; Cereals; Caustic soda; Correlation coefficients; Fourier transforms; Sodium; Pharmaceuticals; Electroactive materials; Sensors; Aluminum oxide; Metformin; Membranes; Nickel; Nanosensors
Location:	United States--US; Saudi Arabia
Editor:	Bishnu Regmi
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-12-01 (Received); 2024-03-14 (Revised); 2024-03-27 (Accepted); 2024-04-12 (Pub)

DOI:	https://doi.org/10.1155/2024/8354311
ProQuest document ID:	3042632891
Document URL:	https://www.proquest.com/scholarly-journals/exploiting-green-synthesized-metal-oxide/docview/3042632891/se-2?accountid=211160
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Last updated:	2024-04-22
Database:	Publicly Available Content Database

Urine Test Strip Quantitative Assay with a Smartphone Camera

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ProQuest document link

ABSTRACT (ENGLISH)

Urine test strips for urinalysis are a common diagnostic tool with minimal costs and are used in various situations including homecare and hospitalization. The coloration scaled by the naked eye is simple, but it is suitable for semiquantitative analysis only. In this paper, a colorimetric assay is developed based on a smartphone digital camera and urine test strips. Assays of pH, albumin, glucose, and lipase activity were performed as a tool for the diagnosis of aciduria, alkaluria, glycosuria, proteinuria, and leukocyturia. The RGB color channels were analyzed in the colorimetric assay, and the assay exerted good sensitivity, and all the particular diagnoses proved to be reliable. The limits of detection for glucose (0.11 mmol/L), albumin (0.15 g/L), and lipase (2.50 U/μL) were low enough to cover the expected physiological concentration, and the range for pH was also satisfactory. The urine test strips with a camera as an output detector proved applicability to spiked urine samples, and the results were also well in comparison to the standard assays which confirms the practical relevance of the presented findings.

FULL TEXT

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1. Introduction

Urine test strips are a common tool in clinical biochemistry that serves to diagnose various diseases, including renal

disease, liver disease, and some metabolic disorders. The analysis of urine for diagnostical purposes is frequently called a urinalysis. Proteins in urine, erythrocytes, specific gravity (urine osmolality), nitrites, leukocyte esterase, glucose, ketones, bilirubin, and pH are the typical markers covered by standard commercial sets. Many relevant articles have widely discussed the role and importance of urinalysis for diagnostic purposes [1–5].

The results achieved using urine test strips cannot fully replace the blood analysis. Some markers analyzed in the urine have less diagnostic importance than the same or similar markers in the blood. Glucose can be mentioned as an example. While the glucose level in the blood (glycemia) gradually increases, the glucose in the urine (glycosuria) occurs only after exceeding the renal glucose threshold. Though urinalysis has some limitations compared to the analysis of blood, it also has some significant advantages. In addition to urinary catheterization, urine collection is a noninvasive process that can be easily performed by the patient itself or by any healthcare provider. Standard urine test strips are also quite inexpensive; they can be used without any laboratory equipment or elaborative sample pretreatment, and they can be performed in the same way as point-of-care tests. Typical urine test strips are manufactured as qualitative or semiquantitative colorimetric sensors; however, the exact determination of marker concentration is not possible without specialized equipment.

Small digital cameras integrated into common electronic devices, such as smartphones and wearable technologies, are not primarily intended as a tool for laboratory analysis. However, analytical applications based on color density channel measurement and other principles have gained popularity, and many applications have been established [6–9]. The use of smartphone cameras appears to be a promising idea in the analyses [10–14]. This paper focuses on the development of a colorimetric urinalysis based on standard commercial test strips with quantification of coloration by a smartphone camera. This approach represents a novel way in analytical chemistry to improve the standard colorimetric tests designed not for instrumental analysis but for scaling by the naked eye. The urinalysis was purposely chosen as a test with a practical impact. It is expected that the use of the standard urinalysis tests in combination with a smartphone camera will provide accurate and more reproducible results suitable for practical use, making the assay more competitive to the standard laboratory methods but still useable, as a point-of-care test.

2. Materials and Methods

2.1. 3D-Printed Holder

The 3D-printed holder was made from black polyethylene terephthalate glycol with a 100 mm height and an internal tube diameter of 40 mm. Prusa Mini+ (Prusa Research; Prague, Czech Republic) printer was used. The printer setting was the following: string diameter of 1.75 mm, infill of s50%, printing layer height of 0.1 mm, nozzle temperature 230°C, and bed temperature of 90°C. The holder is shown in Figure 1.

[figure(s) omitted; refer to PDF]

2.2. Reagents

Glucose, o-phenylenediamine, glucose oxidase, human serum albumin, and lipase from porcine pancreas type VI-S were purchased from Sigma-Aldrich (Saint Louis, Missouri, United States) with activity 20,000 U per protein mg (activity 1 U is equal to hydrolysis of 1.0 microequivalent of fatty acid from a triglyceride in 1 hour at pH 7.7 at 37°C using olive oil). Citric acid, sodium hydroxide, ethanol, and formaldehyde were obtained from Penta (Prague, Czech Republic). The organic and inorganic reagents used in these experiments were of analytical grade. Deionized water was prepared using the Aqua Osmotic 02 device by Aqua Osmotic, Tisnov, Czech Republic.

2.3. Analyzed Samples

(i) Sodium citrate buffer 0.1 mol/L with a pH of 5 and 6, potassium phosphate buffer 0.1 mol/L with pH 7 and 8, and sodium phosphate buffer 0.2 mol/L with pH 9.0 were used as standard samples for the pH assay. The saline served as a blank. Urine samples were used untreated.

(ii) Glucose was solved in pH 7.4 and urine. The solutions served as samples for the glucose assay. Phosphate-buffered saline (pH 7.4) served as a blank.

(iii) The solution of human serum albumin at pH 7.4 and the solution of human serum albumin in urine were used as standard samples for the protein assay. Phosphate-buffered saline (pH 7.4) served as a blank.

(iv) The porcine pancreas was solved in phosphate-buffered saline (pH 7.4) and urine was used as an analyte,

substituting leukocytes. Phosphate buffered saline (pH 7.4) served as a blank.
(v) Urine from anonymized human volunteers was used for validation purposes.

2.4. Urine Test Strips Assay

Standard urine test strips (DekaPhan Leuco, Erba Lachema, Brno, Czech Republic) were used for the experiment (photograph in Figure 1). A test strip contained 10 squares each to test one biochemical marker (specific gravity, leucocytes, nitrite, pH, protein, glucose, ketones, urobilinogen, bilirubin, and blood/hemoglobin). The strip was placed on a white paper surface, covered with the hollow part of the 3D-printed holder, and a smartphone (Redmi Note 11 Pro, Xiaomi Inc., Haidian District, Beijing, China) was located on the upper of the holder in a way as shown in Figure 1. After that, a sample of 10 μl was applied per square and incubated for 60 seconds, respectively, 120 seconds when leukocytase activity was measured and the test strip was photographed. The smartphone camera was set to zoom 1×, automatic flash, and automatic white balance. The camera was focused on the spot where the detecting square was placed, and the picture was collected in an 8 bit jpeg format. Five different test strips were analyzed for every sample. The difference in color depth was calculated from the two photographs.

2.5. Measuring Color Depth

The value of color depth was measured by the software GIMP 2.10.34 (free and open-source software). The spot with the square where the analysis took place was analyzed in five randomly selected spots with a distance higher than 2 mm from the square´s edge. The color depths were measured for the red (R), green (G), and blue (B) channels. The final mean average color depth was determined. Because the 8 bit format of photographs in jpg format was acquired, the color channels can get a value between 0 and 255.

2.6. Standard Assays

The urine test strips and standard assays analyzed the same samples.
(i) The device H160 with the PHW47-SS ISFET electrode (Hach; Loveland, Colorado, United States) served to assay the pH of the samples.
(ii) Glucose was analyzed using glucose oxidase, horseradish peroxidase, and o-phenylenediamine by a spectroscopic assay using standard 1 cm plastic disposable cuvettes and an Evolution 201 spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts, United States) [15].
(iii) Proteins were analyzed using the Bradford spectroscopic assay [16]. The Bradford reagent kit (Sigma-Aldrich) was chosen for the purpose, and the assay was performed in compliance with the protocol provided by the manufacturer. The assay was performed using a standard spectroscopic assay using standard 1 cm plastic disposable cuvettes and a spectrophotometer Evolution 201.
(iv) The porcine pancreas was analyzed as a substance that mimics leukocytes and their leukocytes esterase. Indoxyl acetate served as a chromogenic substrate for an assay in standard 1 cm disposable cuvettes and the Evolution 201 spectrophotometer. Esterase activity can be easily measured by spectrophotometry using indoxyl esters [17]. In this article, the use of indoxyl acetate in the way previously described for cholinesterase and lipase assays was chosen [18–20].

2.7. Data Processing

In the camera-based colorimetric assay, the average color depth (five points randomly selected at a distance from the square side equal to 1/3 of the square side length) was calculated for a sample and a blank (matrix for sample solving). The difference in color depths was calculated from sample and blank assays: Δ Color depth = Color depth (blank) − Color depth (sample).
All samples were measured on a five-time repeat. The mean and standard deviation were calculated from repeated measurements. The limit of detection was determined from the calibration curves using the rule that it is equal to the point in the calibration that numerically corresponds to the triplicate of the blank assay signal (rule $S/N = 3$).

3. Results and Discussion

In this study, assays of four markers typical for urinalysis by a strip test were chosen. The pH assay represented an assay of an organic marker assay, the human serum albumin represented a protein marker, and the assay of lipase as a substitute for leukocytase esterase activity represented an enzymatic marker common in clinical biochemistry.

The pH resulted in the construction of the calibration. Examples of test strip colorations and the calibration curves are shown in Figure 2. The test strips changed colors from orange in acidic buffer pH 5 per light and dark yellow (pH 6 and pH 7) to green (pH 8) and finally blue (pH 9). The sensitivity of the pH assay was very low in the G channel while the R and B channels proved to be applicable. Both R and B channels were well correlated with the change in pH, as the R channel exerted a coefficient of determination of 0.998 and the B channel had a very similar coefficient of determination of 0.997. The difference in color depth had a higher dynamic range for the B channel than for the R channel which also had a lower sensitivity for the acidic buffers. The B channel appears to be optimal for practical use, and the pH based on R and B channels is sensitive enough to cover the physiological range of urine pH and serve to recognize pathologies connected with aciduria or alkaluria.

[figure(s) omitted; refer to PDF]

The glucose assay was performed for the calibration range of 1.4–55 mmol/L and phosphate-buffered saline served as a blank. Examples of cuts from test strips coloring in the presence of glucose and the calibration curve are depicted in Figure 3. The best sensitivity for these cuts from urine test strips was exerted by the assay in the R channel where the limit of detection of 0.11 mmol/L was reached for the glucose and the assay also had the highest dynamic range. The assay in the G channel had a lower dynamic range and limit of detection for glucose was 0.60 mmol/L. The worst result was observed in the calibration of glucose using the B channel where a limit of detection equal to 1.8 mmol/L for glucose was achieved and the dynamic range of color depths was the lowest one. The inverse proportionality of the calibration in the B channel is another notable fact. Nevertheless, calibrations in all three color channels were sensitive enough to provide limits of detection under the value of the renal threshold for glucose, which is around 10 mmol/L depending on the exact type of pathology [21, 22].

[figure(s) omitted; refer to PDF]

The human serum albumin solution was analyzed for a calibration range of 0.3 –5 g/L and phosphate-buffered saline served as a blank. The colors of the cuts from test strips as a result of the protein assays and calibration for human serum albumin are depicted in Figure 4. Limits of detection of 0.15 g/L for the R channel, 0.24 g/L for the G channel, and 0.22 g/L for the B channel were calculated from the calibration. The coloration of the zones for the proteinuria assay by the urine test strips came from yellow (no proteins) to green (maximal concentration of the analyzed protein). The color change was similar to the zones for the glycosuria assay, but while the glucose assay provided contrast color change, the assay of proteins led to a light green, and the dynamic range of the change in color depth was lower. While the change in color depth reached up to 200 for the glucose assay and R channel, the assay of proteinuria reached around 70 in the best R channel. The other channels exerted even lower sensitivity.

[figure(s) omitted; refer to PDF]

Porcine lipase which served as a surrogate for leukocytase esterase is not a common commercially well accessible and the test for leukocyturia was verified this way. Examples of the assay and calibration of the urine test strips for lipase are presented in Figure 5. The part of the urine test strip for leukocyte detection provided quite a weak color change from colorless to a bright violet. The weak coloration resulted in a low dynamic range of the change in color depth. The best response to the color change was observed in the R channel, which was equal to approximately 50; the G channel had the maximal color change around 45; and the B channel around 10. The limit of detection for the lipase assay was equal to 2.50 U/μL for the R channel, 3.40 U/μL for the G channel, and 24.2. for the B channel. The activity of lipase cannot be easily recalculated to the number of lymphocytes, but an activity of 50 U/μL respectively, 500 U per a standard sample 10 μL is roughly equal to 500 lymphocytes considering the color etalon provided by the test strip manufacturer.

[figure(s) omitted; refer to PDF]

The aforementioned assays were validated using intact urine samples (pH measurement) or spiked (glucose added in the glucose assay, human serum albumin added in the protein assay, and porcine lipase added in leukocyte assays). The B channel for the pH assay and the R channels for the remaining three assays were used for the analysis of photographs from the assays by urine test strips. The exact value of the measured parameter was determined from the calibrations above using urine test strips or by standard assays calibrated using the same

standards for the calibration. The results of the validations are summarized in Table 1. When comparing the data reached by the use of urine test strips and the potentiometry for pH and spectroscopy for glucose, human serum albumin, and lipase assay. In the comparison, there were no significant differences by ANOVA at the probability level of 0.05. The selected markers and their expected concentrations that can occur in the urine can be measured with urine test strips with a smartphone camera as a detector quite easily and provide data for diagnosis with similar accuracy as the standard methods.

Table 1

Validation of urine test strips with the digital camera as an output sensor for standard assays.

Marker	Urine test strip	Standard assay	Type of standard assay	Significant difference by ANOVA at p0.05
pH	6.36±0.19	6.41±0.21	Potentiometry	No
Glucose (mmol/L)	10.6±0.7	10.3±0.5	Spectroscopy	No
Human serum albumin (g/L)	2.61±0.31	2.56±0.23	Spectroscopy	No
Leukocyturia surrogated by lipase activity (U/μL)	53.7±4.3	56.2±3.9	Spectroscopy	No

The use of a combination of a smartphone camera and urine test strips, as presented here, falls within the scope of the idea of point-of-care urinalysis with a simple detector device. Instrumentation for point-of-care urinalysis tests has been extensively reviewed [23–26]. Several biosensor applications for particular markers in urine were also presented in recent journal articles [27–31].

4. Conclusions

Urinalysis by standard urine tests has limitations in the low reproducibility and limited quantification of the analyzed markers. The assay can, however, be improved by a colorimetric sensor that can even be a smartphone camera. Point-of-care tests based on this combination remain inexpensive and easy to perform but the subjective scaling of coloration by the human eye is replaced by the smartphone camera. Moreover, such an assay is quantitative and can provide results and support for precision diagnosis similar to standard laboratory methods. The urine test strips can be read by the naked eye as intended by the manufacturers; however, this method provides results highly dependent on individual abilities, sensitivities to distinguish color, and experience with such tests. The combination of smartphone and urine test strips reduces the mentioned drawbacks without raising additional costs, requiring sample processing, or introducing an elaborate assay procedure. The fact that a cheap smartphone camera integrated even into a common device is sufficient for the analysis makes the whole assay more competitive. The necessity to buy even a cheap analytical device can be a substantially limiting factor for the distribution of a point-of-care test. Because the assay needs only a common smartphone without any functions above standards, access to the improved method here is unlimited.

Acknowledgments

A long-term organization development plan Medical Issues of Weapons of Mass Destruction II (DZRO-FVZ22-ZHN II) by the Ministry of Defence of the Czech Republic is gratefully acknowledged. Mr. Vitezslav Vlcek is acknowledged for statistics of datasets.

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DETAILS

Subject:	Digital cameras; pH; Smartphones; Albumins; Pancreas; Proteinuria; Open source software; Biochemistry; Assaying; Calibration; Leukocytes; Urinalysis; Glucose; Urine; Density; Lipase; Colorimetry; Proteins
Business indexing term:	Subject: Smartphones
Location:	Czech Republic; United States--US
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024

Publication year:	2024
Publication date:	2024
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:	2024-01-03 (Received); 2024-02-26 (Revised); 2024-03-12 (Accepted); 2024-03-18 (Pub)
DOI:	https://doi.org/10.1155/2024/6004970
ProQuest document ID:	3020594021
Document URL:	https://www.proquest.com/scholarly-journals/urine-test-strip-quantitative-assay-with/docview/3020594021/se-2?accountid=211160

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Last updated:	2024-04-01
Database:	Publicly Available Content Database

A Simple and Rapid LC-MS/MS Method for the Quantification of Nirmatrelvir/Ritonavir in Plasma of

Patients with COVID-19

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ProQuest document link

ABSTRACT (ENGLISH)

The combined prescriptions of nirmatrelvir/ritonavir and other drugs are limited due to potential drug-drug interactions, so therapeutic drug monitoring (TDM) becomes particularly important. In this study, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established for determination of the nirmatrelvir/ritonavir in plasma of patients with COVID-19, providing technical and theoretical support for the TDM. Plasma samples were processed by protein precipitation using acetonitrile, and analytes were separated on an Agilent Poroshell 120 SB-C18 (2.1 × 75 mm, 2.7 μm) column at 35°C. Acetonitrile and 0.1% formic acid in water (52 : 48) were utilized as the mobile phases at a flow rate of 0.3 mL/min. In the multiple reaction monitoring (MRM) mode, nirmatrelvir and ritonavir were monitored using precursor/product ions: *m*/*z* 500.2/110.1 and 721.3/296.1, respectively, with selinexor as the internal standard. The linear range of both analytes was 2.0 ng/mL to 5000 ng/mL with good inter- and intraday precision and accuracy, and the recovery was 92.0%–107% for nirmatrelvir and 85.7%–106% for ritonavir. Finally, this method was successfully applied to monitor the exposure levels of nirmatrelvir/ritonavir in plasma samples from hemodialysis patients.

FULL TEXT

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1. Introduction

Coronavirus disease 2019 (COVID-19) is a serious infectious disease caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), which has caused a large-scale epidemic worldwide. Multiple generations of variants have occurred during the epidemic, and the fifth generation variant, the Omicron variant, is currently the most common [1]. It has been reported that although the viral genome of SARS-CoV-2 is frequently mutated, nirmatrelvir/ritonavir shows an effective antiviral effect against recent coronavirus mutants [2]. The oral form nirmatrelvir/ritonavir was an effective new drug against COVID-19, compared with remdesivir and molnupiravir [3, 4]. The results of clinical studies conducted on COVID-19 patients with underlying disease factors showed that the risk of hospitalization or death caused by administration of nirmatrelvir/ritonavir was reduced by 89% within 3 d of symptom onset and by 88% within 5 d [5–7].

Ritonavir has been used as a pharmacokinetic enhancer for HIV protease inhibitors, such as darunavir and lopinavir, which are metabolized by cytochrome P450 3A4 (CYP3A4) [7]. Nirmatrelvir is a peptidomimetic inhibitor of SARS-CoV-2 main protease (also referred to as 3C-like protease, 3CLpro). It can prevent virus replication by inhibiting SARS-CoV-2 main protease to make it unable to process multiprotein precursors [8]. CYP3A4 plays an important role in its metabolism, indicating that the combination with ritonavir, a potent CYP3A4 inhibitor, can increase the serum concentration of nirmatrelvir, thereby increasing the therapeutic concentration [5].

However, its potential for enzyme-inhibiting drug-drug interactions should be carefully considered in combination with other prescribed medications. Among them, CYP3A is mainly involved in the metabolism of most

immunosuppressive drugs (ISDs), with narrow treatment scopes [9]. This makes it particularly challenging to administrate the combinations of nirmatrelvir/ritonavir and ISDs. As there is effective inhibition of CYP3A by ritonavir, the exposure of ISD will be significantly increased, which will increase the side effects of the drug. Although toxicity can be prevented by reducing the dose of ISDs, the treatment should be prudent if close monitoring, including TDM, is not feasible [10, 11]. Some recommendations were made by the investigators that tacrolimus should be discontinued or that minimal doses should be administered on the first day and that cyclosporine should be reduced by 80% during antiviral therapy [12].

Hiremath et al. [13] reported that patients with low immune function had higher morbidity and mortality of COVID-19, for example, the patients with advanced chronic kidney disease (CKD) and patients with kidney failure. A study summarized the trial registry which reported that 45% of COVID-19 trials excluded CKD patients [14]. Although some studies have proved the applicability of nirmatrelvir/ritonavir in COVID-19 patients in low dose [15, 16], it is still necessary to pay close attention to the blood concentration of CKD patients. At present, the quantitative methods for ritonavir in human plasma are relatively common, but there are few methods for quantifying nirmatrelvir/ritonavir. In this regard, the latest research is that Martens-Lobenhoffer et al. [17] developed and validated a LC-MS/MS method to simultaneously quantify the content of nirmatrelvir/ritonavir in patients with COVID-19. However, the running time of this method was long, and the total cycle time including balancing was 13 min, which hindered the detection of large batch samples. Before this, there were literature reports [18, 19] about quantifying nirmatrelvir/ritonavir in biological matrix by LC-MS/MS, but these methods were usually narrow in the linear range, which was 10.0 ng/mL to 1000 ng/mL for ritonavir, 40.0 ng/mL to 4000 ng/mL, or 50.0 ng/mL to 500.0 ng/mL for nirmatrelvir.

To better carry out the TDM of nirmatrelvir/ritonavir and ensure the safety and efficacy of medication, a rapid, reliable method with sufficient linear range is urgently needed to simultaneously quantify nirmatrelvir and ritonavir in patients' plasma. Thus, this study presented the development and validation of a fast, sensitive, and reliable LC-MS/MS method with 3.65 min run time for the quantification of nirmatrelvir/ritonavir in human plasma.

2. Materials and Methods

2.1. Chemicals and Reagents

Nirmatrelvir (purity > 98%, lot: N17HS201609) and ritonavir (purity > 99%, lot: H03D9Z76283) were purchased from Shanghai Sunny Biotech Co., Ltd (Shanghai, China). Selinexor (internal standard, lot: C13J11L117641, purity > 99%) was supplied by Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Merck Company, Darmstadt, Germany). HPLC-grade formic acid was from McLean Bio-Tech Co., Ltd (McLean, Shanghai, China). Distilled water was purchased from Shenzhen Watsons Distilled Water Co., Ltd (Shenzhen, China).

2.2. LC/MS-MS Instrumentation

The experiments, which included method development and application, were carried out on the system of Agilent 1290 UHPLC in series with Agilent 6460A mass spectrometer, and the system also included online degasser, binary pump, automatic sampler, and column oven. Agilent MassHunter data processing software (version 6.00) was used to collect and analyze the data.

2.3. Liquid Chromatographic Conditions

The column in this experiment was Agilent Poroshell 120 SB-C18 (2.1 × 75 mm, 2.7 μm, Agilent, USA) with the temperature kept at 35°C. The mobile phase was made up of 48% A phase (water with 0.2% formic acid) and 52% B phase (ACN) in isocratic elution program because the symmetrical peaks and appropriate retention times were obtained for two analytes. The total running time was 3.65 min. The flow rate was 0.3 mL/min.

2.4. Mass Spectrometry Conditions

The analytes were ionized in a positive ionization mode using an ESI source with a capillary voltage of 4500 V. In the MRM mode, all data gathering was done, and Figure 1 showed the product ions and fragmentary structures of nirmatrelvir and ritonavir. The drying gas, sheath gas, and sprayer gas are all nitrogen. The nebulizer pressure was 310.275 kPa (45 psi). The drying gas was moved at a rate of 10 L/min while being heated to 320°C. The sheath gas's temperature was fixed at 300°C, and the flow rate was 12 L/min. The collision gas was a high-quality nitrogen gas,

set at 0.2 MPa. The mass spectrometry parameters for nirmatrelvir, ritonavir, and IS are presented in Table 1.
[figure(s) omitted; refer to PDF]

Table 1

Mass spectrometry parameters of analytes and IS.

Analytes	MRM transition *m*/*z* (*Q*1*Q*3)	Fragmentor (*V*)	CE (eV)
Nirmatrelvir	500.2110.1	125	18
Ritonavir	721.3296.1	140	17
Selinexor (IS)	444.1282.0	120	19

2.5. Preparation of Standard Solution and Quality Control Sample

The stock solutions with concentrations of 1000 μg/mL, 100.0 μg/mL, and 10.0 μg/mL were obtained by accurately weighing 2.0 mg of nirmatrelvir and ritonavir, respectively, and dissolved in MeOH. These stock solutions were transferred into 1.5 mL tubes and frozen at −80°C. The working solutions were prepared by diluting these stock solutions with MeOH, and then working solutions were combined and diluted with blank plasma to obtain calibration standards at the following concentrations: 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, 1000, 2000, and 5000 ng/mL. Quality control (QC) samples of both analytes were also prepared in the same way separately, with concentrations of 5.0, 1000, and 2000 ng/mL (low-middle-high). At −80°C, 1.0 mg/mL of IS stock solution was stored and was diluted freshly with MeOH to obtain 20.0 ng/mL IS solution when needed.

2.6. Sample Pretreatment

A simple protein precipitation method was used for the pretreatment of samples. A 1.5 mL Eppendorf tube was filled with 100 μL of the plasma sample and 200 μL of ACN (containing 20 ng/mL IS), and then the mixture was vortexed for 2 min. After centrifugation at 14 300×g for 10 min, 100 μL of the supernatant was diluted with 100 μL of the initial mobile phase, and then we transferred the diluted solution to HPLC vials with glass inserts, and 5 μL was injected into the system.

2.7. Methodological Study and Data Analysis

The developed method was validated according to the current bioanalytical guidelines from the U.S. Food and Drug Administration (FDA) and China Pharmacopoeia (version 2020). The following items were validated: specificity, linearity, interday and intraday precision and accuracy, linearity, matrix effect and recovery, carry-over, dilution effect, and stability [20, 21].

Specificity was verified using blank matrix, IS spiked, LLOQ, and real samples from 6 different lots.

The linearity of the analytes in three lots was assessed on different days (at least two days), and at least three calibration curves were evaluated in each lot. The low point of standard curve was the LLOQ.

Five QC in three concentration levels and LLOQ samples were prepared in parallel and measured on different days (at least two days) to obtain the interday and intraday precision and accuracy. The precision shows the stability of the instrument, and the accuracy proves the stability of analytes which was calculated using the concentrations of analytes.

The recovery was the percentage of the peak area of spiked samples versus spiked postextraction samples in the same concentration, and the matrix effect was defined as the ratio of the peak area in spiked postextraction samples to the peak area in solvent-substituted samples in the same concentration.

The carry-over was evaluated by injecting the blank sample, and after injection, the highest concentration of the calibration standard was measured.

The dilution effect was assessed by diluting the samples with a concentration higher than the upper limit of quantitation (ULOQ) into the calibration range and comparing the measured concentration with the nominal

concentration.

The stability, including room temperature stability (4 h), standing stability (0 h, 12 h, 24 h), three freeze-thaw cycles, and long-term stability (1 month at −80°C), was evaluated using QC samples at three concentration levels.

2.8. Patient Enrollment and Sample Collection

The research protocol was reviewed and approved by the Ethics Committee of Shanghai Changzheng Hospital, and informed consent was signed by every patient. The hemodialysis patients infected with SARS-CoV-2 were enrolled and treated with nirmatrelvir/ritonavir. The treatment regimen was nirmatrelvir/ritonavir, 150 mg/100 mg, q12 h, D1; nirmatrelvir/ritonavir, 150 mg/100 mg, qd, D2-D5. Blood samples were collected at trough point and 3 h and 12 h after drug administration. To assess the influence of dialysis to the drug exposure, this study further collected the sample in the dialysis procedure. Each blood sample was collected in the EDTA-3K tube, and the plasma was transferred to cryopreservation in −80°C after centrifuge at 4500×g, room temperature for 10 min. Sample preparation and processing were accomplished within 1 h.

3. Results and Discussion

3.1. LC/MS-MS Optimization

In this experiment, the effects of nirmatrelvir/ritonavir in the positive ionization mode and negative ionization mode were compared to find a more suitable detection method, and it was concluded that the response value of the positive ionization mode was higher than that of the negative ionization mode. In the selection of chromatographic columns, different columns (ZORBAX SB-C8, Agilent Poroshell 120 SB-C18, Atlantis T3-C18) were tested. Nirmatrelvir and ritonavir were found to have better peak shapes, responses, and retention times on an Agilent Poroshell 120 SB18 column. The additive ammonium acetate was found to suppress ionization, while formic acid provided higher ionization and therefore promoted higher desirable responses.

3.2. Sample Extraction

Plasma samples were preprocessed using protein precipitator methanol and acetonitrile and so on in different ratios (1 : 2, 1 : 3). The acetonitrile in 1 : 2 ratio resulted in higher extraction recovery (70%–80%) although the matrix effect was slightly less steady than 1 : 3 ratio (RSD % less than 7% vs. RSD % less than <10%). Besides, the retention time was increased by 1 min too compared with other precipitators. The supernatant after protein precipitation was further diluted with diluents MeOH, ACN, mobile phase, and so on in different volume ratios. Dilution of the supernatant with an equal volume of the initial mobile phase showed highest improvements in responses (90%–100%). Other pretreatment methods were not evaluated as satisfactory results for recovery and matrix effects had been obtained. The final sample pretreatment method was determined as 1 : 2 protein precipitation using two-fold volume of ACN, and the supernatant was diluted with initial mobile (1 : 1).

3.3. Method Validation

3.3.1. Specificity

Blank plasma matrices from six different individuals were selected to examine the specificity of the method. Finally, the retention times of nirmatrelvir, ritonavir, and the IS were 1.28 min, 2.62 min, and 1.61 min, respectively. The results showed that the endogenous components interfering with the analytes and IS were not presented in the six different blank plasma matrices, indicating good specificity of the method. The typical chromatogram is shown in Figure 2.

[figure(s) omitted; refer to PDF]

3.3.2. Linearity of Calibration Curves and Lower Limited of Quantification (LLOQ)

The calibration curves of both analytes were regressed using the ratio of peak areas of analyte/IS versus the nominal concentration under the 1/x2 weighing factor, and the equation of the calibration curve of nirmatrelvir was $y = 37.5581 * x + 8.6239$ ($r = 0.9985$) and that of ritonavir was $y = 30.3743 * x - 0.0019$ ($r = 0.9956$). The results showed that the linear relationship of analytes was good in the range of 2.0–5000 ng/mL, and the LLOQs of the method were verified to be 2.0 ng/mL. The deviations of back-calculation of every calibration standard were within ±15%. The regression parameters of the calibration curves are shown in Table 2.

Table 2

The regression parameters of calibration curves of analytes.

Analytes	Regression type	Linear range (ng/mL)	Regression equations	*r*
Nirmatrelvir	Linearity	2.0–5000	$Y=37.5581*x+8.6239$	0.9985
Ritonavir	Linearity	2.0–5000	$Y=30.3743*x-0.0019$	0.9956

3.3.3. Inter- and Intraday Precision and Accuracy

The inter and intraday accuracy and precision were investigated in three batches in three concentration levels along with the linearity assessment. The data showed that the inter- and intra-accuracy (RE %) of nirmatrelvir and ritonavir was within ±15%, and inter- and intraprecision (RSD %) was less than 15%. The data met the requirements of pharmacopoeia, as shown in Table 3.

Table 3

Inter- and intraday precision and accuracy of analytes (*n*=5).

Analytes	Nominal concentration (ng/mL)	Interday measured concentration (ng/mL)±SD	RSD (%)	RE (%)	Intraday measured concentration (ng/mL)±SD	RSD (%)	RE (%)
Nirmatrelvir	5	4.8±0.4	7.9	−3.9	5.0±2.2	9.9	0.1
1000	983.3±89.1	9.1	−1.7	982.3±78.0	7.9	−1.8	2000
2050±105.6	5.2	2.5	2088±104.4	5.0	4.4		
Ritonavir	5	4.8±0.7	13.6	4.1	5.1±0.7	13.4	1.5
1000	1031±118.7	11.5	3.1	1078±18.1	1.7	7.8	2000

3.3.4. Recovery and Matrix Effect

The extraction recovery and matrix effect were performed using QC samples at low, middle, and high concentrations of nirmatrelvir and ritonavir (5.0, 1000, and 2000 ng/mL), in three replicates. It was concluded that the recovery and matrix effect of nirmatrelvir were in the range of 92.0%–107%, 87.1%–97.8% and those of ritonavir were 85.7%–106%, 87.8%–112%. The RSD % of recovery and matrix effect was 1.86%–6.59%, 1.58%–4.23% and that of ritonavir was 7.51%–8.59%, 6.49%–9.90% which were less than 15% and proved that the pretreatment method was feasible. The relevant data are presented in Table 4.

Table 4

Recovery and matrix effect of analytes.

Analytes	Nominal concentration (ng/mL)	Recovery		Matrix effect	
Mean (%)±SD	RSD (%)	Mean (%)±SD	RSD (%)	Nirmatrelvir	5
98.7±6.5	6.6	87.1±3.7	4.2	1000	92.0±4.4
4.8	97.8±1.6	1.6	2000	106.7±2.0	1.9
87.8±3.5	3.9	-			
Ritonavir	5	105.8±8.0	7.5	91.7±9.1	9.9
1000	89.3±7.7	8.6	87.8±5.8	6.6	2000

3.3.5. Stability

The QC samples with low, middle, and high concentrations (5.0 ng/mL, 1000 ng/mL, 2000 ng/mL) were analyzed immediately after pretreatment or storage under the given conditions to investigate their stability. The results showed that after freeze-thaw cycle treatment for three times, stored at room temperature for 4 h, placed in the automatic sampler for 0 h, 12 h, 24 h after pretreatment, stored at −80°C for 90 d, the RE % and RSD % were within the specified range, proving an acceptable stability. The stability data are shown in Tables 5 and 6.

Table 5

Results of stability in the autosampler of analytes (*n*=3).

Analytes	Nominal concentration (ng/mL)	0 h			12 h			24 h		
Mean (%)±SD	RSD (%)	RE (%)	Mean (%)±SD	RSD (%)	RE (%)	Mean (%)±SD	RSD (%)	RE (%)	Nirmatrelvir	5
5.1±0.2	4.5	2.0	4.38±0.21	4.8	−12.5	5.4±0.3	4.9	7.2	1000	1077±32.4
3.0	7.7	908.4±65.5	7.2	−9.2	982.2±29.9	3.0	−1.8	2000	2140±78.1	3.7
7.0	2004±15.6	0.8	0.2	2054±71.3	3.5	2.7	.			

Ritonavir	5	4.6±0.2	3.6	−8.0	4.51±0.16	3.4	−9.8	4.5±0.2	3.7	−9.1
1000	1110±25.9	2.3	11.0	1079±73.1	6.8	7.9	897.5±65.2	7.3	10.3	2000

Table 6

Results of benchtop stability, three frozen-thaw cycles stability, and long-term stability of analytes (*n*=3).

Analytes	Nominal concentration (ng/mL)	Room temperature 4 h			Three frozen-thaw cycles			Long-term stability		
Mean (%)±SD	RSD (%)	RE (%)	Mean (%)±SD	RSD (%)	RE (%)	Mean (%)±SD	RSD (%)	RE (%)	Nirmatrelvir	5
4.6±0.3	6.9	−7.5	4.9±0.6	11.2	−1.2	5.4±0.3	4.8	7.2	1000	908.4±65.5
7.2	−9.2	983.1±67.3	6.8	−1.7	973.4±26.2	2.7	−2.7	2000	1851±26.0	1.4
−7.5	2053±110.3	5.4	2.7	2055±31.9	1.6	2.8	·			
Ritonavir	5	4.5±0.2	3.4	−9.8	4.8±0.6	12.4	−5.0	4.5±0.2	3.7	−9.1
1000	1076±32.0	3.0	7.6	1076±76.6	7.1	7.6	951.7±53.8	5.7	−4.8	2000

3.3.6. Dilution Effect

A spiked plasma sample of 10000 ng/mL was prepared from the stock solutions and then diluted 20-fold with blank plasma. After repeating five times, the results showed that the precision (RSD%, 4.42% for nirmatrelvir and 4.80% for ritonavir) and accuracy (RE, −4.11% for nirmatrelvir and 2.13% for ritonavir) met the standards of the Pharmacopoeia.

3.3.7. Carry Over

Carry over was evaluated by measuring the peak area of the blank sample after the ULOQ of the calibration curve for three cycles. The results showed that the peak areas of analytes in the blank sample after detecting the ULOQ sample of nirmatrelvir/ritonavir were less than to 20% of the peak areas of analytes in LLOQ (2.0 ng/mL), which met the requirements.

3.4. Method Application

Totally four hemodialysis patients were enrolled in this study, and all the patients were treated with nirmatrelvir/ritonavir in the same prescription, and the six plasma samples were collected for every patient at trough, 3 h and 12 h (collecting point 1, 2 and 3) after drug administration, and the other three collecting points were designated in the dialysis process: 0 h, 2 h, 4 h (collecting point 4, 5 and 6), and the dialysis procedure begun at 19 h and finished at 23 h after drug administration. The exposure levels of nirmatrelvir and ritonavir were determined using this method, and the results (Figure 3) showed huge exposure differences between patients (patient 1 compared with other three patients) and patient 3 may suffer from an absorption delay as collecting point 3 (12 h after administration) exhibits a higher exposure level compared with other collecting point. The study reported that within 30 days, nirmatrelvir/ritonavir can significantly decrease the risk of hospital admission or death of a positive outpatient SARS-CoV-2 test [22, 23]. Except for ISDs, many other drugs may have drug-drug interactions with nirmatrelvir/ritonavir, for example, the antiepileptic drugs [24], conazole drugs [25], and so on [26], so the coprescription of nirmatrelvir/ritonavir with other drugs necessitates the close monitoring of their exposure. In renal impairment patients, the exposure of nirmatrelvir increased along with the increasing renal impairment [27], and renal impairment patient may require a dose reduction. In the dialysis patients, nirmatrelvir/ritonavir in this regimen was well tolerated [28]. In our study, the nirmatrelvir/ritonavir exposure levels in plasma could be rapidly eliminated by hemodialysis (collecting points 4, 5, and 6), which was reported by Lingscheid et al. [29]. Nirmatrelvir/ritonavir will not accumulate in the dialysis patients, and dialysis maybe helpful in the rescue of overdose.

[figure(s) omitted; refer to PDF]

4. Conclusion

Here, this study presents a novel, sensitive, and fully validated LC-MS/MS method for the simultaneous quantification of nirmatrelvir and ritonavir in human plasma. The linear ranges were established at 2.0–5000 ng/mL for both nirmatrelvir and ritonavir with only 100 μL plasma sample required for analysis. The sample pretreatment was completed by a simple protein precipitation method, with the running time optimized at 3.65 min. Hemodialysis can rapidly remove nirmatrelvir and ritonavir, which may benefit for the overexposure rescue.

Disclosure

A preprint has previously been published [30].

Authors' Contributions

Xiujing Zhu conducted data curation, methodology, and writing of the original draft. Lin Li conducted conceptualization, writing of the review, and editing. Bing Dai conducted conceptualization, writing of the review, and editing. Zhijun Liu conducted investigation, writing of the original draft, and visualization. Zhipeng Wang performed formal analysis, handled software, writing of the review, editing, and conceptualization. Lili Cui performed methodology and validation. Shouhong Gao conducted writing of the review, editing, and supervision. Wansheng Chen conducted supervision and project administration. Xia Tao performed project administration, conceptualization, and funding acquisition. Deduo Xu collected resources and performed project administration and funding acquisition. The authors Xiujing Zhu, Lin Li and Bing Dai have contributed equally to this work.

Acknowledgments

This work was supported by the Shanghai Yiyuanxinxing Youth Medical Talents Development Program (SHWSRS(2020)_087), Clinical Research Project of Shanghai Municipal Health Commission (202040238), Clinical Medical Technology Incubation Project of Shanghai Changzheng Hospital (no. YLJS2021A-03), and National Natural Science Foundation of China (no. 82274173).

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10.2139/ssrn.4422218, 2023.

DETAILS

Subject:	Mass spectrometry; Acetonitrile; Formic acid; Plasma; Accuracy; Analytical chemistry; Severe acute respiratory syndrome coronavirus 2; Liquid chromatography; Calibration; Quality control; Scientific imaging; Methods; Metabolism; Hemodialysis; Monitoring; Drug dosages; COVID-19
Business indexing term:	Subject: Quality control
Location:	United States--US; China
Company / organization:	Name: Food &Drug Administration--FDA; NAICS: 926150
Editor:	Bishnu Regmi
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-08-04 (Received); 2024-01-11 (Revised); 2024-02-08 (Accepted); 2024-03-06 (Pub)
DOI:	https://doi.org/10.1155/2024/6139928
ProQuest document ID:	2958094139

Document URL:	https://www.proquest.com/scholarly-journals/simple-rapid-lc-ms-method-quantification/docview/2958094139/se-2?accountid=211160
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Last updated:	2024-03-18
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Development of a Novel Multiplex PCR Method for the Rapid Detection of SARS-CoV-2, Influenza A Virus, and Influenza B Virus

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ABSTRACT (ENGLISH)

Objective. A sensitive and specific multiplex fluorescence rapid detection method was established for simultaneous detection of SARS-CoV-2, influenza A virus, and influenza B virus in a self-made device within 30 min, with a minimum detection limit of 200 copies/mL. *Methods*. Based on the genome sequences of SARS-CoV-2, influenza A virus (FluA), and influenza B virus (FluB) with reference to the Chinese Center for Disease Control and Prevention and related literature, specific primers were designed, and a multiplex fluorescent PCR system was established. The simultaneous and rapid detection of SARS-CoV-2, FluA, and FluB was achieved by optimizing the concentrations of Taq DNA polymerase as well as primers, probes, and Mg^{2+}. The minimum detection limits of the nucleic acid rapid detection system for SARS-CoV-2, FluA, and FluB were evaluated. *Results*. By optimizing the amplification system, the N enzyme with the best amplification performance was selected, and the optimal concentration of Mg^{2+} in the multiamplification system was 3 mmol/L; the final concentrations of SARS-CoV-2 NP probe and primer were 0.15 μmol/L and 0.2 μmol/L, respectively; the final concentrations of SARS-CoV-2 ORF probe and primer were both 0.15 μmol/L; the final concentrations of FluA probe and primer were 0.2 μmol/L and 0.3 μmol/L, respectively; the final concentrations of FluB probe and primer were 0.15 μmol/L and 0.25 μmol/L, respectively. *Conclusion*. A multiplex real-time quantitative fluorescence RT-PCR system for three respiratory viruses of SARS-CoV-2, FluA, and FluB was established with a high amplification efficiency and sensitivity reaching 200 copies/mL for all samples. Combined with the automated microfluidic nucleic acid detection system, the system can achieve rapid detection in 30 minutes.

FULL TEXT

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1. Background

The coronavirus disease 2019 (COVID-19) is an emerging acute respiratory infectious disease that has now evolved into a major global public health event. It has been demonstrated that early detection, reporting, and isolation of the disease can effectively contain the spread and dissemination of coronavirus [1–3]. During winter, however, it becomes more difficult to diagnose COVID-19 with increasing transmission of other respiratory viruses with similar symptoms.

COVID-19 and influenza are both respiratory infectious diseases, which can be transmitted through droplets and contact, and the early symptoms of both are similar and difficult to distinguish, such as fever, dry cough, and sore throat [4–6].

COVID-19 is caused by a type B coronavirus (SARS-CoV-2) with a genome of around 30 kb [7–9], which is a single-stranded RNA (+) virus belonging to beta-coronavirus and is capable of infecting both the avian and human species. Influenza is caused by RNA viruses of the *Orthomyxoviridae* family with genomes of about 14 kb, including influenza A (FluA), influenza B (FluB), and influenza C (FluC) viruses [9, 10]. Influenza A and B viruses may cause regional or even large-scale epidemics. Influenza viruses and SARS-CoV-2 can invade the epithelial cells of the upper respiratory tract, and virions are spread by large droplets produced by infected individuals when they cough and sneeze, which leads to the invasion of the epithelial cells of the upper respiratory tract [11, 12].

The Diagnosis and Treatment Protocol of COVID-19 (Trial Version 8) requires that the suspected COVID-19 cases should be diagnosed by methods including rapid antigen testing and multiplex PCR nucleic acid testing to distinguish from influenza virus infection. In addition, the combined assay for SARS-CoV-2, FluA, and FluB is able to detect coinfections. A study involving 93 cases found that 50% of SARS-CoV-2 infections were coinfected with FluA/B, which may lead to earlier organ damage in patients with critical conditions [2]. Concurrent tests for COVID-19, FluA, and FluB not only reduce the number of tests required for patients but also allow a timely clinical treatment plan for coinfected patients.

Differential diagnosis of SARS-CoV-2 and influenza viruses will be helpful in establishing appropriate strategies for public health and patient management, especially in the diagnosis of suspected cases, critical cases, and in the identification of potential outbreak risks. Adding influenza detection to COVID-19 assays can effectively shorten the test time and improve the efficiency of available equipment, personnel, and reagents, which is cost-effective for the containment of the COVID-19 pandemic. A rapid test for SARS-CoV-2, FluA, and FluB is needed during the prevalent seasons of respiratory viruses to control the pandemic and allow timely diagnosis and treatment for patients.

The most cost-effective preventive and control measure in the face of various emerging infectious diseases is to establish rapid and accurate nucleic acid molecular diagnostic methods, which are based on fully automated and integrated molecular diagnostic systems. The fully automated and integrated molecular diagnostic system can automatically complete the entire process of testing, including sample lysis, nucleic acid extraction, nucleic acid rinsing, nucleic acid elution, gene amplification (PCR), and real-time fluorescence quantitative detection, and can realize the “samples-in, result-out” [13].

The integrated molecular diagnostic system has several advantages over the common PCR method: it does not require a nucleic acid extractor or PCR instrument to be used in conjunction with the test, but only one integrated instrument. Compared with the ordinary PCR method, the integrated molecular diagnostic system has multiple advantages: it is not necessary to match the equipment such as nucleic acid extraction instruments, PCR instruments, and other equipment, and only one integrated instrument can be detected; the operator simply needs to add the sample to the kit and insert it into the instrument for testing, without requiring any additional steps during the testing process, thereby optimizing time and effort efficiency; fully closed automated experimental process can avoid sample cross-contamination and environmental pollution to maximize the protection of the operator’s safety.

Nowadays, many organizations are actively carrying out the development and research of fully automated and integrated molecular diagnostic systems [14–19].

In order to achieve rapid nucleic acid detection of SARS-CoV-2, FluA, and FluB, we designed a rapid multiplex real-time fluorescence PCR (RT-PCR) assay based on our self-made prototype Fully Automated Nucleic Acid Amplification Testing System (FANAT-1) for simultaneous detection of SARS-CoV-2, FluA, and FluB.

2. Materials and Methods

2.1. Materials

2.1.1. Samples

(1) Nucleic Acid Samples. Nucleic acid samples include SARS-CoV-2, FluA, FluB, and Psrp Synthetic RNA (Sangon Biotech).

(2) Clinical Samples. Clinical samples include SARS-CoV-2, H1N1, H3N2, H7N9, H5N1, H1N1 (2009) of FluA, the Victoria lineage, and Yamagata lineage of FluB; all clinical samples or viral cultures of different subtypes were obtained from China-Japan Friendship Hospital.

2.1.2. Instruments and Reagents

This study adopted the prototype Fully Automated Nucleic Acid Amplification Testing System (FANAT-1), as well as primers and probes (Sangon Biotech) and nucleic acid extraction kit (QIAGEN). The basic PCR system contains High-Affinity HotStart Taq and TIANSeq M-MLV (defined as N enzyme, TIANGEN Biotech, Cat nos. ET108 and NG212, separately), $MgCl_2$ (25 mM) (Sangon Biotech, Cat no. B601193), One-Step PrimeScript III (defined as A enzyme, Takara Biotech, Cat no. RR601A), Anstart One-Step RT-PCR Mix (heat-labile UDG) (defined as E enzyme, Fapon Biotech, Cat no. MD013P), and 5 × Neoscript RT Premix-UNG (Probe qRT-PCR) (DG) (defined as F enzyme, Biori Biotech, Cat no. FM5254).

2.2. Methods

2.2.1. Design of Primers and Probes

The conserved sequences of the SARS-CoV-2 N gene, ORF1ab gene, FluA M gene, and FluB NS gene were selected as amplification targets, and specific primers and fluorescent probes were designed to detect the sample RNA through the change of fluorescent signals (Table 1).

Table 1

Primers, probe-target genes, and sequence information.

Virus	Primer and probe	Sequence	Modification
SARS-CoV-2[a]	CoV-ORF-F	CCCTGTGGGTTTTACACTTAA	
CoV-ORF-P	CCGTCTGCGGTATGTGGAAAGGTTATGG	5′Texas RED, 3′BHQ2	CoV-ORF-R
ACGATTGTGCATCAGCTGA		CoV-NP-F	GGGGAACTTCTCCTGCTAGAAT
	CoV-NP-P	TTGCTGCTGCTTGACAGATT	5′FAM, 3′BHQ1
CoV-NP-R	CAGACATTTTGCTCTCAAGCTG		

FluA[b]	FluA-F	TAAAGACAAGACCAATCCTGTCACC	
FluA-P	ACGCTCACCGTGCCCAGTGAGCGA	5′CY5, 3′BHQ2	FluA-R
TCCCATTTAGGGCATTTTGGACAAAGC		-	
FluB[c]	FluB-F	AAAGATGGCCATCGGATCCTC	
FluB-P	AAAGCCAATTCGAGCAGCTGAAACTG	5′CY5.5, 3′BHQ2	FluB-R
GCTCTTGACCAAATTGGGAT		-	
Psrp[d]	Psrp-F	GTCCCTTCATCGTCGCTG	
Psrp-P	CACCGTTGCTGTTTTCCTTATCGGTTACGC	5′HEX, 3′BHQ1	Psrp-R

[a]The primer sequences are from “technical guidelines for laboratory testing of novel coronavirus pneumonia” (https://www.chinacdc.cn/jkzt/crb/zl/szkb_11803/jszl_11815/202003/W020200309540843062947.pdf). [b]The primer sequences have been modified from “National Technical Guidelines for Influenza Surveillance (2017 edition),” (https://ivdc.chinacdc.cn/cnic/zyzx/jcfa/201709/P020170930331067634607.pdf). [c]The primer sequences have been modified from “Mokkapati, Anupama., Brown, Bradley., Jones, Robert. 2019, methods of detecting influenza, United States, CEPHEID (Sunnyvale, CA, US), 10480036,” (https://www.freepatentsonline.com/10480036.html). [d]The primer sequences have been designed based on Arabidopsis genome sequences.

2.2.2. Establishment of PCR System

A conventional PCR procedure and a rapid amplification procedure were set up in the experiment for the amplification of the SARS-CoV-2 N gene and FluA M gene to evaluate the effects of enzymes from different manufacturers on the amplification results and to select the best Taq DNA polymerase for the rapid amplification system.

(1) Reagent preparation: four systems of reagent configurations were carried out according to Table 2 with the reaction system of 50 μL.

(2) Amplification template: it includes SARS-CoV-2, FluA, and FluB synthetic RNA.

(3) The amplification reagents were prepared and tested separately on the prototype Fully Automated Nucleic Acid Amplification Testing System (PFANAT-1), with the number of parallel tests being $N=3$ for each condition before calculating the average Ct values and selecting the best rapid amplification system. The amplification procedure was configured according to Table 3. Amplification procedure 1 is the best solution after considering the amplification conditions of the four manufacturers, which is defined as the conventional amplification procedure. Amplification procedure 2 is a fast amplification program optimized for amplification time, which is defined as the rapid amplification procedure.

Table 2

Basic PCR system.

Basic system	N	A	E	F
Composition	Volume (μL)	Volume (μL)	Volume (μL)	Volume (μL)
DNA polymerase	0.5	25	0.75	2
RT enzyme mix	2.5	25	0.4	2
RNase inhibitor	2.5	25	0.5	10
Mg^{2+}(25mM)	3	25	16	10
Reaction buffer	9	25	16	10
Upstream primer (10μM)	1	1	1	1
Downstream primer (10 μM)	1	1	1	1
Probe (10μM)	0.5	0.5	0.5	0.5
Sample	5	5	5	5
Enzyme-free sterile water	Fill in up to 50μL	Fill in up to 50μL	Fill in up to 50μL	Fill in up to 50μL

Table 3

Configuration of different amplification procedures.

Reaction phase	Temperature	Duration		
Amplification procedure 1 (conventional amplification procedure)		Amplification procedure 2 (rapid amplification procedure)	Degradation U-templates	25°C
10min		2min		
Reverse transcription	50°C	15min	3min	
–				
Pre-denaturation	95°C	5min	30s	

–					
Denaturation	95°C	15 s	40 cycles	3 s	40 cycles
Annealing and extension	60°C	30 s	5 s	·	
	Total duration	~1 h 30 min		~30 min	

2.2.3. Amplification System Optimization

(1) Ten different Mg^{2+} concentrations (final concentrations of 0 mmol/L, 1 mmol/L, 1.5 mmol/L, 2 mmol/L, 2.5 mmol/L, 3 mmol/L, 3.5 mmol/L, 4 mmol/L, 5 mmol/L, and 6 mmol/L) were set up, and synthetic RNA from FluA was taken as the test sample to assess the effect of different Mg^{2+} concentrations on the amplification.

(2) The target gene probes of SARS-CoV-2 ORF, SARS-CoV-2 N, influenza A, and influenza B were set with four concentrations each (final concentrations of 0.05 μmol/L, 0.1 μmol/L, 0.15 μmol/L, and 0.2 μmol/L, respectively), and eight concentrations were set for the primers (final concentrations of 0.05 μmol/L, 0.1 μmol/L, 0.15 μmol/L, 0.2 μmol/L, 0.25 μmol/L, 0.3 μmol/L, 0.35 μmol/L, and 0.4 μmol/L); synthetic RNA using SARS-CoV-2, FluA, FluB, and negative control were used as the test samples to examine the effect of different concentrations of probes and primers on the amplification.

2.2.4. Validation of the Minimum Detection Limit

The dilution gradient of *in vitro*-transcribed RNA of SARS-CoV-2, FluA, or FluB at concentrations calibrated by digital PCR was prepared to determine Ct values and create a standard curve; the concentrations of virus cultures were calibrated by the standard curve. Then, serial dilution samples were detected and a 90% positive detection rate was calibrated by the four-parameter fitting algorithm to determine the lowest detection limit, and the clinical samples were diluted to 200 copies/mL using a sample preservation solution to confirm the lowest detection limit. The tests were repeated 20 times under the optimized conditions, and the positive detection rate was obtained based on the tests of three samples of different sources of each virus tested with three different batches of kits. Among them, FluA included five subtypes, H1N1, H3N2, H7N9, H5N1, and H1N1 (2009), and FluB included two subtypes, Victoria and Yamagata.

3. Results

3.1. Effects of Enzymes on SARS-CoV-2 and FluA Tests

The results are shown in Figure 1, which indicates that F enzyme is unable to achieve rapid amplification, E enzyme Ct is delayed, and A enzyme and N enzyme can lead to rapid amplification, with N enzyme working the best.

[figure(s) omitted; refer to PDF]

3.2. Results of FluA Tests at Different Mg^{2+} Concentrations

The Ct value of FluA decreased with the rise of Mg^{2+} concentration, and there was no significant difference when the concentration of Mg^{2+} was at 3 mM or above. Therefore, 3 mM was determined as the optimal concentration of Mg^{2+} considering that excessive concentration would lead to nonspecific amplification (Figure 2).

[figure(s) omitted; refer to PDF]

3.3. Test Results of Different Concentrations of Primer Probes

The Ct value of SARS-CoV-2 N decreases when both primer and probe concentrations are increased, so the final concentration of N probe is set at 0.15 μM and the final concentration of primer can be 0.2 μM since the effect of primer is close at the concentrations ranging from 0.05 μM to 0.4 μM (Table 4); the Ct value of SARS-CoV-2 ORF increases when both primer and probe concentrations are increased, so the final concentration of ORF probe is set at 0.15 μM and the final concentration of primer can be 0.15 μM since the effect of primer is desirable at the concentrations ranging from 0.15 μM to 0.25 μM (Table 4). The Ct value of FluA decreases when both primer and probe concentrations are increased, so the final concentration of FluA probe is set at 0.2 μM and the final

concentration of primer can be 0.2 μM since the effect of primer is desirable at the concentrations ranging from 0.15 μM to 0.4 μM (Table 4); the Ct value of FluB decreases when both primer and probe concentrations are increased, and therefore the final concentration of FluB probe is set at 0.15 μM and the final concentration of primer can be 0.25 μM since the effect of primer is desirable at the concentrations ranging from 0.15 μM to 0.4 μM (Table 4).

Table 4

Ct values under different concentrations of primers and probes.

		Primer concentration (μM)							
0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	SARS-CoV-2 NP probe concentration (μM)	0.05
33.24	32.97	33.34	33.22	33.08	33.20	33.04	33.18	0.10	32.29
32.26	32.33	32.28	32.39	32.20	32.20	32.22	0.15	32.01	31.86
32.15	31.98	32.17	31.94	32.06	31.97	0.20	32.50	32.09	32.00
32.02	31.98	32.20	31.75	32.11	.				
SARS-CoV-2 ORF probe concentration (μM)	0.05	34.14	34.04	34.92	36.63	35.54	39.21	N/A	N/A
0.10	34.20	33.21	33.84	34.98	35.39	37.09	39.82	N/A	0.15
33.19	32.24	33.86	33.73	34.09	36.70	39.31	N/A	0.20	33.72
32.62	35.90	35.14	34.31	35.76	39.69	N/A	.		
FluA M probe concentration (μM)	0.05	31.01	30.69	30.62	30.71	30.61	30.76	30.81	31.07

0.10	30.55	30.27	30.02	30.18	29.98	29.73	29.93	29.98	0.15
30.25	30.02	29.67	29.71	29.45	29.57	29.60	29.75	0.20	30.54
29.91	29.62	29.69	29.61	29.11	29.40	29.63	.		
FluB NS probe concentration (μM)	0.05	33.68	33.29	32.66	33.20	32.33	32.88	32.47	32.60
0.10	33.07	32.77	32.54	32.72	32.67	32.21	32.25	32.76	0.15
32.97	33.38	32.46	32.53	32.01	32.53	32.33	32.60	0.20	34.03

3.4. Comparison of Results between Multiplex Systems and Single Systems

In order to optimize the amplification system, amplification tests were performed using the synthetic RNA of SARS-CoV-2, FluA, and FluB, with Psrp RNA (fragment sequences derived from Arabidopsis genomes) as the internal control. Every assay had parallel tests of 3 samples, and the results showed no significant difference in amplifications between the multiplex system and the single-weight system (Table 5).

Table 5

Ct values of the multiplex PCR system and single PCR system.

High concentration	Multiplex		Single		ΔCt
Mean	SD	Mean	SD	ORF	30.34
0.1	30.79	0.14	−0.45	N	29.77
0.12	29.46	0.05	0.31	FluA	34.14
0.26	33.7	0.36	0.44	FluB	33.97
0.34	33.57	0.30	0.40	Psrp	29.66

3.5. Results of Minimum Detection Limit

The standard curve of the transcribed RNA dilution gradient for SARS-CoV-2, FluA, or FluB is shown in Supplementary Table 1 and Supplementary Figure 1. Using the formula obtained from the standard curve, the concentrations of the clinical samples or viral cultures can be calculated, which are shown in Table 6.

Table 6

The concentrations of the clinical samples or viral cultures of SARS-CoV-2, FluA, and FluB.

Virus	Subtype	Sample	Concentration (copies/mL)

SARS-CoV-2	—	CoV-2-1	1.69*E*+07
CoV-2-2	5.86*E*+09	CoV-2-3	1.70*E*+06
–			
FluA	H1N1	A11-1	1.01*E*+06
A11-2	4.68*E*+06	A11-3	1.20*E*+06
H3N2	A32-1	1.91*E*+09	A32-2
1.28*E*+05	A32-3	2.54*E*+06	H7N9
A79-1	5.82*E*+08	A79-2	1.71*E*+09
A79-3	6.35*E*+08	H1N1 (2009)	A2009-1
4.25*E*+04	A2009-2	3.49*E*+05	A2009-3
1.98*E*+04	H5N1	A51-1	6.17*E*+09
A51-2	2.83*E*+09	A51-3	2.97*E*+09
–			
FluB	Victoria	BV-1	1.23*E*+06
BV-2	4.85*E*+05	BV-3	1.47*E*+05
Yamagata	BY-1	4.91*E*+05	BY-2

The serial dilutions detection data of each target (SARS-CoV-2, FluA, and FluB) are shown in Supplementary Table 2. The lowest detection limit of the SARS-CoV-2N gene was 131.14 copies/mL calculated by the following formula: $y = 0.1969 + 105.2031/(1 + 10\text{^}((1.736\text{-}x) * 2.006))$ from the four-parameter fitting curve (Figure 3(a)). The lowest detection limit of the SARS-CoV-2 ORF gene was 173.47 copies/mL calculated by the following formula: $y = -0.1358 + 107.8358/(1 + 10\text{^}((1.758\text{-}x) * 1.469))$ from the four-parameter fitting curve (Figure 3(b)). The lowest detection limit of FluA was 166.11 copies/mL calculated by the following formula: $y = 0.1509 + 104.14911/(1 + 10\text{^}((1.814\text{-}x) * 1.964))$ from the four-parameter fitting curve (Figure 3(c)). The lowest detection limit of FluB was 102.16 copies/mL calculated by the following formula: $y = -0.0246 + 99.6946/(1 + 10\text{^}((1.747\text{-}x) * 3.694))$ from the four-parameter fitting curve (Figure 3(d)).

[figure(s) omitted; refer to PDF]

As revealed by the results of the three batches of different detection reagents, 200 copies/mL for the clinical samples or viral cultures of SARS-CoV-2, FluA, and FluB all met the requirements of 95%–100% positive detection rate, as shown in Table 7 and Supplementary Table 3. So, the minimum detection limits of SARS-CoV-2, FluA, and FluB can be set to 200 copies/mL.

Table 7

Positive detection rate for viral culture tests of 200 copies/mL for SARS-CoV-2, FluA, and FluB.

Virus	Subtype	Sample	Target	Batch 1 (%)	Batch 2 (%)	Batch 3 (%)
SARS-CoV-2	—	Sample 1	ORF	100	95	100
N	100	100	100	Sample 2	ORF	100
100	95	N	100	95	100	Sample 3
ORF	100	100	100	N	95	100
100	–					
FluA	H1N1	Sample 1	M	100	100	95
Sample 2	M	95	100	100	Sample 3	M
100	95	100	H3N2	Sample 1	M	95
100	100	Sample 2	M	100	100	100
Sample 3	M	100	95	100	H7N9	Sample 1
M	100	100	100	Sample 2	M	100
95	100	Sample 3	M	95	100	100
H1N1 (2009)	Sample 1	M	100	100	100	Sample 2
M	100	100	95	Sample 3	M	100
100	100	H5N1	Sample 1	M	100	95
100	Sample 2	M	100	100	100	Sample 3
M	100	100	100	-		
FluB	Victoria	Sample 1	NS	100	100	95
Sample 2	NS	100	100	100	Sample 3	NS
95	100	100	Yamagata	Sample 1	NS	100
95	100	Sample 2	NS	100	100	100

4. Discussion

In response to respiratory infectious diseases, early and rapid diagnosis can control the development of the disease as early as possible and reduce the number of critical patients. However, the premise of rapid diagnosis is to ensure the sensitivity and accuracy of the detection. Therefore, designing a reaction system to ensure rapid and effective amplification of nucleic acid is the core of this study. Finally, a multiplex fluorescence RT-PCR assay was designed in this study to establish and optimize a multiplex amplification system for COVID-19, influenza A, and influenza B to achieve the differential diagnosis of COVID-19 and influenza in 30 minutes.

This study proved that the enzymes from different manufacturers could affect the amplification results, and N enzymes could achieve rapid amplification through experiments. The detection principle is that the DNA polymerase with 5′~3′ DNA exonuclease activity will degrade the probe when it meets the fluorescence-labeled probe bound to the template strand during the PCR extension, resulting in the release of fluorescence to be detected by the real-time quantitative PCR instrument [20]. On Taq DNA polymerase, the polymerase active region and the 5′~3′ DNA exonuclease active region are found in different structural domains [21], and these two active regions work together to initiate the synthesis of new DNA strands while cleaving the fluorescent probe and releasing the signal. Among the reactions of polymerization and exocytosis, the less efficient reaction directly determines the efficiency of DNA amplification and the release of fluorescent signals. As reported, the Taq polymerase does not degrade the entire probe, and the degraded part is about 5–12 bp from the 5′ ends of the probe; the undegraded probe may participate in the subsequent PCR cycles, so as to inhibit the release of the fluorescent signal; the degradation of the probe is closely associated with the intensity of the Taq enzyme's exonuclease activity [22, 23]. In the case of the limited amount of enzyme (0.38, 0.19 U/reaction), once the Taq polymerase with the same polymerase activity is added to the reaction, the enzyme with higher exonuclease activity shows higher amplification efficiency; when Taq polymerase with the same exonuclease activity is added, the amplification efficiency is basically similar even though the polymerase activity is different. The abovementioned results demonstrate that the exonuclease reaction is a key step for rate control, and the rate is crucial to the efficiency of DNA amplification.

Due to the dependence of enzyme activity on Mg^{2+} concentration in the PCR reaction system, the absence of Mg^{2+} will lead to enzyme inactivation, and the enzyme activity will be inhibited when the concentration of Mg2+ is high. In the study of optimizing the isothermal amplification reaction system, Mg^{2+} concentration in the system has a significant effect on the amplification efficiency [24]. In addition, the divalent cations also affect the dissociation temperature and annealing temperature of the primer and template hybrid. Therefore, the concentration of Mg^{2+} in the system will affect the amplification efficiency. In this study, it was found that the amplification efficiency was the highest and the expansion speed was the fastest when the Mg^{2+} concentration was 3 mmol/L in the multiple amplification system.

The final concentrations of primers and probes for the three respiratory virus assays were determined through the optimization of concentrations of primer and probe in the amplification system. The final concentrations of SARS-CoV-2 NP probe and primer were 0.15 μmol/L and 0.2 μmol/L, respectively; the final concentrations of SARS-CoV-2 ORF probe and primer were both 0.15 μmol/L; the final concentrations of FluA probe and primer were 0.2 μmol/L and 0.3 μmol/L, respectively; the final concentrations of FluB probe and primer were 0.15 μmol/L and 0.25 μmol/L, respectively. The detection of the three viruses using the multiplex assay system constructed in this study did not show a difference from those of the single system amplification, and the minimum detection limits of the present study for SARS-CoV-2, FluA, and FluB could all reach 200 copies/mL. Relevant research results show that the minimum detection limit of single nucleic acid detection of respiratory pathogens (such as SARS-CoV-2) is mostly in the range of 250–1000 copies/mL, and the amplification time is mostly more than 1 h. Although some studies claim that the minimum detection limit of the method can reach 200 copies/mL or lower, the amplification time is close to 2 h [25–29].

In summary, by optimizing the amplification conditions and using a self-made device that integrates sample lysis, nucleic acid extraction, nucleic acid purification, multiplex fluorescent PCR, and result analysis, this study achieves the purpose of 30 min rapid detection without additional operation and the minimum detection limit of 200 copies/mL.

The assay can be used for clinical rapid differential diagnosis, contributing to the combat against the SARS-CoV-2 pandemic as well as the seasonal influenza.

Ethical Approval

Ethical approval was obtained from the Ethical Review Committee of the China-Japan Friendship Hospital.

Authors' Contributions

Liang Ma and Yongtong Cao conceptualized the study. Liang Ma, Haoyan Zhu, Peng Gao, Guoxiong Deng, Xiaomu Kong, Yongwei Jiang, Meimei Zhao, and Yi Liu curated the data. Liang Ma performed funding acquisition. Peng Gao, Yanyan Fan Yongwei Jiang, Meimei Zhao, Yi Liu, and Haoyan Zhu collected the data. Xiaomu Kong performed the statistical analysis. Liang Ma and Yongtong Cao wrote the manuscript.

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DETAILS

Subject:	Infectious diseases; Acids; Reagents; Severe acute respiratory syndrome coronavirus 2; Disease control; Influenza; Viruses; Genomes; Nucleic acids; Viral diseases; Automation; DNA polymerase; Gene amplification; COVID-19; Medical diagnosis; Fluorescence; Gene sequencing; Pandemics; Epidemics; Optimization; Amplification; Multiplexing; Public health; Enzymes; Disease transmission
Business indexing term:	Subject: Automation
Location:	United States--US
Editor:	Waleed Alahmad
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024

Publication date:	2024
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:	2023-05-31 (Received); 2024-02-03 (Revised); 2024-02-10 (Accepted); 2024-02-29 (Pub)
DOI:	https://doi.org/10.1155/2024/4950391
ProQuest document ID:	2954636568
Document URL:	https://www.proquest.com/scholarly-journals/development-novel-multiplex-pcr-method-rapid/docview/2954636568/se-2?accountid=211160

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Last updated:	2024-03-11
Database:	Publicly Available Content Database

Pharmacokinetics of Ziyuglycoside I and Ziyuglycoside II in Rat Plasma by UPLC-MS/MS

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ABSTRACT (ENGLISH)

Ziyuglycoside I and ziyuglycoside II are important active components of *Sanguisorba officinalis* L., which have excellent pharmacological effects, such as antioxidant and anticancer effects. However, the bioavailability of ziyuglycoside I and ziyuglycoside II has not been reported. This work aims to establish a UPLC-MS/MS method to study the pharmacokinetics of ziyuglycoside I and ziyuglycoside II in rats under different administration routes (intragastric and intravenous administration) and to calculate the bioavailability. The concentration of ziyuglycoside I and ziyuglycoside II in rat plasma in the range of 2–2000 ng/mL showed a good linear relationship (r>0.99). The intra-day accuracies of ziyuglycoside I and ziyuglycoside II ranged from 87% to 110%, and the inter-day accuracies ranged from 97% to 109%. The intra-day precision was less than 15% and the inter-day precision was less than 14%. The matrix effects ranged from 88% to 113%. The recoveries were all above 84%. The developed UPLC-MS/MS method for the determination of ziyuglycoside I and ziyuglycoside II in rat plasma was applied to pharmacokinetics. The bioavailability of ziyuglycoside I and ziyuglycoside II was measured at 2.6% and 4.6%, respectively.

FULL TEXT

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1. Introduction

Sanguisorba officinalis L. belongs to the genus *Sanguisorba* in the family Rosaceae [1–3]. It has long been used as a natural remedy for inflammatory and metabolic diseases, such as chronic intestinal infections, bleeding, diarrhea, and duodenal ulcer [4–6]. Ziyuglycoside I and ziyuglycoside II, isolated from *Sanguisorba officinalis* L., are the important active components of *Sanguisorba officinalis* L., which have excellent pharmacological effects. Many studies have shown that saponins extracted from *Sanguisorba officinalis* L. have antioxidant and anticancer effects [7–9]. Among other things, ziyuglycoside I could prevent the formation of collagen fibers, effectively promote the production of type I collagen, and reduce the generation of skin wrinkles, which could be used in the synthesis of beauty and skin care products [7, 10]. Ziyuglycoside II could improve diabetes performance and protect liver and kidney [11].

To date, some studies on the pharmacokinetics of ziyuglycoside I and ziyuglycoside II using LC-MS/MS have been published [12, 13]. Li et al. developed an HPLC-MS/MS method for the quantification of ziyuglycoside I and ziyuglycoside II in rat biological matrices, with a total run time of 6 min for a sample and a lower limit of quantification (LLOQ) of 2 ng/mL [12], and the pharmacokinetics, tissue distribution, and excretion of ziyuglycoside I and ziyuglycoside II were evaluated. Ye et al. developed an LC-MS/MS method for the determination of ziyuglycoside I and ziyuglycoside II in rat plasma using liquid-liquid extraction with n-butanol. Chromatographic separation was achieved using a Thermo Golden C 18 column with a total run time of 10 min for a sample and LLOQ of 2 ng/mL [13]. Wu et al. developed a UHPLC-MS/MS method for determination and pharmacokinetic study of six triterpenes in rat plasma after oral administration of *Sanguisorba officinalis* L. extract [14]. However, only ziyuglycoside I was determined with an LLOQ of 6.05 ng/mL, and ziyuglycoside II was not determined. In addition, these studies on the bioavailability of ziyuglycoside I and ziyuglycoside II have not been published. Therefore, it is necessary to establish

a simple and sensitive UPLC-MS/MS method for determining the bioavailability of ziyuglycoside I and ziyuglycoside II.

The aim of this work is to establish a UPLC-MS/MS method to investigate the pharmacokinetics of ziyuglycoside I and ziyuglycoside II in rats under different routes of administration (intragastric and intravenous administration) and calculate the bioavailability. It is expected to help in the determination of plasma concentration of ziyuglycoside I and ziyuglycoside II, development and research of new ziyuglycoside drugs, and toxicology research.

2. Experimental

2.1. Chemicals

Ziyuglycoside I, ziyuglycoside II, and ginsenoside Rg1 (purity >98%, Figure 1) were purchased from Chengdu Must Biotechnology Co., LTD., China. HPLC-grade acetonitrile, methanol, and formic acid were purchased from Merck, Germany. Ultrapure water was manufactured by Milli-Q Water Systems, USA.

[figure(s) omitted; refer to PDF]

2.2. Apparatus and Conditions

ACQUITY H-Class UPLC system and Xevo TQS-Micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) were used in this work.

A UPLC HSS T3 column (2.1 mm × 50 mm, 1.8 μm) was used with gradient elution at a flow rate of 0.4 mL/min with a mobile phase of acetonitrile-water (containing 0.1% formic acid). The gradient elution conditions were as follows: 0–0.2 min, acetonitrile 10%; 0.2–1.2 min, acetonitrile 10%–90%; 1.2–2.0 min, acetonitrile 90%–90%; 2.0–2.2 min, acetonitrile 90%–10%; and 2.2–3.5 min, acetonitrile 10%.

Nitrogen was used as the desolvation gas (926 L/h) and atomization gas with a capillary voltage of 2.06 kV and an ion source temperature of 147°C, and the desolvation temperature was 499°C. The positive and negative ion mode of ESI was simultaneous monitoring. The positive ion mode of ESI was used for ziyuglycoside II, and the negative ion mode of ESI used for ziyuglycoside I and ginsenoside Rg1. The MRM mode was used for quantitative analysis, m/z 811.291→603.379 was for ziyuglycoside I (cone voltage 34 v and collision voltage 28 v), m/z 605.37→455.36 was for ziyuglycoside II (cone voltage 14 v and collision voltage 28 V), and m/z 845.426→637.529 was for internal standard ginsenoside Rg1 (cone voltage 4 v and collision voltage 28 v).

2.3. Preparation of Reference Solution

5.0 mg of ziyuglycoside I, ziyuglycoside II, and ginsenoside Rg1 was accurately weighed and transferred to a 10 mL volumetric flask and diluted with methanol to the scale line to make a concentration of 0.50 mg/mL, respectively. The working solution of ginsenoside Rg1 was then transferred to a reagent bottle and diluted to 100 mL with methanol at a concentration of 1.0 μg/mL. A series of working solutions were prepared by diluting the stock solution of ziyuglycoside II (0.50 mg/mL) and ziyuglycoside I (0.50 mg/mL) with methanol. All solutions were stored in a refrigerator at −20°C and returned to room temperature before use.

2.4. Preparation of Standard Curves

The ziyuglycoside I and ziyuglycoside II series standards were prepared by adding a suitable working solution to the blank plasma. The standard curve was 2000, 1000, 500, 200, 100, 50, 20, 10, 5, and 2 ng/mL, respectively. Quality control (QC) samples with plasma concentrations (2, 8, 150, and 1500 ng/mL) were prepared using the same method.

2.5. Plasma Sample Pretreatment

The plasma sample was pretreated by liquid-liquid extraction, that is, 100 μL of plasma sample and 20 μL of internal standard (1.0 μg/mL) were added to a 1.5 mL centrifuge tube, vortexed, and mixed for 30 s, and then 0.9 mL ethyl acetate was added for extraction. The samples were then centrifuged at 12000 rpm for 10 min, and the organic phase was transferred to another centrifuge tube and blow-dried at 55°C under a stream of nitrogen. 100 μL of methanol was added to the blown dried centrifuge tube, vortexed, and mixed again, and a small amount of the mixed solution was placed in a bottle for detection.

2.6. Method Validation

The validation method was established in accordance with the US Food and Drug Administration (FDA) Bioanalytical

Method Validation Guidelines [15]. Validation criteria included selectivity, linearity, precision, accuracy, matrix effect, recovery, and stability [16, 17].

2.7. Pharmacokinetics

Sprague Dawley (SD) rats (male, weighing 220–250 g) were purchased from the Animal Experiment Center of Wenzhou Medical University. Before the experiment, food intake was prohibited for 12 hours, but water was freely available. All experimental procedures were approved by the Animal Care Committee of Wenzhou Medical University (wydw2023-0576). 24 rats were divided into 4 groups with 6 rats in each group. In groups 1 and 2, the rats were administered ziyuglycoside I at a dose of 1 mg/kg intravenously (iv) and 5 mg/kg by gavage (ig), respectively. Intravenous administration is administered via sublingual vein. In groups 3 and 4, the rats were administered ziyuglycoside II at a dose of 1 mg/kg intravenously (iv) and 5 mg/kg by gavage (ig), respectively. At 0.0833, 0.25, 1, 2, 4, 6, 8, 12, and 24 h, 0.3 mL of tail vein blood was collected into heparinized tubes and centrifuged at 13,000 rpm for 10 min. Subsequently, 100 μL of the top layer plasma was transferred to a new 1.5 mL centrifuge tube, the corresponding administration times were marked on the centrifuge tube, and it was stored at −20°C. Pharmacokinetic data were determined using DAS (Drug and Statistics) 2.0 software (Shanghai University of Traditional Chinese Medicine, China).

3. Results and Discussion

3.1. Methodology Development

To obtain the best conditions for mass spectrometry, positive ion mode and negative ion mode were used for monitoring [16, 18]. The reactivity of ziyuglycoside I in negative ion mode was higher and the reactivity of ziyuglycoside II in positive ion mode was higher. After optimizing various parameters, the capillary tension and collision energy were finally determined.

To select the appropriate plasma processing method, the extraction efficiencies of analytes by liquid-liquid extraction, solid-liquid extraction, and protein precipitation methods were studied and compared [19]. The solid-liquid extraction method offers better results in extraction yield and matrix effect, but at the expense of higher cost and operational complexity. Using the protein precipitation method, plasma samples could be processed easily and quickly [20], but the extraction efficiency was not high. Liquid-liquid extraction exhibits commendable selectivity [21], minimal matrix effect, and satisfactory extraction efficiency. Ethyl acetate has a lower boiling point than n-butanol and was more volatile, making it more suitable for liquid-liquid extraction. Due to the detection sensitivity requirements, the ethyl acetate liquid-liquid extraction method was selected to process the plasma samples in this work.

The elution systems consisting of methanol, acetonitrile, water, and formate water were compared according to the physicochemical properties and the chromatographic behavior of the analytes [20, 22–24]. The results show that the separation effect of methanol-water or acidic water as system components was not ideal, but the optimized separation effect of acetonitrile-water-formic acid system components was better, and each chromatographic peak separation and the theoretical number of trays met the requirements. In addition, the retention time of the analytes can be changed by selecting the gradient elution conditions, so that the matrix effect can be reduced by separating the analytes from the co-effluent. Only 4 min was needed for a sample, and it was faster than 6 min and 7.5 min as reported in the literature [12, 13]. The retention times of ziyuglycoside I, ziyuglycoside II, and ginsenoside Rg1 were 1.87, 2.24, and 1.68 min using acetonitrile-water-formic acid system as shown in Figure 2, respectively.

[figure(s) omitted; refer to PDF]

In the quantitative analysis of biological samples, the deuterated standard was the best internal standard to correct for loss in the extraction process [25]. However, this internal standard was expensive and not always available for purchase. Therefore, it was preferred to select a compound with similar structure, recovery, and mass spectrometric ion response as the analyte. Ginsenoside Rg1 was chosen as the internal standard in this study because its polarity and ion response corresponded to those of ziyuglycoside I and ziyuglycoside II.

3.2. Method Validation

The selectivity of the method was assessed by analyzing blank rat plasma and blank plasma spiked with

ziyuglycoside I and II as well as an internal standard. Figure 2 shows the UPLC-MS/MS chromatogram of blank rat plasma and blank rat plasma spiked with ziyuglycoside I and ziyuglycoside II. There was no influence of endogenous substances on the determination of the analytes.

The calibration curves were generated by analyzing spiked calibration samples on three different days. The resulting standard curves were well-fitted to the equations through linear regression, employing a weighting factor of the reciprocal of the concentration ($1/x$). The lower limit of quantification (LLOQ) was defined as the minimum concentration observed on the calibration curves. The concentration of ziyuglycoside I and ziyuglycoside II in rat plasma in the range of 2–2000 ng/mL showed a linear relationship, and the standard curve equations were $y = 0.3642x + 1.0438$, $r = 0.9976$; $y = 0.029x + 0.1111$, $r = 0.9981$; y represents the peak area of ziyuglycoside I and ziyuglycoside II, and x represents the concentration of ziyuglycoside I and ziyuglycoside II in plasma. The LLOQ of ziyuglycoside I and ziyuglycoside II in rat plasma was 2 ng/mL. The limit of detection (LOD), defined as a signal-to-noise ratio of 3, of ziyuglycoside I and ziyuglycoside II in rat plasma was 0.5 ng/mL.

The accuracy and precision of the assay were evaluated by analyzing quality control (QC) samples at three different concentration levels in six replicates over a three-day validation period. Precision was quantified as relative standard deviation (RSD). As presented in Table 1, the intra-day accuracies for ziyuglycoside I and ziyuglycoside II ranged from 87% to 110%, while the inter-day accuracies ranged from 97% to 109%. The intra-day precision was below 15%, and the inter-day precision was below 14%.

Table 1

Accuracy, precision, matrix effect, and recovery of ziyuglycoside I and ziyuglycoside II in rat plasma.

Compound	Concentration (ng/mL)	Accuracy (%)		Precision (RSD%)		Matrix effect (%)	Recovery (%)
Intra-day	Inter-day	Intra-day	Inter-day	Ziyuglycoside I	2	89.7	108.6
10.2	7.7	109.6	84.6	8	101.9	99.8	8.9
10.9	88.4	89.0	150	96.0	104.6	9.2	4.8
112.5	88.1	1500	98.0	97.0	4.2	4.8	109.7
94.4	–						
Ziyuglycoside II	2	87.9	106.8	14.1	13.6	95.5	92.0
8	100.9	98.4	5.9	9.0	98.2	89.0	150
109.9	106.2	8.3	10.5	88.4	90.1	1500	106.7

To assess the matrix effect, rat blank plasma was extracted and supplemented with ziyuglycoside I and ziyuglycoside II at concentrations of 2, 8, 150, and 1500 ng/ml ($n = 6$). Subsequently, the corresponding peak areas were compared to those obtained from pure standard solutions at equivalent concentrations. The observed matrix effects ranged from 88% to 113% (Table 1).

The recovery of both ziyuglycoside I and ziyuglycoside II was evaluated by comparing the peak area of extracted quality control samples with that of reference quality control solutions reconstituted in blank plasma extracts ($n = 6$).

All recoveries exceeded 84% (Table 1).

The stability values of ziyuglycoside I and ziyuglycoside II in rat plasma were assessed by analyzing three replicates of quality control (QC) plasma samples, each exposed to distinct conditions. Specifically, the rat plasma samples underwent pretreatment and were stored at room temperature for 24 h, subjected to three freeze-thaw cycles, and their stability was further examined during long-termstorage at −20°C. The results demonstrated an accuracy range of 92%–108% and an RSD within 13%, indicating acceptable stability of both ziyuglycoside I and ziyuglycoside II.

3.3. Pharmacokinetics Study

The plasma concentration-time curve, with the administration time as the *x*-axis and the plasma concentration as the *y*-axis, is depicted in Figure 3. Pharmacokinetic parameters were determined using a noncompartmental model, and the corresponding data are presented in Table 2. Following intragastric administration of ziyuglycoside I, the half-life (t1/2), area under the curve from time zero to infinity ($AUC_{(0-\infty)}$), and clearance (CL) were calculated to be 5.1 ± 2.5 h, 109.0 ± 11.8 ng/mL ∗ h, and 46.3 ± 5.2 L/h/kg, respectively. For intravenous administration of ziyuglycoside I, t1/2 was found to be 1.8 ± 0.7 h while $AUC_{(0-\infty)}$ and CL were measured as 838.3 ± 250 0.3 ng/mL ∗ h and 1 0.3 ± 0.3 L/h/kg, respectively. The reported values for t1/2 after tail vein administration (1.338 ± 0.744 h) and subcutaneous injection administration (6.115 ± 1.92 h) [12] were consistent with our findings. The observed t1/2 value of 19.76 ± 1.59 h for ziyuglycoside I following oral administration of *Sanguisorba officinalis* L. extract at a dose of 2.0 g/kg to rats [13] was not congruent with our results.

[figure(s) omitted; refer to PDF]

Table 2

Main pharmacokinetic parameters after intragastric (5 mg/kg) and intravenous (1 mg/kg) administration of ziyuglycoside I and ziyuglycoside II in rats.

Compound	Group	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	t1/2z	$CL_{z/F}$	Vz/F	Cmax
ng/mL ∗ h	ng/mL ∗ h	h	L/h/kg	L/kg	ng/mL	Ziyuglycoside I	ig
95.4 ± 7.2	109.0 ± 11.8	5.1 ± 2.5	46.3 ± 5.2	329.2 ± 143.6	36.6 ± 5.8	iv	834.7 ± 249.2
838.3 ± 250.3	1.8 ± 0.7	1.3 ± 0.3	3.5 ± 2.0	1268.0 ± 344.8	-		
Ziyuglycoside II	ig	438.9 ± 42.4	458.3 ± 46.3	4.9 ± 1.5	11.0 ± 1.0	77.3 ± 22.5	102.2 ± 14.5

t1/2, $AUC_{(0-\infty)}$, and CL of ziyuglycoside II following intragastric administration were determined as 4.9 ± 1.5 h, 458.3 ± 46.3 ng/mL ∗ h, and 11.0 ± 1.0 L/h/kg, respectively. For intravenous administration, t1/2 of ziyuglycoside II was found to be 6.2 ± 3.1 h, $AUC_{(0-\infty)}$ was measured as 1979.2 ± 185.7 ng/mL ∗ h, and CL was calculated as being equal to 0.5 L/h/kg. The literature reports a t1/2 of ziyuglycoside II after tail vein administration and subcutaneous injection as being equal to approximately 1.027 ± 0.057 h and 7.935 ± 3.264 h [12]. Furthermore, it is reported that t1/2 of ziyuglycoside II after oral administration of *Sanguisorba officinalis* L. extract is approximately 12.16 ± 4.44 h which differs from our results. However, no information regarding the bioavailability of both ziyuglycoside I and ziyuglycoside II has been provided in these literature sources [12, 13]. In this study, we have determined that the bioavailability values for ziyuglycoside I and ziyuglycoside II are estimated at approximately 2.6% and 4.6%, respectively.

4. Conclusion

In this study, a liquid-liquid extraction method was employed to process rat plasma samples and ginsenoside Rg1

served as an internal standard for the establishment of a UPLC-MS/MS method to quantify ziyuglycoside I and ziyuglycoside II. The accuracy, precision, selectivity, and linearity of the method were validated. The developed UPLC-MS/MS method was applied to investigate the pharmacokinetics of ziyuglycoside I and ziyuglycoside II in rats following different administration routes (intragastric and intravenous), resulting in calculated bioavailabilities of 2.6% for ziyuglycoside I and 4.6% for ziyuglycoside II.

Authors' Contributions

Xiuwei Shen and Ziyue Wang contributed equally to this work.

Acknowledgments

This work was supported by the Wenzhou Traditional Chinese Medicine Project (Z2023006).

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DETAILS

Subject:	Pharmacokinetics; Bioavailability; Plasma; Chromatography; Drug dosages; Anticancer properties

Location:	China; United States--US
Company / organization:	Name: Food &Drug Administration--FDA; NAICS: 926150
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-09-28 (Received); 2024-01-21 (Revised); 2024-02-23 (Accepted); 2024-03-01 (Pub)
DOI:	https://doi.org/10.1155/2024/7971021
ProQuest document ID:	2954636562
Document URL:	https://www.proquest.com/scholarly-journals/pharmacokinetics-ziyuglycoside-i-ii-rat-plasma/docview/2954636562/se-2?accountid=211160
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Last updated: 2024-03-11

Database: Publicly Available Content Database

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Validation and Application of Screen-Printed Microchip for Potentiometric Determination of Metformin Hydrochloride in Tablet Dosage Form

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ProQuest document link

ABSTRACT (ENGLISH)

Metformin is an oral biguanides hypoglycaemic agent, which used to lower the blood glucose levels in people with type 2 diabetes mellitus. Many analytical techniques have been used to quantify the drug in different pharmaceutical dosage forms; however, most of these methods have limited throughput in the quality control application. A disposable potentiometric microsensor responsive to metformin has recently been reported. For the first time, herein, this method of analysis has been validated according to IUPAC recommendations and successfully applied in the determination of metformin drug in some dosage form. Different drug formulations of metformin hydrochloride have been collected from the local pharmaceutical stores in Saudi Arabia and analysed using the validated microchip-based method of analysis. Subsequently, the results of this study showed that the validated method was linear, specific, precise, and accurate. The linear range was 1×10^{-1}–1×10^{-5} mol L^{-1} and the correlation coefficient was 0.999. The limit of detection was 2.89×10^{-6} mol L^{-1}, and the limit of quantification was 8.77×10^{-6} mol L^{-1}. This method demonstrated high precision, with an RSD% of less than 2.22%. The accuracy of this method was obtained by comparing the recovery percentage with percentage values less than 5%. The results obtained showed that there was no significant difference between the references, label, and recovery of less than 5%.

FULL TEXT

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1. Introduction

Metformin (MTE) is the most frequently prescribed oral medication as a treatment for people with type 2 diabetes. It lowers blood glucose levels and increases insulin sensitivity in the body, preventing potential diabetic complications such as eye damage, kidney damage, nerve damage, and sexual dysfunction [1–5]. MET is also a preferred antidiabetic drug due to its high efficacy, good safety profile, and low cost [6]. Furthermore, considerable effectiveness of MET on obesity [7], cardiovascular diseases [8], liver diseases [9], cancers [10, 11], and renal diseases [12] were reported. MET hydrochloride (also known as N, N dimethyl imido dicarbonimidic diamide hydrochloride) has the empirical formula C4H11N5.HCl and a molecular weight of 165.63 g/mol. Subsequently, because of its ubiquitous usage, continuous monitoring of MET levels in pharmaceutical formulations and in human plasma has long been a crucial concern. Since the quality of pharmaceutical formulations generally determines the

efficacy and safety of MET treatment.

MET quality control (QC) generally requires an assay with a high throughput capability. For the purpose of determining MET, several instrumental techniques have been developed [13]. These techniques include high-performance liquid chromatography [14, 15], UV-visible spectrophotometry [16–18], LC-MS/MS [19, 20], electrochemical methods of analysis [21–25], spectrofluorimetric methods [26], and varied HPTLC techniques [27–29]. Spectrophotometric assays are considered practical procedures due to their high sensitivity, simplicity, low cost, and wide accessibility in laboratories. However, a majority of these assays have substantial limitations, such as low selectivity because their measurements are made in the UV region [30–32], decreased assay procedure simplicity, and laborious liquid–liquid extraction stages [33–35]. Furthermore, due to differences in the chemical structures of MET, these assays were developed individually. Thin-film microelectrode development, on the other hand, has recently received more interest than previous techniques due to its inherent simplicity, high sensitivity, quick analysis, low cost, large-scale production, and automated and integrated feasibility [36–42].

Consequently, scientists and researchers have been developing analytical techniques with high-throughput capacities to increase the QC analysis and improve its productivity. High-throughput assays enable researchers to efficiently process massive quantities of samples; hence, uniformity of pharmaceutical formulations, rapid identification of active substances, and other pharmaceutical industry operations which could be achieved. Recently, Alfadhel et al. [23] fabricated a novel disposable microchip that demonstrated significant reliability, good credibility, low cost, and rapid determination of MET. Therefore, this research aimed to validate and investigate the realized potentiometric microsensor for the QC application of MET for the first time.

2. Materials and Methods

2.1. Apparatus and Tools

A Jenway (model 3510) pH/mV meter and Jenway combination pH electrode for all pH experiments were used for electrochemical characterization measurements. The metformin-based microchip (Figure 1) has been fabricated, characterized, and used in the metformin analysis as described in our previous work [23]. For MET detection, the microchip was used as the working electrode which based on a tetraphenyl borate/MET ion pair modified with carbon nanotubes in conjunction with the reference electrode (metrohm double junction electrode), as mentioned in the previous teamwork [23]. Double-distilled water was obtained from an Aquatron water distiller (A4000D, Bibby Scientific, UK, 1.0 MΩ cm^{-1}), and it was used to prepare the samples and rinse the glassware.

[figure(s) omitted; refer to PDF]

2.2. Standards Pharmaceutical Formulation and Reagents

The MET hydrochloride raw material (purity: 99.6%) was a gift supplied by Aljazerah Industry from Auro laboratories company (India). Four strengths of MET hydrochloride were purchased from the local pharmacies in Saudi Arabia. The origin of these pharmaceutical formulations was Oman, Saudi Arabia, and France with strengths labelled to containing 500, 750, 850, and 1000 mg MET hydrochloride, respectively.

2.3. Preparation of Standard and Sample Solutions

Stock standard solutions (1×10^{-1} mol L^{-1}) of MET were prepared by dissolving an accurately weighed amount (1.66 g) of the standard material in 100 mL of deionized water. These stock solutions were stable for at least two weeks when kept in a refrigerator at 5°C. The working solutions were prepared by diluting stock solution with deionized water to make different concentrations: 1×10^{-5}–1×10^{-2} mol L^{-1} for MET. Both stock and working solutions were kept in a refrigerator at 5°C.

For the preparation of pharmaceutical formulation sample solutions, three tablets from each of the different studied brands were weighed and finely pulverized. Then, a quantity of 100 mg of the MET from each drug brand powder was transferred into a volumetric flask and dissolved in approximately 100 mL of deionized water, mixed for 15 min, and then sonicated for 30 min. These solutions were then maintained in a refrigerator at 5°C.

2.4. General Procedures

In the electrochemical validation of the used method, the MET microchip and reference electrode were immersed in the calibration standards solutions, and the EMV and mV of the cell were recorded versus the concentration of MET.

The potentiometric validation studies were performed at room temperature (25±2°C). The calibration curves were obtained by plotting subtract logarithm of concentrations against the cell potential, mV. The quantifications of MET samples were achieved under the same conditions. Then, the sample concentrations were calculated by using the linear equations of the calibration curves of MET.

3. Results and Discussion

The metformin-based microchip was characterized in terms of sensitivity, selectivity, effect of pH, and response time and reported in our previous work [23]. The organic layer membrane is frequently employed in chemical electrodes due to its great selectivity, sensitivity, and simplicity. Because of the preceding advantages, a selective microchip electrode was constructed in this work to determine the MET hydrochloride in the solutions. The sensitivity of microchips demonstrates that they have significant merits in detecting MET hydrochloride in solutions and in tablet dosage form. There are numerous advantages to using this method, which are rapid, small size, simple, and costless [23].

3.1. Validation of Proposed Assays

3.1.1. Linearity and Sensitivity

The linearity, selectivity, and sensitivity of metformin hydrochloride are detected by microchips. Calibration graphs were constructed for the detection of MET in aqueous media using a potentiometric microchip (Figure 2). The regression equation of MET was derived, and the results are presented in Table 1. The obtained data shows that the correlation coefficients (r2) of MET was 0.999. The limits of detection (LOD) and limits of quantification (LOQ) were detected. The LOD and LOQ values of MET were found to be 2.89×10^{-6} mol L^{-1} and 8.77×10^{-6} mol L^{-1}, respectively. [figure(s) omitted; refer to PDF]

Table 1

Quantitative parameter of linearity.

Parameter	MET
Linear range (mol L^{-1})	0.00001–0.1
Intercept	56.02
Slope	33.98
Correlation coefficient (r)	0.999
LOD, (mol L^{-1})	2.89×10^{-6}
LOQ, (mol L^{-1})	8.77×10^{-6}

3.1.2. Precision and Accuracy

Replicate analysis of drug solutions at three distinct concentrations was used to assess the precision of potentiometric microchip assays for MET (Table 2). The average relative standard deviation (RSD) of the proposed drug in potentiometric microchips did not exceed 4% for MET (Table 2).

Table 2

MET intra and interday assay precision data (*n*=3).

Component	Theoretical concentration (mol L^{-1})	Measured conc. (mol L^{-1}), RSD (%)

Intra-day	Inter-day	MET	0.0002
0.000205 (3.45)	0.000209 (3.73)	0.002	0.00206 (1.41)
0.00209 (3.06)	0.02	0.0202 (1.78)	0.0206 (1.17)

Eventually, the accuracy of the proposed assays was evaluated by determining the recovery percentage of different concentrations. The values presented in the table show that the recovery percentage of all tested drugs was less than 5% (Table 3).

Table 3

MET % recovery studies and % RSD (*n*=3).

Component	Concentration, (mol L^{-1})	% recovery (average)	SD × 10^{-4}	% RSD
MET	0.0002	102.56	0.071	3.45
0.002	103.13	0.29	1.41	0.02

3.2. Determination of MET in Pharmaceutical Formulations

Commercially available pharmaceutical dosage forms of MET were analysed using the validated method. The mean percentage recovery relative to the label amounts obtained by previous methods is shown in Table 4. The results indicate that there was no significant difference between the references, label, and recovery which was less than 5% (Table 4).

Table 4

Metformin hydrochloride in commercially available pharmaceutical formulations data (*n*=3).

No.	Commercially available pharmaceutical formulations	Origin	Weight of tablet (gm)	Added (nominated) value (mg)	Measured value (mg)	Recovery (%)
1	Tablet, 500	Oman	0.602	83.0	74.5	89.7
2	Tablet, 750	Saudi Arabia	1.093	68.0	68.5	100.7
3	Tablet, 850	France	0.897	94.0	91.1	96.9
4	Tablet, 1000	France	1.071	93.4	88.6	94.8
Average recovery						95.5

4. Conclusions

This study demonstrates the validation of a recently developed disposable potentiometric microsenor which responsive for the measurement of MET hydrochloride in pharmaceutical formulations for the first time. The potentiometric method depends on tetraphenyl borate: a MET ion pair complex ionophore modified with 5% CNTs

sensitive to the MET drug. In addition, this method based on disposable chip assembly, which is used as a low-cost analytical tool (economic), has a rapid response time of less than 10 seconds and is an environmentally friendly “Green” approach. In terms of analytical procedure simplicity, it is a recommended approach for MET hydrochloride and can be employed in high-throughput systems. The proposed approach also offers the merit of determining MET hydrochloride using a single system. These advantages support the use of proposed methodologies as an alternative to current methods in quality control laboratories for regular MET hydrochloride testing.

Acknowledgments

We would like to acknowledge the Deanship of Scientific Research at the Taif University for funding this work.

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DETAILS

Subject:	Mathematical analysis; Quality control; Electrodes; Semiconductors; Calibration; Laboratories; Diabetes mellitus; Recovery; Metformin; Pharmaceutical industry; Methods; Dosage; Correlation coefficients; Pharmaceuticals; Drug dosages; Antidiabetics
Business indexing term:	Subject: Laboratories Pharmaceutical industry
Location:	Saudi Arabia; France; Oman
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-09-17 (Received); 2023-11-29 (Revised); 2024-02-18 (Accepted); 2024-02-27 (Pub)
DOI:	https://doi.org/10.1155/2024/8664723
ProQuest document ID:	2954636557
Document URL:	https://www.proquest.com/scholarly-journals/validation-application-screen-printed-microchip/docview/2954636557/se-2?accountid=211160

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Last updated:	2024-03-15
Database:	Publicly Available Content Database

Method Development for Simultaneously Determining Indomethacin and Nicotinamide in New Combination in Oral Dosage Formulations and Co-Amorphous Systems Using Three UV Spectrophotometric Techniques

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ABSTRACT (ENGLISH)

This research aims to develop methods for simultaneously determining indomethacin (IND) and nicotinamide (NCT) in binary mixtures, immediate-release capsules, sustained-release capsules, and co-amorphous systems, which were designed in 2021 to improve the solubility, dissolution rate, and stability of the amorphous state of indomethacin. Moreover, this new combination may have also other possible medical benefits. Therefore, there is a need to have simple, sensitive, and precise developed methods for simultaneous quantification analysis of IND/NCT in several different ratios. Three UV-spectrophotometry techniques were deployed: zero-crossing point in the second-order derivative, dual-wavelength in the first-order derivative, and ratio subtraction coupled with spectrum subtraction. The limit of detection and the limit of quantifications (LOD and LOQ) for IND were 0.41 and 1.25, 0.55 and 1.66, and 0.53 and 1.62 μg/mL, respectively, while for NCT were 0.53 and 1.59, 0.38 and 1.14, and 0.36 and 1.08 μg/mL, respectively. All methods were linear at least in the range of 2.5–40.0 μg/mL. All proposed methods were validated according to ICH guidelines and their application on the dosage formulations was carried out. Finally, the proposed methods were compared to a reference method for each IND and NCT, and no significant statistical variance was found.

FULL TEXT

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1. Introduction

Indomethacin (IND) is one of the nonsteroidal anti-inflammatory drugs (NSAIDs) and a nonselective inhibitor of COX-1 and COX-2 with antipyretic and analgesic effects. IND is an indole-acetic acid derivative. Its full name is 2-[1(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl] acetic acid with a molecular formula of $C_{18}H_{16}ClNO_4$ as shown in Figure 1(a). It has a molecular weight of 357.08 g/mol. It is an odorless pale-yellow crystalline substance [1].
[figure(s) omitted; refer to PDF]
Nicotinamide (NCT), often known as niacinamide, is 3-pyridine carboxamide as shown in Figure 1(b) [2]. It is an amide form of nicotinic acid or niacin as shown in Figure 1(c). It is also colorless crystals or white crystalline powder. NCT weighs 122.12 g/mol at the molecular level. Additionally, it is known as vitamin B3, one of the hydrophilic B vitamins [3]. NCT may be added to multivitamins products and supplementary oral dosage formulations. It is also found in many dermal preparations, such as solutions, creams, gels, and serums, which are used for skin care and many skin conditions due to its renewal and anti-inflammation properties [4]. Different studies showed that NCT has a mild to moderate anti-inflammation effect that can be combined with anti-inflammatory agents for improved therapy [5–7]. NCT was found to be a hydrotropic solubilizing agent. Hydrotropy is a phenomenon when a solute enhances the solubility of another poorly soluble solute in water and aqueous solutions [8, 9]. Lastly, NCT or its derivative 1-methyl nicotinamide may also have antacid and protective effects to reduce the risk of gastric ulcers related to NSAID intake or stress [10–12].
IND can be delivered using various routes and formulations ranging from oral 25-50 mg immediate-release, 75–100 mg sustained-release capsules, and 25 mg/5 mL suspensions to rectal 50–100 mg suppositories [13]. It can be found alone or with other active ingredients such as paracetamol in ParinCare™ oral capsules or vitamin B1 (thiamine) in local pharmaceutical products, including Indovit® and Indobina® oral capsules. IND, like any other NSAIDs, may cause stomach aches and many patients use antacids to protect the stomach. Orally, IND is fully absorbed through the gastrointestinal tract and has virtually 100% bioavailability [14]. However, due to its poor aqueous solubility, it is classified as class II in the biopharmaceutical classification system (BCS), i.e., it has low solubility and high permeability. This may limit or delay absorption causing a possible decrease in bioavailability. Therefore, there are different methods used to improve the dissolution rate and solubility of class II drugs such as IND. Physicochemical characteristics of drugs can be changed by using crystal engineering techniques such as salt formation, solvate (or hydrate), amorphous forms, and more recently co-crystal and co-amorphous systems, which are common ways to do this for many NSAIDs and other drugs. The co-amorphous system does extra benefit in increasing the stability of the amorphous forms along with increasing solubility and dissolution rate [15, 16].
NCT was used in many studies as an acceptable carrier or coformer with other drugs in co-amorphous drug-drug systems such as atorvastatin and valsartan [15, 17]. In 2021, researchers published a study (Fael and Demirel, 2021) where they suggested and designed the IND and NCT binary combination formula in the form of a co-amorphous drug-drug system, which was prepared for oral administration [18]. The previous work achieved two progressions. First, this co-amorphous system succeeded in increasing the solubility and dissolution rate of IND in the gastrointestinal tract and in limiting the recrystallization of IND to improve the stability of its amorphous form. Second, this new combination itself has promising therapeutical advantages from decreasing the risk of gastric ulcers caused by IND [10, 11] to building synergism in order to increase pain relief depending on the suggested neuroprotective and anti-inflammation effect of NCT [5, 19, 20]. This new suggested formula of IND-NCT could be the first IND-NCT combination that has not been marketed worldwide yet. According to Fael and Demirel, 2021, this IND-NCT co-amorphous mixture was combined by mixing their powders and applying heating then using quench cooling with liquid nitrogen to finally create the co-amorphous system. This was carried out without adding any other ingredient to their mixture. Hence, it is possible to simultaneously estimate this binary mixture using UV spectrophotometric techniques.
In this work, UV spectroscopic techniques were developed, validated, and deployed to simultaneously determine the combination of IND and NCT in the binary mixtures and in the IND dosage formulations that were spiked with NCT with the same IND : NCT molar ratios (M : M) of 1 : 10, 1 : 5, 1 : 3, 1 : 2, 1 : 1, and 1 : 0.5. These ratios were suggested and tested in the previous work of Fael and Demirel, 2021. In this study, a weight/volume ratio (μg/mL) has been

used. Thus, after being approximately converted depending on their molecular weights, mass ratios of 1 : 3, 1 : 2, 1 : 1, 1 : 0.5, 1 : 0.33, and 1 : 0.17, respectively, were used. There are many UV spectrophotometric techniques, including a pharmacopeial method for NCT [21–23] in addition to other methods such as high-performance liquid chromatography (HPLC), including a pharmacopeial method for IND [24–26] and electrochemical techniques [27, 28] to determine NCT or IND in one-component dosage formulations as well as in combinations with other active ingredients. Although there are already developed UV spectrophotometric methods to analyze, only IND in the existence of NCT in the same solution was used in the procedure of analysis, and NCT was used as a hydrotropic solubilizing agent to increase IND aqueous solubility. Usually, this was intended to use an eco-friendly solution capable of dissolving IND using NCT as a hydrotrope in high concentrations like 0.5–2.0 M. In other words, NCT was not a second ingredient with IND in its dosage formulations. These previous methods estimate IND concentration using the specific range of UV spectrum (300–350 nm), where there is only absorption of IND and zero absorption of NCT, while the whole NCT range of absorption interfered with the signal of IND [9, 29]. Our proposed analysis methods are the first ones to simultaneously determine both IND and NCT.

In this paper, few analytical UV spectrophotometric methods have been developed and validated to simultaneously determine both IND and NCT in their mixtures and spiked dosage formulations without prior separation or processing. The results have been shown and discussed. The first and second-order derivative spectra have been deployed using zero-crossing point and dual-wavelength techniques, in addition to ratio subtraction and spectrum subtraction techniques. In total, three UV spectrophotometric methods were developed. These methods have been proven to be accurate, precise, fast, simple, and eco-friendly for many combinations and multicomponent dosage formulations without using any hazardous solvents or invasive materials [30–35]. Additionally, more advanced UV spectrophotometric methods have been developed initially from these reported ones deploying further mathematical processing [36–38].

NCT is freely soluble in water, ethanol, and methanol, while IND tends to be practically insoluble in water. However, IND is sparingly soluble in methanol and ethanol, which were used to dissolve IND and NCT [21, 23]. Additionally, ethanol has a low impact on the environment and a high greenness index. Also, ethanol could be considered a renewable solvent used in many green and sustainable analytical methods and procedures [39, 40]. Thus, ethanol is used as a solvent in the present suggested methods.

2. Materials and Methods

2.1. Instruments

The main instrument was T80+ UV-visible (PG instruments UK), a spectrophotometer device that was coupled with a computer and dedicated software. This device uses 1-centimeter-width quartz cells. Other instruments include an analytical balance (Sartorius, model 2474, Germany), an ultrasonic bath (Power sonic, model 405, Korea), a porcelain mortar, a centrifuge device (90-1 Centrifuge, Shanghai Surgical Instruments Factory, China), volumetric flasks, and pipettes.

2.2. Solvents and Chemicals

Substances used in this work included indomethacin powder with 99.5% purity (BDH Laboratory Supplies, England), nicotinamide powder with 99% purity (BDH Laboratory Supplies, England), and absolute ethanol of analytical grade (Halley Medical/Eurolab, United Kingdom). The tested dosage formulations include Arthacin®, immediate-release (IR) capsules, which contain 50 mg of IND (Oshar Pharma, Syria), Indomed®, and sustained-release (SR) capsules (SR), which contain 75 mg of IND (Medico Labs, Syria).

2.3. Preparation of Standard Solutions

2.3.1. Standard Solution of Each Indomethacin and Nicotinamide

After weighing an equivalent amount to 62.5 mg of pure IND, it was transferred into a 20 mL flask and diluted with ethanol to obtain a standard stock solution of IND with a concentration of 3125 μg/mL. Then, 2 mL was pipetted out to a 25 mL flask to obtain a stock solution of IND with a concentration of 250 μg/mL. Finally, nine volumes of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 mL were pipetted out of the stock solution to nine 10 mL flasks and diluted with ethanol to prepare a set of standard solutions of IND with concentrations of 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0,

40.0, and 50.0 μg/mL, respectively. The same procedure was carried out to obtain NCT standard solution.

2.3.2. Preparation of Standard Mixture of Nicotinamide and Indomethacin

Binary mixtures of IND and NCT were prepared following the same steps demonstrated in the previous Section 2.3.1. However, the 10 mL flasks of the final standard solutions were prepared from the previous 25 mL flasks of both IND and NCT stock solutions. These spiked mixtures with added NCT were prepared in ratios of 1 : 3, 1 : 2, 1 : 1, 1 : 0.5, 1 : 0.33, and 1 : 0.17 (IND : NCT), equivalent to the molar ratios suggested by the designers of the IND/NCT co-amorphous formula (Fael and Demirel, 2021). For either IND or NCT, additional concentrations such as 3.75, 7.50, 22.50, and 45.00 μg/mL were prepared to obtain the previous ratios.

2.4. Preparation of the Mixture from Dosage Formulations

2.4.1. From Immediate-Release (IR) Capsules Formulation

Arthacin®, an oral capsule dosage formulation, is produced by Osher Pharma Co. It is labeled to contain 50 mg of powdered IND in each hard gelatin capsule. Its labeled excipients in the leaflet were colloidal silicon dioxide, magnesium stearate, microcrystalline cellulose, powdered cellulose, sodium lauryl sulfate, and sodium starch glycolate.

Ten capsules were emptied and the powder was weighed. Four quantities of the powder, each of which containing 50 mg of IND, were weighed and transferred to 25 mL flasks separately. One of them will be analyzed after sonicating, diluting, and adding standard additions to determine IND in the presence of the excipients without NCT and to compare the developed methods with a reference method. The other three had NCT added to them in three ratios: 1 : 3, 1 : 1, and 1 : 0.33 (IND : NCT). After adding about 20 mL of ethanol to each of them, they were sonicated for 10 minutes. Then, ethanol was added to the mark. After that, they were transferred to tubes to be centrifuged for 15 minutes. Then, 0.5, 1.0, and 1.5 mL were transferred from the previous 20 mL flasks to other three 20 mL flasks to reach IND concentrations of 50, 100, and 150 μg/mL and NCT concentrations of 150, 100, and 50 μg/mL, and to preserve the ratios of 1 : 3, 1 : 1, and 1 : 0.33 (IND : NCT), respectively. From each of them, 1 mL was pipetted out to four 10 mL flasks. 50, 100, and 150% standard additions of IND and NCT were added to three of them from the pure stock solution flasks of each of IND and NCT from Section 2.3.1. The solutions were then ready to be scanned and analyzed by UV spectrophotometer. Final IND concentrations were between 5 and 15 μg/mL without counting standard additions according to the labeled amount of IND in the capsules.

2.4.2. Sustained-Release (SR) Capsules Formulation

Indomed®, an oral capsule formulation, is produced by Medico Pharma Labs. It is labeled to contain 75 mg of IND pellets, which is a combination of two types (immediate-release and delayed-release pellets) in each hard gelatin capsule. Its labeled excipients in the leaflet were corn starch, hydroxyl propyl cellulose, ethyl cellulose, lactose, sucrose, and titanium oxide.

The same procedures of the IR were carried out here except for some changes. First, capsules of this formulation contain pellets instead of powder. A mortar was used to triturate the pellets into powder after weighing an equivalent of 100 mg of IND. Second, three ratios of 1 : 1, 1 : 0.33, and 1 : 0.17 (IND : NCT) were prepared instead. After centrifuging, 1.0, 1.5, and 1.5 mL were transferred from the previous 20 mL flasks to other three 20 mL flasks to reach IND concentrations of 200, 300, and 300 μg/mL and NCT concentrations of 200, 100, and 50 μg/mL. Finally, from each of them, 0.5 mL was pipetted out to four 10 mL flasks before adding the standard additions (0, 50, 100, and 150%). The solutions were then ready to be scanned and analyzed by a UV spectrophotometer. Final IND concentrations were 10 or 15 μg/mL without counting standard additions, according to the labeled amount of IND in the capsules.

2.5. UV Spectrophotometry Methods

Zero-order UV spectra of IND and NCT show IND's overlapping over the whole NCT absorption region. Three methods were found to simultaneously and quantitatively determine both of them.

2.5.1. Zero-Crossing Point in Derivative Spectrophotometry Methods (ZCD_1 and ZCD_2)

First and second-order derivation (D_1 and D_2) of spectra of IND, NCT, and their mixture were created. One zero-crossing point of IND in the first derivation was found at 223.5 nm where NCT can be determined without any

interference with IND or the excipients of its dosage formulations. However, after further examination and validation and because the absorptivity of both of them is high at this wavelength relative to the other region, it was not possible to simultaneously determine the mixtures probably with some ratios such as 1 : 3, 1 : 0.33, and 1 : 0.17, which made one of the components always reach the limit of the linearity range. Therefore, another zero-crossing point has to be found. In the second derivation, another one was found at 276.5 nm, where NCT can be determined without any interference from IND or the excipients of its dosage formulations. IND has a peak at 318 nm and there is no NCT signal at this wavelength, making it suitable for determining IND.

2.5.2. Dual-Wavelength in First-Order Derivative Spectrophotometry Method (DWD_1)

This method depends on locating two wavelengths λ1 and λ2, where the absorptivity (ε) of one component is the same at both of them (ε1 = ε2), and the second component has different absorptivity between the two wavelengths (ε1 ≠ ε2). The difference in the signal values of the two wavelengths ($\Delta\lambda$) is only dependent on the concentration of the second component (C). A regression equation can be created between C and $\Delta\lambda$ as shown in this equation ($\Delta\lambda = aC + b$) where "a" is the slope and "b" is the intercept with the *Y* axis. In the first-order derivative spectrum, 264.5 and 275.0 nm were found to have equal absorptivity for the NCT spectrum (εNCT1 = εNCT2). Therefore, they can be used to determine IND. 254.0 and 266.0 nm were found to have equal absorptivity for the IND spectrum (εIND1 = εIND2). Thus, they can be used to determine NCT.

2.5.3. Ratio Subtraction and Spectrum Subtraction Methods (RS and SS)

RS and SS are fingerprint techniques that allow us to extract a spectrum that is almost identical to one of the pure solutions of each component of the binary mixture. Zero-order Spectrum of a mixture can be divided by the spectrum of the pure standard solution (divisor) of one component that has a region within the spectrum with only absorption of this component and zero absorption of the other one. The resulting spectrum is called a ratio spectrum and has special characteristics. First, it has a constant region (or plateau region) that represents zero absorption region by the second component. Second, any two points of it, except for the constant region, have different values dependent on the second component only. After creating the ratio spectrum, the value of the constant region is subtracted from the spectrum. The resulting spectrum is then multiplied by the divisor spectrum to create a new resolved spectrum representing the second component and theoretically has a fingerprint spectrum that is almost identical to the one obtained by a pure solution of the second component, whose concentration could be determined at its peak λmax without any effect caused by the first component.

To determine the first component, we can subtract the newly resolved spectrum, which represents the second component, from the spectrum of the mixture to obtain a fingerprint spectrum that is almost identical to the one obtained by a pure solution of the first component, whose concentration could be determined at any proper wavelength without any effect caused by the second component.

IND is the one that has a noninterfering region. Thus, it has been chosen as a divisor. Choosing the best concertation of the standard solution as a divisor can be performed by finding a concentration with a minimum average absolute difference (AAD), which is simply the average of differences between the value of the constant in the ratio spectra created from a series of different concentration of the mixture and the value created from a series of pure standard solutions with same concentrations. The best concentration is the one that gives the minimum AAD. As a result, 15 μg/mL of IND was the most suitable concentration to pick as a divisor. The concentration of NCT was determined at λmax at 261.5 nm, while 268.0 nm was picked for IND determination.

Spectral ratio factor (SRF) was calculated to measure the similarity between the resolved spectra and the corresponding pure standard ones and to ensure the purity of the resolved spectra. SRF is usually a tool to measure the purity of a substance and it is used in this study to test the specificity of RS and SS by testing the purity of the resolved spectra and comparing them to the pure standard ones. Three wavelengths are chosen: λmax, λ1, and λ2 for both the resolved spectra and standard ones [41].(1)R1=AλmaxAλ1,R2=AλmaxAλ2,for the resolved spectrum:(2)Fx=Rx1Rx2=Aλx2Aλx1,for the standard spectrum:(3)Fst=Rst1Rst2=Aλst2Aλst1,SRF=FxFst.

The closer SRF gets to 1, the higher the purity and similarity the resolved spectrum has with the standard one. This indicates high specificity.

3. Results and Discussion

3.1. Finding the Optimal Wavelength for the Proposed Methods

Pure solutions of IND, NCT, and their mixtures have been scanned with the spectrophotometer. The zero-order spectra are shown in Figure 2. Spectra of the IND dosage formulations are shown in Figure 3, which demonstrate that the excipients of both dosage formulations of IND did not have much effect on the spectrum of IND, especially in the region between 250 and 400 nm.

[figure(s) omitted; refer to PDF]

The first-order derivative, which was amplified 10 times (scaling factor/amplifying coefficient was 10), was created for the dual-wavelength method (DWD_1). Dual-wavelength points were found to be 264.5 and 275.0 nm to determine IND (equal absorptivity for NCT) and 254.0 and 266.0 nm to determine NCT (equal absorptivity for IND) as shown in Figure 4. Also in the same figure, the zero-crossing point of IND at 223.5 nm is shown.

[figure(s) omitted; refer to PDF]

In the second-order derivative, which was amplified 40 times (scaling factor/amplifying coefficient was ×40), the best zero-crossing point of IND to determine NCT was found to be 276.5 nm. The best point to determine IND in D_2 was the IND peak at 318.0 nm, where there was no absorption of NCT. This zero-crossing method in D_2 (ZCD_2) is shown in Figure 5.

[figure(s) omitted; refer to PDF]

For the third method (RS and SS), a ratio spectrum was created, and the application of the method to retrieve the NCT and IND pure spectra from their mixture spectrum was carried out. The selected wavelength to determine IND is 268 nm and the one to determine NCT is 261.5 nm at its λmax. This is shown in Figure 6. To calculate the SRF used in specificity tests, two wavelengths (λ1 and λ2), which have the same absorptivity in the standard solutions, were picked. λ1 and λ2 are 295.0 and 318.0 nm, respectively, for IND, and they are 256.0 and 263.5 nm, respectively, for NCT.

[figure(s) omitted; refer to PDF]

3.2. Method Validation

3.2.1. Linearity

The spectra of the IND and NCT series were scanned, and the linearity was tested for the three methods. Additionally, the determination coefficient (r^2), the limit of detection (LOD), and the limit of quantification (LOQ) were calculated. The findings are displayed in Table 1. Additionally, the results of the system suitability test were placed in the last row of Table 1. System suitability was carried out by measuring six repetitions with a concentration of 20 μg/mL for each IND and NCT and calculating the RSD% of them for each method. The results demonstrate low LOD-LOQ for both IND and NCT, acceptable determination coefficients r2, and a wide linearity range. Data were gathered and handled in compliance with ICH guidelines, and the proposed methods were verified in concurrence with ICH criteria with the following tests [42]. For NCT linearity range, it may have a higher upper limit than 50–60 μg/mL in the zero-order spectrum but because whenever NCT exists with IND in any of the studied ratios, their mixtures get out of linearity above that previous concentration, which is true with IND too. IND linearity range may also have a higher upper limit than 50 μg/mL by itself at its 318.0 nm peak but it is not possible to determine NCT when reaching above this concentration.

Table 1

Analytical performance data for the proposed methods.

Parameter	Indomethacin			Nicotinamide		
Method	ZCD_2	DWD_1	SS	ZCD_2	DWD_1	RS
Wavelength (nm)	318.0	264.5–275.0	268.0	276.5	254.0–266.0	261.5

Linearity range (μg/mL)	2.5–50.0	2.5–40.0	2.5–40.0	2.5–50.0	2.5–50.0	2.5–60.0
Determination coefficient r2	0.9998	0.9997	0.9997	0.9997	0.9999	0.9999
Slope	0.00324	0.00761	0.04292	0.01681	0.01602	0.02367
Intercept	0.00015	0.00179	0.00051	0.00029	0.00190	−0.00524
SD of slope	0.00002	0.00006	0.00032	0.00010	0.00007	0.00008
SD of intercept	0.00040	0.00127	0.00694	0.00268	0.00183	0.00255
95% confidence range of slope*	(0.00321–0.00328)	(0.00747–0.00775)	(0.04215–0.04369)	(0.01658–0.01705)	(0.01586–0.01619)	(0.02349–0.02386)
95% confidence range of intercept*	(−0.00081–0.00110)	(−0.00131–0.00489)	(−0.01646–0.01749)	(−0.00604–0.00663)	(−0.00243–0.00623)	(−0.01112–0.00064)
LOD (μg/mL)	0.41	0.55	0.53	0.53	0.38	0.36
LOQ (μg/mL)	1.25	1.66	1.62	1.59	1.14	1.08
System suitability**	0.77	0.33	0.15	0.83	0.23	0.41

*The range is represented by lower limit–upper limit. **RSD% of six repetitions of concentration of 20 μg/mL for each of IND and NCT.

3.2.2. Accuracy Test

It was performed for IND and NCT for the three methods. Three repetitions for each of the six concentrations were analyzed. Table 2 shows the means and the relative standard deviations (RSD%) of the total 18 samples of NCT and IND. Six concentrations from the linearity range instead of three were studied because some ratios of the mixture have one of the components being only useable in either the upper or lower half of the linearity range. Due to this issue with these ratios, six concentrations spread through the whole linearity range were studied in this test. The proposed methods appear to achieve the validation criteria in the accuracy test according to ICH in a wide range of concentrations.

Table 2

Data of the accuracy tests of IND and NCT for the proposed methods.

Concentration (μg/mL)	IND mean recovery ± RSD %*			NCT mean recovery ± RSD %*		
Method	ZCD_2	DWD_1	SS	ZCD_2	DWD_1	RS
5	99.06 ± 0.62	101.71 ± 0.45	99.44 ± 1.78	99.13 ± 1.75	100.15 ± 1.34	99.58 ± 1.41

10	98.79±0.48	100.06±1.44	99.44±0.51	99.92±0.26	99.21±0.75	99.25±1.14
15	99.93±1.75	100.81±1.11	101.59±1.27	99.16±1.53	98.20±0.38	100.46±0.49
20	100.35±0.96	100.22±0.98	100.09±1.01	100.66±0.62	100.46±0.23	100.55±1.52
25	99.78±1.94	100.09±1.66	99.99±0.51	98.48±1.87	100.44±1.30	99.27±0.34
30	99.70±1.88	99.18±0.81	98.98±1.20	100.97±0.55	100.71±0.76	100.13±0.81
Mean recovery± RSD %**	99.60±1.94	100.35±1.25	99.92±1.28	99.72±1.40	99.86±1.17	99.87±1.04

*Three repetitions of each concentration were analyzed with nine total repetitions. **All 18 repetitions of each wavelength.

3.2.3. Precision Test

It was achieved using three repetitions for each of the three concentrations (7.5, 15, and 22.5 μg/mL for IND and 7.5, 15, and 30 μg/mL for NCT) for each method to test the intraday repeatability precision. The same number of samples were analyzed once during each of the following two days to test the intermediate interday precision with a total of 27 samples. RSD% values of the nine samples each day are shown in Table 3 beside the total RSD% of all the 27 samples. The proposed methods appear to achieve the validation criteria in the precision test according to ICH.

Table 3

Data of the precision study of IND and NCT for the proposed methods.

Precision	IND RSD%			NCT RSD%		
Method	ZCD_2	DWD_1	SS	ZCD_2	DWD_1	RS
Intraday day 1	1.49	0.97	0.35	1.21	0.89	0.61
Intermediate day 2	1.44	1.08	0.99	1.57	1.24	0.56
Intermediate day 3	1.37	1.23	1.09	1.66	1.28	0.63
Total repetitions*	1.46	1.18	1.12	1.52	1.28	1.16

*For each category, there are three concentrations and three repetitions for each concentration with a total of 27 repetitions.

3.2.4. Specificity Test

Specificity studies were conducted on the mixture of IND and NCT with six ratios 1:3, 1:2, 1:1, 1:0.5, 1:0.33, and 1:0.17 (IND:NCT) using the three methods. For IND, a concentration of 15 μg/mL was used for six concentrations of NCT, while for NCT, a concentration of 7.5 μg/mL was used for six concentrations of IND. Results are shown in

Table 4 for IND and Table 5 for NCT. For RS and SS, the SPF values were calculated and shown. The methods achieved accepted results in specificity tests for a wide range of the ratios of the binary mixture. Some methods for NCT determination, such as DWD_1 and RS, failed the test only in the ratio of 1:0.17 (IND:NCT) because the absorption of the mixture exceeded the upper limit of the linearity range in the zero-order spectrum due to the high IND concentration of 45 μg/mL along with 7.5 μg/mL of NCT. In the next Figures 7–12, the spectra of the specificity test for IND and NCT are shown, where each of them shows the spectra of six mixtures of IND and NCT. Interchangeably, one of the two components has one constant concentration, while the other has six concentrations to obtain the six ratios of 1:3, 1:2, 1:1, 1:0.5, 1:0.33, and 1:0.17. ZCD_2 is shown in Figure 7 for IND and Figure 8 for NCT. DWD_1 is shown in Figure 9 for IND and Figure 10 for NCT. SS is shown in Figure 11 for IND, where the resolved spectrum of IND is shown with the standard one to be compared with. RS is shown in Figure 12 for NCT, where the resolved spectrum of NCT is shown with the standard one to be compared with.

Table 4

Data of the specificity test of IND for the proposed methods.

Ratio (IND:NCT)	NCT concentration (μg/mL)	IND (15 μg/mL) recovery%*		
ZCD_2	DWD_1	SS	1:3	45.0
100.90	100.68	99.09	1:2	30.0
100.07	100.30	98.13	1:1	15.0
99.65	99.64	99.18	1:0.5	7.5
98.61	99.14	98.08	1:0.33	5.0
101.53	99.60	98.73	1:0.17	2.5
99.24	100.65	100.46	SRF for SS method	

*Mean recovery % of three experiments.

Table 5

Data of the specificity test of NCT for the proposed methods.

Ratio (IND:NCT)	IND concentration (μg/mL)	NCT (7.5 μg/mL) recovery%*		
ZCD_2	DWD_1	RS	1:3	2.50
101.80	100.52	101.41	1:2	3.75
101.40	98.19	101.79	1:1	7.50
98.12	98.10	99.42	1:0.5	15.00
98.44	98.60	98.99	1:0.33	22.50

98.28	100.19	98.39	1:0.17	45.00
101.96	114.41	93.86	SRF for RS method	

*Mean recovery % of three experiments.

[figure(s) omitted; refer to PDF]

3.2.5. Application of the Proposed Method on Dosage Formulations

IR and SR were analyzed for their content of IND in addition to the content of the added NCT from standard solutions. Three ratios were analyzed for each dosage formulation and three repetitions for each ratio. In this application, the standard additions method has been used for both NCT and IND. All amounts and concentrations that were prepared for this test were mentioned in Sections 2.4.1 and 2.4.2. Data of the application on the dosage formulations are shown in Table 6. The results of ZCD_2 were accepted in both dosage formulations in all ratios. The results of both DWD_1 and RS were accepted in both dosage formulations, except for the ratio of 1:0.17.

Table 6

Data of the analysis of IND and NCT in the dosage formulations with the proposed methods.

Dosage formulation + (IND: NCT) ratio	IND mean recovery ± RSD %*			NCT mean recovery ± RSD %*		
Method	ZCD_2	DWD_1	SS	ZCD_2	DWD_1	RS
IR[1] mg (1:3)*	100.42 ± 0.43	100.57 ± 0.72	101.02 ± 0.61	99.52 ± 1.46	100.92 ± 0.61	100.83 ± 0.38
IR (1:1)*	101.28 ± 0.33	101.17 ± 0.64	101.14 ± 1.21	101.27 ± 0.34	99.45 ± 1.18	99.79 ± 0.34
IR (1:0.33)*	101.36 ± 0.37	101.82 ± 0.59	99.98 ± 0.47	101.37 ± 1.18	100.62 ± 1.83	99.58 ± 1.14
IR mean recovery ± RSD %	101.02 ± 0.55	101.19 ± 0.78	100.71 ± 0.90	100.72 ± 1.30	100.33 ± 1.31	100.06 ± 0.85
SR[2] (1:1)*	98.63 ± 0.54	99.00 ± 0.50	99.16 ± 1.51	99.59 ± 1.44	99.91 ± 0.48	100.44 ± 0.65
SR (1:0.33)*	99.63 ± 0.90	99.74 ± 0.48	99.51 ± 0.57	101.26 ± 0.95	101.62 ± 0.34	98.90 ± 0.71
SR (1:0.17)*	98.62 ± 0.87	103.73 ± 0.34	98.63 ± 0.66	101.53 ± 0.89	104.53 ± 0.77	96.79 ± 0.97
SR mean recovery ± RSD %	98.96 ± 0.85	100.82 ± 2.22	99.10 ± 0.95	100.79 ± 1.32	102.02 ± 2.04	98.71 ± 1.75

[1]IR contains 50 mg of IND as labeled. [2]SR contains 75 mg of IND as labeled. *Three repetitions of each ratio.

3.2.6. Comparing with Reference Methods

As there is no previous method for the simultaneous determination of IND and NCT in a mixture, each component was analyzed solely with the three proposed methods and a reference method to compare with. IND IR capsules were analyzed five times using the British pharmacopoeia (BP) HPLC assay method [24] in addition to the proposed methods. NCT pure powder (as there are no local NCT tablets available) was analyzed six times using the UV spectrophotometric assay method in BP [21] in addition to the proposed methods. The resulting data are shown in Table 7 along with statistical values such as standard deviation and variance of each method in addition to the *t*-test and *F*-test compared to the reference methods for each IND and NCT separately. The tests show that there are no statistically significant differences between the proposed methods and the reference ones.

Table 7

Data of the comparison between the proposed methods and reference methods.

	Sample number	Indomethacin in IR capsules[1]				Pure nicotinamide[2]			
ZCD_2	DWD_1	SS	*BP19 HPLC	ZCD_2	DWD_1	RS	*BP19 UV	Recovery %	1
100.04	100.58	101.32	99.55	100.21	100.23	99.13	100.14	2	101.09
100.61	101.22	98.74	99.02	99.95	98.96	99.97	3	101.04	99.79
100.89	100.21	98.04	99.82	98.85	99.86	4	101.27	100.86	100.15
100.80	98.10	99.73	98.53	99.53	5	101.46	101.25	100.42	101.12
98.55	99.54	100.30	99.30	6	—	—	—	—	98.34
99.82	98.07	99.06	Mean recovery%		100.98	100.62	100.80	100.08	98.71
99.85	98.97	99.64	SD		0.55 09	0.53 50	0.505 4	0.96 09	0.81 60
0.2309	0.7500	0.4166	Standard error		0.24 64	0.23 93	0.226 0	0.42 97	0.33 32
0.0943	0.3062	0.1701	Variance		0.30 34	0.28 63	0.255 4	0.92 33	0.66 59

0.0533	0.5625	0.1736	*t*-test with ref method **	1.809	1.086	1.475	—	2.490
1.064	1.908	—	*F*-test with ref method ***	3.043	3.225	3.615	—	3.837

[1]The number of samples of immediate-release capsules was 5 ($n=5$). [2]The number of samples of pure nicotinamide was 6 ($n=6$). *The reference methods to be compared with. **Critical values of the two-tailed test in 5% significance level are 2.776 for $n=5$ and 2.571 for $n=6$. ***Critical values of the *F*-test in 5% significance level are 6.39 for $n=5$ and 5.05 for $n=6$.

4. Conclusion

The results of this work suggest that the developed methods are rapid, easy to use, accurate, precise, and simple. These techniques can be applied in regular quality control tests to simultaneously determine NCT and IND in binary mixtures, immediate-release capsules, and sustained-release capsules. These methods succeeded in determining both IND and NCT in the presence of excipients in the mixture without prior separation. The newly suggested co-amorphous systems have no special excipients or added materials other than the common ones in any oral dosage formulations. Therefore, these methods can be used to simultaneously determine IND and NCT in the co-amorphous systems achieving the same results above.

Authors' Contributions

AS prepared the sample, performed the analysis, and wrote the report and NS oversaw the study's planning and execution as well as its writing and review. The final manuscript was read and approved by both the writers.

Acknowledgments

This work was not funded by any third party.

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DETAILS

Subject:	Spectrophotometry; Ratios; Ulcers; Antacids; Binary mixtures; Formulations; Nonsteroidal anti-inflammatory drugs; Inflammation; Dosage; Drug dosages; Ethanol; Bioavailability; Nicotinamide
Business indexing term:	Subject: Ratios
Location:	United Kingdom--UK; England; Syria
Editor:	Daipayan Roy
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-11-20 (Received); 2024-01-16 (Revised); 2024-01-30 (Accepted); 2024-02-20 (Pub)
DOI:	https://doi.org/10.1155/2024/2035824
ProQuest document ID:	2933994786
Document URL:	https://www.proquest.com/scholarly-journals/method-development-simultaneously-determining/docview/2933994786/se-2?accountid=211160

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Last updated:	2024-03-04
Database:	Publicly Available Content Database

Prioritizing Asthma Treatment Drugs through Multicriteria Decision Making

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ProQuest document link

ABSTRACT (ENGLISH)

Asthma is a medical condition characterized by inflammation, narrowing, and swelling of a person’s airways, leading to increased mucus production and difficulties in breathing. Topological indices are instrumental in assessing the physical and chemical attributes of these asthma drugs. As resistance to current treatments continues to emerge and undesirable side effects are linked to certain medications, the search for novel and enhanced drugs becomes a top priority. In this study, the examination of 19 distinct asthma medications was focused. In this study, quantitative structure-activity relationship (QSAR) and quantitative structure-property relationship (QSPR) modeling, in combination with multicriteria decision-making (MCDM) technique VIKOR (VIekriterijumsko KOmpromisno Rangiranje) were employed on asthma drugs, to achieve the most favorable rankings for each asthma drug, taking into account their distinct properties. The topological indices employed for QSPR modeling were Randic index, reciprocal Randic index, Zagreb indices, hyper-Zagreb index, harmonic index, geometric arithmetic index, and forgotten index.

FULL TEXT

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1. Introduction

Asthma is an intricate, multifaceted, and chronic noncommunicable disease. Asthma is a heterogeneous disease primarily characterized by inflammation and bronchoconstriction, which causes the airway to narrow [1]. Airflow to the lungs becomes difficult as inflamed airways are more sensitive to environmental cues, producing more mucus. As a result, a person may have an “asthma attack,” characterized by intense coughing, wheezing, pressure in the chest, and breathing issues [2]. Exposure to certain allergens can trigger asthma symptoms [3–5]. Immunoglobulin *E* (IgE) which is allergy-specific responds to irritants and allergens by modulating the production of histamine, tryptase, prostaglandins, and leukotrienes, which constricts the airways. Some nonsteroidal anti-inflammatory medications

may also cause the production of mediators, which causes bronchoconstriction [6–8]. Histamine, protease enzymes, tumor necrosis factor (TNF_{α}), prostaglandins (PG_s), leukotrienes (LT_s), and interleukins (IL_s) are among the chemical mediators implicated in asthma. These are all generated from mast cells, which occur in the lungs and inflammatory cells. Together, these mediators constrict bronchial smooth muscle and produce mucosal oedema, hyperemia, and a discharge of viscid secretions, all of which lead to reversible airway blockage. Inhaled corticosteroids, leukotriene modifiers, and other long-term control drugs for asthma can be divided into two categories: quick-relief medications (bronchodilators), which offer rapid relief during an asthma attack, and long-term control therapies (inhaled corticosteroids, etc.), which assist to reduce inflammation and avoid symptoms. The bronchoconstriction, increased vascular permeability, and eosinophil recruitment caused by the CysLT1 (cysteinyl leukotrienes) receptor are competitively antagonised by the mediator-inhibiting drugs, such as montelukast, pranlukast, and zafirlukast. The drugs ramatroban, setipiprant, and fevipiprant treat the symptoms of asthma by suppressing bronchoconstriction, airway hyper-responsiveness, and inflammatory cell infiltration by antagonistically adhering to the DP2 receptor. Toreforant, an antihistamine, suppresses the effects of histamine, which causes bronchoconstriction and therefore reduces asthma symptoms. Salbutamol, terbutaline, and salmeterol are bronchodilators that stimulate the β2 receptor. These trigger bronchial muscle cells to release more cAMP, resulting in muscle relaxation [9]. The 2019 coronavirus disease (COVID-19) has emerged as a significant global health concern, particularly for those with preexisting medical issues. Patients dealing with asthma are deemed to be more vulnerable since COVID-19 can seriously impair respiration. Some research suggests that individuals with asthma may be more vulnerable to COVID-19 [10].

Mathematical chemistry is the transformation of mathematical concepts in the research of the chemistry field. The mathematical modeling of chemical mechanisms is part of it. The field of graph theory known as chemical graph theory currently has several applications in the field of chemistry [11]. In theoretical chemistry, chemical compounds are modeled as molecular graphs with vertices and edges. Topological indices play a crucial role in the field of chemical graph theory and computational chemistry, providing valuable insights into the structural characteristics and properties of chemical compounds [12]. These indices, derived from the connectivity of atoms within a molecular structure, offer a quantitative measure of molecular topology without delving into intricate three-dimensional details. By condensing complex molecular structures into numerical values, topological indices facilitate the prediction of various physicochemical, biological, and pharmacological properties of compounds. Researchers leverage these indices for rational drug design, environmental risk assessment, and the elucidation of structure-activity relationships in diverse chemical and biological systems [13–16]. The significance of topological indices lies in their ability to bridge the gap between chemical structure and properties, thereby enhancing our understanding of molecular behavior and aiding in the efficient design and optimization of chemical entities for specific applications. Quantitative structure-activity and structure-property relationship (QSAR/QSPR) models [17] are the correlation between each numerical descriptor and the attributes related to the referred structure. These models are utilized for the prediction and analysis of the activity, as well as the physical and chemical properties, of chemical structures developed for drugs and materials. This technique partitions a molecule into several numerical concepts called chemical indices [18], each of which independently describes its attributes. First, it has been proven that there is a connection between a chemical compound and a physicochemical or biological property. Next, predictions are made regarding the physicochemical characteristics or biological activities of structurally related compounds [19–27]. Multicriteria decision making (MCDM) [28] is a remarkable field within operational research (OR) [29]. The exploration of diverse objectives using mathematical programming has emerged as a prominent tool for making optimal decisions in specific contexts. There are numerous MCDM methods categorized in different manners. We use the highly compatible VIKOR approach in this research work. Yugoslav researchers Z. S. Jovanovic and M. R. Bozic developed the MCDM method known as VIKOR (VIekriterijumsko KOmpromisno Rangiranje) in 1990 [30]. In a situation when there are several complex criteria to consider when making a decision, the VIKOR technique offers a systematic way to analyze and rank possibilities. It enables decision-makers to pinpoint the most advantageous compromise solution. It considers the simultaneous objectives of increasing the advantages and reducing the

disadvantages. Here, I am going to introduce and develop a mathematical connection between OR (operations research) and the biochem graphical sciences. I have created a decision matrix that represents the performance of each alternative concerning each criterion. The VIKOR approach is a practical and simple way to determine the suitable drugs for a patient. Li et al. used an objective approach to handle criteria weighting [31] while applying the VIKOR method to determine optimal rankings for various anticancer drugs, achieving results for properties such as boiling points and enthalpy of vaporization using QSPR. Guoping Zhang et al. ranked specific networks by applying the SAW method and TOPSIS method [32], and they determined criteria weights using the entropy method.

In this study, the optimal ranking will be determined for asthma drugs that have not been previously investigated. There are 19 alternatives (drug structures) as illustrated in Figure 1.1 in Supplementary Materials and 10 attributes (topological indices). First, criteria importance through intercriteria correlation (CRITIC) is employed for the calculation of objective weights assigned to each criterion associated with the selection of asthma drugs. I have also analyzed the correlation between the calculated topological indices and the rankings obtained through the MCDM process. The CRITIC method takes into account the intensity of contrast and conflict within the decision-making problem's structure [33]. These contrasts between criteria are established through correlation analysis [34]. Readers are urged to explore additional techniques for weight computations, such as BCM and best worst criteria [35, 36]. Lastly, VIKOR is used to provide a ranking of potential asthma drugs. In the present investigation, the VIKOR technique optimizes the application of QSPR modeling to rank the targeted asthma drugs most effectively. The findings are derived from the QSPR analysis conducted on 19 effective asthma drugs [37].

The objective of this research work is to compare asthma drugs and identify the most potent ones by evaluating and ranking the most effective drugs for asthma by considering the characteristics (where flash point and boiling point are considered) they exhibit. The major goal of such a study would be to rank asthma treatment medicines in order of importance. Prioritization could be based on efficacy, safety, cost-effectiveness, availability, and patient preferences. The study could help to find the most effective medications or drug combinations for assisting asthma patients in achieving better control over their condition, reducing symptoms, and improving their quality of life. A study of this nature has the potential to enhance the allocation of resources by healthcare systems and policymakers, as it allows for an evaluation of which pharmaceuticals offer the most favorable combination of cost-effectiveness and positive health outcomes.

The research could contribute to the discovery of medications that demonstrate notable efficacy within specific subgroups of asthma patients. Emphasizing drugs that deliver both effectiveness and cost-efficiency has the potential to generate substantial cost reductions for both healthcare systems and patients. Through the identification of optimal treatment choices, the research has the potential to enhance the overall quality of care for asthma patients, with the possibility of lowering hospitalization rates and mitigating adverse consequences linked to inadequately controlled asthma. The study's results may exert an impact on shaping healthcare policies and formulating clinical practice guidelines about asthma treatment, thereby assisting healthcare practitioners in making well-informed decisions.

The rest of this paper unfolds as follows: In the following section, the author will examine fundamental graph theory terms and introduce the degree-based topological indices that are pertinent to our analysis. This emphasizes the importance of investigating the material and the forthcoming research. By calculating these topological indices, researchers may obtain valuable insight into the molecular structure-activity relationships of these drugs. In Section 3, Microsoft Excel is identified as the data analysis tool used to extract results generated by QSPR modeling via regression analysis. Section 4 discusses the results and the associated discussions. First, the author establishes a framework for integrating QSPR findings into VIKOR analysis. This is achieved by introducing the CRITIC method and providing a detailed explanation of the steps involved in CRITIC. Subsequently, the CRITIC technique is implemented to determine criteria weights. Moreover, both beneficial and nonbeneficial criteria affecting flash point and boiling point are evaluated. Additionally, rankings for flash point and boiling point are presented, and weights are assigned based on correlation coefficients. Section 5 offers additional discussion and concluding remarks and outlines directions for future research.

2. Definitions of Degree-Based Topological Indices of Graph

We consider the molecular graph as an ordered pair, denoted by MVM,EM, with a vertex set VM and an edge set E M, respectively. The edge set is referred to as linkages between the atoms, while the vertex set is referred to as atoms. Let p∈VM, the number of edges that are incident to a vertex p, represented as Rp, determine its degree (or valency). In this study, connected, simple, finite, and loop-free graphs are utilized specifically.

Definition 1.

Randic´ index was given by Milan Randic´ in [38] and is described as the sum of reciprocals of the square root of the product of vertex degrees of all edges in the graph. Mathematically, it can be represented as follows:(1)RM=∑pq∈E M1Rp×Rq.

Definition 2.

The reciprocal Randic index, represented as RR(M), is a modified version of the Randic index that adjusts the calculation by considering the square root of the product of vertex degrees for each edge, rather than using its reciprocal. It was defined by Favaron et al. [39].

The formula for the reciprocal Randic index of a molecular graph M is given as follows:(2)RRM=∑pq∈EMRp×Rq.

Definition 3.

Gutman and Trinajstic´ introduced and defined the first Zagreb index (denoted as M1M) as the sum of the vertex degrees for each edge in the graph and the second Zagreb index (denoted as M2M) in [40–42] as the sum of the products of the vertex degrees for each edge in the graph. Mathematically, it can be represented as follows:(3)M1M =∑pq∈EMRp+Rq,M2M=∑pq∈EMRp×Rq.

Definition 4.

In [43], Shirdel et al. introduced the hyper-Zagreb index and defined it as the sum of the square of the vertex degrees for each edge in the molecular graph. Mathematically, it can be represented as follows:(4)HMM=∑pq∈EMR p+Rq2.

Definition 5.

The harmonic index was proposed by Fajtlowicz [44]. It is calculated based on the harmonic mean of the degrees of adjacent vertices in the graph.

The formula for the harmonic index of a graph M is given as follows:(5)HM=∑pq∈EM2Rp+Rq.

It emphasizes the contribution of less connected vertices to the overall connectivity of the graph. A higher harmonic index value indicates a more complex and well-connected graph structure.

Definition 6.

Vukicevic and Furtula [45] proposed the geometric arithmetic index as the geometric mean and arithmetic mean of the degrees of adjacent vertices in the graph. The formula for the geometric arithmetic index of a molecular graph M is given as follows:(6)GAM=∑pq∈EM2Rp×RqRp+Rq.

It provides insights into the balance between connectivity and degrees adjacent vertices in a molecular graph.

Definition 7.

Furtula and Gutman [46] introduced the forgotten topological index as the sum of squares of the degrees of vertices of a graph. Mathematically, it can be represented as follows:(7)FM=∑pq∈EMRp2+Rq2.

Definition 8.

Zhou and Trinajstić [47] introduced the sum-connectivity index, denoted as SCI(M), which is a measure of the sum of the reciprocal of square roots of the sums of degrees of adjacent vertices in the graph. The formula for the sum-connectivity index of a molecular graph M is given as follows:(8)SCIM=∑pq∈EG1Rp+Rq.

It provides insights into the connectivity patterns within a molecular graph.

Definition 9.

In [48], Estrada et al. introduced and investigated the atom-bond connectivity index (denoted as ABC(M)). It is defined as follows:(9)ABCM=∑pq∈EMRp+Rq−2Rp×Rq.

Atom-bond connectivity index focuses on the contributions of individual bonds to the overall connectivity of a molecular graph.

3. Materials and Methods

The data analysis tool of Microsoft Excel is used to obtain results generated from QSPR modeling via regression analysis. All the data in charts and tables evaluated for the VIKOR technique are computed in Microsoft Excel.

4. Result and Discussions

4.1. Creating a Framework for Incorporating QSPR Findings into VIKOR Analysis

We are evaluating alternative medications for asthma disease based on standardized criteria established during our case study, which aims to generate quantitative structure-property relationship (QSPR) observations. Our goal is to obtain the most optimal outcome as we make our final decision. VIKOR utilizes a ranking system to assess medications for asthma treatment and manages the compromised treatment that closely aligns with the best option.

Step 1: We aim to identify the optimal best si+ and worst si− values for all criterion functions, denoted as i=1,…,n.

For benefit-type functions (i.e., where improvement is desired), the ideal best value si+ is determined as the maxsij:j=1,2,…,J while the ideal worst value si− is determined as the minsij:j=1,2,…,J.

Conversely, for cost-type functions (where higher values are preferred), si+=minsij:j=1,2,…,J while si−=maxsij:j=1,2,…,J.

Step 2: Evaluation of Dj (weighted normalized Manhattan distance) and Bj (weighted normalized Chebyshev distance) values.(10)Dj=∑j=1mwi×si+−sijsi+−si−,Bj=maxwi×si+−sijsi+−si−,

here wi represents the criteria weights of criteria, Dj represents a decision value associated with a specific decision alternative, indexed by j, and Bj represents the maximum value among a set of expressions, each evaluating the performance or desirability of alternative j concerning different criteria in a multicriteria decision making (MCDM).

Step 3: Calculation of values Lj for j=1,2,…,J is achieved using the following equality:(11)Lj=v×Dj−D+D−+D++1−v×Bj−B+B−+B+.

Here, we define D+=minDj,j=1,2,…,J, D−=maxDj,j=1,2,…,J, B+=minBj,j=1,2,…,J, and B−=maxBj,j=1,2,…,J. Additionally, we introduce “v” as the weighting factor for the strategy of maximizing group utility. Here, 1−v represents the weight assigned to individual regret. This strategy may be influenced by a compromise value of v, which could be set at 0.5.

Step 4: We rank the alternatives in ascending order based on the values of D, B, and L, starting with the lowest values.

The alternative with the lowest VIKOR value is identified as the optimal choice. This suggestion aligns closely with the ideal point, as it is ranked best according to the L (minimum) measure.

4.2. Implementation of the CRITIC (Criteria Importance through Intercriteria Correlation) Technique

Careful consideration is essential when choosing drugs for disease treatment, as it constitutes a significant and crucial decision. Multicriteria decision making (MCDM) is used to address this choice, considering both quantitative and qualitative factors. This section employs CRITIC (criteria importance through intercriteria correlation) methods to address the issue of selecting asthma treatment drugs. The CRITIC method is utilized to determine the weights of the criteria for selecting the most suitable asthma drugs, while the VIKOR approach is employed to generate a comprehensive ranking of the available asthma drug alternatives.

Step 1: The decision matrix A is formed. It shows the performance of different alternatives concerning various criteria.(12)A=aijm×n=a11a12⋯a1na21a22⋯a2n⋮am1⋮am2⋱⋮⋯amn i=1,2…,m and j,

where aij presents the performance value of ith alternative on jth criterion.

Step 2: The decision matrix is normalized using the following equation:(13)aij⁎=aij−min aijmax aij−min aij i=1,2,…,m and j=1,2,…,n,

where aij⁎ is the normalized performance value of ith alternative on jth criterion. Here, it should be noted that normalization does not take into account the type of criteria.

Step 3: While determining the criteria weights, both the standard deviation of the criterion and its correlation between other criteria are included. In this regard, the weight of the jth criterion (ωj) is obtained as follows:(14)wj=Cj∑j=1nCj,

where Cj is the quantity of information contained in jth criterion determined as Cj=σj∑j=1n1−rjj′, where Cj represents

the contribution or importance of the *j*th criterion and σj is the standard deviation of the j th criterion and jj′ is the correlation coefficient between the two criteria. It can be concluded that this method gives the higher weight to the criterion which has high standard deviation and low correlation with other criteria. Specifically, a higher value of Bj indicates that a greater amount of information is obtained from the given criterion, so the relative significance of the criterion for the decision-making problem is higher.

Using the technique mentioned above, we have determined the weights of topological indices, as presented in Table 1, whereas standard error as beneficial and nonbeneficial criteria for both cases shown in Table 2.

Table 1

Weight determination through the CRITIC method.

Topological indices	б	Σ (1 − rjj′)	Cj	Wj
ABC (G)	0.288	11.877	3.424	0.110
GA (G)	0.291	8.177	2.381	0.077
SCI (G)	0.282	8.093	2.280	0.074
RR (G)	0.290	13.881	4.026	0.130
R (G)	0.277	8.0755	2.236	0.072
F (G)	0.301	8.158	2.455	0.079
HM (G)	0.286	8.100	2.317	0.075
H (G)	0.284	13.757	3.911	0.126
M_1 (G)	0.282	6.107	1.724	0.056
M_2 (G)	0.284	13.851	3.934	0.127
ABC (G)	0.286	8.107	2.321	0.075

Table 2

Beneficial and nonbeneficial criteria impacting flash point and boiling point (BP).

Topological index	Flash point	Boiling point
ABC (G)	0.946	0.970
GA (G)	0.952	0.968
SCI (G)	0.953	0.971

RR (G)	0.952	0.974
R (G)	0.949	0.967
F (G)	0.909	0.941
HM (G)	0.928	0.954
H (G)	0.949	0.964
M_1 (G)	0.943	0.970
M_2 (G)	0.945	0.966

I have completed the step-by-step calculations for both Steps 1 and 2, as shown in Tables 1.1 and 1.2, respectively, for flash point. Similar calculations for boiling point are performed in Tables 2.1 and 2.2. Furthermore, the final calculations for Steps 3 and 4, focusing on the investigation of flash point, are included in Table 3, whereas the final calculations for Steps 3 and 4, concentrating on the investigation of boiling point, are included in Table 4.

Table 3

Results for Dj, Bj, Lj, and ranking concerning flash point.

Drugs	*D*	*B*	*L*	Ranks
Toreforant	0.514	0.071	0.351	4
Bedoradrine	0.521	0.078	0.417	8
Abediterol	0.536	0.088	0.516	16
Fevipiprant	0.515	0.061	0.280	1
Setipiprant	0.537	0.076	0.435	9
Ramatroban	0.528	0.069	0.368	7
Zafirlukast	0.624	0.120	0.929	19
Pranlukast	0.565	0.106	0.703	17
Montelukast	0.624	0.119	0.926	18
Vilanterol	0.508	0.085	0.443	10
Indacaterol	0.521	0.071	0.364	6

Olodaterol	0.510	0.063	0.282	2
Formoterol	0.466	0.075	0.284	3
Salmeterol	0.486	0.079	0.354	5
Terbutaline	0.408	0.119	0.490	14
Salbutamol	0.414	0.114	0.468	11
Metaproterenol	0.391	0.122	0.476	12
Isoproterenol	0.391	0.122	0.476	13
Epinephrine	0.375	0.130	0.500	15
	D*	0.375	B*	0.061
	D−	0.624	*B*−	0.130

Table 4

Results for Dj, Bj, Lj, and ranking about boiling point (BP).

Drugs	*D*	*B*	*L*	Ranks
Toreforant	0.469	0.056	0.210	8
Bedoradrine	0.453	0.055	0.181	5
Abediterol	0.428	0.058	0.168	3
Fevipiprant	0.450	0.058	0.237	11
Setipiprant	0.455	0.059	0.211	9
Ramatroban	0.476	0.055	0.214	10
Zafirlukast	0.324	0.083	0.172	4
Pranlukast	0.368	0.071	0.165	2
Montelukast	0.323	0.081	0.162	1
Vilanterol	0.440	0.060	0.199	6

Indacaterol	0.475	0.054	0.206	7
Olodaterol	0.497	0.065	0.301	13
Formoterol	0.533	0.085	0.473	14
Salmeterol	0.460	0.070	0.283	12
Terbutaline	0.660	0.124	0.880	16
Salbutamol	0.648	0.121	0.841	15
Metaproterenol	0.666	0.130	0.920	18
Isoproterenol	0.666	0.129	0.920	17
Epinephrine	0.691	0.137	1	19
Toreforant	0.469	0.056	0.210	8
	D*	0.323	B*	0.054
	D−	0.691	*B*−	0.137

It can be seen that fevipiprant is identified as the most suitable drug, considering its proximity to the ideal solution in terms of flash point. On the other hand, montelukast is ranked as the top drug based on boiling point. Weights have been allocated to two criteria, namely, flash point (illustrated in Figure 1) and boiling point (depicted in Figure 2).
[figure(s) omitted; refer to PDF]
Beneficial weights are represented in green color in Figures 1 and 2. Weight allocation for boiling point was derived from correlation coefficient.
The comparison of ranks and drug raking can be seen in Figure 3.
[figure(s) omitted; refer to PDF]

5. Conclusions

Priority ranking of drugs required can be viewed as a multicriteria decision-making (MCDM) challenge. This approach has garnered the interest of numerous researchers in recent times. VIKOR is a valuable approach for addressing MCDM problems, and the resulting solution, which is deemed the closest to the ideal solution, is often deemed acceptable to decision-makers. This study suggests using two criteria, namely, boiling point and flash point, to establish the criteria set. The CRTIC method is employed to calculate weights that aid the decision-maker in determining the priority of drug interventions for various utilities.
Nineteen asthma medications were selected to illustrate an application of the proposed highly effective MCDM technique VIKOR method. The VIKOR methodology heavily relies on evaluations and has been applied within the context of QSPR modeling. We conclude that fevipiprant is determined to be the most suitable drug for being closest to the ideal solution taking flash point into account. However, according to the boiling point, montelukast is the ranked one drug. Theoretical findings of this nature could prove valuable for the future ranking of drug structures using chemical invariants, particularly within the domains of biomedicine and mathematical chemistry, facilitating drug discovery endeavors.

Future research in the field of asthma treatment should focus on utilizing real-world data, patient-centered outcomes, and long-term safety evaluations. Comparative studies will help determine the most effective drugs for different patient groups. Precision medicine, machine learning, patient education, and digital health solutions offer the potential for more personalized and effective asthma management.

The proposed approach can be adapted for various medical decision-making challenges, offering the potential as a valuable decision-support tool for asthma treatment. Additionally, this approach can be applied to other diseases in the future.

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DETAILS

Subject:	Mathematical programming; Research; Patients; Allergens; Side effects; Asthma; Chemical compounds; Multiple criterion; Modelling; Steroids; Multiple criteria decision making; Topology; Histamine; Drugs; Chemistry; Cost analysis; Molecular structure; Decision making; Respiration; Coronaviruses; COVID-19
Business indexing term:	Subject: Multiple criteria decision making Cost analysis
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-11-07 (Received); 2024-01-14 (Revised); 2024-01-20 (Accepted); 2024-02-05 (Pub)
DOI:	https://doi.org/10.1155/2024/6516976
ProQuest document ID:	2928049543
Document URL:	https://www.proquest.com/scholarly-journals/prioritizing-asthma-treatment-drugs-through/docview/2928049543/se-2?accountid=211160

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Last updated:	2024-02-19
Database:	Publicly Available Content Database

Analysis of Quality Differences in Radix Dipsaci before and after Processing with Salt Based on Quantitative Control of HPLC Multi-Indicator Components Combined with Chemometrics

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ABSTRACT (ENGLISH)

Radix Dipsaci (RD) is the dry root of the *Dipsacus asper* Wall. ex DC., which is commonly used for tonifying the kidney and strengthening bone. The purpose of this study was to analyze the difference between raw and salt-processed RD from the chemical composition comprehensively. The fingerprints of raw and salt-processed RD were established by HPLC-DAD to determine the contents of loganin (LN), asperosaponin VI (AVI), caffeic acid (CaA), dipsanoside A (DA), dipsanoside B (DB), chlorogenic acid (CA), loganic acid (LA), isochlorogenic acid A (IA), isochlorogenic acid B (IB), and isochlorogenic acid C (IC). The results showed that after processing with salt, the components with increased contents were LA, CaA, DA, and AVI, and the components with decreased contents were CA, LN, IB, IA, IC, and DB. Then, the chemometric methods such as principal component analysis (PCA) and fisher discriminant analysis (FDA) were used to evaluate the quality of raw and salt-processed RD. In the classification of raw and salt-processed RD, the order of importance of each chemical component was LA>DB>IA >IC>IB>LN>CA>DA>AVI>CaA. These integrated methods successfully assessed the quality of raw and salt-processed RD, which will provide guidance for the development of RD as a clinical medication.

FULL TEXT

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1. Introduction

Radix Dipsaci (RD) is the dry root of the *Dipsacus asper* Wall. ex DC., with the effect of tonifying the liver and kidney, strengthening bones and sinews, renewing fractures, and stopping collapse and leakage. The main chemical components of RD are saponins, alkaloids, iridoids, and lignans [1–5]. In clinical practice, RD is commonly used in the treatment of osteoarthritis, osteoporosis, and kidney-yang deficiency [6–10]. At present, the types of decoction pieces used in clinical practice are mainly raw Radix Dipsaci (RRD), wine-processed Radix Dipsaci, and salt-processed Radix Dipsaci (SRD). Among them, SRD is a processing method developed in modern times. Chemometrics is an emerging interdisciplinary discipline formed by the combination of mathematics, statistics, computer science, and chemistry and is an important means of material-based research of traditional Chinese medicine (TCM). It can introduce multivariate analytical methods into chemical research, process and analyze chemical measurement data in multiple ways, create and optimize various chemical models, and extract the components, structures, and other related information of related substance systems from complex chemical measurement data to the maximum extent. At present, the combination of spectroscopic data and chemometric methods has been used by many scholars to research TCM [11–13]. In this study, HPLC-DAD fingerprints of RD were established, and 10 different components in RD before and after processing with salt were selected for determination and combined with the chemometric methods such as principal component analysis (PCA) and fisher discriminant analysis (FDA) to establish a more comprehensive and quantitative chemical pattern identification and quality evaluation method for the samples of RRD and SRD, providing a certain scientific basis for the later development of studies on spectrum-effect relationship.

2. Materials

2.1. Instruments

U3000 high-performance liquid chromatograph (Thermo, USA), ME-204E electronic analytical balance (0.01 g, Mettler Toledo, Switzerland), NT-xs105 electronic analytical balance (0.01 mg, Mettler Toledo, Switzerland), and DFT-200 portable high-speed traditional Chinese medicine pulverizer were purchased from Wenling Linda Machinery Co., Ltd., China; KQ-500DB CNC ultrasonic cleaner was purchased from Kunshan Ultrasound Instrument Co., Ltd., China; GDC-750 electromagnetic herbal machine roaster was purchased from Hangzhou Haishan Pharmaceutical Equipment Co., Ltd., China.

2.2. Reagents

Loganin (LN, Lot 111640–201808, purity 99%), asperosaponin VI (AVI, Lot 111685–201907, purity 94.3%), caffeic acid (CaA, Lot 110885–201703, purity 99.7%), dipsanoside A (DA, Lot 1647-0025, purity 99%), and dipsanoside B (DB, Lot 1647-0026, purity 99%) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Chlorogenic acid (CA, Lot 120052–201912, purity 98%), loganic acid (LA, Lot 130008–201908, purity 98%), isochlorogenic acid A (IA, Lot 250034-202003, purity 98%), isochlorogenic acid B (IB, Lot 250035-202003, purity 98%), and isochlorogenic acid C (IC, Lot 250036-202003, purity 98%) were purchased from Shanghai Hongyong Biotechnology Co., Ltd. (Shanghai, China).

Methanol (analytical purity) was purchased from Guangdong Guanghua Technology Co., Ltd. (Guangdong, China). Phosphoric acid (analytical purity) was purchased from Zhejiang Hannuo Chemical Technology Co., Ltd. (Zhejiang, China). Acetonitrile (HPLC grade) was purchased from Tedia (Fairfield, USA). The purified water was purchased from Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China).

2.3. Sample Collection

RRD was purchased from different origins in China and was authenticated by Professor Weihong Ge (School of Pharmacy, Zhejiang Chinese Medical University). SRD was prepared from RRD. The processing method of SRD was as follows: mixed RRD with salt water evenly, made it moist, stir-fried for 10 minutes at 160°C in a frying pan, took it out, and let it cool (every 100 kg RRD with 2 kg salt). The samples were stored in the herbarium of Zhejiang Chinese Medical University Chinese Medicine Yinpian Co., Ltd. (Hangzhou, China). The sample information is shown in Table 1.

Table 1

The sample information of raw and salt-processed RD.

No	Origin	Batch number	No	Origin	Batch number
R1	Sichuan	S201908	S1	Sichuan	Y201908
R2	Sichuan	S201903	S2	Sichuan	Y201903
R3	Yunnan	S201911-1	S3	Yunnan	Y201911-1
R4	Yunnan	S201911-2	S4	Yunnan	Y201911-2
R5	Sichuan	S20190157	S5	Sichuan	Y20190157
R6	Yunnan	S20190417	S6	Yunnan	Y20190417
R7	Sichuan	S20190416	S7	Sichuan	Y20190416

R8	Hunan	S20051514	S8	Hunan	Y20051514
R9	Hunan	S20051515	S9	Hunan	Y20051515
R10	Hunan	S20051516	S10	Hunan	Y20051516
R11	Guizhou	S2005054	S11	Guizhou	Y2005054
R12	Hubei	S201910	S12	Hubei	Y201910
R13	Shandong	S202004211	S13	Shandong	Y202004211

3. Methods

The HPLC method for RD was formulated and optimized by our group in a previous work and has been published in other articles by Wu et al. [14]. In this paper, we followed this method for the determination of the content of each component in RD.

3.1. Standard and Sample Solution Preparation

The standard solution was prepared in 80% methanol. The concentrations of LA, CA, LN, AVI, CaA, IA, IB, IC, DA, and DB were 953.54, 477.26, 1185.03, 1952.01, 420.73, 830.06, 602.70, 602.70, 110.85, and 40.22 μg/mL.

The sample powder (80 mesh) was weighed 0.5 g accurately and placed in a conical flask with a stopper, and 25 mL of 80% methanol was added and weighed accurately. The solution was ultrasonicated (power 300 W and frequency 50 kHz) for 30 min and then weighed again. 80% methanol was used to make up the lost weight, and the sample solution was obtained by filtering through a 0.45 μm microporous membrane.

3.2. HPLC Conditions

The chromatographic column was Agilent Zorbax SB-C_{18} column (4.6 × 250 mm, 5.0 μm); the mobile phase was 0.05% phosphoric acid aqueous solution (A)-acetonitrile (B), gradient elution: 0~5 min, 2%~6% B; 5~18 min, 6%~10% B; 18~40 min, 10%~20% B; 40~70 min, 20%~25% B; 70~80 min, 25%~35% B; 80~90 min, 35%~60% B; 90~110 min, 60%~70% B; 110~120 min, 70% B. Column temperature was 25°C, flow rate was 0.8 mL/min, UV detection wavelength was 215 nm, and the injection volume was 10 μL.

3.3. HPLC Methodological Investigation

Linear relationship investigation took the standard solution and diluted it with 80% methanol to make 10 gradient concentration solutions from high to low. According to the chromatographic conditions under "3.2," injected 10 μL of the different concentration standard solution. Drew a standard curve with peak area (y) and concentration (x, μg/mL) to calculate the regression equation. At the same time, we determined the limit of detection (LOD) of the injection concentration when $S/N = 3$ and the limit of quantification (LOQ) of the injected concentration when $S/N = 10$.

Precision took the same R1 sample solution and continuously injected for 6 times according to "3.2"; repeatability took the R1, prepared 6 samples of the sample solution in parallel, and injected according to "3.2"; stable properties took the R1 sample solution and injected according to "3.2" at 0, 2, 4, 8, 12, 16, and 24 hours, respectively (sample solutions were stored at room temperature). The peak areas of the three investigation items were recorded, respectively, and the relative retention time (RRT) and relative peak area (RPA) RSD of each common peak were calculated with AVI as the reference peak.

The recovery rate of sample addition weighed 6 RD samples powders (R1, S1) with known content, respectively, and added 10 standard substances, respectively (according to the content of the component in the sample, we added a certain volume of corresponding dilutions and then evaporated the solvent). We prepared the tested solution according to "3.1," injected according to "3.2," and analyzed it to obtain the average value and RSD of the recovery rates of 10 components.

3.4. Establishment of HPLC Fingerprints and Content Determination

We took each batch of RRD and SRD to prepare the sample solution according to “3.1,” injected according to “3.2,” then imported the collected chromatographic data into “Evaluation of Similarity of Chinese Medicine Fingerprints Software,” respectively, generated the contrast map of raw and salt-processed RD, calculated the similarity, pipetted 10 μL of the standard solution to inject it into HPLC, and used the standard data to determine the content of LA, CA, LN, AVI, CaA, IA, IB, IC, DA, and DB in each sample.

3.5. Chemometric Analysis

The data of 10 components in each sample obtained by the content determination were imported into SPSS for PCA and FDA, and the difference in quality between the RRD and SRD was analyzed.

4. Result and Analysis

4.1. Results of Methodological Investigation

The developed method was used to evaluate the linear range, recovery rate, precision, repeatability, and stability of the method for the determination of 10 components. Table 2 shows that the r of 10 components was all greater than 0.999 in the linear range, presenting a good linear relationship, which meets the experimental requirements. The results of precision, repeatability, and stability showed that the RSD of RRT and RPA were both less than 3%, and the similarity was both greater than 0.995, indicating that the method could be used for HPLC detection of RD. The sample recovery rate results in Table 3 showed that the recovery rates of the 10 components in the raw and salt-processed RD were all within the range of 95% to 100%, indicating that the accuracy was good and met the experimental requirements.

Table 2

The results of linear relation (n=10).

Component	Regression equation	r	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
LA	y=0.0658x−0.1336	0.9997	47.68~953.54	0.15	0.50
CA	y=0.3312x−0.0990	0.9997	23.86~477.26	0.15	0.50
CaA	y=0.6283x+0.3508	0.9997	21.04~420.73	0.12	0.35
LN	y=0.0992x+0.1302	0.9997	59.25~1185.03	0.12	0.35
IB	y=0.3718x+0.2226	0.9997	30.14~602.70	0.15	0.51
IA	y=0.4154x+0.0929	0.9997	41.50~830.06	0.10	0.35
IC	y=0.4199x−0.0743	0.9998	30.14~602.70	0.10	0.35
DB	y=0.0873x+0.0072	0.9997	2.01~40.22	0.10	0.35
DA	y=0.1289x−0.0052	0.9997	5.54~110.85	0.11	0.35
AVI	y=0.0806x+0.0581	0.9999	97.60~1952.01	0.15	0.50

Table 3

The results of sample recovery rate (n=6).

Sample	Component	Initial amount (mg± SD)	Injection amount (mg)	Total amount (mg± SD)	Recovery rate (%±SD)	RSD (%)
RRD	LA	4.38±0.01	4.77	9.17±0.12	100.34±2.40	2.39
CA	1.24±0.00	1.19	2.43±0.01	99.46±2.95	2.96	CaA
0.03±0.00	0.03	0.06±0.00	100.62±4.73	4.70	LN	0.99±0.00
0.95	1.96±0.03	101.88±3.02	3.92	IB	0.14±0.00	0.15
0.29±0.00	103.86±2.81	2.70	IA	1.37±0.00	1.66	3.06±0.03
101.96±1.78	1.75	IC	0.74±0.00	0.60	1.34±0.01	99.10±2.19
2.21	DB	0.34±0.00	0.32	0.60±0.01	99.36±3.24	3.26
DA	0.74±0.00	0.83	1.60±0.01	102.82±1.88	1.83	AVI
17.33±0.03	15.61	33.39±0.28	102.91±1.72	1.67	.	
SRD	LA	5.59±0.01	4.77	10.47±0.09	102.31±1.94	1.89
CA	1.28±0.00	1.19	2.49±0.03	102.34±2.74	2.68	CaA
0.11±0.00	0.11	0.22±0.00	102.92±2.31	2.25	LN	0.91±0.00
0.95	1.89±0.02	103.70±1.59	1.54	IB	0.09±0.00	0.09
0.18±0.00	99.06±3.22	3.25	IA	1.26±0.00	1.66	2.93±0.05
100.56±2.94	2.92	IC	0.73±0.00	0.60	1.34±0.02	101.01±2.70
2.66	DB	0.01±0.00	0.01	0.02±0.00	99.30±3.52	3.54
DA	0.81±0.00	0.83	1.66±0.03	102.32±3.05	2.98	AVI

4.2. HPLC Fingerprints of Samples

The fingerprints of the obtained RRD and SRD are shown in Figures 1 and 2. The results in Tables 4 and 5 showed that the similarity of the fingerprints of raw and salt-processed RD was above 0.900, respectively, and a total of 25 peaks were obtained. Figure 3 shows that compared with the results of the reference solution, 10 components were identified, namely, peak 6-LA; peak 8-CA; peak 9-CaA; peak 10-LN; peak 11-IB; peak 12-IA; peak 14-IC; peak 16-DB; peak 17-DA; peak 20-AVI.

[figure(s) omitted; refer to PDF]

Table 4

The similarity results of 13 batches of RRD.

No	Similarity
R1	1.000
R2	0.912
R3	0.925
R4	0.957
R5	0.923
R6	0.974
R7	0.916
R8	0.947
R9	0.918
R10	0.912
R11	0.916
R12	0.936
R13	0.970

Table 5

The similarity results of 13 batches of SRD.

No	Similarity
S1	0.921

S2	0.910
S3	0.927
S4	0.936
S5	0.973
S6	0.959
S7	0.987
S8	0.958
S9	0.972
S10	0.926
S11	0.974
S12	0.934
S13	0.923

[figure(s) omitted; refer to PDF]

4.3. Result of Contents Determination

As shown in the results from Tables 6 and 7, the LA content in different batches of RRD sample was 1.32~1.92%, CA was 0.34~0.55%, CaA was 0.01~0.03%, LN was 0.24~0.51%, IB was 0.03~0.11%, IA was 0.44~0.60%, IC was 0.21~0.56%, DB was 0.13~0.16%, DA was 0.23~0.53%, and AVI was 4.92~8.86%. The LA content in SRD sample was 1.49~2.72%, CA was 0.27~5.00%, CaA was 0.02~0.11%, LN was 0.05~0.43%, IB was 0.01~0.06%, IA was 0.28~0.58%, IC was 0.15~0.32%, DB was 0.00~0.12%, DA was 0.29~0.42%, and AVI was 6.19~9.35%.

Table 6

Contents of 10 components in RRD (%, *n*=6).

No	LA	CA	CaA	LN	IB	IA	IC	DB	DA	AVI
R1	1.92	0.55	0.01	0.43	0.06	0.60	0.32	0.15	0.33	7.59
R2	1.48	0.52	0.03	0.38	0.05	0.56	0.29	0.13	0.29	6.19
R3	1.49	0.52	0.02	0.24	0.05	0.57	0.30	0.13	0.29	6.23
R4	1.47	0.52	0.02	0.24	0.05	0.56	0.29	0.13	0.28	6.17
R5	1.44	0.34	0.03	0.51	0.05	0.44	0.36	0.16	0.25	5.69

R6	1.32	0.40	0.03	0.41	0.11	0.56	0.54	0.14	0.30	4.92
R7	1.92	0.55	0.03	0.46	0.03	0.60	0.56	0.15	0.23	8.86
R8	1.48	0.52	0.03	0.31	0.04	0.56	0.29	0.13	0.28	5.79
R9	1.49	0.52	0.03	0.31	0.04	0.57	0.21	0.13	0.28	5.79
R10	1.60	0.55	0.03	0.43	0.04	0.60	0.32	0.15	0.28	5.79
R11	1.48	0.52	0.02	0.31	0.04	0.56	0.29	0.13	0.28	5.79
R12	1.32	0.52	0.03	0.41	0.11	0.57	0.54	0.13	0.30	6.23
R13	1.47	0.52	0.02	0.46	0.05	0.56	0.29	0.13	0.23	6.17
X	1.53	0.50	0.02	0.38	0.06	0.56	0.35	0.14	0.28	6.25
SD	0.19	0.06	0.01	0.09	0.03	0.04	0.11	0.01	0.03	0.98

Table 7

Contents of 10 components in SRD (%, *n*=6).

No	LA	CA	CaA	LN	IB	IA	IC	DB	DA	AVI
S1	2.33	0.53	0.04	0.38	0.04	0.53	0.30	0.01	0.34	7.62
S2	2.29	0.51	0.03	0.24	0.04	0.55	0.29	0.01	0.42	7.17
S3	2.72	0.27	0.03	0.06	0.01	0.29	0.15	0.01	0.30	7.22
S4	2.31	0.45	0.02	0.05	0.04	0.51	0.29	0.00	0.31	7.47
S5	1.71	0.30	0.11	0.32	0.03	0.39	0.26	0.09	0.41	8.11
S6	1.64	0.31	0.05	0.38	0.06	0.51	0.28	0.12	0.35	6.66
S7	2.22	0.30	0.04	0.43	0.02	0.58	0.32	0.08	0.36	7.59
S8	1.60	0.37	0.03	0.24	0.03	0.39	0.21	0.12	0.35	6.19
S9	1.58	0.37	0.03	0.24	0.03	0.38	0.28	0.12	0.35	6.23
S10	1.92	0.37	0.04	0.31	0.03	0.39	0.21	0.12	0.33	7.59

S11	1.58	0.37	0.03	0.24	0.03	0.38	0.21	0.12	0.29	6.19
S12	1.49	0.53	0.06	0.24	0.05	0.56	0.30	0.12	0.30	9.35
S13	2.22	0.30	0.04	0.24	0.02	0.28	0.17	0.08	0.31	9.23
X	1.97	0.39	0.04	0.26	0.04	0.44	0.25	0.08	0.34	7.43
SD	0.40	0.09	0.02	0.11	0.01	0.10	0.06	0.05	0.04	1.03

After processing, the contents of 10 components in the corresponding batches of raw and salted-processed RD were significantly different. As shown in Figure 4, the contents of LA, CaA, DA, and AVI in SRD were higher than RRD, the average change rates were 28.80%, 74.78%, 21.38%, and 18.99%, respectively, and the contents of CA, LN, IB, IA, IC, and DB in SRD were lower than those in RRD, with an average change rate of −23.28%, −30.96%, −36.30%, −21.49%, −29.49%, and −45.37%, respectively. This was due to the change in content caused by processing with salt, and it was speculated that the content of phenolic acids and iridoid glycosides in RD might be reduced due to the conversion and degradation of the components after heating.

[figure(s) omitted; refer to PDF]

4.4. Analysis of the Difference between Raw and Salt-Processed RD

4.4.1. PCA

In this experiment, SPSS 25.0 was used to perform PCA on 26 samples. The results are shown in Table 8, the principal components were extracted with the eigenvalue $\lambda > 1$, $\lambda 1 = 4.324$, the contribution rate was 43.237%, $\lambda 2 = 1.512$, the contribution rate was 15.122%, $\lambda 3 = 1.395$, the contribution rate was 13.950%, and the contribution rate of the first principal component was the largest, indicating that it contains the most information. When the number of principal components was 3, the cumulative contribution rate reached 72.310%, and it indicated that the first three principal components could represent most of the information data about the difference between the raw and salt-processed RD. The first, second, and third principal components were used as the coordinate system, and the three-dimensional map of each compound was obtained by projection. As shown in Figure 5, the 10 compounds were divided into 2 categories, one of which was LA, CaA, DA, and AVI and the other type was CA, LN, IB, IA, IC, and DB. This result was consistent with the change law of the content before and after processing with salt, the former was the components whose content increased after processing with salt, and the latter was the components whose content decreased.

Table 8

Principal component eigenvalue and contribution rate.

Principal component	λ	Contribution rate (%)	Cumulative contribution rate (%)
1	4.324	43.237	43.237
2	1.512	15.122	58.359
3	1.395	13.950	72.310
4	0.946	9.456	81.766

5	0.718	7.177	88.943
6	0.529	5.291	94.234
7	0.257	2.566	96.800
8	0.166	1.661	98.461
9	0.122	1.219	99.681
10	0.032	0.319	100.000

[figure(s) omitted; refer to PDF]

The component loading matrix can explain the contribution rate of each variable to the principal component. The greater the absolute value of the compound loading, the greater the contribution to the principal component, indicating that it is more important in the quality control of decoction pieces. According to the data in Table 9, by comparing the absolute value of the load of the compound in the “most informative” first principal component, it could be seen that the importance of the above 10 compounds in the quality control of raw and salt-processed RD was LA > DB > IA > IC > IB > LN > CA > DA > AVI > CaA.

Table 9

Principal component loading matrix.

Compound	Principal component		
1	2	3	LA
−0.767	−0.227	0.450	CA
0.628	−0.389	0.487	CaA
−0.439	0.795	0.062	LN
0.653	0.432	0.067	IB
0.696	0.315	0.103	IA
0.737	−0.103	0.525	IC
0.713	0.305	0.401	DB
0.754	0.194	−0.435	DA
−0.575	0.461	0.202	AVI

4.4.2. Fisher Discriminant Analysis

FDA is one of the methods of discriminant analysis. It uses the idea of variance analysis to project points in a high-

dimensional space to a low-dimensional space to construct one or more linear discriminant functions in different-dimensional spaces. The contents of 10 compounds in RRD and SRD were selected as variables to generate FDA. The results of the coefficients are shown in Table 10, X1, X2, …, X10 in the function expressions were used to represent the normalized data of the content of each compound respectively, and the function expressions were shown as follows:(1)RRD=323.095X1+490.050X2+2368.596X3−167.278X4+1648.097X5+35.132X6−113.313X7+2301.225X8−242.518X9−39.507X10−406.748,SRD=274.028X1+378.002X2+1964.572X3−153.714X4+1382.986X5+17.266X6−102.903X7+1912.431X8−35.987X9−28.381X10−342.073.

Table 10

Fisher linear discriminant function coefficients of raw and salt-processed RD.

Compound	RRD	SRD
LA	323.095	274.028
CA	490.050	378.002
CaA	2368.596	1964.572
LN	−167.278	−153.714
IB	1648.097	1382.986
IA	35.132	17.266
IC	−113.313	−102.903
DB	2301.225	1912.431
DA	−242.518	−35.987
AVI	−39.507	−28.381
Constant	−406.748	−342.073

The above discriminant functions were used to back-substitute the classification. As shown in Figure 6, the samples of raw and salt-processed RD could be well differentiated in the discriminant analysis scatterplot. At the same time, the discriminants of RRD and SRD were consistent with the actual, and the accuracy rates were both 100%.

[figure(s) omitted; refer to PDF]

5. Discussion

The HPLC fingerprints of RD before and after processing with salt were established. There were 25 common peaks in the fingerprints, and the contents of 10 components were determined. The results showed that the contents of LA, CaA, DA, and AVI increased, while the contents of CA, LN, IB, IA, IC, and DB decreased after processing with salt. No new or disappeared components were found in the RD before and after processing with salt, and it was speculated that there might have been intercomponent transformations. For example, the increase in LA content may have been caused by the addition of the -COOH group to LN, which was also consistent with the decrease of LN and organic acids (CA, IB, IA, and IC). The conversion between DA and DB led to an increase in DA content.

The study of the transformation between these components was also an important part of the processing mechanism of TCM. Whether the change in composition after processing with salt was caused by heating or salt processing during frying has not been systematically studied in this section. Subsequent systematic studies will be carried out on the effect of excipient salt on the composition.

Chemometrics, also known as chemical statistics, is a branch of chemistry that combines mathematics, statistics, computer science, and chemistry, which the most important feature of chemistry is the introduction of multivariate analysis methods into chemical research and the multivariate processing and analysis of chemical measurement data. Chemometrics include measurement tests, chemical pattern recognition, regression analysis, and multivariate correction [15, 16]. PCA is an unsupervised pattern recognition analysis which uses the idea of reducing the dimension of the data matrix to convert the original indicators into several comprehensive indicators through a linear transformation under the premise of losing a small amount of information, so as to simplify datasets and visualize differences between samples [17]. Since there is no human involvement in the analysis process, and the calculation model is based on the state of the original variables, PCA is very helpful in reflecting and expressing the overall situation of the variables under analysis and the total control of variables by the researcher, which helps to identify and eliminate problematic samples and abnormal variables, thus improving the accuracy and precision of the mathematical analysis model [18–20]. The use of PCA can simplify complex multivariate data systems, and more studies have reported its use in the study of TCM. Many studies have been reported on its use in the study of Chinese medicine and natural drugs [21–23]. In this study, the results of PCA showed that the order of influence of 10 components in the classification of raw and salt-processed RD was LA>DB>IA>IC>IB>LN>CA>DA>AVI>CaA.

Discriminant analysis is a supervised classification technique belonging to chemometrics, which classifies certain objects studied based on certain observed indicators. In the quality control experiment of TCM, discriminant analysis can establish a discriminant based on the observation data of a batch of known samples of various types and then classify the unknown types of samples. FDA is a common method in discriminant analysis, which is generally used to discriminate two kinds of quantitative data [24]. It uses the idea of one-dimensional ANOVA to reduce the sample points in the n-dimensional space to one-dimensional data by means of linear functions and then classifies the sample points to be judged into different categories according to the distance between samples. FDA can make the differences between sample points in the same category as small as possible and make the differences between sample points in different categories as large as possible, thus effectively improving the discriminant efficiency [25, 26]. This analytical approach of the FDA was applied in this paper, and the results showed that the model and algorithm given in the paper were effective and useful for the classification of raw and salt-processed RD.

6. Conclusion

In this study, the content of 10 chemical components before and after processing with salt of RD was determined by HPLC-DAD and was sorted through PCA to rank the importance of each chemical component. At the same time, the samples were classified and verified by the FDA. The results showed that the components in RD after processing with salt had internal transformation, the contents of LA, CaA, DA, and AVI increased, and the contents of CA, LN, IB, IA, IC, and DB decreased. In the classification of raw and salt-processed RD, the order of importance of each chemical component was LA>DB>IA>IC>IB>LN>CA>DA>AVI>CaA. These components could be used as differential components to identify raw and salt-processed RD. This study provides a comprehensive and quantitative chemical pattern recognition and quality evaluation method for the identification of TCM before and after processing. This method could also provide a scientific basis for further research on the spectrum-effect relationship and mechanism of action.

Authors' Contributions

Hangsha Wu and Yue Lv developed the methodology, investigated the study, and wrote the original draft. Rui Tang, Mingfang Zhao, and Yafei Li conducted the experiment and processed the data. Feiyang Wei conducted the investigation. Changyu Li performed funding acquisition. Weihong Ge reviewed and edited the study and performed funding acquisition. Weifeng Du conceptualized the study, reviewed and edited the study, and supervised the study.

Hangsha Wu and Yue Lv contributed equally to this work.

Acknowledgments

This study was financially supported by Hangzhou Agricultural and Social Development Scientific Research Key Project (202204A06), Ningbo “Science and Technology Innovation 2025” Major Project (20201ZDYF020069), Zhejiang Province Traditional Chinese Medicine Science and Technology Plan Special Project on Traditional Chinese Medicine Modernization (2020ZX006) and National Key R &D Program of China—Research on Key Technology for Quality Identification of Traditional Chinese Medicine (No. 2018YFC1707001).

Glossary

Abbreviations

RD:Radix Dipsaci
LN:Loganin
AVI:Asperosaponin VI
CaA:Caffeic acid
DA:Dipsanoside A
DB:Dipsanoside B
CA:Chlorogenic acid
LA:Loganic acid
IA:Isochlorogenic acid A
IB:Isochlorogenic acid B
IC:Isochlorogenic acid C
PCA:Principal component analysis
FDA:Fisher discriminant analysis
RRD:Raw Radix Dipsaci
SRD:Salt-processed Radix Dipsaci
TCM:Traditional Chinese medicine
LOD:Limit of detection
LOQ:Limit of quantification
RRT:Relative retention time
RPA:Relative peak area
PC:Principal component.

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DETAILS

Subject:	Quality assessment; Chlorogenic acid; Acids; Principal components analysis; Clinical medicine; Discriminant analysis; Methods; Chromatography; Chemical composition; Chinese medicine; Chemometrics
Location:	Switzerland; United States--US; China
Company / organization:	Name: Mettler-Toledo Inc; NAICS: 333998, 334513, 334516; Name: Food &Drug Administration--FDA; NAICS: 926150
Editor:	Charles Wilkins
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United Kingdom, New Yo rk
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:	2022-12-20 (Received); 2023-02-07 (Revised); 2024-01-27 (Accepted); 2024-02-07 (Pub)
DOI:	https://doi.org/10.1155/2024/2109127
ProQuest document ID:	2928049430
Document URL:	https://www.proquest.com/scholarly-journals/analysis-quality-differences-radix-dipsaci-before/docview/2928049430/se-2?accountid=211160

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Last updated:	2024-02-19
Database:	Publicly Available Content Database

Oxidative Polymerization of Aniline on the Surface of Sisal Fibers (SFs) as Defluoridation Media for Groundwater

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ProQuest document link

ABSTRACT (ENGLISH)

Chemical modification of sisal fibers via *in situ* oxidative polymerization of aniline was conducted to examine their defluoridation capacity for fluoride from drinking water. The effects of polyaniline modifications have shown significant changes on the chemical moieties and defluoridation capacity of sisal fibers (SFs). FTIR peaks at 1440 cm^{-1} and 1560 cm^{-1} revealed the presence of benzoid and quinoid structures together with sisal fiber (SF). Thermal profiles confirmed the enhancement of thermal stability of polyaniline-modified sisal fibers (PAniMSFs). SEM microstructure also proved the surface roughening of SFs as a result of polyaniline modifications. Optimal batch

adsorption parameters (pH, contact time, adsorbent dose, and initial concentration) were found to be 5, 60 min, 1 g, and 10 mg/L, respectively. Adsorption kinetics proved that the removal of fluoride follows pseudo-second-order model ($K_2 = 0.18$ g. $(mg \cdot min)^{-1}$), while the adsorption isotherm well described by the Langmuir and Freundlich model with an experimental adsorption capacity of 2.49 mg/g. Hence, modifications and improvements are required to reduce the amount of fluoride to a permissible level and enhance the longevity and activity of adsorbent materials.

FULL TEXT

DETAILS

Subject:	Adsorbents; Thermal stability; Spectrum analysis; Sisal; Polymerization; Adsorption; Surface chemistry; Drinking water; Polyanilines; Parameter modification; Fluorides; Fibers; Roughening; Aniline
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-10-18 (Received); 2023-12-12 (Revised); 2024-01-04 (Accepted); 2024-02-01 (Pub)
DOI:	https://doi.org/10.1155/2024/6941567
ProQuest document ID:	2924793073

Document URL:	https://www.proquest.com/scholarly-journals/oxidative-polymerization-aniline-on-surface-sisal/docview/2924793073/se-2?accountid=211160
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Last updated:	2024-02-12
Database:	Publicly Available Content Database

Document 18 of 25

Development of an On-Column Trace Enrichment Method for the Determination of Sub- μ g/L Bisphenol A in Bottled Water by RP-HPLC with Fluorescent Detection

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ProQuest document link

ABSTRACT (ENGLISH)

A simple extraction-free, on-column trace enrichment liquid chromatographic method for the determination of trace levels of bisphenol A (BPA) in bottled water samples has been developed. It was found possible to determine ng/L (ppt) levels of BPA by the direct introduction of 6 mL of sample water to the HPLC column utilising fluorescence detection (Exλ=274 nm, Emλ=314 nm). Following the loading of the sample and the chromatographic focusing of the BPA on the analytical column, a simple switch from the aqueous sample to the isocratic chromatographic elution step of 50% acetonitrile/deionised water was undertaken. Using a BPA concentration of 0.596 μg/L the effect of sample volume was investigated over the range 1.0 to 12 mL. A linear relationship with the sample volume introduced to the HPLC column and the resulting peak height for BPA was found over the entire range investigated R2=0.999. Using a sample volume of 6.0 mL, a well-defined chromatographic peak was recorded for BPA over the concentration range of 0.1 μg/L to 6.25 μg/L R2=0.9998. A limit of detection of 0.058 μg/L for BPA was calculated based on 3 δ. A mean recovery of 100% with an associated %CV of 7.6% (n=5) was obtained for a bottled spring water sample fortified with 1.25 μg/L BPA. Samples can be processed in under 12 minutes, much faster than that commonly reported for conventional offline extraction and chromatographic-based methods. The results show that the optimised method holds promise for the determination of BPA in such samples.

FULL TEXT

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1. Introduction

Bisphenol A, or BPA, (Figure 1), is used for a number of applications such as a component in synthetic plastic materials, such as poly (vinyl chloride) and as an antioxidant in glues, plastics, and ink. However, its main application is as a monomer in the production of polycarbonate (PC), which is used for a wide range of applications. Exposure to BPA can result in harm during embryonic and foetal development and result in conditions, such as obesity, diabetes, and heart disease [1–8]. Similarly, adverse effects have been reported for both animals [9, 10] and plants [11].

[figure(s) omitted; refer to PDF]

An important source of human exposure is thought to be the ingestion of food and drink that has been in contact with epoxy resins or polycarbonate plastics [12, 13]. This has led to an increasing level of interest in levels of BPA that can be present in both drinking water and bottled water. In a 2009 study [14], it was shown that as little as one week of consuming cold beverages from PC bottles increased urinary BPA concentrations by two-thirds. The amount of BPA leached from PC containers is dependent on a number of parameters, such as the mineral content and pH of the water or food, the surface-to-volume ratio, the exposure time, temperature, and light [15].

The leached BPA would seem to result from the unreacted BPA monomer migrating from the PC, with the various chemical and physical processes accelerating its migration. These processes are also accelerated when reusing PC, particularly when it is damaged, or following washing and exposure to cleaning agents and hypochlorite [16, 17]. Recently, The European Food Safety Authority (EFSA) has dramatically lowered the tolerable daily intake (TDI) for BPA from 4 to 0.2 ng/kg body weight/day [18]. Accordingly, there is great interest in the development of analytical techniques which are capable of determining BPA at low trace levels in a variety of different sample matrices. High-performance liquid chromatography (HPLC) with either UV [19], electrochemical [20, 21], fluorescence detection [22], or mass spectrometry [23, 24] has been reported for the determination of BPA. Gas chromatography following derivatisation [25–27] has also been employed. Methods based on the competitive enzyme-linked immunosorbent assay (ELISA) [28] and molecularly imprinted polymers [29] have also been reported.

Due to the low concentrations commonly encountered, analytical protocols including sample preconcentration steps such as solid phase extraction (SPE) [13, 19, 30, 31] or liquid/liquid extraction [27, 32] are generally employed. Such preconcentration steps can be time-consuming, labour-intensive, suffer from varied recovery efficiencies, and require large sample volumes. This is also potentially problematic, as these sample processing steps can potentially introduce BPA contamination [33, 34].

Conventionally, in HPLC sample extracts are normally obtained by either SPE or liquid/liquid extraction [35–37]. The resulting extract is then taken down to dryness under nitrogen, following, commonly, a preconcentration step, such as rotary evaporation, and the sample residue is reconstituted in a relatively small volume of mobile phase, such as a 1.0 mL. Volumes of between 10 and 20 μL of this sample extract are introduced to the HPLC column via some form of fixed-volume sample loop.

If we calculate the actual percentage of the original sample introduced to the HPLC under these conditions: we see that 20 μL taken from 1.0 mL means that we are effectively only introducing 2% of the sample, and the remaining 98% is effectively wasted. For example, if a 1.0 L sample of water was taken and following extraction, concentrated to 1.0 mL, the introduction of 20 μL of sample to the HPLC would be the same as introducing only 20 mL of the original sample to the instrument.

Nevertheless, one particular alternative approach that overcomes these issues is on-column trace enrichment liquid chromatography. This consists of the direct introduction of a low eluting strength solution such as the water sample itself, directly to the analytical column, where the target analytes are directly focused and concentrated. The introduction of relatively large sample volumes in the hundreds of μL to mL levels [38–46] allows for the determination of low analyte concentrations without the need of time-consuming extraction steps. Following concentration of the analyte on the analytical HPLC column, the target analytes can be eluted and

chromatographically separated via the introduction of the appropriate mobile phase.
This present study has focused on the determination of BPA in bottled water by HPLC single-column trace enrichment (HPLC-SCTR) with fluorescence detection. In the first part of the study, conditions such as sample volume and chromatographic conditions were optimised. The possibility of using the optimised method to determine μg/L levels of BPA in a fortified bottled water sample was then investigated by HPLC-SCTR.

2. Experimental

2.1. Chemicals and Reagents

Solids of BPA, bisphenol F, and bisphenol S were all obtained from Merck (Gillingham, UK). Acetonitrile was obtained from Fisher Scientific (Loughborough, UK). Deionised water was obtained from a Sartorius Arium® mini Ultrapure Water System (Sartorius UK Ltd., Epsom, UK). Polyethylene terephthalate bottled spring water samples (Princes Gate Water, Pembrokeshire, Wales, UK) were obtained from a local commercial outlet. A 100 mL aliquot of this was taken and fortified to be 1.25 μg/L BPA.

2.2. Liquid Chromatography and Single-Column Trace Enrichment

Initial investigations were undertaken to optimise the chromatographic separation of BPA using an injection volume of 20 μL. Separations were achieved using a Hypersil GOLD analytical column (150 × 4.6 mm, 5 μm) with fluorescent detection using an excitation wavelength of 274 nm and an emission wavelength of 314 nm. Mobile phases with acetonitrile concentrations of between 20% and 70% in deionised water were investigated. Further, HPLC-SCTR investigations were made using a TSP Spectra SYSTEM P4000 HPLC pump (Spectra-Physics) connected directly to a Hypersil GOLD (150 × 4.6 mm, 5 μm) analytical column. Fluorescence detection (linear, Fluor LC305) was undertaken using an excitation wavelength of 274 nm and an emission wavelength of 314 nm. The system was first purged with 50% acetonitrile/deionised water and then with the sample water under investigation (Figure 2(a)). Sample aliquots were directly focused on the analytical column by pumping the sample water directly to the analytical column at a flow rate of 2.0 mL/min for 3 minutes (Figure 2(b)). The focused sample components were then eluted from the analytical column by switching directly to a mobile phase of 50% acetonitrile (Fischer, far UV, HPLC grade)/deionised water at a flow rate of 1.0 mL/min (Figure 2(c)). Chromatograms were recorded using a Siemens Kompenosograph X-T C1012 chart recorder.
[figure(s) omitted; refer to PDF]

3. Results and Discussion

3.1. Optimisation of On-Column Trace Enrichment Liquid Chromatography

3.1.1. Liquid Chromatography and Sample Loading Conditions

To optimise the chromatographic separation of BPA, initial studies were undertaken using mobile-phase organic modifier concentrations of 20%, 50%, and 70% acetonitrile/deionised water. A mobile phase of 50% acetonitrile/deionised water was found to give a good balance between resolution and runtime and was used in further investigations. The possibility of accumulating trace concentrations of BPA at the HPLC column was investigated by pumping volumes of between 1.0 and 12 mL of deionised water containing 0.596 μg/L of BPA at a flow rate of 1.0 mL/min to the HPLC column (Table 1). Following loading, the flow to the column was switched directly to 50% acetonitrile/deionised water (1.0 mL/min), maintaining column pressure and overcoming possible issues of column phase collapse [47, 48], and the eluent monitored by fluorescence detection. A linear relationship between the sample volume introduced to the HPLC and the resulting peak height for BPA was found over the entire range investigated $R^2=0.999$.

Table 1

Effect of loading volume on the resulting peak height for a 0.596 μg/L BPA standard.

Loading volume (mL)	Fluorescence units
0	0.00

1	5.00
5	24.5
6	29.0
8	39.6
10	49.5
12	59.4

3.2. Calibration Plot, Precision, Limit of Detection, and Possible Interferences

Standard solutions of 0.10, 0.625, 1.25, 2.30, 6.25, 9.40, and 12.5 μg/L BPA were prepared in deionised water and examined using a loading volume of 6.0 mL using the optimised HPLC conditions. Figure 3(a) shows the typical chromatogram obtained for a 6.24 μg/L BPA standard in deionised water obtained under these conditions. No peaks were determinable for the deionised water run under same conditions (Figure 3(b)). A linear relationship was obtained over the range of 0.10 μg/L to 6.25 μg/L R2=0.9998. A limit of detection, based on 3σ, of 0.058 μg/L for BPA was found based on these values. It should be noted that both the sensitivity and limit of detection for the method could be readily improved by increasing the loading volumes employed [49]. Table 2 shows a comparison of other chromatographic methods for the determination of BPA. This illustrates the relatively large sample volumes required, which all require offline processing steps such as SPE or liquid extraction. Concentrations of 1.2 μg/L of bisphenol F and bisphenol S were investigated as possible interferences and were found to be chromatographically separated from BPA and hence did not interfere (Figure 4).

[figure(s) omitted; refer to PDF]

Table 2

Sample extraction and analytical measurement methods for the determination of BPA in water.

Sample	Sample volume (mL)	Sample extraction	Technique	Limit of detection (μg/L)	Ref.
Drinking water	50	Solid-phase extraction	Ultra-performance liquid chromatography-tandem mass spectrometry	0.01	[13]
River water	50	Automated column switching	High performance liquid chromatography fluorescence detection	0.00009	[49]
Plastic bottled drinking water	3	Solid-membrane extraction based on electro spun nylon 6 nanofibrous membrane	High performance liquid chromatography	0.15	[19]

Tap, lake, and river water	20	Biosorption-based dispersive liquid-liquid microextraction	Gas chromatography/mass spectrometry (GC/MS)	0.15	[27]
Environmental and tap water	5	Ionic liquid dispersive liquid phase microextraction	High performance liquid chromatography mass spectrometry	0.76	[50]
Effluents, soil leachates, food, drinking water and consumer products	—	—	Competitive enzyme-linked immunosorbent assay (ELISA)	5	[28]
Water exposed to polycarbonate animal cages	220	Extracted with dichloromethane in separating funnel	Gas chromatography/mass spectrometry (GC/MS)	0.05–0.1	[51]
Environmental and tap water	1000	Solid-phase extraction	Ultra performance convergence chromatography	10	[30]
Urine, drinking, river and wastewater	20	Magnetic dispersive solid-phase extraction	High performance liquid chromatography with diode array detection	0.6	[52]
Polycarbonate bottled water	25	Solid-phase extraction	High performance liquid chromatography with fluorescence detection	0.30	[31]
Plastic bottled drinking water	0.02	None, direct injection of water sample	High performance liquid chromatography with UV detection	380	[53]
Narrowly dispersible imprinted polymeric microspheres columns	40	None	High performance liquid chromatography single-column trace enrichment UV detection	22.8	[54]
Plastic bottled drinking water	6	None	High performance liquid chromatography single-column trace enrichment fluorescence detection	0.058	This study

[figure(s) omitted; refer to PDF]

3.3. Analytical Application

Concentrations of BPA are reported in waste and environmental water to be, in some cases, as high as the hundreds of μg/L [28, 55]. However, they are generally in the sub to low μg/L concentrations in drinking and bottled water [32, 56]. Consequently, to assess the performance of the optimised HPLC-SCTR approach, five replicate determinations of a bottled spring water sample fortified with BPA at a concentration of 1.25 μg/L were undertaken. Quantification was undertaken through external calibration. Figure 5 shows a representative chromatogram for this water sample fortified with 1.25 μg/L BPA. A mean percentage recovery of 100% was calculated with a

corresponding %CV of 7.6%. The results show that the optimised method holds promise for the determination of BPA in such samples.

[figure(s) omitted; refer to PDF]

4. Conclusions

An assay involving HPLC-SCTR with fluorescence detection has been successfully developed for the determination of trace levels of BPA in spring bottled water, at concentrations commonly previously reported. The detection method is based on the trace enrichment and direct focusing of BPA from the water sample on the analytical HPLC column. This removes the need for solvent or SPE, derivatisation steps or the need for deuterated internal standards, greatly simplifying and reducing the analysis time and expense. Samples can be processed in less than 12 minutes, obtaining performance characteristics comparable to that reported for LC/MS. In this study, a sample volume of 6.0 mL was used, allowing for a limit of detection of 0.058 μg/L BPA. It should be readily simple to improve on this by increasing the volume of sample introduced.

As far as we are aware, this report is the first to describe the use of a HPLC-SCTR assay for the detection of BPA in bottled water. However, we believe that the approach developed here could form the basis of a generic approach for the analysis of other trace contaminates in drinking and bottled water, and in future studies, we plan to investigate this further. Legislation on the usage of BPA is expected to lead to a decline in its occurrence, and the presence of its analogues, bisphenol S, bisphenol F, and bisphenol AF, is likely to increase in the future. It would be readily possible to determine these compounds in water using this same approach.

Acknowledgments

I am grateful to the University of the West of England for the funding of this project.

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DETAILS

Subject:	Acetonitrile; Bisphenol A; Chromatography; Investigations; High performance liquid chromatography; Bottled water; Spring water; Fluorescence; Water sampling
Location:	United Kingdom--UK
Editor:	Danilo Corradini
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-11-18 (Received); 2024-01-09 (Revised); 2024-01-10 (Accepted); 2024-01-24 (Pub)
DOI:	https://doi.org/10.1155/2024/8258123
ProQuest document ID:	2921792743
Document URL:	https://www.proquest.com/s cholarly-journals/development-on-column-trace-enrichment-method/docview/2921792743/se-2?accountid=211160

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Last updated:	2024-02-05
Database:	Publicly Available Content Database

Determination of Major and Trace Metals in Date Palm Fruit (Phoenix dactylifera) Samples Using Flame Atomic Absorption Spectrometry and Assessment of the Associated Public Health Risks

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ProQuest document link

ABSTRACT (ENGLISH)

This study aimed to assess the concentrations of major and trace metals (Na, Ca, Fe, Zn, Ni, Mn, Cu, Cd, and Pb) in date palm fruit samples collected from diverse regions, including Afar (Ethiopia), Iraq, and Saudi Arabia, utilizing flame atomic absorption spectrometry (FAAS). The wet acid digestion method was employed for sample treatment, with optimization of the key parameters such as reagent volume ratio, oven temperature, and digestion time for analytical applications. Under the optimized parameters, average metal concentrations in date palm fruit samples ranged from 205–299, 134–320, 38.8–115, 25.1–42.2, 9.27–27.9, 7.11–16.3, and 0.002–1.15 mg/kg for Ca, Na, Fe, Ni, Zn, Mn, and Cu, respectively. Cd and Pb levels were below detection limits within the linear range. Generally, date palm samples exhibited higher Ca and Na contents and lower concentrations of Cu and Mn than other metals. Pearson correlation analysis revealed very strong positive correlations between Fe and Na, Na and Zn, Na and Mn, Ca and Zn, Fe and Ni, Fe and Mn, and Mn and Ni. Strong negative correlations were observed for Ni and Na, Fe and Cu, and Cu and Ni. Weak correlations were noted among Na and Cu, Ca and Fe, Ca and Ni, Ca and Mn, Ca and Cu, Fe and Zn, Ni and Zn, Zn and Mn, and Zn and Cu. A recovery study using the spiking method demonstrated acceptable percentage recoveries ranging from 91.6% to 97.8%. Health risk assessment, including chronic daily intake (CDI), hazard quotient (HQ), total exposure hazard index (HI), and carcinogenic risk (CR), indicated CDI, HQ, and HI values below 1.0, except for the HI value for Ni. This suggests that the metals pose no probable public health risk, with the absence of Cd and Pb in date palm samples affirming no carcinogenic threats associated with their consumption.

FULL TEXT

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1. Introduction

The date palm (*Phoenix dactylifera*) tree belongs to the family *Arecaceae* and is considered a symbol of life in the desert, as it tolerates high temperatures, water stress, and salinity better than many other trees [1]. It is one of the oldest cultivated trees in arid and semiarid regions. The date palm fruit has tremendous nutritional value due to its rich content of essential nutrients, including minerals, carbohydrates, salts, dietary fibers, vitamins, fatty acids, amino acids, and proteins. It is unique and characterized by certain distinct properties. One of the major features that make the date palm fruit special is that it is the only fruit that can be eaten as a dietary staple in the world and has remained so for thousands of years as a daily diet for millions of people. Another unique property of the date palm fruit is that it can be consumed at each of the three major maturity stages, such as *khalal* (fresh and hard ripe), *rutab* (crisp to the succulent), or *tamar* (fully ripe stage) [2].

The three major maturity stages are based on the changes in color and nutrient composition of the date palm fruit (Figure 1). To reach the fully ripened, or *tamar,* stage, it takes 150–200 days after pollination. At the *tamar* stage, date fruits have the lowest amount of moisture and are ready for shelf preservation. Fruits are fully ripe, very sweet, dark brown or nearly black, soft, and chewy, with the lowest moisture, and rich in reducing sugars such as fructose, glucose, and sucrose [3, 4].

[figure(s) omitted; refer to PDF]

Date palm fruits have an important role in the diet of people from many countries, and the consumption of date fruits is particularly popular in the Middle East, North Africa, and South Asian countries, where about 90% of the global production of dates takes place [5]. The Food and Agriculture Organization of the United Nations (FAO) has been actively engaged in developing the cultivation of date palms. It recognizes the social, economic, and ecological importance of the date palm in countries, with suitable agroclimatic conditions, where it is traditionally grown [6]. Ethiopia has a high demand for date fruit, mainly during the month of Ramadan and other religious ceremonies and traditional rituals [7]. The date palm was introduced to Ethiopia from Middle Eastern countries about 200 years ago by traders from Yemen and Sudan [8]. From that moment on, it is cultivated mainly by agricultural producers in the Afar, Somali, Gambella, Dire Dawa, and Benishangul-Gumuz regions of the country.

There are more than 5,000 date palm species popular all over the globe [9]. They differ in size, color, texture, antioxidant activity, and phenolic content. Therefore, depending on the nutritional and mechanical characteristics of the fruit, it is possible to use it for various purposes. The date fruit is rich in various types of minerals and health-enhancing nutrients [10]. It contains several vitamins (including B1, B2, and C) with antioxidant, antiviral, and anticancer activities [11, 12]. Moreover, it has substantial amounts of minerals, including Na, K, Mg, Ca, P, Fe, Cu, and Zn. [13].

Minerals can be beneficial to plants at certain levels but can be toxic when levels exceed specific thresholds. Some metals, such as Fe, Zn, Mn, Cu, Co, and Ni, are important micronutrients for plants, but others, including Pb, Hg, Cd, As, Cr, Ga, and Ag, are nonessential for plants and have no known physiological function. The presence of these toxic metals in date fruits above the permissible limit may cause severe health problems for people consuming them [14–16]. Thus, the determination of their levels in date palm fruits is very important for the safety of human health and recommendations for human consumption.

Metal contaminants exist as superficial contaminants on pollutant leaflets of date palm fruit, thereby resulting in a suitable biomonitoring indicator for metal pollution in arid and semiarid areas. The date palm fruits are highly prone to contamination with metals [10]. The increasing metal contamination in fruits is due to the impact of fertilizers, pesticides, and various industrial processes that pollute the water and soil [17]. The ability of plants to accumulate metals in tissues depends on plant size, growth speed, and the productivity of biomass [18, 19].

The consumption of heavy metal-contaminated food items (such as date palm fruits) could pose several health risks

to humans, including kidney and liver damage, depletion of immunological defenses and intrauterine growth, psychosocial dysfunctions, anemia, damage to the skin, teeth, and central nervous system, muscular cramps, diseases associated with malnutrition, high blood pressure, and carcinogenic disease. [20–23].

Considering the potential health hazards associated with these metal ions, accurate determination of their concentrations in food samples and comprehensive assessment of the associated public health risks are crucial to safeguard food quality and protect consumer health. The common method for estimating the nature and likelihood of detrimental health effects in individuals exposed to toxic metals is human health risk assessment through chronic daily intake (CDI), hazard quotient (HQ), health risk index (HI), and carcinogenic risk (CR). This approach has been widely used by many researchers to comprehensively estimate the potential hazards due to human health related to exposure to various metals [24–26].

To the best of our knowledge, very few studies have been reported on metal contamination in date palm fruit. Therefore, the aim of this study was (i) to determine the concentrations of major and trace metals (Na, Ca, Fe, Zn, Ni, Mn, Cu, Cd, and Pb) in date palm fruit (*Phoenix dactylifera*) samples collected from three different areas, namely, Afar, Iraq, and Saudi Arabia; (ii) to assess the health risk of trace metals through the determination of chronic daily intake (CDI), hazard quotient (HQ), and hazard index (HI); and (iii) to compare the results of the present study with other previously reported studies on the determination of metals in date palm fruit samples. Furthermore, this experiment aimed at assessing the sugar content of date palm fruit samples.

The present study was limited by the relatively small sample size of date palm fruit (*Phoenix dactylifera*) collected from only three geographical locations and the analysis of only nine major and trace metal elements.

2. Materials and Methods

2.1. Description of the Study Areas

The study areas were selected based on the rank of the world's leading countries growing fresh dates and also on the current availability of the samples in Addis Ababa, Ethiopia. These selected places include the Afar region (Ethiopia), Iraq, and Saudi Arabia, which are favorable for the growth of the date palm fruit. Afar is associated with an ethnic group inhabiting the Horn of Africa. These people primarily live in the Afar region of Ethiopia and in northern Djibouti, as well as the entire southern part of Eritrea [27]. Iraq, officially the Republic of Iraq, is a country in Western Asia, bordered by Turkey to the north, Iran to the east, Syria to the west, and Saudi Arabia to the south. Saudi Arabia, officially the Kingdom of Saudi Arabia, is a country in Western Asia, constituting the bulk of the Arabian Peninsula [28].

2.2. Sample Collection and Preparation

The three varieties of date palm fruit samples were purchased from a local market in Addis Ababa, Ethiopia. The date palm fruit samples were stored in polyethylene bags to keep them free of contaminants. 5 kg of each date palm fruit was cut and deseeded, and the pulp (flesh) portion was carefully washed using tap water and then rinsed using distilled water. The samples were then open-air dried in the laboratory for two weeks to remove as much moisture from the date fruit pulp as possible to inhibit the growth of bacteria, mold, and yeast.

Then, the samples were initially oven-dried at 105°C for 24 hrs to remove the total moisture in the date fruit pulp. The dried date fruit pulp samples were then taken out of the oven and cooled for several minutes. Then, they were ground using a ceramic mortar and pestle to make fine powders, and then the powders were sieved to separate the fine particles of the date fruit pulp from the larger particles. Finally, the finely powdered date fruit pulp samples were then stored in polyethylene bags for further experimental procedures [29].

2.3. Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade. Metals standard solutions (1000 mg/L), namely, Ca, Na, Fe, Ni, Zn, Mn, Cu, Cd, and Pb, were purchased from Sigma-Aldrich (USA). Intermediate and working standards were prepared from these standard stock solutions. HNO_3 and $HClO_4$ were used for the digestion of the date palm fruit samples. All glassware was properly washed and cleaned, and the reagents used were of analytical grade. Distilled water was used throughout the study.

2.4. Sugar Content Determination of Date Palm Fruit Samples

Refractometers measure the degree to which the light changes direction, called the angle of refraction. A refractometer takes the refraction angles and correlates them to refractive index values that have been established. By using this value, one can determine the concentrations of solutions. In the present study, the date fruit pulp pieces (33 g) were placed in 100 mL of distilled water in a glass beaker and heated by using a hotplate (IKA, China) at 85°C for 90 min. The fruits were then separated from the solution by using a metal sieve, followed by squeezing the paste and filtering the solution through fine filters [30]. The solution was passed through a fine metal filter to remove the large fruit tissues. Samples were taken from the solution and cooled for 15 min, and the sugar content was determined by using a J57 automatic refractometer [31].

2.5. Optimization of Digestion Procedures

For the digestion of the date palm fruit, different digestion procedures were tested by using a mixture of HNO_3 and $HClO_4$ and varying the volume of the acids, digestion time, and digestion temperature. An optimized procedure was selected to find the values of these parameters that yield the best performance by using a minimum reagent volume and shorter digestion time that gives a clear and colorless solution at a lower temperature for the digestion of the date palm fruit. In this study, the optimization parameters/conditions for the digestion of the three date palm fruit samples are shown in Tables 1–3. As shown in the tables, for the digestion of the date palm fruit samples, 3 mL of HNO_3 and 1 mL of $HClO_4$ (3 : 1 volume ratio), 300°C, and 3 hrs were chosen as the optimum reagent volume, digestion temperature, and digestion time, respectively [29].

Table 1

Optimization of reagent volume for the digestion of 0.5 g of date palm fruit samples at a constant temperature and digestion time.

Reagent volume ratio (HNO_3 : $HClO_4$) (total volume = 4 mL)	Temperature (°C)	Digestion time (hrs)	Results (solution color)
2 : 1	300	3 : 00	Colorless and turbid
3 : 1	300	3 : 00	Clear and colorless solution
3 : 2	300	3 : 00	Colorless with suspension
4 : 2	300	3 : 00	Colorless with suspension
5 : 1	300	3 : 00	Slightly yellow
6 : 2	300	3 : 00	Colorless with suspension

Table 2

Optimization of temperature for the digestion of 0.5 g of date palm fruit samples at a constant reagent volume and digestion time.

Reagent volume ratio (HNO_3 : $HClO_4$)	Temperature (°C)	Digestion time (hrs)	Results (solution color)

3:1	150	3:00	Colorless with suspension
3:1	180	3:00	Colorless with suspension
3:1	210	3:00	Colorless and turbid
3:1	240	3:00	Colorless with suspension
3:1	270	3:00	Colorless and turbid
3:1	300	3:00	Clear and colorless

Table 3

Optimization of time for the digestion of 0.5 g of the date palm fruit samples at a constant volume and digestion temperature.

Reagent volume ratio (HNO_3:$HClO_4$)	Temperature (°C)	Digestion time (hrs)	Results (solution color)
3:1	300	0.50	Light yellow
3:1	300	1:30	Slightly yellow
3:1	300	2:00	Colorless with turbid
3:1	300	2:30	Slightly yellow
3:1	300	3:00	Clear and colorless solution

2.6. Digestion of the Date Palm Fruit

For the determination of metal contents in the date palm fruit, the wet digestion method was performed by using the Kjeldahl apparatus (Gallenkamp, England). 0.5 g of powdered and homogenized date palm fruit samples were weighed and transferred to a 250 mL round-bottomed flask. Based on the optimized parameters, a mixture of 3 mL of HNO_3 and 1 mL of $HClO_4$ (a total of 4 mL) was added to it, followed by fitting the round-bottomed flask to the reflux condenser and heating at 300°C for 3 hrs until it gives a clear and colorless solution. The digested solution was then allowed to cool for 10 mins and 5 mins before and after removing the flask from the condenser, respectively. The cooled solution was filtered using Whatman filter paper to remove any suspended matter. Finally, the filtrate was transferred to a 50 mL volumetric flask, and the volume was made up by using double distilled water. The blank solution was also prepared by using similar procedures on a triplicate basis [29].

2.7. Calibration of the Instrument

Metal analysis was performed by using atomic absorption spectrometry (AA-7000 Hitachi) with Deuterium Lamp (D2-lamp) background correction and hollow cathode lamps. The air-acetylene flame was used for the determination of all metals. Four different standard concentrations of metal solutions that include 0.25, 0.5, 0.75, and 1 mg/L for Cu, Zn, and Cd; 0.25, 0.5, 1, and 2 mg/L for Ca, Mn, and Fe; and 1, 2, 3, and 4 mg/L for Ni and Pb were prepared for constructing calibration curves. The regression coefficient (R2) exhibited good linearity with a value of 0.99. After calibration of the instrument, the digested sample solutions were aspirated into the flame atomic absorption spectrometer, and metal concentrations were determined. Three replicate determinations were carried out for each

sample. The same analytical procedure was employed for the determination of metals in the blank samples. The parameters for the calibration of the instrument are shown in Table 4.

Table 4

The parameters for the calibration of the FAAS instrument.

Elements	Wavelength (nm)	Standard concentration (mg/L)	Calibration equation	R22
Na	589	1, 2, 4, 8	$y=0.0891x+0.0276$	0.998
Ca	422.7	0.25, 0.5, 1, 2	$y=0.0118x-0.0036$	0.993
Mn	279.5	0.25, 0.5, 1, 2	$y=0.0217x-0.001$	0.999
Fe	248.3	0.25, 0.5, 1, 2	$y=0.0122x-0.0029$	0.992
Ni	232	1, 2, 3, 4	$y=0.0045x+2E-05$	0.996
Cu	324.7	0.25, 0.5, 0.75, 1	$y=0.0118x-0.0036$	0.993
Zn	2139	0.25, 0.5, 0.75, 1	$y=0.051x+0.0008$	0.996
Pb	283.2	1, 2, 3, 4	$y=0.0027x+0.001$	0.998
Cd	213.9	0.25, 0.5, 0.75, 1	$y=0.0273x-0.0016$	0.980

2.8. Method Detection and Quantification Limits

The method detection limit is the smallest concentration of analyte that can be detected with statistical confidence. The International Union of Pure and Applied Chemistry (IUPAC) defines the detection limit as the smallest concentration of analyte that has a signal significantly larger than the signal arising from a reagent blank. The limit of quantification is the smallest concentration of an analyte that can be reliably determined. The limit of detection (LOD) is expressed as three times the standard deviation of absorbance values ($n \geq 10$) obtained from the blank divided by the slope of the calibration curve ($LOD = 3 * Sb/m$), whereas the limit of quantification (LOQ) is calculated as ten times the standard deviation of absorbance values ($n \geq 10$) divided by the slope of the calibration curve ($LOQ = 10 * Sb/m$) [32, 33], where Sb is the standard deviation of absorbance values obtained from the blank, and m is the slope of the calibration curve. In the present study, the detection and quantification limits of the instrument for some metals are given in Table S1.

2.9. Trueness of the Method

Several parameters are taken into account to assess the trueness of the method, including linear dynamic range, LOQ, LOD, reproducibility, repeatability, and measurement uncertainty. Recovery is also one of the most commonly used analytical methods for the validation of results and evaluating whether the analytical method is acceptable for its intended purpose. In this study, the FAAS method validation for analysis was performed by using LOD, LOQ, and trueness tests. The trueness of the proposed method was performed by spiking standard solutions of known concentrations of the elements into the samples. The trueness test for each sample was performed in triplicate.

2.10. Human Health Risk Assessment

Assessment of human health risks due to the transfer of pollutants to the human body is often performed by using many indices, including chronic daily intake (CDI), hazard quotient (HQ), total exposure hazard index (HI), and

carcinogenic risk (CR) [34, 35].

2.10.1. Chronic Daily Intake (CDI)

The daily intake of metals is important to assure the health risks related to the intake of heavy [36] metals from water, food, and exposure to soil. It is expressed in mg/kg/day. The human noncarcinogenic risk effects for each metal can be calculated by using the following equation:(1)CDImg/kgday=IR×CF×EF×EDBW×AT,where IR is the average daily intake rate of the date palm fruit (kg/person/day), which is 100 g/d (0.1 kg/d) [37, 38], CF is the average concentration of heavy metals in the sample (mg/kg), ED is the exposure duration (the mean life expectancy of a person is 67 years), EF is the exposure frequency (365 days per year), BW is the average body weight (70 kg taken for adults), and AT is the mean exposure period for noncarcinogens (365 days per year × exposure number per year) [39].

2.10.2. Hazard Quotient (HQ)

The hazard quotient (HQ) is the ratio of the exposure of a contaminant to the reference oral dose (RfD) of that contaminant (equation (2), [40]). It indicates noncarcinogenic risk over a lifetime and is used to characterize the risk to human health posed by the intake of metal-contaminated food. If HQ(2)HQ=CDIRfD,where CDI represents the chronic daily intake of the sample per day (mg/kg/day) and RfD expresses the reference oral dose of the metal of interest (mg/kg/day). The reference oral dose (RfD) of the metal represents the tolerable daily exposure to any specific contaminant without any major risk of health effects throughout the lifetime of a person [42]. As given by the WHO/FAO (2013) [43], the following RfD values (mg/kg/day) are used in the present assessment: Zn (0.30), Cu (0.04), Ni (0.02), Mn (0.14), Fe (0.70), Pb (0.004), and Cd (0.0005).

2.10.3. Hazard Index (HI)

It is used to evaluate the potential health risks when a person is exposed to more than one heavy metal. The Environmental Protection Agency has developed a hazard index (HI) for the health risk assessment of chemical mixtures [44]. HI can be used as an effective tool to determine the potential health risks that are associated with human exposure to multiple contaminants simultaneously. The HI is the sum of the hazard quotients (HQ) for each contaminant (equation (3), [42]). If the value of HI is greater than 1.0, there would be concern for potential health effects, and if it is below 1.0, it shows no potential health risks of the sample and that the consumers are safe.(3)HI=HQ1+HQ2+HQ3+−−−HQn.

2.10.4. Carcinogenic Risk (CR)

The carcinogenic risk (Cr) for any specific contaminant can be calculated by using the following equation. According to USEPA [45], 10^{-6}–10^{-4} is the range of permitted lifetime risks for carcinogens.(4)CR=CDI×CSF,where CR represents the cancer risk, CDI is the chronic daily intake calculated in equation (1), and CSF is the cancer slope factor.

3. Results and Discussion

3.1. Determination of Sugar Content in Date Palm Fruit Samples

The sugar contents of the date palm fruit samples from the different areas were determined by using refractive index values. The refractive index values of the three date palm fruit samples were measured by using an automatic refractometer. The results for refractive index values of the analyzed date palm fruit samples and reported values are presented in Table 5. As shown in the table, the refractive index values of palm fruit samples from Saudi Arabia exhibited a higher index value than those obtained from other sample areas. This indicates that the date palm fruit samples from Saudi Arabia have a higher sugar concentration because the refractive index value increases when the solution gets thicker, resulting in a denser medium that causes higher refraction [46].

Table 5

The degree Brix and the refractive index values of the three different sample sites.

Sample sites	Degree Brix (°Brix) (w/w)	Refractive index measured (present work)	Refractive index reported [46]

Afar	14.8	1.3554	1.35536
Iraq	15.2	1.3559	1.35600
Saudi Arabia	15.4	1.3563	1.35632

3.2. Metal Contents in Date Palm Fruit Samples

The present work presents the major and trace metal concentrations in date palm fruit samples collected from Afar, Iraq, and Saudi Arabia. The determined metals include Ca, Na, Fe, Ni, Zn, Mn, Cu, Cd, and Pb. The mean concentrations of major and trace metals obtained in the studied date palm samples are summarized in Table 6 and Figure 2. The metal contents were determined based on the dry weight of the samples.

Table 6

The metals contents (mg/kg, *N*=3) in date palm fruit samples collected from different areas.

Elements	Sample areas					
Afar		Iraq		Saudi Arabia		Concentration (mean ± SD)
%RSD	Concentration (mean ± SD)	%RSD	Concentration (mean ± SD)	%RSD	Na	224 ± 6.5
2.9	320 ± 28.1	8.8	134 ± 9.8	7.3	Ca	299 ± 8.35
2.7	205 ± 7.2	3.5	275 ± 6.2	2.25	Ni	25.1 ± 1.59
6.3	28.2 ± 1.94	6.8	42.2 ± 3.87	9.1	Zn	9.58 ± 0.92
9.6	27.9 ± 2.23	7.9	9.27 ± 0.76	8.1	Fe	115 ± 8.53

7.4	114±4.83	4.2	38.8±3.35	8.6	Cu	0.002±0.0001
4.8	0.9±0.04	5.4	1.15±0.02	1.7	Mn	7.11±0.4
5.6	6.66±0.2	3.0	16.3±0.84	5.1	Pb	ND
ND	ND	ND	ND	ND	Cd	ND

ND, not detected.

[figure(s) omitted; refer to PDF]

As shown in Table 7 and Figure 2, the date palm fruit samples from Afar and Iraq contain a relatively higher concentration of Na than date samples from Saudi Arabia. On the other hand, the date palm fruit samples collected from Saudi Arabia have a higher concentration of Ni than those from Afar and Iraq. The amount of Ca in date palm fruit samples from Iraq was relatively smaller than in other areas. However, a higher Ca concentration was obtained in date palm fruit samples from Afar. Fe was found in higher concentrations in the date palm fruit samples from Afar than the date palm fruits from Iraq and Saudi Arabia. The concentration of Zn in the investigated date samples was in the order of Saudi Arabia>Afar>Iraq. A higher concentration of Mn was observed in the date palm fruit from Saudi Arabia than that from Afar and Iraq. The lowest concentration of Cu in date palm fruit was obtained from Afar. Generally, the variation in metal concentrations within the study area can be attributed to factors such as geographical location, geological activities, industrial facilities nearby, and other influential variables. These factors might contribute to the observed differences in metal levels, providing a comprehensive understanding of environmental dynamics.

Table 7

Metal concentrations in date palm fruit (from the present study) and recommended dietary allowances/adequate intakes per adult per day (RDA/AI) values of metals [57].

Metals	Mean concentrations of metals (mg/kg)	Amount of metal a person can get from 100 g of date palm fruit (mg)	RDA/AI values per adult per day (mg)
Na	134–320	13–32	1500
Ca	205–299	21–30	1000
Fe	38.8–115	4.0–12	8.0
Zn	9.27–27.9	0.93–2.8	11

In short, the mean concentration of the major metals in date palm samples from Afar was in the order of Ca>Na>Fe>Ni>Zn>Mn>Cu, while the mean concentrations of the major metals in date palm samples from Afar and Saudi Arabia were in the order of Na> Ca>Fe>Ni >Zn>Mn>Cu, and Ca>Na>Ni>Fe>Mn>Zn>Cu, respectively. Generally, the date palm samples from all areas had higher contents of Ca and Na than other metals. However, all date samples contained lower contents of Cu and Mn. In all cases, the trace metals, Cd and Pb, were not detected

in the analyzed date palm fruit samples.

3.3. Comparison of Results for Metal Contents in Date Palm Fruit Samples in the Present Study with Other Reported Values

As shown in Table 8, in the present investigation, the concentrations of Na and Ca observed in the date palm fruit samples stand out, surpassing the values reported in prior studies, with the exception of samples obtained from Sudan and Nigeria. Similarly, the concentrations of Zn and Mn in the date palm fruit samples obtained in this study were higher than the reported values, except for the samples from Nigeria and Saudi Arabia. Moreover, the Zn concentrations in the date palm fruit samples from the current study were notably elevated compared to the reported values, except for those obtained from Saudi Arabia. It is noteworthy that the amount of Ni in the data samples from this study was higher than the reported values, whereas the Cu content was observed to be the lowest. It is significant to highlight that no traces of the toxic heavy metals Cd and Pb were detected in this study. This distinguishes the date palm fruit samples obtained in the current study from those of Libya, Saudi Arabia, and Egypt, where the presence of Cd and Pb has been reported. The absence of these toxic elements in the examined date palm fruit samples adds a significant dimension to their overall composition, reinforcing their potential for consumption and further emphasizing their safety profile compared to samples from other geographical regions.

Table 8

Comparison of the mean concentration of metals in date palm fruit samples with literature-reported values.

Country	Metal concentration (mg/kg)					Method	Reference
Na	Ca	Zn	Fe		Ethiopia (Afar), Iraq, and Saudi Arabia	134–320	205–299
9.27–27.9	38.8–115		FAAS	This study	Nigeria	912–914	372−371
16.0–19.0	11.50–61.5		FAAS	[47]	Malaysia	123–248	488–707
3.28–3.41	8.08–13.5		ICP-OES	[48]	Saudi Arabia	334–338	143–157
0.34–0.45	80.8–203		FAAS/ICP-MAES	[49]	Sudan	567–1318	222–2381
7.50–10.0	6.91–69.1		FAAS	[50]	Pakistan	83.5–103	75.4–93.4
—	0.8–0.82		FAAS	[51]	Libya	19.1–69.0	24.0–64.0

7.40–13.6	8.40–128		FAAS	[52]	Saudi Arabia	—	—
22.8–45.9	56.9–155		ICP-AES	[53]	Pakistan	—	—
—	0.84–1.9		AAS	[54]	Saudi Arabia	73.8–157	150–339
19.5–21.4	21.2–54.9		ICP-OES	[55]	·		
Country	Metal concentration (mg/kg)					Method	Reference
Ni	Cd	Cu	Mn	Pb	·		
Ethiopia (Afar), Iraq, and Saudi Arabia	25.1–42.2	ND	0.002–1.15	6.66–16.3	ND	FAAS	This study
Nigeria	—	—	9.84–10.2	10.0–21.0	—	FAAS	[47]
Malaysia	0.149–0.194	—	4.47–5.04	1.62–3.36	—	ICP-OES	[48]
Saudi Arabia	9.60–55.6	0.09–28	1.15–1.26	53.4–53.9	—	FAAS/ICP-AES	[49]
Sudan	—	—	7.1–18.6	5.40–7.8	—	FAAS	[50]
Pakistan	—	—	—	—	—	FAAS	[51]
Libya	—	0.14–1.02	0.54–5.5	—	ND–0.94	FAAS	[52]
Saudi Arabia	0.82–2.2	0.15–0.26	22.8–52.2	—	0.002–0.006	ICP-AES	[53]
Pakistan	—	0.01–0.02	0.1–0.26	0.09–0.14	0.21–0.29	AAS	[54]
Saudi Arabia	—	—	6.4–7.7	5.8–8.2	—	ICP-OES	[55]

Egypt	0.93–3.0	ND–0.26	—	—	0.35–2.0	ICP-OES	[56]

ND, not detected.

The permissible amounts of minerals consumed by an adult from date palm fruit are shown in Table 7. The contents of Na, Ca, and Zn in this study are below the recommended values. This suggests that the date palm fruits are a good source of major metals but not sufficient, so an additional diet from other sources is necessary.

3.4. Statistical Analysis

The concept of ANOVA is to compare different sources of variance and make inferences about their relative sizes. In this study, a one-way ANOVA was used to compare the mean concentrations of the metals in the date palm samples from different areas (Afar, Iraq, and Saudi Arabia). Microsoft Excel was also used for data analysis. The results are presented in Table 9. As shown in the table, from the ANOVA results, the values of $F_{(calculated)}$ are less than those of $F_{(critical).}$ This indicates that no significant difference was obtained among the mean concentrations of the metals (Na, Ca, Fe, Zn, Ni, Mn, and Cu) in the date palm fruit samples from the three different sampling areas.

Table 9

One-way analysis of variance (ANOVA) results for the mean concentrations of metals in the date palm fruit samples from the different sampling areas at a 95% confidence level.

Metals	Fcalculated	Fcritical	Remark
Na	0.04	5.14	No significant difference among the sample means
Ca	0.03	5.14	No significant difference among the sample means
Fe	0.04	5.14	No significant difference among the sample means
Zn	0.01	5.14	No significant difference among the sample means
Ni	0.07	5.14	No significant difference among the sample means
Mn	0.03	5.14	No significant difference among the sample means
Cu	0.007	5.14	No significant difference among the sample means

3.5. Pearson Correlation Coefficients of Metals

The Pearson correlation coefficient is a measure of the strength of a linear association between two variables and attempts to draw a line that best fits the data of those variables. The correlation coefficient of experimental analysis indicates how strongly two variables are related to each other. A correlation coefficient of +1.0 indicates a perfect positive correlation, while a correlation coefficient of −1.0 indicates a perfect negative correlation. The correlation values are categorized as no correlation (r = 0.00–0.19), low correlation (r = 0.20–0.39), medium correlation (r = 0.40–0.59), higher correlation (r = 0.60–0.79), and highest correlation (r = 0.80–1.00) [58].

Table 10 presents the correlation coefficient values of the metals in the date palm samples analyzed in this study. Notably, the highest correlation values, encompassing both positive and negative correlations, were observed between Na and Fe, Na and Zn, Ca and Zn, Fe and Ni, Na and Mn, and Fe and Mn. Additionally, significant correlations were observed between Na and Ca, Na and Ni, Fe and Cu, Cu and Ni, and Mn and Cu. Moderate correlations were observed between Fe and Zn, Zn and Mn, and Ca and Cu, while lower correlations were observed in Ca and Fe, Ni and Zn, Ca and Mn, and Zn and Cu. There were no correlations between Ca, Ni, Na, and Cu.

Table 10

Pearson correlation coefficients (*r*) of metals in date palm fruit samples.

	Na	Ca	Fe	Ni	Zn	Mn	Cu
Na	1						
Ca	–0.72	1					
Fe	0.85	–0.26	1				
Ni	–0.75	0.10	–0.98	1			
Zn	0.88	–0.96	0.50	–0.35	1		
Mn	–0.87	0.31	–0.99	0.97	–0.54	1	
Cu	–0.18	–0.53	–0.67	0.78	0.29	0.63	1

From an analytical or physicochemical perspective, understanding these correlations provides valuable insights into the interactions and interdependencies between the different metals present in date palm samples. This information is crucial for elucidating the potential sources of contamination, metabolic pathways, or environmental influences that may contribute to the observed metal associations. Furthermore, such correlations can aid researchers and analysts in refining analytical methodologies and experimental designs for future investigations, ultimately enhancing the precision and relevance of metal concentration assessments in date palm fruit.

3.6. Recovery Study

A recovery study was conducted by spiking known standard solutions of metals to the prepared date palm fruit sample solutions. The percentage recoveries were calculated by using the equation: % recovery = ((amount detected − original amount in the sample)/amount added) ∗ 100. As shown in Table S2, the percent recoveries for the representative four metals analyzed in the date palm fruit samples were found to be within the range of 91.1% –98.5%. This indicates the suitability of the method for real sample analysis.

3.7. Public Health Risk Assessment Associated with the Consumption of Date Palm Fruit

In the present study, in order to assess the health risks of the consumption of date palm fruit, the potential health risk index values such as CDI, HQ, and HI of different date palm samples were calculated for adults as given in Table 11. From the table, it can be seen that the HQ values of all metals in the date palm samples are below 1.0, which pose no public health risks. Furthermore, the HI values of the date palm samples are lower than 1.0, except for Ni. This indicates that the concentrations of metals in the date palm samples are within the permissible limit and have no probable public health risks except Ni (Table 11). Since Cd and Pb were not detected in the date palm samples, the carcinogenic risk (CR) was not calculated and studied in the present study.

Table 11

CDI (mg/kg/day), RfD (mg/kg/day), HQ, and HI for the date palm samples collected from different areas.

Elements	Sampling areas	CDI	RfD	HQ	HI
Ni	Afar	0.0066	0.02	0.33	1.25

Iraq	0.0074		0.37		Saudi Arabia
0.011		0.55		·	
Zn	Afar	0.0025	0.30	0.0083	0.04
Iraq	0.0073		0.024		Saudi Arabia
0.0024		0.008		·	
Fe	Afar	0.03	0.7	0.043	0.1
Iraq	0.03		0.043		Saudi Arabia
0.01		0.014	0.0135	·	
Cu	Afar	0.00000052	0.04	0.000013	
Iraq	0.00024		0.006		Saudi Arabia
0.0003		0.0075	0.057	·	
Mn	Afar	0.0019	0.14	0.014	
Iraq	0.0017		0.012		Saudi Arabia

It should be noted that we determined not only the levels of trace (heavy) metals but also the contents of major metals (such as Ca and Na) in date palm fruit samples in the present study. Thus, the concentrations of Ca and Na in date palm fruit samples were determined as indicated in Section 3.2 and Table 7. However, the potential health risk assessments of these metals were not carried out due to their nontoxic nature and the absence of any known adverse health effects associated with their consumption. Other researchers also performed the potential health risk assessment for only heavy metals, not major metals [20, 24–26, 35, 36, 39, 41, 42].

4. Conclusion

The investigation of date palm fruits from three regions (Afar, Iraq, and Saudi Arabia) revealed a rich mineral profile, particularly in essential minerals such as Ca, Na, Fe, Mn, and Cu. Adequate levels of micromineral nutrients, such as Ni and Zn, were also observed. Notably, the toxic heavy metals Pb and Cd were not detected in any of the samples. The analytical method employed demonstrated exceptional accuracy, with recovery rates ranging from 91.6% to 97.8%. By comparing metal concentrations among the regions, it is observed that a consistent pattern emerged, with Ca, Na, and Fe surpassing Ni, Zn, Mn, and Cu in mean concentrations. The one-way ANOVA test confirmed no significant differences in these mean concentrations between the regions. Notably, Saudi Arabian date palm samples exhibited the highest sugar content, followed by Iraqi, and the lowest sugar content was observed in Afar samples. The potential health risk assessment indicated no probable public health concerns arising from the

consumption of date palm fruits, except for a slightly elevated HI value associated with Ni. Overall, this study provides valuable insights into the mineral composition and safety of date palm fruits from diverse geographical origins. The study provides valuable insights into metal concentrations in date palm fruits, but its limitations include a small sample size, geographical variations, analytical constraints, excluded metals, lack of consideration for temporal variability, and limited exploration of metal sources. These limitations should be considered in interpreting the findings and guiding future research.

Disclosure

This article has been presented as a thesis at Addis Ababa University digital library according to the following link: https://etd.aau.edu.et/bitstream/handle/123456789/22173/Feven20Tamirat202020.pdf?isAllowed=y&sequence=1.

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DETAILS

Subject:	Spectrometry; Food contamination &poisoning; Reagents; Iron; Carcinogens; Public health; Nickel; Absorption spectroscopy; Copper; Cadmium; Calcium; Atomic absorption analysis; Dietary minerals; Polyethylene; Zinc; Risk assessment; Health risks; Trace metals; Lead; Heavy metals; Health risk assessment; Fruits; Manganese; Acid digestion; Parameters; Correlation analysis
Location:	Iraq; West Asia; Addis Ababa Ethiopia; Saudi Arabia; Ethiopia
Editor:	Chanbasha Basheer
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-08-17 (Received); 2023-12-23 (Revised); 2024-01-12 (Accepted); 2024-01-23 (Pub)
DOI:	https://doi.org/10.1155/2024/9914300
ProQuest document ID:	2921792711
Document URL:	https://www.proquest.com/scholarly-journals/determination-major-trace-metals-date-palm-fruit/docview/2921792711/se-2?accountid=211160

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Last updated:	2024-02-05
Database:	Publicly Available Content Dat abase

A Scoping Review on Fungus and Mycotoxin Studies in the Building’s Environment: Mycotoxin Analysis by Mass Spectrometry

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ProQuest document link

ABSTRACT (ENGLISH)

It has been well-established that mycotoxins are poisonous chemical metabolites secreted by certain molds. Some of them significantly affect the health of humans and livestock. Increasing attention is now being paid to uncovering and identifying mycotoxins' presence in the building's environment. However, the main challenge remains in suitable and reliable analytical methods for their identification and detection in infected structures. GC-MS and LC-MS/MS techniques have been used extensively for mycotoxin analysis, and advancement in these techniques enabled a more comprehensive range of mycotoxins to be detected. As such, this study aimed to address a brief overview of various phenomena of existing sample collection, preparation, and analysis to detect mycotoxins in the building's environment. This scoping review includes articles from 2010 to 2020 available from PubMed, Scopus, Cochrane, Wiley, Google Scholar, and ScienceDirect. Duplicate articles were removed, and exclusion criteria were applied to eliminate unrelated studies, resulting in 14 eligible articles. The present study provides an overview of mycotoxin analysis by GC-MS and LC-MS/MS in buildings. Many techniques are available for analyzing and detecting multiple mycotoxins using these methods. Future efforts would focus on rapid assays and tools enabling measuring a broader range of mycotoxins in a single matrix and lower detection limits. In addition, it would assist future findings on new techniques and mycotoxins that existed in the building's environment.

FULL TEXT

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1. Introduction

Mycotoxins are secondary metabolites of fungi associated with various toxicities in humans and animals. They have long been studied because of their extensive exposure to food and feed commodities and their potential use as therapeutic drugs and biological warfare agents. Due to the prevalence of mycotoxin contamination in foods and feeds, many years of research have focused on ingesting these toxic compounds. Inhalation of mycotoxins has yet to attract much attention, and available reports point mainly to occupational and agricultural settings. In the 2000s, mycotoxins were hinted to be toxic agents adverse to human health through inhalation exposure in a nonagricultural indoor environment. Airborne mycotoxins have been reported in moldy buildings other than agricultural settings [1, 2]. They originate from the fungal pollution of the indoor environment, e.g., sterigmatocystin and aflatoxins produced mainly by *Aspergillus* spp. which include *A. versicolor* and *A. flavus*, and macrocyclic trichothecenes produced by *Stachybotrys chartarum*. A comprehensive review of fungal pollution in an indoor environment was documented by Khan and Karuppayil [3]. Wood or wood-based products are susceptible to infestation by *Cladosporium* and *Penicillium* (*Penicillium brevicompactum* and *Penicillium expansum*), *Trichoderma*, and *Aspergillus* [4, 5]. *Paecilomyces variotii*, *Trichoderma harzianum*, and *Penicillium* species attack polyurethanes used in composites for insulation [6]. Also, dust on the surfaces and inner wall materials used in buildings, such as prefabricated gypsum board, paper, and glue, represents an excellent substrate for fungal growth. According to D'Mello [7] and Ciegler and Bennett [8], one mold species can produce several mycotoxins, and vice versa, and different mold genera may produce the same mycotoxins.

Human health effects attributed to the inhalation of mycotoxins in workplaces include mucous membrane irritation, skin rash, nausea, immune system suppression, acute or chronic liver damage, acute or chronic central nervous system damage, endocrine effects, and cancer [9]. Furthermore, some nonspecific symptoms possibly related to mycotoxin production, such as cough, irritation of the eyes, skin, respiratory tract, joint aches, headache, and fatigue, have also been documented [10, 11]. Aflatoxin, trichothecenes, and ochratoxins are the most well-known mycotoxins found in the indoor environment [12, 13]. To date, several hundred mycotoxins have been discovered. Various methods, including high-performance liquid chromatography techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), are widely used for identifying and detecting mycotoxins. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is vital and central for mycotoxin analysis. In contrast, the molecular polymerase chain reaction (PCR) approach and the

enzyme-linked immunosorbent assay (ELISA) were commonly used in fungi identification [14]. The ability and sensitivity of multiple mycotoxin quantifications in various matrices such as food, feed, and biological samples have expanded significantly since 2010, attributed to the progress in LC-MS and the combination of appropriate sample extraction and cleanup procedures [15–17]. This approach is highly relevant as several mycotoxins tend to co-occur with others, regardless of the similarity in their chemical structure [18–20]. The most common mycotoxin extraction method applied for an indoor environment is liquid-liquid extraction (LLE) with a wide variety of solvents such as methanol [21–24], acetonitrile [25–27], and dichloromethane [28, 29]. In addition, the combination of the solvent mixture, e.g., methanol, dichloromethane, and ethyl acetate and chloroform and methanol, in different ratios has also been adopted [23, 30–32].

This selected study is a scoping review that aims to provide insight into the recent mycotoxin study and analysis in the building's environment using GC-MS and LC-MS/MS from the available literature. This review has also enabled us to identify the knowledge gaps and future potential research in this area. Validated and updated evidence from this review can assist professional bodies in the importance of this subject matter on human health and mitigation strategies.

2. Methodology

Scoping reviews provide an excellent approach to analyzing research findings on a particular topic. Scoping offers an overview of the literature, narrowing down the related study to match our targeted case, and finally summarising the main component, concepts, and the available data to give an insight into the gap that is available in the field [33, 34]. Hence, a scoping review was chosen to review research articles on the available analytical techniques that allow the optimum discovery and quantification of targeted mycotoxins relying on the mass and ion charge methods. The mass spectroscopy system offers sensitivity and specificity for challenging and matrix-complex samples. The selected topic accommodated the proposed research study in the Institute for Medical Research (IMR), Malaysia (NMRR-18-962-41809). For the scoping approach, we adopted Arksey and O'Malley's [35] method consisting of six phases to guide the selection process of the suitable literature available for our review purposes. We also considered updated guidelines published by the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) 2020 statement [36].

2.1. Phase 1: Identifying the Research Question

Various methods have been adopted for determining mycotoxins, whether in the air, building materials, or dust, utilizing mass spectrometry or a more advanced tandem mass spectrometry method. As for our scope, we would like to answer "what are the recent methods of LC-MS/MS and GC-MS that are being used to determine the presence of mycotoxin in the targeted samples collected from indoor air in building environments?"

2.2. Phase 2: Identifying the Relevant Studies

During the search, the following criteria were set as guidance:

(1) Articles were written in English only

(2) Articles published from 2010 to 2020

(3) Databases search from PubMed, Google Scholar, Cochrane, Scopus, Wiley, and ScienceDirect

(4) Open-access research articles only, excluding review papers, book chapters, and conference papers

(5) Keywords are indoor air, mycotoxin, LCMS/MS, and GCMS

2.3. Phase 3: Study Selection

PRISMA guidelines were used in our study selection to assist as a guided protocol for the search. At the initial stage, all team members were assigned to a specific database to conduct a web search based on the selected keywords. The search was then transferred to the Microsoft Excel spreadsheet and assigned to team members to scrutinize the obtained papers based on our agreed inclusion criteria. Team members will review the journal papers' abstracts to determine their relevance to our review scope. Furthermore, the full articles will be retrieved, and the information will be transformed to highlight the findings to decide whether a particular paper will be included or excluded for scoping review purposes. When members were unsure about the acceptability of a particular paper during abstracts or full-text screening, a discussion session was conducted to finalize the decision. The flow of the process is shown

in Figure 1.

[figure(s) omitted; refer to PDF]

2.4. Phase 4: Charting/Organizing the Data

The identified, sorted, and selected papers were charted into a table to organize the information systematically. Table 1 shows how the information was tabulated to meet the research questions and scoping review purposes.

Table 1

Search strategy for identification of studies.

No.	Keywords
1	Mycotoxin
2	Indoor air
3	LCMS/MS
4	GCMS
5	Combination of 1 AND 2 AND 3
6	Combination of 1 AND 2 AND 4
7	Combination of 1 AND 2 AND 3 AND 4

2.5. Phase 5: Collating, Summarising, and Reporting the Results

The main aim of a scoping review is to collect the available data, results from findings, and research output into more tangible information to be well-versed on the research that had taken place, the area that can be improvised, and finally give an overview of the research gap that can be tackled for the future research direction (Figure 2).

[figure(s) omitted; refer to PDF]

3. Mycotoxins in Buildings

This review was started with a concurrent investigation on mycotoxins in Peninsular Malaysia's healthcare institutions initiated by researchers from the Institute for Medical Research (IMR), Malaysia. A scoping review was published covering topics on fungal identification in hospital settings, with participants from many nations and locations [38]. The review was generally on fungus profiling in hospitals, with limited papers reviewed on mycotoxin distribution. The findings revealed that the most common fungal genera detected in such settings were *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and *Fusarium* sp. with the most identified *Aspergillus* sp. in hospital wards being *A. flavus*, *A. fumigatus*, and *A. niger*, when compared to several settings such as neonatal intensive care units (NICUs), labour rooms, laboratories, and others. The findings discovered that intensive care units (ICUs) and wards were home to various fungus species, primarily *Aspergillus* spp. (Sham et al., 2021). Additional information on analysis for secondary metabolites in indoor air in building environments reported in this current review believes that more studies in this area can be pursued to gain more knowledge and understanding to solve issues quickly and effectively.

In the past ten years (Jan 2010–Feb 2021), 14 published studies on analysis for secondary metabolites in indoor air in building environments were recorded. Five studies were published in 2016, followed by three earlier publications in 2011. Significant findings from analysis of secondary metabolites in indoor air and studies on building environments based on 14 selected articles are listed in Table 2. Most studies on these areas were conducted in European countries, with four studies in France [21–23, 32]. One of each study was conducted in Italy [39], Croatia

[30], Poland [27], Denmark [31], and Germany [25], respectively. Two studies were conducted in Finland [25], but another was in collaboration with researchers from the Netherlands and Spain [29]. Three studies were reported from outside Europe, one from the USA [24], and the other two were conducted in Malaysia. Both studies collaborated with a researcher from Sweden [28, 40].

Table 2

Highlights on findings of analysis for secondary metabolites in indoor air and studies of building environments based on 14 selected articles.

No.	Country	Samples and study locations	Study aims	Highlights on important findings	Reference
1	France	Fungal aerosols were collected from homes damaged by wood-rotting fungi in Lower Normandy, France. Damaged homes were described as having high moisture content, lack of ventilation, and improper renovation	The fungal contamination in homes harmed by wood-decaying and airborne molds, *Serpula lacrymans*, was described in this study. Mycotoxins in the air were measured using HPLC-MS/MS, and the Ames test was used to determine the mutagenicity of fungal aerosols. Fungal species growing on building materials were studied using cultural and molecular methods, mycotoxins in the air were measured using HPLC-MS/MS, and the Ames test was used to determine the mutagenicity of fungal aerosols	Results showed that *Serpula lacrymans* was detected in the air for one-third of homes with sometimes the co-occurrence of other ligninolytic basidiomycetes species like *Donkioporia expansa*. Various molds in the air (117 species) and on materials (103 species) were identified including a recurrent species like *Aspergillus versicolor* and *Penicillium fellutanum*. Airborne culturable fungal levels were measured up to 5.8×10^5 CFU/m^3. Alternariol and/or ochratoxin A mycotoxins were observed in 4 homes, but no mutagenic activity was found	[21]
–					

2	Italy	Atmospheric particulate matter was sampled in wet indoor environment, workplaces with previous flood and fungal spores detected, and outdoor environments. No specific sampling location was mentioned	Screening of mycotoxins (deoxynivalenol, aflatoxin B1, ochratoxin A, T-2 toxin, zearalenone, and sterigmatocystin) in indoor and outdoor environments, airborne particulate matter was developed, and method performance data were presented	Single-step extraction using optimal solvent mixture (acetonitrile: water at 90 : 10) utilizing the accelerated solvent extraction method and purified using solid-phase extraction via Strata C18-M cartridges. Good linearity was obtained for each mycotoxin at different concentrations by correlation coefficients ranging from 0.994 to 0.999. The apparent recoveries from HPLC/ESI-MS/MS analysis in the MRM mode were >70% with a 10% CV, and the optimized process allowed simultaneous separation of analyte in less than 10 minutes	[39]
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3	France	Bioaerosol samples were collected from cattle sheds located in Normandy	To analyze fungal contamination of bioaerosols collected in a dairy shed during two periods of 10 days each, investigate the toxigenic capacities of *Aspergillus fumigatus* and *Aspergillus flavus* group isolates, and use the Ames test to determine the mutagenic properties of bioaerosol samples	*Aspergillus flavus, Aspergillus fumigatus, Penicillium chrysogenum, Stachybotrys chartarum*, and the allergenic species *Ulocladium chartarum* and *Cladosporium cladosporioides* were the most common in the air. Between the two study periods, the median CFU/m^3 ranged from 6.48 to 76.4, which exhibited significant differences between species. During straw processing, there was a peak of fungal contamination. In vitro *A. flavus* isolates do not produce aflatoxin, while *A. fumigatus* isolates do produce gliotoxin, verruculogen, and fumagillin. Aflatoxin B1 (0.09 ng/filter) and B2 (0.07 ng/filter) were found in bioaerosols at concentrations below LOQ. Results showed that farmers may be exposed to *Stachybotrys chartarum* during ordinary barn work	[22]
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4	Finland	Airborne dust samples were collected from surfaces above floor level of single-family homes across Finland	In this work, sample materials from severely moisture-damaged dwellings were subjected to a multianalyte tandem mass spectrometry-based technique. The goal is to provide a qualitative and quantitative description of the various microbial metabolites found in the samples	Indoor samples for at least one of the 186 analytes analyzed yielded 69 positive results, with up to 33 different microbial metabolites discovered. Hazardous bacterial metabolites were discovered in indoor samples, along with their co-occurrence with mycotoxins. The bacterial chemicals including monactin, nonactin, staurosporine, and valinomycin were found in moist building materials, whereas chloramphenicol was found in high concentrations in house dusts, including settled airborne dust. *Streptomyces* spp., a species of microbes that is considered a moisture damage indicator in indoor environments, produces these bacterial metabolites, which are highly bioactive chemicals	[25]
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5	Malaysia	Airborne dust was collected from randomly selected secondary schools and classrooms in Johor Bahru, Malaysia. A questionnaire with standardized questions was used for health assessment in 15 randomly selected pupils from each class	The researchers wanted to look for specific fungal DNA, hairy pet allergies, and mycotoxins in dust samples from Malaysian schools and see if there were any correlations with kids' respiratory health	Results indicated that fungus DNA and cat allergens were abundant, and students had a high rate of doctor-diagnosed asthma and respiratory problems. The finding of positive associations with specific DNA from the fungal species *A. versicolor* and the bacteria *Streptomyces* spp., despite the lack of a link between total fungal DNA and respiratory health, highlights the importance of analyzing specific microbes when studying respiratory health effects. The use of qPCR to analyze fungal and bacterial DNA collected using the Petri dish sample method appears to be a promising tool for monitoring indoor mold exposure because it can detect both general and DNA sequences regardless of whether organisms are alive or dead	[28]
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6	Finland The Netherlands Spain	The settled dust swab and moldy spot surface swab samples were collected from different locations in school buildings in Spain, the Netherlands, and Finland	The study measured both buildings with and without moisture damage and/or dampness observations. More than 180 analytes were targeted in settled dust and surface swab samples using the LC/MS-based methodology	The results showed that 42%, 58%, and 44% of all samples collected in Spanish, Dutch, and Finnish schools, respectively, were positive for at least one of the metabolites analyzed. The frequency of microbial secondary metabolite determination (except for emodin, certain enniatins, and physcion) was low and ranges from 10% and below of positive samples. Thirty different fungal and bacterial secondary metabolites were found in samples. Some differences in the metabolite profiles were observed between countries and between index and reference school buildings. A major finding was that settled dust from contaminated schools contained additional higher levels of microbial secondary metabolites than dust samples from noncontaminated schools	[29]
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7	Croatia	Indoor air samples of selected apartments, basements, and grain mills were collected in Croatia	The distribution and species diversity of sterigmatocystin (STC)-producing Aspergilli from the section Versicolores in the indoor air of apartments (APs), basements (BS), and grain mills (GMs) in Croatia, as well as their cytotoxic potency, are presented in this study	Total airborne fungal species detected in sampling locations: 0.7–20% in the AP, 11–55% in the BS, and 0–2% in the GM. Dominant species were *A. jensenii* and *A. creber*, followed by *A. protuberus, A. venenatus, A. tennesseensis, A. amoenus, A. griseoaurantiacus,* and 3 undescribed species. Species that produced STC: *A. griseoaurantiacus* (208.29 *μ*g/mL), *A. jensenii* (1.192–133.63 *μ*g/mL), and *A. protuberus* and *A. tennesseensis* (0.117–2.749 *μ*g/mL). Lower species diversity was obtained in the GM with relatively high STC levels (0.06–2.35 *μ*g/g) in 52% samples. STC cannot be fully attributed to Aspergilli (Versicolores). Human lung A549 cells and THP-1 macrophage-like cells were cytotoxic to STC and most STC-producing aspergilli at relatively low concentrations, indicating that humans are at high risk during chronic exposure	[30]
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8	Poland	Urban agglomeration in central Poland: Scrapping and airborne dust samples were collected from residences selected from flats by a simple random selection method. Selection was based on the existence of mycelium on solid surfaces in the flats	The major goal was to determine the toxinogenic potential of fungus isolated from moldy surfaces in residential rooms in an urban agglomeration, remote from flooded areas in a mild temperate zone	This study confirmed on capability of producing sterigmatocystin and roquefortine C by growing *Aspergillus versicolor* and *Penicillium chrysogenum,* respectively, in mixture of fungi from scraping and pure cultures in laboratory conditions. Results shows that sterigmatocystin was produced by 8/13 isolated strains of *Aspergillus versicolor* (2.1–235.9 *μ*g/g), while 4/10 of isolated strains of *Penicillium chrysogenum* produced roquefortine (12.9–27.6 *μ*g/g). Sterigmatocystin was determined (3.1–1683.2 *μ*g/g) in 11/13 scraping samples positive with *Aspergillus versicolor*, while *Penicillium chrysogenum* was determined in 3/10 of samples with roquefortine C (0.9–618.9 *μ*g/g). Mycotoxins in all air dust and scraping samples were detected below LOD, indicating minor exposure to residents in moldy flats of urban agglomeration situated far from flooded territories	[27]
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9	Malaysia	Settled airborne dust was collected from randomly selected 8 primary schools in Johor Bahru, Malaysia. 462 students from four classes of grade two were selected to participate in the questionnaire survey	To see if there was a link between rhinitis and other types of weekly SBS symptoms among junior high school students in Johor Bahru, Malaysia, and levels of cat allergen (Fel d 1), two mycotoxins (verrucarol and sterigmatocystin), and five fungal DNA sequences in the classrooms	Indoor CO_2 levels were 492 ppm (range 380–690 ppm) in all classrooms with open windows. Fungal DNA and cat allergens were common in the studied Malaysian schools, and there were a high prevalence of rhinitis and SBS symptoms among the students, especially headache (20.6%) and tiredness (22.1%). Total fungal DNA in swab samples was significantly associated with rhinitis, ocular symptoms, and tiredness. Positive associations were shown between *Aspergillus versicolor* DNA in Petri dish samples, ocular symptoms, and tiredness. The level of the mycotoxin verrucarol in swab samples was positively associated with tiredness. *Streptomyces* DNA in swab samples and Petri dish samples were negatively associated with tiredness. Mycotoxins may be ubiquitous in tropical indoor environments	[40]
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10	France	Aerosolization of mycotoxins after growth of toxinogenic fungi on wallpaper. No specific sampling location was mentioned	This study investigated mycotoxin production by *Penicillium brevicompactum*, *Aspergillus versicolor*, and *Stachybotrys chartarum* during their growth on wallpaper and the possible subsequent aerosolization of produced mycotoxins from contaminated substrates	Findings showed that mycophenolic acid, sterigmatocystin, and macrocyclic trichothecenes (sum of 4 major compounds) could be produced at levels of 1.8, 112.1, and 27.8 mg/m^2, respectively, on wallpaper, and part of the produced toxins could be aerosolized from substrate. The propensity to aerosolization differed according to the fungal species. Particles were aerosolized from wallpaper contaminated with *P. brevicompactum* when air velocity of just 0.3 m/s was applied, where *S. chartarum* required 5.9 m/s, while *A versicolor* was intermediate at 2 m/s. Quantification of the toxic content revealed the association with particles of size equal to or higher than 3 μm, which may correspond to spores. Some macrocyclic trichothecenes (especially satratoxin H and verrucarin J) can also be found on smaller particles	[32]
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11	Denmark	A water-damaged daycare in the greater Copenhagen area provided fungal biomass and settled dust samples	For known and provisionally identified chemicals, an ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) approach was established using UHPLC-quadruple time-of-flight (QTOF) screening of fungal culture extracts, wall scrapings, and reference standards	This method discovered 12 *Stachybotrys* metabolites, which were measured using either real standards or standards that were similar to authentic standards. The two recognized chemotypes, S and A, coexisted in samples taken from *S. chartarum*-infected walls. A link between mycotoxin concentrations found on contaminated surfaces and in settled dust was established. Results from one dust sample collected from water-damaged room contained 10 pg/cm^2 macrocyclic trichothecenes (roridin E). Primarily, more than one spirocyclic drimane was detected in dust where up to 600 pg/cm^2 was detected in the water-damaged room and 340 pg/cm^2 was detected in the adjacent room. They could be attractive candidates for exposure indicators because of their widespread dispersion in detectable concentrations in dust	[31]
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12	France	Air samples were taken from several locations in France, including mechanical-biological treatment plants, large-scale industrial composting operations, and a material recovery facility	The purpose of this research was to find out how much airborne aflatoxin B1, ochratoxin A, gliotoxin, and sterigmatocystin were present in waste recycling and recovery facilities, as well as the health risks that came with it. Using ultrahigh resolution mass spectrometry and ultraperformance liquid chromatography, targeted mycotoxins were measured in 94 air samples collected in five locations	The results revealed that just 11% of the samples could be quantified. Mechanical separation areas in mechanical-biological treatment facilities and the material recovery facility were used to measure aflatoxin B1 and sterigmatocystin. All the exposure levels were less than 1 $ng \cdot m^{-3}$. This is the first time sterigmatocystin exposure in waste management facilities has been quantified. In any of the air samples, ochratoxin A and gliotoxin were not detected. Approaches to assessing health risks did not reveal a serious hazard to employees' health. Data did not support the requirement for specific preventative actions against the measured biological agent in the air	[23]
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13	Germany	Settled floor dust samples were collected from different sites in Germany: waste management units dealing with municipal waste or paper recycling and severe moisture damage/dampness problems in houses occupied by less than 5 people	The determination of many biological and anthropogenic contaminants in settled floor dust using LC-MS/MS and GC-MS technologies is described in this paper (SFD). The presence of both microbial and nonmicrobial volatile organic molecules was determined using the GC-MS technique. The goal of the targeted LC-MS/MS study is to identify species-specific secondary metabolites	In the SFD matrix, 30 of the 71 discovered volatile organic compounds (VOCs) are new to the database. The results showed that using “AMDIS and SpectConnect” together was helpful in evaluating and identifying prime volatile contaminants in complex environmental samples. Nonanal was found as a possible MVOC marker using principal component analysis (PCA) of peak areas of 18 microbial volatile organic compounds (MVOCs). When considering their possible other origins from paints and cosmetics, respectively, toluene and 1-tetradecanolshowed discriminative influence but are not considered MVOC indicators	[25]

Indoor air samples and study locations were selected from water-damaged home buildings [21, 24, 25, 27], schools and kindergartens [28, 29, 31, 40], workplaces (farms and industries) [22, 23, 39], and few selected buildings and locations [30] (Vishwanat et al., 2011), but one study did not mention their location [32]. Regarding study scope and objective, most studies provided qualitative and quantitative descriptions of the microbial toxins in indoor air and their metabolites found in samples. Lanier et al. [22] conducted an in vitro study on a specific fungus to check on a specific mycotoxin produced. Jeżak et al. [27] determined the toxicogenic potential of fungus isolates from moldy surfaces. Other studies extended into mutagenic properties of bioaerosol samples [21, 22], health risk assessment [23, 28, 40], and cytotoxic potency [30]. The development of mycotoxin screening in airborne particulate matter and method performance using LC-MS was also described [39].

There are studies reported on mycobiota on building materials and bioaerosols collected from different selected locations [21, 22, 25, 30]. Pottier et al. [21] reported that nine out of twenty selected houses contained a fungus identified as *Serpula lacrymans*. Also found in the selected houses were ligninolytic strains like *Donkioporia expansa* , *Serpula himantioides*, and *Coniophora puteana*. Lanier et al. [22] identified 45 fungal species in the cattle shed where *Stachybotrys chartarum* was observed for the first time. Among the common fungal species identified were *Aspergillus fumigatus*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Stachybotrys chartarum*, *Ulocladium chartarum*, and *Aspergillus glaucus*. *Stachybotrys chartarum* was found to be the highest contribution of recurrent strain which was up to 41%. Fourteen fungus species (8 genera) and three yeast species (2 genera) were most frequently isolated on infected surfaces in residential rooms in Poland. They were identified as *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Ulocladium chartarum*, and *Acremonium charticola*. Four identified genera were susceptible to humans (*Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., and *Phoma* sp.). These included two species capable of producing hazardous mycotoxins (*Aspergillus versicolor* and *Penicillium chrysogenum*) [27].

Aspergillus section *Versicolores* producing sterigmatocystin was found in an apartment’s basements and grain mill,

the first study over a year in Croatia. The dominant and highest sterigmatocystin-producing species identified using the calmodulin sequence were *A. jensenii* (1.192–133.63 μg/mL), *A. creber*, and *A. griseoaurantiacus* (208.29 μg/mL). The study also showed that the *Aspergillus* extracts producing positive-sterigmatocystin exert cytotoxicity towards A549 and THP-1 macrophage-like cells in low concentrations [30]. A fungal toxicogenic evaluation was conducted from a mold-infected exterior found in a residential room in the urban agglomeration in Poland without the influence of an environmental factor such as a flood-affected building or area. Mycological analysis showed *Aspergillus versicolor* and *Penicillium chrysogenum* producing sterigmatocystin (Figure 3(a)) and roquefortine C at a range of 2.1–235.9 μg/g and 12.9–27.6 μg/g, respectively, and the detection from air dust and scrapped material was below the limit of detection [27]. Indoor air quality greatly affects respiratory illnesses in which Norbäck et al. [40] found the prevalence of rhinitis and sick-building syndrome among students (n=462; 14–16 years) from the tropical country of Malaysia. Total fungal DNA and Asp/Pen DNA were detected in all classrooms from Petri dishes and swab samples. In the Petri dish samples, 70% detection was obtained for *A. versicolor* DNA, 13% for *S. chartarum*, and 87% for *Streptomyces* DNA. Meanwhile, for swab samples, the detection was recorded at 56% for *A. versicolor* DNA, 3% for *S. chartarum* DNA, and 28% for *Streptomyces* DNA.

[figure(s) omitted; refer to PDF]

An interesting risk association existed between mycotoxin growing on wallpaper and the transfer to indoor air. A study by Aleksic et al. [32] showed mycotoxins growing on wallpaper followed by aerosolization from the infected surfaces produced macrocyclic trichothecenes (112.1 mg/m^2) of satratoxins G and H, roridin L2 (RL2), and verrucarin J (VerJ); mycophenolic acid (1.8 mg/m^2); and sterigmatocystin (27.8 mg/m^2) as shown in Figures 3(a) and 3(b). The mycelium branching from fungal species and conidial morphology contributed to the aerosolization of particles from a substrate with the macrocyclic trichothecenes requiring the highest airspeed, and the total aerosolized toxic load was 5-fold more than others. *Stachybotrys chartarum* required the highest velocity of 5.9 m/s compared to *Aspergillus versicolor* and *Penicillium brevicompactum* to transfer the contaminated substrates. *Stachybotrys chartarum* biomarkers were identified in pure fungal cultures and cotton-tipped swab extracts collected from kindergarten in Greater Copenhagen. The identification revealed 12 *Stachybotrys* metabolites with atranones and macrocyclic trichothecenes (Figure 3(b)) on the gypsum wallboard. Došen et al. [31] also reported that it was the first time the same mycotoxins were found on the contaminated gypsum wallboard and settled dust. Four mycotoxins were targeted at water recycling and recovery facilities in France. Ninety-four air samples revealed quantifiable aflatoxin B1 and sterigmatocystin, while gliotoxin and ochratoxin (Figure 3(b)) were not found in any samples. Mycotoxin exposure was reported to be insignificant and did not give any concerning threat to the workers in a study conducted at waste management facilities [23]. A comparative study using settled floor dust collected from waste management facilities in Germany and residential houses in Finland showed a wider range of metabolites in concentrations of 0.04–49, 1444.0 μg/kg (Vishwanath et al., 2011).

3.1. Sample Collection and Processing

The sample collection and extraction procedures for mycotoxin analysis are summarised in Table 3. Mold and mycotoxins were sampled using a variety of techniques. Building material and dust samples from damaged buildings were collected using a vacuum cleaner [25] to detect multiple microbial toxins from indoor samples and naturally infested materials. Two papers described airborne dust analysis by sampling samples using cotton swabs and Petri dishes. This is performed in a classroom where they are interested in the associations of respiratory symptoms with the levels of selected fungal DNA, furry pet allergens, and mycotoxins in schools [28, 40]. A vacuum cleaner was used to collect settled dust floored from houses inhabited by small groups of people, generally less than 5 (Vishwanath et al., 2011). A foam swab wetted with methanol and swiped across the sampling area was performed for settled dust and moldy spot swab surfaces at different sites in school buildings [29]. Scraping on moldy surfaces and airborne dust samples inside residential rooms were collected using the “aspirator and head with filter” sets. The set consisted of a GilAir-5 (Sensidyne, USA) aspirator, an elastic hose, and an open-measuring head (Two-Met, Poland), with 37 mm diameter and 0.7 μm pore diameter of the GF/F glass fiber filter (Whatman, UK) reported by [27]. Pottier et al. used two methods to collect fungal aerosols in a damaged house: a sterile polytetrafluoroethylene

(PTFE) filter with a 0.2 μm pore size attached to a calibrated vacuum pump and a sterile liquid with a cyclonic air sampler Coriolis® (Bertin Technologies, France). PM4 and PM10 samplings were performed for indoor/outdoor environments [39]. Another study on ambient air PM10 sampling was reported where bioaerosol from a cattle shed monitored revealed the presence of mycotoxins without concentration data due to below quantification unit. Airborne fungi were collected using a MAS-100 Eco air sampler (Merck, Darmstadt, Germany) with 400 holes (hole to agar impactor) and dichloran 18% glycerol agar (DG18) plates [30]. Air sampling was carried out by collecting dust with a CIP 10 sampler. The sampler uses the rotative cup technique with rotation, maintaining a flow rate of 10 L·min^{-1}. The sampler cup had a porous polyurethane foam filter (PUF). After sampling, the rotating cup containing the PUF was removed from the sampler, closed by the cover, and stored at 4°C before analysis [23].

Table 3

Various procedures for mycotoxin studies in indoor air.

No.	Country	Sample collection	Type of extraction	Protocol/modification	Advantages/disadvantages	Reference
1	France	Fungal aerosols were collected for mycotoxin quantification by using a sterile PTFE filter of 0.2 μm pore size mounted in 47 mm diameter filter holders and connected to personal air pumps calibrated to draw 2 L/min for 3 h. Sampling point was selected at room with visible damage and room without apparent damage	The mycotoxin from the fungal aerosol was extracted from the PTFE filters using 10 mL of methanol/water/formic acid (80/20/0.1), which was then maintained in an ultrasonic bath for 3 minutes before being agitated in a multitube vortex. Second extraction was performed with 10 mL of 50/50 dichloromethane/ethyl acetate	The extracts were combined and evaporated to dryness under nitrogen using a Buchi evaporator. The final residue was diluted in 0.5 mL of acetonitrile/water (10/90) and filtered through Millex-HV 0.45 m before being injected into the HPLC-MS/MS system	The study found that a multianalyte tandem mass spectrometry-based technology could be used to look for mycotoxins in fungal aerosols, allowing occupants' exposure to indoor mycotoxins to be assessed. Future research is needed to better understand the amounts of mycotoxins in indoor aerosols over lengthy periods of time	[21]

2	Italy	Indoors, a low-volume universal XR pump, SKC deluxe 224-PCEX8, fitted with an aluminium cyclone, was utilized at a flow rate of 2.5 L·min1 (to collect PM4), while an outdoor dual-channel sampler (HYDRA dual sampler) was employed at a flow rate of 2.3 m^3/h (38.3 L/min) to collect PM10. Private interior environment, public indoor environment, indoor workplace, and outdoor environment sampling points were chosen	ASE extraction and followed by SPE purification	Sampled filters, blank filters, and spiked filters were extracted by ASE using two cycles of a combination of H_2O: ACN (10/90) at 100°C and 1500 psi. The extracted volume (about 20 mL) was evaporated to dryness and redissolved in 1 mL water before loading into an SPE C18-M Strata cartridge that had previously been conditioned with 4 mL MeOH and rinsed with 4 mL water. After washing with pure water, compounds loaded and retained in the cartridge were eluted with 9 mL of MeOH/H_2O 90/10 and 4.5 mL of pure ACN (4 mL). The analytes were swiftly eluted from the SPE cartridge at a steady flow rate using a vacuum manifold. Before the HPLC-MS-MS analysis, the extracts were evaporated under nitrogen stream. Optimal ASE extraction conditions: ACN/H_2O (90/10) solvent mixture with two static extraction cycles (heat-up time 5 min; static time 5; flush volume 60%; purge time 300 s; pressure 1500 psi; T 100°C). The target analytes recovered	Sample cleanup and SPE purification step reduced ion suppression significantly and/or eliminate interferences before HPLC-MS-MS analysis	[39]

				the best with the Strata C18-M cartridge		
3	US	21 dust floor samples from two water-damaged buildings were collected using floor vacuum. Each floor was vacuum covered for a 2 m^2 area for 5 minutes. Samples were homogenized after removing hair, fluff, and larger objects	Extraction with methanol followed by derivatization	HP-5 ms fused-silica capillary column (30 m i.d., 0.25 mm i.d.). The sample injection volume is 1 l. The oven was set to a 70°C beginning temperature, which was then ramped up to 280°C at a rate of 20°C/min. The verrucarol derivative precursor ion was m/z 638, which gave a target product ion of m/z 302 for quantification and two ions of m/z 262 and 213 for qualification (retention time: 9.8 min). Internal benchmark (ISTD, 1, 12-dodecanediol)	Matrix-matched standard curves could be effective for obtaining accurate MCT readings in dust. In the dust extracts, ISTD showed significantly larger matrix effects. None of the 21 dust samples obtained from water-damaged buildings could be detected using standard calibration curves with ISTD modification	[24]

4	France	Ambient air sampling was conducted 24 hours per day in the feeding corridor cattle shed of dairy farm, using a high-volume sampler with PM10 head and 150 mm microfiber quartz filters. Sampling point was selected at feeding corridor	Samples were extracted twice with 30 mL of methanol acidified with acetic acid (0.5%) from the quartz microfiber filter. The solutions were kept in an ultrasonic bath for 3 min and then shaken for 10 min in a multitube vortexer. After evaporation to dryness, the final residue was dissolved in 0.5 mL of a mixture of acetonitrile/water (10/90)	Two approaches were created for the analysis. At 60°C column temperature, the first approach used a Zorbax Eclipse Plus column with a rapid resolution HD-C18 column (1.8 m, 50 2.1 mm; Agilent Technologies). Deoxynivalenol-13C15 was used as an internal standard. The injection volume was 20 μL. Mixture of methanol (solvent A) and water (solvent B) as the mobile phase, linear gradient with 10%–100% solvent A for 10 min, and stay at 100% for 1 min, flow rate of 0.4 mL/min (positive and negative mode). In second approach, Zorbax SB, rapid resolution HT-C18 column (1.8 μm, 50 × 2.1 mm; Agilent Technologies) at 60°C column temperature. Fumonisin B1-13C34 was used as the internal standard. The injection volume was 10 μL. Mixture of acetonitrile (solvent A) and water added formic acid 1% (solvent B) as mobile phase, linear gradient with 10%–100% solvent A for 10 min, and stay at 100% for 1 min, flow rate of 0.4 mL/min (positive	NIL	[22]

				mode)		
5	Finland and Sweden	Settled airborne dust samples were collected from surfaces above floor levels using a conventional vacuum cleaner device and nylon dust collecting socks. To eliminate the coarse fraction, dust bag dust samples were size homogenized by filtering through a sterile strainer. Samples were dried in a desiccator prior to aliquoting and stored at 20°C. Sampling point selected in living room	The raw extracts were diluted and analyzed without further cleaning in a mixture of acetonitrile, water, and acetic acid (79/20/1), and the raw extracts were diluted and analyzed without further cleaning	A method for determining multimycotoxins in food and feed was created, and it was expanded to include a list of 159 fungal and 27 bacterial metabolites for the examination of naturally infected indoor samples. Its purpose is to demonstrate the usefulness of a multianalyte LC-MS/MS-based spiking at many levels for determining extraction performance parameters, matrix effects, and recoveries. Since dust samples absorb a substantial quantity of solvent relative to food and feed matrixes, the fraction of extraction solvent has been increased. In order to completely utilize instrument capacity for data gathering, each analyte's availability time was defined in the sMRM mode, where retention time and dwell time were fiercely generated for each point in time from the target scan time, for each analyte	When compared to pure methanol and ethyl acetate, the acidified mixture of acetonitrile and water provided the optimum compromise for extracting chosen metabolites. Compared to mortar, carton-gypsum board and coarse-soil-containing splints, settled house dust caused severe matrix effects and incomplete extraction led to low recoveries of analytes due to complex composition from cell fragments, and many other organic compounds accumulated on particulate matter HPLC-MS-MS and GC-MS-MS have both been shown to be useful analytical methods for detecting some of the most toxic mycotoxins produced by molds that are commonly found in moist indoor environments. These technologies are sensitive that they can identify STRG, VER, and TRID in both mold-affected building materials and house dust	[25, 26]; Bloom et al. 2007; [41]

6	Malaysia	Airborne dust was collected by both the cotton swab and Petri dish. Settled dust samples were collected by swabbing 60 cm^2 of surface (1 × 60 cm per swab) from the top frame of the blackboard in each classroom. The blackboard top frame was divided into a left and right part, with the left-side dust samples used for fungal DNA analysis and the right-side samples for mycotoxins analysis. Sampling point was selected at the blackboard frame in a classroom	Samples were extracted with methanol, dissolved with DCM, applied to PEI-bonded silica gel column, eluted, evaporated, and redissolved with methanol, and filtered into vials for HPLC injection. For GC-MS injection, methanolic sample extracts are mixed with IS, hydrolyzed and extracted with methanol, and evaporated to dryness. Dried extracts were later derivatized and heated prior to injection	Establishment on GC-MS and GC-MS-MS methods for determination of mycotoxins (verrucarol and trichodermol) and fungal biomass marker (ergosterol) in contaminated indoor environmental samples. Establishment of the LC-MS-MS method for determination of mycotoxins (sterigmatocystin, gliotoxin, aflatoxin B1, and satratoxins G and H) and water-damaged indoor environments samples. Application of different derivative reagents (trimethylsilyl, pentafluorobutyryl, and heptafluorobutyryl) for optimizing determination of mycotoxin with GC-MS and GC-MS-MS. Carryover and ghost peak formation were triggered by adsorption of nonderivatized or semiderivatized mycotoxin in the instrument injector and were overcome by regular injection of a mixture of derivatized reagent with solvent, avoid washing derivatized extracts with water and maximum injection of extracts,	In all samples, mycotoxins were discovered using MSMS and SIM (NICI). Peaks were found in SIM analysis at the correct retention duration but with a significant background noise level due to interference from a partially coeluting chemical. The MSMS analysis, on the other hand, resulted in significantly decreased background noise and higher detection specificity. Heptafluorobutyryl (HFB) derivatives of selected mycotoxins show negligible fragmentation and have good GC-MS and GC-MS-MS detection sensitivity. Combining CI and negative ion (NICI) detection with MS-MS yielded the best detection sensitivity and specificity. Because of its lower detection limit, higher noise reduction, and considerably increased detection specificity, the NICI mode analysis was frequently preferred. HPLC-MS-MS and GC-MS-MS have both been shown to be useful analytical methods for detecting some of the most toxic mycotoxins	[28]; Bloom et al. 2007; [41]

				to minimize risk of injector contamination	produced by molds that are commonly found in moist indoor environments. These technologies are so sensitive that they can identify STRG, VER, and TRID in both mold-affected building materials and house dust	

7	Finland, the Netherlands, Spain	Methanol-soaked foam swabs were pushed around the test area, and adherent dust was carried into methanol-filled vials. A few sampling regions are combined to provide a collective sample per site. The samples were collected at ambient temperature, sealed with parafilm, and stored at −20°C until they were analyzed. Classrooms, hallways, teacher's lounges, libraries, dining halls, bath/shower rooms, storage rooms, and other areas of the school building were chosen as sampling points	The methanolic suspensions were shaken using a rotary shaker, allowed to settle and the clear upper methanolic layers were transferred into glass vials equipped with glass microinserts. The diluted raw extract was directly injected into the HPLC-MS/MS instrument. Then, the methanolic samples were evaporated and reconstituted in DCM. The liquids were applied to preconditioned PEI-bonded silica gel columns. The eluates were evaporated and redissolved in methanol. Methanolic materials were combined with IS, evaporated, hydrolyzed, and extracted with DCM and water. The extract was transferred to fresh vials, evaporated, and kept in a desiccator overnight before being derivatized with HFBI and heated before injection into the GC/MS-MS	For the measurement of 186 fungal and bacterial secondary metabolites, an HPLC-MS/MS technique was developed. A QTRAP 4000 LC-MS/MS system with a TurboIonSpray electrospray ionization (ESI) source and an 1100 series HPLC system were used for detection and quantification in the scheduled multiple reaction monitoring (sMRM) mode. Using a GC-triple-quadrupole MS/MS apparatus, establish GC-MS/MS analyses for the determination of mycotoxins (verrucarol and trichodermol). Carryover and ghost peak formation were triggered by adsorption of nonderivatized or semiderivatized mycotoxin in the instrument injector and were overcome by regular injection of a mixture of derivatized reagent with solvent, avoid washing derivatized extracts with water and maximum injection of extracts, to minimize risk of injector contamination	Since matrix-matched calibration was found to be insufficient for correction of these effects in the very heterogeneous dust matrix, HPLC-MS/MS findings were not corrected for incomplete extraction and/or signal suppression/enhancement due to coeluting matrix constituents. The acquisition of two sMRM transitions per analyte was required for positive identification, and the LC retention time and intensity ratio of the two sMRM transitions had to coincide with the associated values of a genuine standard within 0.1 min and 30% rel., respectively. High prevalence of mycotoxins due to high detection sensitivity offered by the triple-quadrupole mass spectrometers in MS-MS mode. HPLC-MS-MS and GC-MS-MS have proven to be complementary analytical tools for detecting some of the most potent mycotoxins produced by molds frequently encountered in damp indoor environments. These methods are so sensitive that STRG, VER, and	[26, 29, 42]

					TRID can be detected not only in mold-affected building materials but also in house dust	

8	Croatia	Using a MAS-100 Eco air sampler (Merck, Darmstadt, Germany) with 400 holes (hole to agar impactor) and dichloran 18 percent glycerol agar (DG18) plates, airborne fungi were collected in two-month intervals at apartments (APs), basements (BS), grain mill (GM), and open air (ODA) over a one-year period	Growth aspergilli were cut at 3 plugs of 6 mm diameter by using cylindrical drill and transferred into Eppendorf tubes containing 1000 μl of solvent mixture methanol-DCM-ethyl acetate with a ratio (1/2/3) supplemented with 1% (v/v) formic acid. The clean extracted sample was sonicated. Ultrasonically extraction was transferred into clean vials using syringe with 0.45 μm filters (Sartorius, Germany). Then, the extracted samples were dried under stream of nitrogen gas before kept at −20°C. The dried samples were redissolved in 500 μl of methanol/water (3/1) mixture and filtered through 0.45 μm filters into new vials. Collected dust samples were extracted with solvent mixture methanol-DCM-ethyl acetate with a ratio (1/2/3) supplemented with 1% formic acid. The sample mixture was shaken for 1 hour at room temperature and centrifuged. The supernatants were collected and evaporated to dryness. Dried residues were redissolved with	Analysis by using ClassVP 6.2 software and control DGU-14A vacuum degasser, a quaternary LC-10ADVp pump, a CTO-10ASVp column thermostat, an SPD-10ADVp UV-VIS detector, and an SCL-10 system (HPLC Shimadzu). Separation was performed on a LiChroCART Purosphere STAR RP-18 250 mm × 4 mm column with 5 μm particle size (Merck, Hungary) coupled with a Lichospher 100 RP-18 guard column (Merck, Hungary) at 35°C. Mobile phase with setting of flow rate of 0.5 mL/min, and the injection volume was 20 μL. The gradient elution was achieved by changing the ratio of methanol and water. STC was quantified by measuring peak areas in an HPLC chromatogram and comparing them to STC calibration standards	Sharp peak of less polar metabolite eluted right before STC in *A. jensenii* and *A. venenatus* chromatograms was discovered. This study did not analyze it by LC/MS/MS analyses and considering STC-derived metabolites and/or precursors belonging to 5-methoxy-STC. Another research had compared with this study and found the metabolite detected by HPLC-DAD and TLC with $AlCl_3$	[30]

			ration of 5 mL of methanol/water (3/1). All samples were filtered through 0.45 μm filters into clean vials			
9	Poland	Moldy surfaces in form of scrapings and airborne dust from 22 moldy dwellings in winter season	All filters were washed with 4 mL of a solvent ratio of acetonitrile/water/acetic acid (79/20/1). Then, the samples were extracted for 90 min with KL 2 Multipurpose Shaker (Edmund Bühler GmbH, Germany). The samples were centrifuged at 1000g for 15 min. The supernatant was dried under gentle stream of nitrogen at 40°C. 1 mL of acetonitrile was dissolved into dried residue	HPLC analysis was conducted using a symmetry C18 column (150 × 2.1 mm 5 μm) with column temperature set at 40°C and 10 μL of sample injection. Gradient elution using mixture of methanol: acetate buffer 0.1 mol/L pH 4.6 serves as mobile phase. Simultaneous determination was performed at an absorbance of 247 nm and 326 nm. UV spectra at an absorbance of 210–500 nm and retention time of the standards were used to analyze the samples	The method collection like scraping and airborne dust did not indicate quantities exceeding the limit of determination of the method. However, the study revealed that ST and RC were present in the fungi cultivated from scrapings. This demonstrates that toxinogenic strains of *Aspergillus versicolor* and *Penicillium chrysogenum* display this feature when cultivated on MEA medium in the laboratory. All the studies, including this study, used the HPLC approach; therefore, it is unclear why this study was not able to demonstrate the presence of mycotoxins in the air dust and scrape samples from flats	[27]

10	Malaysia	Settled dust samples were collected by swabbing 60 cm^2 of surface (1 × 60 cm) from the top frame of the blackboard in each classroom. The blackboard top frame was divided into a left and right part, with the left-side dust samples used for fungal DNA analysis and the right-side samples for mycotoxin analysis. Sampling point was selected at the blackboard frame in a classroom	Three types of liquid extraction were performed. First, extraction with methanol with three different samples in which the pieces of agar (approximately 5 cm^2), dust sample (~0.4 g), and building material (0.3 to 3 g) were immersed with methanol at room temperature for 72 h. The sample was then centrifuged for 5 min at 3,200 rpm, and the supernatant was poured into new tubes. Second, extraction with heptane 100 μL of sterile water added and the mixture were extracted twice with 2 mL of heptane. Finally, methanolic phases were dissolved in DCM and applied to PEI (1 mL bonded silica gel columns) after being evaporated under a moderate stream of nitrogen. The PEI column already preconditioning the columns with 4 mL of methanol and DCM before eluted with sample. Then, 5 mL of DCM was eluted into the sample through the PEI column and had been evaporated under nitrogen. Prior to analysis, the sample was redissolved with 1 mL of methanol and	HPLC-MS analysis was performed using a ProStar HPLC/1200L triple-quadrupole MS-MS system (Varian Inc., Walnut Creek, CA). 20 μL of each sample was injected, using an autosampler (model 410; Varian), into a Polaris 5 μM C18-A 150 by 2.0 mm RP-18 column equipped with a MetaGuard 2.0 mm Polaris 5-μM C18-A precolumn (Varian). Reserpine was used as the internal standard. GC-MS analysis sample was performed on a CP-3800 gas chromatograph equipped with a fused-silica capillary column (FactorFOUR™, VF-5 ms, 30 m × 0.25 mm i.d., 0.25 mm film thickness) and connected to a 1200 L triple-quadrupole MS-MS detector (Varian Inc., Walnut Creek, CA, USA). Derivatives were analyzed in both EI modes, at an energy of 70 eV and an ion source temperature of 250°C (TMS derivatives) or 200°C (HFB derivatives) and in NICI mode with methane as ionization gas at a pressure of 0.8 kPa and a source	A supplement of 10 mM ammonium acetate and 20 μM sodium acetate was added to the methanol aqueous buffer to increase the cationization in the electrospray ionization mode. Ten microliters of methanol was injected in between samples to minimize cross-contamination. A mix of HFBI and acetone (1/3) was injected in between samples to eliminate any trace of underivatized or semiderivatized VER/TRID	[40], Bloom et al. 2007, [41]

			filtered through 0.45 μm Millex syringe filters. The methodology was modified by the previously mentioned method [41]	temperature of 200°C. Volumes of 1-2 mL were injected in the splitless mode with a helium carrier gas pressure of 69 kPa, using a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The MS-MS conditions were optimized by repeatedly injecting 0.1–1 ng amounts of standards at different collision energy, ion source temperature, and argon pressure in the collision cell. The parameters that gave the largest product ion peak area were selected		

11	France	Aerosolization of produced mycotoxins from contaminated wallpapers. Sampling point was selected at wallpapers	Four MCT (SG, SH, VerJ, and RL2), MPA, and STC were extracted from samples (wallpaper and fiberglass disks) by gentle mechanical agitation on an agitation table in chloroform-methanol (2/1). Mycophenolic acid-d3 and o-methyl sterigmatocystin were added at known concentrations before starting extraction in order to serve as internal standards for MPA and STC, respectively. For MCT, verrucarin A was chosen as an internal standard, as already described. After 4 h, extracts were centrifuged for 5 min at 3,500 rpm and passed through a phase separator (PS) filter (Whatman 1 PS). The filtered extracts were evaporated to dryness and suspended in 1 mL of methanol	Quantification was performed using an Acquity ultraperformance liquid chromatography (UPLC) system coupled to Xevo triple-quadrupole mass spectrometer ethanol. The desolvation temperature and nitrogen flow rate were set at 650°C and 800 liters/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min. Mycotoxins (5 μL of samples) were eluted on an Acquity BEH C18 column (2.1 by 100 mm, 1.7 m; Waters), with ACN-H_2O gradient (0 to 0.5 min), 10% ACN; (0.5 to 4 min), 90% ACN) at a flow rate of 0.35 mL/min. Quantification was carried out by multiple reaction monitoring (MRM) mode in positive electrospray ionization (ESI). Chromatographic data were monitored using the MassLynx 4.1 software	This study showed that three different toxinogenic species produce mycotoxins during their development on wallpaper. These toxins can subsequently be aerosolized, at least partly, from moldy material. This transfer to air requires air velocities that can be encountered under real-life conditions in buildings. Most of the aerosolized toxic load is found in particles whose size corresponds to spores or mycelium fragments. However, some toxins were also found on particles smaller than spores that are easily respirable and can deeply penetrate the human respiratory tract. All of these data are important for risk assessment related to fungal contamination of indoor environments	[32]

12	France	Dust was collected in the air using a CIP 10 sampler (Tecora, France) with an inhalable fraction selector	Mycotoxins were extracted from the porous polyurethane foam filter (PUF) using methanol, and the PUF capsules were rinsed to remove any dust particles	High-resolution mass spectrometry combined with ultrahigh-performance liquid chromatography (UPLC-Q-Orbitrap HRMS). A BEH C18 column (2.1 mm, 10 mm, 1.7 mm) and precolumn were used to separate the analytes (Waters). In positive electrospray ionization mode, mass spectrometry detection was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific™). Analytes were detected by comparing product ion retention durations and mass accuracy	Three waste management sectors were incorporated in the sample strategy, as well as both stationary and personal air samplings. The use of UPLC-HRMS for quantitative analysis of airborne mycotoxins is a very sensitive and specific technology that allowed for the detection of low mycotoxin concentrations in the air. The application of measuring results to the assessment of health risks	[23]
13	Germany	Samples were collected using a vacuum cleaner from different waste management units and houses inhabited by less than 5 people	Headspace volatile extraction procedure fully automated by an autosampler for microbial volatile organic compound using Agilent 6890 GC QTRAP 4000 LC-MS/MS with C18 column	Method for LC-MS-MS was adopted from [26]	The use of LC-MS-MS and GC-MS provides many microbial metabolite and volatile anthropogenic chemical presence in indoor environments. This is the first study to compare individual settled floor dust samples derived from relatively different indoor environments using both LC-MS/MS and GC-MS methods	Vishwanath et al. 2011 [26]

Floor dust mycotoxins were reported by [24] where they used a vacuum attached to a polyethylene filter sock (Midwest Filtration Company, Fairfield, OH, USA) and a precleaned crevice tool on a L'il Hummer™ backpack vacuum sampler (100 ft3/min, 1.5 horsepower; ProTeam Inc., Boise, ID, USA). An Andersen multistage impactor

(Tish 180 Environmental, OH, USA) was used for capturing particles according to 6 ranges of size and 181 aerodynamic characteristics. Each impactor stage had a fiberglass disk to collect particles [32]. Settled dust samples were collected from all available surfaces (shelves, tables, fridges, and tops of the hanging lamps) and other places (excluding the floor) that were regularly cleaned. Each sample was taken from an approximate surface area of 45 × 45 cm using a clean precision Kimwipes® Lite wipe (Kimberly-Clark, GA, USA). Pure agar cultures were extracted using a microscale method modified for *Stachybotrys* metabolites. Three agar plugs (6 mm ID) were cut from a 15-day-old colony from each agar medium (potato dextrose agar (PDA) or malt-extract agar (MEA)) and placed in a 2 mL screw-top vial. Extracts from pure fungal cultures and cotton tip swabs from infected gypsum wallboards were further processed in laboratory before analysis for detection of mycotoxin metabolites by injection directly to an ultrahigh performance liquid chromatography diode array detector quadrapole time-of-flight mass spectrometry method (UHPLC-DAD_QTOF/MS) [31].

Depending on the type of samples collected for sampling, it is important to note that sampling techniques may differ depending on the specific objectives, environment, and suspected mycotoxin contamination sources. The vacuum cleaner can cover a large sampling area and larger sample material. At the same time, cotton swabs and Petri dishes allow for targeted sampling of specific areas where mold growth is visible. The GilAir-5 aspirator is a portable air sampling pump commonly used to collect and analyze various contaminants, including gases, vapors, and aerosols.

The method of collection and the type of samples obtained are the main differences between a sterile PTFE filter with a 0.2 m pore size attached to a calibrated vacuum pump and a sterile liquid with a cyclonic air sampler Coriolis®. A sterile PTFE filter with a pore size of 0.2 m connected to a calibrated vacuum pump is commonly used to collect particulate matter such as dust, pollen, or other solid particles in the air. The filter serves as a barrier, trapping particles while allowing air to pass through. The filter can be removed and analyzed after sampling to determine the types and quantities of particles present. On the other hand, a sterile liquid with a cyclonic air sampler Coriolis® collects microorganisms, such as bacteria and fungi, from the air. The cyclonic action within the sampler separates and concentrates the airborne microorganisms onto a sterile liquid substrate. This liquid is then used for laboratory analysis to identify and quantify the microbial contamination present in the air.

The main difference between PM10 and PM4 air filters lies in the size range of particles they are designed to capture. A PM10 air filter is specifically engineered to capture particles 10 μm or smaller in diameter, including dust, pollen, mold spores, and larger airborne particles. By targeting this size range, the PM10 filter helps monitor and assess air quality, as these larger particles can potentially impact respiratory health and indoor or outdoor air pollution levels. On the other hand, a PM4 air filter is designed to capture particles that are 4 μm or smaller in diameter, including finer particles, such as combustion byproducts, soot, fine dust, and certain allergens. By focusing on this smaller particle size, the PM4 filter provides more detailed information about fine particulate matter, which is known to have potential health implications, especially when inhaled.

The MAS-100 Eco air sampler is an advanced air quality monitoring and analysis device. It is specifically designed to sample and measure microbial contamination in the air, including bacteria, fungi, and other microorganisms. The MAS-100 Eco air sampler utilizes a high-performance filtration system to capture and collect these microorganisms, permitting further analysis and identification in laboratories. A CIP 10 sampler is an individual sampler that traps respirable particles. The physical collection efficiency of CIP 10 equipped with the inhalable fraction selector is estimated to be 50% for particles with an aerodynamic diameter of 1.8 mm and more than 95% for particles with an aerodynamic diameter greater than 2.8 mm.

3.2. Instrumentation Analysis

After sampling, mycotoxins require sample preparation using appropriate analytical instruments. Sample preparation demands using a suitable solvent to extract toxins from the matrix, a cleanup procedure to remove interferences from the matrix, and, if necessary, sample preconcentration before analysis. Selecting an appropriate solvent for mycotoxin extraction depends on the toxin's structure. The most common method is liquid-liquid extraction (LLE), as shown in Table 3. Different types of solvent were used for the extraction of mycotoxins, such as methanol [21–23]

and acetonitrile [25–27], and dichloromethane [28, 29]. Alternatively, a combination of a solvent mixture such as methanol, dichloromethane, and ethyl acetate [30, 31] and chloroform and methanol (2 : 1) [23, 32] was performed to extract different metabolites from samples which are compatible with the solvent. One paper reported on the two-stage extraction (methanol followed by hexane) procedure [40]. One paper conducted sampling on headspace-solid-phase microextraction (SPME), which employs a fiber coated with an extracting phase, which can be a liquid (polymer) or a solid (sorbent), to extract various analytes (volatile and nonvolatile) from various media (Vishwanath et al., 2011). Another paper described an accelerated solvent extractor (ASE) step followed by solid-phase extraction (SPE), ultimately enhancing the purification of analytes, making it possible to eliminate, reduce, and suppress signals from interference [39].

Airborne and bioaerosol samples were analyzed for instrumentation analysis using two HPLC-MS/MS protocols to cover many mycotoxins. Positive and negative ion modes were chosen to obtain a good signal from mycotoxins [21, 22]. One study was reported on the ultraperformance liquid chromatography (UPLC) system connected to Xevo Triple Quadrupole to determine mycophenolic acid, sterigmatocystin, and macrocyclic trichothecenes [32], UPLC-Orbitrap [23], and UHPLC-QTOF [31]. One paper reported mycotoxins in dust analyzed by gas chromatography-MS/MS [24], and one paper described mycotoxins indoor/outdoor airborne particulate matter using LC-MS/MS both in positive and negative ion modes to achieve efficient ionization for the known analytes [39]. Combination analysis using GC-MS/MS and LC-MS/MS was competent to cover volatile and nonvolatile compounds. These instruments analyzed airborne dust, fungi, and moldy surface samples [25, 28, 29, 40]. Interestingly, one paper reported using headspace GC-MS, and detection and quantification were performed on QTRAP LC-MS/MS (Vishwanath et al., 2011). Two papers described the application of HPLC to detect mycotoxins. Concentrations were calculated based on peak areas of the analyte compared to calibrated standards [27, 30].

Table 4 shows that most of the mycotoxins are analyzed using LC-MS/MS compared to the GC-MS method. Twelve publications reported mycotoxin analysis by LC and differentiated by HPLC, HPLC-MS/MS, LC-MS/MS, HPLC/UV-VIS, UPLC, and UHPLC. In general, mycotoxins such as aflatoxins (AFB1, B2, G1, G2, and M1), gliotoxin, mycophenolic acid, ochratoxin, alternariol, zearalenone, sterigmatocystin, patulin, citrinin, fumagillin, and trichothecenes (neosolaniol, T-2 toxin, HT-2 toxin, nivalenol, satratoxin, and roridin) are frequently quantified using liquid chromatography with the detector of a mass spectrometer. All the data acquisition on LC-MS/MS in mycotoxin experiments was performed in the positive or negative ESI (electrospray ionization) mode, using multiple reaction monitoring (MRM) scans.

Table 4

Mycotoxin analysis using GC-MS-MS/LC-MS-MS.

No.	Analyte/metabolite/mycotoxin	System/instrumentation/method	Validation	Reference

Precision/accuracy	LOD/LOQ/CV	Recoveries/R2	1	Fungal aerosols AFB1, B2, G1, G2, M1, diacetoxyscirpenol, gliotoxin, mycophenolic acid, neosolaniol, ochratoxin A, T-2 toxin (method 1); alternariol, deoxynivalenol, deepoxy-deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, HT-2 toxin, verrucarol, zearalenone (method 2)	HPLC-MS/MS, MRM, positive and negative modes, ESI	
Quantification limit (ng/filter): 0.05–0.50	Recovery: 56–101.10%	[21]	-			
2	DON, AFB1, OTA, toxin T-2 (T-2), zearalenone (ZEA), and sterigmatocystin (STE)	HPLC/ESI-MS/MS, MRM, positive and negative modes, ESI, and the ion source temperature of 150°C in splitless mode	The intraday precision: 4% to 8%. The interday precision: 4% to 14%	LOD in the matrix: 0.8 (AFB1) to 8.5 $ng \cdot mL^{-1}$ (T-2 toxin) LOQ in the matrix: 3.2 (AFB1) to 26 $ng \cdot mL^{-1}$ (T-2 toxin)	Recoveries of all tested mycotoxins (72–95%) with a CV% below 10%. when the matrix-matched standard curve without the ISTD was used	[39]
–						

3	Verrucarol	GC-MS/MS, negative chemical ionization mode using methane as ionization gas at the energy of 150 eV	NIL	NIL	Recovery of verrucarol was 94%	[24]
–						
4	3 mycotoxins (HT-2 toxin, nivalenol, patulin) (method 1); 14 mycotoxins (aflatoxins B1, B2, G1, G2, M1, citrinin, diacetoxyscirpenol, fumagillin, gliotoxin, mycophenolic acid, neosolaniol, ochratoxin A, T-2 toxin, verruculogen) (method 2)	HPLC-MS/MS, MRM, positive and negative modes, ESI	NIL	Quantification limit (ng/filter) ranged from 0.05 to 23.49	Recoveries ranged from 42% (HT-2) to 97% (DAS)	[22]
–						

5	A total of 186 compounds comprise 159 fungal metabolites and 27 bacterial metabolites	Liquid chromatography/tandem mass spectrometry (LC-MS/MS), specifically QTRAP 4000 LC-MS/MS equipped with a TurboIonSpray electrospray ionization source and an 1100 series HPLC system	Positive identification was obtained by the acquisition of two sMRM transitions per analyte that made 4.0 identification points according to decision by 2002/657/EC. The LC RT and the intensity ratio of the two sMRM transitions had to agree with the related values of an external standard within 0.1 min and 30% relative, respectively	Generally, in the low μ/Kg range and exceeded 50 μ/kg only for those compounds exhibiting a low apparent recovery or a general low MS/MS sensitivity	29/36 analytes (81%) tested reached within a target range of 70–120%	[25, 26]; [41]; [42]
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6	Aflatoxin B1, gliotoxin, satratoxin G, satratoxin H, and sterigmatocystinTrichodermol and verrucarol	HPLC-MS. A ProStar HPLC/1200L triple-quadrupole MS-MS system was used, 5 μM C18-A 150 by 2.0 mm RP-18 column equipped with a MetaGuard 2.0-mm 5 μM C18-A precolumn. Reserpine was used as the internal standardGC-MS-MS using a CP-3800 GC-triple-quadrupole MS-MS system. The derivatives were analyzed by using MS-MS in negative ion chemical ionization mode, at an energy of 70 eV and an ion source temperature of 150°C, and with ammonia as the ionization gas	The electrospray ionization MS parameters achieved maximal detection sensitivity for each standard when their spectrum showed prominent parent and product ions	Trichodermol and verrucarol 6 pg, sterigmatocystin 12 pg, and aflatoxin B1 and gliotoxin 125 pgSatratoxins G and H were not quantified	The recovery value was 53 ± 6% with 11.2% CV	[28, 42–43]
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7	A total of 186 compounds comprise 159 fungal metabolites and 27 bacterial metabolitesTrichodermol &verrucarol	Liquid chromatography/tandem mass spectrometry (LC-MS/MS), specifically QTRAP 4000 LC-MS/MS equipped with a TurboIonSpray electrospray ionization source (ESI) and an 1100 series HPLC system. Elution was carried out in binary gradient modeGC-triple-quadrupole MS/MS instrument	Positive identification was obtained by the acquisition of two sMRM transitions per analyte that made 4.0 identification points according to decision by 2002/657/EC.The MSMS conditions were optimized by repeatedly injecting 0.1–1 ng amounts of standards at different collision energy, ion source temperature, and argon pressure in the collision cell. The parameters that gave the largest product ion peak area were selected. Detection sensitivity, defined as amounts of standards injected with a signal-to-noise ratio >4	Generally, in the low μ/kg range and exceeded 50 μ/kg only for those compounds exhibiting a low apparent recovery or a general low MS/MS sensitivity	29/36 analytes (81%) tested reached within a target range of 70–120%	[26, 29, 42, 43]
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8	Airborne sterigmatocystin STC	HPLC/UC-VIS	NIL	Quantification limit (0.025–250.0 μg/ml)	R2 values: 0.9999	[30]
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9	Sterigmatocystin roquefortine C	HPLC Waters analytical column	NIL	Limit of determination of the method: 0.2 μg/mlQuantification limit: 0.2–10 μg/ml	NIL	[26]
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10	HPLC-MS-MSAflatoxin BGliotoxinSatratoxin GSatratoxin HSterigmatocystin GC-MS-MSTrichodermolV errucarol	HPLC-MSMS (HPLC/1200L triple-quadrupole MS-MS system). The capillary temperature was 310°C, the capillary voltage was 40 V, the needle voltage was 5,000 V, and the electron multiplier voltage was 2,000 V. The MS spectra were collected as centroid data from m/z 100 to 800, with a scan time of 0.5 s and a scan width of 0.7 sGC-MS-MS (CP-3800): the derivatives were analyzed by using MS-MS in negative ion chemical ionization mode, at an energy of 70 eV and an ion source temperature of 150°C, and with ammonia as the ionization gas (0.4 kPa)	NIL	LOD: 6 pgTrichodermol and verrucarol12 pg sterigmatocystin125 pg aflatoxin B and gliotoxins	NIL	[40]
–						

11	Satratoxin G (SG), satratoxin H (SH), verrucarin J (VerJ), roridin L2 (RL2), mycophenolic acid (MPA), and sterigmatocystin (STC)	UPLC coupled to a Xevo triple-quadrupole mass spectrometer. Quantification was carried out by multiple reaction monitoring (MRM) mode in positive electrospray ionization (ESI)	NIL	LOD:(1) MPA, STC = 0.2 ng/mL(2) RL2 = 0.2 ng/mL(3) VerJ = 5 ng/mL(4) SH, SG = 10 ng/mLLOQ:(1) MPA, STC, RL2, VerJ = 10 ng/mL(2) SG, SH = 100 ng/mL	NIL	[32]
–						
12	Satratoxin HSatratoxin GRoridin L2Roridin EAtranone AAtranone BDolabellaneStachybotrylactamStachybotrylactam (isomer)StachybotryamideStachybotrydialMer-NF-5003-BTrichodermin	UHPLC-QTOF to UHPLC-QqQ	NIL	LOD and LOQ (ng/cm^2), respectively:Satratoxin H (15, 50)Satratoxin G (15, 50)Roridin L2 (0.1, 0.2)Roridin E (0.1, 0.2)Atranone A (2, 6)Atranone B (2, 6)Dolabellane (2, 6)Stachybotrylactam (2, 6)Stachybotrylactam isomer (2, 6)Stachybotryamide (2, 6)Stachybotrydial (2, 6)Mer-NF-5003-B (2, 6)Trichodermin (5, 17)		[31]
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13	Aflatoxin B1Sterigmatocystin GliotoxinOchratoxin	Dionex Ultimate 3000 UPHLC mass spectrometry detection was carried out on a Q Exactive mass spectrometer (Thermo Fisher Scientific™) operated in positive electrospray ionization (ESI (+)) mode	NIL	Mass spectrometry LOQ0.1 ng·mL^{-1}(i) Aflatoxin B1(ii) Gliotoxin(iii) Sterigmatocystin0.2 ng·mL^{-1}(i) Ochratoxin A		[23]
–						
14	71 volatile organic compounds18 microbial volatile organic compounds	Agilent 6890 GC QTRAP 4000 LC-MS/MS with C18 column	NIL	3 μg/kg for(i) Sterigmatocystin	Less than 50%	Vishwanath et al. 2011 [26]

Although some of the mycotoxins like sterigmatocystin are analyzed using detectors other than MS, e.g., UV-VIS [30], one of the advantages of MS over the other detectors is that it is easier to distinguish coeluting compounds using extracted ion chromatograms. LC-MS/MS offers a sensitive, efficient, and multianalyte analysis which is of great importance, especially on mycotoxin determination in various matrices, including indoor environmental samples, for example, ambient air, settled dust, and moldy surface. Attempts to identify these toxins in dust particularly are challenging as it correlates to the amounts present in the sample. Many fungal metabolites possess the same elemental composition and coeluate at the same retention time. Thus, a specific and sensitive instrument is required to distinguish similar compounds which are normally difficult to separate chromatographically. Indeed, several LC-MS/MS multimethods (≥2 mycotoxins) have already been developed for indoor environmental samples [28] (Vishwanath et al., 2011). Developed methods were reported to produce good recovery ranging from 42 to 101.10% with CV around 10% and R2 of 0.994–0.999 (Table 4).

3.3. Method Validation

GC-MS and LC-MS/MS are widely used techniques for mycotoxin analysis in various environmental samples, including building materials, due to their high sensitivity, selectivity, and ability to analyze complex matrices. These techniques have successfully identified and quantified a broad range of mycotoxins, even at low levels, and can differentiate between mycotoxin isomers and closely related compounds. However, the reliability and accuracy of these techniques depend on proper method development, validation, and quality control measures. The lack of such data in studies significantly impacts the interpretation and outcomes. Without proper validation and quality control, findings may be influenced by matrix interferences, extraction efficiency, instrument variability, and method biases. Thorough evaluation and reporting of method performance parameters, such as limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision, and selectivity, are essential. Quality control measures, including calibration standards, matrix-matched standards, and internal standards, are crucial to ensure accuracy and reliability. In addition, using appropriate quality control samples, such as certified reference materials, helps assess measurement uncertainty and ensures comparability across studies.

In mycotoxin analysis, it is crucial to mitigate matrix effects to ensure accurate and reliable results. Matrix effects

occur when the sample matrix interferes with the ionization and detection of analytes, resulting in signal suppression or enhancement. To ensure optimal performance, assessing the practices employed to mitigate matrix effects (e.g., matrix-matched calibration, internal standards, and different sample preparation techniques) is essential.

To obtain an accurate measurement, matrix-matched standards to reduce matrix effects [24], stable isotope-labelled internal standards such as the 13C standard [21], and efficient sample cleanup [39] are normally performed. The detection and quantification of an analyte are significantly influenced by matrix effects associated with heterogeneous components in environmental samples [44]. Coextracted matrix components may cause interference with active sites in the GC inlet liner and the column and produce differential analyte signals between the matrix-containing sample extract and the matrix-free standard extract [45–47]. Efficient sample preparation, for example, solid-phase extraction (SPE) or LLE, is essential and has been found to reduce matrix effects potentially [48]. The effectiveness of these techniques in reducing matrix effects can vary depending on the sample matrix and mycotoxin of interest. This step is crucial, and optimization is needed to minimize sample loss. An internal standard (IS) is frequently used to improve the precision of quantitative analysis in which it compensates for matrix effects or sample loss during preparative procedures. In other words, it monitors and corrects any variations during sample preparation, extraction efficiency, and instrument response. Therefore, the selected IS should be similar to the target analytes regarding ionization properties or chemical structures to ensure it always reacts the same way as the analytes of interest, especially with a matrix [49]. An IS labelled with (13C) or (15N) was commonly employed for each group of mycotoxins, for instance, Fumonisin B1-13C34 and Deoxynivalenol-13C15 [21, 22, 39]. However, a nonlabelled IS, such as reserpine, has also been used as an internal standard [40]. According to Saito et al. [24], the accuracy of the results is largely dependent on the matrix effects, the appropriateness of IS, or the combination of them. On the other hand, only five publications reported analyses have been performed using GC-MS with the negative chemical ionization (CI) mode. Trichodermol and verrucarol, which are in the group of trichothecenes, are the most common mycotoxins tested by GC-MS [24, 28, 29, 40] (Vishwanath et al., 2011).

4. Conclusions

In conclusion, various techniques are available for analyzing and detecting multiple mycotoxins using LC-MS/MS and GC-MS methods. Mold and mycotoxin analysis has evolved in sampling techniques, processing, preconcentration, and instrumentation over the past years. Technological advances are beginning to overcome many challenges posed by the complexity of detecting multiple mycotoxins. Mass spectrometry advancements such as ionization modes, sensitivity, and acquisition speed have increased throughput, the number of mycotoxins that can be simultaneously screened, and the discovery of novel compounds of mycotoxins. Modern technologies, such as hyphenated liquid or gas mass spectrometry, have enabled these analytical methods to be developed and validated for mycotoxin analysis. However, due to the variety of chemical structures, using a single method for mycotoxin analysis is impossible. Routine analysis faces significant challenges due to the demand for rapid, simultaneous, and accurate determination of multiple mycotoxins. Future efforts would concentrate on rapid assays and tools that measure a broader range of mycotoxins in a single matrix and lower detection limits. Highly sophisticated multianalyte methods based on liquid chromatography coupled with multiple-stage mass spectrometry have been developed to identify and determine multiple mycotoxins. This new era of various screening mycotoxin and detection technologies will benefit future research.

5. Future Perspectives

It is anticipated analytical techniques and technologies for mycotoxin detection are likely to advance. This could include creating more sensitive and specific methods, such as advanced chromatographic techniques or rapid screening methods based on biosensors or nanomaterials. These advances will allow for faster and more accurate detection of mycotoxins in a variety of samples.

Moreover, there will be a greater emphasis on developing portable and field-deployable mycotoxin analysis devices. This will enable on-site testing and real-time monitoring, which is especially important in monitoring building environments where rapid decisions are required to prevent mycotoxin contamination.

Authors' Contributions

All the authors contributed equally to each subtopic, reviewing, and final editing of the manuscript.

Acknowledgments

The authors would like to thank the Director-General of Health Malaysia for approval to submit this paper for publication. The authors also thank the Environmental Health Research Center (EHRC) and the Institute for Medical Research (IMR) staff for their assistance. The research herein was funded by the Ministry of Health, Malaysia (NMRR-18-962-41809).

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DETAILS

Subject:	Mass spectrometry; Fungi; Scientific imaging; Metabolites; Chromatography; Keywords; Intensive care; Medical research; Mycotoxins
Location:	Malaysia
Editor:	Waleed Alahmad
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2022-06-13 (Received); 2023-12-08 (Revised); 2023-12-23 (Accepted); 2024-01-27 (Pub)

DOI:	https://doi.org/10.1155/2024/858 1029
ProQuest document ID:	2921792410
Document URL:	https://www.proquest.com/scholarly-journals/scoping-review-on-fungus-mycotoxin-studies/docview/2921792410/se-2?accountid=211160
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Last updated:	2024-02-05
Database:	Publicly Available Content Database

Analytical and Clinical Evaluation of a Chemiluminescent Immunoassay to Detect Serum Chitinase-3-like Protein 1 in HBV-Related Liver Diseases

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ProQuest document link

ABSTRACT (ENGLISH)

Serum chitinase-3-like protein 1 (CHI3L1) is a diagnostic marker for liver diseases, such as hepatocellular carcinoma (HCC). Herein, we aimed to evaluate the analytical performance of a chemiluminescent immunoassay (CLIA) for the quantitative detection of CHI3L1 and its application in hepatitis B virus (HBV)-related liver diseases. The CLIA for CHI3L1 detection presented good analytical performance, with a linear range of 1.50–2000.00 ng/mL and a detection limit of 0.98 ng/mL. To evaluate its clinical application, serum CHI3L1 levels were detected in 82 patients with chronic hepatitis B (CHB) and in 21 healthy controls. The patients with CHB and HCC had higher CHI3L1 levels than the healthy controls and the patients with CHB without HCC. However, CHI3L1 levels did not change significantly with the increase in liver fibrosis stages. The area under the receiver operating characteristic curve for the diagnosis of HBV-related HCC was 0.808, representing a moderate diagnostic value. Correlation analysis revealed a significant association between CHI3L1 and alpha-fetoprotein (AFP) levels, the fibrosis-4 (FIB-4)

index, and the aspartate aminotransferase-to-platelet ratio index (APRI). In conclusion, compared with currently reported methods for CHI3L1 detection, the CLIA has a high sensitivity, a wide linear range, and an acceptable accuracy, precision, and reference intervals, making it valuable in the diagnosis of HBV-related HCC.

FULL TEXT

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1. Introduction

Hepatitis B virus (HBV) is the most common cause of liver diseases in China, which can lead to acute and chronic hepatitis B (CHB), hepatic fibrosis, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [1]. In the clinical setting, timely, accurate, and comprehensive diagnoses are critical to reducing the progression of HBV-related liver diseases. As the gold standard for the diagnosis of liver diseases, liver biopsy has some limitations in clinical application because of its invasive nature [2]. Clinical symptoms, physical examination, imaging screening, and routine laboratory indicators can be insufficiently specific to detect HCC [2]. Therefore, a noninvasive, safe, and specific method would have crucial clinical application in HBV-related liver diseases.

Chitinase-3-like protein 1 (CHI3L1, YKL-40 protein) is a glycoprotein that plays a role in numerous diseases, such as arthritis, idiopathic pulmonary fibrosis, and liver diseases [3–5]. In particular, CHI3L1 is abundantly expressed in liver tissue and is mainly involved in inflammation and tissue remodeling [6]. In clinical applications, studies have shown that CHI3L1 levels were significantly elevated in liver diseases and increased with the severity of the disease [5, 7], which was also emphasized in the “Guidelines on the Management of Hepatic Encephalopathy in Cirrhosis” [8]. Furthermore, serum CHI3L1 has been recommended as a noninvasive marker for liver diseases. A meta-analysis showed that serum CHI3L1 served as an excellent marker to diagnose liver fibrosis, in which the pooled diagnostic values were significantly higher than the clinical indicators such as FibroScan, the aspartate aminotransferase-to-platelet ratio index (APRI), and the fibrosis-4 (FIB-4) index [9]. Moreover, the “Guidelines on the Prevention and Treatment in Chronic Hepatitis B” emphasized the predictive role of CHI3L1 in HBV-related liver diseases [2]. These studies indicated that CHI3L1 could be used as a biomarker for the diagnosis, staging, and prognosis of HBV-related liver diseases.

Various analytical methods have been used to detect serum CHI3L1 [10–12]. The enzyme-linked immunosorbent assay (ELISA) is the most frequently used method to quantify serum CHI3L1 levels; however, its time-consuming operations have limited its clinical application [10]. Later, fluorescent immunoassay methods with simple operation processes were developed. However, the results of the magnetic bead fluorescent immunoassay (MB-FIA) and the fluorescence immunochromatography assay (FICA) were not accurate enough [11, 12]. With the characteristics of a short determination time and high specificity, the chemiluminescent immunoassay (CLIA) has been increasingly used in routine clinical applications [13]. Considering the important clinical value of a CLIA for the rapid and accurate quantification of CHI3L1, it is necessary to explore its analytical performance and clinical application. In this study, we evaluated the analytical performance of a CLIA to quantitatively detect CHI3L1 and the value of CHI3L1 in diagnosing HBV-related liver diseases.

2. Materials and Methods

2.1. Reagents and Instruments

An automatic CLIA analyzer (iFlash3000-A, Shenzhen Yahui Long Biological Technology Co., Ltd., Shenzhen, China) was used to detect serum CHI3L1 levels. The CHI3L1 assay kits (CLIA method) (lot no. 20210201), supporting reagents, including three calibrators (0, 80.05, and 1070.48 ng/ml) and two calibrators at different concentrations from another lot (lot no. 20210601; 70.90 and 864.90 ng/ml), and other materials for the instrument, were also purchased from Shenzhen Yahui Long Biological Technology Co., Ltd. The CHI3L1 assay was a sandwich immunoassay using a direct chemiluminometric technique. According to the manufacturer’s instructions, we loaded the samples, reagents, and other materials related to the CHI3L1 assay into the iFlash3000-A. At least 5 μl of the sample was measured for each determination. Then, the automatic CLIA analyzer performed operations

“incubation-washing-signal triggering” and measurement. The CLIA assay procedure from sample addition to result acquisition was performed in less than 20 min.

2.2. Performance Verification

2.2.1. Linear Range

Following the Clinical and Laboratory Standards Institute’s (CLSI) EP6-A guidelines, three levels of traceable calibration solution (lot no. 20210201; 0, 80.05, and 1070.48 ng/mL) were analyzed in duplicate to obtain the calibration curve for CHI3L1 [14]. Then, to verify the linear range of 1.50–2000.00 ng/mL, as suggested by the manufacturer’s instructions, low- and high-value serum specimens were prepared. The low- and high-value specimens had concentrations that covered the linear range as far as possible. A series of sample concentrations were tested by mixing low (L)- and high(H)-value plasma in certain proportions (5L, 4L + 1H, 3L + 2H, 2L + 3H, 1L + 4H, and 5H). Two replicates of each sample were tested. A scatter diagram and regression analysis were then performed. A linear correlation coefficient of more than 0.99 met the industry-recognized standards.

2.2.2. Functional Sensitivity: Limit of Detection

Following the CLSI’s EP17-A guidelines, the limit of detection (LOD) was determined from an assay of 10 replicates of the zero-level calibration solution (lot no. 20210201; 0 ng/mL) [15]. Based on the calibration curve, the chemiluminescence value of the mean + 2 standard deviations (SD) at the zero-concentration point was converted into the corresponding concentration, which was the LOD concentration.

2.2.3. Accuracy

This procedure followed the EP10-A3 guidelines published by the CLSI [16]. In this study, the detection of two concentrations of traceable calibration solutions (lot no. 20210601; 70.90 and 864.90 ng/mL) was repeated twice a day and for a total of 5 days. The accuracy was accepted when the bias was not more than 12.5%.

2.2.4. Precision

Following the CLSI’s EP15-A2 guidelines, two levels of mixed fresh serum were used [17]. The intraanalysis precision was assessed by calculating the coefficient of variation (CV) (%) of each level three times per run in one day. These samples were analyzed three times for five days to obtain the interanalysis comparison data. The precision was acceptable when the CV was not greater than 10.0%.

2.2.5. Reference Intervals

Following the CLSI’s EP28-A3c guidelines [18], 21 specimens collected from healthy individuals were analyzed for their CHI3L1 levels. On the condition that less than two specimens exceeded the reference interval suggested by the manufacturer, the validation was accepted.

2.3. Clinical Samples

Patients with hepatitis B were diagnosed according to the “2019 Guidelines for the Prevention and Treatment of Chronic Hepatitis B.” The exclusion criteria included the following: (1) aged <18 years; (2) lack of liver biopsy results; (3) lack of sufficient samples for the detection of CHI3L1; (4) complicated with severe diseases; and (5) pregnant or lactating women. The selection criteria for healthy controls were mainly based on negative results for hepatitis B surface antigen (HBsAg) and normal liver function. The sample size required more than 100 cases to meet the requirements of CLSI’s EP9-A3 [19]. Peripheral blood samples were centrifuged at 3000 × g for 10 minutes to obtain the supernatant (serum). Serum samples were stored at −80°C for subsequent use. The routine clinical indicators were provided from clinical electronic databases. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total protein (TP), total bilirubin (TBIL), lactate dehydrogenase (LDH), and alpha-fetoprotein (AFP) levels were measured by using an automatic biochemistry analyzer (Roche Cobas c 602/702, Roche Diagnostics GmbH, Mannheim, Germany). The platelet (PLT) parameters were detected using a Sysmex XN-9000 automatic hematology analyzer (Sysmex, Kobe, Japan). The APRI and FIB-4 indices were obtained by using the following formulae [20]:(1)FIB−4=ageyears×ASTIU/LPLT109/L×ALTIU/L×100,ARPI=ASTIU/L/ULN∗ofASTIU/LPLT109/L×100,where ULN is the upper limit of normal.

This study was approved by the Ethics Committee of the Guangdong Provincial Hospital of Chinese Medicine (ZE2021-084-01). Written informed consent was obtained from the patients and healthy volunteers.

2.4. Statistical Analysis

All statistical analyses were performed using SPSS 23 (IBM Corp., Armonk, NY, USA). Normally distributed data are shown as the mean ± SD; otherwise, M (P25 ~P75) was used. Comparisons of multiple groups were carried out by using a one-way analysis of variance (ANOVA) followed by a Bonferroni test for normally distributed and equal variance data; otherwise, a Kruskal–Wallis test was used. The receiver operating characteristic (ROC) curve was plotted to assess the diagnostic accuracy. We used Pearson's correlation analysis to assess whether the CHI3L1 concentration correlated with the clinical indicators. P values of <0.05 were considered statistically significant.

3. Results

3.1. Performance Verification

3.1.1. Analysis of the Linear Range

The CHI3L1 standard curve equation was $y = 147.345x - 103.971$, R2=0.999 (Figure 1(a) and raw data in Supplementary Table 1). The expected concentration and the experimental results are summarized in Table 1. As shown in Figure 1(b), the linear correlation coefficient was 0.9987, which was greater than 0.9900. These results indicated that the linear range of 1.50 ng/mL–2000.00 ng/mL stated in the reagent instructions was acceptable (Table 1 and Figure 1(b)).

[figure(s) omitted; refer to PDF]

Table 1

Analysis of the CHI3L1 linear range.

Dilution ratio (L : H)	Expected concentration (ng/mL)	Measured value (ng/mL)		Detection average (ng/mL)
1st	2nd	5 : 0	1.53	1.52
1.54	1.53	4 : 1	398.42	395.42
395.41	395.42	3 : 2	795.32	775.26
775.41	775.34	2 : 3	1192.21	1145.00
1125.00	1135.00	1 : 4	1589.11	1524.00
1527.00	1525.50	0 : 5	1986.00	1985.00

L, low; H, high; CHI3L1, chitinase-3-like protein 1.

3.1.2. Analysis of the LOD

The LOD concentration was determined as 0.98 ng/mL (raw data in Supplementary Table 2). Therefore, the detection range was between 0.98 and 2000.00 ng/mL. In addition, several immunoassay methods for serum CHI3L1 levels are summarized in Table 2. Compared with the methods detailed in previous reports [10–12], CLIA presented a shorter detection time, a wider linear range, and a lower detection limit.

Table 2

Immunoassay methods to detect serum CHI3L1 levels.

Methods	ELISA	MB-FIA	FICA	CLIA
Time (min)	>120	>20	>20	>20
Detection range (ng/mL)	60.35–969.98	2.90–111.00	5.00–200.00	0.98–2000.00
LOD (ng/mL)	60.35	2.90	5.00	0.98
Reference	2016 [10]	2015 [11]	2021 [12]	Present study

ELISA, enzyme-linked immunosorbent assay; MB-FIA, magnetic bead fluorescent immunoassay; FICA, fluorescence immunochromatography assay; CLIA, chemiluminescence immunoassay; LOD, limit of detection; CHI3L1, chitinase-3-like protein 1.

3.1.3. Analysis of Accuracy

The biases of the low-concentration calibration solution and high-concentration calibration solution between the instrumental concentration and the theoretical concentration were 0.48% and 0.08%, respectively, which were both less than 12.5% (Table 3 and raw data in Supplementary Table 3).

Table 3

Evaluation of accuracy.

Calibration solution	Instrumental concentration (ng/mL)	Theoretical concentration (ng/mL)	Bias (%)
Low CHI3L1 concentration	71.24	70.90	0.48
High CHI3L1 concentration	864.20	864.90	0.08

CHI3L1, chitinase-3-like protein 1. Bias (%) = ((instrumental concentration − theoretical concentration)/theoretical concentration) × 100%.

3.1.4. Analysis of the Precision of the Assay

As shown in Table 4, the intraassay CVs for levels 1 and 2 were 1.81% and 2.76%, respectively; the interassay CVs were 7.76% and 1.61%, respectively; and the total precision CVs were 7.91% and 2.88%, respectively, which were all less than the acceptable range of not more than 10.0%, indicating that the precision of the testing kit was acceptable (raw data in Supplementary Table 4).

Table 4

Evaluation of precision.

Level	Mean (ng/mL)	Intraassay precision		Interassay precision		Total precision	
SD	CV (%)	SD	CV (%)	SD	CV (%)	Level 1	19.73
0.36	1.81	1.53	7.76	1.56	7.91	Level 2	85.27

SD, standard deviation; CV, coefficient of variation. CV (%) = (SD/mean) × 100%.

3.1.5. Validation of Biological Reference Intervals

In samples from 21 healthy individuals, the results were all <79.0 ng/mL (raw data in Supplementary Table 5). Thus, the reference interval passed the verification standard.

3.2. Serum CHI3L1 Detection in Clinical Samples

To validate the clinical application of the CLIA, serum CHI3L1 levels were detected in samples from 82 patients with CHB and 21 healthy controls (Figure 2 and raw data in Supplementary Table 6). Compared with that in the healthy controls (median: 32.70 ng/mL, P=0.001) and patients with CHB without HCC (median: 38.72 ng/mL, P=0.001), the CHI3L1 concentrations were significantly higher in patients with CHB and HCC (median: 122.14 ng/mL, P=0.001, Figure 2(a)). The CHI3L1 concentrations did not change significantly with increasing liver fibrosis stages (P=0.767, Figure 2(b)), nor did the FIB-4P=0.068 and APRI P=0.055 indices. The AUC of CHI3L1 for the diagnosis of HBV-related HCC was 0.808, with a sensitivity of 76.19% and specificity of 80.49% at a cutoff of 51.00 ng/mL (Figure 2(c)). As shown in Table 5, we found that there were significant correlations between CHI3L1 and AST (r = 0.284, P= 0.005), ALP (r = 0.374, P=0.001), AFP (r = 0.694, P=0.001), LDH (r = 0.431, P=0.001), FIB-4 (r = 0.638, P=0.001), and APRI (r = 0.543, P=0.001), while no correlation was observed between CHI3L1 and the other clinical indicators.

[figure(s) omitted; refer to PDF]

Table 5

Comparison of serum CHI3L1 and clinical indicators.

Indicators	CHI3L1	ALT	AST	ALP	GGT	TP	TBIL	AFP	LDH	FIB-4	APRI
Diagnostic value											
AUC (HCC)	0.808	0.507	0.748	0.698	0.723	0.614	0.522	0.836	0.767	0.884	0.821
AUC (LC)	0.686	0.510	0.589	0.570	0.587	0.552	0.566	0.600	0.516	0.758	0.731
–											
Correlation analysis											
r (CH I3L1)	—	0.032	0.284	0.374	0.074	0.049	0.007	0.694	0.431	0.638	0.543
P	—	0.752	0.005	0.001	0.470	0.630	0.949	0.001	0.001	0.001	0.001

CHI3L1, chitinase-3-like protein 1; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl endopeptidase; TP, total protein; TBIL, total bile acid; AFP, α-fetoprotein; LDH, lactate dehydrogenase; FIB-4, fibrosis-4; APRI, aspartate aminotransferase-to-platelet ratio index; AUC, the area under the receiver operating characteristic curve; HCC, hepatic carcinoma; LC, liver cirrhosis.

4. Discussion

In the present study, we evaluated a simple and rapid CLIA for the quantitative detection of serum CHI3L1, which presented good analytical performance, making it valuable for the diagnosis of HBV-related HCC.

Currently, CLIAs are increasingly being used in the biological analysis because of their extreme sensitivity, high specificity, efficient simplified detection procedures, and short assay time [13]. Therefore, we evaluated the performance of a CLIA to detect serum CHI3L1 according to the CLSI guidelines. The assay kit showed good linearity at CHI3L1 concentrations ranging from 1.50 to 2000.00 ng/mL, with a lower LOD of 0.98 ng/mL (Tables 1 and 2). It has been reported that the upper pathophysiological concentration range of CHI3L1 can exceed 1000.00

ng/mL [21]. In this case, the CLIA for CHI3L1 detection had the advantage of a wide linear range compared with other detection methods (Table 2). Its low background signal meant that the sensitivity of CLIA for CHI3L1 detection was higher than other detection technologies (Table 2). Compared with the widely applied ELISA method, the CLIA assay greatly reduces the detection time because it provides a simplified procedure. However, the available equipment and costs of the CLIA assay should be considered in primary hospitals. In the precision assay, the intraassay, interassay, and total precision CVs were all below 10.00% (Table 4). Compared with previously reported methods [10–12], CLIA presented a simple operation process and shorter detection time, which was the main reason for its excellent precision and avoidance of unpredictable variations. In addition, it has been reported that the CHI3L1 level is approximately 40 ng/mL in healthy blood serum [22], which was consistent with our data (Figure 2(a)). Notably, the CLIA method is highly automated, making it suitable for the detection of CHI3L1 in large-scale clinical samples. The CLIA method to detect CHI3L1 represents a powerful tool to further explore the clinical value of CHI3L1.

Liver diseases are commonly caused by virus infections, especially HBV infections, in China [1]. Considering the important clinical value of CHI3L1, we evaluated the CLIA for serum CHI3L1 detection in HBV-related liver diseases. From the baseline clinical characteristics, no pregnant or lactating women were found at the time of sample exclusion. In addition, we found that HBV infection predominantly occurred in males (71/103), which might be due to their poorer lifestyle habits, such as alcohol consumption and smoking, compared with women [23]. The CHI3L1 concentrations were significantly higher in patients with CHB and HCC than those in the healthy controls and patients with CHB without HCC (Figure 2(a)), which was consistent with the results obtained by Jiang et al. [7] and Liang [24]. Studies have shown that CHI3L1 plays critical roles in cancer cell growth, proliferation, invasion, metastasis, angiogenesis, and immunoregulation [3, 25, 26]. These features might account for the high CHI3L1 levels in HCC; therefore, the effect of CHI3L1 on the occurrence and development of HCC should be further studied. We also observed a three-fold increase in the median level of CHI3L1 in patients with CHB and HCC (Figure 2(a)). This distinct differential expression suggested that CHI3L1 might be highly specific in HBV-related HCC. Indeed, compared with other clinical indicators, both AFP and CHI3L1 had a moderate diagnostic value for HBV-related HCC (Table 5). Serum AFP is a noninvasive marker commonly used in clinical laboratory screening for HCC. Meanwhile, the highest significant correlation was between CHI3L1 and AFP (Table 5, $r = 0.694$, $P < 0.001$). These results indicated that CHI3L1 has a good diagnostic value in HBV-related HCC. CHI3L1 concentrations did not change significantly with the increase in liver fibrosis stages, nor did the FIB-4 and APRI indices, and all three showed poor diagnostic value for liver fibrosis (Figure 2(b)). However, serum CHI3L1, FIB-4, and APRI were recommended indicators to assess liver fibrosis [9, 20]. The main reason for this discrepancy might be that the number of patients was small, especially the sample size of patients at the stage S3-S4 fibrosis (19/61). The early stages of liver fibrosis can be reversible with treatment. Otherwise, liver fibrosis can lead to life-threatening LC or HCC [9]. Therefore, it is necessary to further explore CHI3L1 for the diagnosis of liver fibrosis, including large-scale and multicenter studies, multifactor analysis, and the combined application of multiple indicators or methods. CHI3L1 can be used as a biomarker for prognostic assessment and targeted therapy in liver diseases [27, 28], which also needs to be validated by using the CLIA. Considering the important clinical value of CHI3L1 in other diseases [3], the developed CLIA will be a powerful tool to further explore the clinical relevance of CHI3L1.

5. Conclusions

In conclusion, we validated the CLIA for the rapid determination of serum CHI3L1 levels in HBV-related liver diseases. Compared with the widely applied ELISA, the CLIA assay greatly reduces the detection time. In addition, the CLIA assay has a high sensitivity, a wide linear range, and an acceptable accuracy and precision for the quantitative detection of CHI3L1, which meets the requirements for clinical testing. The CLIA assay for CHI3L1 detection also showed a good diagnostic performance in HBV-related HCC. This CLIA represents a powerful tool to further explore the clinical value of CHI3L1.

Authors' Contributions

Jialing Zhuang and Yanqiang Liao conceptualized and designed the study. Se Peng acquired the data. Jialing

Zhuang, Yanqiang Liao, Lesheng Huang, Jian Hu, Rui Xu, Zhong Li, and Wenzhi Tang analyzed and interpreted the data. Yanqiang Liao, Se Peng, Lesheng Huang, Jian Hu, Rui Xu, Zhong Li, Wenzhi Tang, and Jialing Zhuang drafted and revised the article. Yanqiang Liao and Se Peng contributed equally to this work.

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DETAILS

Subject:	Proteins; Accuracy; Fibrosis; Performance evaluation; Hepatitis B; Mathematical analysis; Reagents; Chitinase; Chemiluminescence; Calibration; Biopsy; Diagnosis; Liver cirrhosis; Liver cancer; Liver; Immunoassay; Liver diseases; Variance analysis; Correlation analysis
Location:	China
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-10-12 (Received); 2023-12-07 (Revised); 2024-01-12 (Accepted); 2024-01-24 (Pub)
DOI:	https://doi.org/10.1155/2024/6688819
ProQuest document ID:	2921792408
Document URL:	https://www.proquest.com/scholarly-journals/analytical-clinical-evaluation-chemiluminescent/docview/2921792408/se-2?accountid=211160

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Last updated:	2024-02-05
Database:	Publicly Available Content Database

Analysis of the Concentration of Heavy Metals in Khat Grown in Meru County and the Assessment of Their Associated Health Risks

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ProQuest document link

ABSTRACT (ENGLISH)

Contamination of farm produce by toxic heavy metals has become a serious global health concern. These metals can bioaccumulate in plant tissues and are precursors for major public health problems such as cancer and neural impairment. Khat (*Catha edulis*) also referred to as miraa has the potential to sequester and accumulate both micronutrients and potentially toxic heavy metals in its consumable parts—tender leaves and soft barks of young shoots which are known to possess psychoactive properties when consumed. Therefore, the motivation behind this contribution is to determine the levels of six heavy metals, namely, cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), iron (Fe), and nickel (Ni) in consumable Meru khat samples, compare these levels with the permissible limits of World Health Organization (WHO) in order to predict associated health risks, and to estimate the noncarcinogenic risks of these metals by total health quotient (THQ) and health index (HI) on khat consumers. 1.0 g of dry ground khat samples was digested in 0.05 M HCl and allowed to stand for 5 hours before being analyzed for heavy metals using inductively coupled plasma atomic emission spectroscopy (ICP-AES). The mean heavy metal concentrations (mg/kg) in dry khat samples of six toxic heavy metals were Cd (7.81 ± 1.56), Cr (15.98 ± 2.22), Cu (15.81 ± 2.84), Fe (97.35 ± 32.67), Ni (0.37 ± 0.02), and Pb (32.36 ± 9.95). Based on the results, the mean levels of Pb, Cd, and Cr exceeded WHO permissible limits. In addition, the Pb and Cd THQ values and the HI of the six heavy metals investigated in the khat samples exceeded the threshold value of 1.0. Furthermore, the THQ and HI values showed that Pb and Cd were potentially the major contributors to noncarcinogenic risks on regular khat consumers. This is a matter of concern on the excessive consumption of Meru khat-based products, which over time may cause a toxicological response. Based on the findings of this study, the use of agrochemicals should significantly be minimized in khat farming. Accordingly, the Meru khat farmers should be sensitized on alternative farming practices that do not potentially cause heavy metal contamination in khat.

FULL TEXT

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1. Introduction

Khat farming and its related activities are a multimillion enterprise for many people in the khat-growing regions and also contribute immensely to the gross domestic product (GDP) for the countries where the plant is grown [1, 2]. In Kenya, Meru County is not only the main producer of khat for local and international markets but also the highest khat consumer [3, 4]. Nonetheless, the consumption of khat leaves for stimulative and psychoactive effects, for instance, is largely unregulated, and the evaluation and monitoring of contaminants such as heavy metals and pesticides in khat before consumption is rarely conducted. This leaves khat consumers vulnerable to the health hazards that can result from contaminated khat. This study is therefore inspired by the potential toxic metals that are considered a major public health hazard due to their bioaccumulation in khat that may have the potential to causesevere etiological risks. Accordingly, the lack of awareness about the presence of heavy metals in food products especially khat has resulted in many health concerns that are detrimental to public well-being [5]. Periodic determination of heavy metals and other micronutrient elements in crops, soils, and water bodies is fundamental in monitoring their concentration levels and maintaining them within allowable limits [6, 7] in order to ensure good health and safety. It is notable that research on the chemical composition of plant-based materials is expanding due to the need for sustainable developments in the agricultural sector, environmental heavy metal pollution, and natural activities including lightning and volcanic eruptions [8]. Therefore, enhanced food security should be aimed at preventing heavy metal contamination [9], potent pesticides, and other agricultural-based synthetic chemicals [10].

Clearly, trace and heavy metals are some of the major food chain contaminants which are precursors for deleterious health effects in humans even at low concentrations [5]. Despite much awareness about heavy metal contamination, anthropogenic activities throughout the world have increased significantly with soil as a sink for toxic metals, which

are not only persistent but also accumulative in soil, and have the potential to increase the toxicity of soils after combining with inorganic and organic matters [11].

The khat plant can absorb both micronutrient elements responsible for its growth and macronutrient elements (heavy metals) into its tissues from contaminated soils such as landfills and industrial effluent [5]. This uptake is continuous and these elements biomagnify in the leafy part of the plant resulting in accumulations of varying concentrations at different plant parts [10, 12]. Important to note is that khat is susceptible to pests; consequently, the use of synthetic pesticides controls them and the application of fertilizers improves the production levels [1]. These practices are well known to introduce heavy metals into the soils which are then absorbed by the plant into its tissues [13]. Their bioaccumulation especially in plant tissues depends on the age and maturity of the plant during harvesting [10]. This agglomeration of toxic heavy metals makes consumable khat parts easily contaminated which in turn can cause serious health risks upon consumption [14].

Of late, heavy metal poisoning is a public health concern that calls for regular monitoring and assessment [10]. Thus, human risk assessment is critical as it estimates the risk of environmental contamination to human health through different modes of exposure such as inhalation, ingestion, and skin contact [15]. Towards this end, research reports from across the world have confirmed severe health problems induced by toxic heavy metals present in plant samples and possible threat to the lifespan of animals [6] and humans. This is due to the fact that these metals are nonbiodegradable, have long-term duration in the environment, and are capable of causing serious public health problems such as cancer, nerve breakdown, and organ failure [13]. However, WHO in one of its reports affirmed that some heavy metals, especially Zn, Mn, Fe, and Cu, are not only essential but also necessary for healthy growth, provided their levels do not exceed acceptable limits beyond which they can cause diseases [6] and result in intoxication and chronic toxicity to humans [9].

The detection and quantification of heavy metals in various samples can be done using analytical techniques such as inductively coupled plasma atomic emission spectrometry (ICP-AES), atomic absorption spectrometry (AAS) [16], and inductively coupled plasma optical emission spectrometry (ICP-OES) [17]. To the best of our understanding, little research has been conducted on heavy metals in the Meru khat. Therefore, the motivation underpinning this study is to determine the concentration profiles of heavy metals in leaf shoots of the Meru khat—Cd, Cr, Cu, Pb, Fe, and Ni—and to compare them with allowable limits of WHO in order to predict associated health risks on consumption of this khat. To assess the magnitude of the risks of these metals, the noncarcinogenic parameters THQ and HI have been estimated. This study provides a basis that will guide alternative agricultural practices in the growing of safer khat plants. Also, these results can enhance the understanding of the public health risks of toxic heavy metals in agricultural crops such as khat in the Meru region. This will provide insights into contamination control, with respect to human health risks and sustainable, human-friendly economic growth [18].

2. Study Area

A total of 11 khat samples used in this study were collected randomly from khat farmers in each of the selected regions—Kangeta, Maua, Laare, Kianjai, and Mutuati in Meru County, Kenya. Miraa referred to locally as *Miwee* among the Ameru people is a type of plant that is cultivated for local and export consumption. The sample collection sites are presented in Figure 1. The collected samples were then directly taken to the laboratory for further treatment and analysis.

[figure(s) omitted; refer to PDF]

Meru County, coordinates 0° 21′ 21″ N/37° 48′ 32″ E, is located in the central part of Kenya and covers a total land area of 7,006 km2. As of the 2019 Kenya Population and Housing Census, the county's population was 1,545,714. The annual rainfall in the county ranges between 300 mm and 2500 mm where the long rains fall from March to May and short rains from October to December. The Meru region is situated close to the equator, making the summers difficult to define. The recorded annual range of temperature is from 12.92 to 23.45 °C characterized by coldest and warmest months. Khat farming is the main economic activity for inhabitants of this county who derive very good returns from this profitable agribusiness venture. This county is part of the country that not only consumes khat in large quantities but also exports it to neighboring countries such as Somalia, Madagascar and Uganda. Khat is an

exceptional plant in the county that can tolerate climatic extremes, particularly drought conditions. The demand for domestic khat consumption and the international markets have caused the khat supply to be severely stressed in the past few years. Agriculture and animal husbandry are the major human activities in most of the study areas and fertilizers (animal manure and chemical fertilizers) and pesticides are widely used in these areas. Intercropping khat with other food crops system is mainly adopted by the khat farmers in this county.

2.1. Experimental

2.1.1. Reagents and Sample Preparation

All reagents used in this study were of analytical grade with % purity ≥99.9. Materials and reagents including commercial 1000 ppm standard mixture solution containing 6 target heavy metal elements were purchased from Kobian chemical Company—a subsidiary of Sigma-Aldrich Ltd., South Africa. Other reagents included 10% nitric (v) acid and 20 volumes of hydrogen peroxide (H_2O_2). Deionized water purchased from Sigma-Aldrich (Germany) was used throughout the study for rinsing, sample preparation, and dilution prior to the analysis.

Khat samples were air-dried in the laboratory for 1 week, followed by oven drying at 70 °*C* for 72 hours to obtain constant mass. The dry samples were ground using a grinder to homogenize and reduce their sizes to 0.25 mm. The samples were then stored in sterilized plastic containers for analysis. The elemental concentrations of the digested khat samples were analyzed using an inductively coupled plasma atomic emission spectrometer (ICPE-9000).

To minimize contamination, stainless steel grinding systems were washed and thoroughly cleaned with acetone before and after each grinding cycle to avoid cross-contamination. The ground khat samples were stored in sterilized plastic containers. All the glassware used for analytical work was thoroughly rinsed using deionized water, followed by concentrated nitric acid wash. The disposable plastic gloves were worn when handling the khat samples during the sampling and analyzing stages. Finally, the digested khat solutions were kept in a refrigerator at all times until analysis was complete.

The digestion procedure of the khat samples was optimized for different parameters such as reagent volume, digestion time, volume ratio of the reagents, and digestion temperature. The trial method optimization was done by varying one parameter at a time and keeping other parameters constant. The parameters that gave clear solutions at lower temperature and shorter reaction time and required minimum reagent volume and ratio were selected as an optimum procedure and were used for the digestion of khat samples. One gram (1.0 g) of finely ground dry khat sample was weighed into sample digestion specimen tubes. The sample was allowed to ash in a muffle furnace for 5 h at 450°C−500 °C before cooling to room temperature in a desiccator. The sample was then transferred into a 100 mL conical flask, followed by the addition of 1 mL mixture of H_2O/HCl (1 : 1 v/v), H_2O/HNO_3 (1 : 1 v/v) and 10 cm^3 of 20 volumes of hydrogen peroxide (H_2O_2), and the mixture evaporated to dryness on a hot plate. The mixture was reconstituted using 25 mL of 0.05 M HCl and allowed to stand for 5 hours awaiting metal analysis. The digests were used to determine the concentrations of Cd, Cu, Pb, Fe, and Ni by ICP-AES.

2.1.2. Analysis of Heavy Metals

Metal ion concentrations in khat plant samples were analyzed using an inductively coupled plasma atomic emission spectrometer (ICPE-9000). All the standard, blanks, and samples were prepared and analyzed in triplicate. An instrument quality control and subsequent tuning were performed using an instrument tuning solution at 0.1 mg/kg in hydrochloric acid. A nine-point calibration curve of multielement standard was drawn in absorbance mode and was used to determine the concentration of heavy metals present in the khat samples. The ICP-AES method developed was validated for parameters such as accuracy, robustness, and precision. The instrumental parameters and working conditions for ICP-AES are given in Table 1. High-purity (99.99%) argon was used as a nebulizer, plasma, and auxiliary gas. The instrument was calibrated for various parameters before the experiments were conducted.

Table 1

Instrumental parameters and operating conditions for ICP-AES.

ICP-AES parameter	Value

Pressure of argon gas	450 ± 10 kPa
Nebulizer flow	0.90 L/min
Plasma power	1300 W
Radio frequency generator	1.2 kW
Pump rate	25 rpm
Coolant flow rate	12.00 L/min
Vacuum pressure	>10 Pa
Exposure time	30.0 seconds
Carrier gas flow rate	0.70 L/min
Auxiliary gas flow	0.60 L/min
Plasma gas flow rate	10.0 L/min

2.1.3. Evaluation of Method Accuracy and Precision

The precision and accuracy of the method were assessed by spiking 10 mL aliquot of 5 μg of each analyte metal ion into 1 g khat sample, followed by the same optimized digestion procedure for the spiked and nonspiked samples. The levels of heavy metal ions in both samples were determined by ICP-AES, and the percentage recovery values were calculated using the following equation:(1)Percentage recovery=Asks−AksAa×100, where Asks is the concentration in spiked khat sample, Aks represents the concentration in nonspiked khat sample, and Aa is the amount added.

2.2. Target Hazard Quotient (THQ)

The heavy metals' noncarcinogenic risk to humans due to regular ingestion of contaminated khat was determined by assessing the THQ [19]. The cumulative risks posed by exposure to all investigated heavy metals in consumable khat were assessed by calculating the HI. The THQ ratio of the investigated heavy metals was evaluated using equation (3) [19].(2)THQ=C°×IR×EF×EDBw×RfD×AT×10−3.

Since EF×ED/AT=1, then equation (2) becomes(3)THQ=C°×IRBw×RfD×10-3.

Here, Bw stands for adult body weight considered at 70 kg; C° denotes the average concentration of heavy metal in the khat (mg/kg dry weight); EF is the exposure frequency taken as 365 days/year; ED represents exposure duration taken as 70 years; AT denotes the average time for potential health risk taken as ED × 365 days; and IR represents the ingestion rate of khat taken as 100 g/person/day as determined in previous studies [20].

The RfD (mg/kg/day) refers to the oral reference dose which is taken as 0.001 for Cd, 0.0035 for Pb, 0.02 for Ni, 0.04 for Cu, 1.5 for Cr, and 0.07 for Fe [19, 21–23]. The HI assumes that the magnitude of adverse effects on target organs and systems of humans will be proportional to the sum of the THQ of multiple heavy metal exposures in consumable khat. This is estimated using the following equation:(4)HI=∑THQ=THQPb+THQCu+THQCd+THQFe+THQCr+THQNi.

If HI is less than 1.0, then the non-carcinogenic effect due to the heavy metal exposure is considered to be negligible. However, if the TH value exceeds 1.0, then these effects are detrimental [15, 24].

2.3. Statistical Analysis

All the statistical tests were carried out using Microsoft Excel spreadsheet and Statistical Package for Social Sciences (SPSS) IBM statistical software, version 25.0. The descriptive statistics such as the means, standard deviation, and relative standard deviation were done using Microsoft Excel. The variation in concentration of toxic heavy metals in khat plant cllected from different farms was subjected to multivariate statistical analysis. Particularly, the Pearson correlation analysis was employed to analyze the correlation between variables (concentration of each heavy metal) with a view to determining data coherence and divergence. The principal component analysis (PCA) was applied to simplify the experimental data and conjecture onthe sources of heavy metals in the Meru khat. PCA allows for the large datasets for dimension reduction while keeping most of the information.

2.4. Method Validation and Recovery Test

The calibration curve prepared using the pure standards of Fe, Cu, Cr, Ni, Pb, and Cd was found to be linear with correlation coefficient, r≥0.998. In order to assess the accuracy of the ICP-AES method, spiked khat samples were used. The relative recovery experiments at different concentrations were evaluated and the results are recorded in Table 2. The relative recovery values ranged between 91.7% and 109.1%, indicating that the method used exhibits commendable accuracy.

Table 2

Percentage recovery test results of heavy metals in khat samples.

Heavy metal	*Concentration in nonspiked khat sample (mg/kg)	Amount added (mg/kg)	**Concentration in spiked khat sample (mg/kg)	Percentage recovery (%)[a]
Cu	16.36	3.50	19.90±0.08	101.1±0.3
Cd	7.64	1.50	9.09±0.11	96.7±0.9
Pb	24.45	2.00	26.43±0.07	99.0±0.2
Fe	91.78	5.00	96.47±0.80	93.8±0.6
Ni	0.36	1.00	1.34±0.01	98.0±0.2
Cr	16.14	2.50	18.59±0.20	98.0±0.7

*Average value of three measurements; **values are mean±SD of triplicate analyses. [a]Values are mean±SD of triplicate.

The sensitivity of the ICP-AES method was assessed by calculating limits of detection (LOD) and limits of quantification (LOQ). As a result, ten separate blank solutions were independently prepared and analyzed. The determination of LOD and LOQ was done based on the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [25], using the following equations:(5)LOD=3δs,(6)LOQ=10δs.where, s is the slope of the calibration curve for each element and δ is the standard deviation of the measurements of the blank solution.

For the evaluation of method linearity, the calibration curves for each element were constructed by plotting the peak area of the optimum emission line to the concentration of the standard solution. Subsequently, least square linear regression analysis was used to evaluate the slope, intercept, and coefficient of determination.

2.5. Calibration

The selection of the optimum emission lines was based on the intensity and their sensitivity as well as the absence of spectral interferences. The selected emission lines for each element in this study were Cd (226.502 nm), Cu (324.752 nm), Ni (232.003 nm), Pb (217.000 nm), Fe (238.204 nm), and Cr (357.869 nm). The performance of the

ICP-AES method was evaluated under the conditions described in Table 1. Table 3 shows the calibration curve, the regression lines, and the LOD and LOQ for all the examined heavy metals in khat samples. From Table 3, the coefficients of determination for all the heavy metals were good ($r2 > 0.9800$) for the experimentally determined values. The LOD and LOQ of the ICP-AES method ranged between 0.072 and 1.193 mg/kg, and 0.220–3.616 mg/kg, respectively. The method applied demonstrated high sensitivity as shown bylow values of LOD.

Table 3

Calibration curves, LODs, and LOQs of the developed ICP-AES method.

Element	Emission line (nm)	Equation	Slope	r2	LOD (mg/kg)	LOQ (mg/kg)
Cd	226.502	$Y = 1.7402x - 371$	1.7402	0.9855	0.979	2.966
Ni	232.003	$Y = 1.1564x + 24$	1.1564	0.9994	0.195	0.296
Pb	217.000	$Y = 1.6231x - 199$	1.6231	0.9967	0.072	0.220
Cr	357.869	$Y = 1.3688x - 67$	1.3688	0.9953	0.552	1.673
Fe	238.204	$Y = 1.3819x - 74$	1.3819	0.9930	1.193	3.616
Cu	324.752	$Y = 1.4488x - 32$	1.4488	0.9958	0.924	2.801

3. Results and Discussion

3.1. Concentration Profiles of Heavy Metals in Khat

In the present study, the concentrations of selected heavy metals, Cu, Cr, Ni, Pb, Cd, and Fe in khat samples collected from the Meru region are reported in Table 4. The results show a wide variation in the concentration of heavy metals in khat samples collected from different khat-growing farms of Kianjai, Laare, Kangeta, Mutuati, and Maua of Meru.

Table 4

Concentration of heavy metal content in khat samples collected from Meru County.

Region in Meru County	Farm code	Metal concentration (mg/kg)					
Cr	Cd	Cu	Pb	Fe	Ni	Kangeta	A
16.40 ± 0.23	5.51 ± 0.11	17.00 ± 0.42	24.14 ± 1.26	92.90 ± 3.12	0.378 ± 0.03	B	19.10 ± 0.12
5.58 ± 0.10	21.18 ± 0.61	31.11 ± 1.52	139.0 ± 3.64	0.386 ± 0.02	-		
Laare	C	13.90 ± 0.14	9.72 ± 0.08	16.79 ± 0.32	36.29 ± 1.02	83.30 ± 2.11	0.361 ± 0.03

D	17.60±0.25	8.88±0.13	14.67±0.35	42.55±2.45	180.5±3.02	0.392±0.02	
Mutuati	E	14.60±0.18	5.48±0.05	13.03±0.26	15.53±0.33	86.80±2.50	0.373±0.01
F	19.00±0.31	8.53±0.06	13.70±0.18	25.92±1.92	83.40±2.10	0.367±0.02	
Maua	G	12.30±0.15	9.23±0.05	17.23±0.43	48.10±2.82	72.10±2.13	0.352±0.02
H	18.00±0.27	8.74±0.12	14.06±0.32	<0.001±0.00	78.50±2.13	0.399±0.04	I
15.60±0.16	8.32±0.23	14.43±0.36	<0.001±0.00	88.50±2.09	0.371±0.03		
Kianjai	J	15.00±0.13	7.67±0.04	19.70±0.52	30.10±2.21	80.40±1.89	0.349±0.02
K	14.30±0.11	8.28±0.05	12.08±0.64	37.47±2.02	85.50±2.05	0.365±0.03	
Mean±SD		15.98±2.22	7.81±1.56	15.81±2.84	32.35±9.95	97.35±32.67	0.372±0.016
–							
RSD (%)		13.9	20.0	18.0	30.8	33.6	4.2

SD, standard deviation; RSD, relative standard deviation.

The average and RSDs percentages of all the heavy metals investigated were calculated and listed in Table 4. The contents of these metals show variations in concentrations for different khat samples. The RSDs percentages ranged from 4.2 to 33.6. These results showed that the most abundant metal in khat samples was Fe, followed by Pb, Cr, Cu, Cd, and Ni, respectively. Generally, iron being the most abundant metal in all khat samples has its concentration ranging from 72.10±2.13 to 180.5±3.02 mg/kg, with a mean of 97.35±32.67 mg/kg as reported in Table 4. The highest value was from the sample drawn from the Laare region (D), while the lowest value was from the Maua region (G). This could be attributed to the fact that khat is grown in different geographical locations with different soil types, different soil pH values, and the application of different agrochemicals. Comparatively, studies carried out on Ethiopian khat indicated that the levels of Fe were lower compared to those of the present study [13]. For instance, this result was lower than the iron content in khat drawn from some regions in Ethiopia which included the Amhara, Oromia, and Southern Nations, Nationalities and Peoples [12]. Nevertheless, the levels of Fe in this work are consistent with those reported from different areas in Ethiopia and Yemen [26].

High amount of organic matter in the soil is known to contribute to high levels of Fe, especially in the khat plant [26, 27]. More importantly, laying harvested maize stalks under khat trees not only prevents water loss from the ground but also decomposes over time and hence is reincarnated into soil composition which then enhances the levels of

Fe in soil. It is estimated on average, that a khat user chews 50–200 g of young khat leaves and shoots per day on wet weight basis [12]. The calculations presented in Table 5 are based on this estimation, which are then compared with allowable limits in order to determine any associated risks. The results in Table 5 indicate that the THQ of iron calculated is less than 1.0; hence, there is no likelihood of causing health effects.

Table 5

The THQ and HI values of investigated heavy metals with respect to khat consumption.

Noncarcinogenic parameter	THQ						HI
Heavy metal	Cr	Cu	Ni	Fe	Cd	Pb	
Values	0.015	0.056	0.027	0.20	11.6	13.2	24.90

Therefore, in all the khat samples analyzed, the concentration of Fe was below the permissible limit (425.0 mg/kg) set by the WHO/FAO [21]. This shows that khat obtained from the regions of Meru could be a good supplement of Fe. Thus, its detection in khat is not only vital to khat users, but managing its levels below permissible limits helps in a range of metabolic processes such as oxygen and electron transport and synthesis of deoxyribonucleic acid (DNA) [28]. Besides, in the biological processes of the human body, Fe provides normal functioning of body cells, other vital organs, and generation of free body radicals which can be useful [29]. Copper is also an essential trace element responsible for healthy plants and animals. This study reports that the concentration of Cu in khat samples ranged from 12.08 ± 0.64 to 21.18 ± 0.61 mg/kg with a mean of 15.81 ± 2.84 mg/kg (Table 4). The lowest and highest concentrations of Cu were found in samples that were obtained from the Kangeta region (sample B) and Kianjai region (sample K), respectively (Table 4).

High levels of Cu could be attributed to the application of Cu-based pesticides meant to control and manage pests, although the khat plant could also absorb some copper from the soil [22]. The levels of Cu from this study are higher than those reported previously in some khat-growing regions from Ethiopia, whose levels were 5.11–9.55 mg/kg [12], but also lower than those obtained from khat-growing regions such as Aweday in Ethiopia with concentrations of 0.10–41.80 mg/kg [22]. Remarkably, the concentrations of Cu in this study are below the permissible limits for vegetables (73.0 mg/kg) set by WHO/FAO [21]. The THQ calculated and estimated in Table 5 shows that khat is not contaminated with Cu and does not pose adverse health challenges during khat consumption from the Meru region. It is notable that below acceptable limits this metal is a very important component of several enzymes such as catalase and metabolic reactions. It is also essential for neurological and hematological systems [30], as well as for some biological processes in living organisms [31].

Lead is a commonly known toxic heavy metal capable of causing environmental contamination and serious etiological risks [32]. The concentration of Pb in khat samples observed in this work ranged from 15.53 ± 0.33 mg/kg to 48.10 ± 2.82 mg/kg with a mean of 32.35 ± 9.95 mg/kg (Table 4). Lead in khat samples collected from sites H and I from farms in the Maua region was noted to be ≤ 0.001 mg/kg. This could be attributed to the low level of lead in the soil and the possible nonapplication of lead-based agrochemicals [33]. Contaminated rivers possibly with detergents and other effluents used for irrigation of khat plants may also be a contributing factor in observed levels of toxic heavy metals in general. A case in point is the high concentration of lead in sample *G* from the Maua region. Application of synthetic pesticides and fertilizers and use of compost manure also contribute to high levels of toxic heavy metals. Moreover, some studies have shown that as the pH decreases, the solubility of heavy metals increases [34], and this increases their absorption by plants. Motor vehicle exhausts containing trimethyl and tetramethyl lead may also contribute to Pb content in khat grown along busy highways [35]. Also, the concentration of Pb can potentially be increased in soils through the disposal of used batteries and waste paints into khat farms [36]. Nonetheless, the concentration of Pb metal in this study is lower than that obtained from some khat-growing regions in Ethiopia such as Aweday, Wendo, Haramaya, Indibir, and Bole which ranged from 5.0 mg/kg to 119.0

mg/kg [22]. The THQ values calculated (Table 5) exceed 1.0, suggesting the likelihood of hazardous consequences of khat consumption. All the samples had a high concentration of lead as compared to the WHO/FAO permissible limit for vegetables (0.3 mg/kg) [22]. This shows that khat samples considered in this work were contaminated by lead metal. Therefore, there is a need to recommend safe khat and safe farming practices that would reduce the concentration levels of lead to be below WHO/FAO set threshold limits.

Cadmium, on the other hand, is a highly toxic element and a well-known potent human carcinogen [37]. Previous studies have reported that high Cd exposure may have significant consequences on skeletal damage, hypertension, and kidney dysfunction [38]. Results of this study show that the levels of Cd in khat samples ranged from 5.48 ± 0.05 to 9.72 ± 0.08 mg/kg with a mean of 7.81 ± 1.56 mg/kg. The highest concentration of Cd was recorded in khat samples collected from the Laare region (site C) and samples from site *G* collected from the Maua region, whereas the lowest concentration was observed in samples collected from sites A and B both from Kangeta region and those collected from site E from Mutuati region which recorded concentrations of 5.51 ± 0.11, 5.58 ± 0.10, and 5.48 ± 0.05 mg/kg, respectively. The presence of Cd in khat and their associated levels could be due to the regular use of phosphate fertilizers and manure especially animal manure from cows, chicken, and pigs that may contain traces of Cd [22]. Also, Cd could be traced to the disposal of used pigments and paints and some electronics discharged into the khat farms through surface run-offs, and which are later absorbed by the plant tissues [36]. Groundwater tapped through wells and boreholes mainly used to irrigate crops are potential candidates which contain Cd from soil sediments [39]. In this work, it is notable that mixed farming is common in Meru County, especially the intercropping of khat with maize and other food crops that necessitate the use of phosphate fertilizers during planting. Through this method of farming, khat may absorb fertilizers contaminated with Cd metal. The levels of Cd reported in this study, according to Table 4, were higher than 1.30–2.90 mg/kg reported in khat samples analyzed in Addis Ababa [40]. The possible reason for this could be due to the cultivation of different khat species in sample collection areas and existing soil types, in addition to agrochemicals applied during khat planting in the Meru region. This could also be due to the application of domestic animal manure containing traces of cadmium as a result of mixed farming. The cadmium levels in this study were lower than those reported from a study that involved vegetables collected from local farms in Kericho West Sub-County which ranged from 10.33 to 29.00 mg/kg [10]. This may be attributed to different agricultural practices, different soil types, and different agrochemicals applied during vegetable farming which differs markedly from khat husbandry. The WHO/FAO through its reports, recommended a standard limit of Cd of 0.2 mg/kg for vegetables [21]. Accordingly, the mean concentration of Cd metal shows exceeding values as well as THQ exceeded 1. This showed that analyzed khat in this study may be moderately hazardous to the consumers.

Nickel is a trace metal released to the environment through natural sources and anthropogenic activities such as steel and cement manufacture [41] and may therefore be present in all soil types and soil profiles [42]. In agricultural soils, it is needed in trace levels for normal plant growth and development otherwise in elevated levels it may be highly toxic to plants, animals, and humans [41, 43]. Nickel metal has shown evidence of carcinogenicity in humans and other mammals, and has been known to cause toxicity to the nervous system, lungs, liver, and reproductive tissues which may result in reduced fertility [44]. The nickel level in khat samples in the present study ranged from 0.349 ± 0.02 to 0.399 ± 0.04 mg/kg with a mean of 0.37 ± 0.02 mg/kg. The maximum concentration of Ni was recorded in sample D obtained from the Laare region and sample H from the Maua region, while the minimum concentration was recorded in sample J from the Kianjai region. This may be ascribed to its levels in different soil types and the farm chemicals applied during khat cultivation. The results of this study present low levels of Ni compared to those recorded in vegetables obtained from Dera Ghazi Khan District in Pakistan which ranged between 1.800 mg/kg and 5.050 mg/kg [21]. Evidently, this can be attributed to different abilities of crops to accumulate and sequester Ni from the environment. Table 4 shows that in all the khat samples, the Ni concentration is below permissible limit (10 mg/kg) set by the WHO [23]. Associated THQ affirms Ni intake through consumption of khat from all the selected regions has no adverse health risk. Especially when its levels are below WHO permissible limits, it serves as an essential element in proper growth and development of the plants such as the germination of seeds as well as in animal species and microorganisms [45]. In addition, it is an essential element for humans because it enhances

hormonal activities, and use in lipid metabolic activities [46].

Another essential element in khat is Cr which is widely known to enhance biological functions such as blood sugar regulation, regulation of cholesterol levels, and fat synthesis in the liver [22]. Chromium levels in khat samples from this work ranged between 12.30 ± 0.15 and 19.10 ± 0.12 mg/kg with a mean of 15.98 ± 2.22 mg/kg (Table 4). High Cr levels in khat may be attributed to its high concentration levels in the soil and its chemical form in the soil, in addition to agrochemicals applied during khat cultivation [22]. In addition, soil redox potential and pH are important parameters that determine the characteristics of Cr in soil, where it has been reported that low soil pH boosts Cr levels in khat [47]. The Cr levels obtained in this work are higher than those obtained from khat samples in Addis Ababa which ranged from 3.10 to 6.76 mg/kg [40] and are fairly compared with khat cultivated in eastern Ethiopia, whose concentration ranged from 9.04 to 14.54 mg/kg [48]. This is due to different growing conditions and different agrochemicals applied during farming [40]. The standard limit for Cr set by the WHO is 2.30 mg/kg in vegetables [22]. The mean concentrations in this work exceeded the permissible limits and the THQ values, indicating that consumption of the Meru khat may cause severe etiological risks mainly through bioaccumulation. However, when maintained below acceptable limits, it is an essential element for biological functions in humans.

Most khat farmers in the Igembe region practice mixed farming such as planting maize, pumpkin, carrot, beans, potatoes, cassava, bananas, cowpeas, millet, and sugarcanes in khat farms [3]. This study indicates that the application of fertilizers containing heavy metals to food crops indirectly become available for khat plants to sequester them into their consumable parts thus increasing its concentration levels.

3.2. Noncarcinogenic Health Risk Assessment of Khat Consumption

The THQ is the measure of possibility used to indicate the likelihood of developing non-carcinogenic health risks when exposed to heavy metal intake. Basically, the standard value of THQ for not developing adverse health problems is ≤1.0; otherwise, if exceeded, it may pose human non-carcinogenic risks [49–51]. This method in the present study considers the likelihood of exposure to heavy metals via consumption of khat but does not necessarily provide a reliable quantitative estimate on exposed population. The THQ values for Pb, Cd, Cu, Cr, Fe, and Ni due to khat consumption habits in the study area were determined and reported in Table 5. Clearly, THQ for lead and cadmium far much exceeded the threshold of 1.0 indicating their high hazardous potential. The THQ for all other heavy metals (cf. Table 5) in khat was found to be safe because they fall below 1.0. Generally, the THQ of heavy metals resulting from khat shoots and leaves intake was found to follow a decreasing order of Pb>Cd>Fe>Cu>Ni>Cr . This suggests that the potential health risks of Pb and Cd through khat consumption are significantly high.

The collective health risks of exposure to multiple metals were determined by taking the summation of the THQ of all heavy metals in khat in the calculation of HI which was used to predict the likelihood of adverse effects. In this study, the HI value recorded for all the analyzed heavy metals in the khat samples was 0.54 as indicated in Table 5. The present findings show that the major contributors to the total HI were Pb and Cd heavy metals. From Table 5, Pb and Cd contributed about 53.01% and 46.59%, respectively, while Fe, Cr, Cu, and Ni collectively contributed 0.4% to the HI.

3.3. Pearson Correlation Analysis

For Karl Pearson correlation analysis, correlation coefficient values and the data obtained are summarized in Table 6. The positive correlation coefficient values indicated a positive correlation among the concentrations of heavy metals, whereas the negative values showed a negative correlation. Correlation coefficient values close to zero (0) indicated non-significant correlation. Correlation coefficient values near 1 indicated a strong and significant correlation among the concentrations of two heavy metals. Fe exhibited the highest number of positive correlations possibly indicating multiple sources of contamination and origins. Iron, the most abundant heavy metal in all the khat samples showed positive correlations with Pb, Cr, Cu, and Ni. This could suggest that its source is uniquely different from those of other heavy metals. Heavy metals Cu, Cr, and Pb had the same number of positive correlations signaling possibly a shared source.

Table 6

Pearson correlation matrix obtained for five heavy metals.

	Ni	Cu	Fe	Cd	Pb	Cr
Ni	1					
Cu	−0.181	1				
Fe	0.565	0.168	1			
Cd	−0.221	−0.264	−0.131	1		
Pb	−0.411	0.293	0.294	0.216	1	
Cr	0.707	0.080	0.495	−0.258	−0.320	1

3.4. Principal Component Analysis

The PCA by varimax rotation method was applied to the dataset of six heavy metals to speculate the sources of these metals in the Meru khat. The data extracted from PCA is presented in Table 7 and consists of a component matrix, initial eigenvalues, a rotated component matrix, and rotation sums of square loadings. The first three principal components (PCs) with eigenvalues greater than 1 accounted for 83.81% of the total variability. Consequently, these PCs demonstrate that heavy metals in the Meru khat originate from three independent sources. The first PC (PC1) explicated 39.55% of the total variance with significantly high loadings of Ni (0.91), Cr (0.88), and Fe (0.65). The regular applications of fertilizers and pesticides might be responsible for Cd and Pb in the study area, signifying that these heavy metals exist as a result of human activities. The contribution of the second PC (PC2) was 25.44% of the total variance with high loadings of Cu (0.75%), Pb (0.78), and Fe (0.57). The result further shows that PC2 was negatively loaded with Ni and Cd. However, the data, especially as reported in Figure 2 further demonstrated that Cu may have a distinct source of origin in the study area. The third PC (PC3) explained 18.83% of the total variance with significant positive high loading of Cd (0.75). The PC3 had negative loading with Cu. The contamination by Fe could be a result of multiple contamination sources as predicted by significant loadings in PC1, PC2, and PC3. From the statistical analysis, significant correlations were found between heavy metals within the same principal components, demonstrating that the results of PCA and Pearson correlation analysis (cf. Table 6) were all important.

Table 7

The principal component analysis of heavy metals in Meru khat samples.

Metals	Principal components matrix			Metals	Rotated components matrix
PC1	PC2	PC3	PC 1	PC2	PC 3

–											
Ni	0.91		−0.16			0.21	Ni		0.90		
−0.30		−0.46		Cr		0.88	0.03		0.03		
Cr		0.83		−0.20		0.19	Fe		0.65		
0.57		0.41		Fe		0.82	0.48				
0.07	Pb		−0.41		0.78		0.35	Pb			
−0.16	0.93			−0.02	Cu		0.01	0.75			
−0.49	Cd			−0.19		0.30		−0.81	Cd		
−0.44	−0.14			0.75		Cu		−0.47	0.45		
0.77	–										
Component	Initial eigenvalues										
Total				Variance (%)				Cumulative (%)			
–											
1	2.37					39.55					
39.55			2			1.53					
25.44				64.98				3	1.13		
18.83				83.81				4	0.56		
9.40				93.21				5	0.27		

4.51			97.72		6	0.14
2.28			100.00		·	
Component	Extraction sums of squared loadings			Rotation sums of squared loadings		
Total	Variance (%)	Cumulative (%)	Component	Total		Variance (%)
Cumulative (%)		-				
1	2.37	39.55	39.55	1	2.23	
37.08	37.08		2	1.53	25.44	64.98
2	1.52		25.36	62.44	3	1.13
18.83	83.81		3	1.28	21.37	83.81

[figure(s) omitted; refer to PDF]

4. Pathophysiological Concerns of Toxic Heavy Metals

The means of human exposure to toxic heavy metals include skin contact, inhalation, ingestion, and direct contact [52]. It is increasingly noticeable that the nonbiodegradability and refractory nature of toxic heavy metals in the environment make them have long-lasting biotoxic consequences in biological systems; hence, there is a need to understand the mechanisms that make them detrimental to public health [36]. Moreover, heavy metals acutely reverse the normal functioning of biological structures and negatively affect biological system functionality which ultimately results in untimely death and other health complications [42]. For instance, heavy metals cause serious problems to the central nervous system such as mental breakdown, kidney damage, and lung activating pathways for disease manifestations [39]. Chromium toxicity in the exposed human population and experimental animals also causes respiratory cancers [36]. Acute Ni toxicity causes symptoms such as vomiting, irritation, body weaknesses, constant cough, nausea, and visual disturbances in humans, while in animals such as rats, it causes renal damage, body weight loss, and instances of kidney failure [42]. On the other hand, chronic Ni poisoning affects the skin and pulmonary as well as cause cancer and dramatis [36], precipitates endocrine disruption in humans, and is a precursor for cardiovascular diseases as well as genotoxicity [42]. A study conducted in North California among children aged 4 to 5 years revealed that they are at a high risk of pediatric obesity due to high levels of Cd in prenatal blood samples [53]. Epidemiological studies showed that accumulation of Cd in body tissues causes development of musculoskeletal diseases such as osteoarthritis, osteoporosis, and kidney impairment [54]. Long-term Pb exposure decreases cognitive performance, causes anemia, hypertension, and causes miscarriages in pregnant women, damages mental faculties, and causes kidney damage [52]. It is recommended that metal chelation therapy, as applied in the treatment of chronic iron toxicity, be employed to minimize heavy metal poisoning arising from khat

consumption and other food products [55].

5. Limitations of the Study

Only 11 khat samples were considered in this investigation for the evaluation of the potential health hazards resulting from khat consumption among the population. Considering the expansive nature of Meru County, the data collected may not be representative of the heavy metal toxicity in khat in the entire Meru region. Moreover, the non-carcinogenic adverse risks due to Pb and Cd cannot be adequately determined based on the small area of Meru County sampled. Basically, the estimation of THQ and HI values was based on the daily ingestion of khat; thus, there is a probability that the values obtained are overestimated. Heavy metal toxic lead was not conducted in this study but was necessary in order to reflect the adverse health effects on khat consumers. Furthermore, the fact that the data were collected during the rainy season cannot entirely be used to give a true picture of heavy metal contamination in the area of study especially during the dry season. Seasonal and temporal variations of heavy metal distribution in the khat-growing region of Meru were not carried out.

6. Conclusions

This study has found that edible parts of khat leaves and shoots collected from the Meru region were contaminated with high levels of lead and cadmium, which is considered a serious public health concern among khat consumers. THQ for lead and cadmium far much exceeded the threshold of 1.0, indicating their high hazardous potential. The THQ for all other heavy metals in khat was found to be within the safe limit of below 1.0. Generally, the THQ for heavy metals resulting from khat shoots and leaves intake was found to follow a decreasing order of Pb>Cd>Fe>Cu >Ni>Cr. Nonetheless, the HI of all investigated heavy metals in khat exceeded the threshold value of 1.0, suggesting possible noncarcinogenic health risks to khat consumers. Statistical treatment of the data reported in this work showed that there were significant variations in the levels of Ni metal in the khat samples from different regions in Meru, although there were no significant variations among toxic selected heavy metals, Fe, Cu, Cd, and Pb. This observation could be attributed to different factors such as methods of farming, chemical composition of the soil, and application of various agricultural practices employed by different khat farmers. Also, principal components (PCs) with eigenvalues greater than 1 accounted for 83.81% of the total variability, therefore, demonstrating that heavy metals in the Meru khat originate from three independent sources. The first PC (PC1) explicated 39.55% of the total variance with significant high loadings of Ni (0.91), Cr (0.88), and Fe (0.65). Consequently, the regular application of agrochemicals including fertilizers and pesticides might be responsible for the observed high levels of Cd and Pb in the study area. On the other hand, Fe showed the highest number of positive correlations, possibly indicating multiple sources of contamination and origins. Iron exhibited positive correlations with Pb, Cr, and Cu but a moderate correlation with Ni and Cd. The main contribution of this study is to provide reliable information for the adoption of better farming practices where the use of agrochemicals should be minimized or avoided completely. Furthermore, Meru khat farmers should be sensitized on appropriate farming practices for safer khat products.

Authors' Contributions

AMO analyzed, wrote, and edited the article. JKK contributed to the method development, edited the article, and provided supervision. JOA edited the article and performed supervision. All authors have read and approved the manuscript.

Acknowledgments

The authors wish to thank the Directorate of Research and Extension of Egerton University for supporting this research.

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DETAILS

Subject:	Farming; Bioaccumulation; Food contamination &poisoning; Toxicity; Reagents; Iron; Public health; Nickel; Emission analysis; Agrochemicals; Copper; Scientific imaging; Cadmium; Inductively coupled plasma; Farmers; Health risks; Lead; Pesticides; Heavy metals; Fertilizers; Health risk assessment; Consumers; Toxicology; Gross Domestic Product--GDP; Rain; Contamination; Chromium
Business indexing term:	Subject: Gross Domestic Product--GDP
Location:	Kenya
Editor:	Kevin Honeychurch
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
Publisher:	Hindawi Limited
Place of publication:	New York
Country of publication:	United K ingdom, 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:	2023-11-15 (Received); 2024-01-03 (Revised); 2024-01-05 (Accepted); 2024-01-19 (Pub)
DOI:	https://doi.org/10.1155/2024/6688184
ProQuest document ID:	2919194299

Document URL:	https://www.proquest.com/scholarly-journals/analysis-concentration-heavy-metals-khat-grown/docview/2919194299/se-2?accountid=211160
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Method Validation and Measurement Uncertainty Estimation for Determination of Multiclass Pesticide Residues in Tomato by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT (ENGLISH)

Method validation is an essential technique for ensuring the reliability and accuracy of an analytical method. This study aimed to optimize and validate a fast, reliable, and accurate method for quantitatively determining pesticide residues of diverse chemical classes in the tomato matrix. Various method performance characteristics were tested and compared with predefined criteria. Twenty-six different pesticides of diverse chemical classes were selected based on their use in tomato cultivation and the availability of reference materials. The pesticide residues in tomato samples were extracted with the QuEChERS technique with some modifications, followed by injection into an LC-MS/MS system operating in an optimized method. The validated method demonstrated reasonable specificity, as there were no interferences from matrix components at the retention times of pesticides. The calibration curves for all pesticides exhibited excellent linearities, with correlation coefficients exceeding 0.99. No significant matrix effect was observed for all pesticides in tomatoes, as the values fell within the range of ±20%. All pesticides were quantified successfully at a concentration of 5 μg/kg except for carbaryl, with an average recovery of more than 70% and a relative standard deviation of less than 20%. Similarly, measurement uncertainties were also estimated based on the validation data, and the values were found below the default limit of 50%. Subsequently, the validated method was applied to analyze 52 locally collected tomato samples. Study findings revealed that only four of the studied pesticides were detected in these samples, and their concentrations were below the maximum residue limits (500 μg/kg each for carbendazim, imidacloprid, and metalaxyl) established for tomatoes by the Government of Nepal and the Codex Alimentarius Commission.

FULL TEXT

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1. Introduction

Pesticides are widely used in agricultural production, including in the cultivation of tomatoes. Several groups of pesticides are used to manage weeds, insects, and other pests. However, some pesticides persist as residues in agricultural produce, and upon consumption of such contaminated produce, these pesticide residues enter the human body, posing potential adverse health impacts [1, 2]. Due to the hazardous nature of these pesticides, their presence is closely monitored in food to inhibit the potential adverse effects on human health. Consequently, many countries have established the maximum residue limits (MRLs) for the residues in various foods. Thus, monitoring the level of pesticide residues in food is desirable to ensure food safety.

Pesticide residue extraction from a food matrix is challenging due to its low concentration and potential interferences from the complex sample matrix [3]. Several sample extraction protocols are employed for this purpose [4]. However, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction technique is the obvious choice for multiresidue analysis due to its ease of use and fast extraction time [5–7]. Additionally, pesticides with a broad range of chemical nature can be extracted simultaneously. It involves two steps: sample extraction and sample cleanup. In the first step, residues are extracted from the homogeneous sample by adding acetonitrile and a blend of salts. The salt mixture allows the normally miscible organic solvent to separate from the water in the sample. In the second step, an aliquot of the organic phase from step 1 is taken for the cleanup through the use of dSPE (dispersive solid-phase extraction), where PSA (primary secondary amine) and anhydrous $MgSO_4$ are used to remove residual water and many potential interfering substances such as sugar and organic acids from the extract. Based on the nature of the sample, other reagents, such as C18 and graphitized carbon black (GCB), can be added in the second step to facilitate the effective cleanup [8–11].

Several analytical techniques, such as gas chromatography-mass spectrometry, gas chromatography coupled to an electron capture detector, high-performance liquid chromatography, and liquid chromatography-tandem mass spectrometry, are available to monitor the level of pesticide residue in a food matrix [7, 12]. Among them, LC-MS/MS is one of the most widely used analytical techniques for pesticide residue analysis in food due to its ability to perform multiresidue analysis quickly with remarkable sensitivity. This study optimized an LC-MS/MS-based method, recognizing the necessity for a fast and reliable method for analyzing multi residue. Various method parameters were optimized to get an optimum response for each of the 26 selected pesticides. These pesticides belong to various chemical classes, including but not limited to carbamates, organophosphates, benzimidazoles, and neonicotinoids. The selection of pesticides in the study was based on the probable use in tomato cultivation and the availability of reference standards. Similarly, tomatoes were chosen as a matrix for this study due to their widespread consumption and availability of already established MRLs for pesticide residues. The Government of Nepal has set MRLs for 75 pesticides in tomatoes, the maximum number of pesticides regulated for any food category in the country. After optimization, the method was validated to assess its applicability. Method validation is an important technique that assesses an analytical method’s suitability for its intended purpose [13]. In validation, various performance criteria are examined to determine the fitness of purpose, and the results are compared against the predefined criteria. If all the tested parameters met the predefined acceptance criteria, then the method is considered fit for purpose. It is essential for ensuring the reliability of the test results. Although several method validation protocols are available depending on the nature of the analytical method, the widely used protocol for pesticide residue analysis is the SANTE guideline [14].

2. Materials and Methods

2.1. Chemicals and Reagents

Individual 16 pesticide standards were obtained from Sigma Aldrich, and a mixed standard of 10 carbamate pesticides was purchased from Restek. HPLC grade of acetonitrile, methanol, formic acid, ammonium formate, and acetic acid and analytical grade of anhydrous magnesium sulfate and primary secondary amine (PSA) were

purchased from local suppliers. The individual stock solution other than the mixed standard solution was prepared at a concentration of 1000 mg/L by dissolving in an appropriate amount of methanol. All standard solutions were stored in a refrigerator at 4°C before use.

2.2. Instrumentation

The study was performed in an Agilent 1290 Infinity LC system connected to an Agilent 6460 triple quadrupole mass spectrometer equipped with Agilent Jet Stream electrospray ionization (AJS-ESI). The chromatographic and mass spectrometric data were acquired and analyzed by using MassHunter software. The chromatographic separation of pesticides was carried out in an Agilent Poroshell 120 EC-C18 analytical column having dimensions of 3.0 × 50 mm and 2.7 μm particle size.

2.3. Method Parameters

The method parameters were optimized before starting actual validation. Separation was carried out in a gradient mode with mobile phase A consisting of 0.1% formic acid and 5 mM ammonium formate in water. In contrast, mobile phase B comprised the same composition of formic acid and ammonium formate in methanol. The gradient started with 5% of mobile phase B, remaining constant until 0.5 minutes. Subsequently, it increased linearly to 65% at 5 minutes and rose to 95% at 6.5 minutes, maintaining the same composition until 9.0 minutes. At 9.1 minutes, the value of mobile phase B decreased sharply to 5% and remained the same until 12 minutes. The optimum flow rate, column temperature, and injection volume were 0.5 mL/min, 40°C, and 3 μL, respectively. Each analysis was completed in 12 minutes.

Pesticides were ionized in positive electrospray ionization (ESI) mode and acquired in dynamic multiple reaction monitoring (dMRM) mode. The optimized flow rates for drying gas and sheath gas were 10 L/min and 11 L/min, respectively, with their temperatures maintained at 250°C and 350°C, respectively. The nebulizer gas pressure was constant at 40 psi, while capillary and nozzle voltages were set at 4000 V and 300 V, respectively.

2.4. Sample Preparation

Pesticide residues incurred in tomato samples were extracted using the QuEChERS AOAC 2007.01 protocol [15] with some modifications. Homogenized samples underwent extraction using a solution comprising 1% acetic acid in acetonitrile. The phase separation of acetonitrile and water layers was achieved using a mixture of anhydrous magnesium sulfate and sodium acetate. The acetonitrile layer containing the extracted pesticides was cleaned using a primary secondary amine and anhydrous magnesium sulfate mixture. Generally, the final extract would undergo evaporation followed by reconstitution in an appropriate solvent that matches the mobile phase composition. However, in this study, the final extract was diluted with water in a 1 : 3 ratio, bypassing the time-consuming evaporation step.

2.5. Method Validation

Method validation is a process of demonstrating the fitness of a method for its intended purpose by examining and providing objective evidence [13]. The method was validated as per analytical quality control and method validation procedures for pesticides in food and feed [14]. Critical method performance parameters such as specificity, linearity, limit of quantification, trueness, and precision were rigorously assessed experimentally in a blank tomato matrix. The obtained data were compared against the predefined criteria outlined in the protocol.

2.6. Measurement Uncertainty

Every measurement is associated with a dispersion known as measurement uncertainty (MU). It gives a range of values within which the true value of the measured quantity is expected to lie. It reflects the inherent variability in a measurement process. In pesticide residue analysis, measurement uncertainty is critical during compliance statements against a standard. Measurement uncertainty values were estimated using a top-down approach based on the validation data [16, 17]. This approach uses trueness and precision data generated in the method validation experiment to estimate the MU value.

3. Results and Discussion

3.1. Optimization of Analyte-Dependent Mass Parameters

The mix working standard solution of all the pesticides was prepared at 1000 ng/mL and used to optimize their

precursor ions, product ions, fragmentor, and collision voltages. The optimization work was performed by injecting pesticide standard solution without a column, and data were acquired using MassHunter Optimizer software. All pesticides showed good response in positive ESI mode, with the majority forming protonated ions $[M+H]^+$, except for aldicarb, aldicarb sulfone, and propargite, which formed ammonium adducts $[M+NH_4]^+$ as a precursor ion. The optimized fragmentor voltages were found in the range of 50–145 V for all precursor ions, while collision energies ranged from 0 to 48 V for all product ions. Two product ions per precursor ion, their ion ratios, and retention time were utilized for pesticide confirmation [14]. The mass spectra and the ion ratio of carbaryl are shown in Figure 1. Initially, pesticides were monitored in multiple reaction monitoring (MRM) mode to obtain the retention times of each pesticide. Subsequently, it was upgraded to dynamic multiple reaction monitoring (dMRM) mode for maximizing sensitivity. In dMRM mode, specific MS transitions are monitored in a narrow time window corresponding to their expected elution from LC rather than throughout the full analysis time. The transition from MRM to dMRM drastically enhances the sensitivity of pesticides, as the MS duty cycle is not wasted by monitoring them when they are not expected to elute from LC [18]. The optimum acquisition parameters of all the target pesticides are summarized in Table 1.

[figure(s) omitted; refer to PDF]

Table 1

Name, ion type, precursor ion, product ions, fragmentor voltage, collision energies, cell accelerator potential, retention time, and ionization polarity of target 26 pesticides.

S. N.	Pesticide	Ion type	Precursor ion (m/z)	Product ions*(m/z)	Frag (V)	CE (V)	Cell acc (V)	Rt (min)	ESI polarity
1	3-Hydroxycarbofuran	$[M+H]^+$	238.1	**181.1**	70	5	6	4.36	Positive
163.1	70	5	6	4.36	Positive	-			
2	Acephate	$[M+H]^+$	184	**142.9**	50	4	6	1.98	Positive
95	50	20	6	1.98	Positive	-			
3	Alachlor	$[M+H]^+$	270.1	**238**	90	4	6	7.07	Positive
162.1	90	16	6	7.07	Positive	-			
4	Aldicarb	$[M+NH_4]^+$	208.1	**116**	60	0	6	4.95	Positive
89	60	12	6	4.95	Positive	-			

5	Aldicarb sulfone	$[M+NH_4]^+$	240	**148**	80	5	6	3.03	Positive
86	80	10	6	3.03	Positive	-			
6	Aldicarb sulfoxide	$[M+H]^+$	207.1	132	55	0	6	2.83	Positive
89.1	55	8	6	2.83	Positive	-			
7	Carbaryl	$[M+H]^+$	202.1	**145**	130	4	6	5.76	Positive
127	130	28	6	5.76	Positive	-			
8	Carbendazim	$[M+H]^+$	192.1	**160**	130	16	6	3.52	Positive
132	130	32	6	3.52	Positive	-			
9	Carbofuran	$[M+H]^+$	222.1	**165**	75	4	6	5.57	Positive
123	75	20	6	5.57	Positive	-			
10	Cyprodinil	$[M+H]^+$	226.1	93.1	130	36	6	7.12	Positive
77.1	130	48	6	7.12	Positive	-			
11	Dimethoate	$[M+H]^+$	230	198.9	70	4	6	4.24	Positive
124.9	70	16	6	4.24	Positive	-			
12	Ethoprophos	$[M+H]^+$	243.1	130.9	85	16	6	7.02	Positive
96.9	85	28	6	7.02	Positive	-			
13	Imazalil	$[M+H]^+$	297.1	**158.9**	120	20	6	6.1	Positive
69.1	120	16	6	6.1	Positive	-			

14	Imidacloprid	$[M+H]^+$	256.1	209	80	12	6	4.02	Positive
175	80	12	6	4.02	Positive	-			
15	Kresoxim-methyl	$[M+H]^+$	314.1	267	60	0	6	7.23	Positive
116	60	8	6	7.23	Positive	-			
16	Metalaxyl	$[M+H]^+$	280.2	220	90	8	6	6.24	Positive
192.1	90	12	6	6.24	Positive	-			
17	Methidathion	$[M+H]^+$	303	144.9	65	4	6	6.33	Positive
85.1	65	16	6	6.33	Positive	-			
18	Methiocarb	$[M+H]^+$	226.1	169	70	4	6	6.67	Positive
121	70	12	6	6.67	Positive	-			
19	Methomyl	$[M+H]^+$	163.1	135	145	8	6	4.34	Positive
107	145	20	6	4.34	Positive	-			
20	Monocrotophos	$[M+H]^+$	224.1	**193**	60	0	6	3.67	Positive
126.9	60	12	6	3.67	Positive	-			
21	Oxamyl	$[M+H]^+$	220.1	163	110	4	6	4.34	Positive
107	110	24	6	4.34	Positive	-			
22	Penconazole	$[M+H]^+$	284.1	158.9	115	28	6	7.21	Positive
70.1	115	12	6	7.21	Positive	-			

23	Propargite	$[M+NH_4]^+$	368.2	**231.1**	80	4	6	7.84	Positive
175	80	12	6	7.84	Positive	-			
24	Propoxur	$[M+H]^+$	210.1	168	60	0	6	5.52	Positive
111	60	8	6	5.52	Positive	-			
25	Thiabendazole	$[M+H]^+$	202	**175**	125	24	6	3.92	Positive
131	125	36	6	3.92	Positive	-			
26	Trichlorfon	$[M+H]^+$	256.9	220.9	90	4	6	4.24	Positive

*Product ions used as quantifier ions are indicated in bold, while qualifier product ions are not in bold. Rt, retention time; CE, collision energy; Frg, fragmentor voltage; Cell acc, cell accelerator potential; ESI, electrospray ionization.

3.2. Optimization of Chromatographic Parameters

Various mobile phase gradient programs were studied in search of optimal resolution and sensitivity. However, due to the diverse chemical nature of multiresidues, some peaks were coeluted even after numerous experiments. The extracted ion chromatogram of mixed standard pesticides in an optimum method (presented in Section 2.3) is shown in Figure 2.

[figure(s) omitted; refer to PDF]

3.3. Modification of Sample Preparation

This study followed the QuEChERS AOAC 2007.01 protocol [15] without deviation until the cleanup step. However, the solvent evaporation of the final extract was skipped to shorten the sample preparation time. The evaporation processes involving a nitrogen turbo evaporator are time consuming and involve the risk of degradation of certain pesticides if temperature is not maintained carefully. An alternative approach was employed to avoid the probable degradation of pesticides and reduce the lengthy evaporation time. In this approach, the final acetonitrile extract was diluted with water in a ratio of 1 : 3 instead of the final evaporation step. This dilution considerably shortened the sample preparation time while ensuring the solvent matching with the mobile phase, resulting in satisfactory peak shapes, especially for early eluting pesticides.

3.4. Method Validation

3.4.1. Specificity

The specificity of an analytical method is examined to verify the absence of potential interfering compounds at the retention time of the target analytes. The tomato blank matrix and matrix-matched standard solutions were analyzed simultaneously to assess the specificity of the method. Comparison of total ion chromatograms (TICs) of both samples, as shown in Figure 3, revealed the absence of any significant interfering peaks at the retention times of all target pesticides, thus indicating the specificity of the method.

[figure(s) omitted; refer to PDF]

3.4.2. Linearity

Linearity was evaluated from the calibration curve constructed from a series of eight duplicate concentrations ranging from 0.5 ng/mL to 100 ng/mL in a solvent with a final composition of 1 : 3 acetonitrile and water. The calibration curves were best fitted to a linear curve with weight 1/x. Good linear relationships were observed with

regression coefficients (R^2) of 0.99 or higher across the examined concentration range for all the pesticides. Calibration curves of some selected pesticides are presented in Figure 4, while regression coefficients (R^2) and slopes of all the pesticides are summarized in Table 2.

[figure(s) omitted; refer to PDF]

Table 2

Regression coefficient (R^2), slope, matrix effect, method LOQ, and method range of the pesticides.

S. N.	Pesticide	Solvent standard calibration curve		Matrix-matched calibration curve		% matrix effect	Method LOQ (μg/kg)	Method range (μg/kg)
R^2	Slope	R^2	Slope	1	3-Hydroxycarbofuran	0.999	177.591	0.999
187.511	6	5	5–400	2	Acephate	1.000	797.581	0.999
783.193	−2	5	5–400	3	Alachlor	0.999	458.473	0.999
438.277	−4	5	5–400	4	Aldicarb sulfone	0.999	663.776	0.999
657.814	−1	5	5–400	5	Aldicarb sulfoxide	0.999	383.474	0.999
388.445	1	5	5–400	6	Aldicarb	1.000	922.285	0.999
864.694	−6	5	5–400	7	Carbaryl	0.998	59.901	0.998

50.906	−15	10	10–800	8	Carbendazim	0.999	3505.077	0.999
3320.299	−5	5	5–400	9	Carbofuran	1.000	2655.585	0.999
2394.499	−10	5	5–400	10	Cyprodinil	1.000	714.738	0.999
692.753	−3	5	5–400	11	Dimethoate	0.999	1627.152	0.999
1570.99	−3	5	5–400	12	Ethoprophos	0.999	1167.623	0.998
1125.412	−4	5	5–400	13	Imazalil	1.000	561.124	0.999
492.480	−12	5	5–400	14	Imidacloprid	1.000	270.316	1.000
325.353	20	5	5–400	15	Kresoxim-methyl	0.999	851.743	0.999
808.224	−5	5	5–400	16	Metalaxyl	0.999	2456.334	0.999
2413.506	−2	5	5–400	17	Methidathion	1.000	894.615	0.999

908.447	2	5	5–400	18	Methiocarb	0.999	1140.993	0.999
1141.137	0	5	5–400	19	Methomyl	1.000	578.232	1.000
576.797	0	5	5–400	20	Monocrotophos	1.000	779.925	0.999
790.445	1	5	5–400	21	Oxamyl	1.000	756.761	0.999
772.839	2	5	5–400	22	Penconazole	0.999	1371.04	0.998
1279.036	−7	5	5–400	23	Propargite	0.999	4822.576	0.999
4457.998	−8	5	5–400	24	Propoxur	1.000	2128.077	0.999
1817.774	−15	5	5–400	25	Thiabendazole	1.000	1222.917	1.000
1209.514	−1	5	5–400	26	Trichlorfon	0.994	501.997	0.999

Matrix effects with negative values indicate signal suppression, whereas positive values indicate signal enhancement.

3.4.3. Matrix Effect

The matrix effect is frequently encountered in LC-MS/MS analysis due to ion suppression or enhancement effect. It arises from coextracted compounds from the sample matrix, influencing analyte concentration measurement [19]. If not adequately compensated for, the matrix effect can significantly affect the trueness of analytical results. The

matrix effect was evaluated by comparing the slopes of the solvent standard (SS) calibration curve with the slopes of the matrix-matched (MM) calibration curve, constructed using standards prepared in a blank sample matrix [20]. The percentage matrix effect was determined using the following formula [21]:(1)matrix effect %=slope of MM curve−slope of SS curve∗100slope of SS curve.

Matrix effect values between 0 and 20% are considered low matrix effects, and the correction for the matrix effect is not necessary. However, if more than a 20% matrix effect is observed, it should be addressed to ensure more accurate results [14]. The negative value represents ion suppression, whereas the positive value represents ion enhancement. The matrix effects for all the analyzed pesticides were found within ±20%, as shown in Table 2, indicating the absence of significant matrix effects caused by the tomato matrix. In such instances, solvent standards could be used for quantifying pesticide residues incurred in unknown samples. This is particularly useful in routine analysis when no blank tomato matrix is available for preparing matrix-matched calibration standards.

3.4.4. Limit of Quantification (LOQ)

The limit of quantification (LOQ) is a critical performance characteristic for pesticide residues. The value should be at or below the maximum residue level (MRL) to draw meaningful conclusions about pesticide residues incurred in a sample. LOQ can be determined using various methods [22, 23]. LOQ was determined by spiking a series of low concentrations in a blank tomato matrix. All pesticides, except carbaryl, were successfully analyzed at the 5 μg/kg level, meeting all identification, recovery, and precision criteria. For carbaryl, it was 10 μg/kg. These LOQs were much lower than the MRLs established for tomatoes by the Government of Nepal [24] and the Codex Alimentarius Commission [25]. The obtained values of LOQ are summarized in Table 2.

3.4.5. Trueness and Precision

Various approaches, such as recovery experiments and analysis of certified reference material, have been employed to assess an analytical method's trueness [13, 26]. In this study, trueness was evaluated by recovery experiments, in which blank tomato samples were spiked at three different concentrations: low (5 μg/kg), medium (10 μg/kg), and high (40 μg/kg), each level in 6 replicates. The recoveries in the 70–120% range were obtained, exhibiting good trueness of the method. Similarly, precision was assessed as repeatability and within-laboratory reproducibility. Both were below 20% relative standard deviation (RSD) for all pesticides. The obtained values of trueness and precision are summarized in Table 3.

Table 3

Trueness as % recovery and precision as % RSD of repeatability (within-laboratory reproducibility) of target pesticides in tomato matrix spiked at 5, 10, and 40 μg/kg.

S. N.	Pesticide	Trueness			Precision as repeatability (within-laboratory reproducibility)		
5 (μg/kg) (n=6)	10 (μg/kg) (n=6)	40 (μg/kg) (n=6)	5 (μg/kg)	10 (μg/kg)	40 (μg/kg)	% recovery	
% RSD			1	3-Hydroxycarbofuran	93	94	86

4.7 (8.2)	3.2 (4.8)	2.3 (1.7)	2	Acephate	81	80	81
2.4 (8.5)	2.2 (6.4)	1.0 (2.8)	3	Alachlor	89	90	87
3.5 (14.5)	3.6 (5.0)	1.2 (1.6)	4	Aldicarb sulfone	89	90	87
3.8 (8.3)	3.3 (4.9)	1.7 (1.6)	5	Aldicarb sulfoxide	87	88	83
3.6 (5.9)	3.5 (3.5)	1.4 (1.5)	6	Aldicarb	87	83	83
2.0 (8.7)	2.2 (6.5)	1.2 (2.2)	7	Carbaryl	*	77	83
*	6.1 (11.7)	5.0 (3.7)	8	Carbendazim	91	86	92
3.1 (9.9)	2.1 (8.3)	1.1 (2.0)	9	Carbofuran	87	85	79
2.5 (12.6)	2.8 (7.5)	2.4 (3.7)	10	Cyprodinil	90	85	83
5.6 (5.5)	3.9 (4.0)	1.1 (1.6)	11	Dimethoate	90	88	88
4.5 (7.0)	2.3 (4.8)	0.9 (1.3)	12	Ethoprophos	91	90	90
2.4 (7.4)	3.0 (4.1)	1.0 (1.2)	13	Imazalil	90	82	78
4.3 (13.3)	4.3 (8.6)	2.9 (4.6)	14	Imidacloprid	104	120	110

5.0 (6.9)	4.9 (4.1)	1.3 (4.6)	15	Kresoxim-methyl	90	90	86
2.5 (6.2)	3.4 (2.8)	1.0 (1.8)	16	Metalaxyl	91	90	89
2.9 (5.6)	2.4 (3.2)	1.2 (1.1)	17	Methidathion	90	93	88
3.2 (6.8)	4.8 (3.6)	1.8 (1.8)	18	Methiocarb	91	93	86
2.1 (4.7)	3.5 (3.1)	1.4 (1.6)	19	Methomyl	90	92	87
5.3 (6.7)	4.0 (4.7)	1.2 (1.9)	20	Monocrotophos	87	90	88
2.9 (6.8)	2.9 (3.2)	1.2 (1.1)	21	Oxamyl	89	93	86
3.9 (7.0)	3.7 (3.2)	1.7 (1.5)	22	Penconazole	88	87	87
3.0 (6.4)	3.2 (4.2)	0.7 (1.4)	23	Propargite	87	83	86
3.1 (5.4)	2.5 (4.5)	1.4 (2.0)	24	Propoxur	90	90	84
1.9 (4.7)	2.1 (1.8)	1.1 (1.3)	25	Thiabendazole	93	88	88
2.4 (6.5)	2.9 (5.1)	1.4 (1.3)	26	Trichlorfon	106	117	113

*For carbaryl, a recovery experiment was performed at the 10 μg/kg level.

3.4.6. Measurement Uncertainty (MU)

Measurement uncertainty (MU) is inherently associated with any measurement. There are various approaches available for the estimation of measurement uncertainty. The most widely used approach is the ISO GUM approach [22, 27, 28]. However, estimating using the bottom-up approach for multiresidue is impractical. Thus, the present study estimated MUs using a top-down approach. Intralaboratory validation data were used to estimate the standard uncertainty and then expressed at a 95% confidence level. Two significant sources, precision and trueness (as bias), were taken as the main contributors to measurement uncertainty. The standard uncertainty of each pesticide

was estimated using the following formula:(2)u=ubias2+uprecision2.

Uncertainty due to bias was estimated from the recovery experiment, while the percentage relative standard deviation of within-laboratory reproducibility was used to estimate the uncertainty arising from precision. The expanded uncertainty was calculated by multiplying the standard uncertainty with a coverage factor (k) of 2, which approximately gives a confidence level of 95%:(3)U=k×u.

The obtained values for all the target pesticides, as presented in Figure 5, were lower than the 50% default value employed by many regulatory authorities for enforcement decisions.

[figure(s) omitted; refer to PDF]

3.5. Application of the Validated Method to the Real Sample

The validated method was further applied to 52 tomato samples collected from various local vegetable markets in Kathmandu, Nepal, for the simultaneous analysis of pesticide residues. The findings of the analyzed samples are shown in Figure 6. None of the target pesticides were detected in 10 (19.2%) samples. Pesticides were detected in 80.8% of the samples analyzed, ranging from one to four pesticides (carbendazim, imidacloprid, metalaxyl, and thiabendazole). Carbendazim, imidacloprid, metalaxyl, and thiabendazole were detected in 34, 28, 7, and 1 samples, respectively, either individually or in combination. Of 42 positive samples, 24 (57.1%) samples were found to contain at least one pesticide residue at or above the LOQ level, while in the remaining 18 (42.9%) samples, pesticides were found below the LOQ level for each detected pesticide. The concentrations of pesticide residues in analyzed tomato samples were found to be in the range of 8–268 μg/kg, with an average of 63 μg/kg for carbendazim, 5–125 μg/kg, with an average of 34 μg/kg for imidacloprid, and 8–67 μg/kg, with an average 32 μg/kg for metalaxyl. These pesticide residue concentrations were significantly lower than the maximum residue limit (MRL) set by the Codex Alimentarius Commission [25] and the Nepal Government [24] for tomatoes (500 μg/kg for each of the pesticides mentioned above). Similar results were observed in a previous study conducted by Bhandari et al. [29] in selected tomato samples (N=32) from Nepal.

[figure(s) omitted; refer to PDF]

4. Conclusions

Pesticide residues in food have always been a public concern, with many people aware of harmful health effects caused by consuming food containing these residues. Analyzing the food samples for pesticide residues is essential to safeguard public health. Routine analysis for residues requires a quick and accurate method with high sensitivity. Thus, we optimized and validated an LC-MS/MS method to analyze 26 pesticide residues in tomato samples. All method validation performance characteristics were satisfactory, indicating the method's reliability.

Furthermore, MUs were also estimated, which would be helpful while deciding the compliance of tomato samples against the established MRL. The validated method was successfully applied to analyze actual tomato samples, and some positive samples were detected and subsequently quantified. Even though 80.8% of the analyzed samples (N =52) had one to four target pesticide residues, these amounts were significantly lower than the MRLs set for tomatoes. The validated method offers several advantages, including simple extraction, cleanup without an additional evaporation step of the final extract, high sensitivity, and good accuracy. Additionally, more pesticides can be included in the validated method to cover a broader range of pesticides. It offers an efficient solution for a quality control laboratory that analyzes multiresidues in tomato samples.

Authors' Contributions

Suraj Shrestha conceptualized and validated study, collected the sample, wrote the original draft, and finalized the manuscript. Bandana Lamichhane validated the study and collected the sample. Nibedita Chaudhary reviewed the draft. All the authors have approved the final manuscript.

Acknowledgments

The authors would like to acknowledge the Director General and Deputy Director Generals of the Department of Food Technology and Quality Control, Nepal, for constant support throughout the research.

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DETAILS

Subject:	Mass spectrometry; Software; Acids; Agricultural production; Food; Tomatoes; Liquid chromatography; Pesticides; Optimization; Residues; Scientific imaging; Chromatography; Uncertainty; Correlation coefficients
Editor:	Mahmood Ahmed
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-11-20 (Received); 2023-12-29 (Revised); 2024-01-04 (Accepted); 2024-01-10 (Pub)
DOI:	https://doi.org/10.1155/2024/3846392
ProQuest document ID:	2916946774
Document URL:	https://www.proquest.com/scholarly-journals/method-validation-measurement-uncertainty/docview/2916946774/se-2?accountid=211160

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Last updated:	2024-01-22
Database:	Publicly Available Content Database

Research Status in the Use of Surface-Enhanced Raman Scattering (SERS) to Detect Pesticide Residues in Foods and Plant-Derived Chinese Herbal Medicines

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ProQuest document link

ABSTRACT (ENGLISH)

Surface-enhanced Raman scattering (SERS) technology has unique advantages in the rapid detection of pesticides in plant-derived foods, leading to reduced detection limits and increased accuracy. Plant-derived Chinese herbal medicines have similar sources to plant-derived foods; however, due to the rough surfaces and complex compositions of herbal medicines, the detection of pesticide residues in this context continues to rely heavily on traditional methods, which are time consuming and laborious and are unable to meet market demands for portability. The application of flexible nanomaterials and SERS technology in this realm would allow rapid and accurate detection in a portable format. Therefore, in this review, we summarize the underlying principles and characteristics of SERS technology, with particular focus on applications of SERS for the analysis of pesticide residues in agricultural products. This paper summarizes recent research progress in the field from three main directions: sample pretreatment, SERS substrates, and data processing. The prospects and limitations of SERS technology are also discussed, in order to provide theoretical support for rapid detection of pesticide residues in Chinese herbal medicines.

FULL TEXT

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1. Introduction

Pesticides are chemicals or biological agents used in agriculture and forestry to prevent and control the growth of pests, including microbes, insects, and weeds, which would alter the growth of desirable plants [1]. Pesticides encompass a wide range of chemicals with broad functions, and their use can greatly improve the yield of crops. However, some pesticides are chemically stable and slow to degrade fully, and they can leave residues on the plant material or in the environment. These residues could then enter the human body through consumption of the plant or via the water or atmosphere. Even small amounts of some residues can impact human health, for example, by inhibiting the immune system or by increasing the risk of cancer [2, 3]. Therefore, it is important to develop tools to sensitively detect pesticide residues in agricultural products to ensure food safety.

Chinese herbal medicines (CHMs) are collected, processed, prepared, and used based on China’s traditional medical theory, and most of them are plant-based medicines that have been used for thousands of years [4]. In modern times, pesticides have been widely used in order to reduce damage caused by pests and diseases during cultivation. The presence of pesticide residues on CHMs can endanger human health, but they can also alter the efficacy of medicinal products. Pesticide residues also affect the trade in CHMs: exported CHMs are frequently returned due to contamination, causing economic losses and negatively affecting the virtuous circle of the Chinese medicinal materials’ market [5]. Because pesticide residues have such an important and multifaceted impact on CHMs, we have focused our analysis on this particular agricultural product.

In this review, the current situation of pesticide residues in foods and plant-derived CHMs is introduced. We summarize existing detection methods, including traditional chromatographic detection methods and emerging rapid detection methods mostly based on spectroscopy. In particular, we focus on the advantages of the use of surface-enhanced Raman scattering (SERS) in the detection of pesticide residues. We introduce current research regarding the application of SERS technology to pesticide residue detection and summarize challenges that remain in the detection process. Finally, we present prospects of future applications of SERS technology in the detection of pesticide residues in CHMs.

2. Methods of Detection of Pesticide Residues in Chinese Herbal Medicines

2.1. Present Status of Pesticide Residues in CHMs

The residual pesticides that contaminate plants cultivated for use in CHMs can be classified based on chemical structure into organophosphorus, organochlorine, carbamate, and pyrethroid pesticides. In the cultivation process, contamination of plants with pesticide residues arises from two factors during the growth process: the direct use of

pesticides and secondary contamination by pesticide residues in soil and water sources. The rate of detection of pesticides in CHMs has been high in multiple batches and types of materials grown in different regions. For example, a recent study surveyed 152 batches of CHMs, including Maidong samples, Baihe samples, Baishao samples, and Chenpi samples, and the rate of detection of glyphosate residue reached 79.61% [6]. In an analysis of 99 batches of Chuanxiong samples, the rate of detection of dichlorodiphenyltrichloroethane (DDT), chlorobenzamide, and other pesticides was 68.69% [7]. The rate of detection of pyrethroid pesticides in 40 batches of wolfberry samples from different sources was 42.9% [8]. Examples of other pesticide residues that have been found associated with CHMs are shown in Table 1.

Table 1

Pesticide residues associated with some Chinese herbal medicines.

Samples	Medicinal part	Analytical techniques	Types of pesticide residues	Ref.
Panax ginseng	Rhizome	GC-MS/MS	Acetochlor, chlorpyrifos, procymidone, triadimefon, propiconazole	[9]
Glycyrrhiza uralensis	Rhizome	GC-MS/MS	*β*-Endosulfan, thiosulphate, deltamethrin	[10]
Lycopus lucidus	Rhizome	GC-MS/MS	Permethrin, propiconazole, benalaxyl, isazofos	[11]
Codonopsis radix	Rhizome	GC-MS/MS	Chlorpyrifos, acetochlor, propiconazole	[12]
Chuanxiong rhizoma	Rhizome	LC-MS/MS	Triazophos, carbofuran, DDT, carbendazim, dimethomorph	[7]
Angelica sinensis radix	Rhizome	GC-MS/MS	Organochlorines, pyrethroids, dinitroanilines	[13]
Panax notoginseng	Root	GC-MS/MS	Dichlorvos, sulfotep	[14]
Fritillaria	Bulb	LC-MS/MS	Carbendazim, permethrin, chlorpyrifos, acetochlor	[15]
Dendrobium officinale	Rhizome	GC	Pyrethroids	[16]
Honeysuckle	Flower	GC-ECD	Cyfluthrin, omethoate, triazophos	[17]
Wolfberry	Fruit	HPLC-MS/MS	Cyfluthrin, fenvalerate, carbendazim, methomyl	[18]

2.2. Methods of Detecting Pesticide Residues in CHMs

The most important traditional methods of detection are chromatography and chromatography coupled to mass spectrometry (MS), while spectroscopic techniques are usually used for rapid detection. The processes of detection can be generally divided into four steps: collection, extraction, purification, and detection.

2.2.1. Traditional Methods

Gas chromatography-MS (GC-MS) couples the strong separation ability of GC with the accurate identification ability

of MS [19]. This technique is associated with high sensitivity and accuracy and is a robust method. Detection limits can be as low as 0.001 $\mu g \cdot kg^{-1}$, which allows for the trace detection of several types of pesticide residues [20]. However, this technique is unable to measure compounds that are strongly polar, nonvolatile, or thermally unstable. In addition, GC-MS is relatively expensive, as it relies on sophisticated instruments and highly trained professionals. Liquid chromatography- (LC-) MS can separate thermally unstable and nonvolatile samples and thus can complement the limitations of GC [21]. LC-MS is also associated with highly accurate, sensitive, and reliable results. The detection limit is as low as 0.01 $\mu g \cdot kg^{-1}$, leading to appropriately qualitative and quantitative capabilities [22, 23]. However, bulky and cumbersome instrumentation has been difficult to adapt to the rapid detection demands of the market.

Thin-layer chromatography uses the adsorption capacity of stationary and mobile phases to achieve the separation of analytes [24]. The method is simple and intuitive, it can analyze multiple samples at the same time, and the detection limit of some pesticides is 0.05 $\mu g \cdot kg^{-1}$ [25]. In general, this method is associated with low sensitivity, though. Its lack of sufficient separating ability often means that it can only serve as an initial means of separation. In short, at present, traditional methods of detection are highly accurate and have low detection limits, but the procedures used to perform detection tend to be complicated, expensive, and time consuming. These deficiencies make them difficult to adapt to the current CHM market demands for rapid application and detection.

2.2.2. Spectroscopic Methods

Compared with traditional methods, spectroscopic methods tend to be associated with rapid and simple operation, allowing them to meet the current need for rapid detection technology. Ultraviolet (UV) spectroscopy is based on transitions of valence electrons in target molecules [26]. This technique tends to be simple to operate and requires short detection times. The detection limits of chlorpyrifos and prothioconazole are 4.0 $\mu g \cdot L^{-1}$ and 0.38 $\mu g \cdot L^{-1}$, respectively [27, 28]. Nonetheless, it has several deficiencies that greatly limit its application in pesticide residue detection, such as high detection limits and a lack of specificity, and its adaptability needs to be improved.

Near-infrared (NIR) spectroscopy is used to analyze information carried by the substance according to the light transmitted or reflected by an NIR light source [29]. It is associated with fast detection speeds, high efficiency, and low cost, and it can realize the qualitative and quantitative analysis of multiple pesticides, such as profenofos, diazinon, and chlorpyrifos [30–33]. However, at present, the range of application of NIR spectroscopy technology is limited, because it has low sensitivity in the detection of some trace substances, and its ability to detect analytes in liquid samples is insufficient.

Terahertz (THz) spectroscopy is a kind of molecular spectroscopy that is associated with good spectral resolution and strong penetration. It can permit the rapid, nondestructive, and label-free detection of pesticide residues [34]. When combined with chemometrics, qualitative and quantitative analyses of pesticides can be realized, and the detection limit of some pesticides is as low as 0.01 $mg \cdot L^{-1}$ [35, 36]. Unfortunately, THz spectroscopy instrumentation tends to be large and expensive, so portable and rapid detection is unavailable. In addition, analytical results are affected by moisture; the sensitivity and speed of detection thus need to be further improved.

Raman spectroscopy, based on the Raman scattering effect discovered by Indian scientist C. V. Raman, yields spectra associated with inelastic scattering and can be used to interrogate the internal structural information of molecules [37]. The signal arising from chemicals can be very specific and is often referred to as a “fingerprint” [38]. Raman spectroscopy is free from water interference, requires short detection times, and is simple to operate. These advantages have led to its adaptation to the analysis of pesticide molecular structures and the detection of pesticide residues [39]. However, the conventional Raman spectrum signal is very weak, which limits its application in analysis. At present, there are two main ways to enhance the Raman signal: one is the improvement in the laser and the optical path and the other is the use of plasmon-enhanced Raman spectroscopy, which combines nanomaterials with electromagnetic radiation [40].

SERS technology, which combines Raman and nanomaterial technologies, can greatly enhance the Raman signal, leading to the trace detection of pesticide residues. SERS also has characteristics typically associated with more conventional methods, including rapid detection, simple operation, instrumentation portability, and low detection

costs. It also has a wide application range and is resistant to interference by off-target molecules.

Currently, SERS is mainly used in the detection of pesticide residues in agricultural products, but there are a few reports regarding its use in detection of pesticide residues in CHMs. For example, SERS was combined with chemometric methods to establish a quantitative prediction model of deltamethrin in *Corydalis yanhusuo*. In this application, gold nanoparticles with a diameter of 75 nm were prepared by a chemical reduction method and then used as a SERS detection enhancement reagent. This technique led to effective predictions and a low detection limit of 0.186 $\mu g \cdot L^{-1}$ [41]. This was the first application of SERS technology to pesticide residues in CHMs, and it provides a new feasible direction for the rapid detection of pesticide residues.

The advantages and disadvantages of various detection methods as applied to the detection of pesticide residues in CHMs are shown in Table 2. To better illustrate the comparison of these detection methods, we developed scores for the application scope, detection limit, cost, sampling time, usability, and portability of a series of detection methods. In this analysis, positive characteristics, including wider application scope, lower detection limit, lower cost, shorter sampling time, and increased usability and portability, are associated with higher scores. As shown in Figure 1, the key strengths of SERS technology are its ability to realize real-time, on-site, and rapid detection of CHMs and its broad application prospects.

Table 2

Comparison of advantages and disadvantages of methods used to detect pesticide residues.

Detection methods	Advantages	Disadvantages
GC-MS/MS	Good sensitivity, accuracy and precision, high analysis efficiency, and wide application range	Unsuitable for analysis of compounds that are strongly polar, nonvolatile, or thermally unstable; expensive instrumentation
LC-MS/MS	Wide analysis range; able to analyze compounds that GC-MS/MS cannot	Complicated, cumbersome, and expensive instrumentation
TLC	Rapid detection and low cost; simple and portable instrumentation; strong selectivity	Low sensitivity and poor separation ability
UV-Vis	High sensitivity, simple operation; can simultaneously analyze multiple compounds	Potential for spectral interference caused by overlapping spectral lines; relatively low selectivity
NIR	Wide application range, can provide structural information	Not suitable for analyzing water-containing samples; data analysis is complex
THz	Rapid and nondestructive detection	Instrumentation is cumbersome and expensive; low detection sensitivity
Raman	Rapid detection, simple and portable instrumentation; provides “fingerprints” of target substances	Weak spectral signal; poor sensitivity and precision
SERS	Fast detection; portable instrumentation with simple operation; high sensitivity	Easily disturbed by external factors; low stability of quantitative calculation models

[figure(s) omitted; refer to PDF]

3. Existing Studies on the Use of SERS in the Detection of Pesticide Residues

Recently, several studies have been performed on the use of SERS technology for detecting pesticide residues (Figure 2). Notably, this technology has been used to detect pesticide residues in CHMs, though further research into this application is warranted.

[figure(s) omitted; refer to PDF]

3.1. Pretreatment Methods

Sample pretreatment can reduce or even eliminate matrix effects and improve the sensitivity and accuracy of SERS-based detection and analysis. However, it is a time-consuming aspect of the detection of pesticide residues, so developing efficient pretreatment methods is of great significance. Common pretreatment methods include solid-phase extraction and solid-phase microextraction, as well as a modified form of solid-phase extraction called “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) technology. The performance of pretreatment methods is typically evaluated based on recovery rates and other indexes [42, 43].

Solid-phase extraction (SPE) technology is based on solid-liquid chromatographic principles and tends to be relatively simple to implement and to yield highly accurate results [44]. Solid-phase extraction technology has been applied to detect pesticide residues in tea, red bean, orange, and apple. It facilitates the removal of interfering pigments, sugars, proteins, and other impurities, and it is associated with high recovery rates [45–47]. However, traditional solid-phase extraction technology is expensive, has limited enrichment capacity, and produces variable results.

Solid-phase microextraction (SPME) can avoid some shortcomings of solid-phase extraction and is associated with short operation times and less sample consumption [42]. The use of SPME prior to detection of pesticide residues in apples, rice, and wheat has been shown to reduce matrix effects with recovery rates ranging from 79.3% to 106.8% [48–50]. This method is not effective in the treatment of substances with similar polarity or those in complex matrixes.

The QuEChERS technique is a simple and rapid pretreatment method related to solid-phase extraction. It minimizes the treatment process, reduces the amount of solvent required, and minimizes environmental pollution (Figure 3) [43]. Using this technique, anhydrous $MgSO_4$, primary secondary amine, C18, and graphitized carbon black (GCB) were used as reagents to purify analytes in the detection of chlorpyrifos residues in tea and soil. The pretreatment was found to eliminate interference by impurities such as organic acids and pigments, and the recovery rate was between 93.2% and 103.8% [51, 52]. Interestingly, when Fe_3O_4 was used in the pretreatment of agricultural residues in tea, the rate of recovery was better than that when GCB was used [53, 54].

[figure(s) omitted; refer to PDF]

Another substrate that has been used in QuEChERS is multiwalled carbon nanotubes (MWNTs), which have nanoscale hollow tubular structures and large specific surface areas [41, 55]. They have been applied to the detection of triazole residues in vegetables and deltamethrin residues in *C. yanhusuo*. These nanotubes were found to remove pigments, fatty acids, and other interfering substances, and they were associated with high recovery rates.

With its advantages of simplicity and speed, QuEChERS technology has become the preferred pretreatment method for SERS detection of agricultural residues. It showed excellent performance in eliminating the matrix effect of CHMs such as *Codonopsis* and *Astragalus* [8, 56], making it possible for QuEChERS-SERS technology to realize rapid detection of pesticide residues in CHMs. The advantages and disadvantages of several common pretreatment methods as applied to the detection of pesticide residues with SERS are shown in Table 3. It is important to note that some purification materials can adsorb pesticides; therefore, it is necessary to pay attention to the amount of material used during processing.

Table 3

Comparison of advantages and disadvantages of pretreatment methods.

Pretreatment methods	Advantages	Disadvantages
SPE	Sample enrichment and purification can be completed at the same time, simple operation	High cost, limited enrichment capacity, poor repeatability
SPME	No need for organic solvents, low environmental pollution; simple instrumentation and easy to operate	Poor repeatability, low accuracy of quantitative detection, few types of commercially available polymers
QuEChERS	Rapid and simple operation, good sensitivity and accuracy, wide application range, less environment pollution	Impurities can be only partially removed, susceptible to matrix effects

3.2. Nanoreinforced Substrates

There are a wide variety of pesticides, and the residues can be present at low concentrations. Fortunately, the Raman signal can be improved by up to 10 orders of magnitude by the use of high-activity enhanced nanoparticle substrates with the optimized size and shape [57]. SERS substrates include both substrates composed of a single precious metal and those composed of composite materials. TEM images of different nanosubstrates are shown in Figure 4.

[figure(s) omitted; refer to PDF]

Single precious metal substrates mainly consist of nanogold or silver. These particles come in different sizes and a variety of shapes, such as spheres, flowers, rods, bipyramids, and stars [58–61]. Gold nanoparticles with an average diameter of 30 nm were used as a reinforcing substrate to detect pesticide residues in Chinese cabbage; here, the limit of detection was 0.5 $mg \cdot kg^{-1}$ [62]. Silver nanoflowers were used to detect the residues of methomyl and acetamiprid in green tea. In this application, voids on the surfaces of the nanoparticles were found to produce local plasmon resonance, which enhanced the Raman signals. In these experiments, the detection limits were 5.58×10^{-4} $\mu g \cdot mL^{-1}$ for methomyl and 1.88×10^{-4} $\mu g \cdot mL^{-1}$ for acetamiprid [44]. When flexible double-cone gold nanoparticles were employed, methylthionine on the surfaces of apples was detected nondestructively with a limit of 31.58 $ng \cdot cm^{-2}$ (Figure 5) [63].

[figure(s) omitted; refer to PDF]

Other highly active SERS composite substrates have been prepared. For example, inert materials and nanomaterials have been used in the assembly of single metal substrates, and amino acids and DNA aptamers have been used as surface modification molecules [64, 65]. The resulting shell-core structure can compensate for shortcomings of raw materials while retaining desirable physical and chemical properties. For example, nanoparticles (NPs) consisting of a silver core and a gold shell (Ag@Au) were found to be stable and to enhance the Raman signal by a factor of 10^7; using this method led to limits of detection of thiram and thiabendazole on apple peel of 1 $mg \cdot kg^{-1}$ [66].

Similarly, cysteamine modification of AuNPs enhances the affinity of acephate to the gold surface, and the resulting detection limit was found to be 0.5 $mg \cdot L^{-1}$ [67]. When a target molecule was linked to a DNA aptamer and then embedded into a nano-tetrahedron based on Au@Ag NPs and a DNA skeleton, as shown in Figure 6, the Raman signal was greatly enhanced, leading to a detection limit as low as 0.0021 $ng \cdot mL^{-1}$ [68, 69].

[figure(s) omitted; refer to PDF]

MOF, SiO_2, Al_2O_3, TiO_2, Fe_3O_4, graphene, molecularly imprinted polymers, and other materials have also been applied to the shells of metal nanoparticles, and these modifications can stably enhance the Raman signal [70–76]. Compared with a Au@Ag substrate, an array of Au@AgNPs had a stronger Raman signal enhancement effect; when they were used to detect thiram on tomato peel with flexible tape, the detection limit was 5 $ng \cdot cm^{-2}$ (Figure 7)

[39]. Several enhancement substrates used for pesticide residue detection are shown in Table 4.

[figure(s) omitted; refer to PDF]

Table 4

Pesticide residues detected by SERS.

Samples	Pesticide	SERS substrate	Detection limit	Maximum residue limit	Ref.
Chinese cabbage	Acetamiprid	AuNPs	1 mg·kg^{-1}	1 mg·kg^{-1}	[62]
Malathion	1 mg·kg^{-1}	8 mg·kg^{-1}	Phosmet	0.5 mg·kg^{-1}	0.5 mg;1kg
–					
Apple	Triazophos	AgNPs	0.02 mg·kg^{-1}	0.2 mg·kg^{-1}	[77]
–					
Green tea	Methomyl	AgNFs	0.6 μg·L^{-1}	0.2 mg·kg^{-1}	[44]
Acetamiprid	0.2 μg·L^{-1}	0.5 mg·kg^{-1}	-		
Tomato/apple peel	Methylthionine	Bipyramid-AuNPs	31.58 ng·cm^{-2}	—	[63]
–					
Tea	Paraquat	Gold nanostars	0.2 mg·kg^{-1}	0.5 mg·kg^{-1}	[49]
–					
Peach	Thiacloprid Profenofos	Au@AgNPs	0.1 mg·kg^{-1}	0.5 mg·kg^{-1}	[78]
0.01 mg·kg^{-1}	0.05 mg·kg^{-1}	-			
Apple	Thiram	Ag@AuNPs	1 mg·kg^{-1}	5 mg·kg^{-1}	[66]
Thiabendazole	1 mg·kg^{-1}	15 mg·kg^{-1}	-		
Rice	Acephate	AuNRs with cysteine	0.5 mg·L^{-1}	1 mg·kg^{-1}	[67]
–					
Tomato peel	Thiram	Au@AgNP array	5 ng·cm^{-2}	5 mg·kg^{-1}	[39]
–					

Cucumber	Acetamiprid	Ag@SiO2	2.66 ng·mL^{-1}	1 mg·kg^{-1}	[71]
–					
Tomato/grape peel	Chlorpyrifos	Ag/TiO2 nanorods	2 ng·cm^{-2}	0.5 mg·kg^{-1}	[73]
5 ng·cm^{-2}	0.02 mg·kg^{-1}	-			
Apple peel	Thiram	AuNR array	0.41 ng·cm^{-2}	5 mg·kg^{-1}	[79]
–					
Centella	Chlorpyrifos	GO-Au nanocomposites	0.1 mg·kg-1	0.5 mg·kg-1	[80]

Thus, both single precious metal substrates and composite substrates have good SERS enhancement effects. The former are simpler to prepare and have been shown to enhance the Raman signal of pesticide residues, but they tend to be less stable and are difficult to store, so they need to be produced on demand. The latter have better adsorption and affinity and higher stability and sensitivity, whereas they are more complicated to prepare.

3.3. Spectral Data Analysis

Prediction models can be established by combining spectral technology with chemometrics. These models involve a rapid spectral analysis that can be completed without sophisticated training, and they can facilitate detection. These processes are accurate and efficient and include spectral preprocessing, model establishment, and model performance evaluation.

An original Raman spectrum contains both chemical information and system disturbance signals. Therefore, it is necessary to preprocess the original spectrum to ensure the accuracy of SERS detection. Common spectral preprocessing methods include the standard normal variate (SNV), multiplicative scatter correction (MSC), mean center (MC), first derivative (D1), and second derivative (D2). When comparing pretreatment performances of SNV, MSC, MC, D1, and D2 in the detection of deltamethrin in wheat, the MC prediction set was found to have the best performance, and the performance following the use of a partial least squares (PLS) model training set was significantly improved [81].

Both single-variable and multivariable models can realize quantitative detection of pesticide residues by SERS. A single-variable model is based on the linear equation relating concentration to peak strength. For example, when the residue of thiram on peach peel was detected with a flexible substrate, a linear equation was established with an R2 of 0.9756, meaning that this model was able to meet the requirements of quantitative detection (Figure 8) [82].

[figure(s) omitted; refer to PDF]

Multivariable modeling methods include PLS, genetic algorithm-partial least squares (GA-PLS), ant colony optimization (ACO), and successive projection algorithm (SPA). The performances of different prediction models are different because models have a variety of spectral intervals and variable selection procedures. At present, the prediction performance of a model is mainly evaluated by the correlation coefficient (R^2), relative percentage difference (RPD), and root mean square error (RMSE), including the root mean square error of calibration (RMSEC) and the root mean square error of prediction (RMSEP). For example, RMSECV and RMSEP have been used as model evaluation indexes, and MC was used as a pretreatment method to compare the performance of PLS, GA-PLS, ACO-PLS, and SPA-PLS. The results showed that MC-SPA-PLS had the best performance and good repeatability in trace detection [81]. Other research on the application of convolutional neural networks and other methods in SERS detection suggest that these methods are efficient in extracting characteristic spectra and lead to improved results [83].

In the actual detection process, a multivariable model can enhance efficiency and accuracy through analyzing the characteristics of different models and selecting an appropriate data processing algorithm according to the scale of required data. However, there is no model database for SERS detection of pesticide residues, and a large amount of preparatory work is required prior to large-scale implementation of this method.

4. Conclusion and Prospects

SERS technology has obvious benefits for the rapid detection of pesticide residues as compared with other detection methods. It allows fast detection with high sensitivity, its operation is relatively simple, and its instrumentation is portable. Effective sample pretreatment and flexible substrates are prerequisites for rapid detection. QuEChERS is the preferred pretreatment method for SERS detection of agricultural residues, because it is simple and fast, can greatly reduce the pretreatment time, and can remove matrix effects to the greatest extent while ensuring a high recovery rate. Flexible enhanced substrates have the advantages of simple preparation, good stability, and strong Raman signal enhancement effects, and they can permit the realization of nondestructive detection of trace contaminants. As shown in Table 4, SERS is presently able to achieve trace detection or even ultratrace detection, and the detection limits are lower than relevant provisions of the maximum residue limit of pesticides in the current national standard for food safety. It is likely, then, that SERS technology can provide conditions for rapid detection of pesticide residues in CHMs. Besides, combining spectral technology with chemometrics can establish prediction models, and the accuracy of quantitative detection can be greatly improved. However, some deficiencies remain in the application of SERS technology for detecting pesticide residues in CHMs. First, CHMs have complex chemical compositions, and some physical and chemical properties are similar to those of pesticide components, which makes extraction, analysis, and detection more difficult. Second, the complicated chemical composition of CHMs means that matrix effects are more complex; importantly, SERS-based detection is associated with an amplified matrix effect, which affects the accuracy of detection results. Third, there are no specific standards available for pesticide residues in the detection of CHMs by SERS, which hinders the implementation of SERS technology.

Research into the detection of pesticide residues in CHMs by SERS should take several forms: (1) optimization of enhanced substrates by changing the size and morphology to develop materials with good reinforcement effects and high stability and to allow additional control over the amount of reactants, reaction temperature, reaction time, and stirring; (2) optimization of pretreatment methods: simple and fast pretreatment methods should be chosen according to the properties of the tested substance to ensure high recovery rates and accuracy; and (3) optimization of algorithm models and the establishment of SERS pesticide residue detection databases, as well as acceleration of the establishment of SERS pesticide residue detection standards for CHMs. With this research progress, it is believed that SERS technology will begin to have a broader market appeal, and it will be possible to realize rapid and real-time detection of pesticide residues in CHMs.

Authors' Contributions

Bing-Yan Chu and Chi Lin contributed equally to this work.

Acknowledgments

This research was funded by the Key R &D Program of Zhejiang Province (2021C02022) and the National Key R &D Program (2022YFD2001801).

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vol. 1,DOI: 10.1016/j.aiia.2019.02.001, 2019.

DETAILS

Subject:	Residues; Raman spectra; Agrochemicals; Nanomaterials; Food; Data processing; Chromatography; Herbal medicine; Substrates; Pesticides
Editor:	Valentina Venuti
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-07-20 (Received); 2023-09-19 (Revised); 2024-01-02 (Accepted); 2024-01-12 (Pub)
DOI:	https://doi.org/10.1155/2024/5531430
ProQuest document ID:	2916946770
Document URL:	https://www.proquest.com/scholarly-journals/research-status-use-surface-enhanced-raman/docview/2916946770/se-2?accountid=211160

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Last updated:	2024-01-22
Database:	Publicly Available Content Database

Study on the Characteristics of Traditional Chinese Medicine Syndromes in Patients with Erosive Gastritis Based on Metabolomics

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ProQuest document link

ABSTRACT (ENGLISH)

According to traditional Chinese medicine theory, tongue coatings reflect changes in the body. The goal of this study was to identify a metabolite or a set of metabolites capable of classifying characteristics of traditional Chinese medicine syndromes in erosive gastritis. In this study, we collected tongue coatings of patients with erosive gastritis with damp-heat syndrome (DHS), liver depression and qi stagnation syndrome (LDQSS), and healthy volunteers. Then, we analyzed the differences in metabolic characteristics between the two groups based on metabolomics. We identified 14 potential biomarkers related to the DHS group, and six metabolic pathways were enriched. The differential pathways included pyrimidine metabolism, pantothenate and CoA biosynthesis, citrate cycle (TCA cycle), pyruvate metabolism, glycolysis/gluconeogenesis, and purine metabolism. Similarly, in the LDQSS group, we identified 25 potential biomarkers and 18 metabolic pathways were enriched. The top five pathways were the TCA cycle, sphingolipid metabolism, fatty acid biosynthesis, pantothenate and CoA biosynthesis, and the pentose phosphate pathway. In conclusion, the DHS group and the LDQSS group have different characteristics.

FULL TEXT

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1. Introduction

Erosive gastritis (EG) is one of the most common diseases in the world [1]. Currently, the main diagnostic method for EG is electronic gastroscopy [2]. However, this method is often too invasive for patients to tolerate [3]. EG

causes great trouble, although it generally does not endanger the life of patients. Therefore, it is worth exploring a simple and noninvasive diagnostic method to identify the characteristics of EG.

According to traditional Chinese medicine theory, tongue coatings reflect changes in the body [4, 5]. Based on this theory, it has been found that tongue features can be used as a stable method for disease diagnosis [6]. Tongue coating evaluation is sometimes even significantly superior to the use of traditional blood biomarkers [7]. In accordance with this theory, EG can be classified into two distinct syndromes: damp-heat syndrome (DHS) and liver depression and qi stagnation syndrome (LDQSS) [8]. Based on the different EG syndromes, traditional Chinese medicine (TCM) can provide more effective personalized treatment according to the features of each syndrome [9]. While previous studies have focused on bacteria from different tongue coatings in EG, variations in their metabolites remain unknown.

As a sensitive indicator for judging the condition of EG, tongue coatings play a pivotal role in the effective diagnosis and treatment [10]. Due to the comprehensive, highly sensitive, specific, and noninvasive nature of metabolomics, it has recently become a new research focus to promote disease diagnosis and is thought to be the most predictive phenotype [11]. In recent years, metabolomics has widely been used in the research of tongue coatings, such as in gastric precancerous lesions, coronary heart disease, and chronic renal failure [12, 13], with a particular focus on chronic gastritis [10]. We have identified changes in the salivary metabolome in patients with EG in our previous study [14]. We carried out the present study to further explore the metabolomics changes in EG patients. Herein, we collected tongue coatings of patients with EG with DHS and LDQSS, the two most common conditions in TCM. Due to their prominence in clinical practice, these two conditions have been listed in the International Classification of Diseases by the World Health Organization [15]. However, their biological characteristics have not yet been systematically studied. Therefore, in this study, we used nontargeted metabolomics methods to study the tongue coating samples of EG patients with DHS and LDQSS and to screen the metabolic products with characteristic significance.

2. Materials and Methods

2.1. Patients

We conducted a case control study to elucidate the composition of tongue coating-related metabolites in EG patients with DHS and LDQSS. A total of 64 patients with EG were selected from the Hebei Provincial Hospital of Traditional Chinese Medicine, including 32 cases with DHS and 32 cases with LDQSS. In addition, we selected 30 healthy volunteers, all of whom were either college students or hospital staff who did not have any digestive system symptoms and who passed the physical examination, tongue coating evaluation, and pulse condition assessment at the Hebei Province Hospital of Traditional Chinese Medicine. The experimental procedure is shown in Figure 1.

[figure(s) omitted; refer to PDF]

2.2. Ethics Approval

All of the subjects signed a written informed consent form before sample collection, and the study was conducted in accordance with the Declaration of Helsinki. In addition, the study was approved by the Ethics Committee of Hebei Province Hospital of Traditional Chinese Medicine (HBZY2021-KY-045-01).

2.3. Diagnostic Criteria of EG

2.3.1. Diagnostic Criteria in Gastroscopy

According to the diagnostic criteria for EG formulated by the Digestive Branch of the Chinese Medical Association in 2017, the diagnosis of EG includes single or multiple erosive lesions in the gastric mucosa that range in size from the size of a needle tip to several centimeters, or else single or multiple verruciform, bulging folds or papuloid protrusions in the gastric mucosa with diameters of 5–10 mm, a mucosal defect or umbilical-like depression at the top, and erosion at the center [16].

2.3.2. Diagnostic Criteria in Pathology

According to the pathological diagnostic criteria in the consensus on the Diagnosis and Treatment of Chronic Gastritis by Integrated Traditional Chinese and Western Medicine, mucosal infiltration with monocytes or neutrophils is the main manifestation of EG [8].

2.3.3. DHS and LDQSS Diagnostic Criteria

According to the TCM syndrome standard in the consensus on the Diagnosis and Treatment of Chronic Gastritis by Integrated Traditional Chinese and Western Medicine [8]:

DHS is defined as follows: having two main symptoms + one secondary disease, or one main symptom + two secondary diseases, combined with an evaluation based on gastroscopic findings.

The main symptoms are ① bloating or stomachache and ② red tongue with yellow, greasy, or thick fur.

Minor symptoms include ① heartburn in the stomach; ② bitter mouth and bad breath; ③ nausea and vomiting; ④ sticky stool; and ⑤ a slippery or wet pulse.

Relevant gastric results include ① thick and turbid mucus and ② obviously congested, edematous, and erosive gastric mucosa.

LDQSS is defined as follows: having two main symptoms + one secondary symptom, or the first main symptom + two secondary symptoms, combined with an evaluation based on gastroscopic findings.

The main symptoms include ① epigastric distension or pain in both flanks; ② pain due to emotional factors; and ③ pulse string.

Minor symptoms include ① frequent belching; ② chest tightness or excessive breathing; ③ lack of appetite; ④ mental depression; and ⑤ a light red tongue with thin white fur; relevant gastric results include ① active or slow peristalsis; ② erythema of the gastric mucosa, in the form of dots, patches, or strips; and ③ bile reflux.

Typical photographs taken from the patients who participated in the research are presented in Figure 2.

[figure(s) omitted; refer to PDF]

2.4. Sample Collection

2.4.1. Tongue Collection

First, we asked the subjects to clean their oral cavity for three times with distilled water free of any impurities. Then, we gently scraped an appropriate amount of tongue coating on the surface of the participant's tongue. Finally, the scraped samples were put into a centrifuge tube and immediately stored at −80°C until further analysis [17].

2.4.2. Sample Extraction

The samples were thawed without any damage. Then, 20 mg of the samples were taken, and 50 μl of water and 200 μl of methanol/acetonitrile solution (1 : 1, v/v) were added before vortex mixing. After that, the samples were sonicated at low temperature for 30 min. Then, they were centrifuged for 20 minutes at 14000 g at 4°C. The supernatant was collected, and 5 μl was injected into the instrument for final detection [18].

2.4.3. UHPLC-Q/TOF-MS Conditions

The samples were separated using an Agilent 1290 Infinity LC Ultra Performance Liquid Chromatography System HILIC column. The AB Triple TOF 6600 mass spectrometer was used to collect the primary and secondary spectra of the samples [19–21].

For HILIC separation, samples were analyzed using a 2.1 mm × 100 mm ACQUIY UPLC BEH 1.7 μm column (waters, Ireland). In both ESI positive and negative modes, the mobile phase contained *A* = 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and *B* = acetonitrile. The gradient was 85% *B* for 1 min and was linearly reduced to 65% in 11 min and then was reduced to 40% in 0.1 min and kept for 4 min and then increased to 85% in 0.1 min, with a 5 min re-equilibration period employed.

For RPLC separation, a 2.1 mm × 100 mm ACQUIY UPLC HSS T3 1.8 μm column (waters, Ireland) was used. In ESI positive mode, the mobile phase contained *A* (water with 0.1% formic acid) and *B* (acetonitrile with 0.1% formic acid); and in ESI negative mode, the mobile phase contained *A* (0.5 mM ammonium fluoride in water) and *B* (acetonitrile). The gradient was 1% *B* for 1.5 min and was linearly increased to 99% in 11.5 min and kept for 3.5 min. Then, it was reduced to 1% in 0.1 min, and 3.4 min of the re-equilibration period was employed. The gradients were at a flow rate of 0.3 mL/min, and the column temperatures were kept constant at 25°C.

The ESI source conditions were set as follows: Ion Source Gas1 (Gas1) was 60 psi, Ion Source Gas2 (Gas2) was 60 psi, curtain gas (CUR) was 30 psi, source temperature was 600°C, and IonSpray Voltage Floating (ISVF) was ±5500 V. In MS only acquisition, the instrument was set to acquire over the m/z range 60–1000 Da, and the

accumulation time for TOF MS scan was set at 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25–1000 Da, and the accumulation time for product ion scan was set at 0.05 s/spectra. The product ion scan was acquired using information dependent acquisition (IDA) with a high sensitivity mode selected. The collision energy (CE) was fixed at 35 V with ±15 eV; declustering potential (DP) was 60 V (+) and −60 V (−); exclude isotopes were set within 4 Da, and candidate ions to monitor per cycle were 10.

2.5. Statistical Analysis

A chi-square test was used for intergroup comparisons of gender, and the Mann–Whitney *U* test was used for intergroup comparison of age. This study relied on MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) to find significant differences between the groups. Data analysis was mainly based on fold-change analysis (FC Analysis), variable importance for the projection (VIP), and orthogonal partial least squares discrimination analysis (OPLS-DA) [22].

3. Results

3.1. Basic Information

A total of 32 EG patients with DHS (15 males and 17 females) and 30 healthy volunteers (15 males and 15 females) were included. There were no intergroup differences in gender distribution ($P>0.05$), and age did not differ significantly between the DHS group with average 49.63 ± 9.76 years and the control group with average 50.13 ± 8.79 years ($P>0.05$). We also included 32 EG patients with LDQSS (15 males and 17 females) and the 30 healthy volunteers mentioned above. There were no intergroup differences in gender distribution ($P>0.05$), and age did not differ significantly between the LDQSS group with average 49.81 ± 9.56 years and the control group with average 50.13 ± 8.79 years ($P>0.05$). The above data can be found in the supplementary material (available here).

3.2. OPLS-DA

There were 481 metabolites by identification, 313 for positive and 229 for negative; they were used to generate the OPLS-DA models. In this study, OPLS-DA was performed between the DHS group and the control group based on the positive and negative ion modes. To avoid overfitting of the supervised model in the modeling process, we used the permutation test to check the model. The results showed that the two groups were well distinguished (Figure 3), and the model had never been fitted. OPLS-DA was also performed between the LDQSS group and the control group. The results showed that the two groups were also well distinguished (Figure 4), and the model had never been fitted.

[figure(s) omitted; refer to PDF]

3.3. Volcano Plot

Based on univariate analysis, the differential metabolites between the DHS group and the control group were determined. We analyzed all metabolites detected in positive and negative ion modes. The differential metabolites with a FC > 2 or FC P value < 0.05 were visualized in the form of a volcano plot. The differential metabolites between the LDQSS group and the control group were also analyzed. The results are shown in Figure 5.

[figure(s) omitted; refer to PDF]

3.4. Differential Metabolites

In this study, strict thresholds of VIP > 1 and P value < 0.05 were used as the screening criteria for metabolites with significant differences between the DHS group and the control group. We demonstrated differential metabolites with FC > 2 or FC

Table 1

Differential metabolites in the positive ion mode.

Mode	Names	VIP value	FC	P value
POS	Cer (d18 : 1/18 : 1(9Z))	2.3520	0.4844	0.00

POS	Arg-Val	2.3244	2.4564	0.00
POS	Arg-Thr	2.2625	3.6149	0.00
POS	3-Hydroxycapric acid	1.9568	0.1426	0.00
POS	N-Stearoylsphingosine (ceramide C18)	2.2985	0.4047	0.00
POS	Arg-Glu	2.0946	2.4844	0.00
POS	Arg-Ile	2.1279	3.0491	0.00
POS	Pro-Arg	1.9208	2.4638	0.00
POS	Arg-Asp	1.7709	2.2461	0.00
POS	Arg-Ala	1.7586	2.4340	0.00
NEG	Cytidine	2.6703	2.7989	0.00
NEG	Phosphoenolpyruvate	2.2585	2.3120	0.00
NEG	Deoxycytidine	2.3083	2.4908	0.00
NEG	Guanosine	1.8122	2.1315	0.00
NEG	Pantetheine	1.7972	0.2491	0.00
NEG	Shikimate	1.8609	0.3931	0.00
NEG	EDTA	1.4562	2.1516	0.01

Table 2

Differential metabolites in the negative ion mode.

Mode	Names	VIP value	FC	P value
POS	Pro-Glu	2.1049	2.0158	0.00
POS	Sphingomyelin (d18:1/18:0)	2.0866	2.1614	0.00
POS	3-Hydroxycapric acid	1.6420	0.2131	0.00
POS	N-Stearoylsphingosine (ceramide C18)	1.9000	0.4935	0.00

POS	Arg-Glu	1.8303	2.1123	0.00
POS	Palmitic acid	1.4477	0.3911	0.00
NEG	L-Tryptophan	2.2527	2.3806	0.00
NEG	Alloxan	2.1289	0.4201	0.00
NEG	Citrate	2.0791	0.4924	0.00
NEG	Phosphoenolpyruvate	1.6457	2.3873	0.00
NEG	Shikimate	1.9389	0.3477	0.00
NEG	Gamma-L-glutamyl-L-glutamic acid	1.7349	2.1103	0.00
NEG	Guanosine	1.6253	2.0845	0.00
NEG	Heptadecanoic acid	1.5218	0.4978	0.00
NEG	Deoxycytidine	1.4530	2.0842	0.00
NEG	O-Phosphoethanolamine	1.6739	0.4994	0.00
NEG	6-Phospho-D-gluconate	1.7636	0.4074	0.00
NEG	Capric acid	1.3734	0.4662	0.00
NEG	Pantetheine	1.6168	0.2956	0.00
NEG	3-Hydroxyisovaleric acid	1.3577	0.3742	0.01
NEG	P-Cresol	1.3360	2.0519	0.01
NEG	p-Hydroxyphenylacetic acid	1.2972	2.0004	0.01
NEG	Citramalic acid	1.4332	0.4531	0.01

3.5. Cluster Analysis

To more comprehensively and intuitively display the differences, we conducted cluster analysis on the above results. Metabolites in the same cluster have similar expression patterns and may have similar functions or participate in the same metabolic process or cellular pathway together. Heat maps were generated to visualize the altered pattern of the significantly different metabolites (Figure 6).

[figure(s) omitted; refer to PDF]

3.6. Biomarker Analysis

Biomarker discovery was achieved through building predictive models of multiple metabolites to classify the patients

into different categories. In this study, we chose random forest (RF) as the multivariate algorithm for ROC curve analysis. ROC curves for biomarkers between the DHS group and the control group were plotted based on the average performance runs (Figures 7(a)–7(c)), and the significant features of the biomarker model were ranked by importance (Figures 7(b)–7(d)). Likewise, ROC curves for biomarkers between the LDQSS group and the control group were plotted based on the average performance runs (Figures 7(e)–7(g)), and the significant features of the biomarker model were ranked by importance (Figures 7(f)–7(h)).

[figure(s) omitted; refer to PDF]

3.7. KEGG Analysis

On the basis of the previous work, we conducted enrichment analysis on the selected significantly different metabolites. The results showed that pyrimidine metabolism, pantothenate and CoA biosynthesis, citrate cycle (TCA cycle), pyruvate metabolism, glycolysis/gluconeogenesis, and purine metabolism played an important role in the comparison between the DHS group and the control group (Figure 8(a)). Of note, the LDQSS group involved more pathways, and the top five were TCA cycle, sphingolipid metabolism, fatty acid biosynthesis, pantothenate and CoA biosynthesis, and pentose phosphate pathway (Figure 8(b)).

[figure(s) omitted; refer to PDF]

4. Discussion

Due to the comprehensive, highly sensitive, specific, and noninvasive nature of metabolomics, it has become a new research focus in disease diagnosis research in recent years. As one of the main methods for finding potential diagnostic biomarkers, metabolomics has extensively been used in the research on tongue coating [23]. There is mounting evidence that metabolic changes are associated with the initiation and development of tongue coatings [10, 12, 13]. Previous studies have demonstrated that gastric precancerous lesions, coronary heart disease, and chronic renal failure [12, 13], but especially chronic gastritis [10], have unique metabolic characteristics. However, although EG is a common type of chronic gastritis, the tongue metabolic profile of EG has not been thoroughly studied, and the underlying mechanism of different syndromes of EG remains unknown. Therefore, we carried out the present study based on metabolomics to discriminate between different features to improve diagnosis.

In this study, the baseline data did not significantly deviate between the DHS group and the control group. The results showed that the differences between the two were not affected by factors such as age and gender. In the comparison between the DHS group and the control group, the OPLS-DA results showed a significant difference between the two groups. The results proved that there was clear differentiation between the two groups. The volcano plot analyses also showed alterations in various metabolites; some were elevated, while others were decreased. In order to obtain more comprehensive and accurate results, characteristic metabolites were detected simultaneously in both positive and negative ion modes. In both modes, different metabolites are detected, but sometimes, there may be duplication. In the positive mode, there were 10 metabolites with an increasing trend. Among them, L-anserine, a bioactive dipeptide found in muscles and brains of vertebrates, was the most elevated metabolite [24]. There were seven metabolites with a downward trend. Among them, 3-hydroxycapric acid was the most significantly reduced metabolite. Along with L-anserine, they were enriched as potential spoilage biomarkers [25], consistent with erosion in the EG. In the negative mode, there were eight metabolites with an increasing trend. Of them, cytidine was the most significantly changed. There were two metabolites with a downward trend. Among them, pantetheine acid was the most significantly changed. Both of them have been used as potential biomarkers for unclassified patients with pediatric-onset multiple sclerosis [26]. The discovery of them may serve as an important potential marker for DHS.

The common goal of this study was to identify a metabolite or a set of metabolites capable of classifying characteristics of TCM syndromes in EG with high sensitivity (true positive rate) and specificity (true negative rate). So, we identified metabolites with significant differences that could act as biomarkers for distinguishing between the two groups by ROC curve analysis. The results showed that these were phenylacetic acid, Cer (d18 : 1/18 : 1(9Z)), Ile-Ser, triethanolamine, albuterol, Pro-Glu, 3-hydroxycapric acid, D-mannitol, Leu-Thr, palmitoyl ethanolamide in the positive mode, and cytidine, guanosine, deoxyguanosine, phosphoenolpyruvate, and deoxycytidine in the negative

mode. We also conducted cluster analysis to enhance the reliability of the results. Based on the enrichment analysis, it was found that pyrimidine metabolism, pantothenate and CoA biosynthesis, TCA cycle, pyruvate metabolism, glycolysis/gluconeogenesis, and purine metabolism played an important role in the comparison between the DHS group and the control group. Pyrimidine metabolism, glycolysis/gluconeogenesis, and purine metabolism are closely linked to inflammation and oxidative stress, the commonly accepted mechanistic pathway associated with marked susceptibility to infection [27–29]. CoA in the pathway of pantothenate and CoA biosynthesis is mainly involved in the metabolism of pyruvate, which can stimulate TCA cycle and provide 90% of the energy requirements for the body. This result indicated that there were differences in energy changes in the DHS group [30].

Similarly, in the comparison between the LDQSS group and the control group, the same operation was performed and satisfactory results were also obtained. OPLS-DA results also showed a significant difference between the two groups, proving that the two groups have significant heterogeneity. The volcano plot results showed up or downregulated differential metabolites. Interestingly, the different metabolites between LDQSS and DHS were the same in the negative mode, while they were different in the positive mode; they were Arg-Thr and 1-palmitoylglycol, respectively. Next, we identified the metabolites with significant differences, which can act as biomarkers for distinguishing between the two groups. The results of ROC curve analysis showed that they were Ile-Ser, D-mannitol, phenylacetic acid, albuterol, 2-phenylbutyric acid, Met-Val, 3-hydroxycapric acid, 2-methylbutyroylcarnitine, Pro-Glu, and 3-butynoic acid in the positive mode, and guanosine, L-tryptophan, alloxan, deoxyguanosine, dodecanoic acid, L-leucine, citrate, lumichrome, 3-methoxy-4-hydroxyphenylglycol sulfate, adrenic acid, L-aspartate, gamma-glutamyl-L-methionine, pantetheine, succinate, and L-alanine in the negative mode. We also conducted cluster analysis to enhance the reliability of the results. Based on the enrichment analysis, it was found that the LDQSS group involved more metabolic pathways, and the top five were TCA cycle, sphingolipid metabolism, fatty acid biosynthesis, pantothenate and CoA biosynthesis, and pentose phosphate pathway. Among them, the TCA cycle, fatty acid biosynthesis, pantothenate and CoA biosynthesis, and pentose phosphate pathways were largely similar to the DHS group. Moreover, the function of sphingolipid metabolism is immense and touches almost all major aspects of cell biology, including roles in cell growth, cell cycle, cell death, cell senescence, inflammation, immune responses, cell adhesion and migration, angiogenesis, nutrient uptake, metabolism, responses to stress stimuli, and autophagy [31].

The abovementioned results fully demonstrated the differences in metabolic characteristics between the LDQSS group and the DHS group. These findings not only provide technical support for the tongue for EG diagnosis but also reflect that different signs of EG have different characteristics, thereby providing support for further precision treatment and research basis for further exploration of the potential mechanisms of different signs based on metabolomics. In the future, the metabolites identified in this study may be used as noninvasive and convenient biomarkers to distinguish DHS and LDQSS of EG patients.

5. Conclusions

Taken together, this study revealed that EG with DHS and EG with LDQSS have different characteristics. In summary, 14 potential biomarkers related to the DHS group were identified and six metabolic pathways were enriched. The differential metabolites were enriched in pyrimidine metabolism, pantothenate and CoA biosynthesis, TCA cycle, pyruvate metabolism, glycolysis/gluconeogenesis, and purine metabolism, pathways related to inflammation, oxidative stress, and energy change. Similarly, in the LDQSS group, 25 potential biomarkers were identified and 18 metabolic pathways were enriched. The top five pathways were TCA cycle, sphingolipid metabolism, fatty acid biosynthesis, pantothenate and CoA biosynthesis, and pentose phosphate pathway. The results showed that the LDQSS group involved more metabolic pathways than the DHS group. There was consistency in the metabolic pathways involved between the two groups, but there were also significantly different pathways. Among them, the function of sphingolipid metabolism is immense and touches almost all major aspects of cell biology. Based on the above results, we hope that this research can provide reference and guidance for follow-up research and even lay the foundation for its application in TCM clinical diagnosis and treatment. However, there

are still many shortcomings in this research. We hope that related research can be carried out in the future.

Acknowledgments

This work was supported by the Natural Science Foundation of Hebei (No. H2020423207) and the Scientific Research Plan Project of Hebei Administration of Traditional Chinese Medicine (2022325).

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DETAILS

Subject:	Medical diagnosis; Coatings; Tongue; Disorders; Biosynthesis; Cardiovascular disease; Hospitals; Traditional Chinese medicine; Biomarkers; Metabolites; Chinese medicine; Pentose; Glycolysis
Business indexing term:	Subject: Hospitals
Location:	Ireland; Hebei China; China
Editor:	Daipayan Roy
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2024
Publication year:	2024
Publication date:	2024
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:	2023-07-10 (Received); 2023-11-30 (Revised); 2023-12-16 (Accepted); 2024-01-02 (Pub)
DOI:	https://doi.org/10.1155/2024/6684677
ProQuest document ID:	2914319181
Document URL:	https://www.proquest.com/scholarly-journals/study-on-characteristics-traditional-chinese/docview/2914319181/se-2?accountid=211160

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Last updated:	2024-01-16
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