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Phytochemical Constituents of Adansonia digitata L. (Baobab) Fruit Pulp from Tekeze Valley, Tigrai, Ethiopia

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ABSTRACT (ENGLISH)

Baobab (*Adansonia digitata* L) is a large tree species growing in semiarid and arid lowlands of Ethiopia and other places. The plant is valued by natives for its contributions as a cash crop and livelihood tree. Previous studies using samples from different countries have documented their phytochemical profiles and nutritional and health benefits. This study explored the phytochemical constituents and biological activities of fruit pulp extracts of baobab collected from Tekeze Valley, Tigrai, Ethiopia. To this end, qualitative phytochemical screening tests, quantitative phytochemical analyses, and gas chromatography-mass spectrometry (GC-MS) analysis were carried out using aqueous extract. Analyses of antioxidant activities were also conducted with aqueous- and methanol-extracts using of 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), and hydroxyl (OH) radical scavenging activity assays. The qualitative screening tests showed the presence of flavonoids, phenols, saponins, tannins, and terpenoids. Quantitative analyses of these phytochemicals at 25, 50, and 100 g/mL aqueous extract resulted in 0.0252 to 0.1000% yields. Yields of flavonoids, phenols, and saponins were higher at 50 g/mL extract, while that of tannins and terpenoids were higher at 100 g/mL. GC-MS analysis resulted in 15 predominant compounds including (1,2bis(trimethylsilyl)benzene (13.17%), 2-methyl-7-phenylindole (11.75%), 2-ethylacridine (10.11%), and benz[b]-1,4-oxazepine-4(5H)-thione,2,3-dihydro-2,8-dimethyl (10.11%). Aqueous and methanol extracts showed concentration-dependent antioxidant activities. In all the assays and concentrations, the antioxidant activities of both extracts were lower than that of the ascorbic acid standard. At equal extract concentrations (e.g., 100 and 250 μ g/mL), methanol extract had higher antioxidant activities than aqueous extract. The findings can encourage future initiatives towards large-scale research for compiling a complete phytochemical profile of the fruit pulp of the Ethiopian baobab.

FULL TEXT

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1. Introduction

Baobab (*Adansonia digitata* L) (Malvaceae) is a large, conspicuous tree, native to semiarid regions of Africa, Asia (in

China and Malaysia), Australia, and the Caribbean (especially Jamaica). Locally called *Dima* (in Tigrinya), the plant is very common in deserts and hot lowlands in Ethiopia. The tree is highly valued by rural communities of arid and hot lowlands of sub-Saharan Africa for its fruits and edible leaves. It is an important cash crop, a source of healthy human food and nutrition with medicinally and pharmaceutically important constituents, and livestock fodder [1–6]. Unfortunately, it is a neglected, locally and globally endangered tree species with no strategies for its conservation, natural and artificial regeneration, and sustainable use [3].

Previous studies in baobab-growing countries have shown that the fruit is the source of nutritionally and medicinally important compounds [7–9]. It is, thus, called the chemist tree because of its health benefits [2, 10, 11]. Baobab fruit pulp has very high vitamin C content (ca. ten times that of orange) [5]. Its seeds have substantial quantities of crude protein, digestible carbohydrates, and oil and high levels of lysine, thiamine, calcium, and iron [5, 6]. Likewise, baobab leaves are superior in nutritional quality to fruit pulp and contain significant vitamin A levels. All parts of the plant have many phytochemical constituents with multiple biological properties including antioxidant and anti-inflammatory activities [5, 12]. Seed, pulp, and seed oil of baobab are also good sources of macro- and micronutrients such as potassium, magnesium, calcium, and phosphorus as well as many health-promoting substances [11, 13]. Hence, the fruit pulp (powder) can be consumed fresh or as a traditionally and industrially processed product. It also has a huge potential for large-scale industrial utilization via producing functional foods and beverages [14, 15].

However, despite the plant grows in the vast area of hot lowlands in Ethiopia, in general, and in Tigrai (northern Ethiopia), in particular, no scientific study was carried out before to explore its chemistry, conservation, and natural and artificial regeneration. No efforts were also made towards its large-scale industrial application. Therefore, the present study aims to describe the qualitative and quantitative phytochemical constituents, identify the major compounds using GC-MS and other methods, and examine the antioxidant properties of the baobab fruit pulp extract. The study's findings will contribute to initiating future research and development programs towards the sustainable use of the plant.

2. Materials and Methods

2.1. Collection Site of Baobab Fruits

Baobab fruits used in the study were collected from Tekeze Valley (11°40′ and 15°12′N and 36°30′ and 39°50′E), Western Zone of Tigrai Region, Ethiopia. The site of fruit collection is located 96 km southwest of Shire-Endaselassie city, stretching between the Asgede-Tsimbla district to the east and the Wolqait district to the west [16].

2.2. Collection and Preparation of Fruits

Fruits were collected during the fruit ripening season of December 2019. Collection of biological materials by Ethiopian researchers for research and development purposes is granted by Article 15, Clause 1 of the Access to Genetic Resources and Community Knowledge, and Community Rights Proclamation of Ethiopia (No. 482/2006). Healthy, ripened, and brownish fruits were collected from five mature trees of riverine forest. The total density in the riverine forest of the area is 5(±0.8) stems per hectare according to one recent study [17]. The fruits collected from each tree were counted and recorded. The fruits were packed in plastic bags and shipped to the tissue culture laboratory of Tigrai Biotechnology Center Pvt. Ltd. Co, Mekelle, Ethiopia, and stored in dry storage boxes until use. Then, healthy, dry fruits with no discoloration were carefully selected and washed using warm tap water (50°C) and sodium hypochlorite to remove any soil, debris, and microbial contaminants. The washed fruits were immediately put within a laminar air flow (LAF) cabinet and were allowed to fully dry before being processed [9].

2.3. Separation of Fruit Pulp

The hard woody shells of the fruits were removed using a sterilized stainless steel knife inside a LAF cabinet. This procedure yielded the white pulp (powder) of the baobab fruit holding several seeds and fibers. The pulp was, then, separated from the seeds and fibers by smashing with a pestle and collected into a sterilized and clean mica bowl. The powdery pulp was sieved using 0.9 mm pore mesh. The pure and fine pulp was transferred into a sterilized clean jar, tightly closed, and kept in a dark and cool place until used for further analyses [9].

2.4. Extraction of *A. digitata* Fruit Pulp

Extraction of fruit pulp was carried out at the geochemical laboratory of the College of Natural and Computational Sciences, Mekelle University, Mekelle, Ethiopia. A 10.0 g sample of dried baobab fruit pulp was macerated in 100.0 mL distilled water with continuous stirring for 24 h. Then, the mixture was filtered and concentrated using a rotary evaporator at 40°C under reduced pressure yielding aqueous extract. Likewise, a 20 g sample of the pulp was macerated in 150.0 mL of 80% methanol through continuous steering for 72 h. Then, the mixture was filtered, concentrated, and dried using a rotary-evaporator at 40°C yielding the methanol extract. The concentrated aqueous and methanol extracts were kept in a refrigerator at 4°C until further analyses [18].

2.5. Qualitative Phytochemical Screening

2.5.1. Alkaloids

Qualitative analysis of alkaloids was carried out using Mayer's test as described in the work of Ansari [19]. A 5.0 mL baobab pulp aqueous extract was evaporated in a test tube leaving a residue. A 1.0 mL of 5% (v/v) HCl was added to the residue in the test tube, shaken well, and filtered. Then, 10 drops of Mayer's reagent were added to the filtrate. The formation of a yellow precipitate signifies a positive test for alkaloids.

2.5.2. Flavonoids

Flavonoids were screened using the Shinoda test as described by Kokate [20]. A 10.0 mL/g aqueous extract of baobab fruit pulp was added to a test tube, and 5.0 mL 95% ethanol and a few drops of conc. HCl were added to it. Then, 0.50 g Mg chips were added to the solution. Pink coloration indicates a positive test for flavonoids.

2.5.3. Glycosides

Identification of glycosides was carried out using the Keller–Killiani test. A 2.0 mL aqueous extract of baobab fruit pulp was added to a test tube. Then, 1.0 mL glacial acetic acid, 1 drop of 5% $FeCl_3$, and 1.0 mL conc. H_2SO_4 were added. The appearance of reddish-brown color at the junction of two liquid layers and the turning of the upper layer into bluish-green indicate the presence of glycosides [19].

2.5.4. Phenols

Screening for phenols was carried out using the ferric chloride test [21]. A 2.0 mL aqueous extract of baobab fruit pulp was added to a test tube. Then, it was diluted to 5.0 mL by adding distilled water, and a few drops of neutral 5% $FeCl_3$ solution were added to it. A dark green color indicates a positive test.

2.5.5. Saponins

The screening for saponins was carried out using the foam test [19]. A 5.0 mL baobab pulp aqueous extract was mixed with 5.0 mL distilled water and shaken vigorously for 10 min. The development of stable foam indicates a positive test for saponins.

2.5.6. Steroids

Qualitative analysis of steroids was carried out using the Salkowski test [22]. A 2.0 mL aqueous fruit pulp extract of baobab was added to a test tube. Then, 2.0 mL chloroform and 2.0 mL conc. H_2SO_4 were added to it, and the solution was shaken well. The turning of the chloroform layer into red and the acid layer into greenish-yellow fluorescence signifies a positive test for steroids.

2.5.7. Tannins

The screening for the presence of tannins was carried out using the lead acetate test [21]. A 5.0 mL aqueous extract of baobab was added to a test tube, and 2.0 mL lead acetate solution was added to it. The development of a white precipitate signifies a positive test for tannins.

2.5.8. Terpenoids

The procedure for detecting terpenoids was carried out using the Salkowski reaction with some modifications [23]. A 0.15 g baobab fruit pulp aqueous extract was mixed with 2.0 mL chloroform followed by careful addition of 4.0 mL conc. H_2SO_4. The mixture was allowed to form a layer, and a reddish-brown coloration in the interface indicates the presence of terpenoids.

2.6. Quantitative Phytochemical Analyses

2.6.1. Total Flavonoid Content

The total flavonoid content (TFC) of the fruit pulp of *A. digitata* was determined according to the aluminum chloride

method using catechin as a standard [24]. A 1.0 mL aqueous extract was poured into a volumetric flask and mixed with 4.0 mL distilled water and was allowed to stand for 5 min. Then, 0.30 mL 5% $NaNO_2$ and 0.30 mL 10% $AlCl_3$ were added and the mixture and was left for 6 min at room temperature. A 2.0 mL 1.0 M NaOH was added to the reaction mixture, and some distilled water was immediately added until the mixture reached the 10.0 mL mark. The absorbance of the reaction mixture was measured using a UV-vis spectrophotometer (Lambda, CE1021, Australia) at 510 nm against a blank. A calibration curve was generated using 10–100 μg/mL of the standard catechin solution ($R^2 = 0.991$). The TFC values were calculated based on the curve, and the contents were expressed as mg of catechin equivalent per gram of the dried extract (mg CE/g dried extract).

2.6.2. Total Phenolic Content

The total phenolic content (TPC) of the fruit pulp aqueous extract was determined using the Folin–Ciocalteu reagent (FCR) [24]. A 1.0 mL aqueous extract of different concentrations was mixed with 0.40 mL FCR (diluted 1 : 10 v/v) in volumetric flasks and was allowed to stand for 5 min. Then, 4.0 mL of 7% Na_2CO_3 solution was added. Upon reaching the 10.0 mL mark by adding distilled water, the mixture was allowed to stand for 90 min at room temperature. The absorbance of each of the samples was measured against a blank at 750 nm using a UV-vis spectrophotometer. A calibration curve was generated using 20–200 μg/mL of the standard gallic acid solution ($R^2 = 0.998$). The TPC values were calculated based on the curve of gallic acid solution. The contents were expressed as mg of gallic acid equivalent per g of the dried extract (mg GAE/g dried extract).

2.6.3. Total Saponin Content

The total saponin content (TSC) was estimated according to the procedure established in the works of Sim [25]. A 1.0 mL sample of aqueous extract was put into a test tube and was mixed with 2.0 mL 8% vanillin, dissolved in ethanol, and agitated until it forms a homogeneous solution. Then, 2.0 mL 72% H_2SO_4 was added to the solution, mixed well, heated in a water bath at 60°C for 10 min, and allowed to cool. The absorbance of the solution was measured at 544 nm against a blank using a UV-vis spectrophotometer. A calibration curve was generated using 10–100 μg/mL of Diosgenin standard solution ($R^2 = 0.992$). The TSC values were calculated based on this calibration curve. The contents were expressed as mg of Diosgenin equivalents per g of the dried extract (mg DE/g dried extract).

2.6.4. Total Tannin Content

The total tannin content (TTC) of the *A. digitata* fruit pulp extract was determined by using tannic acid as a reference compound according to the method described by Saeed et al. [24]. A 1.0 mL sample was put into a test tube and mixed with 5.0 mL vanillin hydrochloride reagent (comprising equal volumes of 8% HCl in methanol and 4% vanillin in methanol). The mixture was allowed to stand for 20 min to complete the reaction, and its absorbance was measured at 500 nm using a UV-vis spectrophotometer. A calibration curve was generated using 20–200 μg/mL of tannic acid standard solution ($R^2 = 0.991$). The TTC values were calculated based on this calibration curve. The contents were expressed as mg of tannic acid equivalents per g of the dried extract (mg TAE/g dried extract).

2.6.5. Total Terpenoid Content

The total terpenoid content was determined according to the method described by Ghorai et al. [26]. A 1.0 mL sample of the aqueous fruit pulp extract was prepared in a test tube, and 3.0 mL chloroform was added to it. The mixture was thoroughly vortexed, left for 3 min, and 200 μL conc. H_2SO_4 was added to it. The mixture was incubated at room temperature for 1.5–2 h in dark to form a reddish-brown precipitate. Then, all the supernatant of the reaction mixture was carefully and gently decanted without disturbing the precipitate. At the end, 3.0 mL 95% (v/v) methanol was added to the precipitate and vortexed thoroughly until the precipitate was dissolved completely. The absorbance was measured at 538 nm using a UV/vis spectrophotometer. A calibration curve was generated using 10–100 μg/mL of linalool standard solution ($R^2 = 0.994$). The values of total terpenoid content were calculated based on this calibration curve. The contents were expressed as mg of linalool equivalents per g of the dried extract (mg LE/g dried extract).

2.7. GC-MS Analysis of Aqueous Extract of *A. digitata* Fruit Pulp Powder

Chemical constituents of the aqueous extract of the baobab fruit pulp were analyzed and identified using a gas

chromatography-mass spectrometer (GC-MS) according to the method developed by Salim [27]. A 2.0 μL sample of the aqueous pulp extract was dissolved in HPLC-grade aqueous solution and subjected to GC. An Agilent 7820AGC system was used for the GC. DB-5 column fused with silica (50 m length × 0.25 mm internal diameter) was used for separation. The column temperature was set to 100°C for 20 min and increased to 270°C for 3 min. Helium was used as the carrier gas with a split ratio of 5 : 4. A 1.0 μL sample was evaporated in a splitless injector at 300°C in 22 min run time. The molecular weight, molecular formula, and structure of the compounds were ascertained by interpretation of the mass spectrum of GC-MS using the database of the NIST library and relevant literature.

2.8. Antioxidant Properties of Extracts of *A. digitata* Fruit Pulp Powder

2.8.1. DPPH Radical Scavenging Activity Assay

The free radical scavenging activities of aqueous and methanol extracts were measured *in vitro* by the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay according to a standard method [28]. A stock solution was prepared by dissolving 24.0 mg DPPH in 100.0 mL ethanol and was stored at 20°C. A working solution was prepared by diluting DPPH stock solution in ethanol, and a 3.0 mL aliquot of the working solution was mixed with 1.0 mL aqueous pulp extract at 100, 250, 500, 750, and 1,000 μg/mL and methanol pulp extract at 50, 100, 150, 200, and 250 μg/mL. Reaction mixtures were shaken well and incubated in the dark for 15 min at room temperature. The absorbance of each mixture was taken at 517 nm using a UV-vis spectrophotometer. A control sample was prepared without the extract, and scavenging activity was estimated based on the percentage of the DPPH radical scavenged as % Inhibition = [(Control OD − Sample OD)/(Control OD] × 100, where OD refers to the optical density.

2.8.2. Nitric Oxide Scavenging Activity

The nitric oxide radical scavenging (NOS) activities of the extracts were determined according to the method described by Erwa and coworkers[29]. A 1.0 mL of 10 mM sodium nitroprusside in phosphate-buffered saline was mixed with 1.0 mL pulp extract of different concentrations (i.e., 100, 250, 500, 750, and 1,000 μg/mL for the aqueous extract and 50, 100, 150, 200, and 250 μg/mL for the methanol extract) and incubated at 25°C for 180 min. Then, 1.0 mL Griess reagent (a mixture of an equal volume of 1% sulphanilamide, 0.1% naphthyl-ethylenediamine dichloride, and 3% H_2PO_4) was added to the incubated solution, and the absorbance was read at 546 nm using a UV-vis spectrophotometer. Ascorbic acid was used as positive control and treated in the same way as the Griess reagent. The percentage inhibition was calculated as Scavenging Effect (%) = [(Control OD − Sample OD)/(Control OD] × 100, where OD refers to the optical density.

2.8.3. Hydroxyl Radical Scavenging Activity

Determinations of hydroxyl radical scavenging (HRS) activities of the extracts were carried out as per the method described in Saeed et al. [24]. Reaction mixtures comprising 0.8 mL phosphate buffer solution (50 mmol/L, pH 7.4), 0.20 mL extract of the different concentrations (i.e., 100, 250, 500, 750, and 1,000 μg/mL aqueous pulp extract and 50, 100, 150, 200, and 250 μg/mL methanol pulp extract), 0.20 mL EDTA (1.04 mmol/L), 0.20 mL $FeCl_3$ (1.0 mmol/L), and 0.20 mL of 2-deoxyribose (60 mmol/L) were prepared in test tubes. The mixtures were kept in a water bath at 37°C. The reaction of each mixture was initiated by adding 0.20 mL ascorbic acid (2.0 mmol/L) and 0.20 mL H_2O_2 (10.0 mmol/L) and was left for 1 h. Then, 2.0 mL cold thiobarbituric acid (10 g/L) and 2.0 mL HCl (25%) were sequentially added to each of the reaction mixtures. The reaction mixtures were heated at 100°C for 15 min and were cooled in a cold water bath. The absorbance of each solution was measured at 532 nm using a UV-vis spectrophotometer. The HRS capacity was evaluated with the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals. The scavenging percentage was calculated as Scavenging Effect (%) = [(Control OD − Sample OD)/(Control OD)] × 100, where OD refers to the optical density.

2.9. Data Analyses

All the tests, experiments, and measurements were carried out in triplicate. The data were analyzed by inferential statistical methods using SPSS Version 20 software. Inferential (sample) data were processed using the analysis of variance (ANOVA) at an *a priori* set p value of ≤0.05. Post-hoc comparisons of the mean (±SD) values were carried out using the least significance difference (LSD). Qualitative data collected by visual observations were used to strengthen the results of the quantitative data analyses.

3. Results and Discussion

3.1. Phytochemical Study of *A. digitata* Fruit Pulp Powder

3.1.1. Qualitative Screening

The qualitative phytochemical screening assay of baobab fruit pulp powder was conducted using the aqueous extract. The assay revealed that five of the eight sought phytochemical groups, namely, flavonoids, phenols, saponins, tannins, and terpenoids were detected in the aqueous extract (Table 1). Previous studies, that employed different methods and extraction solvents, have reported the presence of all or some of these chemical groups [14, 30–33]. The presence of flavonoids and saponins, which promote the antioxidant properties of wild fruits, can extend the shelf lives of derived foods, beverages, and cosmetics [14, 34, 35]. Alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids are important phytochemical constituents with high antioxidant properties and many other therapeutic uses. They are also known for their antimicrobial properties and cholesterol-lowering effects [35–41]. Secondary metabolites, with these and other health benefits by reducing the risks of several chronic diseases, are common in fresh fruits and other plant products. These findings encourage comprehensive research to investigate the Ethiopian baobab fruit pulp for medicinal and nutritional phytochemicals.

Table 1

Phytochemical screening of crude aqueous extract of *A. digitata* fruit pulp compared with previous studies.

No.	Phytochemicals	This study	[14]	[30]	[31]	[32]	[33]
1	Alkaloids	−(WE)	+(WE)	+(WE)	−(WE)	+(AE)	+(ME)
2	Flavonoids	+(WE)	+(WE)	+(WE)	−(WE)	+(ME)	+(ME)
3	Glycosides	−(WE)	+(WE)	+(WE)	+(WE)	+(ME)	NA
4	Phenols	+(WE)	NA	+(WE)	NA	NA	NA
5	Saponins	+(WE)	+(WE)	+(WE)	+(WE)	+(ME)	+(ME)
6	Steroids	−(WE)	NA	+(WE)	+(WE)	NA	+(ME)
7	Tannins	+(WE)	+(WE)	+(WE)	+(WE)	+(AE)	+(ME)
8	Terpenoids	+(WE)	+(WE)	+(WE)	+(WE)	+(HE)	NA

−: absent, +: present; NA: not analyzed; AE: acetone extract, HE: hexane extract, ME: methanol extract, WE: water extract.

3.1.2. Quantitative Analysis

Quantitative analyses of the phytochemicals were carried out with 25, 50, and 100 g/mL fruit pulp extract concentrations. The mean (±SD) concentrations of the phytochemicals increased at statistically significant levels with increasing the concentrations of the extract (Table 2; $p \leq 0.05$). But when the yields are compared, the yields of flavonoids (0.0732%), phenols (0.0652%), and saponins (0.0386%) are higher with 50 g/mL extract, while those of tannins (0.1000%) and terpenoids (0.0636%) are higher with 100 g/mL extract. The lowest yields were observed with 25 g/mL extract in all the phytochemical groups except in flavonoids (Table 2). Thus, higher yields of the phytochemical were observed with 50 and 100 g/mL extract.

Table 2

Quantitative analyses and yield of phytochemicals of *A. digitata* fruit pulp aqueous extract.

No	Phytochemicals	Yield in mg/g (mean ± SD values and percentages)					
25 g/mL extract		50 g/mL extract		100 g/mL extract		Mean ± SD	%
Mean ± SD	%	Mean ± SD	%	1	Flavonoids	15.60 ± 0.08^{c}	0.0624
36.60 ± 1.08^{b}	0.0732	55.30 ± 1.04^{a}	0.0553	2	Phenols	11.00 ± 0.70^{c}	0.0440
32.60 ± 1.08^{b}	0.0652	50.30 ± 1.08^{a}	0.0503	3	Saponins	6.30 ± 1.08^{c}	0.0252
19.30 ± 1.47^{b}	0.0386	31.60 ± 1.08^{a}	0.0316	4	Tannins	16.30 ± 1.08^{c}	0.0652
40.30 ± 1.08^{b}	0.0806	100.00 ± 1.4^{a}	0.1000	5	Terpenoids	11.00 ± 0.70^{c}	0.0440

Means (± SD) in the same row with different letters are statistically significantly different at $p \leq 0.05$.

A study on aqueous fruit extract of Nigerian baobab showed 16.14 mg/g flavonoids, 100.00 mg/g saponins, 351.0 mg/g tannins, and 70.00 mg/g alkaloids [30]. Another study on the hexane fruit extract of Senegalese baobab yielded 5.66 ± 0.18 μg/mg total flavonoids, 103.09 ± 0.63 μg/mg total tannins, and 27.21 ± 0.26 mg/g total polyphenols [33]. A chemical analyses study with aqueous extracts of Saudi Arabian baobab fruit reported 42.70 ± 0.43 mg/g flavonoid and 48.08 ± 1.08 mg/g phenolic contents [42]. Also, a study on fruits extracts collected from various parts of Sudan revealed phenolic contents of 15.50 to 99.66 mg GAE/g and flavonoid contents of 1.03 to 21.53 mg CE/g [43]. A study on the fruit extract of Malawian baobab found a total phenolic content of 1,870 ± 1.61 mg/100 g fresh weight [35]. Such high total phenolic contents were also reported for fruit pulp extracts from Madagascar (1,090 mg GAE/100 g) [44] and Burkina-Faso (3,520 to 4,060 mg GAE/100 g) [45]. Therefore, with 100 g/mL fruit pulp extract, the flavonoid content obtained in the present study was higher than those reported for the Nigerian, Saudi Arabian, Senegalese, and Sudanese baobab fruit pulps. Moreover, the phenolic content was higher than those reported for fruit pulps from Burkina Faso, Malawi, Madagascar, Saudi Arabia, and Senegal. However, the saponin and tannin contents were lower than those reported for the Nigerian and Senegalese fruit pulps.

3.2. GC-MS Analysis *A. Digitata* Aqueous Fruit Pulp Extract

Baobab is known to be the source of several secondary metabolites with multiple nutritional, biological, and pharmaceutical activities and properties [11, 35, 40, 41, 46, 47]. A study on a Malian baobab fruit pulp using high performance liquid chromatography coupled with a photodiode array/UV and electrospray ionization-mass spectrometer and mass spectrometer (HPLC-PDA/UV-ESI-MS/MS) analyses detected citric acid and 14 phenolic compounds [11]. Another HPLC-based study on Malawian fruit also detected high quantities of vitamin C, multiple organic acids, and phenolic compounds [35]. One study on fruit pulp of Nigerian baobab using GC-MS analysis [40] and another on Sudanese baobab using the LC-MS/MS analysis [46] detected 36 and 52 bioactive compounds, respectively. A recent study on the fruit extract using ultrahigh performance liquid chromatography coupled to high-resolution tandem mass spectrometry (UHPLC-HRMS/MS), headspace solid-phase microextraction/gas chromatography coupled to mass spectrometry (HS-SPME/GC-MS), and GC-MS postsilylation detected 77 compounds [41]. In the present study, the GC-MS analysis of the aqueous extract of the Ethiopian baobab fruit pulp

yielded a chromatogram with greater than 200 picks. Fifteen predominant chemical compounds with an area of 5.68 to 13.17% were identified based on the reference compound at pick *m*/*z* 207.10 (100.00%) (Figure 1, Table 3, and Supplementary file, Figure S1). The choices of extraction solvent, retention time, area (%), GC-MS column, reference compound, and NIST data interpretation system determine the qualitative and quantitative results of the GC-MS and other spectrometer methods [45, 46]. The literature study showed that each of the 14 chemical compounds has multiple biological and pharmaceutical activities and/or properties [48–62].

[figure(s) omitted; refer to PDF]

Table 3

Predominant chemical compounds in the aqueous extract of *Adansonia digitata* L. fruit pulp identified by gas chromatography-mass spectrometry (GC-MS).

SN	MF	Compound	Structure	MW	RT (min)	Area (%)	*m*/*z*+	Pharmaceutical or biological activities/properties	Ref
1	$C_{15}H_{13}N$	2-Ethylacridine		207.27*	16.327	10.11	62, 96, 166	(i) Antitumor(ii) Antioxidant	[48, 49]
–									
2	$C_{11}H_{13}NOS$	Benz[b]-1,4-oxazepine-4(5H)-thione,2,3-dihydro-2,8-dimethyl		207.29	16.327	10.11	41, 85, 134, 174	(i) Anticonvulsants(ii) Muscle-relaxant(iii) Daytime sedative(iv) Tranquilizers(v) Anesthetics	[50, 51]
–									
3	$C_{15}H_{13}N$	Benzo[h]quinoline,2,4-dimethyl		207.27*	16.913	8.03	76, 127, 165	(i) Anticancer(ii) Antibacteria1(iii) Antifunga1(iv) Antimalarial	[49]
–									
4	$C_6H_{18}O_3Si_3$	Cyclotrisiloxane, hexamethyl-		222.4618	16.431	9.54	96, 133, 177	(i) Antibacterial(ii) Antimicrobial(iii) Antitumor	[52, 53]
–									
5	$C_7H_6N_2S$	1,2-Benzisothiazol-3-amine		150.2	16.431	7.00	41, 74, 177	(i) Antimicrobial(ii) Antitumor(iii) Promotes human growth and development(iv) Treats skin cancer, atherosclerosis, and migraines(v) Reduces risk of heart disease	[54]
–									

6	$C_{15}H_{13}N$	5-Methyl-2-phenylindolizine		207.27*	16.525	8.92	77, 130, 178	(i) Antibacterial(ii) Antitumor(iii) Treats dysentery and diarrhea	[55, 56]
–									
7	$C_{12}H_{18}O_2Si$	Propiophenone2-(trimethylsiloxy)		222.356	16.705	9.47	45, 75, 151, 177	(i) Antifungal(ii) Anti-leshmanial(iii) Insecticidal	[49, 57]
–									
8	$C_{14}H_{22}O_2$	2,4-Cyclohexadien-1-one, 3,5-bis(1,1-dimethylethyl)-4-hydroxy		222.32	16.705	9.47	57, 123, 179	Not reported	
–									
9	$C_{15}H_{13}N$	1H-Indole, 2-methyl-3-phenyl		207.27*	16.705	9.47	30, 77, 130, 178	(i) Anti-inflammatory(ii) Analgesic(iii) Immune-modulatory(iv) Antioxidant	[58, 59]
–									
10	$C_{16}H_{48}O_7Si_8$	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15, 15-hexadecamethyl		577.2	16.866	9.02	73, 147, 177	(i) Antioxidant(ii) Antibacterial	[48]
–									
11	$C_{15}H_{13}N$	1H-Indole, 1-methyl-2-phenyl		207.27*	16.781	5.68	27, 63, 165	(i) Anti-inflammatory(ii) Analgesic	[49, 59]
–									
12	$C_{10}H_{30}O_3Si_4$	Methyltris(trimethylsiloxy)silane		310.68	16.979	7.30	73, 133, 295	(i) Antioxidant(ii) Antibacterial(iii) Anti-inflammatory	[52]
–									
13	$C_{12}H_{22}Si_2$	1,2Bis(trimethylsilyl)benzene		222.47	17.111	13.17	43, 73, 119	(i) Antibacterial(ii) Antifungal(iii) Anti-inflammatory	[58, 60]
–									

14	$C_{15}H_{13}N$	2-Methyl-7-phenylindole		207.27*	17.263	11.75	30, 102, 165	(i) Colorimetric lipid peroxidation assay(ii) Anti-inflammatory(iii) Antibacterial	[58, 60]
–									
15	$C_{10}H_{13}NO_2$	Propanamide, *N*-(4-methoxyphenyl)		179.2	17.329	7.00	57, 120, 123, 164	(i) Formyl peptide receptor-2 agonists(ii) Antibacterial	[61, 62]

MF: molecular formula; MW: molecular weight; RT: retention time; *m*/*z*+: major fragments; Ref.: references; *: reference compound.

3.3. Antioxidant Properties of *A. digitata* Fruit Pulp Extracts

The antioxidant activities of aqueous and methanol extracts of baobab fruit pulp, analyzed as functions of DPPH radical scavenging, HRS, and NOS showed concentration-dependent increments ($p \leq 0.05$) (Table 4). Concentration-dependent increments of antioxidant activities were also reported by many other researchers [10, 14, 63, 64]. Maximum antioxidant activities of aqueous extract were observed in HRS (62.00 ± 1.41%) followed by DPPH (53.60 ± 0.81%) and NOS (51.60 ± 1.08%) at 1,000 *μ*g/mL. But with the methanol extract, maximum activities were observed in DPPH (72.3 ± 1.08%) followed by HRS (70.6 ± 1.08%) and NOS (68.6 ± 1.47%) at 250 *μ*g/mL. The antioxidant activities of the aqueous and methanol extracts at all concentrations were weaker than that of the ascorbic acid standard except for the methanol extract at 50 *μ*g/mL. The differences between the antioxidant activities of both extracts and the ascorbic acid standard were narrowed down at higher concentrations. Such a trend is a commonly reported observation and is linked to the performance and amount of the antioxidants [63, 64]. The reducing ability of extracts generally depends on the presence of antioxidants (reductones) that exert antioxidant activities by breaking free radical chains by donating hydrogen atoms [65]. But as the reactions progress, the antioxidants can be depleted and become limiting factors for the antioxidant activities. Moreover, the activities of such extracts decline after they passed the inhibitory concentration (IC_{50}) levels of the scavenging processes because the number of free radicals exceeds the number of antioxidants [24].

Table 4

Antioxidant activities of the *A. digitata* fruit pulp extract.

Extracts	Concentration (*μ*g/mL)	Mena (±SD) antioxidant activities of fruit pulp extract (%)			
DPPH	HRS	NOS	AAS	Aqueous	100
12.00 ± 0.70^{e}	13.00 ± 0.70^{e}	8.00 ± 0.70^{e}	44.30 ± 1.47^{e}	250	23.00 ± 0.70^{d}
34.00 ± 1.41^{d}	11.80 ± 0.54^{d}	61.30 ± 1.08^{d}	500	35.00 ± 0.70^{c}	41.00 ± 0.70^{c}
16.00 ± 0.70^{c}	69.30 ± 1.08^{c}	750	44.60 ± 1.08^{b}	52.30 ± 1.08^{b}	30.00 ± 0.70^{b}

72.30 ± 1.47^{b}	1,000	53.60 ± 0.81^{a}	62.00 ± 1.41^{a}	51.60 ± 1.08^{a}	77.60 ± 1.08^{a}
–					
Metha nol	50	22.10 ± 0.73^{e}	19.50 ± 0.35^{e}	18.00 ± 0.70^{e}	21.00 ± 0.70^{e}
100	33.20 ± 0.76^{d}	34.90 ± 0.60^{d}	32.60 ± 0.81^{d}	50.60 ± 1.08^{d}	150
50.00 ± 0.70^{c}	48.00 ± 0.70^{c}	42.16 ± 0.73^{c}	65.10 ± 0.54^{c}	200	62.10 ± 0.73^{b}
64.00 ± 0.70^{b}	56.60 ± 1.08^{b}	76.90 ± 0.63^{b}	250	72.30 ± 1.08^{a}	70.60 ± 1.08^{a}

DPPH: 2,2′-diphenyl-1-picrylhydrazyl; HRS: hydroxyl radical scavenging; NOS: nitric oxide scavenging; AAS: ascorbic acid standard. Means (±SD) in the same column with different letters are significantly different at $p \leq 0.05$.

The present study showed stronger antioxidant activities with the methanol extract. At 100 μg/mL, the antioxidant activities of the methanol extract were 2.77 times stronger for DPPH radical scavenging, 2.68 times stronger for HR scavenging, and 4.08 times stronger for NO scavenging than the aqueous extract. The antioxidant activities at 250 μg/mL were also 3.14 times stronger for DPPH radical scavenging, 2.08 times stronger for HRS, and 5.81 times stronger for NOS with the methanol extract compared to the aqueous extract. Even with the ascorbic acid standard, the antioxidant activities of the methanol extract at 100 μg/mL and 250 μg/mL were 1.14 and 1.43 times stronger, respectively. Even if the concentration of the aqueous extract was four times (i.e., 1,000 μg/mL) than that of the methanol extract (i.e., 250 μg/mL), its antioxidant activities were weaker by about 10–20%. In line with this finding, many studies have shown that types of solvents and further fractionation of the extracts using various solvents affect the antioxidant activities of the fruit pulp extracts [40, 66–68].

Several studies have also shown that the methods of the assay (analysis) [11, 33, 64, 67, 69] and the geographical location or ecology of the baobab fruit [11, 68] affect the antioxidant activities of the pulp extracts. In one study, assays of scavenging capacities were carried out using DPPH, ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)), and nitrite methods with n-hexane, ethyl acetate, chloroform, n-butanol, and residual aqueous fractions of the aqueous fruit extract. Whereas DPPH yielded the best performance with *n*-hexane fraction, ABTS and nitrite yielded best performances with n-butanol and ethyl acetate, respectively [67]. Likewise, a study by Braca et al. [11] using n-butanol fruit pulp extract reported better performance with the ABTS assay compared to DPPH and FRAP (ferric reducing antioxidant power) assays. The present study revealed that HRS was the best method of assay in showing stronger antioxidant activities with the aqueous fruit extract. With the methanol extract, the DPPH and HRS methods were comparable and better than the NOS.

4. Conclusion

The present study has provided us with important data about the phytochemical constituents of the Ethiopian baobab fruit pulp collected from Tekeze Valley. The aqueous extract of the fruit pulp was the source of flavonoids, phenols, saponins, tannins, and terpenoids. Stronger antioxidant activities were observed with the methanol extract than with the aqueous extract. GC-MS analysis generated 15 predominant compounds where 14 of them have multiple biological and pharmaceutical activities and properties. The differences in antioxidant activities due to differences in extraction solvents and methods of free radical scavenging assays would call for a further comprehensive study to develop a complete phytochemical profile of the Ethiopian baobab fruit pulp.

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Effect of Processing Methods on Antinutritional Factors (Oxalate, Phytate, and Tannin) and Their Interaction with Minerals (Calcium, Iron, and Zinc) in Red, White, and Black Kidney Beans

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ABSTRACT (ENGLISH)

The purpose of this study was to assess how different processing techniques affected mineral compositions, antinutritional factors, and their interactions in red, white, and black kidney beans consumed in Ethiopia. Mineral contents were found to be 41–44, 58–78, and 112–126 mg Ca/100 g in the raw, soaked, and cooked samples, respectively. Iron content in the raw, soaked and cooked samples were found to be 2.77–2.97, 1.94–2.20 and 2.87–3.28 mg Fe/100 g, respectively, showing 26–30% loss on soaking followed by 33–48% increase on cooking. While Zn content in the raw, soaked and cooked samples were found to be 2.47–3.26, 3.34–4.68 and 2.83–3.31 mg Zn/100 g, respectively, showing 35–43% increase on soaking followed by 15–29% decrease on cooking. In the case of antinutrients, both treatments showed incredible decrements. Phytate in the raw samples was 178-179 mg/100 g and showed a 12–16% decrement on soaking and a 37-38% decrement up on cooking, oxalate was 1.5–1.8 mg/100 g in the raw samples and showed a 4.4–13% decrement during treatments, and tannin in the raw samples was 102–160 mg/100 g and showed a 23–30% decrement on soaking, followed by 21–41% during cooking. Phytate : Ca and oxalate : Ca molar ratios in soaked and cooked samples were within the critical values in the raw samples. In contrast, phytate : Zn and Ca × phytate : Zn in all treatments were found to be within the critical value, confirming the good bioavailability of zinc in all the samples, while phytate : Fe was found over the critical value, showing its poor availability.

FULL TEXT

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1. Introduction

Kidney bean is one of the main food and income crops in Ethiopia. It is significant for the country's economy and has historically ensured food security in Ethiopia [1]. It ranks third among grain export in Ethiopia, contributing roughly 9.5% of all agricultural exports' total value. In many regions of Ethiopia, it is consumed as the primary traditional cuisine. It is mostly produced in Oromia, South Nations, Nationalities, and Peoples (SNNP), and Amhara regions of Ethiopia, which have respective area coverages of 146,452.41 hectare (ha) (41%), 117,969.97 ha (33%), and 81,235.07 ha (22.74%). It is primarily grown in Ethiopia's eastern, southern, southwestern, and rift valley regions. Remaining 3.25% is produced in other regions of Ethiopia [2]. The southern part of the country, Sidama region, and Gamo Gofa zones produce red and speckled types mainly for home consumption. While in the eastern part, mainly Hararghe highlands, people preferred mostly speckled and white beans [2].

Kidney bean, *Phaseolus vulgaris*, green bean or common bean, is an annual, climbing plant in *Fabaceae* (legume or bean family) in the genus *Phaseolus* L. that sourced in Central and South America and is now cultivated in many parts of the world [3]. Depending on the variety, they may tolerate a wide range of environmental conditions from sea level to about 3000 meters above sea level (m.a.s.l.). However, due to poor pod set brought on by high temperatures, it does not produce fruit below 600 meters [3]. Some of the well-known varieties of kidney beans include red, white, and black ones. These beans are an excellent source of pertinent nutrients and contain a lot of protein, important minerals, and carbohydrates. However, certain antinutritional factors (phytate, oxalate, tannin, and lectin) present in them have an impact on how biologically nutrients are utilized [3].

Minerals are necessary for all living things to be healthy. It is vital to remember that minerals play a combined function in human nutrition and health, just like proteins, carbs, and fats do [4]. One or more of the body's functions are impaired by a mineral shortage. Due to high phytic acid concentration in diets, Zn and Fe are two of the micronutrients that are most frequently deficient in developing nations [4]. The bioavailability of plant-based micronutrients such as iron and zinc is generally influenced more by dietary variables than by macronutrients [4]. The balance between variables that either impede or increase nutrient absorption in the entire diet determines the overall influence on nutrient bioavailability [5].

Phytate is a natural substance known to be antinutritional components in legumes and regarded as a primary storage compound for phosphorus [6]. Essential mineral nutrients are bound by negatively charged phosphate groups in phytic acid, which prevents the body from absorbing them [7]. Phytate particularly affects Zn and Fe, as their deficits have been linked to excessive phytate intake [4, 7].

Oxalate salts are insoluble salts that are naturally found in both the human body and plants. They can exist in the form of soluble salts of potassium and sodium, soluble salts of calcium, magnesium, and iron, or a combination of the two [8]. While soluble oxalate affects the body by blocking the body's ability to absorb dietary calcium and other minerals by building a potent chelate with them, insoluble oxalate is eliminated in urine [9]. The kidney builds up insoluble calcium oxalate in crystal form, generating kidney stones that lead to renal failure. Although water-soluble minerals were also leached out at the same time as the oxalate level was reduced, cooking may produce obvious skin rupture and speed up the leakage of soluble oxalate into cooking water [10].

Tannin is a polyphenolic compound that prevents the body from utilizing vitamins, proteins, and minerals; it is seen to be nutritionally undesirable. It has the capacity to interact with proteins through covalent and hydrogen bonding interactions, which causes proteins to precipitate [11]. They are made up of a remarkably varied assortment of oligomers and polymers [12]. Fortunately, tannins are soluble in water, with the exception of some structures with higher molecular weights.

Despite many advantages that plant-based diets offer, they are less nutrient-dense when ingested improperly [13]. A side effect of eating too much kidney beans may include bloating or gas [14]. However, traditional household practices such as soaking and cooking can decrease the antinutritional content significantly.

The review of the literature reveals that studies on the nutritional and antinutritional composition of red kidney beans have been published from Nigeria, Pakistan, and India [4]. On the other hand, few in-depth studies have been conducted on Ethiopian-originated kidney beans. People are consuming food cooked just after soaking it for a great

deal of time (5–10 hours), but there is no guarantee of the food's quality. Some minerals may not be available in sufficient quantities or may not be processed in the best way possible, preventing people from obtaining all resources found in these plants. This study's objectives were to determine how different processing techniques affected antinutrients phytate, oxalate, and tannin as well as to evaluate minerals calcium, zinc, and iron in samples of red, white, and black kidney beans with respect to their respective molar ratios to gauge the body's mineral absorption. The study significantly advanced our knowledge of the levels of antinutritional compounds such as oxalate, phytate, and tannin contained in red, white, and black kidney beans, as well as how these compounds interact with minerals such as calcium, iron, and zinc to affect health and nutrition.

2. Materials and Methods

2.1. Instruments

An atomic absorption spectrophotometer (AA-6800 AAS Shimadzu, Japan), a UV-Vis spectrophotometer (CECIL, CE 1021, 1000 series, UK), a mechanical shaker (Optima OS-762 Shaker for Incubator 30–300 rpm Load Max 3.0 kg), a vortex mixer (Electro Scientific Industries SI-0286 Vortex Mixer (GENIE2) 3220 rpm/3350 rpm), and a centrifuge (DYNAC II centrifuge, Clay Adams, division of Becton Dickinson and Company, USA) were used.

2.2. Chemicals

All solutions were made using chemicals suitable for analytical grade reagents. $HClO_4$ (70% (Riedel-de Haën, Germany), HCl (37% Riedel-de Haën, Sigma-Aldrich Chemicals GmbH, Germany), HNO_3 (about 69% LR, Eurostar Scientific Ltd., UK), sulfosalicylic acid (Merck, Germany), $FeCl_3.6H_2O$ (iron(III) chloride crystals, Merck, Germany), sodium phytate salt (phytic acid dodeca sodium salt hydrate water 10–15% product, Aldrich, USA), 3 M H_2SO_4 (98%, (Riedel-de Haën, Germany), 0.1 M $KMnO_4$ (min. 99%, Alpha Chemika) solution that was preheated for 1 h in the water bath at 90°C, methanol (30%), and vanillin-HCl solution were used, standard metal ion solutions (1000 mg/L) were purchased from AAS Company Vorna Valley (AAS standard, Germany), and freshly prepared deionized water was used in all experiments.

2.3. Sample Collection

Red, white, and black kidney bean samples were collected from Sidama (Bansa Daye), Amhara (Bure, Gojam), and Oromia (Arsi Negele) regions of Ethiopia. They were transported to the laboratory, cleaned, dried, and stored in hermetically sealed polyethylene bags for future processing.

2.4. Sample Preparation

About 1 kg of red, white, and black kidney beans was manually washed, dried in open air conditions, and divided into two groups (raw and processed). The beans in the processed group were further divided into two equal groups just after soaking for overnight (16 h), and soaking water was drained off. For cooking, half of the soaked samples were cooked in distilled water at 100°C for 60 ± 5 min and allowed to air-dry (at room temperature for 72 h) according to the method used by Ihemeje et al. [15] with little modification of soaking and cooking time. The dried samples (raw, soaked, and cooked) were ground to fine powder using a laboratory electrical grinder and sieved using a sieve of 0.425 mm mesh size, packed into airtight-sealed polyethylene bags, and left for later analysis [15]. The flours of the kidney bean samples were subsequently analyzed for their minerals (calcium, zinc, and iron) and antinutritional factors (phytates, oxalates, and tannins).

2.5. Soaking

The red, white, and black kidney beans were all individually steeped in distilled water for 16 hours at room temperature in the dark with little modification of soaking hours [15]. After that, the soaking water was drained out. The seeds were divided into two halves, with one of them being air-dried for 72 hours to ensure full drying. Samples were ground to pass through a 30-mesh screen. The ground samples were kept in airtight bottles and stored at 4°C for subsequent analysis [15].

2.6. Cooking

The presoaked seeds of red, white, and black kidney beans were cooked on a hot plate (about 60 ± 5 min) until they were readily crushed with the thumb and index fingers. The cooking liquid and seeds were separated by filtration, and the cooked seeds were dried in the same manner as after the soaking phase [15].

Each of the three sets of samples was analyzed in triplicates for their oxalate, phytate, tannin, and minerals (Ca, Fe, and Zn). An analysis of samples was carried out according to the available standard/published methods depending on the availability of lab requirements. UV-Vis spectrophotometry (for phytate and tannin), atomic absorption spectrometry (for calcium, iron, and zinc), and the $KMnO_4$ titrimetry method (for oxalate) were used, respectively [15–17].

2.7. Digestion for Mineral Determination

Exactly, 0.5 g of homogenized and powdered red, white, and black kidney beans was transferred separately into a 250 mL round-bottomed flask and connected to a reflux condenser. 4 mL of 69% nitric acid (HNO_3) and 1 mL of 70% perchloric acid ($HClO_4$) (4 : 1 ratio) at a temperature of 300°C were added to the flask. The mixture was heated for 3 h over a hot plate until the solution was clear and colorless. Then, the solution was allowed to cool for 20 min before the condenser was dismantled and 5 min after dismantling the condenser. Thereafter, 5 mL of deionized water was added, and the solution was filtered through Whatman No. 41 filter paper into a 25 mL volumetric flask. This procedure was followed according to the method developed by Ayele et al. [4] and Maki et al. [16] with some modification in the type and volume of reagents. The filter paper was washed thoroughly, and washing was collected in the flask. To decrease interferences on calcium from phosphates, sulfates, or anticoagulants such as oxalates, citrates, and heparin, 2.5 mL of 10% lanthanum chloride solution was added to the flask since calcium needs to be measured [4]. Finally, it was diluted to the mark (25 mL) with deionized water. The blank was prepared by taking the same amount of reagent through all steps.

2.8. Determination of Phytate, Oxalate, and Tannin

Oxalate was determined by the AOAC 2005 method [17], tannin was determined by the vanillin-HCl assay method using a UV spectrophotometer [18], and the phytate content was determined by the method described by Latta and Eskin [19] and later modified by Vaintraub and Lapteva [20].

The molar ratio was calculated by dividing the mole of antinutrient to the mole of minerals [4, 21]. To assess the impact of high levels of phytate and oxalate on the bioavailability of dietary minerals (zinc, calcium, and iron), the molar ratios of phytate to calcium, phytate to iron, phytate to zinc, oxalate to calcium, and phytate to calcium/zinc computed as the ratios are the better indicators of bioavailability than the amounts of mineral and phytic acid in the diet [16].

2.9. Recovery (Validation and Accuracy)

Accuracy of an analytical method is the degree of agreement of test results generated by the method to the standard true value which is estimated by spiking the sample with a known amount of a standard of 1000 mg/L stock solution [22]. A known concentration of 40% of the analyzed minerals (calcium, iron, and zinc) and antinutrients (phytate, oxalate, and tannin) was added to 0.5 g of powdered kidney bean samples in three treatments (raw, soaked, and cooked). The spiked samples were digested and analyzed at optimum conditions as the method to be validated, and the outcomes were contrasted with the anticipated rise in the parameter to be studied in comparison to the raw data [4].

The limit of detection (LOD) is the lowest analyte concentration that produces response detectable but not necessarily quantifiable above the noise level of the system. It is calculated as $LOD = 3 \times SD_{blank}$ [22]. Low LOD indicates the presence of trace amounts of metals of interest in the sample that can be detected by the given method. The limit of quantification (LOQ) is the smallest quantity of analyte that can be measured with acceptable accuracy and precision, which is calculated as $LOQ = 10 \times SD_{blank}$ [22]. The linear range and precision were tested for the characteristics for the determination of minerals and antinutrients [22]. The wavelength, limit of detection (LOD), limit of quantification (LOQ), correlation coefficient (R^2), and calibration curve equation for mineral determination in kidney bean samples are given in Table S1. As can be seen from the table, the wavelength used for the determination of Ca, Zn, and Fe is 422, 213, and 248 nm, respectively. LOD and LOQ are low enough to determine the trace levels of the three metals of interest. The correlation coefficients (R^2) of the calibration curves are greater than 0.999 for all the three metals which show an excellent relationship between the absorbance and concentration of the three metals.

2.10. Statistical Analysis

Mineral composition and antinutritional factors of the raw and processed samples of three different types of kidney bean were statistically compared using the analysis of variance (ANOVA) and least significant difference (LSD). The statistical package used was SPSS version 25. Significant differences were determined at a $p < 0.05$ level [17]. All the results for minerals (calcium, iron, and zinc) and antinutritional factors (oxalate, phytate, and tannin) were reported as the mean value with their respective standard deviations. The results were reported as mg analyte/100 g of raw, soaked, and cooked samples for consistency and comparison.

3. Results and Discussion

3.1. Calibration Curve for Minerals and Antinutrients

By serially diluting a 1000 mg/L standard stock solution with deionized water and taking precise measurements of the standard solutions and reagents, respectively, the intermediate and working standards solutions of each mineral (zinc, iron, and calcium) and antinutrients (phytate, oxalate, and tannin) were prepared [15]. The calibration curve was obtained by running a series of prepared working standards for all minerals and antinutrients. The correlation coefficient varies from 0.9974 to 0.9996 for minerals and 0.9974 to 0.9975 for antinutrients, which shows a very good linearity of the curves. The calibration curves are given as supporting information (Figures S1 and S2). The correlation coefficients (R^2) of the calibration curves of three metals clearly show that there is a good relationship between the absorbance and concentration of the three metals (Figure S1). Similarly, the correlation coefficients (R^2) of the calibration curves of two antinutrients clearly show that there is a good correlation between absorbance and the two antinutrients (Figure S2).

3.2. Minerals

The mineral contents of the raw, soaked, and cooked red, white, and black kidney bean samples are shown in Figure 1.

[figure(s) omitted; refer to PDF]

3.2.1. Calcium

Calcium content showed a significant difference $p < 0.05$ in both processing conditions (soaking and cooking) for all kinds of kidney bean samples (red, white, and black). The effect of soaking and cooking on the calcium content showed an increment from 41–44 mg/100 g for the raw sample to 58–78 mg/100 g and 112–126 mg/100 g for the soaked and cooked samples, respectively. This implies that the processing method shows a significant increment of calcium from 41.46 to 43.59% during soaking and 38.19 to 48.21% upon cooking, which is similar to the results reported by Akin-Idowu et al. [19] that explained an increment of calcium on soaking followed by cooking. The reason for a significant increment of calcium content during soaking is most probably due to loss of soluble minerals and other components in the soaking water. The Ca-oxalate complex is insoluble in water and hence remains in kidney beans, resulting in the increase in the calcium content in the kidney beans after soaking.

3.2.2. Iron

The iron content shows a significant difference $p < 0.05$ between the raw, soaked, and cooked conditions in all the samples (red, white, and black kidney beans). Iron content in the raw, soaked and cooked samples were found to be 2.77–2.97, 1.94–2.20 and 2.87–3.28 mg Fe/100 g, respectively, showing 26–30% loss on soaking followed by 33–48% increase on cooking. This might be because of leaching of iron. Upon cooking, the content of iron increased from 1.94 to 3.28 mg/100 g, 2.20 to 2.87 mg/100 g, and 2.21 to 3.01 mg/100 g for the red, white, and black kidney beans, respectively, which means it showed a 32.62–47.93% increment during cooking, which is similar to the result reported by Omoruyi et al. [23]. This might be due to the releasing of iron from antinutritional factors, mainly phytate.

3.2.3. Zinc

The levels of zinc in all samples showed increments during soaking. The results obtained were 2.82–3.78 mg/100 g, 3.26–4.68 mg/100 g, and 2.47–3.34 mg/100 g for the red, white, and black kidney bean samples, respectively, which means it showed a 26.04–43.55% increment during soaking. The result is consistent with the one reported by Akin-Idowu et al. [19]. But loss of zinc was observed after cooking. The results were 3.78−2.83, 4.68−3.31, and 3.34−2.89 mg/100 g for the red, white, and black kidney bean samples, respectively, which means a 15.3–29.27%

decrement was observed during cooking that might be due to leaching with the cooking water.

3.3. Antinutrients

Antinutritional contents of red, white, and black kidney bean samples in raw, soaked, and cooked conditions are shown in Figure 2.

[figure(s) omitted; refer to PDF]

3.3.1. Phytate

From all the antinutritional factors, phytic acid is considered as one of the main problems for human health and nutrition [15]. Soaking was previously reported to reduce phytates [16] due to leaching of phytate ions into the soaking water. The same thing happened in our research for both treatments. Furthermore, water consumption activates the bean's phytase enzymes, which decrease and breakdown phytates [10].

In our study, the effect of soaking and cooking showed a significant difference at $p < 0.05$, and the loss of phytate during both treatments is 37.3%, 35.9%, and 61.7 39.6%, respectively, for the red, white, and black kidney bean samples, which is higher than in the result reported by Bhandari and Kawabata [24]. They reported the average loss of phytates during cooking to be 20% which coincides with the result reported by Ihemeje et al. [15] that reported a 32.8–44.7% decrement of phytate upon cooking. In this study, the highest amount of phytate is observed in red kidney bean, which is 179, 157, and 98.2 mg/100 g for raw, soaked, and cooked beans, respectively. Phytate mainly reduces the bioavailability of dietary zinc by forming insoluble mineral chelates [22].

3.3.2. Oxalate

Oxalate is reported to have an effect comparable to that of phytate [22]. Oxalate mainly binds calcium and makes it unavailable for absorption by the body, causing the formation of kidney stones [25]. It significantly affects the availability of calcium only when the ratio of oxalate: Ca is greater than one [26].

In our findings, almost the same amounts of oxalate are found in all types of kidney beans, and a significant loss of oxalate is not expected if the soaking and cooking water is not discarded after all [27] because the higher percentage of oxalate reduction during cooking may also be due to its solubility in boiling water.

3.3.3. Tannin

Tannin shows a significant difference $p < 0.05$ in both treatments. The highest amount of tannin in all treatments is observed in red kidney bean, which is 160, 112, and 88.8 mg/100 g for raw, soaked, and cooked beans, respectively. The values were very small as compared to 3833–4533 mg/100 g as reported by Ruchi and Sheet [28] on red kidney beans (raw and processed). The reduction in the tannin content may be due to leaching of polyphenols into the soaking water [29, 30].

3.4. Molar Ratios and Bioavailability of Minerals

Bioavailability is the percentage of a mineral's total amount that may be absorbed in a form that is metabolically active [31]. The calculated values of the molar ratios were also compared with the reported critical values for these ratios [31]. Phytate : Ca, phytate : Fe, phytate : Zn, and Ca × phytate/Zn molar ratios of red, white, and black kidney bean samples under three processing conditions are given in Table 1.

Table 1

Phy : Ca, Phy : Fe, Phy : Zn, and Ca × Phy/Zn molar ratios of red, white, and black kidney bean samples under three processing conditions.

Samples	Phytate (mmol/100 g)	Ca (mmol/100 g)	Zn (mmol/100 g)	Fe (mmol/100 g)	Phytate : Ca molar ratio	Phytate : Zn molar ratio	Phytate : Fe molar ratio	(Ca × phytate/Zn)
RR	0.27	1.02	0.043	0.051	0.26	6.30	5.31	6.40
SR	0.24	1.5	0.058	0.035	0.16	4.10	6.77	6.21

CR	0.15	2.8	0.044	0.059	0.051	3.36	2.51	9.54
RW	0.27	1.1	0.050	0.049	0.24	5.4	5.51	5.94
SW	0.23	1.94	0.072	0.039	0.12	3.14	5.79	6.25
CW	0.15	2.95	0.051	0.051	0.051	2.84	2.84	8.63
RB	0.27	1.07	0.038	0.053	0.25	7.13	5.11	7.63
SB	0.24	1.45	0.051	0.039	0.16	4.63	6.05	6.86
CB	0.14	3.14	0.044	0.054	0.04	3.23	2.63	10
Critical values					<0.24	<15	<1	<0.5 mol/kg

Sample code: RR: raw red; RW: raw white; RB: raw black; SR: soaked red; SW: soaked white; SB: soaked black; CR: cooked red; CW: cooked white; CB: cooked black.

3.4.1. Phytate:Ca

The phytate:Ca molar ratios in two treatments (soaked and cooked) are less than 0.24, which shows the good bioavailability of calcium [25]. But they vary from 0.24 to 0.26 for raw samples which will have less bioavailable calcium for the body because they are trapped by antinutrients mainly by oxalate and phytate and released during the treatments [19].

3.4.2. Phytate:Fe

The phytate:Fe molar ratios are greater than one in both for all kinds of samples, which is a sign of poor iron bioavailability [32]. Processing methods showed a bit increment on iron content, especially cooking, but do not make it sufficiently available.

3.4.3. Phytate:Zn

The phytate:Zn molar ratios of both processing conditions are less than 15 and indicate good bioavailability of zinc [25, 32]. The bioavailability of zinc is reduced by phytate [32]. Hence, the phytate:Zn molar ratio is considered a better signal of zinc bioavailability than total dietary phytate levels alone [25]. For the good bioavailability of zinc, at least phytate:Zn molar ratios need to be within a range of 10–15 [32].

3.4.4. Phytate × Calcium to Zinc (Ca × Phytate/Zn)

The molar ratios of Ca × phytate/Zn of samples range from 5.94 to 10.0. The potent effect of calcium on zinc absorption in the presence of high phytate intakes has led to the suggestion that the phytate × Ca/Zn millimolar ratio may be a better index of zinc bioavailability than the phytate:Zn molar ratio alone [26]. High calcium levels in foods can promote the phytate-induced decrease in zinc bioavailability when the Ca × phytate/Zn molar ratio exceeds 0.5 mol/kg [32]. In this study, the values of all the samples were within the critical molar ratios of Ca × phytate/Zn, which indicates the bioavailability of zinc is not affected by the kinetic synergism between calcium and zinc.

3.4.5. Oxalate to Calcium Ratio (Oxalate:Ca)

To know the progress of availability of minerals from reduction of oxalate content after soaking followed by cooking, the oxalate to calcium (oxalate:Ca) molar ratio is calculated, and it is all within the critical values (<1), which implies that oxalate cannot have any adverse effects on bioavailability of dietary calcium in all types of samples just after proper treatments. The results are given in Table 2.

Table 2

Oxalate:calcium molar ratios of red, white, and black kidney bean samples.

Samples	Calcium (mol/100 g)	Oxalate (mol/100 g)	Oxalate : Ca molar ratio
RR	1.02	0.019	0.02
SR	1.5	0.017	0.01
CR	2.8	0.014	0.005
RW	1.1	0.016	0.01
SW	1.94	0.015	0.007
CW	2.95	0.014	0.005
RB	1.07	0.019	0.02
SB	1.45	0.016	0.012
CB	3.14	0.015	0.005
Critical values	<1		

Sample code: RR: raw red; RW: raw white; RB: raw black; SR: soaked red; SW: soaked white; SB: soaked black; CR: cooked red; CW: cooked white; CB: cooked black.

3.5. Comparison of Mineral (Ca, Fe, and Zn) and Antinutrient (Phytate, Oxalate, and Tannin) Contents of Ethiopian Red, White, and Black Kidney Beans with Those of Other Reported Literature

It has been observed that a food crop's mineral and antinutritional compositions are directly correlated with its genetic background, geographic origin, and soil characteristics [29]. Therefore, it is crucial to see our results of Ethiopian kidney beans compared with those of other reported literature from different parts of the world. The comparison between this study (kidney beans from Ethiopia) and other reported literature (kidney beans from India, Nigeria, Kenya, Pakistan, and West Africa) is given in Tables 3 and 4.

Table 3

Comparison of minerals (Ca, Zn, and Fe) found in Ethiopian red kidney bean with other reported values mg/100 g.

Countries	Processing conditions	Amount of minerals (mg/100 g)			References
Calcium	Iron	Zinc	Ethiopia	Raw	41
2.85	2.82	This study	Soaked	60	1.94
3.78	Cooked	112	3.28	2.83	·
Pakistan	Raw	54.9	11.5	2.7	[33]

Soaked	54.2	7.4	2.3	Cooke d	56.3
10.5	3.0	-			
Nigeria	Raw	28	1.5	NR	[15]
Soaked	30	1.2	NR	Cooke d	32.5
1.9	NR	-			
India	Raw	58	0.89	3.0	[34]
Soaked	NR	NR	NR	Cooke d	112
1.25	6.4	-			
Kenya	Raw	NR	6.5	1.8	[32]
Soaked	NR	4.9	3.7	Cooke d	NR

NR = not reported.

Table 4

Comparison of antinutrients (phytate, oxalate, and tannin) found in Ethiopian red kidney bean with other reported values mg/100 g.

Countries	Processing conditions	Antinutrients (mg/100 g)			References
Phytate	Oxalate	Tannin	Ethiopia	Raw	179
1.79	160	This study	Soaked	157	1.60
112	Cooked	98.2	1.32	88.4	·
Pakistan	Raw	610	NR	610	[33]
Soaked	610	NR	630	Cooked	630
NR	110	-			
Nigeria	Raw	320	18.3	78	[15]

Soaked	215	15.2	87	Cooked	177
10.0	56	-			
Kenya	Raw	207	NR	163	[35]
Soaked	189	NR	106	Cooked	178
NR	63	-			
West Africa	Raw	341	115	112	[36]
Soaked	291	109	110	Cooked	194

NR = not reported.

The general trend in the calcium content in the raw, soaked, and cooked kidney beans found in the present study is similar to that reported from India and Nigeria. However, there is a wide variation in the values of calcium contents in the kidney beans from the three countries (Ethiopia 41–112, India 58–112, and Nigeria 28–32.5 mg/100 g). These values are different from that reported from Pakistan (54.2–56.3), which shows no change in the calcium content upon soaking and cooking. The general trend in the iron content in the raw, soaked, and cooked kidney beans found in the present study is similar to that reported from all the other countries. Although there is a variation in the value of iron contents among the five countries, the iron content of Ethiopian kidney beans (1.94–3.28 mg/100 g) is lower that from Pakistan (7.4–11.5 mg/100 g) and Kenya (4.9–6.8 mg/100 g) but higher than that from India (0.89–1.25 mg/100 g) and Nigeria (1.2–1.9 mg/100 g). The zinc content in the kidney beans from Ethiopia (2.82–3.78 mg/100 g) and other countries (Pakistan 2.3–3.0, India 3.0–6.4, and Kenya 1.8–3.7 mg/100 g) does not show any clear trend. The zinc content is not reported in the kidney beans from Nigeria.

The general trend in the phytate content in the raw, soaked, and cooked kidney beans found in the present study is similar to that reported from Nigeria, Kenya, and West Africa. While kidney beans from Pakistan showed a very high phytate content (610–630 mg/100 g) and did not show any effect on the phytate content upon soaking and cooking. The phytate content of Ethiopian kidney beans (179−98.2 mg/100 g) is lower than that reported from Nigeria (320−177 mg/100 g), Kenya (207−178 mg/100 g), and West Africa (341−194 mg/100 g). The general trend in the oxalate content in the raw, soaked, and cooked kidney beans found in the present study is similar to that reported from Nigeria and West Africa. However, the oxalate content of Ethiopian kidney beans (1.79−1.32 mg/100 g) is much lower than that from Nigeria (18.3−10.0 mg/100 g) and West Africa (115−94 mg/100 g). The oxalate content in kidney beans from Pakistan and Kenya is not reported. The general trend in the tannin content in the raw, soaked, and cooked kidney beans found in the present study is similar to that reported from Nigeria, Kenya, and West Africa but different from that of Pakistan. The tannin content of kidney beans from Ethiopia (160−80.4 mg/100 g) is higher than that from Nigeria (78−56 mg/100 g) and similar to that from Kenya (163−63 mg/100 g) and West Africa (112−59 mg/100 g).

4. Conclusion

This study gives information on the mineral contents (Zn, Fe, and Ca) present in samples of red, white, and black kidney beans of Ethiopian origin and the effects of antinutritional factors (phytate, oxalate, and tannin) found in them under two treatments. In addition, the relative bioavailability of the minerals is assessed by calculating molar ratios of antinutrients to the minerals, and the results are compared with the critical values to confirm the bioavailability of minerals. The results obtained from the study showed that both treatments (soaking and cooking) significantly reduce antinutritional factors and increase the bioavailability of the minerals. Processing methods resulted in a significant increment of calcium (41.46–43.59%) during soaking and 38.19–48.21% upon cooking. Iron showed a

decrease just after soaking (26–30%), followed by a 33–48% increment upon cooking. While an increment of zinc was observed upon soaking, it resulted in a 15–29% decrease during cooking. In contrast to minerals, both treatments displayed an astounding decrease in antinutritional factors. Phytate showed a 12–16% decrease upon soaking, followed by a 37–38% decrease upon cooking. Oxalate resulted in a 4.4–13% decrease in both treatments. Tannin showed a 23–30% decrease upon soaking, followed by a 21–41% decrease during cooking. Phytate : Ca and oxalate : Ca molar ratios in soaked and cooked samples were within the threshold ranges in the raw samples. The phytate : Zn and Ca × phytate : Zn molar ratios in all treatments were found to be within the critical value, proving the good bioavailability of zinc in all samples, while phytate : Fe was shown to be above the critical value, demonstrating its poor availability. All kinds of kidney beans are used as a source of calcium and zinc, while the phytate : Fe molar ratio indicates that iron from all kinds of kidney beans is not bioavailable. The results obtained from the study showed that both treatments (soaking and cooking) significantly reduce antinutritional factors and increase the bioavailability of the minerals. The measured antinutrient to mineral ratio also demonstrated the effectiveness of the strategies for lowering antinutrients that might improve the nutritional characteristics of kidney beans. The results found showed that there is no significant difference between the three types of kidney beans in terms of nutritional quality (minerals concentrations) and antinutritional factor contents.

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DETAILS

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Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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Structural Identification of Impurities in Pioglitazone Hydrochloride Preparations by 2D-UHPLC-Q-Exactive Orbitrap HRMS and Their Toxicity Prediction

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ABSTRACT (ENGLISH)

Pharmaceutical companies and regulatory agencies have more and more concerns for impurities in pharmaceuticals and their toxicity. In this work, heart-cutting two-dimensional ultrahigh-performance liquid chromatography (2D-UHPLC) in combination with high-resolution mass spectrometry (HRMS) was used, setting HRMS as positive mode of electrospray ionization to identify five impurities in pioglitazone hydrochloride preparations. With the heart-cutting 2D-UHPLC and online desalting technique, the structures of five impurities were deduced in an analysis of MS^n data. And three of them, Impurity-2, Impurity-3, and Impurity-5, have never been reported before. The fragmentation patterns of five impurities were proposed on a basis of accurate mass and fragment ions in this study. Since the toxicity of impurities is relevant to their structures, toxicology of all five impurities was predicted by three software tools, and the result showed that these compounds have good safety profile.

FULL TEXT

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1. Introduction

As a thiazolidinedione insulin sensitizer, pioglitazone can activate peroxisome proliferator-activated receptor-gamma (PPAR-*γ*), which is distributed predominantly in the adipose tissue, skeletal muscle, and liver [1–3]. PPAR-*γ* regulates lipogenesis, fatty acid storage, insulin sensitivity, and glucose metabolism [4]. Pioglitazone, a potent and selective PPAR-*γ* agonist, can improve insulin sensitivity and enhance hyperglycemia [5]; thus, it has been widely used for treatment on type 2 diabetes mellitus (T2DM) [6]. Pioglitazone hydrochloride manufactured by Takeda Pharmaceutical Company Limited was approved in 1999 in the US and marked as ACTOS® [7].

The impurities of drug product have negative impaction for its safety and its efficacy. This study focused on the related substances of pioglitazone hydrochloride preparations from the surveillance sampling of Shandong Medical Product Administration. The method described in European Pharmacopeia (EP) 10.0 Pioglitazone Hydrochloride was applied to analyze the impurities in inspected samples. Excepting eight identified impurities, five unidentified impurities were detected with high frequency in 54 batches of inspected pioglitazone hydrochloride preparations that were produced by 13 manufacturers.

UHPLC-Q-exactive orbitrap HRMS is widely used for the detection and identification of impurities, as UHPLC (ultrahigh-performance liquid chromatography) provides a rapid and effective separation, while HRMS (high-resolution mass spectrometry) offers accurate mass and fragment ions, which are beneficial for structural elucidation [8, 9]. It is known that all LC-MS methods are based on a volatile salt in mobile phase. Although ammonium acetate, used for separation and determination of the related substances in EP 10.0 for pioglitazone hydrochloride, is a volatile salt, its high concentration leads to strong ion suppression effect which may decrease the MS sensitivity of the impurities. Therefore, no peak was detected in the total ion chromatogram (TIC) when target impurities were directly injected into MS without desalting. Over the past decades, two-dimensional liquid chromatography (2D-LC) technique has been developing rapidly and been applied extensively in the field of pharmaceutical analysis [10–12]. Generally, 2D-LC can be classified into two types: comprehensive two-dimensional liquid chromatography which transports continuous stream of effluent from 1D column into 2D column, and heart-cutting two-dimensional liquid chromatography which transfers targeted portion (the peak of aimed impurity) of the 1D effluent into the 2D column. For heart-cutting 2D-LC, the first chromatographic dimension is utilized to trap the targeted impurities into quantitative loop by a switching valve, while the second chromatographic dimension serves as an online desalting segment which can remove the nonvolatile salt or high concentration of volatile salt from the first dimension with a low concentration of volatile mobile phase [10]. And the present study employed a heart-cutting 2D-LC coupled with high-resolution mass spectrometry (HRMS) to characterize the structures of five unidentified impurities, and three of them was never been reported before.

Assessing the biological toxicity of impurities is beneficial for the quality control of drug products. While animal model

for toxicity accessing has many constrains, researchers focus on computational methods. Since impurity toxicity is closely relevant to its molecular structure, structure-activity relationships (SARs) have been normally used in the pharmaceutical industry to estimate their toxicity by computer [13]. In this work, ADMET Predictor™ 8.0, Derek Nexus 5.0.1 (knowledge-based), and Sarah Nexus 2.0.1 (statistics-based) were employed to evaluate the toxicity of target impurities based on the speculated structures.

2. Experimental Methods

2.1. Samples and Reagents

54 batches of investigated pioglitazone hydrochloride preparations (including pioglitazone hydrochloride tablets and pioglitazone hydrochloride capsules) were obtained from the surveillance sampling of Shandong Medical Product Administration. The reference substance pioglitazone hydrochloride (purity 100.0%, batch number 100634–201703) was purchased from the National Institute for Food and Drug Control (Beijing, China). Ammonium acetate (purity 98.3%) and acetonitrile (HPLC grade) were supplied by Fisher Chemical (USA). Water used for all analyses was purchased from Watsons (China).

2.2. Instrumentation

The Agilent 1260 Infinity II HPLC (Agilent Technologies, USA) was used for screening impurities for investigation. The UHPLC-HRMS System (Thermo Fisher Scientific, Germany) consists of ultimate 3000 pump, autosampler, column compartment, and orbitrap high-resolution mass spectrometer. Tune 2.9 software (Thermo Fisher Scientific, USA) was used to control the mass spectrometer. XCalibur 4.0 software (Thermo Fisher Scientific, USA) was used for instrument control and data processing. Compound Discoverer 3.1 was adopted to analyze the molecular formulas of investigated impurities. Mass Frontier 7.0 software was utilized to analyze the fragmentation mechanism of mass spectrometry. Chromatographic separation was achieved by an Inertsil ODS-3 C18 column (250 mm*4.6 mm, 5 mm) (Thermo Fisher Scientific, USA). The desalting was achieved by an Inertsil ODS-SP C18 column (150 mm*4.6 mm, 5 mm) (Thermo Fisher Scientific, USA). The centrifugation was performed on a 5804R refrigerated centrifuge (Eppendorf, Germany). The ultrasonic process was operated on a KQ-500DE Thermostat Ultrasonic Instrument (Kunshan, China). MS105DU Analytical Balance (Mettler Toledo, Switzerland) was used to weight. ADMET PredictorTM 8.0 software (Simulations plus Inc., USA), Derek Nexus 5.0.1 software (Lhasa Limited, UK), and Sarah Nexus 2.0.1 software (Lhasa Limited, UK) were employed to predict the toxicity of impurities.

2.3. Sample Preparation

54 batches of pioglitazone hydrochloride preparations were in the form of tablets or capsules. For tablets, 20 tablets were grinded into homogeneous powder, whereas for capsules, the shells of 20 capsules were removed and then the powder was mixed. The powder (containing about 20 mg pioglitazone) was accurately weighed and transferred into a 100 mL volumetric flask, followed by adding 20 mL of methanol to dissolve by sonication. Then, the sample was diluted with mobile phase to volume and mixed well, followed by centrifugation at 8000 rpm for 10 min. Finally, 20.0 μL of the top supernatant was taken for screening target impurities, and 100.0 μL of the supernatant was injected for online two-dimensional UHPLC-HRMS analysis.

2.4. Chromatographic Conditions

First-dimensional separation conditions are as follows: chromatographic column: Inertsil ODS-3 C18 column (4.6 × 250 mm, 5 μm); column temperature: 35°C; mobile phase: 0.1 mol/L solution of ammonium acetate, acetonitrile, and glacial acetic acid (25 : 25 : 1, V/V/V); detection wavelength: 269 nm; flow rate: 0.7 mL/min. This first-dimensional separation condition was also performed on Agilent 1260 Infinity II HPLC to analyze 54 batches of samples, subsequently screening targeted impurities.

Two-dimensional separation conditions are as follows: chromatographic column: Inertsil ODS-SP C18 column (4.6 × 150 mm, 5 μm); column temperature: 35°C; mobile phase: 5 mmol/L solution of ammonium acetate (A) and acetonitrile (B); flow rate: 0.3 mL/min; the gradient elution program is shown in Table 1.

Table 1

The gradient elution program of the second dimension.

Run time (min)	A% (%)	B% (%)	
0	95	5	Equilibrate column in second dimension
t	95	5	-
t+8	95	5	Online desalting step
–			
t+23	5	95	Transfer the desalted impurities into MS
t+28	5	95	-
t+29	95	5	Re-equilibrate column

t: the time of target impurities is completely trapped into the two-dimensional separation system.

Mass spectrometry conditions: The Q-exactive orbitrap HRMS was equipped with an HESI ion source and was operated in a positive mode. The ionization parameters were set as follows: spray voltage of 3.8 kV, capillary temperature of 320°C, and vaporizer temperature of 250°C, and the sheath gas, auxiliary gas, and S-lens RF levels were set at 40 arb (arbitrary units), 10 arb, and 50 arb, respectively. Spray stabilization and collision-induced dissociation in the higher energy collision dissociation (HCD) cell adopted high purity nitrogen gas (purity 99.9%). The MS analysis was operated in full MS/dd-MS^2 (data-dependent MS^2) mode. The selected scan range of full MS scan was from *m/z* 50 to 750, and the resolution was 70,000. For the dd-MS^2 scan, the mass resolution was set to 17,500; AGC target was set at 1e5, maximum injection time (IT) was set at 50 ms, and stepped NCE was set to 10, 20, and 30.

2.5. 2D-UHPLC System and Online Desalting Procedure

2D-UHPLC system and online desalting procedure are illustrated in Figures 1(a)–1(d). The trapping of target impurities and online desalting was achieved by valve switching. A loop of 500 *µ*L was equipped on Valve 1 (Figure 1), which was used to collect target impurities eluting from the first dimension. In the beginning of the analysis, the first dimension with the high concentration salt was used to separate all impurities, while the second dimension only started to equilibrate Column 2 (Figure 1(a)). When the peak of target impurity was detected at the first-dimensional UV detector, the target impurity was transferred into a loop of 500 *µ*L by Valve 1 switching (Figure 1(b)). After the trapping of target impurity was finished, Valve 1 was switched back (Figure 1(c)). Then, the mobile phase from the second dimension transferred the impurity in the loop into Column 2. This step sustained for 8 min, and the high concentration salt was flushed out into waste. After that, Valve 2 was switched (Figure 1(d)), the target impurity was introduced into MS.

[figure(s) omitted; refer to PDF]

2.6. Toxicity Prediction

Based on the speculated structures, Gastroplus 9.0 ADMET Predictor™ 8.0 software was used to predict the toxicity of target impurities, while Derek Nexus 5.0.1 (knowledge-based) software and Sarah Nexus 2.0.1 (statistics-based) software were applied to evaluate the genotoxicity.

3. Results and Discussion

3.1. Selection of Target Impurities

The method for detecting related substances in pioglitazone hydrochloride preparations was performed referring to the analytical method of European Pharmacopoeia 10.0 Edition for pioglitazone hydrochloride, as described under Section 2.4. Five unidentified impurities were detected with high frequency in 54 batches of pioglitazone

hydrochloride preparations, especially with high-level in 2 batches of products from 2 manufacturers (Figure 2). Therefore, their structures became the objective of this study.

[figure(s) omitted; refer to PDF]

3.2. Identification of Target Impurities

Fragmentation pattern of pioglitazone is beneficial to elucidate the structures of target impurities. HRMS analysis of pioglitazone showed a protonated molecular ion peak at *m/z* 357.1260 $[M+H]^+$ corresponding to molecular formula $C_{19}H_{20}N_2O_3S$ (exact mass: 356.1189). Figure 3 shows MS^2 spectrum of *m/z* 357.1254. Figure 4 shows fragmentation patterns of $[M+H]^+$ for pioglitazone. The cleavage product at *m/z* 240 is a loss of 2,4-thiazolodinedione ($-C_3H_3NO_2S$) from $[M+H]^+$, and *m/z* 286 can be attributed to the ring opening of 2,4-thiazolodinedione to remove oximide ($-C_2HNO_2$). The product ions at *m/z* 134, 119 were the characteristic product ions derived from the cleavage of ethoxy phenyl ether bond.

[figure(s) omitted; refer to PDF]

A protonated molecular ion peak at *m/z* 258.1480 $[M+H]^+$ observed in the HRMS spectrum of Impurity-1 was matched to the molecular formula $C_{16}H_{19}NO_2$ (exact mass: 257.1410). Figure 5 shows the MS^2 spectrum of *m/z* 258.1480. Characteristic product ions of Impurity-1 at *m/z* 134 and 240 indicated that its structure is unchanged compared with pioglitazone except 2,4-thiazolodinedione unit. The characteristic product ions at *m/z* 240 and 228 were corresponding to the loss of H_2O, CH_2=O from $[M+H]^+$, respectively, indicating the presence of hydroxymethyl group ($-CH_2OH$) in the structure of Impurity-1. The structure of Impurity-1 is shown in Table 2. Figure 6 shows fragmentation patterns of $[M+H]^+$ for Impurity-1.

[figure(s) omitted; refer to PDF]

Table 2

Molecular formula, accurate mass, and elucidated structure of target impurities.

Impurity	Molecular formula	$[M+H]^+$/(*m/z*)		Deviation (ppm)	Elucidated structure
Theoretical	Experimental	Impurity-1	$C_{16}H_{19}NO_2$	258.1489	258.1480
3.5		Impurity-2	$C_{16}H_{17}NO_3$	272.1281	272.1272
3.3		Impurity-3	$C_{19}H_{20}N_2O_4S$	373.1216	373.1205
2.9		Impurity-4	$C_{19}H_{20}N_2O_2S$	341.1318	341.1311
2.1		Impurity-5	$C_{19}H_{20}N_2O_3S$	357.1267	357.1259

[figure(s) omitted; refer to PDF]

Impurity-1 is a process impurity. As shown in Figure 7, Intermediate-1 reacted with 2,4-thiazolidinedione to form Intermediate-2 via Knoevenagel condensation, and then Intermediate-2 was reduced to pioglitazone. Meanwhile, the remaining Intermediate-1 could be reduced to Impurity-1.

[figure(s) omitted; refer to PDF]

The HRMS spectrum of Impurity-2 showed a protonated molecular ion peak at *m/z* 272.1272 $[M+H]^+$ corresponding to the molecular formula $C_{16}H_{17}NO_3$ (exact mass: 271.1203). Figure 8 shows the MS^2 spectrum of *m/z* 272.1272. Characteristic product ions of Impurity-2 at *m/z* 134, 119, and 228 indicated that its structure is unchanged compared with pioglitazone except 5-methylene-2,4-thiazolodinedione unit. The characteristic product ions at *m/z* 254 and 228 were the loss of 18 Da and 44 Da from protonated molecular ion of Impurity-2, respectively, indicating that the structure of Impurity-2 contains carboxyl group (-COOH). The structure of Impurity-2 is shown in Table 2. Figure 9 shows fragmentation patterns of $[M+H]^+$ for Impurity-2.

[figure(s) omitted; refer to PDF]

Intermediate-1 may be oxidized into Impurity-2 under alkaline conditions. Thus, Impurity-2 is a process impurity. The HRMS analysis of Impurity-3 displayed a protonated molecular ion peak at *m/z* 373.1205 $[M+H]^+$ which is compatible to the molecular formula $C_{19}H_{20}N_2O_4S$ (exact mass: 372.1138) which has the same molecular formula of impurity A (Figure 10) listed in European Pharmacopeia 10.0. The retention time of Impurity-3 was earlier than impurity A in the chromatogram (Figure 2). Figure 11 shows the MS^2 spectrum of *m/z* 373.1205. The existence of characteristic product ions at *m/z* 134, 119, and 240 demonstrated that the structure of Impurity-3 is consistent with pioglitazone besides 5-methylene-2,4-thiazolodinedione unit. The characteristic product ion at *m/z* 355 was the loss of 18 Da from protonated molecular ion of Impurity-3, which showed the presence of a hydroxyl (-OH) group. Therefore, substituted position of the hydroxyl group was altered comparing with impurity A. The structure of Impurity-3 is shown in Table 2. Figure 12 shows fragmentation patterns of $[M+H]^+$ for Impurity-3.

[figure(s) omitted; refer to PDF]

According to the mechanism of Knoevenagel condensation reaction, Impurity-3 was the by-product of the synthetic process of Intermediate-2 (Figure 13). Therefore, it belongs to process impurity.

[figure(s) omitted; refer to PDF]

A protonated molecular ion peak at *m/z* 341.1311 $[M+H]^+$ obtained in the HRMS spectrum of Impurity-4 was consistent with the molecular formula $C_{19}H_{19}N_2O_2S$ (exact mass: 340.1240). Figure 14 shows the MS^2 spectrum of *m/z* 341.1311. The exact mass of Impurity-4 was 18 less than pioglitazone, which denoted the loss of H_2O. Furthermore, the distinction between Impurity-4 and pioglitazone was the 2,4-thiazolodinedione unit owing to the presence of typical fragment ions at *m/z* 134 and 240. Therefore, a loss of H_2O occurred on the 2,4-thiazolodinedione. The presence of a fragment ion at *m/z* 114 manifested that the thiazole ring became more stable corresponding to the increased stability by the formation of carbon-carbon double bond after the dehydration of the C-4 position carbonyl. The structure of Impurity-4 is shown in Table 2. Figure 15 shows fragmentation patterns of $[M+H]^+$ for Impurity-4.

[figure(s) omitted; refer to PDF]

During the process of Intermediate-2 being reduced to pioglitazone, it may be over-reduced and then dehydrated to form Impurity-4 (Figure 16). Therefore, Impurity-4 is a process impurity.

[figure(s) omitted; refer to PDF]

The HRMS data of Impurity-5 showed a protonated molecular ion peak at *m/z* 357.1259 $[M+H]^+$ corresponding to the molecular formula $C_{19}H_{20}N_2O_3S$ (exact mass: 356.1189), and this showed that Impurity-5 is the isomer of pioglitazone. Figure 17 shows the MS^2 spectrum of *m/z* 341.1311. The presence of fragment ions at *m/z* 134 and 240 indicated that its structure is unchanged compared to pioglitazone except 5-methylene-2,4-thiazolodinedione unit. The possible structure of Impurity-5 is deduced (Table 2) by referring the synthetic route of pioglitazone. Figure 18 shows fragmentation patterns of $[M+H]^+$ for Impurity-5.

[figure(s) omitted; refer to PDF]

Impurity-5 is a process impurity. The 4-position carbonyl group of the 2,4-thiazolodinedione ring might be reduced in the process of Intermediate-2 reduction (Figure 19).

[figure(s) omitted; refer to PDF]

3.3. Toxicity Prediction of Target Impurities

As exhibited in Table 3, the genotoxicity of all target impurities belonged to Class 5 which was defined as "no structural alerts or alerting structure with sufficient data to demonstrate lack of mutagenicity or carcinogenicity" [14].

Table 3

The results of toxicity prediction of target impurities.

Impurity	Structure	TOX_Risk	TOX_Code	Derek prediction	Sarah prediction	ICH M7 class
Impurity-1		0				Class 5
Impurity-2		0				Class 5
Impurity-3		1	Xr			Class 5
Impurity-4		1	Xr			Class 5
Impurity-5		2	Xr, Hp			Class 5

ADMET Predictor™ provided predictions of TOX_Risk and TOX_Code; Xr: carcinogenicity in rat; Hp: hepatotoxicity. The value of TOX_Risk predicted by ADMET Predictor™ indicated the number of potential toxicity problems that a compound might have. The predicted compounds with the value below 3.3 are considered as safe. Table 3 shows that the values TOX_Risk of five target impurities are all less than 3.3. Overall, five target impurities were predicted as safe compounds.

4. Conclusions

The online desalting technique achieved by heart-cutting 2D-LC coupled with HRMS showed several advantages in structural identification of impurities. First, the aimed impurity can be transported into mass spectrometry without changing the mobile phase of the analytical method which may contain nonvolatile salt or high concentration salt. In addition, HRMS can offer information of accurate mass and secondary fragment ions which are helpful for structural elucidation. In this study, fragmentation patterns of pioglitazone and five unidentified impurities were investigated and applied to obtain structural information of these impurities. Two impurities, Impurity-1 and Impurity-4, were reported previously, whereas the remaining three were first reported in this article. The toxicity assessments of these five impurities were predicted, which indicated that they all have a good safety profile. This study may provide a reference for the quality control of pioglitazone hydrochloride preparations.

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DETAILS

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Liquid Chromatography-Tandem Mass Spectrometry Detection of Human and Veterinary Drugs and Pesticides in Surface Water

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ABSTRACT (ENGLISH)

Antibiotics and pesticides are widespread in most rivers and lakes due to the overuse of antibiotics and pesticides, but there are few methods for simultaneous analysis of antibiotics and pesticides in aquatic environments. To address this knowledge gap, a concise and sensitive analytical method is proposed in which three classes of human and veterinary drugs (sulfonamides, macrolides, and hormones) and two classes of pesticides (organophosphorus and neonicotinoids) are simultaneously extracted and determined in surface water. The solid-phase extraction column with Cleanert PEP-2 was preconditioned sequentially with 6 mL of methanol, ultrapure water, and citric acid buffer (pH 3.0) each for simultaneous extraction and further purification. The forty-seven target analytes were analysed by LC-MS/MS in positive and negative ion modes. The LC separation was performed using a Sigma-Aldrich C_{18} column with 0.1% formic acid in water and acetonitrile as a gradient eluting mobile phase in positive ion mode. The internal standard method was used to overcome the inevitable matrix effects in LC-MS/MS analysis. The matrix effects of most target analytes were in the range of 27–151%. The recoveries of forty analytes in the three concentrations (10, 50, and 100 ng L^{-1}) of surface water spiked samples ranged from 41 to 127%. The method quantitative limits of the analytes were in the range of 0.40–5.49 ng L^{-1}. Application of the method to analyze samples in the eight runoff outlets of the Pearl River Delta showed that some antibiotics and pesticides were detected, and the concentration of parathion was as high as 154 ng L^{-1}. A powerful tool for quickly and efficiently screening for contaminants in surface water has been presented.

FULL TEXT

DETAILS

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Rapid Separation of Asiatic Acid, Quercetin, and Kaempferol from Traditional Chinese Medicine Centella asiatica (L.) Urban Using HSCCC-Semi-Prep-HPLC and the Assessment of Their Potential as Fatty Acid Synthase Inhibitors

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ABSTRACT (ENGLISH)

The main objective of this study was to rapidly separate asiatic acid (AA), quercetin (QCN), and kaempferol (KPL) from *Centella asiatica* (L.) Urban using high-speed counter-current chromatography (HSCCC) in tandem with the UV detector of semipreparative high-performance liquid chromatography (Semi-Prep-HPLC) and to evaluate their potential as inhibitors of fatty acid synthetase (FAS). To efficiently prepare large amounts of AA, QCN, and KPL from *Centella asiatica* (L.) Urban, rapid and simple methods by HSCCC were established respectively based on the partition coefficients (*K* values) of crude samples. The conditions of HSCCC-Semi-Prep-HPLC for the large-scale separation of AA, QCN, and KPL from *Centella asiatica* (L.) Urban were established and optimized. This included selecting the solvent system, flow rate, rotation speed, and so on. HSCCC-Semi-Prep-HPLC was successfully applied to separate and purify AA, QCN, and KPL, with *n*-hexane-*n*-butanol-methanol-water (3 : 1 : 3 : 3, V : V : V : V) as the solvent system for AA, which was detected at a wavelength of 210 nm with the stationary phase retention of 70%, and with *n*-hexane-ethyl acetate-methanol-water (0.8 : 0.9 : 1.2 : 1, V : V : V : V) as the solvent system for the co-separation of QCN and KPL, which was detected at a wavelength of 254 nm with the stationary phase retention of 65%. AA could be isolated at a large scale with high purity (>91.0%) in only one-step HSCCC-Semi-Prep-HPLC separation (within 150 min) under the optimized conditions. Meanwhile, QCN and KPL could be simultaneously isolated at a large scale with high purity (>99.1%) by another one-step HSCCC-Semi-Prep-HPLC separation (within 240 min) under the optimized conditions. The assessment of inhibition potential revealed that AA exhibited the strongest inhibitory effect on FAS, with an IC_{50} of 9.52 ± 0.76 μg/mL. Madecassic acid (MA) followed closely with IC_{50} values of 10.84 ± 0.92 μg/mL. QCN and KPL showed similar and relatively weaker inhibitory effects on FAS, with IC_{50} values of 43.09 ± 2.98 μg/mL and 36.90 ± 1.83 μg/mL, respectively. Overall, the HSCCC-Semi-Prep-HPLC method proved to be a highly efficient and reliable technique for separating AA, QCN, and KPL from *Centella asiatica* (L.) Urban, and the isolated compounds showed potential as FAS inhibitors.

FULL TEXT

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1. Introduction

Centella asiatica (L.) Urban, also known as Gotu Kola [1], is a traditional Chinese medicine that has been recorded as a medicinal herb in Shennong's Classic of Materia Medica more than 2000 years ago. According to the 2020

edition of the Chinese Pharmacopoeia, *Centella asiatica* is cold in nature and has a bitter and pungent taste, with the functions of clearing heat and dampness, detoxification, and reducing swelling. It is commonly used in the treatment of damp-heat jaundice, heatstroke, diarrhea, hematuria and dysuria, abscesses and sores, bruises and sprains, infectious hepatitis, skin diseases, epidemic cerebrospinal meningitis, etc. [2, 3]. Asiatic acid (AA), the aglycones of ursane-type pentacyclic triterpenoids, is the major active component that was isolated and identified from *Centella asiatica* (L.) Urban, and our previous research has shown that *Centella asiatica* (L.) Urban is rich in AA, with a content of 1.115 $g \cdot kg^{-1}$ [3, 4]. Quercetin (QCN) and kaempferol (KPL), representative of the flavonoids, are also the main components of *Centella asiatica* (L.) Urban, with the content of >1.28 $g \cdot kg^{-1}$ and >7.82 $g \cdot kg^{-1}$, respectively [5]. AA has been found to have various pharmacological effects, such as promoting the healing of skin wounds [6], anti-inflammatory [7, 8], antioxidant [9, 10], antidepressant [11], anti-Alzheimer's disease [12, 13], antitumor [14, 15], nerve repair [16, 17], antiobesity [18, 19], and protection of cardiovascular and cerebrovascular systems [20, 21]. QCN and KPL have been shown to have antioxidant [22, 23], anti-inflammatory [22, 24], antihypertensive, cardioprotective [24, 25] effects.

To further explore the pharmacological effects and research mechanisms of AA, QCN, and KPL, there is a need to isolate these compounds. However, the availability of the reports on the isolation of these compounds from the *Centella asiatica* (L.) Urban is , at present, very scarce. Our research group has previously reported the isolation and purification of AA from *Centella asiatica* (L.) Urban using silica gel column chromatography [26]. However, this method is found to be tedious and time-consuming for the isolation and purification of AA from *Centella asiatica* (L.) Urban. Moreover, when the AA extract was subjected to three rounds of separation and purification using silica gel column chromatography, we were only able to achieve a yield of 79.0% [26]. Additionally, this method has the drawback of compound loss due to the highly irreversible adsorptive property of the solid matrix [27]. High-speed countercurrent chromatography (HSCCC) is an advanced liquid-liquid partition chromatography technology that has been widely used for the separation and purification of various natural products due to its several advantages [27]. One of its well-known benefits is that it does not require a solid support matrix for the stationary phase, allowing the preparative separation of solutes in a two-phase solvent system [28]. This feature eliminates the irreversible adsorption loss of the sample caused by the solid support matrix in traditional chromatography columns [29]. In addition, HSCCC offers several advantages, such as high recovery, high efficiency, simple pretreatment operation, high reproducibility, and ease of scaleup [27–29]. However, selecting an appropriate solvent system for separation is a critical operation.

To date, as far as we know, no report has been reported on the use of HSCCC for the separation and purification of QCN and KPL from *Centella asiatica* (L.) Urban. Du et al. [30] described the separation of four ursane triterpenoids (containing AA) from a crude extract of *Centella asiatica* using HSCCC. The separation was achieved by employing a solvent system consisting of a three-step gradient with a time-dependent increase in the eluting strength of the mobile phase, which involved four solvent systems [30]. In this experiment, the lower phase of the solvent system composed of *n*-hexane/*n*-butanol/0.05M NaOH (5/1/6, V/V/V) served as the stationary phase, while the upper phase was used as the initial mobile phase [30]. The flow rates were significantly reduced from the initial 5.0 mL/min to 3.0 mL/min (step 1), 2.0 mL/min (step 2), and 1.5 mL/min (step 3), which was accompanied by a stepwise increase in the *n*-butanol content in the mobile phase, starting from a ratio of 5 : 1 and progressing to 1 : 1 (step 1), 1 : 2 (step 2), and 1 : 4 (step 3) of *n*-hexane/*n*-butanol [30]. Thin-layer chromatography (TLC) with an ethyl acetate-methanol-water (8 : 2 : 1, V/V/V) solvent system was employed to evaluate the HSCCC fractions. The triterpenoids were visualized by spraying with 3% sulfuric acid in ethanol and heating to 110°C for 5 minutes on a hot plate [30].

However, this HSCCC method had several limitations. It required manual changes of solvent systems and adjustments of flow rates, making it relatively cumbersome and time-consuming. Real-time online monitoring was not feasible, and TLC was used for triterpenoid detection instead. Furthermore, the study did not measure the distribution coefficients of the samples in their respective solvent systems, did not screen for the optimal solvent system, and did not report the purity of the samples obtained after a single HSCCC separation. The lack of real-time tracking for the distillate represented a significant limitation. Therefore, there is a need to develop a simple, rapid,

and real-time trackable HSCCC-Semi-Prep-HPLC method to achieve fast separation of AA.

The purpose of this work, therefore, was to establish an effective and convenient HSCCC method for preparative isolation and purification of the target compound AA, QCN, and KPL directly from the crude extract of *Centella asiatica* (L.) Urban. Furthermore, potential inhibiting effects of these ingredients on fatty acid synthetase (FAS) were evaluated by the respective FAS inhibitory test. The chemical structures of AA, QCN, and KPL are shown in Figure 1.

[figure(s) omitted; refer to PDF]

2. Materials and Methods

2.1. Materials and Reagents

Centella asiatica (L.) Urban was purchased from Tong Ren Tang Pharmacy in Beijing. Acetyl CoA, malonyl CoA, NADPH, and reference substance of AA (purity >95%) were purchased from Sigma-Aldrich. The reference substance of QCN (purity >98%), KPL (purity >98%), and madecassic acid (MA, purity >97%) were purchased from Tauto Biotech, Shanghai, China. All organic solvents used for the preparation of enriched extract and for HSCCC separation were of analytical grade (Beijing Chemical Works, Beijing, China). The methanol and acetonitrile used for HPLC were of chromatographic grade (Thermo Fisher Scientific, Waltham, MA, USA), and the water used was deionized. Fatty acid synthetase was isolated and purified from fresh duck liver that had been prepared and stored according to the method reported [31] and was identified as a single band by polyacrylamide gel electrophoresis (SDS-PAGE). The high-speed frozen centrifuge used in this manuscript is the MIKRO-22R (Hettich GmbH, Germany).

2.2. Apparatus

The HSCCC equipment utilized in this paper was a TBE-300C HSCCC system (Tauto Biotech, Shanghai, China), consisting of three multilayer coil separation columns connected in series (inner diameter of tubing = 1.6 mm, total volume = 300 mL, β-values = 0.5–0.8), and a 20 mL sample loop. The instrument’s revolution speed can be regulated using a speed controller within the range of 0–1000 rpm. The HSCCC system was equipped with a TBP-5002 constant-flow pump (Tauto Biotech, Shanghai, China), a HX-1050 thermostatic circulating instrument (BIOCOOL, Beijing, China), and a 1525 semipreparative high-performance liquid chromatography system (which included a binary gradient pump, UV-2487 dual-wavelength UV detector, empower workstation, which were all manufactured by Waters Corporation, USA). Additionally, a Waters 1525 series analytical HPLC system (which included a binary gradient pump, UV-2487 UV detector, online vacuum degasser, and automatic sampler) was used for analytical determination. The UV-2550 UV-visible spectrophotometer (Shimazu Corporation, Japan), the LABOROTA type 4000 rotary evaporator (Heidolhp, Germany), the SHZ-III circulating water vacuum pump (Yarong Biochem, Shanghai, China), the KQ-250DB type CNC ultrasonic cleaner, and the Mill (Tasite Instrument, Tianjin, China) were also used in this paper. The ESI-MS^n system, controlled by Xcalibur® software (version 1.3), consisted of a Finnigan LCQ Deca XP ion-trap spectrometer equipped with an electrospray source (Thermo Finnigan, San Jose, CA, USA).

2.3. Methods

2.3.1. Chromatographic Conditions for the Determination of AA

Based on the chromatographic conditions established earlier by our group, the chromatographic column used was Symmetry C18 (4.6 mm × 250 mm, 5 μm, Waters, USA) with acetonitrile-10 mmol/L ammonium acetate aqueous solution (19 : 31, v/v) as the mobile phase. The flow rate was 1.0 mL/min, the detection wavelength was set to 210 nm, the column temperature was maintained at 25°C, and the sample size was 20 μL. Figure 2 displays the chromatogram of the AA reference substance (purity >95%).

[figure(s) omitted; refer to PDF]

To determine the most suitable mobile phase, various ion-pairing reagents were added to improve the resolution of the AA peak and impurity peak in the HPLC chromatogram of the *Centella asiatica* (L.) Urban extract. Two binary solvent systems, methanol-water and acetonitrile-water, were used as mobile phases. Our team previously investigated the effect of methanol or acetonitrile as organic phases on the separation and analysis of AA. We found that methanol has significant end absorption at 210 nm, while acetonitrile does not. Therefore, acetonitrile was

chosen as the organic phase.

Several mobile phases, including acetonitrile-acetic acid aqueous solution, acetonitrile-formic acid aqueous solution, acetonitrile-phosphate buffer solution, acetonitrile-ammonium chloride aqueous solution, and acetonitrile-β-cyclodextrin aqueous solution, were screened for their effects on the HPLC chromatograms of the *Centella asiatica* (L.) Urban extract. The proportions of the mobile phases and their solution pH (pH = 3–6) were further adjusted for analysis. However, none of the above mobile phases effectively separated AA and the separation degree between the AA peak and impurity peak was <1.5.

We then used acetonitrile-10 mmol/L ammonium acetate aqueous solution (19 : 31, V/V), which resulted in a good separation effect. The separation degree between the AA peak and impurity peak was >2, with a good peak shape and no front or tailing peak, and an AA retention time of about 17 min. Therefore, the acetonitrile-10 mmol/L ammonium acetate aqueous solution (19 : 31, V/V) was chosen as the mobile phase for qualitative and quantitative analyses of AA in the *Centella asiatica* (L.) Urban extract. Figure 3 shows the HPLC chromatograms of the AA-containing samples before and after optimizing the chromatographic conditions.

[figure(s) omitted; refer to PDF]

In addition, we investigated the effects of flow rate and column temperature on the retention time and separation degree of AA. We found that both flow rate and column temperature significantly affected the retention time of AA and had a certain effect on the separation degree between AA and impurities. When the flow rate and column temperature were low, the retention time of the sample significantly increased, and the separation effect was better. Considering the effects of retention time and separation degree, a flow rate of 1.0 mL/min and a column temperature of 25°C were ultimately chosen.

2.3.2. Chromatographic Condition for the Determination of QCN and KPL

The chromatographic column used in the study was also Symmetry C18 (4.6 mm × 250 mm, 5 μm, Waters). A binary linear gradient elution method was employed for the mobile phase, with acetonitrile as phase A and 0.3% (V : V) acetic acid in water as phase B. The gradient program for chromatographic separation, as indicated in Table 1, was as follows: T_{min} A : B): T_0 20 : 80; T_4 20 : 80; T_{20} 60 : 40; T_{30} 60 : 40. Detection was performed at a wavelength of 254 nm, and the flow rate was set as 1.0 mL/min. Additionally, the column temperature was kept constant at 25°C, and the injection volume for all samples was 5 μL. Figure 4 depicts the chromatogram of QCN (purity >98%), KPL (purity >98%) reference substance, and crude extract A from *Centella asiatica* (L.) Urban, respectively.

Table 1

Gradient elution procedure for the mobile phase.

Time (min)	A (%)	B (%)
0	20	80
4	20	80
20	60	40
30	60	40

[figure(s) omitted; refer to PDF]

2.3.3. ESI-MS^n Condition for the Determination of AA, QCN, and KPL

For AA, ESI-MS^n was operated with a sheath flow rate of 40 psi, an ion spray voltage of 4.5 kV, and a heated capillary temperature of 350°C. The peristaltic pump flow rate was maintained at 10 μL/min. MS^n product ion spectra were generated through collision-induced dissociation (CID) of the deprotonated molecule ion $[M\text{-}H]^-$ of the analyte at an isolation width (*m/z*) of 1.0. The collision energy for the analyte fell within the range of 25% to 38%. For QCN

and KPL, ESI-MS/MS was operated with a sheath flow rate of 20 psi, an ion spray voltage of 4.0 kV, and a heated capillary temperature of 350°C. The peristaltic pump flow rate was also consistently set at 10 μL/min. MS/MS product ion spectra were generated through collision-induced dissociation (CID) of the deprotonated molecule ion $[M-H]^-$ of the analyte at an isolation width (*m/z*) of 1.0. The collision energy for the analyte was fixed at 40%.

2.3.4. Preparation of Crude Extract from *Centella asiatica* (L.) Urban

The preparation of *Centella asiatica* (L.) Urban extract was carried out with a slight modification to the previous method established in our laboratory [4]. *Centella asiatica* (L.) Urban was dried to a constant weight at 40°C, crushed, and sifted through a 40-mesh sieve. Ultrasonic-assisted extraction was performed according to the optimized extraction process established by our group, using a solid-liquid ratio of 1 : 15, an ethanol concentration of 75%, and ultrasonic treatment at 40°C for 1 hour. The extracted liquid was filtered on a Brinell funnel with a filter paper, and the residue was extracted twice under the same conditions. The filtrate was combined and reduced pressure concentration was carried out with a rotary evaporator at 40°C. The resulting ethanol extract of *Centella asiatica* (L.) Urban is the *crude extract A*, which is used for the isolation and purification of QCN and KPL. An appropriate amount of crude extract A was extracted using an ethyl acetate-water (2 : 1, v/v) system in a liquid separation funnel, and the ethyl acetate layer was collected. The water layer was extracted twice with twice the volume of ethyl acetate, combined with the ethyl acetate layer, and then decompressed and concentrated using a rotary evaporation instrument at 40°C. The resulting extract was dried in an oven at 40°C to a constant weight (*crude extract B*) and stored in vacuum dry storage for further use (used for the isolation and purification of AA).

2.3.5. Selection of a Solvent System

The optimal solvent system for AA was selected based on the K value of different solvent systems. Three potential solvent systems, namely, *n*-hexane-*n*-butanol-methanol-water (solvent system A), *n*-hexane-*n*-butanol-ethanol-water (solvent system B), and *n*-hexane-*n*-butanol-acetonitrile-water (solvent system C), were selected for further screening based on the average polarity of the solvent system and the physicochemical properties of AA [32]. Ten different solvent systems were prepared according to the conditions listed in Table 2 and screened one by one.

Table 2

Screening of the solvent system for AA.

Serial number	Solvent system	Component ratio (V : V : V : V)
1	A-1	1 : 1 : 1 : 1
2	A-2	1 : 1.1 : 1 : 1
3	A-3	1 : 1.1 : 0.9 : 1
4	A-4	1.5 : 1 : 0.9 : 1
5	A-5	1.5 : 1 : 0.8 : 1
6	B-1	1.1 : 1 : 1.1 : 1
7	B-2	1.5 : 1 : 0.8 : 1
8	C-1	1 : 1 : 1 : 1

9	C-2	1.5 : 1 : 0.9 : 1
10	C-3	1.8 : 1 : 0.8 : 1

Solvent system A: *n*-hexane-*n*-butanol-methanol-water. Solvent system B: *n*-hexane-*n*-butanol-ethanol-water. Solvent system C: *n*-hexane-*n*-butanol-acetonitrile-water.

The *K* value was used as an assessment indicator for selecting the two-phase solvent system that was ultimately used for the HSCCC separation [32]. The *K* value of the target components was estimated through HPLC analysis as follows:

The solvent system was mixed in a liquid separation funnel and shaken thoroughly at room temperature. Then, 10.0 mL of upper phase solution and 10.0 mL of lower phase solution were transferred into another liquid separation funnel, and approximately, 20 mg of crude extract B was added. The funnel was plugged and shaken vigorously for about one minute to allow the sample to equilibrate between the two phases. Equal volumes (2.0 mL) of solution from the upper and lower phase solutions were dried out by rotary evaporators at 40°C. The residues were diluted with methanol, and the analytical HPLC system was used to determine the peak area (A) of AA in the two phases of the solvent system. The partition coefficient, K, was the ratio of AA's peak area A_s in the stationary phase to A_m in the mobile phase, that is, $K = A_s / A_m$.

Based on the physicochemical properties of QCN and KPL, the classical *n*-hexane-ethyl acetate-methanol-water system was selected as the solvent system to screen these compounds. This solvent system is commonly used for extracting plant secondary metabolites with different polarities. The solvent system screening methods for the separation of QCN and KPL were similar to those used for AA.

2.3.6. Selection of Other Separation Conditions

To ensure the best HSCCC separation, the selection of other separation conditions such as rotation speed and the flow rate of the mobile phase was investigated [32]. Three different rotational speeds (800 rpm/min, 900 rpm/min, and 1000 rpm/min) were tested in countercurrent chromatography experiments to determine their effect on the retention rate and separation efficiency of the stationary phase. Similarly, two different flow rates of the mobile phase (1.5 mL/min and 2.0 mL/min) were tested to determine their impact on the retention rate and separation efficiency of the stationary phase. These experiments were conducted to ensure the optimal conditions for the HSCCC separation process.

2.3.7. Preparation of the Solvent System and Sample Solution

In this study, the selected two-phase solvent system was fully equilibrated in the separation funnel by vigorously shaking it for 2 minutes at room temperature. Prior to use, the two phases were separated and degassed through sonication for 15 minutes. To prepare the sample solutions for HSCCC separation, 200.0 mg of crude extract B was dissolved in 10 mL of the upper phase (solvent system for AA) and 500.0 mg of crude extract A was dissolved in 10 mL of the upper phase (solvent system for QCN and KPL).

2.3.8. HSCCC Separation Procedure

First of all, the semipreparative HPLC system was connected to the TBE 300A HSCCC system. After starting the circulating water bath, the multilayer spiral chromatographic column was completely filled with the upper phase (stationary phase) at a flow rate of 25.0 mL/min. Then, the HSCCC host was started, and the appropriate rotation mode (forward/reverse rotation) was selected. The rotation speed was slowly adjusted to the optimized speed, and the mobile phase was pumped at the optimized flow rate. When a clear mobile phase flowed out from the tail end, it indicated that the two-phase solvent had reached hydrodynamic equilibrium in the instrument, and the sample could be injected through the injection valve at this point. Once the system reached hydrodynamic equilibrium, 10.0 mL of the test solution was injected into the sample valve and eluted in an appropriate elution mode. The effluent was monitored and recorded online at 210 nm (for AA) or 254 nm (for QCN and KPL) using the Waters Empower Workstation of the semipreparative HPLC system. Based on the HSCCC spectrum monitored at 210 nm or 254 nm, the column effluents between the half-peak widths of each peak were continuously collected. After that, the

generated fractions were dried at 40°C under reduced pressure and then dried in an oven at 40°C to a constant weight. The powder was then stored in vacuum-dry storage for further use. Once the separation was complete, the solvents in the column were expelled to estimate the retention ratio of the solvent system. The retention ratio (*R*) of the stationary phase was calculated using the equation $R\,(\%) = (320 - V_L)/320$, where *R* represents the retention ratio of the stationary phase (upper phase) and V_L is the volume of stationary phase that flowed out of the column [27, 33].

2.3.9. Determination of FAS Enzyme Activity and Inhibitory Activity

The activity of FAS was determined using ultraviolet spectrophotometry, following the method reported by Tian et al. [34]. The procedure is as follows: At 37°C, 1.85 mL of phosphate buffer (pH = 7.0), 25 μL of acetyl-CoA (0.2 mmol/L), 50 μL of malonyl CoA (0.4 mmol/L), 50 μL of NADPH (1.3 mmol/L), and an appropriate amount of blank solvent were added into 2 mL colorimetric dishes successively. Then, 20 μL of FAS solution was added and mixed to start the enzymatic reaction. The activity of FAS enzyme A_0 was determined by UV spectrophotometry at 340 nm.

2.3.10. Determination of FAS Inhibitory Activity

All samples were dissolved in 50% ethanol. Each crude extract (A and B), as well as AA, MA, QCN, and KPL, were prepared in six series of solutions with concentrations of 3.12 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25.0 μg/mL, 50.0 μg/mL, and 200 μg/mL, respectively. Using the method described in Section 2.3.9, each sample solution was substituted for the blank solvent to measure FAS enzyme activity (A_i) at different concentrations. A_i/A_0 represents the remaining activity after FAS combines with the sample solution.

2.3.11. Determination of Half-Inhibitory Concentration (IC_{50})

Regression analysis was performed using the SPSS 19.0 software. The Probit analysis logistic model was then used to calculate the IC_{50} value of the sample solution and its corresponding confidence interval, based on the logarithm of the sample mass concentration corresponding to the residual activity value. The IC_{50} value represents the inhibitory capacity of the inhibitor, with a smaller value indicating a stronger inhibitory capacity. Therefore, IC_{50} can be considered an important parameter for evaluating the FAS inhibition activity of the samples.

3. Results and Discussion

3.1. Optimization of the HSCCC Two-Phase Solvent System

The key factor in achieving a successful HSCCC separation is identifying an appropriate two-phase solvent system. The search for a suitable two-phase solvent system accounts for 90% of the entire work in HSCCC [27, 35]. The selection of the solvent system for HSCCC separation is based on golden rules for selecting optimum conditions for high-speed counter-current chromatography and the difference in partition coefficients (*K*) of the target compound between the two-phase systems [32]. The *K* value in the range of 0.5–2.0 for the target compound is generally considered appropriate for HSCCC separation [31, 35]. As mentioned previously, selecting an appropriate solvent system is one of the most crucial steps in achieving successful HSCCC separation. The partition coefficient (*K*) value is the most significant indicator for determining the resolution in HSCCC. If the *K* value is less than 0.5, the material being separated will be eluted too quickly, resulting in low resolution. Conversely, if the *K* value is greater than 2.0, the peak retention time of the material will be too long, leading to peak broadening and a reduction in separation efficiency.

3.1.1. Selection of a Two-Phase Solvent System for the Isolation of AA

The partition coefficients (*K* values) of AA in crude extract B were measured in ten different two-phase solvent systems selected from three potential solvent systems using HPLC with UV detection at 210 nm. The results are summarized in Table 3.

Table 3

Distribution coefficient of AA in 10 different solvent systems.

Serial number	Solvent system	Component ratio (V : V : V : V)	*K* value

1	A-1	1:1:1:1	0.49
2	A-2	1:1.1:1:1	0.61
3	A-3	1:1.1:0.9:1	0.96
4	A-4	1.5:1:0.9:1	0.75
5	A-5	1.5:1:0.8:1	0.11
6	B-1	1.1:1:1.1:1	0.94
7	B-2	1.5:1:0.8:1	1.01
8	C-1	1:1:1:1	0.25
9	C-2	1.5:1:0.9:1	0.43
10	C-3	1.8:1:0.8:1	0.27

Solvent system A: *n*-hexane-*n*-butanol-methanol-water. Solvent system B: *n*-hexane-*n*-butanol-ethanol-water. Solvent system C: *n*-hexane-*n*-butanol-acetonitrile-water.

According to Table 3, it can be seen that for solvent systems A and B, there exist solvent systems with a distribution coefficient *K* within the range of 0.5–2 as the proportion is adjusted. However, the distribution coefficients *K* of samples in the C1, C2, and C3 systems are all less than 0.5. Additionally, it was found that system C easily forms three layers upon adjusting the proportion, making it unsuitable for this experiment. Then, solvent systems with *K* values within the range of 0.5–2 in Table 3 were selected for the HSCCC fixed phase retention test. However, the results showed that the stationary phase retentions of systems A2, A3, A4, B1, and B2 were all below 30%, failing to meet the requirement of the HSCCC stationary phase retention of more than 40%. Therefore, the above conditions are not desirable.

It is speculated that the low retention rate of the fixed phase may be due to the occurrence of emulsification between the two incompatible phases of the solvent system during the high-speed repeated extraction process. Reducing the emulsification phenomenon can increase the retention rate of the fixed phase. After multiple screenings, it was found that the stationary phase retention could be significantly increased by decreasing the proportion of butanol or increasing the proportion of methanol in the solvent system. Moreover, the solvent system containing ethanol was found to be more prone to emulsification than the solvent system containing methanol.

Based on the above experimental results, system A was further screened to optimize the optimal solvent system. The composition and distribution coefficient *K* of the new solvent system are shown in Table 4.

Table 4

The partition coefficient *K* of AA in solvent system A.

Serial number	Component ratio (V:V:V:V)	*K* value
A_1	2:1:2:2	0.48

A_2	3:1:3:3	1.02
A_3	3:2:3:2	0.17
A_4	3:1:2:3	1.56

Solvent system A: *n*-hexane-*n*-butanol-methanol-water.

According to Table 4, both solvent systems A_2 and A_4 meet the requirements for distribution coefficients *K*, and the further stationary phase retention tests indicate that the stationary phase retention *s* of both systems are above 70%. Taking into account the polarity and solubility of the sample, solvent system A_2, composed of *n*-hexane, *n*-butanol, methanol, and water (3:1:2:3, V:V:V:V), was chosen as the experimental condition for HSCCC separation and purification of AA.

3.1.2. Selection of a Two-Phase Solvent System for the Isolation of QCN and KPL

The partition coefficients (*K* values) of QCN and KPL in crude extract A were measured using HPLC with UV detection at 254 nm. Based on the polarity of QCN and KPL, the classical solvent system of *n*-hexane-ethyl acetate-methanol-water was selected for separation. The proportion of the solvent system was adjusted through the method given in Section 3.4 and repeatedly screened based on the respectively distribution coefficient *K* of QCN and KPL. Finally, the optimal solvent system was determined to be *n*-hexane-ethyl acetate-methanol-water (0.8:0.9:1.2:1, V:V:V:V).

3.2. Selection of Other Separation Conditions

Various factors were analyzed, including the rotation speed and the flow rate of the mobile phase. The flow rate of the mobile phase directly affects the amount of stationary phase fixed in the column, separation time, and peak resolution [29, 32]. In this study, the effect of solvent systems at rotation speeds of 800 rpm/min, 900 rpm/min, and 1000 rpm/min on the retention of the target compound's stationary phase was investigated by comparing the retention of AA, QCN, and KPL in HSCCC. Results showed that an increase in rotation speed led to a slight increase in the retention of the stationary phase and the separation effect of AA. However, there was no significant difference in the influence of the solvent systems *n*-hexane, *n*-butanol, methanol, and water (3:1:3:3, V:V:V:V) on the stationary phase retention and separation effect. Since a high rotation speed can cause emulsification between immiscible phases and affect the instrument's service life, a rotation speed of 800 rpm/min was selected for the separation of AA.

Further exploration was conducted on the flow rate of the mobile phase, and the effect of flow rates of 1.5 mL/min and 2.0 mL/min on the separation of AA, QCN, and KPL was examined. The results showed that both flow rates had no significant effect on the separation of AA, QCN, and KPL, but the separation time was longer at a flow rate of 1.5 mL/min. Therefore, a flow rate of 2.0 mL/min was chosen as the flow rate of the mobile phase. Furthermore, a slight increase in the stationary phase retention of QCN and KPL was observed with an increase in rotation speed, but the effect was not significant. However, the separation effect of the sample was significantly improved with an increase in rotation speed. Therefore, a rotation speed of 1000 rpm/min was selected for this experiment. Under the aforementioned conditions, the solvent system used for the separation of AA exhibits a stationary phase retention rate of 70%, whereas the solvent system used for the simultaneous separation of QCN and KPL displays a retention rate of over 65%.

3.3. Separation of AA from Crude Extract B by HSCCC

To perform the HSCCC separation, 200 mg of crude extract B was dissolved in 10.0 mL of the upper phase of a *n*-hexane-*n*-butanol-methanol-water (3:1:3:3, v/v/v/v) system. The stationary phase used was the upper phase, and the lower phase was used as the mobile phase, with a head-to-tail (forward) elution mode according to the procedure described in Section 2.3.7. The eluent was collected at the half-peak width of the absorption peak in the HSCCC chromatogram and was subsequently filtered and analyzed by HPLC under the conditions described in Section 2.3.1, as shown in Figures 5 and 6.

[figure(s) omitted; refer to PDF]

Then, ESI-MS^n was used for further verification of AA under the conditions described in Section 2.3.3. To ensure an adequate number of fragment ions, a solution containing 10 μg/mL of the effluent separated from crude extract B by HSCCC in methanol was utilized for the fragmentation pattern study. The quasi-molecular ion of the effluent in negative mode exhibited an *m/z* value of 487.39. Its MS/MS spectra and the data of the MS^n spectra for its primary fragment ions are presented in Figure 7 and Table 5.

[figure(s) omitted; refer to PDF]

Table 5

ESI-MS^n negative ions of the effluent separated from crude extract B by HSCCC.

$[M-H]^-$	Scan mode (*m/z*)	Main fragmentation ions (*m/z*)
487	MS^2 (487)	487, 473, 441, 423, 409, 391, 379
MS^3 (487441)	421, 409, 379, 233	MS^3 (487423)
405, 393, 347	MS^3 (487409)	391, 379, 375

The results demonstrated that the purified sample obtained through HSCCC exhibited a peak shape and retention time in the HPLC chromatography spectrum, UV spectrum, and ESI-MS/MS spectrum, and the data of the ESI-MS^n spectrums were consistent with those of the AA reference substance. These findings strongly indicate that the compound is highly likely to be AA. Based on the data presented in Figure 6, the purity of AA was calculated to be 91.0%.

3.4. Separation of QCN and KPL from Crude Extract A by HSCCC

To perform the HSCCC separation, 500 mg of crude extract A was dissolved in 10.0 mL of the upper phase of a *n*-hexane-ethyl acetate-methanol-water (0.8 : 0.9 : 1.2 : 1, v/v/v/v) system, and elution was carried out using the upper phase as the stationary phase and the lower phase as the mobile phase in a head-to-tail (forward) manner according to the method described in Section 2.3.7. The HSCCC chromatogram of crude extract A at 254 nm is shown in Figure 8; the eluent was collected from the half-peak width of the absorption peaks IV and V. After filtration through a 0.22 μm filter membrane, the appropriate amount of distillate was taken and analyzed by HPLC under the conditions described in Section 2.3.2, as shown in Figures 9 and 10.

[figure(s) omitted; refer to PDF]

Then, ESI-MS/MS was employed for the further verification of distillates IV and V, which were separated from crude extract A using HSCCC, under the conditions described in Section 2.3.3. To ensure an adequate number of fragment ions, solutions containing 10 μg/mL of distillates IV and V were respectively prepared in methanol. The quasi-molecular ions of distillates IV and V in negative mode were observed at *m*/*z* 301.25 and *m*/*z* 284.97, respectively. The MS/MS spectra of distillates IV and V in negative mode are presented in Figures 11 and 12, respectively.

[figure(s) omitted; refer to PDF]

The results showed that peaks IV and V in the HSCCC were all single components. Among them, the purified samples corresponding to peaks IV and V had a peak shape, retention time, UV spectrum, and ESI-MS/MS spectrum consistent with those of the QCN and KPL reference substances, respectively, indicating that peak IV was indeed QCN and peak V was indeed KPL. In addition, the HPLC analysis of QCN and KP showed retention times of 14.0 min and 17.0 min, respectively, with purities of 99.1% and 99.2%, respectively. Furthermore, peaks I and II in the HSCCC corresponded to a mixture of multiple components with retention times within 4 min in the HPLC spectrum and did not contain QCN and KPL components. In this study, the HSCCC technique was used to isolate and purify QCN (peak IV) and KPL (peak V) from the crude extract A of *Centella asiatica* (L.), with purities >99%

measured by HPLC (as shown in Figures 9 and 10).

In this study, the HSCCC technique was successfully used in isolating and purifying AA, QCN, and KPL with high purity levels. In the previous stage of this study, our team employed silica gel column chromatography with a petroleum ether: acetone system to separate and purify asiatic acid from *Centella asiatica* (L.) Urban [26]. The results showed that a single separation by column chromatography only yielded AA with a purity of 4.6%, and even after repeating the separation by column chromatography three times, the purity was only 79.0% (shown in Figure 13), which is much lower than the purity achieved by HSCCC in this study.

[figure(s) omitted; refer to PDF]

3.5. FAS Inhibition Activity of Each Sample from *Centella asiatica* (L.) Urban

This study investigated the inhibitory effects of seven substances extracted from traditional Chinese medicine *Centella asiatica* (L.) Urban on FAS activity at different concentrations. The residual activity value of FAS was used to determine the sample's ability to inhibit FAS. The results are illustrated in Figure 14, indicating a dose-response relationship between the sample concentration and FAS activity inhibition. Of the six substances, AA and MA were found to have stronger inhibitory effects on FAS than crude extracts A and B, QCN, and KPL. Additionally, QCN and KPL exhibited similar inhibitory effects, while MA and AA had similar but stronger effects. The relatively weak inhibitory effects of crude extract A and B were attributed to the presence of inactive impurities. Lastly, the study revealed a positive correlation between dose and effect, with the inhibitory effect of the sample on FAS activity becoming stronger at higher concentrations.

[figure(s) omitted; refer to PDF]

SPSS 19.0 software was used for statistical analysis, and the IC_{50} values of effective components of *Centella asiatica* (L.) Urban FAS inhibition were obtained, as shown in Table 5. The results showed that AA and MA had the strongest inhibitory effect on FAS with IC_{50} values of 9.52 ± 0.76 μg/mL and 10.84 ± 0.92 μg/mL, respectively. MA is the hydroxylation product of AA and is also an important component of *Centella asiatica* (L.) Urban. These results could confirm the existence of a variety of compounds with strong inhibitory effects on FAS activity in *Centella asiatica* (L.) Urban. As shown in Table 6, triterpenoids exhibited stronger FAS inhibition activity compared to flavonoids QCN and KPL, as well as AA and MA.

Table 6

IC_{50} value of each ingredient extracted from *Centella asiatica* (L.) Urban in the inhibition of FAS ($n = 3$).

No.	Sample solution	IC_{50} (μg/mL)
1	Crude extract A	48.15 ± 2.51
2	Crude extract B	57.02 ± 3.59
3	QCN	43.09 ± 2.98
4	KPL	36.90 ± 1.83
5	AA	9.52 ± 0.76
6	MA	10.84 ± 0.92

4. Conclusions

The establishment of efficient and effective methods for the isolation of bioactive compounds from natural sources is crucial for their potential use in various applications. This study successfully demonstrated the potential of HSCCC-Semi-Prep -HPLC separation in the rapid and large-scale isolation of AA, QCN, and KPL from *Centella asiatica* (L.)

Urban. These methods resulted in high purity (>91.0% for AA and >99.1% for QCN and KPL) and large-scale isolation of the compounds by only one-step HSCCC-Semi-Prep-HPLC separation under the optimized conditions. The study also identified that QCN and KPL had weaker inhibitory effects on FAS compared to AA and MA. Overall, the study provides valuable information for the efficient extraction and separation of bioactive compounds from *Centella asiatica* (L.) Urban and their potential use as inhibitors of FAS.

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DETAILS

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Measurement of Magnesium, Zinc, and Copper in Human Serum by Using Isotope Dilution Inductively Coupled Plasma Mass Spectrometry (ID ICP-MS)

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ABSTRACT (ENGLISH)

In order to evaluate the reliability of the ID ICP-MS method for the measurement of magnesium, zinc, and copper in human serum, we investigated the traceability, precision, trueness, and uncertainty of the method. This method

traces the contents of magnesium, zinc, and copper in human serum to the standard materials NIST SRM3131a, SRM3168a, and SRM3114 respectively, thus completing the traceability to SI unit. The repeatability of this method for measuring magnesium, zinc, and copper in the human serum reference material GBW09152 was found to be 0.2%, 0.7%, and 0.6% ($n=9$), respectively. The measurement, when employed to measure the magnesium, zinc, and copper in standard materials, had caused a maximum deviation of less than 0.88%, 1.35%, and 1.15%, respectively. The measurement results are within the stated uncertainty range of standard materials. The expanded uncertainties were 0.2 mg·kg^{-1}, 0.04 mg·kg^{-1}, and 0.08 mg·kg^{-1} ($K=2$) for magnesium, zinc, and copper, respectively. Therefore, this method has high trueness, good reproducibility, and simple operation and is suitable for tracing the values of magnesium, zinc, and copper in human serum.

FULL TEXT

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1. Introduction

Isotope dilution mass spectrometry, which has many advantages over conventional methods, is the only authoritative method that can directly provide trace and ultratrace values [1]. In common analysis methods, the accuracy of the results may be affected by the loss of the elements to be tested in the process of sample pretreatment, matrix effects, and instrument signal drift [2–5]. Isotope dilution mass spectrometry is the method which measures only the isotope abundance ratio in the sample. The abundance ratio becomes a constant value when the concentration of the isotopic spike is added to the sample and reaches the equilibrium with the absence of external contamination, and the sample loss in the process of sample separation and concentration does not affect the abundance ratio [6]. Isotope dilution method can greatly eliminate interference and errors caused by sample pretreatment and has thus been confirmed by the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) as one of the five methods with absolute measurement properties [7, 8]. This isotope dilution mass spectrometry originated from the UK Government Chemist Laboratory (GCL) [9, 10]. We have established isotope dilution mass spectrometry for the detection of potassium and selenium in human serum using this principle [11]. Compared with conventional isotope dilution mass spectrometry, it has the advantage of not having to calibrate the concentration of the isotopic spike during the standardization process. It can determine the chemical composition in serum only by means of measuring the selected pair of isotope abundance ratios, thus reducing the requirements on the performance of the mass spectrometry instrument [12–17].

Trace elements of magnesium, zinc, and copper in the human body are closely related to people’s health [18–22]. Currently, there is no reference method for measuring zinc and copper in serum by using isotope dilution mass spectrometry in the Joint Committee on Traceability on Laboratory Medicine (JCTLM) list. Only the measurement of the concentration of magnesium in serum by using traditional isotope dilution mass spectrometry is available [23–25]. A precise method for analyzing magnesium, zinc, and copper in human serum by using two-step isotope dilution mass spectrometry was established in this laboratory. The repeatability of this method for measuring magnesium, zinc, and copper in the human serum reference material GBW09152 was found to be 0.2%, 0.7%, and 0.6% ($n=9$), respectively. The expanded uncertainties were 0.2 mg·kg^{-1}, 0.04 mg·kg^{-1}, and 0.08 mg·kg^{-1} ($K=2$) for magnesium, zinc, and copper, respectively. With the quality of easy operation, high trueness, and good reproducibility, this method is suitable as a reference method for measuring magnesium, zinc, and copper elements in human serum.

2. Materials and Methods

2.1. Materials and Reagents

The laboratory is a class 100,000 cleanroom. The experimental water was provided by a water purification system: Milli-Q Advantage (Millipore, USA). The nitric acid (Ultrapure-BVIII) and hydrochloric acid (MOS grade) used in the experiment were produced by the Beijing Institute of Chemical Reagents, China. The microanalytical balance used for sample weighing was Mettler Toledo XS205 (Switzerland), and the sample analysis was performed on an ICP

mass spectrometer: ELAN DRC-e (PerkinElmer, USA). The standard materials used for method tracing were magnesium (Mg) standard solution SRM3131a (NIST, USA), copper (Cu) standard solution SRM3114 (NIST, USA), and zinc (Zn) standard solution SRM3168a (NIST, USA). The isotope spikes used in the method were ^{25}Mg (assay: 97%), ^{65}Cu (assay: 99%), and ^{67}Zn (assay: 94%), concentrated isotopes from the Oak Ridge National Laboratory, USA. Trueness verification was performed by using inorganic components in frozen human serum GBW09152 (National Institute of Metrology, China), ERM-DA120a (LGC, UK) and electrolytes in frozen human serum SRM956d (NIST, USA).

2.2. Instrument Parameters

The instrument parameters for this research are listed in Table 1.

Table 1

Instrument parameters.

Parameter	Mg	Cu	Zn
ICP RF power (W)	1100	1100	1100
Gas flows (L/min)	0.97	0.97	0.97
Lens voltages (V)	6.75	6.75	6.75
Analog stage voltage (V)	−1700	−1700	−1700
Pulse stage voltage (V)	900	900	900
Dwell time per AMU	2 ms	2 ms	2 ms
Scan mode	Peak hopping	Peak hopping	Peak hopping
Sweeps/reading	25	25	25
Readings/replicate	15	20	25
Replicates	15	20	25
Cell Gas A: CH_4 (mL/min)	1.4	0	0.45
RP q: Ar (mL/min)	0.8	0.25	0.25

2.3. Methods

(1) A two-step dilution of NIST SRM3131a was performed by using the weighing method in a ratio of approximately 1 : 20, and a three-step dilution of NIST SRM3168a and NIST SRM3114 in a ratio of approximately 1 : 20. The final dilution must be prepared on the day of the experiment.

(2) The concentrated isotopic metal chips of ^{25}Mg, ^{65}C, and ^{67}Zn were dissolved with BVIII grade nitric acid and diluted to an appropriate concentration with ultrapure water, followed by a two-step dilution on the day of the experiment.

(3) A mixed solution of magnesium standard solution and ^{25}Mg isotope diluent, a mixed solution of zinc standard

solution and ^{67}Zn isotope diluent, and a mixed solution of copper standard solution and ^{65}Cu isotope diluent were prepared, respectively, by using the weighing method. A mixed solution of the serum sample and ^{25}Mg isotope diluent, a mixed solution of the serum sample and ^{67}Zn isotope diluent, and a mixed solution of the serum sample and ^{65}Cu isotope diluent were also prepared, respectively, by using the weighing method. The isotopic ratio ($^{24}Mg/^{25}Mg$ or $^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$) in the mixture solution was close to 1, and at the same time, the cps signal intensities of the corresponding isotopes in the mixture of the standard solution and the isotope diluent as well as in the mixture of the serum sample and the isotope diluent were close.

(4) Solutions of magnesium, copper, and zinc with appropriate concentrations were prepared on the day of the experiment so that the signal intensity of ^{24}Mg, ^{66}Zn, and ^{63}Cu in the solution is consistent with that of the corresponding isotopes in the mixed solution in (3).

(5) Solutions of ^{25}Mg, ^{67}Zn, and ^{65}Cu diluents with appropriate concentrations were prepared on the day of the experiment so that the signal intensity of the isotopes in the solution is consistent with that of the corresponding isotopes in the mixed solution in (3).

(6) Mass spectrometric procedures: the concentrations of magnesium, zinc, and copper elements in the serum sample were calculated according to the concentration formula (1) in the isotope dilution mass spectrometry [9]:(1)C x=Cz·mZcmYc·mYmX·RY−RBRB−RZ·RZ−RBcRBc−RY−CB.

In this formula, Cz is the concentration of the standard solution, m_Y is the mass of the enriched isotope added to the serum sample, m_X is the mass of the serum sample added to the mixture of the serum sample and the enriched isotope, m_{Yc} is the mass of the enriched isotope added to the standard solution, and m_{zc} is the mass of the standard solution added to the mixture of the standard solution and the enriched isotope. Rz is the isotope ratio of $^{24}Mg/^{25}Mg$ or $^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$ in the standard solution, and R_Y is the isotope ratio of $^{24}Mg/^{25}Mg$ ($^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$) in the enriched isotope. R_B is the isotope ratio of $^{24}Mg/^{25}Mg$ ($^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$) in the mixture of serum sample and the enriched isotope, and R_{Bc} the isotope ratio of $^{24}Mg/^{25}Mg$ ($^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$) in the mixture of standard solution and the enriched isotope. C_B is a blank in the measurement process.

It was found that the fluctuation of the isotope ratio in the standard solution or the enriched isotope had little impact on the final results, while the measurement fluctuation of the isotope ratio in the mixed solution would lead to changes in the final results. Therefore, the mixed solution of the standard solution and the enriched isotope and the mixed solution of the serum sample and the enriched isotope must be alternately measured six times so as to reduce the error introduced by instrument measurement drift.

3. Results and Discussion

3.1. Determination of Instrument Measurement Conditions

In this experiment, the dynamic reaction cell mode of inductively coupled plasma mass spectrometry (ICP-MS) was used to eliminate interference. When analyzing magnesium, interference from NaH was severe, and interference from Ca^{++} and LiO may also exist. The condition optimization was aiming to obtain the best analysis effect when analyzing ^{24}Mg, and the appropriate flow rate of the reaction gas CH_4 and argon. When analyzing zinc, interference from SO_2, ClO_2, and ArP may exist, and interference on ^{67}Zn was more likely to occur. The condition optimization was aimed at obtaining the best analysis effect when analyzing ^{67}Zn and the appropriate flow rate of the reaction gas CH_4 and argon. When analyzing copper, interference from SO_2 and PO_2 may exist, while all these interferences can be ignored in actual analysis. Therefore, this experiment analyzed copper in blood under standard mode.

3.2. Deduction of Signal Background in the Experimental Method

Solutions of serum magnesium with 6 concentration gradients ranging from 0.5 μg/ml to 9 μg/ml were measured. The response signals of ^{24}Mg and ^{25}Mg are linearly related to the concentration range of magnesium (see Figures 1(a) and 1(b)), with a linear correlation coefficient (r) of 0.99999. However, the ratio of ^{24}Mg response signal to ^{25}Mg response signal was not constant but gradually tended to be constant with the increase of concentration (see Figure 1(c)). This is because the straight lines in Figures 1(a) and 1(b) have a nonzero intercept, indicating the presence of a blank response signal for ^{24}Mg and ^{25}Mg. As the concentration becomes smaller, the relative proportion of the response signal becomes higher, and the impact on the ratio of ^{24}Mg to ^{25}Mg response signal is more significant.

Therefore, this blank response signal cannot be ignored. After subtracting the blank response signal from the measured impact signal, a constant value is obtained for the ratio of the ^{24}Mg to ^{25}Mg·g response signals. The same is true for the determination of $^{66}Zn/^{67}Zn$ and $^{63}Cu/^{65}Cu$, so in this experiment, the response signals used are all values after deducting the blank signal.

[figure(s) omitted; refer to PDF]

3.3. Effect of Solution Reaction System on Measurement Precision

It was found in the experiment that using a 0.02% hydrochloric acid system can improve the stability of $^{66}Zn/^{67}Zn$ measurement. Therefore, the determination of zinc in serum was indeed carried out in a 0.02% hydrochloric acid system.

3.4. Process Blank (LOB) and Detection Limits (LOD)

While preparing the mixture of serum sample and the enriched isotope, an appropriate amount of ^{25}Mg (^{67}Zn or ^{65}Cu) solution was taken into the blank sample tube as a process blank, so that the $^{24}Mg/^{25}Mg$ ($^{66}Zn/^{67}Zn$ or $^{63}Cu/^{65}Cu$) in the process blank was approximately equal to 2. The process blank was determined along with the samples in the same batch. The process blanks for magnesium, zinc, and copper in serum were 0.5 mg/kg, 0.09 mg/kg, and 0.010 mg/kg, respectively. When the confidence interval of 95% was determined, the detection limits of magnesium, zinc, and copper in serum were 0.7 mg/kg, 0.11 mg/kg, and 0.016 mg/kg, respectively.

3.5. Method Precision

The method precision was the relative standard deviation of the measurement results of 6 bottles of human serum reference materials. The experiments were carried out in 2 consecutive days, with 3 bottles each day, and 3 parallel for each bottle. The results are shown in Table 2. The precision for magnesium, zinc, and copper in different concentrations of human serum reference materials was lower than 0.3%, 0.9%, and 0.6%, respectively.

Table 2

Repeatability of magnesium, zinc, and copper in human serum by ID ICP-MS.

		Mg ($mg \cdot kg^{-1}$)			Zn ($mg \cdot kg^{-1}$)			Cu ($mg \cdot kg^{-1}$)	
Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Day 1	1#
18.0	23.3	28.7	0.625	0.831	1.597	1.06	1.61	18.0	23.3
28.7	0.627	0.834	1.583	1.06	1.62	18.1	23.2	28.9	0.646
0.835	1.610	1.06	1.62	2#	18.0	23.3	28.8	0.631	0.834
1.597	1.07	1.60	18.0	23.3	28.8	0.627	0.825	1.594	1.06
1.62	18.0	23.2	29.0	0.634	0.832	1.595	1.06	1.62	3#
18.0	23.3	28.7	0.628	0.818	1.607	1.06	1.61	18.0	23.3
28.7	0.634	0.819	1.599	1.06	1.63	18.0	23.3	28.9	0.625
0.826	1.596	1.06	1.61	-					
Day 2	4#	18.0	23.3	28.7	0.644	0.828	1.601	1.05	1.60

18.0	23.2	28.7	0.634	0.827	1.606	1.05	1.60	18.0	23.3
28.7	0.633	0.825	1.616	1.05	1.60	5#	18.0	23.2	28.7
0.634	0.827	1.599	1.05	1.61	18.0	23.3	28.7	0.632	0.830
1.596	1.06	1.60	18.0	23.3	28.7	0.629	0.826	1.600	1.06
1.62	6#	18.0	23.3	28.7	0.628	0.823	1.606	1.06	1.60
18.0	23.2	28.7	0.633	0.830	1.591	1.06	1.59	18.0	23.3
28.8	0.632	0.825	1.602	1.06	1.60	-			
s		0.03	0.03	0.09	0.006	0.005	0.008	0.005	0.010
–									
Avg		18.0	23.3	28.8	0.632	0.828	1.600	1.06	1.61
–									
CV		0.2%	0.1%	0.3%	0.9%	0.6%	0.5%	0.4%	0.6%

3.6. Method Trueness

Magnesium, zinc, and copper in standard substances NIST956D, ERM-DA120a, and GBW09152 were analyzed by using isotope dilution mass spectrometry. Parallel analysis was conducted three times a day for three consecutive days, and the results were good, as shown in Table 3.

Table 3

Analysis of standard reference material by two-way ID ICP-MS.

		Mg (mg·kg^{-1})				Zn (mg·kg^{-1})		Cu (mg·kg^{-1})	
NIST956d			GBW	ERM	GBW	ERM	GBW	Level 1	Level 2
Level 3	09152	-DA120a	09152	-DA120a	09152	Day 1	Test 1	34.98	22.91
10.49	20.59	0.649	1.156	1.126	1.064	Test 2	34.84	22.70	10.38
20.54	0.664	1.148	1.131	1.069	Test 3	34.60	22.72	10.41	20.61
0.660	1.140	1.126	1.073	-					

Day 2	Test 1	35.08	22.51	10.37	20.56	0.653	1.140	1.145	1.082
Test 2	34.60	22.95	10.43	20.63	0.652	1.156	1.140	1.077	Test 3
34.66	22.83	10.51	20.52	0.650	1.144	1.143	1.080	·	
Day 3	Test 1	34.74	22.59	10.36	20.50	0.665	1.147	1.131	1.069
Test 2	34.67	22.66	10.25	20.60	0.658	1.137	1.130	1.067	Test 3
35.04	22.73	10.28	20.56	0.651	1.156	1.135	1.072	·	
Avg		34.80	22.73	10.39	20.57	0.656	1.147	1.13	1.072
−									
cv		0.5%	0.6%	0.8%	0.2%	0.9%	0.7%	0.6%	0.6%
−									
Certified values		34.96 ± 0.24	22.83 ± 0.16	10.30 ± 0.08	20.75 ± 0.44	0.658 ± 0.033	1.132 ± 0.056	1.130 ± 0.033	1.085 ± 0.044
−									
Bias (%)		0.46%	0.42%	−0.84%	0.88%	0.33%	−1.35%	−0.37%	1.15%

3.7. Uncertainty Evaluation

In this research, the uncertainty caused by factors such as the experimental reagents, the samples, the laboratory environments, the solution preparation, the instrument measurement, and the data processing has been evaluated as the source of uncertainty in the measurement process. It can be concluded that by evaluating the uncertainty of each parameter in formula (1), the uncertainty caused by each factor in the measurement process can be fully included. Each parameter in formula (1) is an independent parameter, and the uncertainty ucy related to measurement is calculated as follows:(2)ucy=∑i=1N∂f∂xi2u2xi.

The formula of the sensitivity coefficient (∂f/∂xi) of each parameter in formula (1) is as follows:(3)∂Cx∂Cz=mZcmYc·mYmX·RY−RBRB−RZ·RZ−RBcRBc−RY,∂Cx∂mY=Cz·mZcmYc·1mX·RY−RBRB−RZ·RZ−RBcRBc−RY,∂Cx∂mx=Cz·mZcmYc·−mYmX2·RY−RBRB−RZ·RZ−RBcRBc−RY,∂Cx∂mYc=Cz·−mZcmYc2·mYmX·RY−RBRB−RZ·RZ−RBcRBc−RY,∂Cx∂mZc=Cz·1mYc·mYmX·RY−RBRB−RZ·RZ−RBcRBc−RY,∂Cx∂Rz=Cz·mZcmYc·mYmX·RY−RBRB−RZ2RZ−RBcRBc−RY+RY−RBRB−RZ·1RBc−RY,∂Cx∂RY=Cz·mZcmYc·mYmX·RZ−RBcRB−RZ·RBc−RY+RY−RBRBc−RY2,

∂Cx∂RB=Cz·mZcmYc·mYmX·RZ−RBcRBc−RY·−RB−RZ−RY−RBRB−RZ2,∂Cx∂RBc=Cz·mZcmYc·mYmX·RY−RBRB−RZ·−RBc−RY−RZ−RBcRBc−RY2,∂Cx∂CB=−1.

Therefore,(4)ucy=∂Cx∂Cz2·ucCz2+∂Cx∂mY2·ucmY2+…+∂Cx∂CB2·ucCB2.

This research has evaluated the uncertainty of measurement and Table 4 is the source of uncertainty for measuring the parameters. The uncertainty of the measurement of Type A is the experimental standard deviation of the 6 repeated instrument measurements, taking the worst result in the experiment as the evaluation data. The uncertainty of the measurement of Type B is the uncertainty of solution preparation, which is synthesized from the uncertainty resulting from the electronic balance calibration and the uncertainty resulting from weighing.

Table 4

Sources of uncertainty in the determination.

Sources of uncertainty	Mg		Zn		Cu	
GBW09152		GBW09152		GBW09152		Value (x_i)
u_c (x_i)	Value (x_i)	u_c (x_i)	Value (x_i)	u_c (x_i)	Type A uncertainties	
R_Y	0.014	0.000	0.034	0.000	0.004	0.000
R_z	6.667	0.021	6.208	0.014	2.107	0.003
R_{Bc}	0.920	0.002	1.041	0.003	1.080	0.004
R_B	0.885	0.003	1.004	0.003	1.049	0.002
C_B (mg·kg^{-1})	0.5	0.3	0.09	0.05	0.010	0.005
Type B uncertainties						
C_z (mg·kg^{-1})	19.398	0.003	1.290	0.002	1.008	0.002
m_{zc} (mg)	3.0×10^2	0.040	2.6×10^2	0.040	5.4×10^2	0.040
m_{Yc} (mg)	5.5×10^2	0.040	2.0×10^2	0.040	4.0×10^2	0.040
m_Y (mg)	3.4×10^2	0.040	200×10^2	0.040	4.0×10^2	0.040
m_x (mg)	2.0×10^2	0.040	300×10^2	0.040	4.8×10^2	0.040
Combined types A and B	—	0.25	—	0.045	—	0.011
Degrees of freedom (Veff)		8		8		8

Coverage factor (k)	—	2	—	2	—	2
Measured value ($mg \cdot kg^{-1}$)	20.57	—	1.147	—	1.072	—
Expanded uncertainty ($U(\bar{x})$, $k=2$) ($mg \cdot kg^{-1}$)	—	0.2	—	0.04	—	0.08

4. Conclusions

This isotope dilution mass spectrometry established in our laboratory with the quality of easy operation, high trueness, and good reproducibility was used to accurately analyze the contents of magnesium, zinc, and copper in human serum. In the isotope dilution mass spectrometry determination process, there is no need to measure the accurate amount of isotope, the enriched isotope, which reduces the requirements for mass spectrometry instruments [26, 27]. This method is suitable as a reference method for assigning values of magnesium, zinc, and copper in human serum.

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Comparing Jaffe and Enzymatic Methods for Creatinine Measurement at Various Icterus Levels and Their Impacts on Liver Transplant Allocation

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ABSTRACT (ENGLISH)

The Model for End-Stage Liver Disease (MELD) scoring system is used to prioritize liver transplantations and assess disease severity. This includes the international normalized ratio (INR), creatinine, and total bilirubin. Since there are several ways to measure creatinine, MELD scores can produce inconsistent results. The objectives of this study were to define a valid cut-off for bilirubin interference in creatinine measurement and to assess the effects of various icteric levels on creatinine measurement and liver transplant allocation. A total of 400 serum samples were categorized into four groups based on their icteric indices and total bilirubin levels, including non-, mild, moderate, and severe icteric samples. Both chemical Jaffe and enzymatic techniques were used to determine the creatinine levels in all four groups, and the findings were compared. In parallel, serum samples from 83 liver transplant candidate patients were divided into three groups depending on their bilirubin levels and then similarly evaluated and interpreted. The MELD scores were then computed for each group and compared. In icteric samples, the enzymatic method produced higher results for the creatinine concentrations than the Jaffe method did, and the

mean creatinine difference rose from 0.08 in nonicteric group to 1.95 in groups with severe icterus. In addition, the enzymatic approach yielded higher findings for creatinine and subsequently for MELD scores in patients who were liver transplant candidates. When the bilirubin concentration was above the 4 mg/dL threshold, there were differences between the approaches for both the creatinine and the MELD score (p values: 0.0001 and 0.027, respectively). The chemical Jaffe is a readily available and considerably cost-effective method for measuring creatinine. However, it is influenced by a variety of known and unknown interfering substances, and it should be applied cautiously when working with icteric samples. Alternate techniques such as the enzymatic method should be considered when the bilirubin level exceeds 4 mg/dL. Though this cut-off is instrument and kit-dependent, each laboratory is advised to have its cut-off for bilirubin interference.

FULL TEXT

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1. Introduction

The Model for End-Stage Liver Disease (MELD) scoring system was introduced in the USA in 2002 and is used in many countries to prioritize liver allocation for most patients who require transplantation and to differentiate the severity of liver diseases. In fact, it is based on the “sickest first” principle. According to multiple studies, MELD can predict three-month mortality for patients on the liver transplant waiting list with an accuracy of about 80%. Pretransplant mortality was observed to increase exponentially rather than linearly with a change in the MELD score; as a result, changes of one or two points near the upper end of the MELD score are very clinically significant. Moreover, the MELD is a helpful clinical aid in a wide range of hepatic disease severity and variety. It incorporates commonly used laboratory tests, including the International Normalized Ratio (INR), serum creatinine, and serum total bilirubin. Unlike the objectivity of these three variables, the MELD score may be subject to some limitations based on how the parameters, especially creatinine, are measured [1–5].

Creatinine is measured using different automated methods, which include chemical Jaffe and enzymatic methods on automated analyzers, high-performance liquid chromatography (HPLC), and isotope dilution-mass spectrometry (IDMS). IDMS is the reference method of creatinine measurement, but it is not practical for routine usage [6–8].

The chemical Jaffe is one of the earliest methods for creatinine measurement, in which creatinine reacts with picrate under alkaline conditions to produce a yellow-red substance that is spectrophotometrically measured at a wavelength of 505 nm. It was first introduced in 1886 and is still in use today with some modifications due to its greater availability and cost-effectiveness [9–12]. However, major analytical problems are associated with the Jaffe reaction, particularly *those relating to positive and negative* interference by chromogens. More than 50 chromogenic interferents have been documented [13]. Glucose, uric acid, antibiotics, keto acids, bilirubin, and other chromogens interfere with creatinine measurement, and it may be measured higher or lower than the actual value. The original Jaffe method has undergone numerous modifications to reduce interference by such substances, with varying degrees of success [14, 15]. Although these modifications can correct interference from slow-reacting noncreatinine chromogens (glucose, acetone, and ascorbic acid), fast-reacting substances such as alpha-keto compounds and cephalosporin antibiotics give positive interference. In contrast, serum bilirubin negatively interferes with creatinine results and is a serious concern for clinical labs. Both conjugated and unconjugated bilirubin are disturbing factors as well as bilirubin breakdown products [3, 4, 10, 14].

Prior studies have demonstrated poor agreement and significant variation (low and high) between different creatinine measurement methods in specimens with high bilirubin concentration (icteric samples) and the MELD scores subsequently [4, 9, 10]. In a study by Evangelos Cholongitas et al., four different creatinine assays, including O'Leary modified Jaffe, compensated kinetic Jaffe, enzymatic, and standard kinetic Jaffe, were compared in patients with aberrant liver function tests. There was poor agreement between different methods, and increased variability in creatinine results and MELD scores occurred with increasing bilirubin concentrations [10]. Moreover, Carol Goulding et al. showed a lack of reproducibility of creatinine measurement and MELD scoring among four liver transplant

units, and in two studies by Thorsten Kaiser et al., the Jaffe-based method showed greater creatinine levels than the enzymatic methods [4, 9, 16]. These discrepancies are worse with more severe jaundice and are sufficient to allow a patient to die while on the waiting list who may otherwise have received a transplant if his blood had been analyzed by a different method.

Similarly, the estimated glomerular filtration rate (GFR) calculation for chronic kidney disease (CKD) is another issue with the diversity of methods to measure serum creatinine. A patient's estimated GFR-based classification can vary significantly depending on small analytical changes in serum creatinine [17–24].

Since the current method for measuring creatinine (chemical Jaffe) is affected by high serum bilirubin, we conducted this study to compare the chemical Jaffe method with the more precise enzymatic method in icteric samples and assess the impact of various icteric levels on liver transplant allocation. Furthermore, we aimed to establish a trustworthy cut-off for bilirubin interference in the Jaffe method.

2. Materials and Methods

This cross-sectional study was conducted in the Clinical Chemistry Laboratory of Abu-Ali Sina Hospital, Shiraz, Iran, a transplantation center, from May 2022 to November 2022. The study was designed following the Declaration of Helsinki after obtaining approval from the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1400.105).

The icteric index was set up on an autoanalyzer for a simpler selection of icteric samples, using 56 serum samples with different levels of bilirubin, absorbance measurement at various specific bichromatic wavelength pairs (480 and 505 nm), and 0.9% sodium chloride as a reagent. The icteric index is a cost-effective, quick, and simple method for estimating hyperbilirubinemia [25, 26]. The relationship between total bilirubin level and icteric index is depicted in Figure 1.

[figure(s) omitted; refer to PDF]

Next, over a month, 400 residual serum samples from 356 individuals who were referred to the lab for various clinical issues were selected and categorized into four groups based on their icteric indices and total bilirubin levels, including nonicteric (bilirubin: ≤1.3 mg/dL), mild (bilirubin: 1.4 –4 mg/dL), moderate (bilirubin: 4.1–15 mg/dL), and severe (bilirubin: >15 mg/dL) icteric serum samples. Then, the specimens of all four groups were analyzed for creatinine using both chemical Jaffe and enzymatic methods, and the results were compared.

Concurrently, the specimens of patients who were candidates for liver transplantation (83 patients) were analyzed and interpreted similarly. All samples were stored at −20°C before analysis. Then, the MELD scores were calculated and compared in three groups, based on bilirubin level, with the formula, according to the guidelines of the United Network for Organ Sharing [27].(1)10×00.957×LogecreatinineμmolL*A+0.378×LogebilirubinμmolL*B+1.120×Loge INR+0.643,A=00.01131=creatininemg/dLcreatinineμmol/L,B=00.05848=bilirubinmg/dLbilirubinμmol/L,*The upper limit of serum creatinine was capped at 4 mgdL.

The measurements for creatinine were performed concurrently by the manufacturer's instructions using a DIRUI 1200 autoanalyzer after the two methods had been calibrated and quality control results had been confirmed. The reagents for the measurement of bilirubin and creatinine (Jaffe and enzymatic methods) were obtained from Biorex (Table 1). The INR was derived from prothrombin time (PT) measured using a Stago coagulation analyzer. None of the patients were taking either ascorbic acid or antibiotics. In addition, low-volume serum specimens and those with concurrent hemolysis and/or lipemia were excluded from the study.

Table 1

Characteristics of methods used in the study.

Analytes	Method	Reagent	Wavelength (nm)	Analytical sensitivity (mg/dL)	Linearity limit (mg/dL)	Limit of icterus interference, bilirubin (mg/dL)

Creatinine	Jaffe	BIOREX	500	0.2	20	4
Creatinine	Enzymatic (creatinine deiminase)	BIOREX	340	0.2	20	15
Bilirubin	Jendrassik- Grof	BIOREX	546	0.1	25	—

IBM SPSS (version 25.0) was used to analyze all the data. Quantitative variables were expressed as mean ± SD and/or median (range). Significance testing was 2-sided and set to less than 0.05. The Wilcoxon signed-rank test was used for a nonparametric comparison between paired *Cr* values and paired MELD scores. The Mann–Whitney *U* test was used to determine how the mean values differed.

3. Results

In nonicteric samples, there were no discernible differences between the Jaffe and enzymatic methods for measuring creatinine; however, in icteric samples, the enzymatic approach indicated a substantial increase (p value 0.0001), with a rising trend from the mild to severe icteric group (Table 2). Figure 2 depicts the connection between the Jaffe and enzymatic approaches in these groupings.

Table 2

Comparison of 4 groups regarding Jaffe and enzymatic creatinine results.

Groups	Number of samples	Degree of icterus	Total bilirubin (mg/dL) (mean ± SD)	Creatinine Jaffe (mg/dL) (mean ± SD)	Creatinine enzymatic (mg/dL) (mean ± SD)	P value
1	100	Nonicteric	0.64 ± 0.24	1.71 ± 1.41	1.79 ± 1.38	0.237
2	100	Mild icteric	2.2 ± 0.77	1.39 ± 1.06	1.81 ± 1.14	<0.0001
3	100	Moderate icteric	8.3 ± 5.34	1.2 ± 0.95	1.91 ± 1.20	<0.0001
4	100	Severe icteric	30.8 ± 17.31	1.33 ± 1.04	3.28 ± 1.89	<0.0001

[figure(s) omitted; refer to PDF]

Similar alterations in creatinine and MELD scores were found in 83 samples from liver transplant candidates during the second investigation (Table 3). The mean MELD score differences between the two approaches are shown in Figure 3.

Table 3

Data from specimens of liver transplantation candidates.

Total bilirubin (mg/dL)	Number of cases	Bilirubin range (mean) (mg/dL)	Mean difference of creatinine

<1.5	34	0.12–1.48 (0.75)	0.08 ± 0.42
1.5–4	21	1.51–3.82 (2.37)	0.10 ± 0.28
>4	28	4.53–58.4 (17.40)	0.78 ± 0.80
Total	83	0.12–58.4 (6.78)	0.32 ± 0.64

[figure(s) omitted; refer to PDF]

4. Discussion

It is generally known that bilirubin negatively affects the Jaffe method's estimate of serum creatinine. The exact mechanism of bilirubin interference is not well known. However, bilirubin is converted to biliverdin under alkaline conditions, which results in a drop in absorbance at 510 nm (the absorbance peak of the creatinine picrate complex) and an increase at 630 nm (the absorbance peak of biliverdin), underestimating the concentration of creatinine. So, excess bilirubin results in a negative interference (lower creatinine values) that increases with increasing serum bilirubin concentrations and is typically found in the sickest patients with the greatest priority for liver transplantation [3, 4, 10, 14]. However, bilirubin interference in the Jaffe method appears to be more manufacturer-dependent, and few researchers have found positive interference when using compensated Jaffe methods [7, 28, 29].

This interference can be solved in several ways, including sample dilution, rate-blanking, the addition of oxidizing agents (ferricyanide), and deproteinization of the serum. The serum dilution and rate-blanking methods are currently applied to some reagents available, with varying degrees of success. However, pretreatment by deproteinization of patients' serum and oxidizing agents cannot be routinely utilized because it cannot be automated and requires manual operation [13, 14, 30].

Alternatively, creatinine concentrations can be measured enzymatically. Several enzymes, such as creatinine amidohydrolase and creatinine kinase, can convert creatinine to creatine with a subsequent absorbance change at 340 nm. This method has been reported to be more resistant to bilirubin interference and improve the specificity of the measurement. According to previous studies and the manufacturer's specifications, the enzymatic approach appears more appropriate as a routine laboratory technique for measuring icteric serum creatinine [31]. However, it is considerably more expensive than the kinetic Jaffe method [7, 9, 32, 33].

In this study, the effectiveness of the Jaffe and enzymatic methods in icteric samples was compared at various icterus levels. The creatinine concentrations showed higher results using the enzymatic method than the Jaffe method, and as bilirubin levels rose, the mean differences in creatinine widened. Furthermore, the enzymatic method produced higher results for creatinine and MELD scores in patients who were candidates for liver transplantation. The differences between the methods for creatinine and MELD scores were significant when bilirubin concentration crossed the border of 4 mg/dL, which is consistent with the manufacturers' claim regarding the degree of bilirubin interference. Likewise, various limits for bilirubin interference have been established by previous research (i.e., 25 mg/dL) using different reagents and analyzers [1].

The lower creatinine and MELD scores by the Jaffe method will cause patients to be misplaced on the waiting list and delay receiving liver transplants. These findings restrict the application of the Jaffe method for creatinine measurement in icteric samples.

In a laboratory, a test's cost-effectiveness is just as essential as its accuracy. The cost-effectiveness of a test is significant when it is sensitive and specific enough to make a diagnosis [34, 35]. As a result, in our laboratory, the enzymatic approach should be reserved just for instances where the bilirubin level is greater than 4 mg/dL.

5. Conclusion

The chemical Jaffe is a readily available and considerably cost-effective method for measuring creatinine. However, it is influenced by a variety of known and unknown interfering substances, and it should be applied cautiously when working with icteric samples, and alternate techniques such as the enzymatic method should be considered when

the bilirubin level exceeds 4 mg/dL. Though this cut-off is instrument and kit dependent, each laboratory is advised to have its cut-off for bilirubin interference.

Authors' Contributions

SN, DS, and AMH developed the concept of the study and the study design and wrote the draft manuscript. DS and SN set up the tests on autoanalyzers. EMJ and EM did the analyses. AFE and ZSHA selected the cases and samples. SN, DS, and MS with the input of all authors interpreted the data. All authors read and approved the final manuscript.

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Retracted: Application of Isotope Tracer in Cross-Well Nanometre Tracer Testing

Chemistry International Journal of Analytical

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FULL TEXT

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(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Peer-review manipulation

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Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] Y. Pang, L. Zhao, Y. Song, G. Wei, Y. Han, Q. Wang, "Application of Isotope Tracer in Cross-Well Nanometre Tracer Testing," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6387030, 2022.

DETAILS

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Retracted: Influence of Nanosemiconductor Materials on Thermal Stability of Solar Cells

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References

[1] A. Ma, "Influence of Nanosemiconductor Materials on Thermal Stability of Solar Cells," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6805501, 2022.

DETAILS

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Place of publication:	New York
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Retracted: Preparation and Optical Properties of Compound Nanopowder Art Ceramics

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[1] Y. Li, "Preparation and Optical Properties of Compound Nanopowder Art Ceramics," International Journal of

Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5415922, 2022.

DETAILS

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Retracted: Multiobjective Optimization Design of Green Building Energy Consumption Based on Inorganic Thermal Insulation Nanomaterials

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[1] Y. Yuan, S. Li, "Multiobjective Optimization Design of Green Building Energy Consumption Based on Inorganic Thermal Insulation Nanomaterials," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6993034, 2022.

DETAILS

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Retracted: Impact Sound Insulation Performance Testing of Nano-Inorganic Composite Floor Slabs for Green Buildings

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References

[1] W. Luo, Y. Yang, H. Zhou, "Impact Sound Insulation Performance Testing of Nano-Inorganic Composite Floor Slabs for Green Buildings," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5642361, 2022.

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Subject:	Research; Acoustic insulation; Green buildings
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Retracted: Broadband Design of Midinfrared Chiral Metamaterials Based on the Indium Tin Oxide Conical Helix

Chemistry International Journal of Analytical

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References

[1] W. Zhang, "Broadband Design of Midinfrared Chiral Metamaterials Based on the Indium Tin Oxide Conical Helix," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/3644004, 2022.

DETAILS

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Document 15 of 79

Retracted: Application of Carbon Nanofiber-Modified Concrete in Industrial Building Design

Chemistry International Journal of Analytical

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References

[1] T. Liu, "Application of Carbon Nanofiber-Modified Concrete in Industrial Building Design," International Journal of Analytical Chemistry, vol. 2023,DOI: 10.1155/2023/2587551, 2023.

DETAILS

Subject:	Research; Industrial buildings; Carbon fibers; Building design
Publication title:	International Journal of Analytical Chemistry; New York
Volume:	2023
Publication year:	2023
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Publisher:	Hindawi Limited
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Publication subject:	Chemistry--Analytical Chemistry
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Language of publication:	English
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Retracted: Preparation and Synergistic Anti-Tumor Effect of Iridium Oxide Nanocomposites under Microscope

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In addition, our investigation has also shown that one or more of the following human-subject reporting requirements has not been met in this article: ethical approval by an Institutional Review Board (IRB) committee or equivalent, patient/participant consent to participate, and/or agreement to publish patient/participant details (where relevant).
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References

[1] X. He, F. Xiang, Z. Xu, "Preparation and Synergistic Anti-Tumor Effect of Iridium Oxide Nanocomposites under Microscope," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/9694425, 2022.

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Publication title:	International Journal of Analytical Chemistry; New York
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Retracted: Human Health Risk Prediction Method of Regional Atmospheric Environmental Pollution Sources Based on PMF and PCA Analysis under Artificial Intelligence Cloud Model

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[1] S. Zhang, X. Sun, N. Liu, J. Mi, "Human Health Risk Prediction Method of Regional Atmospheric Environmental Pollution Sources Based on PMF and PCA Analysis under Artificial Intelligence Cloud Model," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/7207020, 2022.

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Wastewater Treatment Using Wood Ash and Cement as Chemical Coagulant

Milkessa Ingida [1] ; Bedane, Gurmesa [1] ; Firanbon Adugna [1] ; Nigusu, Degefa [1] ; Hussen, Mohammed [1] ; Chala Hailu Sime [1]

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ABSTRACT (ENGLISH)

Water is essential for daily activities and maintaining human well-being. However, in many less-developed countries, including Ethiopia, the lack of a well-developed wastewater treatment system leads to contaminated surface water. This poses significant risks to human health. To address this problem, wastewater can be treated using locally

available materials such as wood ash and cement as chemical coagulants. The objective of this study was to treat wastewater using these materials. The study involved analyzing a 20-liter sample of wastewater from the Awetu River in Jimma City, Ethiopia. The materials used for the treatment included wood ash, cement, and lemon. Various doses of cement and wood ash were prepared and added to the wastewater. The results showed that 5 g was the optimum dosage for effectively treating the wastewater. The treated water at the optimum dosage exhibited significant improvements in turbidity, total dissolved solids, conductivity, and color, meeting drinking water criteria. Overall, the study concludes that locally available materials such as wood ash and cement can be successfully utilized as chemical coagulants for wastewater treatment. This approach offers a viable solution for improving water quality and reducing the risk of waterborne diseases.

FULL TEXT

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1. Introduction

Water contamination can occur due to natural environmental factors as well as human activities. Contaminated water can cause health risks to individuals through microbiological, chemical, and physical contamination [1]. Microbiological contamination includes various disease-causing organisms such as bacteria, fungi, and viruses, which can lead to the spread of pathogens and even epidemics, potentially resulting in fatalities [2, 3]. Waterborne diseases refer to infections transmitted through the consumption of contaminated drinking water. While various pathogens can be transmitted through water, bacteria and protozoa are among the most common culprits [4]. Apart from specific substances such as cyanide and nitrate, chemical contamination usually presents a prolonged threat to health. When water quality is compromised in terms of clarity, appearance, or flavor, it might be considered unsatisfactory for consumers. Physical contamination also poses a potential health hazard, as numerous microorganisms are frequently linked with solid particles in water, thus elevating the likelihood of survival and dissemination of microbiological impurities [5].

In order to ensure the safety of water for consumption, all wastewater needs to undergo treatment to remove any potential health risks associated with it [6]. Most treatment systems are designed to address microbiological contamination and physical constituents that hinder acceptability or support microorganism survival, mainly related to suspended solids in the water. Additionally, disinfection is a standard practice included in water treatment processes to further enhance safety [7].

The contamination of drinking water with pathogens presents a significant and widespread health risk to humans globally, leading to numerous disease outbreaks and instances of poisoning resulting from exposure to untreated or inadequately treated water throughout history [8]. Efforts to enhance water quality and sanitation have seen substantial investments from international donors and governments. However, the extension of water supply systems in developing countries has faced challenges, and there are still over 780 million people worldwide lacking access to improved sources of drinking water. Specifically, within this statistic, a significant portion, exceeding 605 million individuals, resides in sub-Saharan Africa, highlighting the region as particularly affected by inadequate access to safe water [9].

As a developing country, Ethiopia has embraced the Millennium Development Declaration, which primarily aims to reduce poverty [10]. Previous studies have highlighted the importance of clean water, proper sanitation, and hygiene in poverty alleviation [11]. The provision of safe drinking water and appropriate treatment methods is a global concern. However, developing countries, including Ethiopia, have been facing insufficient access to safe drinking water from improved sources and inadequate sanitation services [12].

Consequently, individuals continue to rely on unsecured water sources like rivers, streams, springs, and hand-dug wells. Due to their open nature, these sources are highly vulnerable to contamination from floods, birds, animals, and human activities [13, 14]. Moreover, many of these water sources are located near gullies where open defecation is prevalent, leading to the contamination of water by flood-washed waste materials [15]. To ensure

environmental safety, a considerable portion of the water utilized by households, industries, and businesses requires treatment before it is discharged back into the environment [16].

The issue of water quality has direct or indirect implications for health. This alarming revelation emphasizes the critical need for global attention and research on water treatment [17]. A substantial portion of the population in developing countries, such as Ethiopia, primarily residing in rural areas, faces significant challenges in accessing safe drinking water and adequate water quality for various purposes, including irrigation. The problem of water quality is pervasive, affecting both urban and rural populations [18].

Access to improved sources of drinking water in Ethiopia is limited, with only 54% of households having access to such sources [19]. Furthermore, a mere 8% of households have improved toilet facilities that are not shared with other households [20]. This issue is prevalent in the Jimma community, resulting in numerous individuals being affected by waterborne diseases like diarrhea and cholera.

Assessing water treatment measures is crucial in determining the availability of improved water sources. The quality of drinking water is deemed acceptable when it meets the requirements in terms of its physical, chemical, and bacteriological parameters, ensuring safety [21]. Chemical coagulants are commonly employed in community drinking water treatment systems, although some applications can be observed in household water treatment methods.

The primary chemicals utilized for coagulation in water treatment include aluminum sulfate (alum), polyaluminium chloride (PAC), alum potash, and iron salts (ferric sulfate or ferric chloride). Additionally, lime ($Ca(OH_2)$), lime soda ash (Na_2CO_3), and caustic soda (NaOH) are occasionally employed to soften water [22]. Ensuring access to clean drinking water is crucial for human health, national security, and economic prosperity. The coagulation process in water treatment aims to eliminate colloidal particles from wastewater, which can encompass suspended matter and various sizes of solid particles [23].

Ethiopia's current focus, as outlined in the Millennium Development Goal declaration, is poverty alleviation through the effective utilization of water treatment resources [24]. To accomplish this objective, one of the priority areas is the provision of sufficient and high-quality water [18, 25]. Hence, it becomes imperative to find a cost-effective approach for treating contaminated surface water in both rural and urban communities. This research aims to address this issue by utilizing wood ash and cement as chemical coagulants for wastewater treatment.

Obtaining and ensuring a sufficient water supply has always played a critical role in the development of human settlements. In order to make water suitable for drinking and other purposes, various forms of treatment are necessary for all water sources. The main objective of this research aligns with this need and aims to treat wastewater for potable use or drinking and other purposes by employing cement and wood ash. These materials have been chosen due to their effectiveness and easy availability locally. Thus, this study utilizes cement and wood ash as coagulants for wastewater treatment.

2. Materials and Methods

The study was conducted in the southwest of the Oromia region, specifically within Jimma City of Ethiopia. The wastewater samples for laboratory analysis were collected from Awetu River, which is situated within Jimma City. The river is located roughly 3 kilometers away from the Jimma Institute of Technology. Its geographic coordinates are approximately 7°40′3″N latitude and 36°50′5″E longitude.

2.1. Collection of Raw Material

For this study, a wastewater sample was collected from the Awetu River in Jimma, Ethiopia. The laboratory facilities at Jimma Institute of Technology, specifically the Water Supply and Environmental Engineering Laboratory, were utilized to analyze and measure different properties of the collected wastewater samples. The properties of the water, both before and after the coagulation process, were tested. This allowed for the assessment of the efficiency and effectiveness of the coagulation treatment on the sampled wastewater collected.

2.2. Materials and Chemicals Used

In this research, various materials were used to conduct the experiments effectively. These included instruments like a balance for precise measurements, a turbidity meter to assess water clarity, a Crison conductivity meter to

measure conductivity, cuvettes for holding samples, a pH meter for acidity testing, beakers for mixing, filter papers for separating particles, specialized jar test equipment, pipettes for accurate liquid transfer, plates for organizing samples, an oven for controlled drying, an incubator for specific environmental conditions, crucibles for heating samples, and various chemicals like lemon for adjustments and treatments. Each of these tools played a crucial role in obtaining accurate data and insights during the study.

2.3. Cement and Wood Ash as a Chemical Coagulant

Cement and wood ash, both of which are white fine powders, were employed in the treatment of wastewater in this study. Ash can come in various forms, including wood ash, volcanic ash, coal ash (fly ash), cremation ash, and seaweed ash. For this research, wood ash was specifically used after undergoing a sieving process. In addition, wood ash can be used as a chemical coagulant. It contains various minerals, including alumina (Al_2O_3), up to 15 percent iron oxide (Fe_2O_3), and ideally no more than about 6 percent silica (SiO_2). The primary compound in wood ash responsible for its coagulating properties is calcium aluminate ($CaO{\cdot}Al_2O_3$). The primary compound responsible for cement's binding properties is calcium aluminate ($CaO.\ Al_2O_3$) [26].

Wood ash, on the other hand, contains several compounds such as calcite ($CaCO_3$), lime (CaO), and calcium chlorate hydrate ($Ca(ClO)_2.3H_2O$) [27]. The primary compound found in wood ash is calcium carbonate or calcite.

When cement and wood ash are used together in water treatment processes, their combined properties and chemical compositions can contribute to the coagulation of particles and the removal of impurities from wastewater. The specific characteristics of cement and wood ash make them effective materials for treating contaminated water and improving its quality.

Wood ash and cement are derived from different sources and production processes. Wood ash is obtained through the burning of wood, while cement is produced in industrial settings and comprises lime, silica, alumina, and iron oxide. The particle size of wood ash may vary depending on the source, with some ashes being finer or coarser than cement particles. When a mixture of cement and wood ash was added to high turbidity water or wastewater, they settled at the bottom of the water, allowing for the purification of water, with the purified water being collected from the top.

When only wood ash was used as a sole coagulant, it tended to form gel-like lumps when mixed with wastewater, which impeded the treatment process. To address this issue and minimize the formation of gel-like lumps, a mixture of both cement and wood ash was utilized as a chemical coagulant. The addition of cement helped to prevent the formation of gel-like lumps. Moreover, using cement alone as a coagulant would be more expensive compared to wood ash.

2.4. Preparation of Cement and Wood Ash as a Coagulant

In this study, the powder of cement and wood ash was prepared separately. The fine powder of wood ash was obtained by using a mortar and pestle to grind the ash into a finely powdered form. This wood ash powder was then directly mixed with cement, which acted as a coagulant for wastewater treatment purposes. The ratio of cement to wood ash can vary depending on specific treatment goals, characteristics of the wastewater, and local conditions. Generally, the mixture may consist of cement and wood ash in proportions ranging from 1:1 to 1:3 by weight. However, it is crucial to note that precise ratios should be determined through laboratory testing and empirical observation to ensure optimal performance in a given wastewater treatment application. For this study, cement and wood ash of 1:3 by weight were used as a coagulant in the wastewater treatment process. When cement and wood ash are mixed and added to wastewater, it forms a weak base and part of salt is settled down. The prepared cement and wood ash powder mixture was added to this fixed volume of wastewater for coagulation. This allowed for the assessment of the effectiveness of the coagulation process in treating the wastewater.

To determine the appropriate dose for wastewater treatment, various amounts of cement and wood ash (5, 7, 10, 15, and 25 grams) were measured using a balance and mixed with the sample wastewater. The mixture was then placed on a shaker and agitated for several minutes. This ensured thorough mixing and interaction between the coagulant and the wastewater. Figure 1 shows dosage of wood ash and cement.

[figure(s) omitted; refer to PDF]

2.5. Coagulation Experiments

In this study, a total of 20 liters of wastewater samples were collected from the Awetu River for coagulation-flocculation analysis. To conduct the analysis, the collected water sample was dispensed into five separate beakers, with each beaker containing 500 ml of the sample water. Dividing this sample into beakers of the same volume is a deliberate choice to establish controlled experimental units. This fixed volume not only facilitates precise dosage measurements but also allows for consistent treatment across samples. Such careful handling of the sample ensures that any observed variations in the coagulation process are attributable to the dosage levels and not influenced by sample size.

The coagulation-flocculation process was carried out using a jar test apparatus. The measured dosage of cement and ash (5 g, 7 g, 10 g, 15 g, and 25 g) was added to the five individual beakers, each containing 500 ml of the water sample. This setup enabled the evaluation of the effectiveness of different coagulant dosages in achieving coagulation-flocculation in wastewater treatment. Figure 2 shows the jar test and dosage.

[figure(s) omitted; refer to PDF]

Once the desired amount of coagulant mixture was added to the turbid water sample, the blades of the jar test apparatus were adjusted based on the intended mixing speed for both the coagulation and flocculation tests.

For the coagulation test, which involves rapid mixing, the blades were set to rotate at a speed of 260 rotations per minute (rpm). This speed was maintained for 5 minutes to ensure thorough mixing of the added coagulant with the colloidal particles present in wastewater. This rapid mixing promotes the destabilization of the particles and initiates the coagulation process.

Following the coagulation process, the next step involved flocculation, which requires slower mixing. To allow the destabilized particles to agglomerate and form larger flocs that can settle more rapidly, the rotation speed of the blades was reduced. By decreasing the rotation speed of the stirrers, the particles were encouraged to agglomerate and form flocs, facilitating their settling. In this case, stirrers were allowed to rotate at a speed of 90 rpm for 15 minutes.

Following the flocculation process, the samples were allowed to settle for 30 minutes. During this settling period, the particles settled at the bottom of the container, allowing the clear supernatant to form on top. After the settling processes were completed, samples of the clear water were collected for the analysis of their turbidity, pH, conductivity, color, and total dissolved solids (TDS).

2.5.1. Water Parameter Conducted

pH tests were conducted to assess the effect of the coagulation process on the water pH level. Each coagulant has an optimal pH range at which it works best, typically between 6.5 and 8.5. Lower pH levels tend to favor organic removal, while higher pH levels promote inorganic removal. pH levels were monitored and controlled by adjusting the coagulant dosage levels. The pH of the water samples is measured using a calibrated Crison pH meter, ensuring accurate pH readings for analysis.

The turbidity test is a method used to quantify the presence of suspended matter in a water sample, which can include both organic and inorganic substances. Turbidity serves as an important indicator of contamination levels in water, making it crucial to minimize turbidity throughout the treatment process. To conduct the turbidity test, a sample of turbid water is poured into a 25 ml cuvette and inserted into a turbidity meter. The turbidity meter measures the intensity of light scattered by the suspended particles in the water sample. The resulting turbidity value is displayed on the instrument's LCD panel and is typically expressed in Nephelometric Turbidity Units (NTU).

By evaluating the turbidity levels before and after treatment, the efficiency of the coagulant dosage can be determined, allowing for adjustments to be made as needed to achieve the desired reduction in turbidity. Minimizing turbidity is crucial to ensure water quality and remove potential contaminants. The percentage of turbidity removal is given by(1)$\%\text{Turbidity removal}=\frac{\text{Initial turbidity}-\text{Final turbidity}}{\text{Initial turbidity}}\times 100\%$.

A conductivity test is conducted to measure the total dissolved solids (TDS) in water. This test helps determine the presence of both cations and anions in the water sample before and after treatment. To perform the conductivity test, a conductivity meter is used. The water sample is poured into a beaker, ensuring that the meter probe does not

touch the sides or bottom of the beaker. The meter is then carefully inserted into the water, allowing it to stabilize. The reading of conductivity is displayed on the LCD panel of the meter once it has reached equilibrium. Conductivity tests provide valuable information about the level of dissolved ions, salts, and other substances in the water.
By comparing the conductivity measurements before and after treatment, the effectiveness of the treatment process in reducing the presence of dissolved solids can be determined. To ensure accurate conductivity measurements, it is important to use a properly calibrated Crison Conduct meter. Regular calibration of the meter helps ensure reliable and precise readings, allowing for accurate monitoring of the water's conductivity levels throughout the treatment process.
Total dissolved solids (TDS) refer to all the solid substances that are dissolved in water. In potable water, TDS mainly consist of inorganic salts, minute amounts of organic matter, and dissolved gases. The presence of high TDS levels in water can be reduced through processes like oxidation, settling, and filtration and can also be completely removed through distillation. TS refer to total dissolved solids and suspended solids in raw water. The calculation result shows the TS of water before and after adding coagulants. The percentage turbidity removal for the added different dosage of CWA coagulant was determined from the relation(2)TS=W2−W1Vt,where TS = total dissolved solids in mg/l, $W1$ = mass of crucible in grams, $W2$ = mass of crucible with sample water after oven-dried in grams, and V_t = total volume of sample water in liter (l).
Color, taste, and odor are important characteristics to consider when assessing water quality. The color of wastewater collected from the Awetu River, for example, is determined using a spectrometer. Prior to using the spectrometer, calibration is performed using distilled water as a reference. The wastewater sample is then placed in the spectrometer using a sample set, and the peak absorbance of the sample water is read from the graph displayed on the LCD spectrum. Taste and odor are subjective assessments, referring to any taste or smell in water that deviates from what is considered acceptable by the consumer.

2.5.2. The Sample Wastewater Parameters

The measurements of various water quality parameters provide valuable insights into the condition of the water being examined. The collected wastewater has a turbidity of 145 NTU. A turbidity level of 145 indicates that the water may contain suspended particles or impurities. It has a salinity of 151.8 mg/l, which indicates a relatively high level. It has a resistivity of 0.32 kΩ; a low resistivity signifies that the water is not very resistant to electrical flow. The dissolved oxygen level of 2.17 mg/l present in the sample water may be a concern for aquatic organisms that rely on higher oxygen concentrations. An absorbance value of 0.863 suggests the presence of substances that can absorb light. With a transitivity of 13.7, the water allows a moderate amount of light to pass through. The measurement of total solids at 6.4 indicates the presence of solid particles in the water. These parameters collectively offer a comprehensive assessment of the water quality, highlighting its suitability for various purposes and its potential impact on the environment. Table 1 shows the parameters of sample wastewater before the addition of coagulant.

Table 1

Parameters of sample wastewater before the addition of coagulant.

Test	Measurement	Unit
Turbidity	145	NTU
pH	7.91	pH meter
TDS	1161.5	mg/l
Conductivity	580	μs/cm

Salt	151.8	mg/l
Resistivity	0.32	kΩ
Dissolved oxygen	2.17	mg/l
Absorbance	0.863	—
Transitivity	13.7	—
Total solid	6.4	mg/l

3. Results and Discussion

3.1. Cement and Wood Ash in Turbidity Removal of Wastewater

Experimental results after coagulation and flocculation processes with different dosages are shown in Figure 3. During the jar test experiment, different coagulant dosages were added to a 500 ml of sample water with an initial turbidity of 145 NTU. After undergoing the coagulation-flocculation and clarification processes, the supernatant sample water was collected for turbidity analysis. The results showed that the addition of the coagulant dosage led to a reduction in turbidity. The turbidity values after treatment were measured as follows: 5.22 NTU for the 5 g dosage, 7.98 NTU for the 7 g dosage, 13.63 NTU for the 10 g dosage, 22.00 NTU for the 15 g dosage, and 27.84 NTU for the 25 g dosage. As shown in Figure 3, it is clear that the turbidity of the wastewater is significantly decreased.

[figure(s) omitted; refer to PDF]

The experiment has shown that the turbidity of the raw water was reduced to 5.22 NTU with a removal efficiency of 96.4% using a 5 g dosage of cement and wood ash. At this dosage, cement and wood ash are active coagulants. Therefore, 5 g is the optimum dosage for turbidity removal. This indicated that cement and wood ash can be used as chemical coagulants in turbidity removal.

Similarly, as the concentration of the coagulant increased, we see a gradual decrease in removal efficiency, indicating that higher concentrations do not necessarily yield better results. Even at the highest concentration of 25 g, the removal efficiency was 80.8% as indicated in Table 2.

Table 2

Percentage of turbidity removal with different dosages of CWA.

CWA coagulant concentration (g/500 ml)	Initial turbidity (NTU)	Final turbidity (NTU)	Percentage of turbidity removal
5	145	5.22	96.4
7	145	7.98	94.8
10	145	13.63	90.6
15	145	22.00	86.8
25	145	27.84	80.8

3.2. Cement and Wood Ash Coagulant on the pH

The experiment involved testing the pH levels of treated water before and after the addition of lemon, along with varying dosages of coagulants, as shown in Table 3. The initial measured pH value of sample wastewater was 7.91 at room temperature. After adding coagulants, the pH levels increased for each dosage: 5 g resulted in 8.7, 7 g in 8.92, 10 g in 9.16, 15 g in 9.32, and 25 g in 9.67. These values slightly exceeded the recommended pH range of 6.5 to 7.5 according to WHO standards [28]. So, it cannot satisfy the recommended values. To address this, an acid solution was introduced to the water samples to neutralize them. For instance, 5 ml of lemon was added to 500 ml of water treated with 5 g of coagulant, resulting in a pH of 6.97. This adjustment brought the water within the acceptable pH range for domestic use, demonstrating the effectiveness of the treatment process.

Table 3

pH of the treated water before and after addition of lemon.

Treated water sample (g/500 ml)	pH value	Efficiency of the lemon on the pH
5	8.7	6.97
7	8.92	7.23
10	9.16	7.45
20	9.32	7.62
25	9.67	7.95

3.3. Cement and Wood Ash Coagulant on Conductivity

The ability of water to conduct electricity is determined by its conductivity, which is influenced by the presence of positively charged ions (cations) and negatively charged ions (anions) in the water. When water contains high levels of ions, it typically exhibits lower electric conductivity. Before the coagulants were added to the turbid water, the conductivity value of the water sample was measured as 580 μs/cm. After the addition of the coagulants with varying dosages per 500 ml of water as 5 g, 7 g, 10 g, 15 g, and 25 g, the resulting conductivity values of the treated water are 265, 300, 381, 437, and 517 μs/cm, respectively. A lower value of conductivity is the property of clean water. The values of the conductivity of treated water with different dosages show the feasible results to be required for water quality as per WHO standards which are in the range of 200 μs/cm to 800 μs/cm [28]. The conductivity of the treated water sample is shown in Table 4.

Table 4

The conductivity of the treated water with different coagulant dosages.

Treated water sample (g/500 ml)	Conductivity value (μs/cm)
5	265
7	300
10	381

15	437
25	517

3.4. Cement and Wood Ash Coagulant on Color Removal

The experiment involved treating 500 ml of turbid water with an initial turbidity of 145 NTU with a light absorbance value of 0.863. The absorbance of light was measured using an electrophotometer, and the results displayed varying degrees of absorbance. As shown in Table 5, when 5 g of coagulant was used, the absorbance was only 0.007, resulting in an impressive color removal of 99.18%. Similarly, with 7 g of coagulant, the absorbance was 0.009, indicating a color removal of 98.95%. As the dosage increased, the absorbance values also rose, but the percentage of color removal slightly decreased. For instance, at 25 g of coagulant, the absorbance was 0.097, and the color removal percentage was 88.76%. It can be observed that the absorbance values increased as the dosage of the coagulant increased. This indicates that the percentage of color removed from the water decreased with higher coagulant dosages. In other words, higher doses of the CWA coagulant were more effective in removing unnecessary color from the raw water.

Table 5

Absorbance and percentage color removal of different coagulant dosages added.

No.	Sample	Coagulant added (g/500 ml)	Absorbance	% color removal
1	S1	5	0.007	99.18
2	S2	7	0.009	98.95
3	S3	10	0.073	91.53
4	S4	15	0.081	90.6
5	S5	25	0.097	88.76

This finding demonstrates that the CWA coagulant is particularly effective in addressing and reducing color-related issues in wastewater, providing valuable information for optimizing the treatment process for improved water quality.

3.5. Cement and Wood Ash Coagulant on the Removal of Dissolved Oxygen

Dissolved oxygen (DO) is used to describe the amount of oxygen dissolved in a unit volume of water. It is essential for the maintenance of healthy lakes and rivers. In healthy water bodies such as lakes and rivers or streams, the dissolved oxygen is about 10 ppm. The minimum level of 3 to 5 mg/l is desirable for the survival of aquatic life. In the experiment of wastewater, the DO value before treatment was 2.17 mg/l. After treatment, as the dosage of coagulant increased, the DO value also increased, and at 5 g of dosage, the DO value after treatment was 3.26 mg/l. The test results show that the treated water is in a healthy condition and suitable for aquatic life. After the addition of coagulant to wastewater, the dissolved oxygen of the sample water was discussed as listed in Table 6.

Table 6

Value of dissolved oxygen after treated with different dosages of CWA.

No.	Sample of coagulant (g/500 ml)	Initial dissolved oxygen (mg/l)	Final dissolved oxygen (mg/l)

1	5	2.17	3.26
2	7	2.17	3.95
3	10	2.17	5.25
4	15	2.17	5.46
5	25	2.17	5.52

3.6. Cement and Wood Ash Coagulant on the Salt

Salinity refers to the measurement of dissolved salt content in water. Seawater is known to have a salinity of approximately 3500 mg/l, while freshwater typically has a salinity of about 1000 mg/l [28]. In the experiment conducted, prior to the addition of the coagulant, the initial salt concentration in the water was measured to be 151.8 mg/l at room temperature. After the coagulant was added, the salt concentrations varied depending on the dosage used. Specifically, the salt concentrations were measured as 897 mg/l, 960 mg/l, 1186 mg/l, 1600 mg/l, and 2203 mg/l for different doses of the coagulant in 500 ml of the sample water.

These results indicate that the addition of the coagulant had an impact on the salt concentration in the water. The varying doses of the coagulant resulted in different levels of salt in the treated water sample. It is important to note that the specific coagulant used and the dosages applied may have contributed to these variations in salt concentration. From Figure 4, as the amount of the dosage increased, the salinity of the water also increased. When the salinity is less than 250 mg/l, it causes diseases like cardiovascular disease, heart disease, and kidney disease, and when it is above 1000 mg/l, it also causes diseases. So, for the sample of 5 g dosage with 500 ml of raw water, the obtained value was 897 mg/l and it satisfies the WHO ranges.

[figure(s) omitted; refer to PDF]

3.7. Cement and Wood Ash Coagulant on the Resistivity of the Sample Water

Resistivity in water is the measure of the ability of water to resist an electrical current, which is directly related to the number of dissolved salts in the water. Water with a high concentration of dissolved salts will have a low resistivity. The appropriate resistivity of clean water is recommended as 500 Ω–1500 Ω. As shown in Figure 5, it is noteworthy that initially, the water exhibited a low resistivity of 0.32 kΩ across all coagulant dosages. However, after the coagulation process, there was a significant increase in resistivity values. For instance, with a 5 g coagulant dosage, the resistivity surged to 688 Ω. Similarly, as the dosage increased, the final resistivity values followed suit, showcasing a trend of improved resistance to electrical flow in the treated water. The results suggest that the use of cement and wood ash coagulant can significantly contribute to achieving the desired resistivity levels for quality water treatment.

[figure(s) omitted; refer to PDF]

3.8. Cement and Wood Ash Coagulant on the Transitivity

Transitivity is the ability of water to transmit light. A high percentage of transparency indicates more transmitted light. Table 7 illustrates the initial and final transitivity percentages for various coagulant dosages. Initially, the water exhibited a transitivity of 13.7%, indicating a moderate ability to transmit light. Following the coagulation process, there was a notable increase in transitivity percentages across all dosages. For example, with a 5 g coagulant dosage, the final transitivity reached an impressive 98.3%. Similarly, as the dosage increased, the final transitivity percentages remained notably high. This demonstrates the effectiveness of the coagulation process in enhancing the water's ability to transmit light, which is crucial for maintaining water clarity. The results suggest that the use of cement and wood ash coagulant can significantly contribute to achieving the desired transitivity levels for quality water treatment.

Table 7

Transitivity of treated water with different dosages of CWA.

No.	Sample of coagulant (g/500 ml)	Initial transitivity (%)	Final transitivity (%)
1	5	13.7	98.3
2	7	13.7	97.9
3	10	13.7	84.6
4	15	13.7	82.9
5	25	13.7	80.0

3.9. Experimentation of Cement and Wood Ash Coagulant in Reducing TDS

The TDS values of sample water after the addition of coagulant are presented in Table 8. TDS refer to the materials that are completely dissolved in water; this solid is filterable, and it is the residue after evaporation of the filterable sample. As indicated in Table 8, as the coagulant dosage increased, there was a notable rise in TDS concentrations. For instance, at a 25 g coagulant dosage, the TDS reached 3767 mg/l. This indicates that higher coagulant dosages led to an increase in the concentration of dissolved solids in the treated water. It is important to note that while the coagulation process effectively reduced turbidity, it also resulted in an elevation of TDS levels. The amount of TDS for freshwater is <1500 mg/l. The TDS value is between 1500 mg/l to 5000 mg/l in brackish water and >5000 mg/l in saline water [28]. At a dosage of 5 g of CWA coagulant in 500 ml, the obtained TDS value was 987 mg/l. This demonstrates that CWA is effective in removing TDS from raw water.

Table 8

TDS of treated water after adding coagulant.

No.	Sample	Coagulants added (g/500 ml)	TDS (mg/l)
1	B1	5	987
2	B2	7	1126
3	B3	10	2065
4	B4	15	2439
5	B5	25	3767

3.10. Total Solids (TS) of Wood Ash and Cement

Another significant characteristic of clarified (treated) water is its reduced total solids (TS). After applying the coagulant to clear the initially turbid water with a turbidity of 145 NTU, the TS level was 6.4 mg/l. However, at the ideal dose of 5 grams per 500 ml of raw water, the TS value notably dropped to 0.88 mg/l. This demonstrates the coagulant's effectiveness in reducing the total solids content in the treated water, underlining its role in enhancing water quality. Table 9 indicates the TS of wastewater before adding CWA.

Table 9

TS of wastewater before adding CWA.

Sample of water before treatment	Volume of sample (ml), V_t	Mass of crucible (g), W_1	Mass of crucible with 25 ml of sample water after oven-dried (g), W_2	TS (mg/l)
S-0	25	56.069	56.229	6.4

The TS value of wastewater before adding coagulant and after oven-dried for 1 hr at 105°C was 6.4 mg/l. After using various amounts of CWA coagulant on a 500 ml sample of wastewater, the sample was dried in an oven at 105°C for 1 hour. The results, shown in Table 10, indicate that as the dosage of CWA increased, the total solids (TS) content also increased. At 5 grams per 500 ml of raw water, the TS concentration was 0.88 mg/l. This indicates that the CWA coagulant effectively reduced the total solids content in the treated water, which is crucial for enhancing water quality and suitability for various purposes. The trend in the data underscores the potential of CWA as an effective coagulant in wastewater treatment.

Table 10

TS of treated wastewater after adding CWA with different dosages.

Sample water after treated	Dosage of CWA (g)	The volume of sample water (ml), V_T	Mass of crucible (g), W_1	Mass of crucible with 25 ml of sample water after oven-dried (g), W_2	TS (mg/l)
S-1	5	25	50.426	50.448	0.88
S-2	7	25	52.644	52.684	1.60
S-3	10	25	52.997	53.054	2.28
S-4	15	25	49.513	49.598	3.40
S-5	25	25	49.523	49.639	4.64

3.11. Recommended Value with Obtained Value at 5 g Dosage of CWA

Based on the experimental results obtained at the optimum dosage, all the parameters measured were found to be within the standard range for drinking water quality. The comparison between the recommended values and the obtained values is presented in Table 11.

Table 11

Comparison of recommended values with obtained values.

Parameters	Recommended value	Obtained value	Unit
Turbidity	5 to 15	5.22	NTU
pH	6.5 to 7.5	6.9	—
Conductivity	200 to 800	265	μs/cm

Dissolved oxygen	3 to 5	3.26	mg/l
Absorbance	100%	99.18%	—
Transitivity	100%	98.3%	—
TDS	<1500	987	mg/l
Resistivity	500 to 1500	688	Ω
Salt	<1000 m	897	mg/l

Based on these results, it can be concluded that the treatment process, particularly at the optimum dosage, successfully met the recommended values for drinking water. The turbidity, pH, conductivity, dissolved oxygen, absorbance, transmittance, TDS, resistivity, and salt content were all within acceptable limits for safe drinking water quality. This indicates the effectiveness of the treatment in achieving suitable water quality standards.

4. Conclusions

This study was conducted in Jimma City of Ethiopia, specifically focusing on wastewater samples collected from the Awetu River, situated approximately 3 kilometers away from the Jimma Institute of Technology. The study aimed to evaluate the effectiveness of cement and wood ash as chemical coagulants in treating wastewater. Through a series of experiments, various properties of the water before and after treatment were analyzed. The results showed that the addition of the coagulant led to a reduction in turbidity, indicating improved water clarity. Additionally, the treatment process had positive effects on pH levels, conductivity, dissolved oxygen, color removal, and total dissolved solids (TDS), all of which are crucial factors in assessing water quality. Notably, at an optimal dosage of 5 grams per 500 milliliters of raw water, the total solids content was significantly reduced, demonstrating the coagulant’s effectiveness in purifying the water. Overall, this study provides valuable insights into the potential use of cement and wood ash as coagulants for wastewater treatment, showing promising results in achieving safe and suitable water quality standards.

Authors’ Contributions

Milkessa Ingida, Gurmesa Bedane, Firanbon Adugna, and Degefa Nigusu collected data, conducted the experiments, and wrote the main manuscript. Mohammed Hussen and Chala Hailu Sime supervised all the work. All authors reviewed the manuscript.

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DETAILS

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A Highly Selective Analytical Method Based on Salt-Assisted Liquid-Liquid Extraction for Trace-Level Enrichment of Multiclass Pesticide Residues in Cow Milk for Quantitative Liquid Chromatographic Analysis

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ABSTRACT (ENGLISH)

In this study, a simple, inexpensive, selective, and fast salting-out assisted liquid-liquid extraction (SALLE) technique coupled with high-pressure liquid chromatography-diode array detection (HPLC-DAD) was developed for the extraction, preconcentration, and analysis of trace level seven multiclass pesticide residues in pasteurized and raw cow milk samples. The significant factors that affect the extent to which the target analytes are extracted, such as the type of extraction solvent and its volume, the type and concentration of salting-out salts, the pH of the solution, and the extraction time, have been investigated. Under optimum conditions, the correlation coefficient (r^2) was obtained within a range of 0.9982–0.9997 for a broad linear range concentration of 2–1500 ng·mL^{-1}. Reliable sensitivity was achieved with limits of detection (LODs) and limits of quantification (LOQs) ranging from 0.58–2.56 ng·mL^{-1} and 1.95–8.51 ng·mL^{-1}, respectively. While precision with interday and intraday in terms of relative standard deviations (RSDs) was observed in the range of 1.97– 7.88% and 4.52– 8.04%, respectively. The results of the precision studies reveal that good repeatability and reproducibility (RSDs <9) were achieved, thus showing a low variability extraction of the developed method. Finally, the proposed and validated approach was effectively used to extract and determine pesticide residues in real milk matrices; however, the target analytes were not detected in all samples.

FULL TEXT

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1. Introduction

Pesticides are chemical compounds used all over the world to control, prevent, or eliminate pests that threaten plants, animals, and human environments. It is well documented and known that their use increases agricultural productivity, though their residues greatly contaminate environmental components [1]. Nowadays, pesticide use has significantly increased throughout the world and similarly in Ethiopia [2] mainly as a result of the country's continuous agricultural reform. Out of the enormous quantities of pesticides applied, only less than 0.1% actually reach the intended pests; the remainder may end up on other environmental surfaces and accumulate in grasslands and feed additives given to cattle and other animals [3–5]. Pesticide residues at trace levels can be hazardous to unanticipated targets, posing a serious threat to human health and the ecosystem [2, 6, 7]. Humans come into contact with these chemicals through unsafe use, food, or the environment [5, 8]. Food security is a condition in which everyone, at all times, have physical, social, and economic access to sufficient quantities of wholesome foods to meet their dietary needs and food preferences for an active and healthy life [9]. Due to these facts, monitoring for pesticides in food matrices on a regular basis is crucial and has become one of the hot research topics these days [10, 11].

Pesticides typically exist in low concentrations in the environment and food matrices, and determination of these trace quantities requires various analytical instruments, including gas chromatography-mass spectrometry (GC-MS) [7, 10, 12], gas chromatography-tandem mass spectrometry (GC-MS/MS) [13], high-performance liquid chromatography (HPLC) combined with a diode array detector (DAD) [14–16], tandem mass spectrometry (MS/MS) [1, 5, 17–19], and ultraviolet detector (UVD) [20–22]. Most of these instruments provide good selectivity, sensitivity, low detection capacity, and so on; however, there are financial limitations to acquire them at laboratories that are not well equipped to meet the demanded requirements. However, relatively less expensive techniques, such as HPLC-DAD, are routinely used for monitoring of pesticide and other pollutant residues. In addition, when combined with a DAD detection system, HPLC procedures are typically favored over GC ones since HPLC is used without derivatization and is a sufficiently selective and sensitive analytical method [23]. Therefore, HPLC-DAD was chosen for monitoring of multiclass pesticides in milk samples for the designed sample preparation methods in the current study.

Dairy farming is one of the most profitable businesses in Ethiopia, particularly in the central Oromiya regional state. Furthermore, Ethiopia has one of the highest populations of cattle in Africa, estimated at 60 million heads, and around 90% of milk products are obtained from cows [24]. Milk is one of the required food item for mankind, but the question of its contamination with trace-level pesticides must be given attention, particularly when its handling personnel are untrained farmers and agricultural extension workers who lack knowledge of pesticide management, how to use for agronomy, and veterinary care are involved [8, 25]. Studies have revealed that despite the fact that most pesticides are often present in low concentrations, their persistence causes them to accumulate in animal tissues where they enter the food chain [5, 10, 11, 19]. Contamination of milk and milk products is extremely concerning because these foods hold a very special place in the diets of infants, young children, and the elderly for whom milk is a complete diet enriched with proteins, vitamins, fats, and essential minerals [26, 27].

The health concerns posed by trace pesticide residues in food can be significant, especially for young children whose enzymatic and metabolic systems are still developing [28–30]. Research on pesticide residues in the environment and various foods that have detrimental effects on human health is receiving special attention [30]. Because milk has dissolved proteins, carbohydrates, and minerals, it is difficult to recover trace-level multiclass pesticide residues with different physicochemical properties, and thus developing an amenable sample extraction technique and cleanup step is very crucial before chromatographic analysis [26].

Among numerous sample preparation techniques that have been performed to achieve efficient extraction of pesticides from milk and milk products, liquid-liquid extraction (LLE) [13, 31], solid-phase extraction (SPE) [16], dispersive solid-phase extraction (DSPE) [19], magnetic solid-phase extraction (MSPE) [28, 32], and solid-phase

microextraction (SPME) [33, 34], Quick, easy, cheap, effective, rugged, and safe (QuEChERs) [14], pressurized liquid extraction (PLE) [12], and cloud point extraction [35, 36] were some of the reported works in the literature. The majority of these methods are labor intensive, time-consuming, and environmentally unfriendly, despite the fact that they offer clear advantages for extraction of pesticides from milk. Besides, as stated explicitly in published literature, industrially produced QuEChERs kits, SPME needles, and SPE cartilage materials are quite expensive [5, 6, 37]. Preconcentration of multiclass pesticide residues in food samples nowadays needs the development of analytical techniques that are miniaturized, efficient, simple, fast, and affordable. The most popular method, dispersive liquid-liquid microextraction (DLLME), is limited to the use of nonpolar, water-immiscible solvents with low dielectric constants and poor extraction efficiency of polar organic and inorganic compounds [38]. As a result, the introduction of salt-assisted liquid-liquid extraction (SALLE), an efficient extraction method for polar to moderately polar organic compounds, was made feasible by using more polar and water-miscible organic extraction solvents like acetonitrile, isopropanol, acetone, ethanol, and methanol, among others. In the SALLE method, organic solvent is separated from the mixture, and a two-phase system is created as a result of addition of inorganic salt [39]. When using inorganic or organic salts, the salting out effect increases the ionic strength of the solution and decreases the solubility of the weak electrolyte in water, which causes the target analyte to be extracted into the organic solvent, resulting in high extraction efficiency of polar or slightly polar target analyte in an aqueous sample [39]. The SALLE method produces extracts with solutes in organic solvent that may be evaporated and reconstituted with an appropriate solvent for preconcentration and analysis by HPLC or GC [40, 41]. On the other hand, in the SALLE methods, extraction solvents are compatible with the majority of analytical instruments, particularly chromatographic ones, making it possible to directly inject the extract into these methods of analysis [36, 42, 43].

SALLE has been used successfully to analyze pesticides in foods [20, 40, 44], biological matrices [36], and environmental water [45–47]. Researchers put a lot of work into making the method automated and high throughput during the development step to reduce processing time and chemicals required [35, 37]. Though various pretreatment technologies have been developed, the method of SALLE has still been widely used, since it integrates sample cleanup, preconcentration, and extraction in one single step and shares the advantages of the sample pretreatment technique gained from QuEChERS [48, 49]. Even though numerous advantages were reported, the application of SALLE for enrichment of multiclass pesticides in milk samples is scarce in the reported literature.

To the best of our knowledge, there are no reports in the literature on the use of the SALLE technique coupled with HPLC-DAD for simultaneous extraction and determination of multiclass pesticide residues including carbamate (carbrayl), organophosphate (methidathion), triazines (cyanazine, atrazine, and propazine), neonicotinoid (thiamethoxam), and strobilurin (azoxystrobin) in cow milk samples. Therefore, the present study was designed to develop, optimize, and validate a simple, fast, inexpensive, and an environment friendly (green) analytical technique based on SALLE, as an alternative for preconcentration and extraction of seven multiclass pesticide residues in cow milk samples.

2. Experimental

2.1. Chemicals and Reagents

The standards used in this study are of analytical reagent grade substances; methidathion was obtained from Sigma-Aldrich (St. Louis, MO, USA), and atrazine, cyanazine, and propazine were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Azoxystrobin, carbrayl, and thiamethoxam were the products of Sigma-Aldrich (Steinheim, Germany). All the pesticide standards were of the highest purity, viz., >98%. Other common chemicals used in the study were also analytical-grade reagents while the solvents utilized including acetonitrile (ACN), dihexyl ether, ethyl acetate, and acetone acquired from Sigma-Aldrich (Steinheim, Germany), methanol (MeOH) received from Carlo Erba (Rodano, Italy), and iso-propanol (IPA) obtained from Sigma-Aldrich (Seelze, Germany) were HPLC-grade reagents. Magnesium sulphate anhydrous and ammonium sulphate were from Fine Chem Industries (Mumbai, India, 99%). Ammonium acetate (BDH Chemical Ltd, England, 96%) was obtained from VWR International (Radnor, PA, USA). Sodium sulphate and sodium acetate anhydrous were from (BDH Chemical Ltd, England, 96%). Common chemicals such as NaCl were obtained from Sigma-Aldrich (Steinheim, Germany), hydrochloric acid (HCl)

was purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium hydroxide (NaOH) was the product of Merck chemicals (Darmstadt, Germany). Ultrapure water was prepared by purifying with a double distiller, a 8000 Aquatron water Still (Bibby Scientific, Staffordshire, UK), and a deionizer (EASY Pure LF, Dubuque).

2.2. Instruments and Equipment

Chromatographic analyses were carried out using the Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) outfitted with a quaternary pump, vacuum degasser, standard and preparative autosampler, thermostated column compartment, autosampler thermostat, and a diode array multiple wavelength detector. LC Chemstation software (B.02, 01-SR1) was used for sample processing and data acquisition. Chromatographic separation was performed using a ZORBAX ODS-C_{18} (150 × 3 mm, i.d., 3.5 μm particle size) analytical column from Agilent Technologies. The sample solution pH was measured using an Adwa pH meter, model 1020, made by Adwa Hungary Kft. in Szeged, Hungary. For sample preparation, a centrifuge, Model 800, Jiangsu Zhenji insturuments Co., Ltd. (Jiangsu, China), a 15 mL centrifuge tube, Corning integrated (Corning, NY, Mexico), and an ultrasonic heater, Dacon®, were utilized.

2.3. Chromatographic Conditions

Chromatographic separations were achieved using the isocratic condition of a binary mobile phase, consisting of solvent A (40% ultrapure water) and solvent B (60% methanol). Prior to the sample injection, the HPLC column was equilibrated with the mobile phase for 10 min. Analysis was performed with a flow rate of 1 mL/min, column temperature of 35°C, injection volume of 15 μL, and UV detection at 224 nm for all the target analytes. Peak area was used as instrumental response and comparison of the responses. Under these chromatographic conditions, baseline separation was maintained for all the target analytes.

2.4. Standard Solution Preparation

The stock standard solution of each target analyte, with the concentration of 0.1 mg/mL, was prepared by weighing the appropriate amount and dissolving it in methanol. Intermediate standard solutions of 10 μg/mL were obtained by diluting the stock solution with ultrapure water. Other working solutions of lower concentrations were also prepared by diluting the intermediate solution in the ultrapure water. All standard solutions were stored in the refrigerator below 4°C, when not in use. The chemical structures, common names, abbreviations, the octanol-water partition coefficient (logP; at pH 7 and 20°C), and other relevant physicochemical properties of the target pesticides considered are shown in Figure 1.

[figure(s) omitted; refer to PDF]

2.5. Milk Samples

A total of 7 milk samples (one fresh raw milk collected from a dairy cattle farm and three pasteurized milk processed and packed by two dairy product suppliers) were taken. Pasteurized milk samples were bought from randomly selected local supermarkets in Addis Ababa, and raw milk samples were donated from a randomly selected dairy cattle farm in Sheger city (in sululta subcity) in April 2023 for the multiclass pesticide residue analysis. After arrival at the laboratory, the pasteurized milk samples in their original packing and raw milk in a brown bottle were kept in a refrigerator at 4°C until the time of analysis, when not in use. Note that the names of the producers have been kept confidential to protect their business and reputation.

2.6. Procedure of SALLE

Aliquots of 0.5 mL of milk sample were placed in a 15 mL falcon centrifuge conical bottom tube and then diluted to 5.0 mL with ultrapure water (pH 8.0) to reduce the matrix effect of the sample. The sample solution pH was adjusted using 0.1 M HCl or 0.1 M NaOH solution and spiked with appropriate amount of mixed standard solutions of the pesticides. The sample solution was then kept to stand for 20 min to equilibrate, and 1.0 mL ACN was added and vortexed for 0.5 min. This was followed by the addition of 2.0 g $MgSO_4$ to the mixture and vortexed for an additional 2 min to dissolve the salt to be used as a salting out agent. After centrifuging the resulting content at 4000 rpm for 5 min, 500 μL of the supernatant was carefully withdrawn with a micropipette and transferred to a vial filtering through a 0.22 μL filter membrane. Then, 15.0 μL was injected into the HPLC–DAD system for extract analysis.

2.7. Statistical Analysis

Descriptive statistical analysis of means, standard deviations, and relative standard deviations for data obtained during parameter optimizations and validations of the method was performed using Microsoft Office Excel 2010 software, and figures were drawn using Origin 2019b software.

3. Results and Discussion

3.1. Optimization of the SALLE Procedure

This research work was designed with the interest of developing an efficient analytical methodology which is miniaturized, simple, fast, and cost-effective for the analysis of multiclass pesticide residues. Attainment of the desired efficiency was achieved by making use of a single sample preparation process to be able to analyze seven multiclass pesticide residues simultaneously. During method development, experiments were conducted to optimize different extraction parameters including the type and volume of the organic solvent, type and amount of salt, pH of the sample solution, and vortex time. These experimental conditions were evaluated by spiking reagent water at concentrations of 100 μg/L for CAR; 130 μg/L for THE, CYZ, ATZ, and PRZ; 260 μg/L for AZO; and 390 μg/L for MET. All the experiments were performed in triplicate (experimental) and doublet reading (instrumental). The mean peak area studies that may have impacts on the SALLE extraction efficiency were taken as instrument response when establishing the optimum experimental conditions for the following parameter under study.

3.1.1. Selection of Extraction Organic Solvent

Selection of an appropriate extraction solvent is the critical step in a SALLE procedure. The organic solvents with the desired characteristics such as high capability to dissolve the analyte, miscibility with water, ease of inducing phase separation upon addition of the appropriate salt and having good chromatographic behavior were tested as extraction solvent. Moreover, the solvent peak should not interfere with the analyte peak under the selected HPLC conditions. In this work, solvents such as MeOH, ACN, IPA, acetone, diethyl ether, and ethyl acetate were tested. A series of experiments were performed using a 5 mL ultrapure water sample containing 30% NaCl (m/v) and 2 mL of each organic solvent with the exception of methanol and acetone, in which the two phase systems were not observed. Similar observations were also noted for methanol and acetone and reported in literature by other workers [20, 22, 50]. The reason for the absence of phase separation in methanol could be due to the high polarity of methanol caused by its hydroxyl group and the hydrogen bond formed between this solvent and water which as a result increases its solubility [51]. Figure 2 depicts the observed maximum peak area when ACN was used as the extraction solvent. This might be attributed to its closer polarity with water and its promising protein precipitation reagent for milk [52]. Additional advantages of using ACN as an extraction solvent are its ability to extract a wide range of compounds [53] caused by its higher polarity and its less toxic and less harmful nature compared to other common extraction solvents. These characteristics also make it more suitable from the viewpoint of green chemistry. Thus, ACN is selected to be used as extraction solvent in this study.

[figure(s) omitted; refer to PDF]

3.1.2. Volume of the Extraction Solvent Effect

The volume of the extraction solvent is a very crucial parameter that influences the extraction performance of the SALLE technique since it affects the amount of analyte solubility in the sample solution [51]. Generally, the volume of extraction solvent used should be as low as possible to achieve the highest possible enrichment and the least toxicity hazards for environment. In this context, the influence of the ACN volume on the extraction efficiency was investigated between 700 and 1800 μL. As shown in Figure 3, peak areas of all the analytes increased with the volume of ACN from 700 to 1000 μL and then decreased upon further increase in the volume of the ACN. With low volumes, i.e., lower than 700 μL, the interface between the extraction solvent and the aqueous phases was not clear, and collection of the organic layer was found to be difficult. A decrease in extraction efficiency above 1000 μL may be due to the dilution effect resulting from the higher volume of the organic phase obtained after extraction, and hence further higher volumes were not performed [22]. Hence, based on the observed experimental results, 1000 μL ACN was selected as the optimum volume in all the subsequent experiments.

[figure(s) omitted; refer to PDF]

3.1.3. Effects of the Salt Type

The solubility of both the analytes as well as the extraction solvent in the aqueous phase could be decreased by salt addition, and this in turn enhances the analytes transfer into the organic phase [44, 48]. As different salts have the capacity to cause different degrees of phase separation [52], in this study, the effect was evaluated by addition of the salts such as NaCl, $(Na)_2SO_4$, $MgSO_4$, $(NH_4)_2SO_4$, and $\mathbf{NH_4CH_3CO_2}$, using 30% (m/v) of each salt, as potential salting out agent. It was observed that all salts could induce phase separation, but as it can be seen from Figure 4, the highest instrumental response for all analytes was obtained when $MgSO_4$ was used as the salting out agent. This could be due to its high ionic strength per unit concentration in the aqueous phase [43].

[figure(s) omitted; refer to PDF]

3.1.4. Effect of Salt Concentration

Varying salt concentrations may cause the degrees of phase separation to vary [43, 54]. A salting-out study was carried out by adding different amounts of $MgSO_4$ in the range of 0.75 g–2.5 g (or 15–50%, m/v) in the aqueous sample solution. It was shown that in Figure 5, the peak area of the target analytes was slightly increased as the concentration of the salt increases from 1 g to 2 g. However, at higher concentrations, the peaks were observed to slightly decrease for all the target analytes, and thus 40% m/v (2 g) was chosen to be the optimum for the following experiments. Similar quantities of this salt were found to cause a significant salting-out effect in the SALLE analytical method, reported in the literature and employed for fruit juice, yogurt, and carbonated drink matrices [41, 44].

[figure(s) omitted; refer to PDF]

3.1.5. Effect of Sample pH

In SALLE, the sample solution pH also has a significant role on the extraction efficiency of the multiclass pesticides, as it affects the extent of their ionization as well as the solubility in aqueous media [35, 51, 55]. The effect of this parameter was evaluated by carrying out a series of experiments varying the pH values from 3.0 to 9.0 in the aqueous solution. These pH values were adjusted using HCl and NaOH. The experimental results obtained revealed that pH 8 was the optimum value, as shown in Figure 6. This could mainly be associated with the enhanced stability of the target analytes in the weakly alkaline solution, while they were easily degraded in acidic and strongly alkaline environments [4]. Therefore, pH 8 was selected as the optimum value for the subsequent studies.

[figure(s) omitted; refer to PDF]

3.1.6. Effect of Centrifugation Time

In SALLE procedures, optimizing the time required for phase separation is also an important analytical step, in order to obtain a clear extract [44]. In order to establish the optimum conditions: centrifugation time was varied between 1 and 7 min, with a 2 min interval, at a constant speed of 4000 rpm. Based on the peak areas representing the target analytes, the highest results were obtained at the centrifugation time of 5 min (shown in supplementary material Figure S1). Therefore, centrifugation time of 5 min was selected as the optimum time for the subsequent studies.

3.1.7. Effect of Vortex Agitation Time

Mass transfer is a time-dependent process and one of the most important factors in most of the extraction procedures [44]. Vortex was performed to strengthen the contact between acetonitrile and the aqueous sample solution (i.e., influence the kinetics of the extraction), thus facilitating the formation of the two-phase system. Besides, in the present study, vortex agitation was also employed to enhance the dissolution of the salting-out salt. Therefore, a vortex time was evaluated in the range of 0.25–4 min, at the maximum vortex speed, and thus a slight increase of peaks was obtained when the vortex time increased from 15 sec to 30 sec. This indicates that the diffusion of pesticides from the sample to the acetonitrile medium was found to require a short time. A decrease in extraction efficiency after 30 sec (Figure 7) may be associated to the back extraction. Thus, extraction time of 30 sec was chosen in the present study.

[figure(s) omitted; refer to PDF]

3.2. Analytical Performance of the Proposed Method

3.2.1. Calibration Curves and Precision Study

The proposed analytical method was validated through linearity and analytical figures of merit under optimal conditions. Linearity validation of the method was performed with the establishment of the linear calibration using

external standard, and the corresponding curves (supplementary information, Figure S2) were generated by plotting the area of the analyte peak against the standard concentration (ng/mL). Good linearity, with a correlation coefficient >0.998 was obtained for all the target analytes considered in this study over the studied concentration range (Table 1). Individual chromatograms of the target analyte considered in this study are given in Figure S3.

Table 1

Analytical figures of merit for the SALLE technique combined with HPLC-DAD for multiclass pesticide residues under study.

Analyte	Linear range (ng/mL)	Regression equation	LOD (ng/mL)	LOQ (ng/mL)	r^2	[a]Repeatability (RSD %, $n=6$)		[a]Reproducibility (RSD%, $n=12$)	
THE	10–750	$y=0.116x+2.3401$	2.27	7.58	0.9993	[b]2.02	[c]3.38	[b]5.56	[c]7.77
CYZ	3–1000	$y=0.1776x+2.7101$	1.43	4.76	0.9997	3.54	2.05	6.57	4.52
CAR	2–750	$y=0.3345x+2.8145$	1.76	5.88	0.9992	5.50	5.13	7.32	5.13
ATZ	10–1000	$y=0.2883x+6.8847$	0.68	2.26	0.9994	3.11	1.97	8.13	6.57
MET	8–1500	$y=0.0384x+1.8033$	2.56	8.51	0.9991	4.40	4.04	7.52	6.65
AZO	5–1500	$y=0.1033x+2.7038$	2.05	6.84	0.9991	6.44	7.88	5.99	8.04
PRZ	3–1000	$y=0.4143x+8.486$	0.58	1.95	0.9982	2.88	5.13	7.67	7.59

[a]Validated using a pasteurized milk sample one (PSM) sample. [b]Level 1 : 10 μg/L for CAR; 15 μg/L for THE, CYZ, ATZ, and PRZ; 30 μg/L for AZO; 45 μg/L for MET. [c]Level 2 : 100 μg/L for CAR; 130 μg/L for THE, CYZ, ATZ, and PRZ; 260 μg/L for AZO; 390 μg/L for MET.

The precision of the proposed method was also evaluated in terms of repeatability (intraday precision) and reproducibility (interday precision). To study repeatability of the method, pasteurized milk sample one (PSM) was spiked with the mixture of seven pesticides at two concentration levels (μg L^{-1}): Level 1 : 10 for CAR; 15 for THE, CYZ, ATZ, and PRZ; 30 for AZO; and 45 for MET, and Level 2 : 100 for CAR; 130 for THE, CYZ, ATZ, and PRZ; 260 for AZO; and 390 for MET. The sample was extracted in triplicate and injected in duplicate on the same day under the optimized experimental conditions. The reproducibility of the method was also validated using the same milk sample at concentration values used above to evaluate reproducibility for four consecutive days, following single extraction and injection. As shown in Table 1, the RSD % of the method was in the range of 1.97–7.88 for intraday and 4.52–8.04 for interday. The results of the precision studies reveal that good repeatability and reproducibility (RSD <9) were achieved, thus showing a low variability extraction technique [35, 56].

3.2.2. Sensitivity

The sensitivity of the method guaranteed the detection and confirmation of pesticide residues in milk found at levels below or above the limits of detection (LODs). The calculations for LODs and limits of quantitation (LOQs) were based on the standard deviation (σ) of the seven extraction responses of blank milk for each type of milk samples and the slope of the calibration curve (S) using equations $3 \times \sigma/S$ and $10 \times \sigma/S$, respectively [55]. The results are given in Table 1, showing that the LODs ranged from 0.58 to 2.56 $ng \cdot mL^{-1}$ while LOQs from 1.95 to 8.51 $ng \cdot mL^{-1}$.

3.3. Applications of the SALLE Method to Real Milk Samples

The suggested method's accuracy was validated using three real milk samples including pasteurized milk sample one (PMM), pasteurized milk sample two (PSM), and raw sululta milk sample (RSM). None of the tested milk samples produced signals corresponding to values above the LODs when the unspiked sample was evaluated to determine whether the seven selected target analytes were identified or not. The observed results may indicate that the samples tested were either free of pesticide residues or contained amounts below the detected limits. The average relative recovery (RR%) of each sample spiked at two concentration levels and extracted in triplicate was used to determine the accuracy of the proposed SALLE technique (Table 2). Relative recovery was intended using the standard addition on the blank real samples to evaluate the methods accuracy [21, 22, 35]. Mean relative recoveries (RR %) at two concentration levels were in the range of 85.9–108.8%, with %RSD <11.5 for the studied milk samples. The results obtained for recovery were in the acceptable range [56], indicating that the matrices of milk samples have no intense effect on the performance of the proposed method. Similar results were also reported by other workers both for accuracy and precision for the analysis of pesticides in the studied milk [35].

Table 2

Relative recovery (RR) values of the proposed method in the milk samples.

Sample	Spiked level	Analytes						
THE	CYZ	CAR	ATZ	MET	AZO	PRZ	%RR (%RSD, $n = 3$)	
PSM	Level 1	95.7 (2.6)	93.0 (4.9)	92.8 (4.3)	87.7 (7.3)	90.2 (5.5)	92.8 (6.1)	108.8 (3.3)
Level 2	93.3 (4.2)	94.2 (3.0)	104.6 (5.5)	92.3 (8.0)	87.5 (4.8)	89.7 (5.4)	96.7 (2.7)	·
PMM	Level 1	97.9 (7.3)	93.2 (4.6)	91.7 (4.6)	87.9 (7.0)	91.6 (4.1)	85.9 (4.6)	108.3 (8.9)
Level 2	92.9 (7.0)	94.3 (3.1)	97.5 (5.0)	91.3 (3.4)	90.0 (4.0)	88.5 (7.0)	92.9 (3.3)	·
RSM	Level 1	96.0 (7.8)	86.3 (8.9)	88.4 (11.4)	88.2 (8.4)	85.3 (10.3)	88.6 (5.2)	94.2 (11.3)

PSM, pasteurized milk sample one; PMM, pasteurized milk sample two; and RSM, raw sululta milk sample. Level 1: 10 μg/L for CAR; 15 μg/L for THE, CYZ, ATZ, and PRZ; 30 μg/L for AZO; 45 μg/L for MET. Level 2: 100 μg/L for CAR; 130 μg/L for THE, CYZ, ATZ, and PRZ; 260 μg/L for AZO; 390 μg/L for MET.

The chromatograms of the target multiclass pesticide residues in the PSM milk sample before and after spiking at concentration (level 2) used for precision study using the developed SALLE methods are shown in Figure 8. The separation of target analytes in the chromatogram obtained using reversed-phase high-performance liquid chromatography is in the order of their polarity in which the more polar elute first and the less polar one retained more (Figure 1, $\log K_{ow}$ value). The chromatograms selectivity was assessed by comparing blank and fortified sample peaks. It is evident from these chromatograms that absence of the chromatographic peaks from coextracted components and is well resolved for all analytes, demonstrating a high level of selectivity at the same retention time

as the target pesticides. Therefore, the reported chromatogram endorses the selectivity of the developed SALLE technique. The other milk samples evaluated by this study also had the same profiles (supplementary information, Figures S4 and S5).

[figure(s) omitted; refer to PDF]

3.4. Comparison of the Proposed Method to Other Previously Reported Methods for the Analysis of Milk Samples

The presented analytical method, i.e., SALLE-HPLC-DAD for preconcentration and determination of multiclass pesticide residues was compared with other methods reported in the literature, such as dispersive liquid-liquid microextraction with gas chromatography mass spectrometry (DLLME-GC-MS) [17], solid-phase extraction with high performance liquid chromatography coupled with ultraviolet detector (SPE-HPLC-UV) [16], cloud point extraction with HPLC-UV (CPE-HPLC-UV) [3], head space solid-phasemicroextraction with GC-MS (HS-SPME-GC-MS) [33], quick, easy, cheap, effective, rugged, and safe (QuEChERS) coupled with HPLC and diode array detector (QuEChERS-HPLC-DAD) [14], and dispersive solid-phase extraction combined DLLME with HPLC-DAD (DSPE-DLLME-HPLC-DAD) [57], and the results are shown in Table 3. As can be seen, in terms of the LODs, precisions and accuracy of the present method were better than or comparable to those of the other methods applied for extraction of pesticides from the same type of matrices, i.e., milk sample. For HS-SPME [33] and SPE [16] methods, there may be the problem of facing sample carryover effects which leads to false-positive results. The proposed SALLE is simple, and unlike the SPE method, it does not require multisteps conditioning, washing, loading, and elution [5, 6]. In addition, the proposed method is found to use simpler equipment and exhibits a wider linear range, integrated pretreatment and preconcentration in a single step, which would make the procedure simpler, cost-effective, time saving, and eco-friendly.

Table 3

Comparison of the proposed method with other methods applied for the extraction and determination of pesticides in milk samples.

Methods	Detection	Extraction time (min)	LR (μg L^{-1})	LOD (μg L^{-1})	RSD	RR	Ref.
CPE	HPLC–UV	30	50–2000	6.79–11.2	1.41–5.99	70.5–96.9	[3]
QuEChERS	HPLC–DAD	1	—	20–60	1–23	35–131	[14]
SPE	HPLC–UV	20	1–320	0.12–0.40	5.1–6.3	86–110	[16]
DLLME	GC–MS	0.5	2–1000	0.9–5.0	1.02–4.18	86.15–112.45	[17]
HS-SPME	GC–MS	45	6.5–56	2.2–10.9	6.1–29.5	—	[33]
DSPE–DLLME	HPLC–DAD	11	0.57–1000	0.17–0.36	3.3–7.2	79–92	[56]
SALLE	HPLC–DAD	0.5	2−1500	0.58–2.56	1.97–8.04	85.9–108.8	This work

"—", not reported.

4. Conclusions

In this study, the SALLE-HPLC-DAD analytical technique, that is, simple, fast, and green, was developed and

optimized for routine monitoring and quantitative determination of seven multiclass pesticide residues with a wide range of physicochemical properties including methidathion, atrazine, azoxystrobin, cyanazine, carbrayl, thiamethoxam, and propazine in samples of raw and pasteurized milk. Compared with more traditional extraction techniques like LLE and SPE, this method uses a significantly smaller extraction solvent and sample volume. For all of the experimental factors taken into account during the investigation, the approach was optimized utilizing univariate methods. The optimized method offers sufficient accuracy, precision, linearity, and sensitivity under optimum extraction conditions in a short extraction time. No matrix interferences were coextracted or seen in the analysis at their respective retention times while this method was being used to extract trace-level pesticides from milk samples. Comparatively to other reported research that used hazardous halogenated organic solvents as extraction solvents, the extraction solvents used in the current extraction approach are more environmentally benign. As a result, the trace level enrichment of multiclass pesticides using the SALLE analytical technique could be thought of as a good alternative for selective and sensitive extraction and practical assessment of multiclass pesticide residues in milk as well as enrichment of other trace compounds in complex samples in routine laboratory analysis.

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DETAILS

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Next-Generation Sequencing Analysis of 3 Uterine Adenosarcomas with Heterogeneously Differentiated Genomic Mutations

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ABSTRACT (ENGLISH)

Uterine adenosarcoma (UA) is an uncommon mixed tumor containing a benign to at most mildly atypical epithelial component and a sarcoma-like stroma, usually a low-grade, stromal component, with rare heterogeneous elements. Currently, tumor etiology is largely unknown. To better understand the gene mutations in UA, next-generation sequencing (NGS) technology analysis was performed. This study showed that two low-grade UAs with heterologous components had ATRX gene frameshift mutation, and one patient had a MED12 missense mutation. Copy number amplification genes were mainly observed on chromosome $12q^{13-15}$. In this study, PIK3/AKT/PTEN pathway mutations were found to be common in adenosarcoma. In addition, a rare BCORL1-PRR14L fusion

mutation was also identified. These findings provide a basis for future research into these molecular changes in tumorigenesis and targeted therapy.

FULL TEXT

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1. Introduction

Uterine adenosarcoma (UA) is an uncommon mixed tumor containing a benign to at most mildly atypical epithelial component and a sarcoma-like stroma and accounts for approximately 5% of uterine sarcomas [1]. The epithelial component is Müllerian-derived, mainly endometrioid epithelium and a few visible tubal epithelium and squamous epithelium; the glandular epithelium may be accompanied by different atypical degrees. The stroma is generally a low-grade uterine sarcoma, mostly endometrial stromal sarcoma [2], which may contain heterologous components, such as the skeletal muscle, cartilage, and smooth muscle, among others. However, adenosarcoma with heterologous components is rare [3], and the molecular mechanism is unclear. Two of the three UAs reported in this study had heterologous components, and the molecular changes were detected by next-generation sequencing (NGS) to understand the pathogenesis and identify diagnostic or prognostic biomarkers.

2. Materials and Methods

2.1. Case Selection

The specimen-related and existing clinical data of three patients with pathologically diagnosed UA treated in our hospital from 2017 to 2022 were obtained.

2.2. Next-Generation Sequencing (NGS)

DNA was extracted from tissue samples based on the instructions in the QIAGEN DNA FFPE extraction kit (QIAGEN, Valencia, CA, USA). The quality control samples were arranged for the construction of the library. The 481 gene probe was used for targeted enrichment of the DNA samples, and the target enrichment library was sequenced on the NovaSeq 6000 platform (Illumina). Finally, comprehensive information regarding genetic mutations, such as point mutation, insertion and deletion mutations, and gene copy number change, was obtained from the sequencing data using the genome analysis toolkit.

3. Results

3.1. Clinicopathological Features

The clinicopathological characteristics of the patients are summarised in Table 1. The age of the three patients ranged from 57 to 76 years (average, 67 years). The sites of occurrence of the tumors were the uterine fundus, corpus, and cervix. Three patients were admitted because of abnormal vaginal bleeding. All three patients underwent abdominal hysterectomy and bilateral adnexectomy. The postoperative pathological macroscopic image revealed that the uterus was enlarged and single or multiple polypoid masses protruded into the uterine cavity or cervical canal. The average diameter of the tumors was 6.9 cm (range, 2.8–14 cm). The surface of the tumors was smooth, and the cut surface was fish flesh-like, with grey red color and tender texture. The tumors had a saccular shape with local bleeding and necrosis.

Table 1

Clinical features of uterus adenosarcoma.

Case no.	Age (years)	Symptoms	Tumour size (cm)	Stage	SO	Microscopy (hd)	Surgery	Adjuvant therapy	Time to recurrence (months)	Site(s) of recurrence	follow-up(months)

1	57	Abnormal vaginal bleeding	4	IA	(−)	Cartilage	TAH, BSO	None	N/A	N/A	2
2	76	Abnormal vaginal bleeding	14	IA	(−)	N/A	TAH, BSO	None	N/A	N/A	26
3	68	Abnormal vaginal bleeding	2.8	IA	(−)	Smooth muscle	TAH, BSO	None	17	Vagina	36

N/A = not applicable; TAH = total abdominal hysterectomy; BSO = bilateral salpingo-oophorectomy; SO = sarcomatous overgrowth.

Microscopically, the tumor tissue was composed of endometrioid glands and proliferative spindle cells. A large number of tumor cells were diffused and distributed in a woven and cord-like arrangement, and local tumor cells were concentrated around the gland, forming a cuff structure around the gland (Figure 1). Fusiform nuclei with mild to moderate atypia and local mitotic images are easy to see (Figure 2). One case had heterogeneous differentiation of tumor cells into chondrocytes (Figure 3), and another case had smooth muscle differentiation. No definite tumor thrombus was found in the vasculature.

[figure(s) omitted; refer to PDF]

Immunohistochemical examination showed positive expression of CK7 and EMA in glandular epithelium. CD10 and vimentin were positive in the interstitium; desmin, SMA, and PR were partially positive in one patient, and ER was partially positive in two patients. P53, inhibin, caldesmon, myogenin, and MyoD1 were negative in all three patients, and the positivity rate of Ki67 was 5%–15%. Combined with the histopathological and immunohistochemical findings of the patients, adenosarcoma of the uterus was diagnosed. None of the patients received radiotherapy or chemotherapy after surgery. The follow-up time ranged from 2 months to 36 months. One patient had vaginal recurrence after 17 months.

3.2. Overview of Genomic Changes

The genomic variants identified in the patients are summarised in Figure 4, including genes with pathogenic mutations (ATRX, PTK2, PTCH1, and KRAS) and novel variants of unknown significance (FANCA, CREBBP, FOXL2, WRN, and DOT1L). Two patients with heterologous differentiation had the ATRX gene frameshift mutation (p.S788, case 1; p.P2141S, case 3). The other type was missense mutations in DOT1L (p.E262K, p.K401N, patient 2), KRAS (12p12.1, patient 2), and MED12 (p.Q2097L, case 2). The present study found that copy number amplification of genes (CDK4, GLI1, HMGA2, MDM2, and TSPAN31) mainly occurred on chromosome 12q[13–15] (Figure 5). In addition, this study found gene fusion mutations, including PLEKHA7-BRCA1, PPFIA2-ARID2, RAB22A-TEK, DOT1L-ZNF57, and BCORL1-PRR14L fusions. Furthermore, we found that PIK3/AKT/PTEN pathway mutations were the most common in adenosarcomas through the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (Figure 6).

[figure(s) omitted; refer to PDF]

4. Discussion

UA, also known as Müllerian adenosarcoma [3], was defined by the World Health Organization as a mixed epithelial and mesenchymal tumor [4]. UA occurs most frequently in the uterine body, followed by the cervix, and about 1/4 showed other heterologous interleaflet components, with striated muscle differentiation being the most common [5,

6]. In this study, it is rare that one patient had tumors in the uterine cavity and cervix simultaneously. The age of onset of UA ranges from 10 to 94 years (median age, above 50 years) [7] and is more common in postmenopausal women. Overall, UA has a better prognosis than other uterine sarcomas and carcinosarcomas [8, 9]. However, Heveder et al. [10–12] suggested a 50% reduction in survival in UA patients with adverse prognostic factors. Although surgery is the major treatment [13], the therapeutic effect of chemotherapy in UA has been reported in the literature [14].

Some commonalities were identified between copy number variations (CNVs) and mutations. A study has shown that UA and its variants are genetically heterogeneous with frequent CNVs in SO [15]. Meanwhile, MDM2, CDK4, HMGA2, and GLI1 gene amplifications are common molecular events in Müllerian adenosarcoma, often seen in patients with SO [16]. Consistently, in this study, gene amplification was seen in recurrent UAs and UAs with heterologous differentiation without SO.

Furthermore, the MED12 gene is closely related to uterine leiomyoma [17]. The 70% of uterine leiomyomas have point mutations in the MED12 gene, and all relevant point mutations are concentrated in exon 2 [17]. MED12 mutations alter the functioning of the MED12 protein, thus disrupting normal cell signalling and repair regulation of cell growth and other functions, resulting in uncontrolled cell growth and tumorigenesis [17]. In contrast, this study has detected a missense mutation in exon 43 of the MED12 gene, p.Q2097L, resulting in the change of the 6290th base from A to T and the change of the 2097th amino acid from glutamine to leucine. However, the clinical significance of this mutation is unclear. Nevertheless, if the protein functions abnormally, it may affect downstream signalling pathways and be involved in tumorigenesis and progression of cancer.

Moreover, ATRX (located on chromosome Xq21.1) encodes a chromatin remodelling protein which is thought to be important in regulating DNA methylation and telomere stability [18]. Howitt et al. [16] reported that ATRX mutations were present in 50% of UAs with SO (including one case with distant metastasis), while ATRX mutations were not present in UAs without SO, suggesting that ATRX may be a poor prognostic feature of MA [19, 20]. This is inconsistent with findings of this study, in which ATRX mutations were occurred in low-grade UAs with heterologous components but not with SO. Therefore, there requires further studies to confirm these findings.

In addition, structural variation (SV) is relatively low in UAs. This study reported a fusion mutation in the BCORL1-PRR14L gene. Meanwhile, the diagnosis of BCOR overexpressing uterine sarcomas and high-grade endometrial mesenchymal sarcomas carrying these mutations is suggested by identifying ZC3H7B-BCOR fusion mutations or BCOR-ITD [21]. However, the clinical significance of this mutation in UAs is currently unclear. If the protein functions abnormally, it may affect downstream signalling pathways involved in tumor development and progression. Analysis of these gene fusions may provide clues to further understanding of the genetic mechanisms involved in UA tumorigenesis.

PIK3/AKT/PTEN pathway mutations are most common in adenosarcoma [16]. The findings of this study consistently indicated that targeting this pathway may be a potential therapeutic target in UA treatment. Moreover, surgical resection is the main treatment for UA, and hysterectomy with bilateral adnexectomy is the basic surgical method [22]. Previous studies have reported that the recurrence rate of UA is 14.3–46%, and the local recurrence rate is higher than the distant recurrence rate [8, 23]. The high-risk factors might affect the prognosis of adenosarcoma [24] and therefore individualized treatment should be considered. In terms of adjuvant therapy, it has been shown that adenosarcoma recurrence with or without sarcoma overgrowth responds to the treatment regimen of ifosfamide or doxorubicin [25]. However, radiotherapy does not benefit the overall survival of patients, and there is insufficient evidence regarding the benefits of chemotherapy and hormone therapy [25]. Further studies are needed to determine the most effective adjuvant therapy.

5. Conclusion

In conclusion, UA is a rare uterine sarcoma. This study showed that the ATRX gene was mutated in low-grade UA with a heterozygous component, possibly having important prognostic implications. Meanwhile, molecular evaluation of mutations in the BCOR and BCORL1 genes in the diagnosis of uterine sarcomas overexpressing BCOR is recommended to differentiate high-grade endometrial mesenchymal sarcomas with BCOR fusions from rare

adenosarcomas with BCORL1 gene rearrangements and BCORL1-PRR14L fusions, potentially broadening the genetic spectrum of adenosarcomas. Future studies should be conducted on a larger sample, along with a detailed study of the mutated genes.

Ethical Approval

This case report was approved by the Ethics Committee of the Affiliated Hospital of Zunyi Medical University.

Consent

The signed informed consents were obtained from all patients.

Authors' Contributions

Yao Li wrote the original draft. Xiang Huang analysed the data. Yao Li, Xue Meng, YuQing Luo, and Shuai Luo performed the study and JinJing Wang revised the manuscript. All authors finally approved the submitted and published versions of the manuscript.

Glossary

Abbreviations

CNVs:Copy number variations
ER:Estrogen receptor
H &E:Hematoxylin and eosin
IHC:Immunohistochemistry
IRB:Institutional Review Board
NGS:Next-generation sequencing
PR:Progesterone receptor
SO:Sarcomatous overgrowth
SV:Structural variation
UA:Uterine adenosarcoma.

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DETAILS

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QSPR Modeling of Fungicides Using Topological Descriptors

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ABSTRACT (ENGLISH)

A topological index is a real number that is obtained from a chemical graph's structure. Determining the physiochemical and biological characteristics of a variety of medications is useful since it more accurately represents the theoretical characteristics of organic molecules. This is accomplished using degree-based topological indices. The QSPR research has improved the structural understanding of the physiochemical properties of fungicides. Thirteen fungicides are examined for some of their physiochemical properties, and a QSPR model is built using nine of the drugs' topological indices. Here, we examine the degree to which the topological indices and physiochemical attributes are connected. To do this, we create networks connecting each of the topological indices to the properties of fungicides and computationally construct topological indices of the drugs mentioned above. According to this QSPR model, the melting point, boiling point, flash point, complexity, surface tension, etc. of fungicides are strongly connected. It was discovered that the topological indices (TIs) applied to the fungicides more accurately represent their theoretical features and show a strong correlation with their physical attributes.

FULL TEXT

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1. Introduction

For many decades, fungicides have predominantly been used to control fungal-caused plant diseases that threaten human health and crop production [1]. Losses in crops reached almost one billion dollars. The pathogen (fungus) is highly aggressive under field conditions when the environmental conditions favor the disease development [2]. Currently, due to the unavailability of cultivars with complete resistance, the application of fungicides is the main recommended tool for disease control along with cultural practices [3].

There are presently nine and forty seven groups of contact fungicides with multisite and single-site modes of action, respectively. Single-site active fungicides are less toxic to nontarget organisms. Modern systemic fungicides are typified by the triazoles. This group of fungicides is still the basis of cereal disease management strategies worldwide. Their antifungal activity is based on their ability to inhibit CYP51 (lanosterol 14-demethylase), a key enzyme for sterol biosynthesis in fungi [4]. Each triazole substance may have a somewhat different effect on the metabolic process that produces sterols [5], while the outcomes—abnormal fungal growth and death—are identical in different fungi. Triazole chemicals are crucial because of their outstanding antifungal effectiveness, comparatively low risk of resistance, and long-term stability in soil and water [1]. Triazoles can be used as early infection treatments or as a preventative measure. Some triazole fungicides have antisporulant qualities. However, these are ineffective once a fungus starts to develop spores as spores have enough sterol to form germ tubes. Within the triazole family, the principal compounds are difenoconazole, fenbuconazole, tebuconazole, cyproconazole, myclobutanil, penconazole, propiconazole, tetraconazole, triadimenol, prothioconazole, triticonazole, bromuconazole, epoxiconazole, fluquinconazole, flutriafol, ipconazole, metconazole, paclobutrazol, flusilazole, bitertanol, and triadimefon [6].

Topological indices (TIs) are quantitative descriptors obtained from a chemical graph that thoroughly characterize the chemical system and are widely employed in the study on the physiochemical features of numerous drugs. The chemical graph theory makes extensive use of polynomials and TIs, which are extensively used to depict the chemical structure. Graph invariants (TIs) have recently attracted a lot of attention in studies of quantitative structure-property relationships (QSPRs) and quantitative structure-activity relationships (QSARs) and are used in a wide range of mathematical fields, including bioinformatics, mathematics, informatics, and biology. For further study on QSPR modeling on certain drugs, we encourage readers to read [7–10].

We examined some of the physiochemical characteristics of thirteen fungus therapy medications and created a QSPR model utilizing nine topological indices. For this, we compute topological indices of the drugs analytically and depict graphs relating each of these topological indices to the characteristics of fungus drugs. The melting point, boiling point, flash point, complexity, surface tension, etc. of fungus medicines are closely related according to this QSPR model.

2. Preliminaries

In drug configuration, atoms depict vertices, and the associated bonds connecting the atoms are termed as edges. Graph GV,E is thought to be simple, finite, and connected, whereas *V* and *E* in the chemical graph are referred to as vertex and the edge set, respectively. The degree of a vertex u in the graph *G* is the number of vertices adjacent to u in *G* is denoted by du. In chemistry, the valence of a compound and the degree of a vertex in a graph are concepts that are inextricably linked [11, 12]. The inspiration for this article comes from the idea that different medications (structures) may be identified, and that when they are examined for various factors while keeping topological indices in mind, their dominance can be rated. The QSPR model has been applied for the 9 topological indices, which are given in the following.

Definition 1.

The ABC index [13] is given under(1)$ABCG=\sum uv\in EGdu+dv-2dudv$.

Definition 2.

The first degree-based TI is Randic index RAG calculated by Milan Randic in 1975 [14] is given under(2)$RAG=\sum uv\in EG1dudv$.

Definition 3.

The sum connectivity index [15] is given under(3)$SG=\sum uv\in EG1du+dv$.

Definition 4.

The GA index [16] is given under(4)GAG=∑uv∈EG2dudvdu+dv.

Definition 5.

First and second Zagreb indices [17] are given under(5)M1G=∑uv∈EGdu+dv,M2G=∑uv∈EGdudv.

Definition 6.

Harmonic index [18] of *G* is given under(6)HG=∑uv∈EG2du+dv.

Definition 7.

Hyper Zagreb index [12] is defined as(7)HMG=∑uv∈EGdu+dv2.

Definition 8.

Forgotten index [16] is given under(8)FG=∑uv∈EGdu2+dv2.

3. Quantitative Structure Analysis and Regression Model

In this section, TIs of the fungicides are computed. The relationship between QSPR analysis and TIs suggests that the physiochemical characteristics of the fungus are highly connected. Thirteen medicines are used in the analysis. The drug edifices are exhibited in Figure 1. We implement regression analysis calculations for this study. Drug computable structure analysis of nine TIs for QSPR modeling tenacity is performed. The topological indices of the respective drugs are computed in Table 1. The ten physical properties, such as solubility in water, boiling point (BP), density, melting point (MP), molar mass, flash point (FP), topological polar surface area, heavy atom count, complexity, and refractive index, are listed in Table 2. We impose a linear model by using the following equation:(9)P =α+βTI.

[figure(s) omitted; refer to PDF]

Table 1

The TIs values of candidate drugs.

Names of drug	ABC(*G*)	RA(*G*)	$M_1(G)$	$M_2(G)$	HM(*G*)	*H*(*G*)	SCI(*G*)	*F*(*G*)	GA(*G*)
Paclobutrazol	15.40229	9.376029	102	116	508	8.852381	9.613811	276	19.96016
Tebuconazole	16.20668	9.800443	108	123	548	9.216667	10.03661	302	20.79001
Flutriafol	17.1846	10.59317	116	137	580	10.2381	11.04456	306	23.27251
Myclobutanil	14.34883	9.137977	94	107	458	8.819048	9.34952	244	19.34277
Propiconazole	17.08729	10.62696	116	137	580	10.28571	11.06044	306	23.29099
Prothioconazole	16.62446	9.935071	116	140	608	9.45	10.38075	328	21.98206
Epoxiconazole	17.63074	10.71518	124	154	640	10.45714	11.38356	332	24.46225
Triadimefon	15.40229	9.376029	102	116	508	8.852381	9.613811	276	19.96016
Cyproconazole	15.77039	9.593172	108	129	548	9.238095	10.04456	290	21.27251
Flusilazole	17.22504	10.57634	116	136	578	10.20476	11.03074	306	23.2321

Hexaconazole	15.12489	9.548661	100	115	494	9.152381	9.757768	264	20.20879
Flucanozole	17.24621	10.5663	116	135	576	10.18571	11.02206	306	23.20537
Voriconazole	19.48426	11.98945	131	154	657	11.47143	12.40002	349	25.98436

Table 2

Physical properties of drugs.

Names of drugs	Solubility in water (mg/L at 20°C)	Boiling point (°C)	Density (g/cm^3)	Melting point (°C)	Molar mass (g/mol)	Flash point (°C)	Topological polar surface area ($Å^2$)	Heavy atom count	Complexity	Refractive index
Paclobutrazol	22.9	460.9 ± 55	1.23	165	293.8	232.6 ± 31.5	50.9	20	300	1.58
Tebuconazole	36	476.9 ± 55	1.249	102.4	307.82	242.2 ± 31.5	50.9	21	326	1.58
Flutriafol	130	506.5 ± 60	1.3	130	301.29	260.1 ± 32.9	50.9	22	365	1.6
Myclobutanil	142	465.2 ± 55	1.2	65	288.78	235.2 ± 31.5	54.5	20	345	1.589
Propiconazole	100	480.0 ± 55	1.39	−23	342.22	244.1 ± 31.5	49.2	22	377	1.623
Prothioconazole	300	486.7 ± 55	1.36	141.5	344.2	248.2 ± 31.5	80	21	458	1.698
Epoxiconazole	7.1	463.1 ± 55	1.374	134	329.76	233.9 ± 31.5	43.2	23	421	1.659
Triadimefon	64	441.9 ± 55	1.22	82	293.75	221.0 ± 31.5	57	20	338	1.579
Cyproconazole	140	479.1 ± 55	1.32	106.2	291.77	243.6 ± 31.5	50.9	20	331	1.633

Flusilazole	41.9	392.5 ± 52	1.2	52	315.39	191.2 ± 30.7	30.7	22	333	1.563
Hexaconazole	18	490.3 ± 55	1.3	111	314.21	250.3 ± 31.5	50.9	20	308	1.549
Fluconazole		579.8 ± 60	1.5	138–140	306.271	304.4 ± 32.9	82	22	358	1.683
Voriconazole		508.6 ± 60	1.4 ± 0.1	127–130	349.3	261.4 ± 32.9	77	25	448	1.617

P denotes the physiochemical property of the given drug. TI stands for topological index, α stands for constant, and β stands for regression coefficient. MATLAB and R-language software are helpful for results. Linear models are used to analyze nine TIs of the fungicides and their properties. ChemSpider and PubChem are used to get the information given in Table 2. The 2D and 3D graphs of the medicines with TIs are given in Figures 2 and 3, respectively.

[figure(s) omitted; refer to PDF]

3.1. Regression Models and Statistical Parameters Comparison between TIs and Correlation Coefficient of Properties

Relation between TIs and physical properties of fungicides is successfully analyzed by imposing QSPR modeling. This sort of analysis can be useful for the model. It is eminent the value of p is less than 0.05 and *r* is greater than 0.6. Hence it is concluded that the entire properties given in Tables 3–11 are significant. Figure 4 depicts the graph.

Table 3

Statistical parameters used in QSPR model for ABC(*G*).

Physiochemical property	*N*	*A*	*b*	*r*	r^2	*F*	P
Solubility in water	11	86.314	0.295	0.004	0.000	0.000	0.992
Boiling point	13	343.135	8.246	0.261	0.068	0.804	0.389
Density	13	0.626	0.041	0.610	0.372	6.514	0.027
Melting point	13	49.859	3.180	0.087	0.007	0.083	0.779
Molar mass	13	125.565	11.392	0.720	0.519	11.863	0.005
Flash point	13	161.403	4.983	0.261	0.068	0.803	0.389
Topological polar surface area	13	6.608	2.991	0.269	0.072	0.857	0.374
Heavy atom count	13	3.597	1.077	0.963	0.927	139.079	0.000

Complexity	13	−65.744	25.904	0.684	0.467	9.654	0.010
Refractive index	13	1.386	0.014	0.399	0.159	2.086	0.177

Table 4

Statistical parameters used in QSPR model for RA(*G*).

Physiochemical property	*N*	*A*	*b*	*r*	r^2	*F*	P
Solubility in water	11	187.306	−9.686	0.067	0.004	0.040	0.846
Boiling point	13	328.548	14.870	0.278	0.077	0.920	0.358
Density	13	0.610	0.069	0.601	0.361	6.218	0.030
Melting point	13	104.598	−0.217	0.003	0.000	0.000	0.991
Molar mass	13	120.460	19.059	0.712	0.506	11.280	0.006
Flash point	13	152.595	8.984	0.278	0.077	0.919	0.358
Topological polar surface area	13	11.439	4.395	0.233	0.054	0.633	0.443
Heavy atom count	13	2.545	1.858	0.981	0.961	274.362	0.000
Complexity	13	−56.575	41.290	0.643	0.414	7.770	0.018
Refractive index	13	1.428	0.018	0.312	0.098	1.190	0.299

Table 5

Statistical parameters used in QSPR model for SCI(*G*).

Physiochemical property	*N*	*A*	*b*	*r*	r^2	*F*	P
Solubility in water	11	122.660	−3.065	0.026	0.001	0.006	0.940
Boiling point	13	344.671	12.804	0.267	0.072	0.848	0.377
Density	13	0.631	0.065	0.628	0.395	7.173	0.021
Melting point	13	97.816	0.435	0.008	0.000	0.001	0.980
Molar mass	13	134.489	17.041	0.711	0.506	11.276	0.006
Flash point	13	162.321	7.738	0.267	0.072	0.847	0.377

Topological polar surface area	13	19.082	3.511	0.208	0.043	0.499	0.494
Heavy atom count	13	4.041	1.649	0.973	0.947	197.308	0.000
Complexity	13	−42.909	38.510	0.671	0.450	9.011	0.012
Refractive index	13	1.403	0.020	0.383	0.147	1.893	0.196

Table 6

Statistical parameters used in QSPR model for GA(G).

Physiochemical property	N	A	b	r	r^2	F	P
Solubility in water	11	83.186	0.365	0.007	0.000	0.000	0.983
Boiling point	13	361.053	5.359	0.254	0.065	0.761	0.402
Density	13	0.667	0.029	0.645	0.415	7.818	0.017
Melting point	13	91.378	0.499	0.020	0.000	0.005	0.947
Molar mass	13	148.492	7.486	0.710	0.504	11.194	0.007
Flash point	13	172.207	3.239	0.254	0.065	0.761	0.402
Topological polar surface area	13	25.199	1.396	0.188	0.035	0.404	0.538
Heavy atom count	13	5.637	0.713	0.957	0.915	119.159	0.000
Complexity	13	−24.023	17.495	0.693	0.480	10.149	0.009
Refractive index	13	1.390	0.010	0.439	0.193	2.627	0.133

Table 7

Statistical parameters used in QSPR model for $M_1(G)$.

Physiochemical property	N	A	b	r	r^2	F	P
Solubility in water	11	−9.471	0.920	0.098	0.010	0.087	0.775
Boiling point	13	370.704	0.975	0.237	0.056	0.656	0.435
Density	13	0.677	0.006	0.644	0.415	7.806	0.017
Melting point	13	42.770	0.535	0.112	0.013	0.140	0.716

Molar mass	13	141.046	1.549	0.754	0.568	14.480	0.003
Flash point	13	178.030	0.589	0.237	0.056	0.657	0.435
Topological polar surface area	13	15.411	0.364	0.252	0.064	0.746	0.406
Heavy atom count	13	6.361	0.135	0.927	0.860	67.373	0.000
Complexity	13	−50.995	3.707	0.753	0.567	14.383	0.003
Refractive index	13	1.355	0.002	0.517	0.267	4.010	0.070

Table 8

Statistical parameters used in QSPR model for HM(*G*).

Physiochemical property	*N*	*A*	*b*	*r*	r^2	*F*	P
Solubility in water	11	−60.409	0.275	0.174	0.030	0.280	0.610
Boiling point	13	393.103	0.154	0.209	0.044	0.504	0.493
Density	13	0.749	0.001	0.634	0.402	7.385	0.020
Melting point	13	25.196	0.138	0.161	0.026	0.293	0.599
Molar mass	13	154.568	0.284	0.772	0.596	16.218	0.002
Flash point	13	191.549	0.093	0.209	0.044	0.505	0.492
Topological polar surface area	13	17.964	0.068	0.262	0.069	0.813	0.387
Heavy atom count	13	8.695	0.023	0.870	0.757	34.253	0.000
Complexity	13	−30.761	0.701	0.795	0.633	18.934	0.001
Refractive index	13	1.351	0.000	0.585	0.342	5.709	0.036

Table 9

Statistical parameters used in QSPR model for $M_2(G)$.

Physiochemical property	*N*	*A*	*b*	*r*	r^2	*F*	P
Solubility in water	11	−20.127	0.868	0.142	0.020	0.185	0.677
Boiling point	13	402.839	0.585	0.203	0.041	0.473	0.506

Density	13	0.786	0.004	0.648	0.420	7.959	0.017
Melting point	13	48.750	0.410	0.122	0.015	0.167	0.690
Molar mass	13	172.188	1.083	0.751	0.564	14.214	0.003
Flash point	13	197.430	0.354	0.203	0.041	0.474	0.505
Topological polar surface area	13	28.830	0.208	0.205	0.042	0.482	0.502
Heavy atom count	13	9.699	0.089	0.876	0.768	36.388	0.000
Complexity	13	7.449	2.714	0.785	0.617	17.696	0.001
Refractive index	13	1.373	0.002	0.584	0.342	5.706	0.036

Table 10

Statistical parameters used in QSPR model for *F*(*G*).

Physiochemical property	N	A	b	r	r^2	F	P
Solubility in water	11	−98.456	0.645	0.202	0.041	0.381	0.552
Boiling point	13	386.780	0.310	0.211	0.044	0.512	0.489
Density	13	0.740	0.002	0.605	0.366	6.357	0.028
Melting point	13	1.929	0.336	0.197	0.039	0.443	0.519
Molar mass	13	143.313	0.570	0.776	0.602	16.662	0.002
Flash point	13	187.734	0.187	0.211	0.045	0.513	0.489
Topological polar surface area	13	7.378	0.163	0.315	0.099	1.211	0.295
Heavy atom count	13	8.273	0.044	0.844	0.713	27.269	0.000
Complexity	13	−52.270	1.387	0.788	0.620	17.985	0.001
Refractive index	13	1.340	0.001	0.572	0.327	5.336	0.041

Table 11

Statistical parameters used in QSPR model for *H*(*G*).

Physiochemical property	N	A	b	r	r^2	F	P

Solubility in water	11	170.472	−8.336	0.063	0.004	0.035	0.855
Boiling point	13	339.625	14.367	0.274	0.075	0.891	0.365
Density	13	0.636	0.069	0.615	0.378	6.693	0.025
Melting point	13	125.068	−2.332	0.038	0.001	0.016	0.901
Molar mass	13	136.206	18.255	0.695	0.483	10.290	0.008
Flash point	13	159.276	8.682	0.274	0.075	0.891	0.365
Topological polar surface area	13	22.495	3.446	0.187	0.035	0.397	0.542
Heavy atom count	13	3.821	1.806	0.972	0.946	190.910	0.000
Complexity	13	−29.312	40.254	0.640	0.409	7.625	0.019
Refractive index	13	1.428	0.019	0.332	0.110	1.365	0.267

[figure(s) omitted; refer to PDF]

3.1.1. Regression Models for ABC(*G*)

(10)Solubilityinwater=86.314+.295ABCG,Boilingpoint=343.135+8.246ABCG,Density=0.626+.041ABCG,Melting point=49.859+3.180ABCG,Molarmass=125.565+11.392ABCG,Flashpoint=161.403+4.983ABCG,Topologicalpolar surfacearea=6.608+2.991ABCG,Heavyatomcount=3.597+1.077ABCG,Complexity=−65.744+25.904ABCG, Refractiveindex=1.386+.014ABCG.

3.1.2. Regression Models for RA(*G*)

(11)Solubilityinwater=187.306−9.686RAGBoilingpoint=328.548+14.870RAGDensity=0.610+0.069RAGMeltingpoint =104.598−0.217RAGMolarmass=120.460+19.059RAGFlashpoint=152.595+8.984RAGTopologicalpolarsurface area=11.439+4.395RAGHeavyatomcount=2.545+1.858RAGComplexity=−56.575+41.290RAGRefractiveindex= 1.428+.018RAG.

3.1.3. Regression Models for SCI(*G*)

(12)Solubilityinwater=122.660−3.065SCIGBoilingPoint=344.671+12.804SCIGDensity=0.631+.065SCIGMeltingpoint =97.816+.435SCIGMolarmass=134.489+17.041SCIGFlashpoint=162.321+7.738SCIGTopologicalPolarSurface Area=19.082+3.511SCIGHeavyAtomCount=4.041+1.649SCIGComplexity=−42.909+38.510SCIGRefractiveindex= 1.403+.020SCIG.

3.1.4. Regression Models for GA(*G*)

(13)Solubilityinwater=83.186+.365GAGBoilingpoint=361.053+5.359GAGDensity=.667+.029GAGMeltingpoint= 91.378+.499GAGMolarmass=148.492+7.486GAGFlashpoint=172.207+3.239GAGTopologicalpolarsurfacearea= 25.199+1.396GAGHeavyatomcount=5.637+.713GAGComplexity=−24.023+17.495GAGRefractiveindex=1.390+. 010GAG.

3.1.5. Regression Models for $M_1(G)$

(14)Solubilityinwater=−9.471+0.920M1GBoilingpoint=370.704+.975M1GDensity=0.677+.006M1GMeltingpoint= 42.770+.535M1GMolarmass=141.046+1.549M1GFlashpoint=178.030+.589M1GTopologicalpolarsurfacearea= 15.411+.364M1GHeavyatomcount=6.361+.135M1GComplexity=−50.995+3.707M1GRefractiveindex=1.355+.002M 1G.

3.1.6. Regression Models for HM(*G*)

(15)Solubility in water=−60.409+.275HMGBoiling point=393.103+.154HMGDensity=.749+.001HMGMelting point= 25.196+.138HMGMolar mass=154.568+.284HMGFlash point=191.549+.093HMGTopological polar surface area= 17.964+.068HMGHeavy atom count=8.695+.023HMGComplexity=−30.761+.701HMGRefractive index=1.351+.000 HMG.

3.1.7. Regression Models for $M_2(G)$

(16)Solubility in water=−20.127+.868M2GBoiling point=402.839+.585M2GDensity=.786+.004M2GMelting point= 48.750+.410M2GMolar mass=172.188+1.083M2GFlash point=197.430+.354M2GTopological polar surface area= 28.830+.208M2GHeavy atom count=9.699+.089M2GComplexity=7.449+2.714M2GRefractive index=1.373+.002M2G .

3.1.8. Regression Models for *F*(*G*)

(17)Solubility in water=−98.456+.645FGBoiling point=386.780+.310FGDensity=0.740+.002FGMelting point=1.929+. 336FGMolar mass=143.313+.570FGFlash point=187.734+.187FGTopological polar surface area=7.378+.163FG Heavy atom count=8.273+.044FGComplexity=−52.270+1.387FGRefractive index=1.340+.001FG.

3.1.9. Regression Models for *H*(*G*)

(18)Solubility in water=170.472−8.336HGBoiling point=339.625+14.367HGDensity=.636+.069HGMelting point= 125.068−2.332HGMolar mass=136.206+18.255HGFlash point=159.276+8.682HGTopological polar surface area= 22.495+3.446HGHeavy atom count=3.821+1.806HGComplexity=−29.312+40.254HGRefractive index=1.428+.019HG .

3.2. Standard Error of Estimate (SEE), Correlation Determination, and Comparison

A measure of variation for an observation calculated around the computed regression line is said to be the standard error estimate. It examines the extent of accuracy of predictions made about the calculated regression line in Table 12.

Table 12

Standard error of estimate.

Topological indices	*Standard error of estimate*									
Solubility in water	Boiling point	Density	Melting point	Molar Mass	Flash point	Topological polar surface area	Heavy atom count	Complexity	Refractive index	ABC (*G*)
90.3417	42.7913	0.075526	51.3723	15.38515	25.8647	15.0241	0.425	38.783	0.044068	RA(*G*)
90.1415	42.5820	0.076173	51.5652	15.58503	25.7384	15.1681	0.308	40.682	0.045658	$M_1(G)$
89.9110	43.0604	0.072886	51.2414	14.57363	26.0265	15.0952	0.588	34.984	0.041146	$M_2(G)$

89.4259	43.4031	0.072591	51.1779	14.65046	26.2331	15.2673	0.756	32.902	0.039002	HM(G)
88.9703	43.3450	0.073716	50.8925	14.10078	26.1981	15.0522	0.774	32.215	0.038999	$H(G)$
90.1651	42.6330	0.075143	51.5279	15.94348	25.7689	15.3246	0.366	40.841	0.045334	SCI(G)
90.3127	42.7115	0.074144	51.5640	15.58663	25.8162	15.2560	0.361	39.401	0.044396	$F(G)$
88.4891	43.3293	0.075867	50.5571	13.98699	26.1888	14.8049	0.841	32.738	0.039441	GA(G)

4. Conclusions

It is noted that Randic index RA(G) provides high correlated value of heavy atom count at $r=0981$. $F(G)$ index provides maximum correlated value for molar mass $r=0.776$ and complexity $r=0.788$. No correlation was found between TIs and density, polar surface area, flash point, boiling point, melting point, refractive index, and solubility in water.

In this work, the TIS for fungicides were computed, and they were contrasted with a linear QSPR model. Using the data gathered in this manner, the pharmaceutical industry will be able to create new medications to discover preventative treatments for the aforementioned illness. The variety of topological indicators for these medications is strongly affected by the correlation coefficient. The results offer a technique to evaluate physiochemical features for new discoveries of other disorders and are eye-opening for researchers working on drug science in the pharmaceutical sector.

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Determination of Tenacissoside G, Tenacissoside H, and Tenacissoside I in Rat Plasma by UPLC-MS/MS and Their Pharmacokinetics

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ABSTRACT (ENGLISH)

An ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method was developed for the determination of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma. The rat plasma was treated with liquid-liquid extraction using ethyl acetate. The determination was performed on the UPLC HSS T3 column (50 mm × 2.1 mm, 1.8 μm) with a mobile phase consisting of acetonitrile-water (containing 0.1% formic acid) and gradient elution at a flow rate of 0.4 mL/min. Electrospray (ESI) positive ion mode detection and multireaction monitoring (MRM) quantitative analysis were performed. A total of 36 rats were given tenacissoside G, tenacissoside H, and tenacissoside I, respectively, orally (5 mg/kg) and intravenously (1 mg/kg), with 6 rats in each group, to evaluate the pharmacokinetic difference of tenacissoside G, tenacissoside H, and tenacissoside I in rats. The calibration curves showed good linearity in the range of 5–2000 ng/mL, where *r* was greater than 0.99. The results of precision, accuracy, recovery, matrix effect, and stability met the requirements of biological sample detection methods. The established UPLC-MS/MS method was successfully applied to pharmacokinetic studies of tenacissoside G, tenacissoside H, and tenacissoside I, and the bioavailability was 22.9%, 89.8%, and 9.4%, respectively.

FULL TEXT

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1. Introduction

Marsdenia tenacissima, also known as *glaucescent fissistigma* root, *Rosa banksiae* f. *lutea* (Lindl.) Rehd, etc., is the dry stem and rattan of *Marsdenia tenacissima* (Roxb) Wight et Am., a plant belonging to the family Asclepiadaceae [1–3], which was originally published in the Herbal Medicines of Southern Yunnan and is now published in the Chinese Pharmacopoeia 2010 edition [4, 5]. It is mainly distributed in Guizhou, Yunnan, Sichuan, Guangxi, and other places. *Marsdenia tenacissima* tastes bitter and slightly cold. It has the effects of clearing heat and detoxifying, relieving cough, and asthma, dispersing knots and relieving pain, and fighting cancer. The series of preparations made from its single medicinal material are widely used in clinics [6–8].

The roots, stems, and leaves of *Marsdenia tenacissima* can be used as medicine. Its effective components are steroidal glycosides, alkaloids, and polysaccharides [9–11]. Its active antitumor components are mainly steroidal components. It is reported that *Marsdenia tenacissima* polysaccharides and some fat-soluble components also have antitumor effects [12]. At present, dozens of steroids have been isolated from this plant. The C21 steroidal glycosides are mostly white crystals or powder, which mainly exist in the plants of Asclepiadaceae. The main antitumor active component of *Marsdenia tenacissima* is C21 steroidal glycosides [13]. Polysaccharide and some fat-soluble components also have antitumor effects. At present, more than 40 kinds of C21 steroidal glycosides have been isolated from the plant, which are a class of compounds formed by a class of steroidal derivatives of glycosides and 2-deoxysugars, and the sugar chain contains up to 6 sugars. These compounds contain a variety of aglycones with different structures [8, 9], of which there are 6 main configuration aglycones. In *vivo* pharmacokinetics of tenacissoside H and tenacissoside I have been reported in literature [14–16], but bioavailability has not been reported.

High-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) technology has the advantages of

high sensitivity, low detection limit, and small sample consumption and is widely used in drug analysis of chemical composition, drug metabolism, and impurity identification [17, 18]. UPLC, which columns with small particle sizes and under ultra-high pressure, maintains the basic principles of a traditional HPLC system but demonstrates improved separation efficiency and speed [19, 20].

Therefore, this study was to establish an UPLC-MS/MS for the determination of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma and study the pharmacokinetics and bioavailability to provide a scientific experimental basis for the basic research of clinical pharmacy.

2. Experimental

2.1. Reagents and Animals

Tenacissoside G, tenacissoside H, tenacissoside I, and astragaloside IV (internal standard) (purity ≥98%, Figure 1) were all purchased from Chengdu Master Pharmaceutical Co., Ltd. Acetonitrile and methanol in chromatographic purity were purchased from Merck. Ultra-pure water (resistance >18 mΩ) was prepared by the Milli-Q purification system in the United States. Sprague–Dawley (SD) rats (220–250 g) were from the Animal Experimental Center of Wenzhou Medical University.

[figure(s) omitted; refer to PDF]

2.2. Instrument Condition

A Waters XEVO TQ-S microtriple quadrupole series mass spectrometer was used for the detection of tenacissoside G, tenacissoside H, and tenacissoside I.

Chromatographic conditions were as follows: UPLC HSS T3 column (50 mm × 2.1 mm, 1.8 μm) and column temperature set at 40°C. The mobile phase was acetonitrile-water (containing 0.1% formic acid) with gradient elution at a flow rate of 0.4 mL/min and elution time of 6 min. 0–0.2 min, acetonitrile 10%; 0.2–2.4 min, acetonitrile 10%–75%; 2.4–5.0 min, acetonitrile 75%–90%; 5.0–5.1 min, acetonitrile 90%–10%; and 5.1–6.0 min, acetonitrile 10%.

Mass spectrometry conditions: nitrogen as conical gas (50 L/h) and desolvated gas (900 L/h); capillary voltage set at 2.5 kV; ion source temperature at 150°C; and desolvent temperature at 450°C. ESI positive ion mode detection and MRM were used for quantitative analysis: *m*/*z* 815.5 755.5 for tenacissoside G (cone voltage 96 v, collision voltage 26 v), *m*/*z* 817.4 757.5 for tenacissoside H (cone voltage 96 v, collision voltage 40 v), *m*/*z* 837.4 777.5 for tenacissoside I (cone voltage 86 v, collision voltage 30 v), and *m*/*z* 785.4 143.0 for astragaloside IV (cone voltage 6 v, collision voltage 46 v).

2.3. Standard Curve

Tenacissoside G, tenacissoside H, tenacissoside I, and astragaloside IV reserve solution (500 μg/mL) were prepared with methanol, respectively. Tenacissoside G, tenacissoside H, and tenacissoside I working solution was obtained by diluting the reserve solution with methanol. Both the reserve solution and working solution were stored at 4°C. Appropriate amount of tenacissoside G, tenacissoside H, and tenacissoside I working solution was added to the blank rat plasma, and then the tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma were 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL. Three quality control (QC) samples with plasma concentrations (8, 180, and 1800 ng/mL) were prepared by the same method.

2.4. Sample Handling

A plasma sample of 100 μL was added to a 1.5 mL microcentrifuge tube, then 10 μL of astragaloside IV (1.0 μg/mL) and 1.0 mL ethyl acetate were added, vortex was mixed for 1.0 min, and it was centrifuged (13,000 rpm, 4°C, 5 min). The organic phase was transferred into another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL methanol and centrifuged at 13,000 rpm for 5 min. The supernatant was pipetted to an auto sampler vial, and 2 μL was injected into the UPLC-MS/MS for analysis.

2.5. Pharmacokinetic Study

Tenacissoside G, tenacissoside H, and tenacissoside I were given sublingual intravenous administration (iv) of 1 mg/kg and oral administration (po) 5 mg/kg, respectively, with 6 rats in each group, for a total of 36 rats. All experimental procedures and protocols were approved by the Animal Care Committee of Wenzhou Medical University (xmsq 2023-0689). The 0.4 mL blood was collected from the caudal vein at 0.083 3, 0.5, 1, 2, 3, 4, 6, and

8 h for tenacissoside G; 0.083 3, 0.5, 1, 2, 3, 4, 6, 8 and 12 h for tenacissoside H; and 0.083 3, 0.5, 1, 2, 3, 4, and 6 h for tenacissoside I, collected in heparinized test tubes and centrifuged at 13000 r/min for 10 min. 100 μL of the plasma was then transferred to a new 1.5 mL microcentrifuge tube and held at −80°C prior to analysis. Pharmacokinetic parameters were statistically calculated using the pharmacokinetic software (DAS 2.0 version).

3. Result

3.1. Selectivity

The retention times of tenacissoside G, tenacissoside H, tenacissoside I, and astragaloside IV were 3.32, 3.42, 3.41, and 2.63 min, as shown in Figure 2, respectively. The optimized gradient elution procedure was used to isolate tenacissoside G, tenacissoside H, and tenacissoside I, effectively, and no interference of endogenous components was observed in the retention time of tenacissoside G, tenacissoside H, and tenacissoside I. This method has good selectivity.

[figure(s) omitted; refer to PDF]

3.2. Standard Curve

The calibration curves of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma showed good linearity in the range of 5–2000 ng/mL, with r greater than 0.99. The typical regression equation of the tenacissoside G in rat plasma was as follows: $y1 = 0.0045x1 + 0.0102$ ($r = 0.9976$), $x1$ was the concentration of tenacissoside G in plasma, and $y1$ was the ratio of tenacissoside G peak area to the internal standard. The typical regression equation for tenacissoside H in rat plasma was $y2 = 0.0046x2 + 0.0022$ ($r = 0.9986$), where $x2$ was the concentration of tenacissoside H in plasma, and $y2$ was the ratio of the tenacissoside H peak area to the internal standard. The typical regression equation for tenacissoside I in rat plasma was $y3 = 0.0020x3 + 0.0097$ ($r = 0.9977$), $x3$ was the concentration of tenacissoside I in plasma, and $y3$ was the ratio of tenacissoside I peak area to internal standard. The lower limit of quantitation of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma was 5 ng/mL, and the detection limit was 1.5 ng/mL.

3.3. Precision, Accuracy, Recovery, and Matrix Effect

The intraday and interday precision of tenacissoside G was within 10%, the accuracy was 90% to 111%, the recovery was over 92%, and the matrix effect was in the range of 94% to 109% (Table 1).

Table 1

Accuracy, precision, matrix effect, and recovery of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma.

Compound	Concentration (ng/mL)	Accuracy (%)		Precision (RSD%)		Matrix effect (%)	Recovery (%)
Intraday	Interday	Intraday	Interday	Tenacissoside G	5	90.6	110.5
10.0	10.0	108.5	92.9	8	108.8	99.0	8.4
4.8	94.5	92.2	180	99.4	105.5	5.3	9.3
102.3	97.3	1800	104.4	104.9	8.1	4.8	99.8
94.4	–						

Tenacissoside H	5	91.1	97.9	13.0	11.8	106.9	97.2
8	88.2	109.8	10.2	9.1	101.8	96.9	180
100.9	92.8	11.0	8.3	102.1	90.6	1800	96.2
114.5	6.8	2.0	107.8	88.6	·		
Tenacissoside I	5	95.3	88.8	8.8	14.1	92.8	83.5
8	93.2	105.8	7.2	6.4	91.3	83.0	180
109.8	101.6	10.0	10.7	94.1	80.6	1800	101.3

The intraday and interday precision of te nacissoside H was within 13%, the accuracy was 88% to 115%, the recovery was above 88%, and the matrix effect was in the range of 101% to 108% (Table 1).
The intraday and interday precision of tenacissoside I was within 15%, the accuracy was 88% to 110%, the recovery was above 80%, and the matrix effect range was 91% to 99% (Table 1).

3.4. Stability

The plasma samples of rats were stored in an automatic injector for 2 h, pretreated, and placed at room temperature for 24 h. The plasma samples underwent three freeze-thawing cycles, and the stability test was conducted at −20°C for 30 days. The accuracy of tenacissoside G was 88%–112%; RSD was within 13%. The accuracy of tenacissoside H was 88%–111%; RSD was within 15%. The accuracy of tenacissoside I was 88%–111%; RSD was within 15% (Table 2). The results indicated that tenacissoside G, tenacissoside H, and tenacissoside I were stable.

Table 2

Stability of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma.

Compound	Concentration (ng/mL)	Autosampler (4°C, 12 h)		Ambient (2 h)		−20°C (30 d)		Freeze-thaw	
Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Tenacissoside G	8
102.1	11.3	104.9	12.9	106.8	12.9	100.0	9.1	180	97.8
6.7	95.0	4.9	99.9	1.4	111.3	11.5	1800	100.2	2.2
100.1	8.9	93.4	5.7	88.7	12.3	·			

Tenacissoside H	8	102.6	9.5	102.3	7.8	102.2	12.3	94.8	10.9
180	101.5	4.7	95.5	11.8	88.2	3.6	110.9	14.5	1800
95.9	7.5	102.2	7.9	109.6	3.9	94.2	7.1	·	
Tenacissoside I	8	94.8	5.1	105.5	10.9	90.4	14.3	88.0	8.9
180	103.0	4.5	95.3	5.0	103.1	11.2	109.2	13.0	1800

3.5. Pharmacokinetic Study

The concentration-time curves of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma are shown in Figure 3. The main pharmacokinetic parameters are listed in Table 3, and oral bioavailability was 22.9%, 89.8%, and 9.4%, respectively.

[figure(s) omitted; refer to PDF]

Table 3

Main pharmacokinetic parameters after intravenous (IV, 1 mg/kg) and oral (PO, 5 mg/kg) administration of tenacissoside G, tenacissoside H, and tenacissoside I in rats.

Compound	Group	$AUC_{(0-t)}$ (ng/mL·h)	$AUC_{(0-\infty)}$ (ng/mL·h)	$t_{1/2z}$ (h)	$CL_{z/F}$ (L/h/kg)	$V_{z/F}$ (L/kg)	C_{max} (ng/mL)
Tenacissoside G	po	2037.0 ± 630.4	2046.0 ± 639.2	0.9 ± 0.3	2.7 ± 0.9	3.6 ± 1.6	900.2 ± 246.3
iv	1778.5 ± 419.6	1801.4 ± 418.2	0.8 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	1137.1 ± 386.1	·
Tenacissoside H	po	1336.5 ± 146.1	1359.8 ± 127.7	1.8 ± 0.6	10.1 ± 4.0	325.5 ± 18.2	1336.5 ± 146.1
iv	361.2 ± 35.7	364.6 ± 36.5	1.3 ± 0.1	5.0 ± 0.5	303.7 ± 30.9	361.2 ± 35.7	·
Tenacissoside I	po	113.0 ± 8.5	116.9 ± 9.9	1.1 ± 0.3	43.0 ± 3.8	69.6 ± 16.0	60.3 ± 7.7

4. Discussion

In order to obtain the best mass spectrum conditions, the positive and negative ion modes were used for monitoring. The responses of tenacissoside G, tenacissoside H, and tenacissoside I were higher in the positive ion mode than the negative ion modes. After optimizing various parameters, the mass spectrum parameters can meet the requirements of accurate quantification of effective substances in biological samples. Through the standard sample, the capillary voltage and the collision energy were optimized.

The commonly used biological sample pretreatment methods were liquid-liquid extraction (LLE) [21–24], solid-phase

extraction (SPE) [25, 26], and protein precipitation method (PPT). The SPE method has complicated operation steps and a high extraction column price. LLE could efficiently extract target substances, especially for low-concentration samples, with higher extraction efficiency than PPT. LLE and PPT methods were tried in this work, and LLE with ethyl acetate was found to be a better extraction efficiency (around 90%) than PPT with acetonitrile (around 60%). During quantitative analysis, it is necessary to add an internal standard substance of known concentration as a quantitative reference for the compounds to be measured in the sample. The internal standard substance should have similar physical and chemical properties to the compound to be tested, and be stable in the sample, and be easy to detect and quantify. Commonly used internal standard substances include isotope-labeled compounds and structural analogues. Astragaloside IV has similar physical and chemical properties to tenacissoside, and it was selected as the internal standard.

Zhao et al. studied the plasma concentration and pharmacokinetic process of tenacissoside A in rats by the LC-MS/MS method [27]. Medroxyprogesterone acetate was used as the internal standard. The oral bioavailability of the drug was low (2.6%), the elimination was faster, and the first-pass effect was obvious. Li established a LC-MS/MS method for simultaneous determination of garcinia extract in rat plasma samples of tenacissoside B, tenacissoside H, tenacissoside I, caffeic acid, cryptochlorogenic acid, chlorogenic acid, and neochlorogenic acid [16]. Digoxin was used as an internal standard reference. Zeng et al. have developed a LC-MS/MS method for simultaneous determination of three isomerized gestrins (17*β*-tenacigenin B, tenacigenine B, and tenacigenine A) and their corresponding glycosides (tenacissoside A and tenacissoside B) in rat plasma [14]. After dexamethasone acetate was added as an internal standard, a simple liquid-liquid extraction technique was used. This method was successfully applied to the pharmacokinetic study after intravenous injection of Xiao-Ai-Ping in rats. However, these methods did not study the bioavailability of tenacissoside B, tenacissoside H, and tenacissoside I.

5. Conclusion

In this study, the UPLC-MS/MS technique was established for the determination of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma in the range of 5–2000 ng/mL. The rat plasma was treated with liquid-liquid extraction using ethyl acetate, and astragaloside IV was used as internal standard. The selectivity, linearity, precision, accuracy, recovery, and stability of this method have been verified, and it has been applied to the pharmacokinetic study of tenacissoside G, tenacissoside H, and tenacissoside I in rats, and the bioavailability was calculated to be 22.9%, 89.8%, and 9.4%, respectively.

Authors’ Contributions

Fan Chen and Yizhe Ma contributed equally to this work.

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DETAILS

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Retracted: Prediction of Dissolved Oxygen Concentration in Sewage Treatment Process Based on Data Recognition Algorithm

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FULL TEXT

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Peer-review manipulation

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] L. Ma, J. Liu, "Prediction of Dissolved Oxygen Concentration in Sewage Treatment Process Based on Data Recognition Algorithm," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/1525902, 2022.

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Wild Mushrooms: A Hidden Treasure of Novel Bioactive Compounds

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ABSTRACT (ENGLISH)

Secondary metabolites are hidden gems in mushrooms. Understanding these secondary metabolites’ biological and pharmacological effects can be aided by identifying them. The purpose of this work was to profile the mycochemical components of the extracts of *Auricularia auricula judae*, *Microporus xanthopus*, *Termitomyces umkowaani*, *Trametes elegans*, and *Trametes versicolor* to comprehend their biological and pharmacological capabilities. Mushroom samples were collected from Kenya’s Arabuko–Sokoke and Kakamega National Reserved Forests and identified using morphological and molecular techniques. Chloroform, 70% ethanol, and hot water solvents were

used to extract the mycochemical components. Gas chromatography mass spectrometry (GC-MS) was used to analyze the chloroform, 70% ethanol, and hot water extracts of all the species examined. A total of 51 compounds were isolated from all extracts and classified as carboxylic acids, esters, phenols, fatty acids, alcohol, epoxides, aldehydes, fatty aldehydes, isoprenoid lipids, and steroids. Tetracosamethyl-cyclododecasiloxane (18.90%), oleic acid (72.90%), phenol, 2, 6-bis (1, 1-dimethylethyl)-4-methyl-, and methylcarbamate (26.56%) were all found in high concentrations in *A. auricular judae*, *M. xanthopus*, *T. umkowaani*, *T. elegans*, and *T. versicolor*, respectively. Fatty acids make up the majority of the compounds isolated from the *T. elegans* chloroform extract and the *T. umkowaani* 70% ethanol extract, respectively. Particularly, these fatty acids play crucial roles in the anti-inflammatory, hypocholesterolemic, anticancer, and antibiofilm formation activities. These bioactive elements indicate that the extracts of five wild mushrooms may be reliable sources of secondary metabolites for therapeutic development. Therefore, additional research is required to comprehend the usefulness of these chemicals in many functional areas and to improve the present understanding of macrofungi.

FULL TEXT

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1. Introduction

Macrofungi are sources of a wide range of physiologically active substances [1, 2]. Despite being a significant source of numerous bioactive chemicals that can be used to produce innovative medications, macrofungi have not yet been extensively utilized [3]. A growing area of research is the hunt for fungus-derived bioactive substances [4]. In light of treatment failures and the global multidrug resistance crisis, a constant hunt for new molecules with therapeutic value has become imperative [5]. For downstream applications and bioprospecting, mushrooms have a wide range of compounds [6]. Natural chemicals obtained from mushrooms can be thoroughly investigated and evaluated, which can be extremely beneficial for treating both infectious and noninfectious disorders [7, 8]. Medicinal mushrooms and fungi are believed to have 130 different therapeutic properties, including antiallergic [9], antiarthritic, antiasthmatic [10], anticancer [11], pesticide [12], antidepressive [13], antidermatophytic [14], antidiabetic [15], antifertility, antifungal [15], antihelminthic [16], cytotoxic [17], antihypercholesterolemic [18], antihyperlipidemic [19], antihypertensive [20], antihypocholesterolemic [21], anti-inflammatory [15], antimalarial [22], antimicrobial [15], antioxidant [11], antiparasitic [23], antispasmodic [24], antiviral [13], anticardiovascular illnesses [18], hepatoprotective [10], immunomodulator [22], immunostimulant [13], insecticidal [22], larvicidal [25], nematicide [26], nephroprotective, neuroprotective [13], osteoprotective [4], and vasodilator [27], among others.

Auricularia auricular judge (Bull.) belongs to phylum-basidiomycota, class: agaricomycetes, order: auriculariales, family: auriculariaceae, and genus: *Auricularia*. *A. auricula-judae*, also known as black fungus, wood ear, Jew’s ear, or jelly ear, is a species of edible mushroom that is very nutrient-dense [28, 29]. It contains a variety of nutrients that are worthy for our health, including polysaccharides, melanin, polyphenols, flavonoids, amino acids, carbs, vitamins, and trace minerals. Furthermore, it has a variety of chemical combinations with antioxidant, anticoagulant, and anticancer properties [30].

Microporus xanthopus (Fr.) kuntze is a member of the genera *Microporus* and phylum-basidiomycota, class: agaricomycetes, order: polyporales, and family: polyporaceae. It is a polypore medicinal mushroom that cannot be eaten. It contains a variety of chemical components, including alkaloids, flavonoids, steroids, triterpenoids, and coumar, which have the potential to have positive pharmacological effects with applications in agriculture, medicine, and other fields [31]. According to reports, it has anthelmintic [32], antibacterial [33], anticancer [1], and antiangiogenic properties.

Termitomyces umkowaani is a member of the phylum-basidiomycota, class: agaricomycetes, order: agaricales, and family: lyophyllaceae. *Termitomyces* species are obligate mutualistic edible mushrooms that coexist with fungus-growing termites [34]. *Termitomyces*’ geographic range matches that of termites, and it gives its hosts vitamins and digesting enzymes [35, 36]. *Termitomyces*’ bioactive substances may be able to treat diseases including Alzheimer’s, hyperlipidemia, cancer, and gastroduodenal disorders [34, 37].

*Trametes elegans*is a member of the phylum-basidiomycota, class: agaricomycetes, order: agaricales, and family: polyporaceae. *T. elegans* is an endophytic and saprotrophic fungus that brought white rot on wood [38]. Due to its extensive use in the culinary and pharmaceutical industries, *T. elegans* (also known as Turkey tail) has become incredibly well-known [39]. Its capacity to break down dead organic matter and utilize a variety of substrates has resulted in a diversity of its biological and metabolic processes [40]. It is also well-known for its therapeutic benefits, commercial applications (such as in the food sector), and roles in bioremediation and the biodegradation of cellulosic waste [41, 42].

Numerous biologically active polysaccharides found in *Trametes versicolor* (L) Lloyd (family: polyporaceae) are used to treat a variety of ailments, including rheumatoid arthritis, chronic hepatitis, infections of the respiratory tract, urinary tract, and digestive system, and tumors. It consists of 18 different amino acids, including aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine, as well as a wide range of other substances, including proteins, fatty acids, polysaccharides, polysaccharopeptides, glucans, vitamins, and inorganic salts [43, 44]. All of these amino acids are necessary for growth and repair because they play various roles in cellular, tissue, and organ structure [45, 46].

Only a small or nonexistent amount of research has been carried out on the identification of bioactive chemicals that confer these therapeutic capabilities on the very few species of Kenyan wild mushrooms that have been claimed to have therapeutic potential. Determining the bioactive substances in wild mushroom extracts that are responsible for their therapeutic benefits is crucial. Therefore, the objective of this study was to investigate the bioactive substances found in the chloroform, ethanol, and hot water extracts of five wild mushrooms and to ascertain their biological and pharmacological therapeutic capabilities that may shed light on their usage in both traditional and contemporary medicine.

2. Materials and Methods

2.1. Wild Mushrooms Collection and Identification

In the national reserved forests of Kakamega and Arabuko–Sokoke, mushrooms were collected. They were randomly collected from tree bark or other substrates (wood, soil, or leaf litter). To keep them structurally sound and wet, they were wrapped in aluminum foil and put in an icebox. Following that, they were recognized using both morphological and molecular techniques. Specimens were identified using spore print color (white, black, brown, pink, purple, etc.) and macroscopic and microscopic methods (form and size of basidiospores, basidia, cystidia, and generative hyphae) [47]. In addition, *Species Fungorum* and associated literature were used to compare the morphological traits of the specimens [48]. The size and form of the gill, the color and shape of the cap, the color and shape of the stipe, and other morphological characteristics of the mushroom are some of the parameters utilized for identification. For the sake of identification, the gill margin, stipe location, stipe base, and pileal margin and surface were also applied. Other morphological details of the mushrooms, such as the cap's structure, the gills' margin and placement, the stipe's surface and form, and the pileus' margin and surface, were also noted. The ornamentation of the pileus and stipe surfaces, the presence or absence of an annulus on the stipe, and the presence or absence of a volva at the base of the stipe were additional characteristics that were utilized to describe and identify the mushrooms (Table 1). The samples were then preserved for additional analyses after being dried in an electric drying oven at 50°C for 168 h [49].

Table 1

Morphological characters and keys used for the identification of the wild mushrooms.

Mushrooms species	Cap structure	Location of stipe	Pileus margin	Pileal shape	Pileal surface	Annulus (veil/ring)	Volva (cup)	Stipe surface	Stipe base/shape	Gill margin	Gill attachment

Auricularia auricula judae	N/A	N/A	Incurved	Ear shaped	N/A	—	—	N/A	No stipe	N/A	N/A
Microporus xanthopus	Half funnel	N/A	Smooth	Hemispherical	Smooth	—	—	N/A	Substipitate	N/A	Sinuate
Termitomyces umkowaani	Umbonate	Central	Smooth	Knobbed	Smooth	—	—	Smooth	Swollen	N/A	Adnexed
Trametes elegans	Funnel-like	N/A	Smooth	N/A	Rough	—	—	N/A	Nonstipe	N/A	Adnate
Trametes versicolor	N/A	N/A	N/A	N/A	N/A	—	—	N/A	Substipitate	N/A	Free

Note. N/A-not applicable, —denotes the absence of annulus and volva.

The dried fruiting body of mushrooms was used to extract gDNA using the cetyl trimethyl ammonium bromide (CTAB) technique [12]. Highly conserved portions of the ITS1 and ITS4 of the mushroom rDNA genes were amplified using the PCR amplification technique by using specialized markers [50]. PCR products that had been amplified were separated using gel electrophoresis and seen under UV illumination. Each PCR product's presence and quantity were calculated by contrasting it with the control (1 kb DNA ladder).

2.2. Extraction of Bioactive Compounds

Chloroform, 70% ethanol, and hot water solvents were used to extract bioactive chemicals, with a few changes from other investigations [51, 52]. A 100 g of powdered mushroom was combined with 1L of each of distilled hot water (heated at 60°C for 2 h.), 99.8% chloroform (Sigma Aldrich, USA), and 70% ethanol (ECP Ltd, New Zealand) separately in an Erlenmeyer flask at 25°C and shaken using an incubator shaker (SK-727, Amerex Instruments, Inc., USA) at 150 rpm for 72 h. The extracts were concentrated and dried using a rotary evaporator (EV311, Lab Tech Co., LTD, UK) at 50°C after being centrifuged at 3000 rpm for 15 min (Eppendorf centrifuge 5810 R, Germany). The extracts were freeze-dried (mrc freeze dryer, Model, FDL-10N-50-8M) and stored in a −80°C deep freezer. Finally, unprocessed extracts were placed in amber-colored bottles and placed in a refrigerator at +4°C for further analysis.

2.3. GC-MS Analysis of Extracts

The GC-MS analysis was conducted using a silica capillary column (30 × 0.25 mm ID × 1 μm, composed of 100% dimethylpolysiloxane) and operated in an electron impact mode at 70 eV (Agilent Scientific, Palo Alto, CA). Helium (99.999%) was a carrier gas at a constant flow of 1 mL/min. Extracts were dissolved in dichloromethane and 1 μL solution was injected into the column at 250°C and ion-source temperature 280°C. The oven temperature was programmed at 110°C for 2 min. The temperature was increased from 110°C to 200°C (10°C/min) then to 280°C (5°C/min) and finally ended at 280°C for 9 min. The total run time was 28 min. The compounds were identified from the MS data, by comparing the spectra of known compounds stored in the National Institute of Standards and Technology (NIST) library with the mass spectrometry (MS) of unknown compounds. The relative % amount of each compound was calculated by comparing its average peak area to the total areas. Measurement of peak areas and data processing were carried out by the Turbo-Mass-OCPTVS-Demo SPL software [53].

2.4. Statistical Analysis

All the tests, experiments, and measurements were carried out in triplicate. Microsoft Excel software package was used to analyze quantitative data [52].

3. Results and Discussion

3.1. GC-MS Analysis of Wild Mushroom Extracts

Five extracts of wild mushrooms were analyzed using GC-MS, and fifty-one (51) chemicals were found. Some of the compounds obtained are acyclic monoterpenoids, alcohol, aldehyde, alkene, alkyl benzene, aromatic organic heterocyclic, benzoic acid ester, cycloalkane methanol, cyclohexane, epoxides, ester, fatty acid, fatty acid ester, fatty alcohol, fatty aldehyde, isoprenoid lipid, organosiloxane, phenol, phthalate, pyrrolidines, siloxane, steroid, and *β* -carotene. Tables 2–6 list the many compounds and the pharmacological and biological actions of each one.

Table 2

GC-MS analysis of *A. auricula judae* hot water extract.

Peaks	RT (min)	PA (%)	IUPAC name and MF of compounds	Nature of compounds	Pharmacological and biological activities	Ref.
1	19.98	10.65	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- ($C_{16}H_{50}O_7Si_8$)	Siloxane	Antidepressant and antimicrobial	[54, 55]
2	18.43	7.12	Salicylic acid, diethyl bis (trimethylsilyl) ester ($C_{10}H_{28}O_4Si_3$)	Ester	Antioxidant, antimicrobial, antimalarial, and anti-inflammatory	[56, 57]
3	17.19	4.43	Di-n-octyl phthalate ($C_{24}H_{38}O_4$)	Phthalic acid	Antimicrobial and insecticidal	[58, 59]
4	16.87	6.12	Di-n-decylsulfone ($C_{20}H_{42}O_2S$)	Phthalate	Antimicrobial, anticancer, antihelminthic, antagonistic, and larvicidal	[60, 61]
5	16.38	7.98	2-Methyl-6-methylene-octa-1,7-dien-3-ol ($C_{10}H_{16}O$)	Acyclic monoterpenoids	No activity reported	
6	16.18	5.65	1-Heptanol, 2,4-dimethyl- (R, R)- (+)- ($C_9H_{20}O$)	Alcohol	Antifungal, antioxidant, and anticholinesterase	[62–64]
7	15.34	4.31	Cyclohexanol, 2,4-dimethyl- ($C_8H_{16}O$)	Cyclohexane	Anticancer	[65]
8	14.65	3.43	Carbonic acid, methyl octyl ester ($C_{10}H_{20}O_3$)	Ester	Hepatoprotective, antihypertensive, antioxidant, antimicrobial, antidiabetic, cholesterol-lowering, antiurolithiasis, and antifertility	[66]
9	14.06	5.25	1-Allylcyclopropyl methanol ($C_7H_{12}O$)	Cycloalkane methanol	No activity reported	

10	13.64	7.23	2-Methyl-1-ethylpyrrolidine ($C_7H_{15}N$)	Pyrrolidines	Antitumor	[67]
11	13.01	6.33	Oxirane, 2,2′-(1,4-dibutanediyl) bis- ($C_8H_{14}O_2$)	Epoxides	Antibacterial	[68]
12	12.47	11.34	2-Nonanol, 5-ethyl- ($C_{11}H_{24}O$)	Fatty alcohol	Anticancer	[69]
13	11.91	5.86	1-Hexene, 4, 5-dimethyl- (C_8H_{16})	Alkene	Antimicrobial	[70]
14	11.34	14.21	Phenol, 2,6-bis (1,1-dimethyl ethyl)-4-methyl-, methylcarbamate ($C_{17}H_{27}NO_2$)	Alkylbenzene	Antioxidant, antibacterial, anti-inflammatory, and temporarily treat pharyngitis	[71, 72]

MF: molecular formula; RT: retention time; PA: peak area.

Table 3

GC-MS analysis of *M. xanthopus* hot water extract.

Peaks	RT (min)	PA (%)	IUPAC name and MF of compounds	Nature of compounds	Pharmacological and biological activities	Ref.
1	6.42	8.11	1-Heptanol, 2,4-dimethyl-, (2S, 4R) -(-)- ($C_9H_{20}O$)	Alcohol	Antifungal	[62, 63]
2	7.28	4.34	Oxirane, 2,2′-(1,4-butanediyl) bias- ($C_8H_{14}O_2$)	Epoxides	No activity reported	
3	10.48	3.67	3-Methyl-2-(2-oxopropyl) furan ($C_8H_{10}O_2$)	Aldehyde	Antioxidant and antimicrobial	[73, 74]
4	11.32	5.50	7-Hexadecenal, (Z)- ($C_{16}H_{30}O$)	Fatty aldehyde	Antiviral and antibacterial	[75, 76]
5	12.09	7.87	1,2,3,3a-Tetrahydro-7-methyl-10-4-methylphenyl) benzo [c] cyclopenta [f] −1,2-diazepine ($C_{20}H_{20}N_2$)	Aromatic organic heterocyclic	No activity reported	
6	12.81	4.41	Tetradecane, 2,6,10-trimethyl- ($C_{17}H_{36}$)	Isoprenoid lipid	Antifungal, antibacterial, and nematicidal	[77]

7	13.19	4.19	Heptacosane ($C_{27}H_{56}$)	N-alkanes	Antibacterial, antifungal, antioxidant, antimalarial, and antidermatophytic	[78, 79]
8	13.47	11.39	Didodecyl phthalate ($C_{32}H_{54}O_4$)	Phthalate	Vasodilator, antihypertensive, uric acid excretion stimulant and diuretic, antimicrobial, and antifouling	[80, 81]
9	14.18	1.17	Acetamide, N-[3-(10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5-ylidene)propyl] −2,2,2-triflouro-N-methyl ($C_{21}H_{20}F_3NO$)	Unknown	Reducing depressive symptoms	[82]
10	14.97	13.76	2,2′-Divinylbenzophenone ($C_{17}H_{14}O$)	Unknown	Antimicrobial, anti-inflammatory, and antioxidant	[83]
11	15.95	14.18	Trans-1, 1′-bibenzoindanylidene ($C_{18}H_{16}$)	Unknown	No activity reported	
12	17.18	16.32	1-Monolinoleoylglycerol trimethylsilyl ether ($C_{27}H_{54}O_4Si_2$)	Steroid	Antidiuretic, anti-inflammatory, antidiabetic, antimicrobial, antioxidant, antiarthritic, and antiasthma	[84, 85]

MF: molecular formula; RT: retention time; PA: peak area.

Table 4

GC-MS analysis of *T. umkowaani* 70% ethanol extract.

Peaks	RT (min)	PA (%)	IUPAC name and MF of compounds	Nature of compounds	Pharmacological and biological activities	Ref.
1	4.88	5.68	Butanedioic acid diethyl ester ($C_8H_{14}O_4$)	Fatty acid	Antimicrobial, antispasmodic, and anti-inflammatory	[86]
2	7.87	4.11	Octadecanoic acid, ethyl ester ($C_{20}H_{40}O_2$)	Fatty acid esters	Hypocholesterolemic5-alpha-reductase inhibitor, lubricant, and antimicrobial	[87, 88]
3	9.86	2.45	h-Hexadecanoic acid ($C_{16}H_{32}O_2$)	Fatty acid (aka palmitic acid)	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, antibacterial, anti-inflammatory, antitumor, immunostimulant, hemolytic 5-α reductase inhibitor, and lipooxygenase inhibitor	[5, 89]

4	10.04	7.90	Hexadecanoic acid, ethyl ester ($C_{18}H_{36}O_2$)	Fatty acid ester (akapalmitic acid ester)	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, and hemolytic 5-*α* reductase inhibitor	[5]
5	10.24	8.78	i-Propyl hexadecanoate ($C_{19}H_{38}O_2$)	Fatty acid	No activity reported	
6	10.97	9.98	9,12-Octadecadienoic acid (Z, Z)-($C_{18}H_{32}O_2$)	Fatty acid (aka conjugated linoleic acid)	Anti-inflammatory, antioxidant, hypocholesterolemic, antimicrobial, antitumor, insecticide, antiarthritic, antieczemic hepatoprotective, antiandrogenic, nematicide, antihistaminic, antiacne, hemolytic 5-*α* reductase inhibitor, and anticoronary	[5, 66, 89–91]
7	11.09	13.43	9,12-Octadecadienoic acid, ethyl ester ($C_{20}H_{36}O_2$)	Fatty acid ester (aka omega-6)	Hypocholesterolemic, nematicide, antiacne, antiarthritic, hepatoprotective, antimicrobial, antiandrogenic, hemolytic 5-*α* reductase inhibitor, antihistaminic, anticoronary, and insecticide, antieczemic	[5, 56, 66]
8	11.27	0.89	Isopropyl linoleate ($C_{21}H_{38}O_2$)	*β*-carotene	Antimicrobial and antioxidant	[31, 92–94]
9	13.19	1.50	1-Monolinoleoylglycerol trimethylsilyl ether ($C_{27}H_{54}O_4Si_2$)	Steroid	Antimicrobial, antiasthma, antidiuretic, antioxidant, anti-inflammatory, and antidiabetic	[84]
10	14.18	15.90	12-Methyl-E, E-2, 13-octadecadien-1-ol ($C_{19}H_{36}O$)	Alcohol	Antimicrobial	[95]
11	14.97	1.12	7-Hexadecenal, (Z)-($C_{16}H_{30}O$)	Fatty aldehyde	Antiviral and, antibacterial	[75, 76]
12	15.95	3.60	1, 2-Benzenedicarboxylic acid, diisooctyl ester ($C_{24}H_{38}O_4$)	Ester	Antimicrobial and antifouling	[96]
13	17.20	18.90	Tetracosamethyl-cyclododecasiloxane ($C_{24}H_{72}O_{12}Si_{12}$)	Siloxane	No activity reported	
14	18.53	5.76	Heptasiloxanehexadecamethyl ($C_{16}H_{48}O_6Si_7$)	Organosiloxane	No activity reported	

MF: molecular formula; RT: retention time; PA: peak area.

Table 5

GC-MS analysis of *T. elegans* chloroform extract.

Peaks	RT (min)	PA (%)	IUPAC name and MF of compounds	Nature of compounds	Pharmacological and biological activities	Ref.
1	9.86	16.89	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	Fatty acid	Antioxidant, antiandrogenic, hypocholesterolemic, nematicide, pesticide, and antibiofilm formation	[89, 97]
2	10.97	72.90	Oleic acid ($C_{18}H_{34}O_2$)	Fatty acid	Antioxidant, apoptotic activity in tumor cells, anticancer, and antibiofilm formation	[97, 98]
3	11.12	10.21	Octadecanoic acid ($C_{18}H_{36}O_2$)	Fatty acid	Antimicrobial and antibiofilm formation	[97, 99]

MF: molecular formula; RT: retention time; PA: peak area.

Table 6

GC-MS analysis of *T. versicolor* hot water extract.

Peaks	RT (min)	PA (%)	IUPAC name and MF of compounds	Nature of compounds	Pharmacological and biological activities	Ref.
1	6.42	26.56	Phenol, 2,6-bis (1,1-dimethyl ethyl)-4-methyl, methylcarbamate ($C_{17}H_{27}NO_2$)	Phenol	Antioxidant, antibacterial, anti-inflammatory, oral anesthetic/analgesic, and temporarily treat pharyngitis	[71, 72]
2	9.86	2.20	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	Palmitic acid	Antioxidant, nematicide, pesticide, hypocholesterolemic, and antiandrogenic	[100]
3	10.73	3.40	Nonadecane ($C_{19}H_{40}$)	Hydrocarbon	No activity reported	
4	11.12	8.41	9,12-Octadecadienoic (Z, Z)- ($C_{18}H_{32}O_2$)	Polyunsaturated fatty acid	Anti-inflammatory, hypocholesterolemic, antitumor, hepatoprotective, nematicide, insecticide, antibiofilm formation, antihistaminic, antieczemic, antiacne, hemolytic 5-*α* reductase inhibitor, antiandrogenic, antiarthritic, and anticoronary, antimicrobial	[84, 97, 101–103]

5	11.34	5.73	7-Hexadecenal, (Z)- ($C_{16}H_{30}O$)	Fatty aldehyde	Antiviral and antibacterial	[75, 76]
6	13.19	12.20	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl) oxy]-1-[[(trimethylsilyl) oxy] methyl] ethyl ester (Z, Z, Z)- ($C_{27}H_{52}O_4Si_2$)	Polyunsaturated fatty acid	Antimicrobial, antioxidant	[104, 105]
7	15.97	22.40	1-Momolinoleoylglycerol trimethylsilyl ether ($C_{27}H_{54}O_4Si_2$)		Antimicrobial, antiasthma, antidiuretic, antioxidant, anti-inflammatory, and antidiabetic	[84]
8	18.11	19.10	1,2-Benzenedicarboxylic acid, diisooctyl ester ($C_{24}H_{38}O_4$)	Benzoic acid ester	Biopesticides, antibacterial	[106, 107]

MF: molecular formula; RT: retention time; PA: peak area.

3.1.1. GC-MS Analysis of *Auricularia auricula-judae*–AAJ

The hot water extract (HWE) of AAJ found fourteen (14) bioactive chemicals, as shown in Figure 1(a). These substances have shown a wide range of biological and pharmacological functions. Phenol, 2,6-bis (1,1-dimethyl ethyl)-4-methyl-, methylcarbamate (14.21%), 2-nonanol, 5-ethyl- (11.34%), octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (10.65%), 2-methyl-6-methylene-octa-1,7-dien-3-ol (7.98%), 2-methyl-1-ethylpyrrolidine (7.23%), salicylic acid, and diethyl bis (trimethylsilyl) ester (7.12%) were identified as major compounds Table 2. These compounds were classified into 1-heptanol, 2,4-dimethyl- (R, R)- (+)- (alcohol), 1-hexene, 4, 5-dimethyl- (alkene), octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (siloxane), Carbonic acid, methyl octyl ester and salicylic acid, diethyl bis (trimethylsilyl) ester (ester), di-n-octyl phthalate (phthalic acid), and di-n-decylsulfone (phthalate). The fruiting body of AAJ is reported to contain large amounts of fiber, carotenes, minerals (calcium, phosphorous, and iron), and vitamins in addition to proteins, carbs, and lipids [28]. In addition, polysaccharides, melanin, and polyphenols—vital categories of secondary metabolites that are synthesized in response to biotic (pathogens) and abiotic stresses—salinity, water, and climatic stress—are present in AAJ as bioactive constituents [28]. According to one study, siloxanes have been well-acknowledged to possess substantial antibacterial and antioxidant effects [54]. Thus, the compounds (di-n-decyl sulfone, cyclohexano, 2,4-dimethyl-, salicylic acid, diethyl bis (trimethylsilyl) ester, carbonic acid, methyl octyl ester, phenol, 2,6-bis (1,1-dimethylethyl)-4-methyl-, methylcarbamate, etc.,) found in the HWE of AAJ could prevent diseases such as aging, cancer, cardiovascular disease, inflammation, and other disorders that are dangerous to humans' health occurred due to the overabundance of free radicals in our body [108]. In addition, phenolic compounds can influence biological processes such as cell cycle control, apoptosis induction, and antiproliferation, which are primarily mediated through interactions between receptors and ligands [92].

[figure(s) omitted; refer to PDF]

Many biological and pharmacological activities, including antidepressant, antimicrobial, antioxidant, antimalarial, anti-inflammatory, insecticidal, hepatoprotective, antihelminthic, larvicidal, antihypertensive, anticancer, antidiabetic, cholesterol-lowering, antiurolithiasis, and antifertility, have been demonstrated by the HWE of AAJ, as shown in Table 2. The anticoagulant, antidiabetic, antioxidant, anticancer, hypolipidemic, antiobesity, anti-inflammatory, antiradiation, immunomodulatory, and antibacterial properties of AAJ extracts have also been established by earlier investigations [28, 109, 110]. According to a study, the phenolic substances epicatechin, catechin, chlorogenic acid, quercetin, and rutin are among the phenolic compounds found in the HWE of AAJ. Significant scavenging ability was shown by these phenolic compounds against hydroxyl radicals, superoxide anions, and DPPH-free radicals [111].

Crude AAJ extracts have greater antioxidant activity, control blood pressure, and reduce blood lipid and cholesterol levels [28].

Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl, salicylic acid, diethyl bis (trimethylsilyl) ester, di-n-octyl phthalate, di-n-decyl sulfone, carbonic acid, and methyl octyl ester have all demonstrated antibacterial action in this study. The antibacterial activity of crude polysaccharides derived from AAJ has been demonstrated in the past against *E. coli*, *S. aureus*, *B. cereus*, *S. typhi*, *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans*, and *C. parapsilosis* [28, 109]. Many secondary metabolites, including *β*-glucans, chitin, and derivatives of the sterol ergosterol, have been detected in numerous *in vitro* and *in vivo* studies. These metabolites demonstrate potential anti-inflammatory activity by reducing the production of proinflammatory cytokines, promoting the production of anti-inflammatory cytokines, and preventing both immune response and the development of cancer cells in the body [28, 30, 110, 112]. They also protect the body by lowering blood cholesterol, boosting our immune system, preventing inflammatory disorders, and delaying the development of cancer [28, 112, 113].

By regulating pancreatic insulin secretion, mushroom polysaccharides have demonstrated antidiabetic properties that help to maintain blood glucose homeostasis [114]. A prior study claimed that polysaccharides derived from AAJ extracts significantly reduced the risk of diabetes in streptozotocin-induced diabetic rats. After giving AAJ polysaccharides to streptozotocin and high-fat diet-induced diabetic rats, low-density lipoprotein, and total cholesterol levels in the blood were markedly decreased [115]. In addition, streptozotocin-induced diabetic mice improved the insulin resistance islet damage in diabetes-induced rats treated with AAJ polysaccharides, which changed glucose metabolism, elevated insulin levels, and decreased blood glucose levels [116, 117]. These results supported the idea that AAJ-derived polysaccharides could be employed as possible diabetic treatment agents by modifying blood glucose levels [28].

High concentrations of insoluble fibers can be found in the HWE of AAJ [118]. Through the modification of gut microbiota, these fibers may have positive effects on health [119, 120]. Insoluble fibers have a crucial role in regulating the environment of the gut microbiota and influencing their metabolic activities [121, 122]. They also serve as prebiotics. By fighting for food and preventing adhesion to the gut wall, the beneficial gut microbiota is essential in defending our body against a variety of disease-causing pathogenic bacteria [123]. These gut bacteria also aid in the formation of short-chain fatty acids, such as acetate, propionate, and butyrate, which are crucial for our epithelial cells during their digestion and fermentation processes [124, 125]. By upholding a healthy gut environment and acting as the only carbon source for intestinal bacteria during fermentation, *β*-glucans derived from HWE of AAJ have many health-promoting effects. They also raised the quantity of good bacteria, such as *Bifidobacteria* and *Lactobacillus*, which aid in the synthesis of short-chain fatty acids in our intestines [126]. During the oral treatment of mice, they also saw an increase in serum IgA and IgG levels [127]. Furthermore, they stop the development of harmful bacteria in our gut, which may eventually shield our bodies against a variety of disorders linked to the gut [128, 129].

Biological defenses against cardiovascular disease exist in edible mushrooms. There have been claims that certain *Auricularia* species contain substances that decrease cholesterol [130]. AAJ extracts have reportedly been shown to lower low-density lipoprotein cholesterol levels, which are the cause of cardiovascular disease [121]. AAJ extract effectively decreased serum and liver total cholesterol (TC), total triglyceride (TG), and serum lactate dehydrogenase C (LDH-c) levels in mice using hyperlipidemic mice as a model [131].

Natural immunomodulators can be found in large quantities in medicinal mushrooms. They contain a variety of immune-regulatory substances, including immunomodulatory proteins, lectins, polysaccharides, and terpenes. Immunomodulators can act as immunological adjuvants, immune stimulants, or immune suppressants [132]. For instance, an active substance from AAJ called AF1 with a 1, 3-d-glucan main chain and two 1, 6-d-glucosyl residues has been shown to cause apoptosis in cancer cells [133].

3.1.2. GC-MS Analysis of Hot Water Extract of Microporusxanthopus–MX

Twelve chemicals were found in the hot water extract (HWE) of *M. xanthopus* (MX), as shown in Figure 1(b). Table 3 lists the twelve compounds and their relative abundances. These include trans-1, 1′-bibenzoindanylidene (14.18%),

2, 2′-divinylbenzophenone (13.76%), and didodecyl phthalate (11.39%). Alcohol, epoxides, aldehydes, fatty aldehydes, isoprenoid lipids, n-alkanes, and steroids were the several classifications given to the substances. These substances have been demonstrated to have antioxidant, antimicrobial, nematicidal, antimalarial, antidiuretic, antiasthmatic, vasodilator, antifouling, antidermatophytic, antihypertensive, uric acid excretion stimulant and diuretic, lowering depressed symptoms, and anti-inflammatory properties. In addition, the steroid 1-monolinoleoylglycerol trimethylsilyl ether exhibits antidiuretic, antidiabetic, anti-inflammatory, antimicrobial antioxidant, antiarthritic, and antiasthma properties Table 3.

In line with the current findings, HWE of MX, numerous mushroom extracts including *Agaricus bisporus*, *Cyclocybe aegerita*, *Cyclocybe cylindracea*, and *Tremella fuciformis* have been investigated for the treatment or prophylaxis of type-2 diabetes, which develops when insulin production is unbalanced as a result of the dysfunction of insulin-secreting beta cells in the pancreas [134, 135]. Mushrooms assist patients in avoiding excessive blood sugar levels since they are the food with the least quantity of digestible carbohydrates [136]. Diabetes can be treated with bioactive compounds that are extracted from medicinal mushrooms [137, 138]. Extracts from *Inocutis levis* and *Antrodia cinnamomea* have been suggested as treatments for diabetes because they improve insulin resistance, insulin sensitivity, and tissue uptake of glucose, which helps to regulate blood sugar levels [135, 139].

Most of the substances discovered in the HWE of MX in the current results demonstrated antibacterial action. Another investigation confirmed that HWE of MX-derived oligosaccharides, polysaccharides, and polyphenols had antibacterial effects on *S. aureus* strains that were resistant to methicillin and *E. coli* strains that produced Shiga toxin [33]. Similar to this, MX’s CE has shown increased antibacterial activity against *S. aureus* (ATCC 25923), MRSA (ATCC 33591), and *K. pneumoniae* (ATCC 13883) [52].

The majority of mushrooms are known to produce several bioactive compounds that are employed as potential treatments for cardiovascular disorders [140, 141]. Although the mechanism of action/treatment of these bioactive compounds is still unknown, it may be related to changes in phospholipid metabolism, bile acid secretion, and LDL receptor expression [142]. The presence of chemicals in mushrooms that can alter cholesterol metabolism, absorption, and gene expression has also been noted in other investigations [141, 143]. For example, *Grifola frondosa*, *Hypsizigus marmoreus*, and *Pleurotus ostreatus* extracts have been shown to alter the gene expression patterns in mouse livers [135, 144].

3.1.3. GC-MS Analysis of 70% Ethanol Extract of *Termitomyces umkowaani* (TU)

The 70% ethanol extract (EE) of *T. umkowaani* (TU) (Figure 1(c)) was used to identify fourteen different components. These substances were categorized into acids, alcohols, esters, ethers, ketones, aldehydes, and other categories. Tetracosamethyl-cyclododecasiloxane (18.90%), 12-methyl-E, E-2, 13-octadecadien-1-ol (15.90%), 9, 12-octadecadienoic acid, and ethyl ester (13.43%) were the most prominently seen compounds Table 4.

Many fatty acids (FAs) such as linolenic acid, butanedioic acid diethyl ester, octadecanoic acid, ethyl ester, h-hexadecanoic acid, hexadecanoic acid, ethyl ester, i-propyl hexadecanoate, 9, 12-octadecadienoic acid (Z, Z)-, 9, 12-octadecadienoic acid, ethyl ester, and 7-hexadecenal, (Z)- were noticed in EEofTU. These FAs demonstrated antimicrobial, pesticide, antioxidant, antispasmodic, antitumor, antihypocholesterolemic, antiarthritic, anti-inflammatory, nematicide, immunostimulant, antiacne, insecticide, antieczemic, hepatoprotective, antihistaminic, and anticoronary properties [145–147]. In addition to FAs, the EE of TU revealed additional bioactive substances, such as isopropyl linoleate (-carotene), 1-monolinoleoylglycerol trimethylsilyl ether (steroid), and 12-methyl-E, E-2, 13-octadecadien-1-ol (alcohol). These substances also have antibacterial, antioxidant, antiasthma, antidiuretic, anti-inflammatory, and antidiabetic activities. By preventing the proliferation of bacterial cells and the development of biofilms, linoleic and oleic acids displayed an antibacterial activity against *S. aureus* [148].

The EE of TU contains hexadecanoic acid, and ethyl ester (palmitic acid ester), which has antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, antibacterial, anti-inflammatory, antitumor, immunostimulant, hemolytic 5-reductase inhibitor, and lipoxygenase inhibitor effects. Dietary fats contain palmitic acid (PLA), which ensures an intake of 20 g on average per day. Its important nutritional function [149] justifies the relatively high need for these fatty acids in the human body (20–30% of total fatty acids). It was discovered through

transcriptomic research that palmitic acid affected many signaling pathways, including lipid metabolism in neurons. On the other hand, excessive ingestion of palmitic acid has been linked to neurodegenerative conditions, such as Parkinson's disease [150, 151]. To prevent harmful effects such as oxidative stress, PLA has a critical role in low levels of stress, which can activate the stress response pathway.

The EE of TU contained the 9, 12-octadecadienoic acid (Z, Z)-, that is also known as conjugated linoleic acid. Omega-3 and omega-6 fatty acids are present in linolenic acid (LA). LA can minimize risk factors for arthritis and heart disease as well as assist in reducing bodily inflammation. Prostaglandin E1, a product of omega-3 fatty acids, increases immunity and lowers blood cholesterol [152, 153]. The heart's health is improved by omega-3 fatty acids, which also lower the risk of stroke, heart attack, and high blood pressure [153, 154]. In general, mushrooms have more unsaturated fatty acids than saturated ones [155]. These polyunsaturated fatty acids preserve the liver's ability to produce bile acids, prevent hormonal imbalance, and affect prostaglandin synthesis [156].

LA currently exhibits antibacterial action. In support of the present findings, *Termitomyces* species extract in methanol and ethanol demonstrated strong antibiotic action against pathogenic microorganisms such as *E. coli, B. cereus*, *S. aureus*, *P. aeruginosa*, *S. typhimurium*, and *C. albicans* [157]. The dichloromethane extract of *Termitomyces striatus* also demonstrated antibacterial action against fungi (*C. albicans* and *S. cerevisiae*) and bacteria (*P. aeruginosa*, *E. coli*, *B. subtilis*, and *S. aureus*) [158]. Numerous species of *Termitomyces* have notable antibacterial action against various harmful pathogens. For instance, the water extract of *T. clypeatus* demonstrated antibacterial and antifungal activities against *C. albicans*, *E. coli*, *S. typhi*, and *S. aureus*. The water extract of *T. heimii*also demonstrated antibacterial and antifungal activities against *E. coli*, *K. pneumoniae*, *Pseudomonas* sp., *S. aureus*, *S. pyogenes*, and *Ralstonia* sp.

Fatty acids from the EE of TU, such as octadecanoic acid, ethyl ester, h-hexadecanoic acid, 9, 12-octadecadienoic acid (Z, Z), 9, 12-octadecadienoic acid, and ethyl ester, have demonstrated hypocholesterolemic action. Edible mushrooms include large levels of dietary fiber and other nutrients such as eritadenine, guanylic acid, and ergosterol that can prevent diseases associated with nutrition, including atherosclerosis, by reducing hypocholesterolemic levels [159, 160]. Total and LDL cholesterol levels in the blood were found to decrease with dietary TU intake [161]. Rats that were fed diets containing a combination of mushrooms saw a decrease in their triglyceride and total cholesterol levels [156]. By changing lipid metabolism and preventing both the buildup of liver lipids and the rise of serum lipids, polysaccharides and fibers extracted from aqueous extract of edible mushrooms also reduced the serum triglyceride concentration inhypertensive and hyperlipidaemic rats [162].

3.1.4. GC-MS Analysis of Chloroform Extract of *Trametes elegans* (TRE)

Three substances were found in the *T. elegans* chloroform extract (CE), as shown in Figure 1(d). N-hexadecanoic acid (16.89%), oleic acid (72.90%), and octadecanoic acid (10.21%) are among the chemicals that have been discovered. These substances fall under the category of essential fatty acids, which are required for their anti-inflammatory, antioxidant, and hypocholesterolemic properties. In rats, a lack of the normal necessary fatty acid linoleic acid results in hair loss [21], minor skin scaling, and slow wound healing [22].

According to Table 5, the majority of the discovered compounds show antimicrobial, antioxidant, anticancer, antiandrogenic, hypocholesterolemic, nematicide, pesticide, and antibiofilm formation capabilities. Numerous substances found in the extract, including tocopherols, flavonoids, polyphenols, tannins, and lignins, may be associated with these wide-ranging activities [163]. The oxidizing cascade of free radical reactions in molecules is blocked by the TRE extract's antioxidant activity, which also lessens oxidative damage brought on by oxidative stress [164]. Antioxidants shield our bodies from serious health problems such as diabetes, cancer, aging, atherosclerosis, and others [165].

From the CE of TRE, three isolated essential fatty acids have shown antibiofilm-forming activity. By preventing pathogenic microorganisms from forming biofilms, fungal metabolites have antiquorum-sensing properties that have the potential to reduce the development of drug resistance. Numerous secondary metabolites with biofilm-inhibitory properties are found in many edible mushrooms, according to earlier investigations. For instance, active antibiofilm inhibitory activity against *Pseudomonas*, *S. aureus*, and *C. albicans* has been demonstrated by coprinuslactone,

roussoellenic acid, and microporenic acid A obtained from *Coprinus comatus*, *Roussoella* sp, and Kenyan basidiomycete, respectively [166, 167]. By facilitating antibiotics' capacity to enter biofilms, biofilm inhibitors improve the effectiveness of the antibiotics [168].

The CE of TRE possesses anticancer properties. The extracts combat cancer cells in a variety of ways, such as immune system regulation and cell death [169]. Preclinical and clinical testing for several promising anticancer drugs based on fungi is now underway [170]. Irofulven, for instance, is a semisynthetic medication made from illudin S, a toxin discovered in the *Omphalotus illudens*. Irofulven has been tested in phases I and II clinical studies, showing promise in its ability to treat malignancies of the breast, blood, colon, sarcoma, prostate, lungs, ovary, and pancreas, as well as the brain and central nervous system [171, 172]. Another anticancer substance discovered in fungi *Akanthomyces muscarius* and *Nigrospora sphaerica* is called aphidicolin. Aphidicolin has not yet been marketed as an anticancer medication, even though it specifically targets the binding site on the DNA polymerase and enzymes [135].

One of the fatty acids, n-hexadecanoic acid, found in the CE of TRE showed nematicidal action as shown in Table 5. Even though chemical nematicides (like methyl bromide) are effective and have been commercialized, they can have detrimental effects on the environment by destroying all soil life and thinning the ozone layer. Finding ecologically sound substitutes has recently been the focus of intense research in both academia and industry [135]. Edible mushrooms have been found to contain several nematotoxic substances, including fatty acids, alkaloids, peptide compounds, terpenes, condensed tannins, phenolic compounds, and proteases [173]. One of the nematicidal substances that have been identified from *Arthrobotrys* species and other fungi is linoleic acid [174]. *Pleurotus pulmonarius* and *Hericium coralloides*, on the other hand, are two basidiomycetes that have demonstrated potent nematicidal actions against *Caenorhabditis elegans* [175]. From a *Sanghuangporus* species obtained in Kenya, metabolites (3, 14′-bihispidinyl and hispidin and phelligridin L) with mild nematicidal activity against *Caenorhabditis elegans* have been identified [176]. Recent studies have shown that chaetoglobosin A and its derivative 19-O-acetylchaetoglobosin A, which were isolated from *Ijuhya vitellina*, have nematicidal activity against the eggs of *Heterodera filipjevi* [177].

3.1.5. GC-MS Analysis of Hot Water Extract of *Trametes versicolor* (TRV)

T. versicolor (TRV) hot water extract (HWE) was used to identify eight different chemicals (Figure 1(e)). The most prevalent substances were phenol (26.56%), 2, 6-bis (1, 1-dimethyl ethyl)-4-methyl, methylcarbamate (22.40%), 1, 2-benzene dicarboxylic acid, and diisooctyl ester (19.10%), as shown in Table 6.

In TRV, a polyunsaturated fatty acid called 9, 12-Octadecadienoic (Z, Z)- has demonstrated antitumor action [107]. The TRV extract contains substances that fight cancer and boost the immune system, such as polysaccharides, *β*-glucans, lignins, and ergosta-7, 22-dien-3 beta-ol [178]. Cytotoxic action against cancer cells was shown by polysaccharides extracted from the TRV extract [45]. In addition to significantly improving the quality of life of cancer patients receiving chemotherapy or radiation therapy, polysaccharides containing peptides also help patients with hepatitis, hyperlipidemia, and other chronic diseases live longer and have better quality of life [178, 179]. In mice bearing xenografts, an aqueous extract of TRV inhibited the migration and invasion of 4T1 breast cancer cells and downregulated the activity of the xenograft-inducing molecules tumor necrosis factor, interferon, interleukin-2, interleukin-6, and interleukin-12 [180]. The polysaccharides linked to the TRV protein displayed tumor necrosis factor-dependent antiproliferative activity towards MCF-7 cells and enhanced the proliferative response of blood lymphocytes, which was connected to the upregulation of interleukin-6 and interleukin-1 mRNA [181].

4. Conclusion

Bioactive compounds identified from the five wild mushroom extracts possess anti-inflammatory, antioxidant, nematicide, antimicrobial, anticancer, hypocholesterolemic, antihypertensive, pesticide, and antibiofilm formation properties. The wild mushroom extracts are rich in essential fatty acids and other many bioactive compounds that could have high industrial potential and biological activities. Phenol, 2, 6-bis (1, 1-dimethylethyl)-4-methyl-, methylcarbamate (*A. auricular-judae*), 1-monolinoleoylglycerol trimethylsilyl ether (*Microporus xanthopus*), tetracosamethyl-cyclododecasiloxane (*T. umkowaani*), oleic acid (*T. elegans*), and phenol, 2, 6-bis (1, 1-

dimethylethyl)-4- methyl, methylcarbamate (*T. versicolor*) are the most abundant compounds. These compounds can be deployed to discover novel drugs against various noninfectious diseases such as cancer, hypertension, and diabetes. The identified compounds shall be subjected to further studies to utilize their usefulness in the prevention and treatment of infectious and noninfectious human diseases. To understand the mechanisms of action of the active ingredients, rigorous chemical analyses as well as *in vivo* pharmacokinetics and pharmacodynamics of individual compounds are needed. Future investigation is needed to clarify the long-term effects of taking medicinal mushroom products with other drugs.

Authors' Contributions

GG and DBS were involved in the study conception and design. GG performed the laboratory tests and the data analysis. GG and DBS wrote and refined the manuscript. Both authors read and approved the final manuscript.

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Glossary

Abbreviations

AAJ:*Auricularia auricula judae*

CE:Chloroform extract

EE:70% ethanol extract

FAs:Fatty acids

GC-MS:Gas chromatography mass spectrometry

HWE:Hot water extract

LA:Linolenic acid

LDL:Low-density lipoprotein

MF:Molecular formula

MX:*Microporus xanthopus*

PA:Peak area

PLA:Palmitic acid

RT:Retention time

TRE:*Trametes elegans*

TRV:*Trametes versicolor*

TU:*Termitomyces umkowaani*

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A Novel miRNA Detection Method Using Loop-Mediated Isothermal Amplification

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ABSTRACT (ENGLISH)

A novel ligation-based loop-mediated isothermal amplification has been developed for miRNA detection. Two stem-loop structure DNA linker A/B probes which hybridized with miRNA were designed to establish a rapid and ultrasensitive miRNA-LAMP system for miRNA detection. Target miR-200a was used to template the ligation of Linker A/B probes with SplintR Ligase and used as a dumbbell-shaped amplicon. By adding BIP/FIP and Bst 2.0 DNA polymerase, the LAMP reaction was carried out, which brought greatly improved amplification efficiency. The double-stranded DNA fluorescent dye EvaGreen was added for the detection of amplification product to achieve the quantification of the target miRNA. This method can detect miRNA in a linear range of seven orders of magnitude, with a detection limit of 100 fM. Therefore, this ultrasensitive miRNA-LAMP assay provides a new path for the highly sensitive quantitative analysis of miRNA, thereby bringing convenience to clinical diagnosis and prognostic research.

FULL TEXT

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1. Introduction

MicroRNAs (miRNAs) comprise a family of small single-stranded non-protein-coding RNAs (20–25 nucleotides) that have recently emerged as post-transcriptional regulators of gene expression [1, 2]. In addition, miRNAs play critical roles in various biological processes and genetic pathways. They are also involved in a number of human diseases including cancer [3–6], which have been hypothesized to play an important role in the control of tumor growth and eventually lead to more roles and progression [7]. miRNAs have been regarded as promising biomarkers for clinical diagnosis and treatment [8, 9]. However, miRNA detection is very challenging because of the intrinsic characteristics of these molecules, such as small size, sequence similarity among various members, and lack of common features. Currently, more than 2000 miRNAs have been annotated in humans (miRBase: http://www.mirbase.org/index.shtml), and computational predictions indicate that miRNAs may regulate the expression of 60% of all human protein-coding genes [10]. MiRNAs play a pivotal role in critical biological processes, including cellular growth, proliferation, and differentiation [11, 12]. A growing body of evidence has demonstrated the importance of miRNAs in managing chemotherapy efficacy in multiple human cancers, and they might also function as tumor suppressors and oncogenes. miRNAs have opened a new window to an important area of biology that was previously unexplored and also have important implications in human development and diseases. Thus, the rapid and sensitive quantification of miRNAs is of great importance to the understanding of their biological functions and clinical applications.

At present, the most conventional methods used for detecting miRNA include northern blot analysis [13, 14], stem-loop quantitative reverse transcription polymerase chain reaction (RT-qPCR) [15–17], and microarrays [18, 19]. However, these methods are limited by inaccurate quantification, slow and complicated operation, time-consuming process, and high instrument requirements. Recently, several isothermal amplification tests, e.g., primer extension [20], isothermal exponential amplification techniques such as strand displacement amplification [21–24], rolling circle

amplification (RCA) [25–27], nucleic acid sequence-based amplification [28], helicase-dependent amplification [29], recombinase polymerase amplification [30], and loop-mediated isothermal amplification (LAMP) [31–34] have been developed. These methods have been successfully tested on several techniques for miRNA detection and are more efficient than the typical PCR. Thus, they can be applied as alternative methods. Moreover, these methods have great potential for on-site, point-of-care, and in situ assay applications. Isothermal amplification techniques eliminate the need for temperature cycling for PCR, enhancing the PCR amplification yield. Among them, LAMP and RCA are the most commonly used isothermal amplification techniques.

LAMP is an isothermal method that was first described by Notomi et al. [35]. In a typical LAMP reaction, there are at least four primers: two inner and two outer primers. These primers were designed to recognize a set of six distinct sequences, displaced by the on the autocycling strand displacement activity DNA polymerase and generates a large amount of amplified products within 1 h; moreover, the method can be simplified by running at a constant temperature, eliminating the need for temperature cycling during amplification [34]. The mechanism of LAMP amplification reaction includes three steps: production of starting material, cycling amplification and elongation, and autocycling strand displacement. Therefore, the limit of detection and dynamic range of the nucleic acid will be highly useful depending on the nature of the bio-fluid tested from patients (e.g., whole blood, serum, or plasma) [36]. In the last decade, most studies determined whether LAMP with biosensor could distinguish between nucleic acid that differs by a single base mismatch.

We have previously developed a reverse transcription-based LAMP (RT-LAMP) to provide a highly selective and sensitive platform for miRNA [37]. In this work, we also used the LAMP reaction for miRNA detection, but reverse transcription process was replaced by ligation reaction to make the method simple and sensitive. Two stem-loop structures DNA (Linker A/B probes) were designed, which were hybridized with miRNA and linked by SplintR Ligase when the target miRNA appeared. The LAMP reaction was carried out by adding backward inner primer (BIP)/forward inner primer (FIP) and Bst 2.0 DNA polymerase. Compared with other self-assembly amplification methods, LAMP greatly improved the amplification efficiency. Finally, we added the double-stranded DNA fluorescent dye EvaGreen to detect the amplification product and achieve the quantification of the target miRNA, as shown in Scheme 1. The selectivity of this method was evaluated by testing miR-200a and other miRNAs from the miR-200 family. Our method can effectively distinguish miR-200a from other homologous family miRNAs with high sequence similarity. This method has also been successfully applied to quantitatively detect the amount of miR-200a in cell samples, indicating its clinical value. Therefore, this novel detection method could open a new path for the highly sensitive quantitative analysis of miRNA, thereby bringing convenience to clinical diagnosis and prognostic research.

[figure(s) omitted; refer to PDF]

2. Materials and Methods

2.1. Material and Apparatus

miRNA 1st Strand cDNA synthesis kit was purchased from Vazyme (Nanjing, China), and Bst 2.0 DNA polymerase and the corresponding isothermal buffer were purchased from New England Biolabs (USA); dNTPs and RNase-free water were all obtained from Takara (Dalian, China). All nucleic acid in this work including Linker A, Linker B, and inner primer (FIP and BIP) were synthesized and purified by Sangon Biotech (Shanghai, China). miRNAs were synthesized and purified by Genepharma (Shanghai, China). The sequences of all nucleic acids are listed in Table S1. The real-time fluorescence measurements of the LAMP reactions were proceeded on a StepOne (Applied Biosystems) real-time PCR instrument and had the following sequences.

2.2. Procedure of LAMP Assay

In a typical experiment, 9 μL reaction mixture containing 1 μL target miRNA, 1 μL each Linker A and B probes 1, 25 nM and 1 μL of 0.8 mM dNTPs, 1x mM in SplintR Ligation buffer was heated to 95°C for 5 min and chilled on ice for 2 min, then 1 μL 7 U of SplintR Ligase was added and incubated at 16°C for 30 min. The SplintR mixture solution (10 μL) was added into a total volume of 20 μL in 1x isothermal amplification buffer to form LAMP Mix composed of forward inner primer (FIP), backward inner primer (BIP) 80 nM, and 8 U of Bst 2.0 DNA polymerase. 1 × EvaGreen

dye was added to the reactions following primer annealing but prior to the addition. The reactions were analyzed using the Step OnePlus real-time PCR machine that was set up to incubate the samples for 60 cycles of two-step incubations: step 1: incubation at 61.5°C for 150 s and step 2: incubation at 61.5°C for 30 s (total incubation time of 3 min/cycle unless otherwise indicated). The resulting data were analyzed using the Step OnePlus analysis software to generate Ct (quantification cycle) values for each amplification, and Ct was redefined by multiplying Ct by 3 in this paper.

2.3. Cell Lysis and RNA Preparation

The human colon cancer cells (HT29) and human hepatoma cells (BEL7402) were gifts from Department of Pharmacology, College of Pharmaceutical Sciences, Zhejiang University. Cell lines were maintained according to instructions from the ATCC. HT29 and BEL7402 were collected and centrifuged at 3000 rpm for 5 min in culture medium, washed once with PBS buffer, and then spun down at 3000 rpm for 5 min. Total RNA was extracted from human colon cancer cells using miRNeasy Kit according to the manufacturer’s procedures. The sample of miR-200a in these cells was diluted and then analyzed with the proposed miRNA detection method.

3. Results and Discussion

3.1. Design Principle of this Method Based LAMP

This miRNA detection strategy contains two linker probes and two primers; both of them are from the typical LAMP reaction. The LAMP system, which consists of two hairpin DNA probes (Linker A and Linker B) are designed and constructed as a dumbbell DNA initiator to get an active amplicon. The Linker A/B probes have an overhang complementary to the half of target miRNA. Only when it is perfectly hybridized with miRNA, the ligation of these two probes would be carried out in the presence of SplintR Ligase to afford high specificity in identifying the mutations in target miRNA. The LAMP process would start with the coming of FIP/BIP primer where intact inner primers bind and extend. The fluorescent dye (1x EvaGreen) was added to the reactions following primer annealing with the addition of Bst 2.0 DNA polymerase. In the absence of miRNA or there is a mismatch between miRNA, no dumbbell-shaped amplicons structured product is obtained, therefore, precluding the LAMP amplification with merely giving a low and a late increase of the fluorescence signal. This miRNA-LAMP assay could get a high sensitivity and selectivity according to the real-time fluorescence curve and Ct value which were recorded in LAMP reaction.

3.2. Design of Linker A/B Probes

To explore the feasibility and more investigate of this method in practical use, we designed and compared two templates (MERS 1a and MERS 1b), which came from the original LAMP. This two Linker A/B probes extension mechanism of the miRNA-LAMP system were tested for miR-200a detection, which is well a known biomarker overexpressed in the luminal breast cancer (strongly downregulated during oncogenic EMT) [42]. The switch from loop structure is a key factor for the amplification mechanism, thus the design of Linker A/B probes is important for the detection. The real-time fluorescence intensity distribution curves were used to investigate the efficiency of Linker A/B probes from two templates. After we tested in parallel with different concentrations from 1 nM and 10 pM of target miR-200a, the probes from MERS 1b template showed high sensitivity and changed in the fluorescence intensity function of target concentration by real-time RT-PCR, as shown in (Figure 1).

[figure(s) omitted; refer to PDF]

3.3. Amplification Reaction Steps for Detection Method

To achieve a highly sensitive detection of miRNA, we optimized the miRNA-LAMP reaction by investigating different operation strategies. One-step reaction method where we put all the enzyme, target, and primers in one tube and then start the amplification directly were compared with two-step method (ligation and amplification). The quality of the Ct value for different concentrations of the target are shown in Figures 2(a)–2(c); the two-step method showed better performance in the amplification curve and enhanced sensitivity for miRNA detection.

[figure(s) omitted; refer to PDF]

3.4. Ligase Enzyme Selection

To achieve the highest-efficiency ligation between the Linker A/B probes mediated by ligase enzyme to form the dumbbell-shaped, the approach was optimized by testing three different types of ligase enzymes. SplintR Ligase,

Taq DNA Ligase, and T4 DNA Ligase were applied miRNA-LAMP detection. As shown in the (Figures 3(a)–3(d)), results showed that the SplintR Ligase demonstrated high efficiency compared with the other two enzymes (T4 DNA Ligase and Taq DNA Ligase), which ligated the two adjacent single-stranded DNA splinted fragments with high efficiency by a complementary miRNA strand.

[figure(s) omitted; refer to PDF]

3.5. Optimization for the miRNA-LAMP Assay

To establish the detection method and reduce the number of false-positive results, several parameters were systematically investigated for miRNA-LAMP detection. These parameters included the concentrations of Linker A/B, FIP and BIP primers, SplintR Ligase, Bst 2.0 DNA polymerase, and the reaction temperature. The concentration of Linker A/B led to an increase in the ΔCt value, which reached the maximum at 50 nM and then decreased gradually (Figure S1). The ΔCt value reached the maximum when primers FIP/BIP concentration is 800 nM and then decreased gradually (Figure S2). The ΔCt value also reached a maximum for a Bst 2.0 DNA polymerase amount of 8 U (Figure S3) and SplintR Ligase amount of 4 U (Figure S4). Furthermore, we investigate the LAMP amplification temperature ranging from 59°C to 65°C; the ΔCt values for each different amplification temperature were compared when the amplification temperature is 61.5°C and the results show lower ΔCt values from miRNA (Figure S5).

3.6. Selectivity

To evaluate the selectivity of this miRNA-LAMP method, interference assays were performed under identical conditions using other miRNA from miR-200 family, such as miR-200b, miR-200c, and miR-429, due to the high sequence homology (Table S1). As shown in Figure 4, the *Ct* value was measured from perfectly complementary targets miR-200a (1 pM) to other miR-200 family miRNAs (10 pM). The results suggested that high amplification and lowest Ct value for miR-200a target compared to other members of the family, which demonstrated that this approach for detection miRNA had high selectivity and sensitivity. Our miRNA assay can effectively discriminate between members of multiple closely related sequences of miRNAs from the miR-200 family.

[figure(s) omitted; refer to PDF]

3.7. Quantification of miRNA-LAMP Assay

Under the optimized conditions, different concentrations of miR-200a were analyzed by monitoring the changes in the *Ct* value. The real-time fluorescence intensity curves all exhibited a sigmoidal shape, and gradual increases of the *Ct* values were observed with decreased miR-200a concentrations from 100 fM to 10 nM. A fluorescence signal produced by a miR-200a concentration as low as 100 fM could be clearly discriminated from that of the blank control (Figure 5). The miR-200a ranged between 100 fM to 5 nM, with the correlation equation $Ct = -3.7485 \log C + 57.364$ ($R^2 = 0.9859$). The sensitivity of this assay compared favorably with previous efforts for miRNA detection, as summarized in (Table S2).

[figure(s) omitted; refer to PDF]

3.8. Assay of miRNA in Cell Samples

The proposed methods were successfully used to quantify the amount of miR-200a in the total RNA sample that was extracted from cells. Human colorectal cancer cells HT29 were used as positive control and human liver cancer cell BEL-7402 as a negative control (low expression miR-200a). We detected the miR-200a level from the total RNA extracted concentration not exceeding 250 ng; the results of miRNA-LAMP assay were in good agreement with those obtained from other stem-loop RT-PCR. miR-200a in two types of cells HT29 and BEL-7402 were detected. The results indicate that HT29 and BEL-7402 cells contain 73.3 pM and 6 fM of miR-200a in total RNA 415 ng and 316.8 ng.

4. Conclusion

In this section, a simple and efficient miRNA-LAMP detection method based on development self-assembly amplification to LAMP is established. In this method, the smart design of the two probes with the loop was specifically designed to form two different stem-loop structures before and after binding to the target miRNA. The sensitivity of the LAMP method is comparable to that of the self-assembly amplification method, but the upper limit of quantification of the miRNA-LAMP method is higher and showing a wider range of quantification, which is feasible

for the accurate detection of miRNAs down to the 100 fM level in real samples. Moreover, this proposed miRNA-LAMP assay does not require any modified or labeled DNA probes, and only one type of ligase enzyme with DNA polymerase is needed, which should significantly reduce the cost and simplify the experimental procedure. At the same time, the miRNA-LAMP-based miRNA detection method we just need to modify the complementary sequences of the probe and target miRNA can easily be extended to the detection of other small RNAs, including miRNA, so it has universal value. The versatile miRNA-LAMP miRNA assay not only remarkably simplifies the probe design for efficient LAMP amplification but also leads to high sensitivity and specificity.

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Determination of Methanol, Acetaldehyde, and Ethyl Acetate in Thousand Folds of Ethanol Sample by Headspace Gas Chromatography with Mass Spectrometry

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ABSTRACT (ENGLISH)

Alcohol beverages have been widely consumed in several parts of the world. In this study, volatile organic compounds in alcoholic beverages including acetaldehyde, ethyl acetate, methanol, and higher ethanol were investigated and evaluated using a headspace gas chromatograph equipped with a mass spectrometer. This study evaluated the suitability of the chromatographic system, linearity, limit of detection, and limit of quantification, accuracy, and precision of the single and simultaneous determination of acetaldehyde, ethyl acetate, and methanol in thousand folds of ethanol. Results showed that the acetaldehyde concentration in local beer samples and local manual product liqueur samples ranged from 4.65 to 13.22 mg/L and from 5.55 to 75.96 mg/L, respectively, but in local industrial product liqueur samples, acetaldehyde was not detected. Methanol was only detected in a few local beer samples and locally manually produced liqueur samples within low concentrations. Ethyl acetate was only detected in all local beer samples, but it was not present in local industrial product liqueur samples.

FULL TEXT

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1. Introduction

Ethanol level is important to consumers for the mouthfeel and flavor of alcoholic beverages. Beer, wine, and liqueur have ethanol levels ranging from 3 to 6% (v/v), 7 to 21% (v/v), and 20 to 50% (v/v), respectively [1, 2]. This compound is presented in alcoholic beverages as a consequence of the carbohydrates' fermentation by yeasts. Volatile flavor compounds were presented in the alcoholic beverages caused by distillation procedure [2, 3]. Among these compounds, acetaldehyde is one of the naturally occurring compounds that could be found in diverse beverages (such as alcoholic drinks and juices) and foods (such as dairy products and vegetables) as well as liqueurs, wines, and brandies [4]. This compound was a flavor enhancer that was added at low concentrations to provide a pleasant fruity bouquet [5, 6]. Acetaldehyde in the most of 18 craft beer samples was from 2.02 to 19.64 mg/L and in 8 industrial beer samples was from 2.91 to 13.28 mg/L [7]. In alcoholic beverages or drinks, besides acetaldehyde, methanol is also one of the naturally occurring compounds that can be found at various levels because methanol is a byproduct that was produced due to the degradation of pectin during liqueurs' fermentation [8]. Methanol in most of the 18 craft beer samples was from 4.52 to 11.13 mg/L and in 8 industrial beer samples was not detected [7]. In the case of ethyl acetate, this is the most frequent ester in liqueurs and it is produced by the acetic bacteria metabolism and yeast during the liqueur fermentation. One of the symptoms of liqueur spoilage is a high level of ethyl acetate. It is well known that the high level of ethyl acetate in liqueur is a factor conditioning liqueur quality [9]. Ethyl acetate in most of the 18 craft beer samples was from 13.30 to 42.33 mg/L and in 8 industrial beer samples was from 13.68 to 27.79 mg/L [7].

On the other hand, ethanol, methanol, acetaldehyde, and ethyl acetate are volatile compounds whose detection and quantification in alcoholic beverages can be used as a biological indicator to identify the origin of several kinds of alcoholic drinks because the volatile compounds which characterize the beverage type are diverse and originated from raw materials. And these volatile compounds are generated during production, maturation, and storage [10]. Ethanol, acetaldehyde, methanol, and ethyl acetate in alcoholic beverages were determined by several methods. Ana Catarina et al. determined acetaldehyde, ethyl acetate, and methanol in wine spirits, brandy, and grape marc spirits by the GC-FID method [11]. Acetaldehyde and methanol in alcoholic beverages were determined by GC-MS [12], GC-FID, and GC-TCD [13, 14]. Methanol and ethanol concentrations in alcoholic drinks were detected using GC-TCD [15] and HS-GC-MS [16]. These volatile compounds were simultaneously determined by GC-FID [17]. There are such methods for the simultaneous determination of methanol, acetaldehyde, and ethyl acetate in a high level of ethanol in alcoholic drinks. This method, which is proposed in the present study, enriches the scientific literature and has a practical, applied value. Thus, the simultaneous determination of methanol, acetaldehyde, and ethyl acetate in thousand folds of ethanol in alcoholic drinks by headspace gas chromatography with mass spectrometry is a necessary method to evaluate liqueur quality.

2. Materials and Methods

2.1. Chemicals

Methanol, acetaldehyde, ethyl acetate, ethanol standard, n-butanol internal standard, acetonitrile, and water were purchased from Merck, Darmstadt, Germany. All chemical standards used for the analysis were of analytical grade. Nitrogen, hydrogen, and helium were of extrapure grade 4.5 (Air Liquide, Ho Chi Minh City, Vietnam).

Methanol, acetaldehyde, ethyl acetate, ethanol, acetonitrile standard solutions, and n-butanol internal standard solution were prepared in water solvent and stored at −18°C. These solutions were sonicated for 5 min before use.

2.2. Sample Preparation and Equipment

5 mL of samples was poured into 20 mL of glass headspace vials, n-butanol was added as internal standard, and then, the vials were sealed with caps lined with a silicon PTFE membrane. The vial samples were incubated for 10 min at 70°C in order to facilitate the transfer of analytes to the sample's volatile fraction. All samples were analyzed by GC-MS Thermo Fisher Scientific ISQ 72008051 gas chromatography with a mass spectrometric detector equipped split/splitless inlets, and a headspace sampling system. For separation of impurities, 1 μL of the sample's volatile fraction in the headspace was injected into the GC inlet. A TG-WAXMS 30 m × 0.25 mm × 0.5 μm (polyethylene glycol) capillary column was used to separate the volatile fractions under a constant flow of helium 1.2 mL/min. The duration of the analysis was 8 minutes. For increased sensitivity, other method parameters were optimized. All experimental samples were analyzed in triplicate.

2.3. Validation of the Method of a Single Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol

Each of the acetaldehyde, methanol, and ethyl acetate standard solutions was prepared for calibration at 5 to 240 mg/L and 50 to 2400 mg/L of ethanol standard solution. Every single standard solution was determined in triplicate. All prepared standards were analyzed at optimized parameters by HS-GC-MS Thermo Fisher Scientific ISQ 72008051 gas chromatography. The validation method of single determination was determined by the limit of detection (LOD), the limit of quantification (LOQ), repeatability, and recovery.

The LOD is the smallest sample concentration at which the substance signal may be consistently recognized when compared to a blank run's baseline noise. For evaluating the detection limit, a signal-to-noise ratio of 3 : 1 is typically regarded adequate [18].(1)$LOQ=3.3\,LOD$.

Intra-assay and inter-assay precision levels were assessed by analyzing the quality control samples. Intra-assay was assessed using a minimum of 9 determinations at 3 concentrations/6 replicates each for the procedure. Inter-assay precision levels were evaluated in three concentrations at 5, 80, and 240 mg/L for acetaldehyde, ethyl acetate, and methanol and 50, 800, and 2400 mg/L for ethanol for six consecutive days. Intra-assay and inter-assay precision was evaluated in terms of relative standard deviation (% RSD).

Acetaldehyde, methanol, ethyl acetate, and ethanol recoveries were measured based on accuracy at 5, 80, and 240 mg/L for acetaldehyde, ethyl acetate, methanol, and 50, 800, and 2400 mg/L for ethanol.(2)$\%\,Recovery=\frac{Actual\,amount}{Theoretical\,amount}\times100$.

2.4. Optimization of HS-GC-MS Parameters for the Simultaneous Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol

The experiment was carried out by varying the incubation temperature of headspace at 40°C, 50°C, 60°C, 70°C, and 80°C; incubation time of the headspace at 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min; inlet temperature at 150°C, 170°C, 190°C, 210°C, 230°C, and 250°C; column temperature program at 38°C and increased to 50°C with various heating rate of 3°C/min, 10°C/min, 15°C/min, and 20°C/min, to 25°C/min, where it was held for 1 min and then 50°C and increased to 170°C at 35°C/min; detector temperature at 150°C, 170°C, 190°C, 210°C, 230°C, and 250°C; carrier gas flow rate was surveyed at 1.0 mL/min, 1.2 mL/min, 1.4 mL/min, 1.6 mL/min, 1.8 mL/min, and 2.0 mL/min; split ratio at 1 : 10, 1 : 20, 1 : 40, 1 : 60, and 1 : 100. The chromatogram was recorded, and the area of the peak was calculated to choose the optimal condition.

2.5. Validation of the Method for Simultaneous Determination of Acetaldehyde, Methanol, Ethyl Acetate, and Ethanol through HS-GC-MS by Using Optimized Parameters

For calibration, the mixer of acetaldehyde, methanol, ethyl acetate, and ethanol standards at 5 to 240 mg/L of acetaldehyde, methanol, and ethyl acetate standard solutions and 50 to 2400 mg/L of ethanol standard solution were prepared. Every mixer standard solution was determined in triplicate. All prepared samples were analyzed at optimized parameters by HS-GC-MS Thermo Fisher Scientific ISQ 72008051 gas chromatography. The validated method simultaneous determination was determined by the selectivity factor (α), the limit of detection (LOD), the limit of quantification (LOQ), repeatability, and recovery.

2.6. Application of the Optimized Method

The optimized method was applied to 5 beer samples, 5 industrial liqueur samples, and 5 manual liqueur samples (Table 1) purchased from shops in Ho Chi Minh City, Vietnam. All samples were stored at room temperature (21°C) and protected from light. All samples were added with internal standard prior to the analysis.

Table 1

Sample name, number of samples, and number of individuals for each sample.

No.	Sample	Production year	Alcohol content of manufacturer’s data (%, v/v)	No. of the analysis sample	No. of individuals per sample
Beer samples					
1	BBV	2022	5	20	30
2	BHD	2022	5	20	30
3	BLR	2022	5	20	30
4	BSG	2022	5	20	30
5	BTG	2022	5	20	30
–					
Industrial liqueur samples					
6	VKNH	2022	30	20	30
7	RDBT	2022	30	20	30
8	RNUL	2022	40	20	30
9	VKM	2022	30	20	30

10	VK G	2022	30	20	30
–					
Manual liqueur samples					
11	TCH Đ1	2022	40	20	30
12	TCH Đ2	2022	25	20	30
13	TCH H1	2022	30	20	30
14	TCH H2	2022	30	20	30
15	TCH V	2022	30	20	30

3. Results and Discussion

3.1. Single Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol by HS-GC-MS

The linearity of the headspace gas chromatography mass spectrometry method was determined at seventh concentration levels ranging from 5 to 240 mg/L of acetaldehyde, methanol, and ethyl acetate standard solutions and from 50 to 2400 mg/L of ethanol standard solution. Each of these standard solutions was incubated in the headspace at 70°C for 10 minutes, and then, the evaporative part was injected into the chromatographic system ($n = 3$). The peak area and retention time of acetaldehyde, methanol, ethyl acetate standard, and internal standard were recorded, and the mean values of the peak area ratio were plotted against the concentrations to obtain the calibration curves. Linear regression of acetaldehyde, methanol, ethyl acetate, and ethanol were $y = (0.0164 \pm 0.0008)\ x + 0.0151 \pm 0.0098$ (mg/L),$y = (0.0025 \pm 0.00004)\ x + 0.0008 \pm 0.0003$ (mg/L), $y = (0.0298 \pm 0.0064)\ x + 0.0167 \pm 0.0138$ (mg/L), and $y = (0.0033 \pm 0.0002)\ x + 0.0501 \pm 0.0135$ (mg/L), respectively (Figure 1). The good coefficients of acetaldehyde, methanol, ethyl acetate, and ethanol were 0.9998, 0.9998, 0.9999, and 0.9999, respectively.

[figure(s) omitted; refer to PDF]

The results of validating the determination of acetaldehyde, methanol, ethyl acetate, and ethanol are shown in Table 2. As presented in Table 2, the limit of detection (LOD) and limit of quantification (LOQ) were 0.55 mg/L and 1.83 mg/L for ethanol, 0.63 mg/L and 2.09 mg/L for methanol, 0.52 mg/L and 1.72 mg/L for acetaldehyde, and 0.51 mg/L and 1.70 mg/L for ethyl acetate, respectively. The LOD in this research was better than that in other documents. Helena et al. presented a LOD of 0.85 mg/L for acetaldehyde and 0.75 mg/L for acetone, ethanol, and methanol [18]. Pontes et al. showed that the LOD was 0.87 mg/L for methanol, 0.51 mg/L for acetaldehyde, and 0.82 mg/L for ethyl acetate [19]. The method in this study has good accuracy, precision, linearity, and efficiency for the quantification of acetaldehyde, methanol, ethyl acetate, and ethanol.

Table 2

The results of validating the determination of acetaldehyde, methanol, ethyl acetate, and ethanol.

The validated values	Ethanol	Methanol	Acetaldehyde	Ethyl acetate
Linear range (mg/L)	5–2500	5–240	5–240	5–240
–				
Linear relationship (r^2)	0.9999	0.9998	0.9998	0.9999
–				
Limit of detection (mg/L)	0.55	0.63	0.52	0.51
–				
Limit of quantification (mg/L)	1.83	2.09	1.72	1.70
–				
Relative standard deviation (%RSD) at:				
(1) Low concentration	50 mg/L: 2.11	5 mg/L: 2.53	5 mg/L: 2.78	5 mg/L: 2.15
(2) Middle concentration	800 mg/L: 0.98	80 mg/L: 1.43	80 mg/L: 2.13	80 mg/L: 2.38
(3) High concentration	2400 mg/L: 0.42	240 mg/L: 1.70	240 mg/L: 2.93	240 mg/L: 2.34
–				
Reproducibility (%RSD) at:				
(1) Low concentration	50 mg/L: 3.56	5 mg/L: 1.15	5 mg/L: 2.42	5 mg/L: 2.78
(2) Middle concentration	800 mg/L: 1.31	80 mg/L: 1.74	80 mg/L: 2.68	80 mg/L: 2.75
(3) High concentration	2400 mg/L: 1.00	240 mg/L: 1.78	240 mg/L: 1.32	240 mg/L: 2.94
–				
Assay recovery (%H) at:				
(1) Low concentration	50 mg/L: 94.14–104.11	5 mg/L: 96.28–103.25	5 mg/L: 95.89–106.15	5 mg/L: 97.68–101.95
(2) Middle concentration	800 mg/L: 98.42–101.43	40 mg/L: 98.94–103.88	80 mg/L: 95.49–103.35	80 mg/L: 98.8–104.72
(3) High concentration	2400 mg/L: 98.76–101.64	240 mg/L: 97.42–102.00	240 mg/L: 97.38–101.13	240 mg/L: 97.87–101.94

3.2. Simultaneous Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol by HS-GC-MS

3.2.1. The Optimized Parameters

In HS-GC-MS, the samples must be held in the headspace system at high temperatures and a reproducible equilibrium must be established between a solution sample and a headspace. The chromatographic process depends on the analytical conditions including detector temperature, inlet temperature, column temperature program, flow rate, split ratio, headspace temperature, and incubation time of the headspace. Therefore, the study of headspace conditions and GC-MS parameters in the multicomponent analysis is necessary for the HS-GC-MS method.

3.2.2. Optimization of Incubation Temperature of Headspace

In the headspace technique, the sample was put into a sealed vial which was heated to an essential temperature for a period of time. More volatile compounds will tend to move into the headspace above the solution sample. The incubation temperature of headspace was one of the factors affecting the volatile ability of compounds. In this study, after some trials of different headspace temperatures for 10 min, 80°C was selected as a headspace temperature because of the great peak surface. The results are shown in Figure 2.

[figure(s) omitted; refer to PDF]

3.2.3. Optimization of Incubation Time of the Headspace

Solvent-vapor equilibria in the headspace play an important role in the headspace analysis. The more volatile compounds can be evaporated into the headspace, and more volatile compounds will be injected into the column of the GC. If the sample is incubated for a too short time, less of the volatile compounds will be in the headspace, which can affect the overall peak area. In this study, the incubation time of the headspace was in the range of 5–30 minutes. The results shown in Figure 3 show that those equilibration time periods longer than 10 min do not yield a significant increase in the peak area. In the case of ethanol, methanol, ethyl acetate, and acetaldehyde, the incubation time longer than 20 minutes shows a high peak area. It is of interest to note that the incubation time of the samples was 10 minutes.

[figure(s) omitted; refer to PDF]

3.2.4. Optimization of Inlet Temperature

The inlet was to set an optimal temperature which helped the analytes quickly vaporize. It was not only too high to cause the breakdown of these compounds but also not too low to decrease the sensitivity due to the compounds which did not vaporize. Inlet temperature was not adjusted higher or lower to optimize the performance in each different analysis. In our study, the inlet temperature was set in the range from 150 to 250°C. The results are presented in Figure 4. Figure 4 presents that the peak area of ethanol, methanol, ethyl acetate, and acetaldehyde in the chromatogram decreased with increasing temperature in the inlet. The optimal inlet temperature was set at 150°C.

[figure(s) omitted; refer to PDF]

3.2.5. Optimization of GC Oven Temperature

The programmed temperature was related to the analysis which was performed on the instrument. For acetaldehyde, ethyl acetate, methanol, and ethanol, the program can be operated at an inlet temperature of 150°C, with the column program starting from 38°C and increased to 50°C at several heating rates from 3°C/min to 25°C/min, where it was held for 1 min and then from 50°C and increased to 170°C at 35°C/min. In these chromatograms, the peaks of ethyl acetate and methanol overlapped at heating rates of 10, 15, 20, and 25°C/min. Otherwise, the peak of acetaldehyde, ethyl acetate, methanol, and ethanol can be separated at a heating rate of 3°C/min. So, the optimization of temperature for the column program to operate was an inlet temperature of 150°C with column program from 38°C and increased to 50°C at 3°C/min, where it was held for 1 min and then 50°C and increased to 170°C at 35°C/min. This temperature column program was set for the simultaneous determination of methanol, acetaldehyde, ethyl acetate, and ethanol in alcohol. The chromatogram of this temperature column program is shown in Figure 5.

[figure(s) omitted; refer to PDF]

Table 3 shows the chromatographic system data of simultaneous analysis of acetaldehyde, ethyl acetate, methanol, ethanol, and butanol at heating rates of 3, 5, 10, 15, 20, and 25°C/min. The results showed that when the heating rate increased, the retention time of the analytes decreased. It was because gas chromatography relies on the evaporation temperature of the analytes to separate the analytes contained in the sample background. Therefore, when the temperature increases rapidly, the analytes will be eluted out of the column faster, which causes the peak of analytes, which takes similar retention time to overlap each other. It is easy to completely overlap the low-intensity peaks. So, the chromatographic spectrum will only show high-intensity peaks.

Table 3

Chromatographic system data: retention times, selectivity factor, peak area, resolution, symmetry, and theoretical plates at several heating rates.

Compounds	Heating rate (°C/min)	Retention time t_R (min)	Peak area	Symmetry	Theoretical plates (N)	Selectivity factor (α)	Resolution (Rs)
Acetaldehyde	3	1.81	132912975	1.08	14560	4.14	26.67
5	1.81	141684515	1.00	20967	3.90	26.91	10
1.79	131957565	1.17	14240	3.78	19.43	15	1.78
148895708	1.08	10346	3.21	17.07	20	1.78	143766294
1.00	14082	2.81	17.57	25	1.76	150403601	1.17
10115	2.34	12.67	-				
Ethyl acetate	3	3.41	401766478	1.00	51680	1.07	2.55
5	3.29	401137946	1.17	48107	1.36	12.00	10
3.15	364177594	0.92	24806	1.30	7.86	15	3.06
412759798	1.22	23409	1.25	6.71	20	3.01	383032531
0.96	22650	1.22	6.00	25	2.71	426593713	1.22
18360	1.18	4.29	-				
Methanol	3	3.55	290731692	1.13	80656	1.29	11.00
–							

Ethanol	3	4.21	29073 1692	0.96	57875	2.31	54.29
5	4.01	210622985	0.85	71467	1.96	43.33	10
3.70	169468067	1.08	60844	1.78	33.82	15	3.53
18774705 7	0.92	55382	1.72	30.36	20	3.43	142977205
0.92	52288	1.69	29.09	25	3.01	135532318	0.92
40267	1.74	26.36	-				
Butanol	3	8.01	89132 249	0.92	209502	—	—
5	6.61	87923903	0.82	19418 7	—	—	10
5.56	20142136	0.82	19784 7	—	—	15	5.20
22625365	1.00	173059	—	—	20	5.03	22851934
0.82	161926	—	—	25	4.46	22713765	1.22

3.2.6. Optimization of Detector Temperature

After the volatile compounds moved the length of the GC column, they were entered into the mass spectrometer and were fragmented into an ion by an electron ionization technique. In the electron ionization technique, an electron that was produced by a filament was accelerated and knocks an electron out of the molecule to produce a molecular ion. These molecular ions were detected by an electron multiplier, which essentially turned the ionized mass fragment into an electrical signal that was then detected. In this study, the detector temperature was set at 150, 170, 190, 210, 230, and 250°C. The effect of the detector temperature on the peak areas of ethanol, methanol, ethyl acetate, and acetaldehyde is shown in Figure 6. The results showed that at 250°C, the peak area of ethanol, methanol, ethyl acetate, and acetaldehyde was the highest. The optimal detector temperature was set at 250°C.

[figure(s) omitted; refer to PDF]

3.2.7. Optimization of Mobile Phase Flow Rate

The mobile phase flow rate changed from 1.0 to 2.0 mL/min. The results showed that the flow rate increased and the retention time of the analytes decreased. It was due to the fact that the carrier gas plays the role of the mobile phase eluting the analytes out of the column, the analytes move out of the column as soon as possible. The change in the mobile phase flow rate causes the change of peak areas of ethanol, methanol, ethyl acetate, and acetaldehyde (Table 3). This may be caused by the decrease in ionization efficiency due to the nonstoichiometric ratio of air and the dilution of gas eluted from the column. Thus, the mobile phase flow rate of 1.2 mL/min was selected as the optimal value (Table 4).

Table 4

Chromatographic system data: retention times, selectivity factor, peak area, resolution, symmetry, and theoretical

plates at several mobile phase flow rates.

Compounds	Mobile phase flow rate (mL/min)	Retention time t_R (min)	Peak area	Symmetry	Theoretical plates (N)	Selectivity factor (α)	Resolution (Rs)
Acetaldehyde	1.0	1.98	132912975	1.08	9801	4.23	24.43
1.2	1.81	263343533	1.06	10625	4.14	21.33	1.4
1.68	231957565	1.04	12544	4.38	21.71	1.6	1.58
248895708	1.08	11095	4.25	22.00	-		
Ethyl acetate	1.0	3.69	40166478	0.96	60516	1.07	2.00
1.2	3.41	562827701	1.02	29070	1.07	10.15	1.4
3.20	574141983	1.08	25600	1.38	54.29	1.6	3.01
588579335	1.04	29584	1.39	9.73	1.8	2.86	575987017
1.02	20449	1.39	9.33	2.0	2.73	549110283	1.08
18632	1.39	9.07	-				
Methanol	1.0	3.84	20731692	1.04	94372	1.29	11.00
1.2	3.55	43102960	1.00	56011	1.29	10.15	·
Ethanol	1.0	4.54	290731692	1.08	51529	2.17	51.86
1.2	4.21	746495376	1.04	57875	2.31	54.29	1.4
3.95	484352051	1.13	39006	2.44	55.86	1.6	3.74

451993075	1.00	34969	2.53	53.20	1.8	3.56	485375812
0.96	41383	2.62	62.46	2.0	3.41	312404571	0.98
37969	2.70	63.38	-				
Butanol	1.0	8.17	89132249	1.13	296661	—	—
1.2	8.01	205425091	1.04	209502	—	—	1.4
7.86	205427572	1.01	274576	—	—	1.6	7.73
204673487	1.04	195111	—	—	1.8	7.62	212624219
1.06	258064	—	—	2.0	7.53	20666047	0.95

3.2.8. Optimization of Slip Ratio

One of the substantial parameters affecting method sensitivity was the split ratio. Increasing the split ratio leads to a decrease in the amounts of analytes introduced into the column. Therefore, the splitless injection technique will be the highest sensitivity. In the case of the headspace technique, ethanol, methanol, ethyl acetate, and acetaldehyde were evaporated into the headspace upper of the solution, so that the impurity compounds could be removed. In an alcohol sample, the ethanol amount was steadily higher than other compounds. Injection in the splitless mode or at low split ratios may potentially lead to problems with the methanol peak which can be overlapped by a large ethanol peak, analyte peak shape, and efficiency of resolution. Split ratios were studied at 1∶10, 1∶20, 1∶40, 1∶60, and 1∶100. Figure 7 shows the effect of the split ratio on the peak area of ethanol, methanol, ethyl acetate, and acetaldehyde. At a split ratio 1∶10, the peak area of these analytical compounds was large and this ratio was set at the optimal parameter.

[figure(s) omitted; refer to PDF]

3.2.9. System Suitability

The mixture of ethanol, methanol, ethyl acetate, and acetaldehyde was injected into HS-GC-MS in six replications under the optimal conditions mentioned above. The peak width, retention time, resolution, symmetry, and theoretical plates were recorded to assess the suitability of the analytical instrumentation conditions. The results in Table 5 show the theoretical plates >2000, resolution >1.5, symmetry in the range 0.9–1.1, and the relative standard deviation of retention time <2%. These system suitability parameters were obtained as acceptance criteria from the International Conference on Harmonization (ICH) [20, 21]. The method was suitable for the simultaneous analysis of ethanol, methanol, acetaldehyde, and ethyl acetate.

Table 5

System suitability.

		Peak width	Retention time t_R (min)	Resolution (Rs)	Symmetry	Theoretical plates (N)
Acetaldehyde	1	0.08	1.82	17.71	0.92	8252
2	0.09	1.82	15.94	0.90	6522	3
0.08	1.81	17.82	0.89	8162	4	0.09
1.82	15.94	0.94	6521	5	0.09	1.82
15.94	0.96	6521	6	0.08	1.81	17.78
0.97	8190	-				
Ethyl acetate	1	0.10	3.41	1.61	1.00	18613
2	0.11	3.41	1.53	1.02	15386	3
0.10	3.41	1.71	1.04	18613	4	0.11
3.41	1.53	1.06	15384	5	0.11	3.41
1.53	1.04	15384	6	0.10	3.41	1.65
1.03	18605	-				
Methanol	1	0.08	3.56	5.96	1.08	31614
2	0.08	3.56	6.55	1.11	31621	3
0.07	3.56	6.56	1.21	41287	4	0.08
3.56	5.96	1.06	31613	5	0.08	3.56
5.70	1.04	31613	6	0.07	3.55	6.62
1.07	41151	-				
Ethanol	1	0.14	4.21	37.98	1.07	14479
2	0.12	4.21	39.98	1.07	19709	3

0.13	4.21	39.98	1.04	16792	4	0.14
4.21	37.98	1.03	14479	5	0.15	4.21
34.53	1.09	12613	6	0.13	4.21	39.98

3.2.10. Optimized Parameters

Headspace injector conditions include the following:

(i) Incubation temperature of headspace: 80°C

(ii) The incubation time of the headspace: 10 min

Chromatographic conditions include the following:

(i) Column: TG-WAXMS 30 m × 0.25 mm × 0.5 μm (polyethylene glycol)

(ii) Detector temperature: 250°C

(iii) Inlet temperature: 150°C

(iv) GC oven temperature: 38°C and increased to 50°C at 3°C/min, where it was held for 1 min and then 50°C and increased to 170°C at 35°C/min

(v) Injector volume: 400 μL

(vi) Slipt ratio: 1 : 10

(vii) Mobile phase flow rate: 1.2 mL/min

3.3. Validation of the Method of Simultaneous Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol by HS-GC-MS

3.3.1. Linear Regression

The linearity of simultaneous determination of methanol, acetaldehyde, ethyl acetate, and ethanol in the headspace gas chromatography mass spectrometry method was determined at seven concentration levels ranging from 5 to 240 mg/L of acetaldehyde, methanol, and ethyl acetate standard solutions and from 50 to 2400 mg/L of ethanol standard solution. Each of these standard solutions was incubated in the headspace at 70°C for 10 minutes, and then, the evaporative part was injected into the chromatographic system ($n = 3$). Recording the peak area of acetaldehyde, methanol, ethyl acetate, ethanol standard, and n-butanol internal standard, the mean values of the peak area ratio was plotted against the concentrations to obtain the calibration curves. Linear regression and good coefficients of acetaldehyde, methanol, ethyl acetate, and ethanol are shown in Table 6.

Table 6

Linear regression of acetaldehyde, ethyl acetate, methanol, and ethanol.

Compound	Concentration range (mg/L)	Linear regression	Linear relationship (r^2)
Acetaldehyde	5–240	$y = (0.01360 \pm 0.00093)x + (0.00501 \pm 0.00036)$	0.9999
Ethyl acetate	5–240	$y = (0.0790 \pm 0.00568)x - (0.02641 \pm 0.00053)$	0.9998
Methanol	5–240	$y = (0.00125 \pm 0.00006)x - (0.00043 \pm 0.00011)$	0.9998
Ethanol	50–2400	$y = (0.00363 \pm 0.00015)x - (0.01855 \pm 0.00053)$	0.9999

Table 5 shows that the concentration and peak area of acetaldehyde, ethyl acetate, and methanol in the concentration range from 5 to 240 mg/L and ethanol in the concentration range from 50 to 2400 mg/L correlated with linear relationship $r^2 > 0.999$.

3.3.2. Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification of the method are presented in Table 7. Table 7 presents the results obtained from the simultaneous determination of acetaldehyde, methanol, ethyl acetate, and ethanol in alcohol samples using the proposed method.

Table 7

Limit of detection and limit of quantification of the HS-GC-MS method.

	Acetaldehyde	Ethyl acetate	Methanol	Ethanol
Limit of detection (mg/L)	0.52	0.51	0.72	0.61
Limit of quantification (mg/L)	1.74	1.72	2.41	2.03

3.3.3. Repeatability

Intra-assay and inter-assay precision levels were assessed by analyzing the quality control samples. Intra-assay was assessed using a minimum of 9 determinations at 3 concentrations/6 replicates each for the procedure. Inter-assay precision levels were evaluated in three concentrations at 5, 80, and 240 mg/L for acetaldehyde, ethyl acetate, and methanol and 50, 800, and 2400 mg/L for ethanol for six consecutive days. Intra-assay and inter-assay precisions were evaluated in terms of relative standard deviation (% RSD). The RSD of intra-assay and inter-assay precisions in Table 8 was less than 4% at three concentrations of acetaldehyde, methanol, ethyl acetate, and ethanol.

Table 8

The repeatability of the HS-GC-MS method.

Compound	Acetaldehyde			Ethyl acetate			Methanol			Ethanol		
Concentration (mg/L)	5	80	240	5	80	240	5	80	240	50	800	2400
–												
Intra-day precision of the method												
RSD (%)	1.88	1.77	1.00	1.45	2.39	0.88	2.96	1.77	0.64	2.37	1.69	0.64
–												
Inter-day precision of the method												
RSD (%)	2.24	1.29	0.72	2.60	1.23	0.46	1.28	2.82	0.96	1.20	1.94	1.30

3.3.4. Recovery

Acetaldehyde, methanol, ethyl acetate, and ethanol recovery were measured based on accuracy at 5, 80, and 240 mg/L for acetaldehyde, ethyl acetate, and methanol and 50, 800, and 2400 mg/L for ethanol. Recovery of acetaldehyde, methanol, ethyl acetate, and ethanol was found to be 95–102%, 97–103%, 98–105%, and 99–107%, respectively (Table 9).

Table 9

Recovery of the HS-GC-MS method.

Concentration level	% recovery			
Acetaldehyde	Ethyl acetate	Methanol	Ethanol	Low concentration
94.73–101.39	96.65–102.94	100.7–104.21	103.79–106.79	Middle concentration
98.45–101.36	99.02–102.31	97.85–104.68	99.19–103.63	High concentration

The results showed that the limit of detection, the limit of quantification, linear range, repeatability, and recovery efficiency of the simultaneous method were good, meeting the requirements for method validation of AOAC by GC-MS equipment.

3.3.5. Advantages and Disadvantages of Simultaneous Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol by HS-GC-MS

(i) Saving analysis time: By only one injection, the simultaneous concentration of methanol, acetaldehyde, ethyl acetate, and ethanol could be determined in samples. The total time required for the chromatographic analysis was 9 min. Several authors have developed GC methods for the simultaneous determination of methanol, acetaldehyde, ethyl acetate, and ethanol. One comparable study was conducted by Schlatter et al. who developed and validated a method for the simultaneous determination of acetaldehyde, methanol, acetone, and ethanol. The total time required for the chromatographic analysis was 15 min, which was 1.5 fold higher than our method [22].

(ii) Cost savings: By only a single injection, the concentration of methanol, acetaldehyde, ethyl acetate, and ethanol in the samples can be determined simultaneously, so the cost of the analysis is smaller than in the case of individual substances.

(iii) Minimizing errors in the analysis process: In the simultaneous analysis process, the steps of the analysis were minimized compared to the single analysis process, thereby limiting the errors arising from the manipulation process.

(iv) The analytes in the sample have different concentrations. So, it is difficult to process the samples, especially with trace concentration. To minimize this drawback, a standard addition method was performed to decrease the difference in concentration between the analytes.

3.3.6. Application of Simultaneous Determination of Methanol, Acetaldehyde, Ethyl Acetate, and Ethanol in Alcohol by HS-GC-MS

Because the ethanol concentration in the alcohol samples was at 5–40%, the samples should be diluted to analyze at the appropriate ratio. Meanwhile, methanol, acetaldehyde, and ethyl acetate concentrations were lower than ethanol concentrations. Therefore, the dilution samples have done a standard addition at 5 mg/L for each methanol, acetaldehyde, and ethyl acetate, and an internal standard was added into the dilution sample. Table 10 presents the actual sample analysis results.

Table 10

Concentrations of methanol, acetaldehyde, ethyl acetate, and ethanol in alcohol samples in Ho Chi Minh City.

No		Samples	Acetaldehyde (mg/L)	Ethyl acetate (mg/L)	Methanol (mg/L)	Ethanol (%)
1	Local beer samples	BBV	9.88	33.53	0.63	4.53
2	BHD	4.65	41.36	n/d	4.80	3

BLR	13.22	27.53	1.43	4.51	4	BSG
7.59	8.83	n/d	5.86	5	BTG	6.89
35.41	n/d	5.46	-			
6	Local liqueur samples (industrial products)	VKNH	n/d	n/d	n/d	33.07
7	RDBT	n/d	n/d	n/d	33.24	8
RNUL	46.14	40.03	n/d	39.60	9	VKM
n/d	n/d	n/d	29.96	10	VKG	n/d
n/d	n/d	29.57	-			
11	Local liqueur samples (manual products)	TCHD1	39.80	n/d	1.77	37.77
12	TCHD2	6.95	n/d	n/d	25.68	13
TCHH1	5.55	4.57	n/d	32.41	14	TCHH2
8.83	32.00	3.69	29.21	15	TCHV	75.96

Note. n/d: not detected (below detection limit).

Ethanol concentration in liqueur product samples varies for each product group. Ethanol concentration in beer was in a range from 4.5% to 5.9%; meanwhile, the ethanol concentration in local liqueur samples (industrial products and manual products) was in a range from 25.7% to 39.6%. These results were in accordance with the declaration of the manufacturers. Most of the beer samples did not contain methanol or contained a little methanol level. In contrast, the content of ethyl acetate in beer samples was high, in the range of 30–40 mg/L. Meanwhile, the content of acetaldehyde ranged from 4.6 to 13.2 mg/L. Beer was produced through fermentation and then filtration without distillation. So, beer samples have a lot of impurities in them, such as acetaldehyde and ethyl acetate.

Industrial liqueur products have no or contain very little methanol, acetaldehyde, and ethyl acetate (except for RNUL products which contain above 40 mg/L of acetaldehyde and ethyl acetate). Most industrial liqueur products were produced using multistage distillation towers to distillate and help separate and decrease impurities in the liqueur. In the group of manual liqueur products, the ethanol concentration is from 25.6 to 37.8%, which depends on the needs of each product. Most of the manual liqueur product samples contained acetaldehyde, but its significant difference lies in the levels of different production facilities. In contrast, ethyl acetate and methanol concentrations did not present or were found very little in manual liqueur products. The reason is that all local artisanal distilleries use simple or self-designed distillation equipment. So, the process of separating ethanol from impurities does not completely depend on the system and conditions of the distillation process. Therefore, the results in the group of manual liqueur products are significantly different in impurity concentration between production facilities. The

methanol, acetaldehyde, and ethyl acetate concentrations in these liqueur products were less than these concentrations in several documented previous studies. According to Kokkinakis et al., the concentrations of acetaldehyde, ethyl acetate, and methanol in bottled spirits and in-bulk spirits were 297.58 mg/L, 429.16 mg/L, and 698.02 mg/L and 199.75 mg/L, 1067.66 mg/L, and 781.20 mg/L, respectively [23]. The methanol and acetaldehyde contents of fermented plant beverages in Thailand were less than 29 mg/L and 45 mg/L, respectively [24].

4. Conclusions

This study was taken to analyze the simultaneous concentration of acetaldehyde, methanol, ethyl acetate, and ethanol in alcohol, which are most commonly consumed by the habitants of Ho Chi Minh City, Vietnam. The HS-GC-MS parameters for simultaneous determination of these volatile compounds were optimized. The simultaneous method was validated. In beer samples, there were impurities such as acetaldehyde and ethyl acetate because the production process did not go through distillation. In industrial liqueur products, there is very little methanol, acetaldehyde, and ethyl acetate because multistage distillation towers were used to distillate to help separate and decrease impurities in the liqueur. For manual liqueur products, there are still impurities such as methanol and acetaldehyde because simple or self-designed distillation equipment was used to separate impurities during the production process.

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DETAILS

Subject:	Mass spectrometry; Acetaldehyde; Fermentation; Alcoholic beverages; Quality standards; Methanol; Ethanol; Gas chromatography; Liqueurs; Alcohol; Scientific imaging; Volatile organic compounds--VOCs; Chromatography; Beer; Beverages; Ethyl acetate; Low concentrations
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(6) Peer-review manipulation

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[1] Z. Guo, R. Han, Y. Li, Y. Zhu, B. Zhang, H. Zhang, "Mo-Si-B Alloy Formed by Optional Laser Melting Process,"

International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/4996265, 2022.

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Retracted: Application of Particle Swarm Algorithm in Nanoscale Damage Detection and Identification of Steel Structure

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[1] Y. Zhang, "Application of Particle Swarm Algorithm in Nanoscale Damage Detection and Identification of Steel Structure," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/4300840, 2022.

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Retracted: Stability Analysis and Construction Parameter Optimization of Tunnels in the Fractured Zone of Faults

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References

[1] B. Huang, H. Chen, C. Duan, W. Li, "Stability Analysis and Construction Parameter Optimization of Tunnels in the Fractured Zone of Faults," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/2211499, 2022.

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Document 30 of 79

Retracted: Application of Nanometer Heavy-duty Coating in the Optimization of Process Parameters for Power Generation Machinery

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[1] W. Long, "Application of Nanometer Heavy-duty Coating in the Optimization of Process Parameters for Power Generation Machinery," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5600230, 2022.

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Document 31 of 79

Retracted: Blasting Law of Liquid CO_2 Phase Change in Coal Mine Based on Numerical Simulation

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[1] B. Fang, "Blasting Law of Liquid CO 2 Phase Change in Coal Mine Based on Numerical Simulation," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6866925, 2022.

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Retracted: A Systematic Study on the Extraction and Image Reproduction of Ceramic Sculpture Artworks

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Retracted: Application of Carbon Fiber Cement-Based Composites in Improving Construction Durability

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[1] X. Xu, "Application of Carbon Fiber Cement-Based Composites in Improving Construction Durability," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/2323534, 2022.

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Retracted: Effect of Nano Titanium Oxide with Different Surface Treatments on Color Stability of Red-Tinted Silicone Rubber

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Assessment of Drinking Water Quality in Urban Water Supply Systems: The Case of Hawassa City, Ethiopia

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ABSTRACT (ENGLISH)

In many developing countries, such as Ethiopia, water quality and the risk of water-related diseases are serious public health issues. The present study goal was to assess the drinking water quality from source to household tap water. To characterize and analyze drinking water quality parameters, 21 water samples were collected, of which 11 water samples were collected from sources (spring, borehole, and river), 4 from service reservoirs, and 6 from tap water. The mean values of the parameters were as follows: total dissolved solids (TDS) (142.79 mg/L), temperature (22.08°C), turbidity (9.49 NTU), electrical conductivity (EC) (250.14° μS/cm), pH (7.45 mg/L), fluoride (1.15 mg/L),

nitrate (NO_3^-) (2.91 mg/L), total hardness (TH) (57.45 mg/L), calcium (41.7 6 mg/l), magnesium (10.74 mg/L), phosphate (0.44 mg/L), sulfate (3.99 mg/L), residual chlorine (1.53 mg/L), alkalinity (196.39 mg/L), and microbiological (total coliform and coliform/CFU) which were the main physiochemical parameters analyzed for the study. The findings revealed that the majority of the water quality parameters tested were within the WHO and National Drinking Water Quality Standards (NDWQS). However, some of the parameters such as temperature, turbidity, fluoride, and residual chlorine did not meet the standards. The mean temperatures at the source, reservoir, and tap water were 22.01°C 22.5°C,and 21.83°C, respectively. Turbidity levels in source samples ranged from 10 to 45 NTU, with a mean of 24.5 NTU, exceeding the WHO's recommendation of less than 5 NTU. The Boko Alamura well had a high fluoride content (3.9 mg/l), which was above the WHO and NDWQS permissible limits. There was no free residual chlorine in the tap water sample. The results show that the Hawassa drinking water supply did not contain total or fecal coliform in any of the samples tested. The overall WQI for the water source, reservoir, and tap water was also determined to be 89, 71, and 69.7 points, respectively. Therefore, based on the WQI result, Hawassa drinking water quality is good for the source, reservoir, and tap water.

FULL TEXT

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1. Introduction

Water is a natural resource that is critical to human survival[1–3]. It sustains all forms of life and generates jobs and wealth in the water, tourism, and recreation industries. The global slogan "Water is Life" implies that water is one of the most basic human needs. Life as we know it on our planet would be impossible without water [4, 5]. Water distribution networks are critical in modern communities because their proper operation is directly related to the well-being of the population [6, 7]. In spite of such importance, water crises and quality are major concerns in many countries, particularly in arid and semiarid regions where water scarcity is common, and water quality assessment has received little attention [8–10]. Water quality is constantly under attack because it is essential to the human body and ecosystem. Globally, the growing human population has a negative impact on surface waters and watersheds. As the demand for freshwater rises with the growth of the human population, the degradation of the water quality in aquatic ecosystems has become a global concern [11]. Although urbanization is a global phenomenon with far-reaching consequences for natural ecosystems, the ecosystem's primary constituent is water, a valuable natural resource and national asset. Water sources include rivers, lakes, glaciers, rainwater, groundwater, and so on. Water resources are important in many sectors of the economy, including agriculture, livestock production, forestry, industrial activities, hydropower generation, fisheries, and other creative activities [12]. In order to achieve the desired goal, it is crucial to use a variety of physical, chemical, and biological variables for different purposes (drinking, industrial, agricultural, recreational, and habitat). [13, 14]. Groundwater is a critical component of human development because it is the primary source of drinking water in many countries around the world [15–17]. The insufficiency in surface water resources makes the people dependent on groundwater for the regular water supply [18]. Monitoring water quality is an essential tool in the management of freshwater resources. The International Organization for Standardization (ISO) defines monitoring as "the programmed process of sampling, measurement, and subsequent recording or signaling, or both, of various water characteristics, frequently with the goal of assessing conformity to specified objectives." The most popular definition of water quality is "it is the physical, chemical, and biological characteristics of water" [19]. It was prudent to conduct research on the city's water supply system in order to determine the quality of drinking water.

Several major issues affecting human survival on Earth are caused by a lack of clean water for a large number of communities, as well as environmental aesthetics [20, 21]. In many developing countries, such as Ethiopia, water quality and the risk of water-related diseases are serious public health issues. It can be directly or indirectly linked to public health due to the low or high concentrations of numerous contaminants in drinking water [22–24]. Access to improved water supply and sanitation has been very low, and hence, the majority of communicable diseases are associated with unsafe and inadequate water supply [25]. In Ethiopia, the safety of potable water and the risk of

waterborne diseases are major public health concerns. A communicable disease associated with unsafe and inadequate water and poor human excreta disposal accounts for approximately 60% of the health problem [26]. Waterborne diseases, particularly diarrhea, coliforms, and *E. coli* microorganisms, were prevalent in SNNP. This is because there was insufficient investigation and subsequent control of water quality parameters. Water-related diseases are frequently reported as being among the top ten diseases in the region's health sector, and there are several signs that the region's population is suffering from water-related diseases, most likely as a result of poor drinking water quality [27, 28]. Contamination can significantly change the chemical properties of water, compromising the overall balance of the system, causing economic losses, and making its consumption impractical [29–31].

So far, no research activity has been conducted on the city's drinking water quality that may enable us to know the quality of drinking water; however, it has been observed that some people in the study area did not drink tap water and complained that the water has a salty taste. They generally distrust the quality of tap water and prefer to drink bottled water. The main objectives of this study are to investigate the drinking water quality in Hawassa City utilizing on-site and laboratory experiments and to assess the findings by contrasting and comparing them to prior studies, national and international standards, and guidelines. Based on the aforementioned study goals, it not only assesses the safety of the source, reservoir, and tap water for consumption but also offers a foundation for their management strategy toward them.

2. Research Methodology

2.1. Description of the Study Area

This study was carried out in Hawassa, a city in Ethiopia's Sidama regional state. The city is situated between 7°3′1.3464″N latitude and 38°29′43.8144″E longitude, at a height of 1708 meters above sea level. Addis Ababa is located 273 kilometers to the south of the city. The city is the capital of the Southern Nations, Nationalities, and Peoples' Region, as well as a special zone. Figure 1 depicts the location of the study area.

[figure(s) omitted; refer to PDF]

2.2. Climate and Hydrology

Hawassa town has a hot temperature, fluctuating between 10°C in winter and 30°C in summer. The town average annual rainfall is 956 mm. The average maximum rainfall during the rainy season is about 126 mm in September. The number of sunny hours in a day ranges from 4 hours in the rainy season to 9 hours in the dry season. Relative humidity varies from 40% to 90% during the year. The average wind speed recorded ranges from 0.6 m/s to 1.1 m/s. According to the National Weather Service, the estimated annual PET intake for the Hawassa station is about 1599 mm, with a minimum of 102 mm in July and a maximum of 173 mm in December.

2.3. Geology and Hydrogeology

The Hawassa Basin is a volcanic tectonic collapse located in the central part of Ethiopia's main Rift Valley. There are several rift system faults that tend to the north and northeast along Lake Hawassa. These errors are extensive and often constitute step errors. They mainly dominate the south and southwest of the lake. The collapsing structure of the volcano forms an almost circular pattern around the Hawassa Lake Basin. This collapse intersects several Main Ethiopian Rift (MER) fault systems, suggesting that the collapse occurs after the fault.

Lake Hawassa covers an area of 100 square kilometers, while Cheleleka Wetland covers an area of 12 square kilometers. Recent lake and alluvial deposits, coal cones, rhyolite lava flows, and related igneous rocks, tuffs, and volcanic ash form this basin. Rhyolitic lava flows and related igneous and ash rocks belong to recent rhyolite volcanic centers and coal cones to basalt of recent highlands. The cliffs and mountains at the eastern edge of the Hawassa Lake Basin comprise the Nazareth Series, consisting of ignimbrite, unwanted tuff, ash stream, rhyolitic stream, dome, and trachyte. The northern, southwestern, and western margins include the Dino Formation, which is characterized by lava rock overlaid by coarse pumice of tuff ignimbrite with a rare alternation of lake sediments. The Hawassa Basin strata are based on the Dino Formation, also known as the Nazareth Series.

2.4. Data Collection Process

Personal observation and field measurement were used to collect data. This was accomplished by employing the

primary data collection method to obtain the information required to meet the objective. On both primary and secondary data, qualitative and quantitative analyses were performed. Tables, maps, and/or phrases were used to evaluate the data qualitatively. In contrast, quantitative data were analyzed in Excel.

2.5. Sampling Methods

Samples were collected from raw water source locations such as reservoirs and water taps where customers receive water. The tap water sample was collected twice, from two different kebeles (it is collected randomly from ketena one and two of the kebele). The total sample was collected in three phases. In the first phase (10/12/2013 E. C to 18/12/2013 E. C), 11 water samples were collected from sources. In the second phase (18-19/12/2013 E. C), the samples were collected from the reservoir.

In the third phase, the samples were collected from the water tap in 29/12/2013 E. C. Precautions were taken for sampling. Contaminant-free containers were used, devices or instruments used for sampling were calibrated, and the time and the type of samples were leveled. The location of the sampling points is shown in Figure 2.

[figure(s) omitted; refer to PDF]

2.6. Water Quality Parameter Analysis and Instruments

Water samples were collected from Hawassa's drinking water supply system's 21 drinking water supply stations. Four water samples were taken from service reservoirs, and three kebeles (small administrative) of water taps were also used to obtain six samples. Taps were turned on or left running for at least a few minutes prior to sampling to ensure a representative sample (temperature and electrical conductivity were monitored to verify this). The other 11 samples were collected from the source water. Various physicochemical parameters (electrical conductivity, TDS, pH, and temperature) of the water samples were measured in the field using portable meters at the time of sampling. Water samples were taken in clean containers provided by the laboratory.

2.7. Physicochemical Test Procedures

Sensitive water quality parameters such as temperature, pH, EC, and TDS were determined using on-site measurements. A thermometer and a portable digital pH meter were used to measure temperature and pH. The pH meter was calibrated with pH 4.0 and pH 7.0 before being used for the analysis, and it was washed with distilled water between samples as directed in the pH meter operation guide. A portable digital conductivity meter was used to measure electrical conductivity and total dissolved solids (TDS). Their measurements were taken immediately after the samples were collected at each location. The remaining indicators of water quality were measured in accordance with the standards. The equipment was thoroughly cleaned and disinfected before each use to prevent secondary contamination and ensure accurate results.

2.8. Bacteriological Parameter Analysis

To avoid the growth or death of microorganisms in the sample, bacteriological tests were done on the same day the sample was collected. Using the membrane filtration method, a 100 ml water sample was sucked through a filter with a little hand pump. After filtration, the bacteria on the filter paper were placed in a Petri dish with a nutritive solution (also known as culture media, broth, or agar). The temperature and period of incubation differed based on the type of indicator bacteria and culture media applied (for example, total coliforms were incubated at 35°C and fecal coliforms were cultured at 44.5°C with some types of culture media).

2.9. Calculation of Water Quality Index (WQI)

The water quality index (WQI) is a straightforward and effective method for determining water quality. It is also an excellent way to disseminate information about water quality. The WQI method is a straightforward and practical way to assess the general quality of surface/groundwater and its appropriateness as drinking water [32, 33]. The water quality index (WQI) is a measure of the acceptability of water for human consumption that takes into account the combined effects of various water quality factors [34]. It was calculated using the weighted arithmetic index method adopted from [35]. The quality rating scale for each parameter qi was calculated by using the following equation:(1)qi =CiSi×100.

A quality rating scale (q_i) for each parameter is assigned by dividing its concentration (C_i) in each water sample by its respective standard (S_i), and the result is multiplied by 100. The inversely proportional value of the recommended

standard (S_i) of the corresponding parameter is used to calculate the relative weight:(2)Wi=1Si.
The overall water quality index (WQI) was calculated by aggregating the quality rating (Qi) with unit weight (Wi) linearly:(3)WQI=∑i=1i−nWiqi.
Generally, WQI is discussed for a specific and intended use of water. In this study, the WQI for drinking purposes is considered, and permissible WQI for the drinking water is taken as 100:(4)overallWQI=∑qiwi∑wi.

3. Results and Discussion

3.1. Physicochemical Analysis Results of Source, Reservoir, and Tap Water Samples

The physicochemical parameters such as total dissolved solids (TDS), turbidity, electrical conductivity (EC), temperature, pH, calcium, magnesium, total hardness, alkalinity, fluoride, nitrate (NO_3), sulfate (SO_4), phosphate (PO_4), and residual chlorine at different sample locations are shown in Table 1. Figures 3–15 depict the detailed analysis.
[figure(s) omitted; refer to PDF]

Table 1

Mean values and standard deviations of physicochemical parameters at the source, reservoir, and tap water samples.

Parameters	Units	11 samples from source		4 samples from reservoir		6 samples from tap water		Standard	
Mean	Std.	Mean	Std.	Mean	Std.	ES	WHO	TDS	Mg/l
190.90	58.82	67.30	32.77	170.17	42.95	1000	1000	Temp.	°C
21.91	0.98	22.50	1.29	21.83	0.75	—	<15	EC	μS/cm
339	99.68	72.75	38.39	338.67	85.17	1500	1000	Turbidity	NTU
24.45	16.21	1.55	0.45	2.48	0.38	7	5	pH	—
7.13	0.37	7.54	0.07	7.69	0.24	6.5–8.5	6.5–8.5	Ca	Mg/l
71.04	52.08	32.50	3.42	21.73	9.66	—	75	Mg	Mg/l
9.90	3.40	12.00	3.83	10.33	1.86	50	50	F	Mg/l
1.10	1.06	1.30	0.31	1.04	0.37	3	1.5	NO_3	Mg/l
3.776	2.43	2.73	0.38	2.23	0.58	50	50	SO_4	Mg/l

4.633	4.95	7.00	6.24	0.35	0.81	—	250	PO_4	Mg/l
0.337	0.22	0.43	0.34	0.54	0.11	0.02	0.05	TH as $CaCO_3$	Mg/l
89.864	25.95	30.00	24.15	52.50	8.22	300	300	Alkalinity	Mg/l
162.5	40.88	187.50	46.64	239.17	42.83	—	200	Residual chlorine	Mg/l

[figure(s) omitted; refer to PDF]

3.1.1. Total Dissolved Solids (TDS)

TDS in drinking water has no health-based limit. As a result, TDS occurs in drinking water at concentrations far below those that are harmful. Water with TDS levels less than 100 mg/L, on the other hand, is considered to be good in terms of palatability [36]. Figure 3 shows that the mean concentration of TDS in water samples in the study area ranged from 67.3 to 190.9 mg/l. The source has the highest TDS value (190.9 mg/l). TDS levels are higher in the source and water tap samples than in the reservoir samples. However, the health risks are minimal because the TDS value is much lower than 1,000 mg/l, which is the WHO and NDWQS maximum permissible limit. The TDS values of water in this study are higher than those in previous studies' results, i.e., the mean TDS records of various cities' water sources; the TDS at Nekemte is 48 mg/l, at Damot Sore Woreda is 67.79 mg/l, and at Tula subcity is 150.7 mg/l.

3.1.2. Turbidity

The turbidity levels in the source samples ranged from 10 to 45 NTU, with a mean of 24.5 NTU, which was higher than the WHO and NDWQS recommendation of 5 NTU and 7 NTU. The mean turbidity values at the reservoir and tap water, on the other hand, are determined to be within the permissible limits of 1.55 NTU and 2.48 NTU, respectively (Figure 4). Turbidity in water is caused by sewage matter, which increases the risk of pathogenic organisms being shielded by turbidity particles and thus escaping the disinfectant's effect.

3.1.3. Electrical Conductivity (EC)

Electrical conductivity (EC), a measure of water's ability to conduct an electric current, is proportional to the amount of dissolved minerals in the water but does not indicate which element is present. In contrast, a higher EC value indicates the presence of pollutants such as sodium, potassium, or chloride [37]. As shown in Figure 5, the samples from the Hawassa water source have a mean EC value of 339, with maximum and minimum values of 243 and 569 (μS/cm). The Hawassa water reservoir's average EC is 72.75 μS/cm, with a range of 35 to 115 μS/cm. Similarly, Hawassa tap water has an average EC value of 338.67 μS/cm, with a range of 166 to 388 μS/cm. The tested values for Hawassa drinking water at the source and tap water are within permissible limits when compared to WHO and NDWQS standards.

3.1.4. Temperature

Temperature is one of the physicochemical factors used to determine drinking water quality. As the temperature of the water rises, so does the demand for disinfectants and microbial activity, reducing the palatability of the water [25]. However, the results show that all of the temperature values for the Hawassa water samples from several samples are above the WHO recommended limit. The temperature range of the source was 21–22.8°C, which corresponded to the minimum and maximum temperatures of the water source. Similarly, the reservoir and tap water

samples have temperatures ranging from 21 to 24°C and 21 to 23°C, respectively, which are outside of the acceptable temperature range set by the World Health Organization [36]. The majority of the sampled sites had temperature variations from the sources to the water taps, which did not meet the WHO requirement of 15°C. The reservoir (new reservoir 1) sample had the highest temperature (24°C) (Figure 6). The tropics have a hot climate with lots of rain, which may have contributed to the high temperatures found in water samples from various Ethiopian cities [38]. Similarly, earlier research in the Damot Sore Woreda of the south regional state [39] reported a mean temperature of 23.27°C.

3.1.5. pH

As a starting point for the pH scale, neutral chemicals are used. Alkaline or basic compounds have a pH greater than 7.0 (7.1–14.0). Acidic compounds have a pH value less than 7.0 (0–6.9). pH adjustment is a common method in water treatment and one of the most critical operational elements for water treatment processes such as disinfection and flocculation [40]. The WHO defines the minimum and maximum permissible pH for drinkable water as 6.5 to 8.5 [36]. All water samples had a pH range of 6.5–7.99, but the mean pH increased from source to tap water (Figure 7). There were no statistically significant differences between sampling stations, and the pH levels in this study area are within WHO and national guidelines.

3.1.6. Calcium and Magnesium

Calcium comes from both natural and man-made sources. Water that flows within an aquifer could be internal. The average calcium levels in the study's source, reservoir, and tap waters are 72.31 mg/l, 32.1 mg/l, and 21.3 mg/l, respectively (Figure 8). The maximum calcium value of the source water (Abella Wondo No. 2 well, 160 mg/l) does not meet the WHO's calcium limit for drinking water [36]. These variations could be caused by the geological contents of the well. All reservoir and tap water samples, on the other hand, are within the recommended level of 75 mg/l. Magnesium levels in this study's source, reservoir, and tap water samples were found to be 9.9 mg/l, 12 mg/l, and 10.33 mg/l, respectively (Figure 8). This means that the magnesium level is within an acceptable range and has no negative health implications.

3.1.7. Total Hardness

It denotes the total amount of calcium and magnesium ions present in the body. Initially, hardness was measured and analyzed in raw water samples as a proxy for water quality in terms of precipitating soap. The highest permissible limit of total hardness as $CaCO_3$, according to the World Health Organization [36], is 300 mg/l. The mean total hardness at the source, reservoir, and tap water is 89.86 mg/l, 30 mg/l, and 52.50 mg/l, respectively, according to the laboratory results of this study (Figure 9). According to WHO standards, the degree of hardness of the Hawassa City water supply is moderately soft, which is not harmful to users.

3.1.8. Alkalinity

Water sources tolerate extremes in these ranges, with alkalinity values ranging from 5 to 125 mg/l considered normal. According to the WHO standard guideline for drinking water potability, the maximum acceptable permitted value of $CaCO_3$ should not exceed 200 mg/l. According to laboratory test results, the total alkalinity of the Hawassa City water supply samples ranged from 124 to 280 mg/l of $CaCO_3$ at the source sample, 125 mg/l to 230 mg/l at the reservoir sample, and 195 mg/l to 310 mg/l at the tap water sample (Figure 10). According to the findings of this study, one source sample, samples from new reservoir 1 and 2, and a sample from pissa kebele sample 2 did not meet the standards established.

3.1.9. Fluoride

The fluoride concentration in Hawassa City's water sources ranged from 0 to 3.9 mg/l (Figure 11). The fluoride concentration in the Boko Alamura well was 3.9 mg/l, which was higher than WHO and national standards. The WHO recommends a fluoride concentration of 1.5 mg/l, but Ethiopian drinking water recommendations require less than 3 mg/l [41]. Other water tests (reservoir and water tap samples) came up short of the acceptable limit. The fluoride levels in this study exceeded the maximum values of Damot Sore Woreda (1.13 mg/l) [5].

3.1.10. Nitrate (NO_3)

The main sources of nitrates in drinking water are fertilizer runoff, sewage leakage, and erosion of natural deposits

[42, 43]. According to laboratory results, the mean nitrate levels of Hawassa's water source, reservoir, and water tap are 3.78, 2.73, and 2.23 mg/l, respectively (Figure 12). The WHO and Ethiopian standards were found to be met by all of the samples tested. Water with nitrate concentrations greater than 10 mg/l nitrate-N will cause methaemoglobinaemia in users, according to the guidelines [41]. As a result, referring to the guideline, there is no nitrate problem in Hawassa's drinking water supply, according to the findings.

3.1.11. Sulfate (SO_4)

Sulfates have no health-based recommendations. However, because drinking water with a high sulfate concentration can cause gastrointestinal effects, drinking water sources with a sulfate concentration of more than 500 mg/l should be reported to health authorities. Sulfate in drinking water can also cause a noticeable taste and contribute to distribution system corrosion [36]. The study's laboratory results show that the mean sulfate level in the Hawassa water supply's source, reservoir, and tap water is 4.63 mg/l, 7 mg/l, and 0.31 mg/l, respectively (Figure 13). The reservoir sample has the highest mean value. However, according to WHO standards, there is no sulfate problem in the study area.

3.1.12. Phosphate (PO_4)

The three most common forms of phosphorus in water are orthophosphate, condensed phosphate, and organically bound phosphate. Phosphorus is released in the form of phosphate by the microbial decomposition of organic materials. The significance of phosphorus stems from its ability to promote eutrophication in the presence of other nutrients, particularly nitrogen. The phosphorus quality criterion in water serves only to prevent undesirable algal growth [44]. The mean phosphate concentrations in this study for source, reservoir, and water tap samples were 0.38 mg/l, 0.43 mg/l, and 0.54 mg/l, respectively. Phosphate concentrations in tap water were found to be higher (0.54 mg/l). The observed value was higher than the permissible level for drinking water recommended by WHO and ES. The phosphate concentration in household tap water was higher than that in source and reservoir samples, indicating that there is phosphate ion pollution in the supply network, as shown in Figure 14. The mean phosphate value in Hawassa's water supply, on the other hand, is not significantly different from previous findings [38] in Nekemte, Oromia, and [5] in Damot Sore Woreda drinking water supply).

3.1.13. Residual Chlorine

The World Health Organization recommends a minimum free chlorine residual of 0.2 mg/L and a maximum residual chlorine of 0.5 mg/L in any water supply distribution network (http://www.Safewater.Org). Several studies have discovered that when residual chlorine levels fall below recommended levels, a variety of water quality issues can occur. Bacteria and viruses known as bacteriophages can multiply in water that has not been thoroughly disinfected. It may also be capable of causing waterborne infections, depending on the species.

The Ethiopian drinking water standard also recommends a residual chlorine level of 0.5 mg/l in drinking water. However, the mean free residual chlorine (FRC) concentration of water samples from the reservoir and the tap in this study was 0.08 mg/l and 0 mg/l, respectively (Figure 15). These values were lower than the WHO and ES maximum concentrations. This indicates that the water can be recontaminated and that there is no reserved chlorine to disinfect it, which could lead to a water-related disease in the consumer. The discovered result is also lower than the findings reported in previous studies, for example, at the Nekemte main distribution tank (0.23 mg/l and 0.28 mg/l, respectively) [38].

3.2. Bacteriological Analysis

The total coliform group has been chosen as the primary indicator bacteria for the presence of pathogens in drinking water [26]. It is a primary indicator of water's suitability for consumption. If a large number of coliforms are discovered in water, it is highly likely that other pathogenic bacteria or organisms exist. Total coliform must be absent in public drinking water supplies, according to the WHO and Ethiopian drinking water feces. In this study, no coliform bacteria were found at any of the sampling sites. Figure 16 depicts the mean total coliform bacteria levels in drinking water collected from the study area.

[figure(s) omitted; refer to PDF]

3.3. Evaluation of Water Quality Index in the Study Area

WQI is a well-known and effective tool widely used in water quality assessment [32]. Water quality data are extremely important for policy adjustment, and the water quality index (WQI) is the most convenient way to transmit the quality of drinking water resources. Several water quality indices have been developed over the years by national or international organizations and are used to assess water quality in a variety of scenarios. Figure 17 depicts the WQI and overall WQI of all samples obtained, as determined by equations (1)–(4). According to the findings of this study, the WQI of Hawassa's drinking water supply is within acceptable limits (100). The WQI was divided into five categories, ranging from “excellent water quality” to “unfit for use water.”

[figure(s) omitted; refer to PDF]

The indices were developed primarily to reflect changes in the physicochemical quality of surface water. They can, however, be used as components of environmental change. There are temporal variations within an aquatic system. The system impact of this change can be measured by linking water quality to potential water use [45, 46]. In this study area, average WQI scores (ranging from 67.5 to 89) indicated that drinking water quality is good.

4. Conclusions

The study's goal was to assess the drinking quality of Hawassa, Ethiopia, by looking at physical, chemical, and bacteriological drinking water parameters. The drinking water quality parameters from the Hawassa City water supply's source, main reservoirs, and tap water were examined using on-site measurement and experimental analysis. The findings revealed that the majority of the water quality parameters were within the WHO and Ethiopian drinking water quality standards. Total dissolved solids (TDS), electric conductivity (EC), pH, total hardness (TH), phosphate (PO_4), nitrate (NO_3), sulfate (SO_4), calcium (Ca), and magnesium (Mg) are among them. However, some physiochemical parameters (temperature, turbidity, fluoride at one well source, and residual chlorine) do not meet standards. The temperature of all water samples from the source, reservoir, and tap water exceeded 15°C. The source sample has the highest mean turbidity (24.5 NTU). However, the turbidity levels in reservoir and tap water samples are within acceptable limits (1.55 NTU and 2.48 NTU, respectively). The presence of 0.08 mg/l and 0 mg/l of free residual chlorine in tap water samples indicates that an insufficient amount of chlorine is added at the treatment plant, which could lead to recontamination of drinking water and health issues for the user. The results, on the other hand, showed that the sample analyzed was not contaminated with both total and fecal coliform, indicating that the water supply is well protected from human excreta and animal waste. In this study area, the overall average values of WQIs for source, reservoir, and tap water were 89, 71, and 67.5, respectively. As a result of the study's findings, the drinking water quality in Hawassa City can be classified as good or fair based on the water quality index classifications. Quality analysis and operational changes will be critical in improving Hawassa City's water supply system. To further guarantee that the water is fit for human use, frequent drinking water quality tests should be conducted at the source, primary distribution tanks, distribution systems, and pipelines. The investigation was limited to evaluate bacteriological and physiochemical parameters of the water delivery system from the source to household tap connections during the dry season. A comparable investigation ought to be carried out during the rainy season of the year. In addition, additional water quality factors such as heavy metals and their sources should be taken into account in future research.

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DETAILS

Subject:	Magnesium; Alkalinity; Waterborne diseases; pH; Water supply; Lava; Public health; Water sampling; Electrical resistivity; Drinking water; Water quality; Turbidity; Freshwater resources; Quality assessment; Groundwater; Boreholes; Consumption; Physiochemistry; Data collection; Dissolved solids; Chlorine; Water supply systems; Parameters; Quality standards; Rain; Developing countries--LDCs
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Retracted: Electrochemical Preparation of Nanocatalysts and Their Application in Electrocatalysis

Chemistry International Journal of Analytical

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FULL TEXT

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(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Peer-review manipulation

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We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

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References

[1] H. Zhou, "Electrochemical Preparation of Nanocatalysts and Their Application in Electrocatalysis," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/9884302, 2022.

DETAILS

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[1] J. Mi, X. Sun, S. Zhang, N. Liu, "Residential Environment Pollution Monitoring System Based on Cloud Computing and Internet of Things," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/1013300, 2022.

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Retracted: Clinical Observation of MRI Scanning Combined with Clinical Nursing for Surgical Breast Cancer Patients

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In addition, our investigation has also shown that one or more of the following human-subject reporting requirements has not been met in this article: ethical approval by an Institutional Review Board (IRB) committee or equivalent, patient/participant consent to participate, and/or agreement to publish patient/participant details (where relevant).
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[1] H. Zhang, Y. Yin, W. Tao, L. Liu, "Clinical Observation of MRI Scanning Combined with Clinical Nursing for Surgical Breast Cancer Patients," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6863281, 2022.

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Retracted: Influence of Nano-Cutting Fluid in New Cutting and Forming Processes on Heat Transfer Performance of Mechanical Engineering

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[1] W. Liu, "Influence of Nano-Cutting Fluid in New Cutting and Forming Processes on Heat Transfer Performance of Mechanical Engineering," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5603355, 2022.

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Retracted: Effect of Free Formaldehyde on Chemical Structure and Thermal Properties of Nano-Titanium Dioxide Resin

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References

[1] L. Lv, A. Wang, Y. Zhong, "Effect of Free Formaldehyde on Chemical Structure and Thermal Properties of Nano-Titanium Dioxide Resin," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/7306597, 2022.

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References

[1] Z. Yang, "3D Modeling of Sculpture Nano-Ceramics under Sparse Image Sequence," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5710535, 2022.

DETAILS

Subject:	Research; Statuary; Three dimensional models
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Retracted: Application of Realistic 3D Model in Building Prefabricated Nanomaterial Structure

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FULL TEXT

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(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

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[1] S. Li, "Application of Realistic 3D Model in Building Prefabricated Nanomaterial Structure," International Journal

of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/1726948, 2022.

DETAILS

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Retracted: Calculation and Analysis of Nonlinear Algorithm for Stability of Nanosilica Powder Soft Soil Pile Foundation

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References

[1] J. Sun, L. Guo, L. Gong, F. Zheng, H. Hao, "Calculation and Analysis of Nonlinear Algorithm for Stability of Nanosilica Powder Soft Soil Pile Foundation," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/1451633, 2022.

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Retracted: Load Test Analysis of a Long-Span Prestressed Nano-Concrete Highway Bridge

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[1] L. Yan, "Load Test Analysis of a Long-Span Prestressed Nano-Concrete Highway Bridge," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5169548, 2022.

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Retracted: Effect of Aeolian Sand Powder Addition on Frost Resistance of Concrete Pavement

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References

[1] O. Zhao, X. Deng, "Effect of Aeolian Sand Powder Addition on Frost Resistance of Concrete Pavement," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/5087896, 2022.

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Retracted: Test and Detection of Antifreezing and Anticorrosion Performance of Carbon Nanofiber Bridge Concrete

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References

[1] H. Yang, D. Wang, H. Yu, "Test and Detection of Antifreezing and Anticorrosion Performance of Carbon Nanofiber Bridge Concrete," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/4055128, 2022.

DETAILS

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Retracted: Application of Cement-Based Composite Nanomaterials in Prefabricated Thin-Wall Light Steel Structure Composite Wall

Chemistry International Journal of Analytical

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References

[1] X. Chen, H. He, L. Huo, Z. Chen, "Application of Cement-Based Composite Nanomaterials in Prefabricated Thin-Wall Light Steel Structure Composite Wall," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6015826, 2022.

DETAILS

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Retracted: Electrochemical Intelligent Recognition of Mineral Materials Based on Superpixel Image Segmentation

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[1] W. Liu, F. Jin, "Electrochemical Intelligent Recognition of Mineral Materials Based on Superpixel Image Segmentation," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/6755771, 2022.

DETAILS

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Volume:	2023
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Retracted: Mining Geological Environment Monitoring and Real-Time Transmission Based on Internet of Things Technology

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Retracted: Mechanical Performance Test and Numerical Simulation Analysis of Building Steel Plate and Concrete Composite Structure

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[1] Q. Liu, "Mechanical Performance Test and Numerical Simulation Analysis of Building Steel Plate and Concrete Composite Structure," International Journal of Analytical Chemistry, vol. 2022,DOI: 10.1155/2022/2156921, 2022.

DETAILS

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Simultaneous Determination and Stability Analysis of Ten New Psychoactive Substances including Synthetic Cathinones, Phenethylamines, and Ketamine Substitutes in Urine Using Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT (ENGLISH)

Knowing the stability of drugs is important to ensure accurate and reliable results of drug concentrations. This study evaluated the stability of ten new psychoactive substances (NPSs) in urine and methanol/water at different storage temperatures. Quantitative analyses were performed using liquid chromatography-tandem mass spectrometry.

Three replicates of each storage condition were analyzed at day 0 and after 7, 14-, 30-, 60-, and 90 days with storage at +25°C, +4°C, and −20°C. For each analyte, the percent difference at each time interval from day 0 was calculated for each storage condition. Para-methoxyamphetamine (PMA), para-methoxymethamphetamine (PMMA), deschloroketamine (DCK), and 2-fluorodeschloroketamine (2-FDCK) were stable in urine, even when stored for 90-day periods at various temperatures. For synthetic cathinones, the concentrations declined over time at room temperature (+25°C) in urine but were relatively stable in methanol solvent with 0.1% formic acid. The significant degradation was found at +25°C, and the most excellent stability was shown by samples stored at −20°C. Phenethylamines (PMA and PMMA) and ketamine substitutes (DCK and 2-FDCK) were relatively more stable than synthetic cathinones (mephedrone, butylone, pentylone, ephylone, 4-MEAPP, and eutylone).

FULL TEXT

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1. Introduction

New psychoactive substances (NPSs) are a complex and diverse group of substances often known as either designer or synthetic drugs or “legal highs.” NPSs are associated with several health and social harms on an individual and societal level [1, 2]. The number of NPSs is constantly growing, and trends and patterns of use change over time. Abuse of NPSs has become a critical threat to health and security in recent decades [3]. Some NPS, such as synthetic cathinones, para-methoxymethamphetamine, and ketamine, have posed a risk to public health and a challenge to drug policy [4]. Chewing khat leaves is common in the Arabian Peninsula and Eastern Africa. The primary psychoactive compositions in khat leaves cause sympathomimetic activity, mild euphoria, and excitation [5]. Synthetic cathinones are designer analogs of the natural active principle of khat. In recent years, synthetic cathinones have become the most seized class of NPS reported to the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory System [6]. Para-methoxymethamphetamine (PMMA) and para-methoxyamphetamine (PMA) are the para-methoxylated analogs of methamphetamine and amphetamine and have been found in tablets and capsules of the 3,4-methylenedioxymethamphetamine (MDMA) sold as “ecstasy.” A number of deaths have been attributed to tablets that contained PMMA and PMA [7–9]. 2-fluorodeschloroketamine (2-FDCK) and deschloroketamine (DCK) as substitutes for ketamine have emerged among drug abusers in recent years and are considered to have similar abuse potential as ketamine [10]. Due to its recent appearance, little research has been done on the compound.

The urine specimen is commonly used for NPS analysis. The time from sample collection to analysis might be several days due to transportation time. Sometimes, repeat testing due to unexpected results or additional drug analysis makes the time between collection and analysis increase by more than several days. Because of this time interval, to ensure that results are accurate, it is necessary to demonstrate that specimens are stable during this time frame [11, 12]. Moreover, the preparation of quality control and calibration samples in the process of illicit drug analysis is time-consuming. The working solutions of the analyte reference materials were often prepared using methanol and water and then stored for several days to months at refrigerator or freezer temperature. If preanalytical changes in concentration occur between specimen collection and analysis or between the initial and retests due to drug instability or degradation, the results of the re-test may vary from the original test results [13, 14]. Therefore, understanding the stability of analyzed drugs in testing samples is critically important.

Most published studies on NPS stability focused on single-category [14–18]. Few studies addressed the stability among different NPS categories. The stability studies among different solvents were also scarce. Methods to detect NPSs in biological matrices are challenging due to their low concentrations. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a tool of choice for quantitative bioanalytical assays due to its inherent selectivity and sensitivity and has proven to be fast and accurate [19, 20]. In this study, we used LC-MS/MS to develop a quantitative method for simultaneous determination of multiple NPSs and evaluate the stability of these NPSs, classified as synthetic cathinones, phenethylamines, and ketamine, in 50% methanol/water with or without

0.1% formic acid, and urine, at different storage temperatures.

2. Materials and Methods

2.1. Chemicals and Reagents

Reference materials were used for the validation and stability analysis. All chemicals and solvents used were of analytical grade. Methanol was purchased from Fisher Chemicals (Loures, Portugal), formic acid was purchased from Merck (Darmstadt, Germany), and ethanol was purchased from Merck (Darmstadt, Germany). 4-methylmethcathinone (mephedrone), butylone, pentylone, eutylone, ephylone, 4-methyl-α-ethylaminopentiophenone (4-MEAPP), 4-methoxyamphetamine (PMA), para-methoxymethamphetamine (PMMA), deschloroketamine (DCK), mephedrone-D3, butylone-D3, 3,4-methylenedioxyamphetamine-D5 (MDA-D5), and ketamine-D4 were purchased from Cerilliant (Texas, USA). 2-fluorodechloroketamine (2-FDCK) was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

2.2. Sample Preparation

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20221077). After informed consent was obtained, the midstream urine was obtained from five healthy volunteers simultaneously and pooled together for validation and stability experiments. Therefore, the urine matrices in this study were the same in all test samples. Pooled blank urine was confirmed to be negative for any medications using LC-MS/MS testing and stored at −20°C. The calibration standards and quality control samples were prepared using pooled blank urine. The working solution of ten analytes (10 mg/L) was prepared in 50% methanol/water with 0.1% formic acid from reference materials of each analyte. Spiking solutions were prepared from the working solutions as mixtures of the ten NPSs. The internal standards (ISs), including mephedrone-D3, butylone-D3, MDA-D5, and ketamine-D4, were prepared in 50% methanol/water with 0.1% formic acid for each final concentration with 25 ng/mL.

Protein precipitation was performed before LC-MS/MS analysis. In a microcentrifuge tube, 50 μL of urine was mixed with 50 μL methanol. After centrifugation at 14,000 × g for 10 min to separate layers, 20 μL of the supernatant was transferred to a new microcentrifuge tube and mixed with 160 μL of 25 ng/mL internal standard and 20 μL of 50% methanol/water and 0.1% formic acid solution. Then, 20 μL of the sample was injected into the LC-MS/MS system.

2.3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Chromatographic separation was performed using an ACQUITY® UPLC® H-Class UPLC system (Waters, USA) with a Kinetex Biphenyl LC column (100 mm × 2.1 mm, 2.6 μm, Phenomenex) preceded by a Security Guard™ ULTRA Cartridge UHPLC Biphenyl 2.1 mm ID column. The mobile phases comprised solvents A and B: 2% and 99.9% methanol in 0.1% formic acid solution, respectively. The gradient elution profile is shown in Table 1. The analytes were then quantified using a Xevo® TQ-XS tandem mass spectrometer (Waters Corporation, Milford, MA US). The mass spectrometer was operated in multiple reaction monitoring modes (MRMs). The MRM transitions and conditions for the analytes and ISs, as well as the retention times, target ions, and qualifier ions used for identification and quantification, are shown in Table 2.

Table 1

Time program for the chromatographic separation procedure.

Total time (min)	Flow rate (mL/min)	Mobile phase solvent A (%)	Mobile phase solvent B (%)
0	0.4	95	5
1	0.4	95	5
4	0.4	70	30

7	0.4	50	50
13.5	0.5	5	95
14	0.5	5	95
14.5	0.4	95	5
16	0.4	95	5

Solvent A: 97.9% water with 2% methanol and 0.1% formic acid. Solvent B: 99.9% methanol with 0.1% formic acid.

Table 2

Retention time, multiple reaction monitoring transitions, dwell time, cone voltage, collision energy for analytes, and internal standards.

Compound name	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Cone voltage (V)	Collision energy (V)	Internal standard
Mephedrone	5.38	178.00	160.00	0.003	18	12	Mephedrone-D3
		145.00		18	22		·
Butylone	5.54	222.10	174.10	0.003	25	15	Butylone-D3
		204.10		30	10		Pentylone
6.47	236.00	188.00	0.008	24	20	Butylone-D3	
	218.00		24	12		·	
Eutylone	6.01	236.00	188.00	0.008	36	25	Butylone-D3
		218.00		36	19		·
Ephylone	6.89	250.00	202.00	0.008	31	26	Butylone-D3
		174.00		31	41		·
4-MEAPP	7.43	220.00	105.00	0.022	12	26	Butylone-D3
		144.00		12	38		·
PMA	4.60	166.00	149.00	0.003	25	8	MDA-D5

		121.00		25	20		·
PMMA	5.15	180.10	120.91	0.003	30	22	MDA-D5
		91.10		30	30		·
DCK	6.04	204.00	173.00	0.008	21	17	Ketamine-D4
		145.00		21	24		·
2-FDCK	5.88	222.00	109.00	0.008	22	25	Ketamine-D4
		163.00		22	25		·
Mephedrone-D3	5.37	180.79	147.89	0.003	40	18	—
		144.83		38	20		·
Butylone-D3	5.53	225.03	177.07	0.003	36	18	—
		148.94		36	26		·
MDA-D5	4.47	185.00	168.00	0.003	20	10	—
		110.00		20	20		·
Ketamine-D4	6.59	242.00	129.00	0.008	20	30	—

LC-MS/MS: liquid chromatography-tandem mass spectrometry; mephedrone: 4-methylmethcathinone; ephylone: N-ethylpentylone; 4-MEAPP: 4-methyl-*α*-ethylaminopentiophenone; PMA: para-methoxyamphetamine; PMMA: para-methoxymethamphetamine; DCK: deschloroketamine; 2-FDCK: 2-fluorodeschloroketamine; MDA-D5: 3,4-methylenedioxyamphetamine-D5.

2.4. Method Validation

The validation procedures were based on guidelines for bioanalytical method validation [21, 22] and the SWGTOX validation guidelines for urine [23]. All method validations were carried out by LC-MS/MS, and the data were analyzed by Microsoft Excel software (version 15.0.5363.1000).

2.4.1. Specificity and Selectivity

Specificity and selectivity were assessed by spiking each drug and individual IS to test for interference. Exogenous interferences were evaluated by spiking blank matrices with twelve analytes, including dehydronorketamine, ethylone, dibutylone, flunitrazepam, nimetazepam, nitrazepam, phenylpropanolamine, ephedrine, pseudoephedrine, phentermine, cocaine, and ecgonine methyl ester, to ascertain if exogenous analytes may interfere with the method

analytes.

2.4.2. Sensitivity and Linearity

The lower limit of quantification (LLOQ) was determined as the lowest concentration with chromatographic signal-to-noise (S/N) value of 10 : 1 and met the accuracy limit of ±20%. Linearity was assessed by analyzing ten separate calibration curves by spiking blank urine with concentrations at 3.1, 6.2, 12.5, 25, 50, 62.5, 75, and 100 μg/L for all compounds. Calibration curves produced per batch were generated by plotting the peak area ratio (PAR) versus the spiked analyte concentration. A blank matrix containing only IS was analyzed with each batch but not included in the calibration curves. The correlation coefficient (R^2) was calculated and with an acceptability criteria of >0.99.

2.4.3. Precision and Accuracy

Accuracy and precision were calculated by running calibration standards alongside five replicates of 25, 50, and 75 ng/mL. Both intra- and interday precision were assessed across five batches with an acceptability criterion of ≤10%. A method accuracy limit of ±15% was used [24].

2.4.4. Carryover and Matrix Effects

Carryover was assessed by running the highest calibration standard (H) and analyzing subsequent blanks of 50% methanol/water with 0.1% formic acid (L) for the presence of any analytes in the sequence L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10, and L11. The difference between the high-low and low-low means needs to be less than three times the low-low SD.

According to the guideline on bioanalytical method validation [22], the matrix effects were estimated with ten urine samples from ten individuals. For each analyte and the IS, the matrix factor (MF) was calculated for each sample by calculating the ratio of the peak area in the presence of the matrix (measured by analyzing a matrix blank spiked after extraction with an analyte) to the peak area in the absence of the matrix (pure solution of the analyte). The IS-normalized MF was also calculated by dividing the analyte's MF by the IS's MF. The CV of the IS-normalized MF from the ten different urine samples should not be greater than 15%.

2.5. Sample Stability

Analyte stability was analyzed in different matrices and temperatures, including solutions of 50% methanol, 50% methanol/water with 0.1% formic acid, and urine, and storage at 25°C (room temperature), 4°C (refrigerator), and −20°C (freezer). Stability was assessed by monitoring the PARs of each analyte to the IS and the individual peak areas themselves. Three replicates at each concentration were analyzed at day 0 and after 7, 14, 30, 60, and 90 days. For each analyte, the percent difference at each time interval from day 0 was calculated and averaged for triplicate samples at each storage condition. There were no exclusion criteria; the percent difference calculations included every specimen concentration result. A concentration change of ±20% was deemed unstable.

3. Results and Discussion

3.1. Validation Experiments

3.1.1. Specificity and Selectivity

The retention time, MRM transitions, dwell time, cone voltage, and collision energy for the analytes and ISs are shown in Table 2. The total ion chromatogram (TIC) of the analytes and internal standards are shown in Figure 1. No endogenous or exogenous interferences were observed from the pooled drug-free matrices analyzed. None of the analytes or ISs interfered with the other analytes' peak areas or retention times within this method.

[figure(s) omitted; refer to PDF]

3.1.2. Sensitivity and Linearity

The LLOQs of all analytes in this method validation are shown in Table 3. 4-MEAPP, eutylone, and 2-FDCK were linear over a concentration range of 3.1–100 μg/L with a correlation coefficient (R^2) > 0.99. The calibration curves for other analytes were linear over a concentration range of 6.3–100 μg/L (R^2 > 0.99). The linearity curves of all analytes are shown in Figure S1.

Table 3

Lower limit of quantification (LLOQ), calibration curve, correlation coefficient (R^2), precision, and accuracy of all analytes in urine by LC-MS/MS.

Analytes	LLOQ (ng/mL)	Calibration curve (ng/mL)	R^2	Intraday precision(n=5, %CV)			Interday precision(n=5, %CV)			Accuracy (%)(n=5)		
25 ng/mL	50 ng/mL	75 ng/mL	25 ng/mL	50 ng/mL	75 ng/mL	25 ng/mL	50 ng/mL	75 ng/mL	Mephedrone	6.3	6.3–100.0	0.9969
5.66	3.85	3.08	2.23	4.16	2.31	100.00	101.20	108.80	Butylone	6.3	6.3–100.0	0.9991
1.80	3.27	4.37	6.63	1.90	5.81	99.20	100.40	97.80	Pentylone	6.3	6.3–100.0	0.9984
2.83	3.59	3.24	4.87	9.21	4.66	100.00	99.60	98.40	Eutylone	3.1	3.1–100.0	0.9986
4.90	2.30	3.09	4.83	3.03	5.20	100.00	96.20	98.40	Ephylone	6.3	6.3–100.0	0.9994
3.64	2.95	3.37	8.33	9.15	9.30	98.40	100.40	98.40	4-MEAPP	3.1	3.1–100.0	0.9991
3.37	2.83	3.75	4.53	7.35	4.04	99.20	100.00	97.33	PMA	6.3	6.3–100.0	0.9990

4.67	1.08	1.20	5.48	9.58	3.04	97.60	101.20	99.47	PMMA	6.3	6.3–100.0	0.9997
2.23	1.77	0.94	8.70	6.96	5.24	98.40	100.80	100.00	DCK	6.3	6.3–100.0	0.9997
0.89	0.90	2.00	3.52	3.04	4.34	101.60	99.96	98.93	2-FDCK	3.1	3.1–100.0	0.9994

LC-MS/MS: liquid chromatography-tandem mass spectrometry; mephedrone: 4-methylmethcathinone; ephylone: N-ethylpentylone; 4-MEAPP: 4-methyl-α-ethylaminopentiophenone; PMA: para-methoxyamphetamine; PMMA: para-methoxymethamphetamine; DCK: deschloroketamine; 2-FDCK: 2-fluorodeschloroketamine; CV: coefficient of variation.

3.1.3. Precision and Accuracy

According to quantitative mass spectrometry for pharmacokinetic studies, the bioanalytical method of choice must be specific, precise, and reproducible to the intended analyte in a given matrix . The current best industry practice of validating an LC-MS/MS method and applying it for sample analysis suggested that accuracy is determined by replicate analysis of samples (QCs) containing known amounts of the analyte. The mean measured value should be within 15% (bias) of the nominal value for all QC concentration levels except for the LLOQ, where the bias (%) should be within 20% [24, 25].

The accuracy and precision results are shown in Table 3. All analytes in urine had precision values (%CV) <10%. The means of intraday precision (%CV) of all analytes in urine were 3.32, 2.58, and 2.85% at 25, 50, and 75 ng/mL, respectively. The interday precision across all analytes averaged 5.32%, 5.84%, and 4.76% at 25, 50, and 75 ng/mL, respectively. The accuracy of each analyte in urine fell within the ±15% criterion with a range from 96.20% to 108.80%.

3.1.4. Carryover and Matrix Effects

No carryover was found in the blank samples following duplicate injection of the highest calibrator. The concentrations of all blank samples were 0 μg/L. Matrix effect data of all analytes are shown in Table 4. The matrix effects of these analytes ranged from 84%–109%.

Table 4

Matrix factor (MF) in % (range) of all analytes in urine.

Compound name	MF (50 ng/mL)		CV (%) of the IS-normalized MF*
Mephedrone	94.8	[82–110]	8.6
Butylone	99.1	[92–109]	4.9

Pentylone	94.9	[90–104]	3.8
Eutylone	98.1	[86–109]	7.2
Ephylone	93.5	[86–101]	4.5
4-MEAPP	95.0	[90–104]	4.6
PMA	87.8	[82–103]	8.6
PMMA	95.5	[85–103]	5.5
DCK	93.3	[84–103]	5.1
2-FDCK	95.5	[91–99]	2.6

*The CV(%) of the IS-normalized MF calculated from ten different samples of the matrix should not be greater than 15%. CV: coefficient of variation; IS: internal standard; mephedrone: 4-methylmethcathinone; ephylone: N-ethylpentylone; 4-MEAPP: 4-methyl-α-ethylaminopentiophenone; PMA: para-methoxyamphetamine; PMMA: para-methoxymethamphetamine; DCK: deschloroketamine; 2-FDCK: 2-fluorodeschloroketamine.

3.2. Stability of the Analytes in Different Matrices and Temperatures

The stability of each analyte in different matrices and temperatures are shown in Figures 2 and 3. The details of the percentage of the target concentration for the analytes in different matrices and storage temperatures are shown in Table S1.

[figure(s) omitted; refer to PDF]

3.2.1. The Stability Study of Mephedrome

The stabilities of the synthetic cathinones in different matrices and storage temperatures are shown in Figure 2. For mephedrone, when the samples were stored at freezer temperature (−20°C), the percent differences were within 20% of the original measurement for all solvents, even by day 90. When the samples were stored in 50% methanol/water with 0.1% formic acid, the percent differences were within 20% of the original measurement for all storage temperatures for 90 days (Figure 2(a)). Mephedrone in urine stored at 25°C showed a 68.65 ± 1.21% loss of the initial concentration by day 7 and a 96.43 ± 0.06% loss of the initial concentration by day 14. Mephedrone in urine stored at 4°C showed a 20.9 ± 3.66% loss of the initial concentration by day 14 and a 42.66 ± 3.10% loss of the initial concentration by day 30 (Table S1).

3.2.2. The Stability Study of Butylone, Pentylone, and Eutylone

For butylone, pentylone, and eutylone, when the samples were stored at −20°C, the percent differences were within 20% of the original measurement for all solvents, even by day 90. When stored in 50% methanol/water (with or without 0.1% formic acid), the percent differences were within 20% of the original measurements at 4°C and −20°C (Figures 2(b)–2(d)).

Butylone in urine stored at 25°C showed a 48.42 ± 1.90% loss of the initial concentration by day 14 and an 88.15 ± 0.73% loss of the initial concentration by day 30. Butylone in urine stored at 4°C showed a 30.01 ± 1.64% loss of the initial concentration by day 90. Butylone in 50% methanol/water without 0.1% formic acid stored at 25°C showed a 24.43 ± 1.61% loss, a 40.74 ± 1.03% loss, and a 64.50 ± 0.31% loss of the initial concentration by day 30, day 60, and day 90, respectively.

Pentylone in urine stored at 25°C showed a 27.22 ± 2.19% loss of their initial concentration by day 7 and a 60.16 ± 1.44% loss of the initial concentration by day 14. Pentylone in urine stored at 4°C showed a 35.54 ± 1.24% loss of the initial concentration by day 90. Pentylone in 50% methanol/water without 0.1% formic acid stored at 25°C

showed a 27.84 ± 1.19% loss, a 61.03 ± 1.70% loss, and a 79.37 ± 0.69% loss of the initial concentration by day 30, day 60, and day 90, respectively.

Eutylone in urine stored at 25°C showed a 42.74 ± 3.10% loss of the initial concentration by day 14 and a 79.26 ± 1.09% loss of the initial concentration by day 30. Eutylone in urine stored at 4°C showed a 23.92 ± 0.64% loss of their initial concentration by day 90. Eutylone in 50% methanol/water without 0.1% formic acid stored at 25°C showed a 25.78 ± 2.29% loss, and a 50.21 ± 0.37% loss of the initial concentration by day 60, and day 90, respectively (Table S1).

3.2.3. The Stability Study of Ephylone and 4-MEAPP

For ephylone and 4-MEAPP, when the samples were stored at −20°C, the percent differences were within 20% of the original measurement for all solvents, even by day 90. When the samples were stored in 50% methanol/water with 0.1% formic acid, the percent differences were within 20% of the original measurement for all storage temperatures for 90 days (Figures 2(e) and 2(f)).

Ephylone in urine stored at 25°C showed a 24.32 ± 0.90% loss of the initial concentration by day 7 and a 53.00 ± 1.85% loss of the initial concentration by day 14. Ephylone in urine stored at 4°C showed a 36.11 ± 1.47% loss of the initial concentration by day 90. Ephylone in 50% methanol/water stored at 25°C showed a 25.17 ± 2.29% loss of the initial concentration by day 30 and a 71.02 ± 0.41% loss of the initial concentration by day 90.

4-MEAPP in urine stored at 25°C showed a 37.81 ± 1.52% loss of the initial concentration by day 7 and a 74.29 ± 1.69% loss of the initial concentration by day 14. 4-MEAPP in urine stored at 4°C showed a 29.59 ± 1.28% loss of their initial concentration by day 60 and a 53.98 ± 1.09% loss of the initial concentration by day 90. 4-MEAPP in 50% methanol/water stored at 25°C showed a 21.05 ± 3.44% loss of the initial concentration by day 14 and a 90.3 ± 0.19% loss of the initial concentration by day 90 (Table S1).

3.2.4. The Stability Study of PMA and PMMA

PMA and PMMA samples were stable for more than 90 days at 4°C and −20°C in all solvents evaluated in this study (Figures 3(a) and 3(b)). In PMA samples, the percent differences were all within 20% of the original measurements in all solvents, even at room temperature by day 90. In PMMA samples, a more than 20% difference was found only in urine at room temperature by day 60.

3.2.5. The Stability Study of DCK and 2-FDCK

DCK and 2-FDCK samples were stable for more than 90 days at 4°C and −20°C in all solvents evaluated in this study (Figures 3(c) and 3(d)). In the DCK and 2-FDCK samples, more than 20% differences were found only in 50% methanol/water with 0.1% formic acid at room temperature by day 90 and day 60, respectively.

The stability study showed that phenethylamines (PMA and PMMA) and ketamine substitutes (DCK and 2-FDCK) were relatively stable than synthetic cathinones (mephedrone, butylone, pentylone, ephylone, 4-MEAPP, and eutylone). PMA, PMMA, DCK, and 2-FDCK were stable in urine, even when stored for 90-day periods at various temperatures (Figure 4). Similar results were reported previously about the long-term stability of amphetamine-type stimulants (ATS) [26, 27]. Thus, delayed testing, repeat testing, and add-on testing for PMA, PMMA, DCK, and 2-FDCK in urine specimens can yield reliable results for up to 90 days following the urine collection date.

[figure(s) omitted; refer to PDF]

The stability results for synthetic cathinones showed that the detectable concentrations would decline over time (Figure 2). In urine samples, all the synthetic cathinones tested in this study declined within 7–14 days at room temperature. Most synthetic cathinones, except mephedrone, are stable in urine for at least 30 days when stored at refrigerator temperature. Mephedrone concentrations in urine significantly declined within 14 days, even at refrigerator temperatures (Figure 4). All tested synthetic cathinones were stable at freezer temperature in both urine and 50% methanol/water, no matter with or without 0.1% formic acid. Synthetic cathinones dissolved in 50% methanol with 0.1% formic acid were relatively more stable than those stored without formic acid in the present study. Previous studies reported similar results that synthetic cathinones are relatively stable under acidic conditions [14].

Many commercially available NPS reference substances are dissolved in methanol. Considering the polarity and

solubility distribution of NPSs, we chose 50% methanol as one of the solvents for drug stability evaluation. Some previous studies that investigated the stability of synthetic cathinones used preservatives or buffers containing metal ions for different pH solutions [14, 28]. However, metal ions may interfere with mass spectrometry or damage the instrument. In this study, we used formic acid to maintain the pH of solvents.

The chemical behavior of synthetic cathinone is mainly determined by the ketone and amine groups in its structure. Synthetic cathinones have a “beta-keto” structure and two tautomers of “keto” and “enol” form in solution. Many factors, such as solvent properties, matrix, pH value, aromaticity, conjugation, hydrogen bonds, and substitutions of synthetic cathinones, would affect the equilibrium position between the keto and enol forms [29]. A previous study considered that solvent effects are due to the strong tendency of the enol form to hydrogen bond intramolecularly, while the keto form may hydrogen bond with protic solvents, providing stabilization [30]. Acidic conditions favor the formation of an enol form. It has been reported that acid makes the carbonyl group more electrophilic, increasing the acidity of alpha-protons and facilitating enol formation [29]. Previous studies demonstrated that mephedrone could form dihydro-mephedrone through carbonyl reduction of its ketone group [31, 32]. We speculate that synthetic cathinones might partially be transformed into the enol form due to low pH in 50% methanol with 0.1% formic acid. The reaction of the ketone functional group will not be able to proceed under acidic conditions, thus leading to an increase in stability (Figure S2).

Using unstable reference material might lead to unreliable results that could significantly impact the justice system and the individuals whose samples are tested in casework. Storing the samples with 0.1% formic acid would improve the stability of the analytes, especially in synthetic cathinones. The most significant degradation was found at room temperature (25°C), and the most excellent stability was stored in the freezer (−20°C). Although unopened reference material may be stable for long, this work highlights the importance of regularly updating reference material once opened.

4. Conclusion

In this study, we simultaneously determined ten NPSs, including synthetic cathinones, phenethylamines, and ketamine substitutes, and evaluated their stability in various solvents and storage temperatures. Mephedrone is the least stable analyte tested in this study. Phenethylamine and ketamine substitutes are more stable than synthetic cathinones, synthetic cathinones are more stable under acidic conditions, and all analytes are stable within 90 days at freezer (−20°C) temperature. Knowing the stability of drugs is essential to ensure accurate and reliable results of drug concentrations. These findings highlight the importance of the storage environment for reference materials and biological samples in forensic laboratories when performing NPS analysis.

Ethical Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20221077).

Consent

Informed consent was obtained from all subjects involved in the study.

Authors’ Contributions

Yi-Ching Lin conceptualized the study; Yi-Ching Lin, Li-Ping Tseng, and Yung-Hung Lee contributed to methodology; Yung-Sheng Lan and Yi-Cheng Lee performed formal analysis; Yi-Ching Lin and Feng-Shuo Yang investigated the data; Hei-Hwa Lee, Li-Ping Tseng, Yung-Hung Lee, Yung-Sheng Lan, Yi-Cheng Lee, and Yi-Cheng Chou performed data curation; Feng-Shuo Yang and Hei-Hwa Lee prepared the original draft; Yi-Ching Lin reviewed and edited the manuscript. All authors read and approved the final manuscript. Feng-Shuo Yang and Hei-Hwa Lee contributed equally to this work.

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DETAILS

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Rapid Determination of Three Organic Acids in Polygonum Vivipari Rhizoma via One Marker by HPLC-UV at Equal Absorption Wavelength and Effervescence-Assisted Matrix Solid-Phase Dispersion

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ABSTRACT (ENGLISH)

A rapid HPLC-UV method for the determination of three organic acids (neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid) in Polygoni Vivipari Rhizoma (PVR) by one marker was developed. The sample was prepared by effervescence-assisted matrix solid-phase dispersion (EA-MSPD). The separation of compounds was performed on a Poroshell column. The equal absorption wavelength was set as follows: 292 nm (0~7 min) and 324 nm (7~10 min). The analytical time including sample extraction and HPLC separation time was 12 min. The analytical method validation such as accuracy (recoveries 99.85%–106.29% and RSD <2.9%), precision (RSD <1.3%), reproducibility (RSD <1.7%), and stability tests (RSD <0.7% in 24 h) proved that the established HPLC method was suitable for determination of three organic acids in PVR. The contents of three analytes obtained by the external standard method with three markers and the equal absorption wavelength method with one marker were similar (RSD ≤ 2.0%). The developed method, which is rapid and reference compound saving, is an improved quality evaluation method of PVR.

FULL TEXT

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1. Introduction

Polygoni Vivipari Rhizoma (PVR), also called “Zhuyaliao” in Chinese, is a famous Tibetan folk medicine. Its dried root is usually used in checking diarrhea and activating blood circulation to dissipate blood stasis [1]. According to the literature studies, phenolic compounds (organic acids and flavonoids) are the main active constituents responsible for the antioxidant and bacteriostatic activities [2–4]. Among the phenolic compounds, the chlorogenic acid series compounds such as neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid have been found

to exhibit good pharmacological activities [5]. Furthermore, these three compounds are also the primary organic acids found in PVR [6]. Therefore, simultaneous determination of three organic acids is crucial for the quality evaluation of PVR due to their good bioactivities and high contents.

To date, several HPLC methods for the determination of the three organic acids were reported by the external standard method (ESM) based on three reference compounds applied [7–10]. The chlorogenic acid is cheap, which is about 14 dollars per 20 mg, while the prices of neochlorogenic acid and cryptochlorogenic acid are relatively more expensive, which are both about 140 dollars per 20 mg. In order to reduce the cost of PVR sample test and simplify the method, it is necessary to develop an analytical method for the determination of the three organic acids by one cheap reference compound (chlorogenic acid). The quantitative analysis of multicomponents by single marker (QAMS) method has been applied in herbal medicines [11–14]. However, the relative calibration factor (RCF) is necessary to be established, which increases the operational complexity and limits the wide application of QAMS. Therefore, developing an HPLC method for determination the three compounds by one reference compound without RCF is preferable. These three organic acids performed different UV absorptions at different wavelengths. The chlorogenic acid may have the equal UV absorption with two other compounds at certain wavelengths. It is the equal absorption waveslength (EAW) of chlorogenic acid with neochlorogenic acid or cryptochlorogenic acid. Hence, developing a HPLC-UV method at the EAWs could realize simultaneous determination of the three components by chlorogenic acid without RCF.

In addition, due to the complex matrix of PVR and similar structures of chlorogenic acid and two other organic acids, the reported HPLC-UV methods for the determination of three organic acids, including extraction and separation, are always time-consuming (more than 25 min) [7–10]. In order to develop a rapid HPLC method for determining the three organic acids in PVR, the rapid sample extraction and HPLC separation should be considered. Effervescence-assisted matrix solid-phase dispersion (EA-MSPD), a modified MSPD method, is proved to be a simple, fast, and effective extraction technique. It promotes the microextraction process by generation of carbon dioxide in situ from the effervescent mixture consisting of a carbon dioxide source and an acid component dissolved in water [15–17]. Hence, EA-MSPD is a potential rapid extraction method for extracting organic acid from PVR. On the other hand, the Poroshell column is a kind of rapid HPLC column [18–20], which can provide the rapid separation of organic acids in PVR.

In the present study, a rapid and reference compound saving HPLC-UV method for the determination of neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid via one cheap marker (chlorogenic acid) in EAW is developed. The developed HPLC-UV method was successfully applied in the determination of organic acids in ten batches of PVR samples.

2. Materials and Methods

2.1. Chemicals and Materials

Neochlorogenic acid (99.9%), chlorogenic acid (99.0%), and cryptochlorogenic acid (99.2%) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). HPLC-grade methanol was bought from Energy Chemistry Co., Ltd. (Shanghai, China). HPLC-grade acetic acid was purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Analytical grade methanol, sodium dihydrogen phosphate, and sodium carbonate were bought from Xilong Scientific Co., Ltd. (Shantou, China). Oxalic acid was obtained from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Citric acid was obtained from Chengdu Kelong Chemical Co., Ltd. (Sichuan, China).

10 batches of PVR samples were collected from Sichuan, Yunan, and Guizhou Provinces and authenticated as the dried root of Polygonum Viviparum by Dr. Zheng-Ming Qian. Voucher specimens were deposited at Key Laboratory of State Administration of Traditional Chinese Medicine, Dongguan, Guangdong. All crude samples were smashed into powder using a tube mill (IKA, Guangzhou, China) and passed over 50 meshes.

2.2. Preparation of Reference Compound Solutions

1.5 mg/mL neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid were dissolved in 50% methanol, respectively. Mixed reference compound solutions were prepared by mixing them and diluted to the intended concentration with 50% methanol. All solutions were stored at 4°C.

2.3. Preparation of Sample Solution

The sample solution was prepared by the EA-MSPD method. In order to obtain the good extraction efficiency, different extraction conditions (composition of effervescent mixture, ratio of sample and effervescent mixture, milling time, polarity, and volume of extraction solvent) were studied by the single-factor method. The contents of three organic acids were used to evaluate the extraction efficiency.

The sample powder (0.25 g) and the effervescent mixture (0.592 g sodium carbonate and 0.658 g oxalic acid, molar ratio about 10 : 13) were precisely weighed and milled with the Retsch MM400 ball milling instrument (Retsch, Shanghai, China) for 1 min to obtain the homogeneous mixture. The 0.4 g mixture was accurately weighed into a 50 mL centrifuged polypropylene tube, and 4 mL of 20% methanol was added. The effervescence occurred instantly and lasted about 30 s. When the process ended, the extraction solution was vortexed (5 s) and filtered through a 0.22 μm membrane before HPLC injection.

2.4. UV Condition

Three reference compounds were dissolved with 10% methanol containing 0.1% acetic acid (the HPLC mobile phase) to 19 μg/mL (neochlorogenic acid 19.08 μg/mL, chlorogenic acid 19.09 μg/mL, and cryptochlorogenic acid 19.07 μg/mL). The ultraviolet spectra of the three analytes were obtained by scanning the three reference compound solutions from 200 nm to 400 nm with Agilent Cary 60 ultraviolet spectrophotometer (Agilent Technologies, USA). 10% methanol containing 0.1% acetic acid was used as blank.

2.5. HPLC Condition

An Agilent 1260 II Series HPLC system (Agilent Technologies, USA) was employed for the analysis. The separation of compounds was achieved on an Agilent Poroshell 120 EC-C18 column (50 × 4.6 mm, 2.7 μm) (batch number: B18386) at a column temperature of 35°C and eluted with 10% methanol containing 0.1% acetic acid at a flow rate of 1.0 mL/min in the isocratic mode. The detection wavelength was set at 0~7 min (292 nm and 2 nm) and 7–10 min (324 nm and 2 nm). The injection volume was 2 μL.

2.6. Method Validation

The method validation, including linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, repeatability, and stability tests, was carried out.

2.6.1. Linearity, LOD, and LOQ

A series of concentrations of reference component solutions were prepared for the evaluation of linearity. The neochlorogenic acid (0.32 to 477.00 μg/mL), chlorogenic acid (0.64 to 477.18 μg/mL), and cryptochlorogenic acid (0.95 to 476.65 μg/mL) were analyzed by HPLC. The standard curve was constructed by plotting the peak area (y) versus the concentrations of reference compounds (x). The LODs and LOQs were determined by reference components and recorded as the corresponding concentrations, which gave the signal-to-noise (S/N) ratios approximately 3 and 10, respectively.

2.6.2. Precision and Repeatability

The intra- and interday assays were used to assess the precision of the developed method. The intraday precision was determined by analyzing the reference components solution six times within one day. The interday precision was determined by analyzing the reference components solution twice per day for three days. The relative standard deviation (RSD) was used as a measure of precision. The repeatability of the developed method was evaluated by six replicates of the PVR sample analysis. The samples were extracted as “2.3” and analyzed as “2.4.” The RSD of the contents was used as a measurement of repeatability.

2.6.3. Recovery

A recovery test was used to evaluate the accuracy of the developed method. Known amounts of three organic acids were added to the PVR sample powder and then extracted and analyzed by the developed method. The PVR sample was analyzed six times. The recovery rates were calculated as 100% × (found amount − original amount)/spiked amount.

2.6.4. Stability and Robustness

The stability was assessed by analyzing PVR sample solution five times within 24 hours. Variation was evaluated by

the RSD. The robustness studies were carried out by analyzing the reference solution with the developed method with small changes in method parameters as follows: flow rate (1.0 ± 0.1 mL/min) and column temperature (35 ± 3°C). The developed method was also tested on 3 different Agilent Poroshell 120 EC-C18 columns (batch number: B15046, B18386, and B19476) and 2 different instruments (Agilent 1260 I and Agilent 1260 II). With chlorogenic acid as the reference compound, the RRT values and the content of neochlorogenic acid and cryptochlorogenic acid in PVR samples were calculated for evaluation. Furthermore, the resolution of the 3 target peaks in PVR sample solutions was also used to evaluate the robustness.

3. Results and Discussion

3.1. Optimization of EA-MSPD Extraction Conditions

In order to obtain the good EA-MSPD method, different extraction conditions (composition of effervescent mixture, ratio of sample and effervescent mixture, milling time, polarity, and volume of extraction solvent) were studied by the single-factor method. The contents of three organic acids were used to evaluate the extraction efficiency. Tukey’s honestly significant difference test was carried out to compare the organic acid contents at different levels of the investigated parameter.

The effervescent mixture, a combination of the carbon dioxide source and acid component, had direct effect on the effervescent effect and extraction efficiency. Three commonly effervescent mixtures were tested, including sodium carbonate-oxalic acid, sodium carbonate-citric acid, and sodium carbonate-sodium dihydrogen phosphate [21–23]. It was observed that the effervescence effect of sodium carbonate-oxalic acid was more intense than two others, and the effervescence time (30 s) was faster than two others (more than 60 s). The ratio of sodium carbonate and oxalic acid and the ratio of sample and effervescent mixture were also important to the PVR sample extraction. According to the chemical reaction of sodium carbonate and oxalic acid, the molar ratio of sodium carbonate and oxalic acid is 1 : 1 (mass ratio of 100 : 85). The effervescent mixture (sodium carbonate: oxalic acid = 100 : 85) would make the sample solution in a weak alkaline environment. The organic acids are unstable in alkaline solution [24, 25]. So, more oxalic acid was added in the effervescent mixture to keep the sample solution in acid environment. Four different ratios of sodium carbonate and oxalic acid (100 : 85, 100 : 90, 100 : 95, and 100 : 100) were compared. As shown in Figure 1(a), the ratios of sodium carbonate and oxalic acid in 100 : 90, 100 : 95, and 100 : 100 showed better extraction efficiency. Stability tests also revealed that three organic acids were stable in 24 h at these ratios. Consequently, 100 : 90 was selected as the condition because of the less material cost. Three different ratios of sample and effervescent mixture (1 : 5, 1 : 10, and 1 : 20) were evaluated. As shown in Figure 1(b), the content of analytes was similar in the three conditions. The ratio of sample and effervescent mixture (1 : 5) was chosen for less material consume. After the composition of the effervescent mixture and the ratio of sample-effervescent mixture were fixed, the milling time (1, 2, and 3 min) was examined. The results (Figure 1(c)) of the three tests were similar, and 1.0 min was used in this study.

[figure(s) omitted; refer to PDF]

The polarity of the extract solvent would influence the solubility of the analytes. Methanol was selected as the extract solvent because of its wide practicability and superior capacity for extracting components from herbal medicine [4, 6–10, 26]. Different concentrations of methanol (0, 20, 40, and 60%) were compared. The results (Figure 1(d)) showed that 20%, 40%, and 60% methanol had better extraction efficiency than water. The 20% methanol was chosen based on the methanol cost. Different solvent volumes (4 mL, 8 mL, and 12 mL) were also examined. Figure 1(e) reveals that 4 mL was sufficient to extract the analytes from PVR.

Compared with the votexing extraction method (non-EA-MSPD sample preparation), the extraction efficiency of the developed EA-MSPD method was improved 26.2% for neochlorogenic acid, 33.0% for chlorogenic acid, and 34.9% for cryptochlorogenic acid. To further confirm the extraction efficiency of the developed EA-MSPD, PVR sample S1 was extracted by the proposed EA-MSPD method and the reported ultrasonic extraction method [10], respectively. Three replicates were performed. The contents of neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid tested by the developed EA-MSPD method were 4.49 ± 0.01%, 8.88 ± 0.03%, and 0.76 ± 0.01% while the contents of these three analytes by the reported ultrasonic extraction method were 4.48 ± 0.05%, 8.59 ± 0.04%, and 0.71 ±

0.02%. These results show that the extraction efficiency of the developed EA-MSPD method is similar to that of the reported ultrasonic extraction method, which could be used for extracting three organic acids from PVR.

3.2. Optimization of HPLC Conditions

In order to develop a rapid HPLC separation of the three target compounds, the rapid HPLC column (Poroshell column) was employed. 0.1% acetic acid methanol based on the literature was used as the mobile phase system [10]. Three different mobile phases (8%, 10%, and 12% methanol with 0.1% acetic acid, respectively) were tested for separation. 10% methanol with 0.1% acetic acid was chosen as the eluting solvent for the good resolution and short separation time. The flow rate of 1.0 ml/min was used according to the literature [10]. Three different column temperatures (30, 35, and 40°C) were tested. The separations of analytes in three temperatures were similar, and 35°C was used in the current experiment as easy control and less energy consume.

Traditional QAMS often employs the maximum absorption wavelength of analytes, at which different compounds have different UV responses. So, the RCF is used for the determination of multiple compounds with one standard. In this study, three organic acids are detected at the EAW. The three analytes have the same response, and the RCFs are close to 1.0. So, it can test three compounds with one standard without RCF. Therefore, the selection of EAW is the key factor in the present HPLC method, which includes two steps (find and confirm EAW). First, screening the EAW by UV, three reference compound solutions at the same concentration were scanned from 200 nm to 400 nm with an ultraviolet spectrophotometer to get the UV spectrum for the three analytes. As shown in Figure 2, the UV response of cryptochlorogenic acid was lower than the other two compounds at the same UV wavelength. In order to obtain the better UV response of analytes, the maximum absorption wavelength (at 326 nm) was selected as the HPLC detection wavelength of cryptochlorogenic acid. The UV response of cryptochlorogenic acid (at 326 nm) was equal to noechlorogenic acid (at 296 nm and 338 nm) and chlorogenic acid (at 294 nm and 340 nm). Second, confirming the EAW by HPLC-UV, the mixed reference compound solution (neochlorogenic acid 63.60 μg/mL, chlorogenic acid 63.62 μg/mL, and cryptochlorogenic acid 63.55 μg/mL) was injected to HPLC and detected at different wavelengths for confirming the EAW. The cryptochlorogenic acids were detected around 326 nm (±0 nm, ±1 nm, ±2 nm, and ±3 nm). It was found that cryptochlorogenic acid had the maximum peak area at 324 nm (Table S1). So, the detection wavelength of cryptochlorogenic acid was set at 324 nm. The noechlorogenic acid was detected around 296 nm (±0 nm, ±1 nm, ±2 nm, ±3 nm, and ±4 nm) and 338 nm (±0 nm, ±1 nm, ±2 nm, ±3 nm, and ±4 nm). The chlorogenic acid was detected around 294 nm (±0 nm, ±1 nm, ±2 nm, ±3 nm, and ±4 nm) and 340 nm (±0 nm, ±1 nm, ±2 nm, ±3 nm, and ±4 nm). The results (Table S1) showed that the peak areas of noechlorogenic acid (at 292 nm and 338 nm) and chlorogenic acid (at 292 nm and 339 nm) had the same peak areas with cryptochlorogenic acid (at 324 nm). Considering less detection wavelengths used, 292 nm was chosen as the detection wavelength for noechlorogenic acid and chlorogenic acid. In addition, the different bandwidths (1 nm, 2 nm, 4 nm, and 8 nm) were compared for the detection of three reference compounds at EAW. The results showed that the lowest RSD of peak areas could be obtained at 2 nm. The EAW conditions were as follows: 0~7 min (292 nm, 2 nm) for detecting noechlorogenic acid and chlorogenic acid and 7–10 min (324 nm, 2 nm) for detecting cryptochlorogenic acid.

[figure(s) omitted; refer to PDF]

3.3. Method Validation

The validation of the current methods is summarized in Tables 1–4. The analytical method showed good linearity in the tested range with correlation coefficient $R = 0.9999$. The LODs and LOQs of the three analytes were less than 0.7 μg/mL and 1.0 μg/mL, respectively. The RSDs of intraday and interday precision were less than 1.3%. The RSDs of repeatability were less than 1.7%. The RSDs of stability were less than 0.7% within 24 hours. The recoveries of three analytes were 99.85~106.29% (RSD less than 2.9%). In the robustness test, the RSDs of both contents and RRTs of noechlorogenic acid and cryptochlorogenic acid (determinated by chlorogenic acid) were all less than 2.0%. The resolutions of the 3 target peaks to the adjacent peaks were all larger than 1.5.

Table 1

The linearity, LODs, and LOQs of analytes.

Analytes	Calibration curves	*R*	Test range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Neochlorogenic acid	$y=4.3597x-3.8990$	0.9999	0.32~477.00	0.16	0.32
Chlorogenic acid	$y=4.4316x-5.5305$	0.9999	0.64~477.18	0.32	0.64
Cryptochlorogenic acid	$y=4.4577x-2.9198$	0.9999	0.95~476.65	0.64	0.95

Table 2

Precision, repeatability, and stability of the analytes.

Analytes	Precision (RSD %)		Repeatability (RSD %, $n=6$)	Stability (RSD %, 24 h)
Intraday ($n=6$)	Interday ($n=6$)	Neochlorogenic acid	0.36	0.85
0.63	0.27	Chlorogenic acid	0.56	0.54
0.58	0.38	Cryptochlorogenic acid	1.28	1.12

Table 3

The recoveries of the analytes.

Analytes	Sample	Original (mg)	Added (mg)	Found (mg)	Recovery (%)	Average recovery (%)	RSD (%)
Neochlorogenic acid	1	0.4542	0.2396	0.6976	101.55	100.99	2.69
2	0.4530	0.2396	0.6889	98.44	3	0.4533	0.2396
0.6859	97.03	4	0.4551	0.2396	0.7041	103.90	5
0.4530	0.2396	0.6968	101.73	6	0.4529	0.2396	0.7004
103.29	–						
Chlorogenic acid	1	0.3956	0.2052	0.6026	100.90	99.85	2.87
2	0.3945	0.2052	0.5937	97.06	3	0.3948	0.2052

0.5919	96.06	4	0.3964	0.2052	0.6072	102.75	5
0.3946	0.2052	0.5986	99.43	6	0.3944	0.2052	0.6055
102.89	–						
Cryptochlorogenic acid	1	0.1101	0.0631	0.1767	105.54	106.29	0.78
2	0.1098	0.0631	0.1773	106.90	3	0.1099	0.0631
0.1763	105.17	4	0.1103	0.0631	0.1781	107.30	5
0.1098	0.0631	0.1768	106.15	6	0.1098	0.0631	0.1771

Table 4

Robustness tests for the sample solution.

Parameters		RRTs*		Content (mg/g)**	
Neochlorogenic acid	Cryptochlorogenic acid	Neochlorogenic acid	Cryptochlorogenic acid	Current method	
0.438	1.397	4.63	0.78	·	
Flow rate (mL/min)	0.9	0.439	1.394	4.55	0.77
1.1	0.440	1.391	4.55	0.77	·
Column temperature (°C)	32	0.428	1.429	4.59	0.77
38	0.448	1.365	4.51	0.79	·
Column	B15046	0.434	1.375	4.56	0.76
B19476	0.442	1.387	4.59	0.77	·
Instrument	Agilent 1260 I	0.442	1.385	4.60	0.77

*RRT was calculated as the retention time of the tested compound/the retention time of chlorogenic acid. **Content was calculated based on the peak area of the tested compound/peak area of chlorogenic acid × concentration of chlorogenic acids.

3.4. Analysis of Sample

The developed HPLC-UV EAW method was successfully applied in the determination of the target components in PVR samples. The chromatograms of the reference compounds and sample are shown in Figure 3, and the results are listed in Table 5. To confirm the feasibility of the developed HPLC-UV EAW method, the contents of three organic acids in ten PVR samples were determined by the ESM (with three reference compounds) and EAW method (with chlorogenic acid), respectively. The RSDs of the results obtained by the two methods were not more than 2.0%. These results indicated that the developed HPLC-UV EAW method could be used for quantitative analysis of three organic acids in PVR sample. The contents of noechlorogenic acid (1.24~8.25 mg/g), chlorogenic acid (1.75~13.75 mg/g), and crptochlorogenic acid (0.65~2.10 mg/g) in PVR samples were agreed with the literature data [10].

[figure(s) omitted; refer to PDF]

Table 5

The content of three organic acids in PVR samples (*n* = 2).

No.	Source	Chlorogenic acid	Neochlorogenic acid			Cryptochlorogenic acid		
ESM (mg/g)	EAW (mg/g)	ESM (mg/g)	RSD* (%)	EAW (mg/g)	ESM (mg/g)	RSD* (%)	S1	Sichuan
8.83 ± 0.04	4.46 ± 0.04	4.45 ± 0.03	0.50	0.77 ± 0.01	0.76 ± 0.02	1.99	S2	Yunan
7.09 ± 0.13	8.25 ± 0.14	8.21 ± 0.16	1.65	2.10 ± 0.00	2.11 ± 0.00	0.21	S3	Sichuan
6.40 ± 0.01	4.23 ± 0.01	4.22 ± 0.00	0.12	0.86 ± 0.00	0.85 ± 0.00	0.74	S4	Sichuan
4.86 ± 0.04	2.95 ± 0.04	2.92 ± 0.02	0.83	0.81 ± 0.01	0.80 ± 0.01	1.14	S5	Guizhou
5.50 ± 0.01	2.05 ± 0.01	2.01 ± 0.01	1.02	0.65 ± 0.01	0.64 ± 0.01	1.93	S6	Yunan
8.61 ± 0.01	1.24 ± 0.01	1.20 ± 0.00	2.00	0.71 ± 0.00	0.70 ± 0.00	0.91	S7	Guizhou
4.29 ± 0.01	2.27 ± 0.01	2.24 ± 0.01	0.89	0.75 ± 0.01	0.74 ± 0.01	1.04	S8	Yunan
1.75 ± 0.00	2.32 ± 0.00	2.29 ± 0.01	0.86	0.72 ± 0.00	0.71 ± 0.00	0.89	S9	Guizhou

6.08± 0.05	2.48± 0.05	2.45±0.01	0.79	0.66±0.00	0.65± 0.00	0.95	S10	Sichuan

*RSD was obtained from the tested contents by the EAW method ($n=2$) and the ESM method ($n=2$).

3.5. Comparisons of the Developed and Previously Reported Methods

Several HPLC methods for analyzing these three organic acids have been reported [7–10]. Compared with these reported methods, the developed method is reference compound saving, simple, and fast.

The reported methods employed ESM with three reference compounds applied. In addition, the traditional QAMS for the determination of the three target analytes with one marker, often performed at the maximum absorption wavelength 330 nm, at which neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid respond differently, resulted in which the RCFs of neochlorogenic acid and cryptochlorogenic acid to chlorogenic acid are required. In this study, the EAW method uses only chlorgenic acid for determination of three target analytes. No RCF is applied because EAW is employed, at which neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid have the same UV response, and RCFs are close to 1.0. So, the developed HPLC-UV EAW method was much simpler than the traditional QAMS method.

The literature's methods for analyzing the three organic acids consume more than 25 min. For example, the HPLC method developed by Haghi et al. [7] costs 120 min in sample extraction and takes 35 min in HPLC separation with a total time of 155 min. Another HPLC method developed by Honda et al. [9] consumes 60 min including sample extraction (30 min) and HPLC separation (30 min). In the current method, EA-MSPD is applied in the PVR sample extraction and the Poroshell column is executed in HPLC separation. The whole process only costs 12 min in total, including about 2 min of sample preparation and 10 min of HPLC separation. It is faster compared to the reported methods [7–10].

4. Conclusions

In the present study, a rapid HPLC-UV EAW method for simultaneous determination of three organic acids in PVR samples by chlorogenic acid is established. Compared with the reported methods, the developed method is rapid, simple, and reference compound saving. It would be a good improved method for quality evaluation of the major organic acids in PVR samples.

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DETAILS

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Evaluation of Antibiotics Residues in Milk and Meat Using Different Analytical Methods

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ABSTRACT (ENGLISH)

Veterinary drugs are pharmacologically and biologically active chemical agents. At present, veterinary drugs are extensively used to prevent and treat animal diseases, to promote animal growth, and to improve the conversion rate of feed. However, the use of veterinary drugs in food-producing animals may leave residues of the parent compounds and/or their metabolites in food products resulting in harmful effects on humans. To ensure food safety, sensitive and effective analytical methods have been developing rapidly. This review describes sample extraction and cleanup methods, and different analytical techniques are used for the determination of veterinary drug residues in milk and meat. Sample extraction methods, such as solvent extraction, liquid-liquid extraction, and cleanup methods such as dispersive solid-phase extraction and immunoaffinity chromatography, were summarized. Different types of analytical methods such as microbial, immunological, biosensor, thin layer chromatography, high-performance liquid chromatography, and liquid chromatography–tandem mass spectrometry were discussed for the analysis of veterinary drug residues in animal-derived foods. Liquid chromatography–tandem mass spectrometry is

the most widely used analytical technique for the determination of antibiotic drug residues. This is due to the powerful separation of LC and accurate identification of MS, and LC-MS/MS is more popular in the analysis of veterinary drug residues.

FULL TEXT

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1. Introduction

Veterinary antibiotics are extensively applied for therapeutic and prophylactic infections of different animals. By definition, veterinary drugs are any substances applied or administered to any food-producing animals, such as meat or milk-producing animals whether used for therapeutic, prophylactic, or diagnostic purposes [1]. Veterinary drugs can be divided into six classes such as antimicrobials antiparasites, anti-inflammatory drugs, tranquillizers, drugs with growth promotional effect, and others [2].

Antimicrobials are the dominant class of veterinary drugs used after 1950s to treat bacterial infectious diseases in animals. However, after the use of veterinary drugs in food-producing animals, parent compounds and their metabolites may accumulate in the products of animal origin. The normal use of veterinary drugs is acceptable in risk analysis by dietary intake assessment. But, unreasonable use of veterinary drugs due to the lack of scientific knowledge and the blind pursuit of economic benefits by husbandry personnel may lead to the existence of high drug residue in animal-derived food products [3]. The existence of veterinary antibiotic residues in animal products such as milk and meat may cause allergies in humans, and in the extensive and long run may facilitate the development of resistant pathogens. The existence of resistant bacterial strains produces severe health consequences on the human body [4]. These reasons make it important to effectively control antibiotic residues in animal-derived food products, and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number of anti-infective agents in milk and meat as described in Table 1. National residue monitoring programs to control veterinary drug residues in different animal-derived food products including meat and milk are compulsory in all nations. Effective veterinary antibiotic monitoring program requires specific, sensitive, and reliable analytical methods that can detect all antibiotic drug residues below regulated levels (maximum residue level) [6]. Various analytical methods have been described to determine antibiotic drug residues in milk and meat, such as chromatographic, immunochemical, and biosensor tests [7]. The methods used in the detection usually depend on the type of antimicrobials targeted, the expected time limitations, selectivity, and its cost. These methods that are used for the measurement of antimicrobial residues may be qualitative and quantitative [4]. Therefore, the objective of this review is to explain the large range of analytical methods developed and used for the determination of antimicrobial drug residues in meat and milk.

Table 1

Maximum antibiotic drug residue limits for commonly used antimicrobials in food staffs of animal derived (from annex I [5]).

Antimicrobial	Muscle (μg/kg)	Liver (μg/kg)	Kidney (μg/kg)	Fat (μg/kg)	Cow milk (μg/kg)
Amoxicillin	50	50	50	50	4
Benzyl penicillin	50	50	50	50	4
Chlortetracycline/oxytetracycline/tetracycline	100	300	600	—	100

Streptomycin/dihydrostreptomycin	600	600	1000	600	200
Closantel	1000	1000	3000	3000	—
Spiramycin	200	300	300	300	—
Cefquinome	50	100	200	50	20
Tilmicosin	50	1000	1000	50	50
Tylosin	100	100	100	100	50

2. Antimicrobial Drug Residues

Antimicrobial residues are defined as all active ingredients or its metabolites of those drugs or degradation products that remain in animal-derived food products [8]. The use of veterinary antibiotics for food-producing animals may cause the existence of residues in foodstuffs of animal origin like meat and milk [9].

3. Risk Factors for Antibiotic Residue Occurrence

Most of the time, livestock producers treat entire groups of livestock animals. This activity unintentionally and unnecessarily can expose healthy animals to antimicrobials. Additionally, many livestock producers administer subtherapeutic doses of veterinary antibiotics to treat infectious diseases, and this will cause the antibiotic residue to enter the human food chain [10]. Animal fecal recycling, where the drug residues can excrete in the feces of treated animals, which will contaminate the feed of untreated animals, can also be the cause of the occurrence of veterinary antibiotic residues [10]. The followings are the major risk factors for antibiotic residue occurrence.

3.1. Disease Status

The disease status of an animal can affect the pharmacokinetics of the drugs administered, which can influence the potential for residues [11]. This can occur either when the disease affects the metabolic system (and consequently drug metabolism) or when the presence of infection and/or inflammation causes the drug to accumulate in affected tissues. The changes in liver function by fasciolosis result change in the drug metabolism. The kidney is the most important site of drug excretion. Renal disease usually significantly affects drug excretion (retard drug removal from the body). The systemic clearance and elimination half-life are important parameters referring to the overall rate of elimination (metabolism and excretion). Although most compounds are excreted primarily by the renal, some drugs are partially or completely excreted through the bile. It has been reported that there is an extensive species variation among animals in their general ability to excrete drugs in the bile; for example, chickens are characterized as good biliary excretes, whereas sheep and rabbit are characterized as moderate and poor excretes [11].

3.2. Extra-Label Drug Use

Extra-label drug use (ELDU) refers to the use of an approved drug in a manner that is not in accordance with the approved label directions. It occurs when a drug only approved for human use is used in animals when a drug approved for one species of animal is used in another, when a drug is used to treat a condition for which it was not approved, or the use of drugs at levels in excess of recommended doses [12]. For instance, the use of enrofloxacin solution as a topical ear medication (only approved for use as an injection) is the common ELDU in veterinary medicine [13].

3.3. Improper Withdrawal Time

Withdrawal time is the time for the residue of toxicological concern to reach a safe level of drug concentration. The withdrawal time can be varied for different veterinary drug products depending on different conditions, such as the type of drug, dosage form, and route of drug administration. Withdrawal time can also be defined as the interval between the last administration of a veterinary drug to the animals under normal conditions of use and the time when a treated animal can be slaughtered for the production of safe foodstuffs [14]. Therefore, failure to wait for the

withdrawal period causes the occurrence of residue in foods of animal origin such as meat and milk, which are used for human consumption [10].

4. Safety Evaluation of Veterinary Antibiotic Residues

The following parameters are used to evaluate the safety of veterinary antibiotic residues.

4.1. Acceptable Daily Intake (ADI)

By definition, acceptable daily intake for a given drug is the amount of a drug that can be ingested every day over a lifetime without appreciable health risks to the consumer [15]. Acceptable daily intake is also defined as a maximum amount of drug residues or chemicals, which can be consumed every day by the most sensitive classes in the population with any outward effects on their health [16].

The acceptable daily intake can be calculated by using a safety or uncertainty factor, which is commonly 100, to the no observed adverse effect level (NOAEL) obtained from the most sensitive test species. The 100-fold safety factor is based on the need to take into account both the variations in species and variations in toxicokinetics and toxicodynamics [17].(1) $ADI = \frac{\text{Long-term NOAEL lowest value}}{100}$.

4.2. Maximum Residue Limits (MRL)

The term maximum limit for residues of veterinary antibiotics or drugs is the maximum concentration of veterinary drug residues resulting from the use of veterinary drugs legally permitted or recognized as acceptable in animal food products. The concentration of drug residue can be expressed in milligrams/micrograms per kilogram of the commodity (or milligrams/micrograms per liter in the case of a liquid commodity) or ppm/ppb [18]. A residue at or below the stated MRL is considered safe when animal-derived food at that level is consumed daily for a lifetime (Table 1). The MRLs are specified for several animal-derived food products (different edible tissues and other food commodities). When veterinary drugs are used according to the period of treatment and the withholding period specified before slaughter or milking, the concentration of drug residues should be at levels that will not cause an adverse effect on the health of the consumer. Therefore, animals are suitable for food production if the amounts of veterinary antibiotic residues in animal food products are below levels which could cause a health risk for consumers [19].

Nowadays, regulatory bodies have been established for veterinary drugs used in food-producing animals to ensure regular monitoring of veterinary drug residues in livestock products. The regulatory laws can help the government’s policies in managing animal-derived food safety, prevention, and control of food safety incidents [18].

5. Impact of Antimicrobial Residues

The incidence of veterinary antibiotic residues in animal-derived foods produces a significant health risk for consumers because of the emergence of microbial resistance noticed in recent years [20]. Extensive use of antibiotics might increase the risk of an adverse effect of residues on the customer and the occurrence of antibiotic resistance as well as hypersensitivity reactions in consumers [10]. Therefore, ingenuity in the use of veterinary antibiotics in the manner of preventing animal feed and food contamination is required [16]. The followings are some of the impacts of antibiotic residues.

5.1. Antimicrobial Resistance

The emergence of antimicrobial resistance has been observed due to different factors. Some of the factors include repeated use and exposure to sublethal doses of antimicrobials [20]. In addition, the application of animal manure for soil fertilization can be a contemplated contributor to environmental contamination and transmission of antimicrobial drug residues through animal feces. Currently, the development of antimicrobial-resistant bacterial genesis is frequently described owing to the overuse of veterinary antimicrobials all over the world. The utilization of veterinary antimicrobials in food-producing animals causes selection for bacterial resistant to antimicrobials. Administering these antibiotics to humans will result in poor response to treatment during illness [21].

5.2. Drug Hypersensitivity

Drug hypersensitivity is defined as an immune arbitrated response to a drug agent in a sensitized patient, and drug allergy is constrained to a reaction mediated by IgE. Allergic reactions to drugs may include anaphylaxis, serum sickness, and cutaneous reaction, and a delayed hypersensitivity response to drugs seems to be more frequently

linked with antibiotics, especially penicillin. Penicillin residues in milk could provoke allergic reactions in sensitized individuals [22]. About 10 percent of the human population is considered hypersensitive to an amount of a substance, including penicillin, but in animals, the extent of hypersensitive to the drug is not well known. Certain macrolides might also in exceptional be responsible for liver injuries, triggered by a specific allergic response to macrolide-modified hepatic cells [23].

5.3. Teratogenic Effect

The term teratogen applies to a drug or chemical agent that produces a toxic effect on the embryo or foetus during a critical phase of gestation. Consequently, a congenital malformation, which affects the structural and functional integrity of the organism, is produced [24].

6. The Extent of Veterinary Antibiotic Residue in Ethiopia

In most African countries, veterinary antibiotics are used to treat infectious diseases or feed domestic animals. The current threat of antimicrobial residue is a major challenge for public health. This challenge faced the human population worldwide, including in Africa [25]. These veterinary antibiotic residues are escalating rapidly, disregarding topographical, biological, or legitimate variations among countries [25, 26].

A study was conducted in Ethiopia for the determination of oxytetracycline and penicillin G residues in milk samples from farms (Nazareth dairy farms). From the total 400 milk samples, 48 milk samples were found to contain oxytetracycline and penicillin G residues.

Further investigation was also carried out in Ethiopia in 2007 to determine the proportion of tetracycline residual levels in cattle. Among the meat samples collected from the three sampling sites (Addis Ababa, Debre Zeit, and Nazareth slaughterhouses), 93.8%, 37.5%, and 82.1% tested positive for oxytetracycline residues, respectively [27].

7. Codex and Food Safety System in Ethiopia

By definition, the Codex Alimentarius Commission is the international body that is responsible for the execution of the joint FAO/WHO food standards program [28]. It was established in 1962 by FAO and WHO. The program is aimed at safeguarding the health of customers and facilitating international trade in foods [29].

The Ethiopian National Codex Committee (ENCC) was established under the auspices of the Quality and Standards Authority of Ethiopia (QSAE) in 2003 [28]. The NCC member organizations are Addis Ababa University, the Ministry of Health, the Ministry of Agriculture, the Ministry of Trade and Ministry of Industry, the Ethiopian Public Health Institute and Consumers Association and the Ethiopian Chamber of Commerce, and QSAE [30].

The principal responsibilities of the National Codex Committee are endorsement of recommended Codex standards as Ethiopian standards, representing the country’s interest in selected international Codex meetings, detecting priority areas on food safety, expanding fundable projects, and conducting national awareness program on food safety and codex standards [31].

8. Analysis of Antibiotic Drug Residues

8.1. Sample Pretreatment

The occurrence of antibiotic residues can vary within a single organ, and it is a major factor to consider before sample preparation. For instance, residue differences can occur in the kidney between the medulla and the cortex [32]. Accordingly, it is important to take a characteristic aliquot of the biological sample. This may need the removal of some portions throughout the composite sample to get a representative sample [33]. Homogenisation with a blender is often important to get a homogenous biological sample. Liquid biological samples like milk are generally easier to process than solid samples, and antibiotic residues are more homogenously distributed throughout [33].

8.2. Sample Extraction Techniques

Drug residue extraction is the removal of an active agent (antibiotic residue) from a solid (animal tissues and organs) or liquid mixture (from milk) with an extraction solvent. Residues are typically extracted from samples using simple solvent extraction or liquid-liquid extraction (LLE). The extraction technique adopted may depend on the nature of the samples (i.e., liquid or solid) and the physicochemical properties of the residues (polarity and pKa) [33]. The major goal of the sample extraction process is to get a suitable sample for analytical instruments, commonly for chromatographic analysis, that will not contaminate the analytical instrument. The method of biological sample

preparation and extraction technique selected is generally dictated by the analytical methods accessible and the physical characteristics of the residues in the process of investigation [33]. The following methods are used to describe the extraction of antibiotic residues in biological samples.

8.2.1. Solvent Extraction Technique

In the solvent extraction method, the biological sample (most of the time, meat) is mixed with the selected extraction medium or solvent. The solvent helps to dissolve veterinary drug residues and other biological extractives. The extractive solvent also promotes the deproteinization of biological samples [30]; most of the time, organic extraction solvents are distinctly important in veterinary drug residue analysis because they enable the extraction of protein-associated veterinary drugs from biological samples. Factors to be considered during the selection of an extraction solvent are the thermodynamic properties and its ability to interact with the analyte [34].

Some organic solvents, such as acetonitrile, methanol, and ethanol, are water miscible and frequently applicable in veterinary drug residue extraction. This is due to the polar behavior of the majority of veterinary antibiotic drugs. Proteins from biological samples are generally not soluble in organic solvents. Therefore, organic solvents help to precipitate proteins, so veterinary drug residues can be left at protein binding sites [35].

For polar antibiotic residues, aqueous extraction solvents can be applied. Assorting the pH of an extraction solvent can increase the polarity of the extraction solvent. Therefore, it may have a greater capacity for solubilizing polar veterinary antibiotic residues. Mineral acids were utilized for the extraction of tetracycline residues since this drug is not acid labile [19]. Popelka et al. [36] used an acidic buffer and heat to extract neomycin from tissues.

8.2.2. Liquid-Liquid Extraction

One of the most useful techniques for residue extraction from the biological matrix is liquid-liquid extraction (LLE) [37]. It is a technique applied for the isolation and extraction of analytes from a mixture using two immiscible extraction solvents [37]. The concept “like dissolves like” works well in the LLE method of antibiotic residue separation from the biological matrix. The capacity to isolate analytes from a mixture using this technique depends upon how differently the compounds in the sample mixture partition themselves between the two immiscible phases (solvents). By carefully choosing an extraction solvent, the analyte of interest (antibiotic residue) is meticulously divided into one of two immiscible or partially miscible phases [34]. LLE separates analytes from interferences by partitioning the sample between two immiscible liquids or phases. First, the component mixture is dissolved in a suitable solvent, and a second solvent that is immiscible with the first solvent is added. Next, the contents are thoroughly mixed (shaking), and the two immiscible solvents are allowed to separate into layers [38]. The less dense solvent will be on the upper layer, while the denser solvent will be on the lower layer. The components of the initial mixture will be distributed amongst the two immiscible solvents as determined by their partition coefficient. The relative solubility that a compound has in two given solvents can provide an estimation of the extent to which a compound will be partitioned between them [39]. A compound that is more soluble in the less dense solvent will preferentially reside in the upper layer. Conversely, a compound more soluble in the denser solvent will preferentially reside in the lower layer. Lastly, the two immiscible layers are separated and transferred, and the component in that solvent is isolated. Generally, after extraction, hydrophilic compounds are seen in the polar aqueous phase, and hydrophobic compounds are found mainly in the organic solvents [38]. Liquid-liquid extraction (LLE) has been exploited as an extraction procedure for aminoglycosides and macrolides from complex matrices. In a method published on determination of the streptomycin and dihydrostreptomycin, milk samples were prepared using LLE [40].

8.3. Sample Cleanup Methods

Most biological sample matrices contain endogenous compounds that have a negative impact on the detection of antibiotic residues, so after extraction, different cleanup techniques can be used to remove interferences. Interference is defined as any component of biological samples that can prevent or hinder the process of determining the analyte or drug residues [15]. The followings are important techniques used for the cleanup of drug residues from interferences.

8.3.1. Dispersive-Solid Phase Extraction (DSPE)

It is a cleanup method that requires the mixing of sorbent with the sample, which has been pre-extracted using proper extraction techniques. Proper sorbents adsorb matrix coextractives onto their surface, leaving analytes of interest in the solvent [41]. In the process, magnesium sulfate ($MgSO_4$) can be added to get extra cleanup by withdrawing residual water and some other components *via* chelation [41]. Subsequently, the mixture can be centrifuged, and the resulting supernatant or filtrate can be analyzed directly or can be subjected to a concentration and solvent exchange step [42]. DSPE is an extremely rapid, simple, and cheap process that provides high recovery and reproducibility for many liquid chromatography and gas chromatography-amenable analytes [42].

8.3.2. Immunoaffinity Column Chromatography

Nowadays, immunochemical methods are mostly applied for the separation of antibiotic residues from biological materials. This method gives high specificity, sensitivity, and sample throughput [43]. In this technique, antibodies against the analytes or residue of interest will be immobilized on the surface of a solid sorbent support that is packed into a syringe barrel. For the development of the immunoaffinity column method, different parameters may be adjusted to attain ideal separations. Some of the parameters include the properties of the sorbent, the integrating mechanism for immobilization of the antibody on the surface of the sorbent, and the property of the antibody. Optimal sample loading, proper cleaning, and elution techniques should be determined after the preparation of the immunoaffinity column. The effectiveness of the technique depends on the functionality of the antibody to bind the residue or analyte of interest. For better antibody-antigen interaction, it is necessary to work under optimal conditions, which are as close as possible to physiological conditions. Such characteristics limit the application of this technique to polar veterinary antibiotics. Maximal heat, pH, and organic solvent content may cause the denaturation of antibodies on the solid support. Throughout sample loading, some conditions must behave to the establishment of the antigen and antibody complex. The formed antigen-antibody complex should not be overblown by washing solvents and elution conditions, which are essential for the dissociation of the antigen-antibody complex [44]. Additionally, the dissociation of antibody/antigen complex must ideally be reversible. Therefore, the antigen-antibody complex can be comfortably reformed, which helps reuse the immunoaffinity column. Hou et al. [45] used immunoaffinity chromatography cleanup for the simultaneous analysis of avermectins in bovine tissues by the LC-MS-MS method [45].

9. Screening Methods

Several tests have been described for the screening of antimicrobial residues in various biological samples. Bio-based screening methods applied for the detection of antimicrobials in animal-derived food products have been reviewed [36, 46, 47]. The most frequently used bio-based screening methods for antimicrobials are microbiological inhibition assays, immunoassays, and biosensor tests [48].

In the process of screening methods, compliant samples are accepted, and suspected noncompliant samples have to be rechecked and confirmed using other confirmatory methods. A scheme of the typical screening analysis procedure is shown in Figure 1. In antibiotic residue determination, high-throughput methods with low cost and the ability to identify an analyte or class of analytes at the level of interest are needed [49]. In the event, antibiotics have a maximum residue limit, and the screening analytical method should be able to identify the residue under the maximum limit. The screening analytical methods must also avoid false negative results because they will be considered as compliant samples and will not be analyzed or determined by confirmatory analytical methods. Additionally, the screening analytical method must not give an excessive number of falsely noncompliant samples that will be later confirmed as compliant, despite the extra cost and time involved [50].

[figure(s) omitted; refer to PDF]

The followings are the important terminologies used to describe the screening analytical methods and to evaluate antibiotic residues in milk and meat.

9.1. Microbiological Inhibition Assays

Microbiological inhibition assays are one of the most widely used screening analytical methods. The principle of this technique is based on a reaction between bacteria and antimicrobials that are present in biological samples. Various biological tests were expanded to screen various antibiotic residues from animal-derived food products [36]. There

are two most common formats for microbiological inhibition assays, such as the tube and plate tests [51].
The tube test of a microbiological assay comprises of a growth medium inoculated with a bacterium, supplemented with a pH or redox indicator. Then, biological samples are added to the tube, and if there are no particular antimicrobials present in the biological sample, the bacteria begin to grow and produce acid, which will cause a detectable color change. Conversely, if antimicrobials are present in the biological sample that inhibits bacterial growth, no color change will occur in the tube [52].
The plate microbiological test consists of a layer of nutrient agar inoculated with bacteria, and the biological samples are brought onto the surface. If there is no specific antimicrobials are present in the biological sample, the bacteria begin to grow throughout the plate. If a specific antibiotic is present in the biological sample (meat or milk), no bacterial growth will take place on the sample that can be observed from the bacterial-free inhibition zone [51].
Now, microbiological inhibition tests are available in kits that can test a lot of samples quickly. This is called “high productivity.” Microbiological tests need restricted laboratory capacity to make certain reproducible situation of application. Microbiological tests are extensively used to perform antibiotic residue control [53].
The advantage of microbiological inhibition assays compared to immunoassays and instrumental analytical methods is that microbiological tests can detect any antibiotic residues that show antibacterial activity [54]. Moreover, these tests have the potential to cover the entire antibiotic spectrum within a single test [53]. The limitation of these techniques is their lack of selectivity, especially the tube microbiological inhibition test, relatively high detection limits, and the long bacterial incubation time. Consequently, microbiological inhibition assays are not suitable for the detection of banned antibiotic compounds like chloramphenicol [51].

9.2. Immunological Techniques

Antigen-antibody interaction has been used for many years to identify a wide variety of food constituents, including substances responsible for adulteration and contamination [55]. The interaction of antigen and antibody is very specific and useful for the detection of veterinary drug residues in animal-derived food products. The most widely used method comprises of the enzyme-linked immunosorbent assay (ELISA). Detection sensitivity depends on the strength of the signals during the reaction [56]. The ELISA-based detection system is usually based on enzyme-labeled reagents. There are various formats for the enzyme-linked immunosorbent assay (ELISA) technique. The first form of the ELISA technique is sandwich ELISA tests; in this technique, a primary antibody is bound to the plate well. Then, the antigen of the sample extract was added to the well complexes with the bound antibody and remains bound to the plate after washing. Then, a secondary antibody, which is labeled with an enzyme such as peroxidase, is added to the well followed by additional washing of the well. The quantity of conjugate bound to the plate is detected after incubation with a specific substrate [32]. The color is developed during incubation and measured with a microplate reader, which is proportional to the amount of analyte in the sample [32].
The second type of ELISA technique is direct competitive ELISA; in this technique, a primary antibody is coated onto the plate wells and incubated with the sample extract containing the antigens. After equilibrium is reached, an enzyme-labeled antigen can be added. This conjugate will bind to the free binding sites of the primary antibody. Thus, the more antigen in the sample (biological sample in this case), the lower the amount of enzyme-labeled antigen bound will be formed. Then, the appropriate specific substrate is added, and the plate is incubated for color development. In this case, there is an inverse relationship between the color developed and the concentration of the analyte in the sample [57].
The ELISA technique is an extensively used and specific test for the screening of veterinary antimicrobial residues in animal-derived food products. The competitive ELISA technique is frequently applied for the quantitative determination of antibiotic residues in meat and milk [57, 58]. Gaurav et al. [59] reported that tetracycline residues were detected in milk by competitive ELISA. From 133 cattle milk samples, 18 samples were found to be contaminated with tetracycline. The concentration of tetracycline residues in milk samples was found to be in the range 16–134.5 μg/l. According to the report, three samples exceeded the maximum recommended tetracycline antibiotic residue levels (MRLs). Sultan [60] reported that enrofloxacin residues in liver sample of poultry, sheep, and cattle collected from slaughter house Iraq. Out of 30 samples from each species, 17 poultry samples, 8 cattle

samples, and 5 sheep samples exceeded the maximum residue limits. The concentration of enrofloxacin in liver sample of poultry, cattle, and sheep was 10–10690, 30–3610, and 20–1320 μg/kg, respectively.

9.3. Biosensors

Biosensor is one of the screening analytical methods for veterinary drug residue analysis. Various types of biosensors (such as immunobiosensor, bacterial biosensor, optical biosensor) have been developed to determine antimicrobial drug residues. Biosensors employ biological molecules, such as enzymes or antibodies, which are efficient for recognizing particular targeted analytes or residues. In the detection process, the molecules are paired to a transducer, which response to the reaction between the residue and the bound biological molecule. The resulting biochemical alert is observed optically or changed to an electronic signal, which is additionally clarified by a suitable instrument. Biosensor tests are capable of identifying concurrent multiclass antibiotics and pesticides in biological samples at the same time. As some authors described, there is no need for sample cleanup for biosensor analytical technique [61]. A report from Möhrle et al. [62] showed that the biosensor method was used for the screening of macrolide antibiotics. Using an electrochemical biosensor, Ferrini et al. [63] determined β-lactams in milk samples by means of CO_2 measurement. The production of CO_2 was related to the microbial growth of the test microorganism, and the presence of β-lactams in milk inhibits the microbial growth.

9.4. High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC analytical technique allows the qualitative and quantitative determination of multidrug residues in animal-derived food products (meat and milk), but nowdays, its applicability has rapidly decreased due to the development of other advanced techniques like high-performance liquid chromatography (HPLC) [64]. Reported uses of HPTLC applied to meat include the determination of veterinary drug residues such as clenbuterol and other agonists [65], nitroimidazole and sulphonamides, and thyreostatic drugs [66]. Bartolucci et al. [67] presented a method using a TLC plate precoated with silica gel and eluting chemicals with 0.5 ml methanol-acetic acid-acetone (1 : 5 : 94, v/v/v) to analyze the sulfamethoxazole residues in milk. Reimer and Suarez [68] developed a TLC method with a high-performance TLC plate eluting with ethyl acetate-n-butanol-methanol-aqueous ammonia (35 : 45 : 15 : 2, v/v/v/v) to analyze sulphonamides in salmon muscle tissue. During the detection process, the HPTLC plates are sprayed with a proper chromogenic reagent or viewed under UV light for visualization of compounds. Detection by fluorescence is also applied. Quantitative analysis is achieved by measuring the relative intensity of the spot of the sample *vs* that of the internal standard by scanning densitometry [66].

9.5. High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is a separation analytical technique. The principles of HPLC involve the injection of a small volume of liquid samples into a tube (column) packed with tiny particles called the stationary phase. Individual components of the sample are moved down the packed column with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. The sample components are separated from one another by the column packing that involves various chemical interactions between the molecules and the packing stationary phase. The separated components are detected at the exit of the column by a flow-through device (detector) that measures their amount. An output from this detector is called a liquid chromatogram [69].

High-performance liquid chromatography is one of the most powerful analytical instruments in pharmaceutical analysis and analytical chemistry. HPLC has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Compounds at very low concentrations (as low as parts per trillion) may be easily identified by this technique. HPLC can be and has been applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, and forensic samples [69].

In the field of analyzing drug residues in biological samples from animals, HPLC is being used more and more every day. HPLC has different mobile phases, a vast library of column packings, and various modes of operations [70]. HPLC was used for veterinary drug residue determination of oxytetracycline and penicillin G in milk in Ethiopia, specifically samples collected from Nazareth dairy farms [71]. From 400 milk samples, 48 milk samples were found to contain oxytetracycline and penicillin G in the range of 45–192 μg/l and 0–28 μg/l, respectively.

HPLC technique was also used for the determination of sulphanilamide, tetracycline, streptomycin, and ciprofloxacin

In South Africa. A total of 150 samples of raw meat from sales points were analyzed, and the concentration ranges of 20.7–82.1, 41.8–320.8, 65.2–952.2, and 32.8–95.6 for sulphanilamide, tetracycline, streptomycin, and ciprofloxacin, respectively [72]. A study was also conducted in Iran to determine the residues of tetracycle groups (tetracycline, oxytetracycline, and chlortetracycline) from cattle tissue and organs, by using a high-performance liquid chromatography technique. The tetracycline concentrations in the triceps muscle, gluteal muscle, diaphragm, kidney, and liver were 176.3, 405.3, 96.8, 672.4, and 651.3 ng/g, respectively. The concentrations of tetracyclines were higher in liver and kidney samples compared to other samples [73] and were higher in cured meat products [74].

10. Confirmatory Methods for Antibiotic Residues

Confirmatory methods, mainly based on liquid chromatography combined with tandem mass spectrometry (LC-MS^2), are required for unequivocal identification and, if necessary, quantification of the analyte of interest. Only after a confirmatory analysis, a suspected contaminated sample will be declared noncompliant. However, Commission Decision 2002/657/EC still accepts detection techniques such as diode-array (DAD) or fluorimetric detection (FLD) as possible confirmatory techniques; nevertheless, from the practical point of view, confirmation of antibacterial residues in food is performed by LC-MS techniques since they can provide information about the chemical structure of the analyte [75].

Confirmatory analytical methods or techniques for determining veterinary drug residues or contaminants should give real information about the chemical structure of the residue or analytes. Methods that only use chromatographic analysis and do not use spectrometric detection are not good enough to be used as confirmatory methods on their own. However, if a single technique lacks sufficient specificity, the desired specificity may be achieved by analytical procedures consisting of suitable combinations of cleanup, chromatographic separations, and spectrometric identification [64].

LC-MS is the most commonly employed method for the determination of veterinary drug residues in animal-derived foods products [76]. The LC-MS technique uses LC as the separation system and MS as the detection system [77]. In order to quickly separate and identify numerous residues, it combines the high selectivity, high sensitivity, and relative molecular mass information of MS with the great separation capacity of chromatography for complicated materials.

LC-MS technique was applied for the determination of macrolides [78], sulphonamides [79], tetracyclines [80, 81] penicillins [82], quinolones and fluoroquinolones [83], and aminoglycosides [84] in the food of animal origin. High-resolution liquid chromatography combined with TOF-MS was used for the multiresidue determination of about 100 veterinary drugs in egg, fish, and meat [85]. LC/MS method was also developed and used for the identification and quantification of 30 antibiotics from four different chemical classes (sulphonamides, tetracyclines, quinolones, and beta-lactams) in Lebanon (Table 2). Out of 80 chicken muscle samples collected, 77.5% of samples were contaminated with antibiotic residues, out of which 53.75% were exposed to co-occurrence of multidrug residues [72].

Table 2

Antibiotic residues in different animal products.

Name of the antibiotic	Matrix	Extraction technique	Purification technique	Detection system	Recovery (%)	Reference
Tetracycline	Muscle	MSPD	Elution solvent: H_2O (70°C)	LC-MS/MS	99–103	[86]
Tetracycline	Milk	LLE	SPE (oasis HLB)	LC-MS/MS	74–101	[87]

Tetracycline	Porcine kidney	MIPs	Elution solvent: MeOH : 1 M KOH (9 : 1, v/v)	HPLC-UV		[88]
Sulfonamide	Milk	LLE	Ultrafiltration	LC-MS/MS	90–125	[89]
Sulfonamide	Muscle	LLE	LLP (H_2O: EtOAc)	UPLC-MS/MS	68–114	[90]
Quinolones	Bovine tissues	MSPD (sand)	Elution solvent: H_2O (100°C)	LC-MS/MS	87–109	[91]
Quinolones	Milk	MSPD (sand)	Elution solvent: H_2O (100°C)	LC-MS/MS	93–110	[77]
Quinolones	Eggs and tissue	MIPs	Elution solvent: ACN : TFA (99 : 1, v/v)	HPLC-FL	86–105	[92]
Aminoglycosides	Milk	MSPD (sand)	PLE	LC-MS/MS	70–92	[93]
Aminoglycosides	Muscle, liver	LSE	SPE (WCX)	LC-MS/MS	61–116	[94]
β-Lactams	Bovine kidney	LSE	DSPE (C_{18})	LC-MS/MS	58–75	[76]
β-Lactams	Milk	LLE	LLP	HPLC-UV	94–103	[95]
β-Lactams	Muscle	LSE	Ion-exchange SPE	LC-MS/MS	87–103	[96]

11. Conclusion

The repeated application of veterinary drugs to animals resulted in the occurrence of residues at various concentration levels in animal-derived food products. In particular, antibiotic residues in the dairy and meat industries may result in antibiotic resistance, which has an extensive public health consequence. The harmful effects of drug residues residing in animal-derived food products may also induce carcinogenic and mutagenic effects and lead to the condition of antimicrobial allergy in individuals who consume animal-derived food products. Accordingly, it is important to effectively control antibiotic residues in animal-derived food products. Effective residue monitoring requires specific, sensitive, and reliable analytical methods that can identify all veterinary drug residues under-regulated levels (MRL). In this review, sample extraction methods and analytical techniques for the determination of veterinary drug residues are summarized. For sample extraction techniques, LLE is widely used for the extraction of residue from biological samples. Solvent extraction especially organic solvent extraction is also applicable for residue extraction. The two types of analytical methods such as screening and confirmatory methods are discussed. Screening methods comprise microbiological, immunological, biosensor, thin-layer chromatography, and high-performance liquid chromatography. Liquid chromatography–tandem mass spectrometry is the most widely used confirmatory analytical technique for the determination of antibiotic residue in animal-derived food products. To sum up, determination methods for veterinary drug residues are developing toward high speed, high sensitivity, high throughput, and multiple residues.

Disclosure

A preprint of this article has been previously published [97].

Authors' Contributions

Feleke MG carried out the selection of kinds of literature on antibiotic residues using different instrumental analyses, designed the study, and participated in the write-up. Kasahun AE participated in the selection, conceptualizing, coordination, write-up, and final draft of the manuscript. All authors validate the final manuscript.

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DETAILS

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Design and Fabrication of a High Performance Microfluidic Chip for Blood Plasma Separation: Modelling and Prediction of System Behaviour via CFD Method

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ABSTRACT (ENGLISH)

This paper presents a single-step microfluidic system designed for passive separation of human fresh blood plasma using direct capillary forces. Our microfluidic system is composed of a cylindrical well between upper and lower channel pairs produced by soft photolithography. The microchip was fabricated based on hydrophobicity differences upon suitable cylindrical surfaces using gravitational and capillary forces and lateral migration of plasma and red blood cells. The plasma radiation was applied to attach the polymeric segment (polydimethylsiloxane (PDMS)) to the glass. Meanwhile, Tween 80 was used as a surfactant to increase the hydrophobicity of the lateral channel surfaces. This led to the higher movement of whole blood, including plasma. Fick’s law of diffusion was validated for this diffusion transfer, the Navier–Stokes equation was used for the momentum balance, and the Laplace equation was utilized for the dynamics of the mesh. A model with high accuracy using the COMSOL Multiphysics software was created to predict the capillary forces and chip model validation. RBCs (red blood cells) were measured by the H3 cell counter instrument, by which 99% plasma purity was achieved. Practically, 58.3% of the plasma was separated from the blood within 12 min. Correlation between plasma separation results obtained from software and experimental data showed a coefficient of determination equal to 0.9732. This simple, rapid, stable, and reliable microchip can be considered as a promising candidate for providing plasma in point-of-care diagnostics.

FULL TEXT

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1. Introduction

Blood plasma is a primary source for the preparation of biological products. The presence of biomarkers in blood plasma has given its diagnostic value in the clinic. Plasma separation is a prerequisite for blood analysis in some diagnostic studies [1–3]. Differences in the particle sizes of blood and plasma have been used to separate blood components [4]. Several methods, such as centrifugation and hemapheresis, have been utilized to separate plasma from blood. However, conventional plasma separation processes have not been favorable for patients who require

regular blood monitoring due to limitations of high cost, time-consumption, and inaccessibility [5]. The need for high blood volume and energy consumption is the other disadvantage of this method. So, simple and inexpensive alternative methods are needed for plasma separation. One of the new and efficient methods for separating blood compounds is using microfluidic devices with microfluidic-based systems, attempting to minimize the disadvantages of conventional methods [6–8].

It is well known that rapid blood tests at the beginning and over the course of treatment are significant. By using microfluidic device technology, not only has the whole blood analysis method been integrated into small devices but also the sample size, response time, and costs for large-scale production have been reduced [9]. Nowadays, two methods, named active [10] and passive [11] methods, have been reported for the development of chips to separate plasma, besides paper-based [12] and CD shape-based [13] microfluidic systems.

In an active strategy, the energy required for plasma separation is provided by the acoustic or electromagnetic fields, making the system more complicated. Active method-based devices have reduced the time needed for samples to reach the required situation. However, hydrodynamic forces and cell responses to various biophysical effects have been reported as the basis for passive-based devices. Nonstop operating, easy manufacturing, low cost, and simple design have been the factors that have led many researchers to focus on the importance of finding more efficient methods. Various techniques, including sedimentation, microfiltration, and hydrodynamic forces, have been used in passive devices based on the mechanical properties of particles. Passive devices have induced less stress on red blood cells than active ones, resulting in less hemolysis and more reliable diagnosis [14].

In addition, the necessity of a pump to inject blood samples into the device has been reported to be one of the major limitations of both active and passive devices. The major drawbacks reported in both studies were a low plasma recovery rate of about 3.4%, and challenging and expensive microchip construction. Some studies have used a vacuum desiccator method to inject the blood samples into the microchannel [15].

In the last decade, principles of capillary force, blood cell deposition, and cross-flow filtration have been applied to design and construct plasma separation by microfluidic systems. One study has extracted plasma by a “capillary flow and cross-flow filtration” method after PDMS modification of microfluidic channels by a surfactant without any external forces [16]. In another study, the asymmetric capillary flow of a microfluidic chip was designed through surface modification of the channel bonds by a multilayered spray coating of silica nanocrystals [17].

Some researchers have fabricated a bilayer PDMS microchip in which the upper membrane filter separates the plasma from the blood using gravimetric force [18]. In a similar study, a pump and vacuum were used to separate plasma and blood samples from the bottom of a PDMS cylindrical channel during plasma separation moved upwards. Meanwhile, the authors have used diluted blood as an inlet into the system. In addition, the time gap between the blood inlet in the first step and vacuum insertion in the final step showed the limited performance of the device. Valuable efforts have been made to develop a model for non-Newtonian fluid flow upon growing needs for diagnostic applications [19]. Liu et al. have interpreted the flow of non-Newtonian fluids through a direct channel using an altered Lucas–Washburn equation. Danilov et al. have investigated theoretical and experimental aspects of fluid flow through the capillary channels using non-Newtonian behaviour and a dynamic contact angle [20].

Some efforts have been made to commercialize microfluidic chips for plasma separation. CD-based microfluidic chips proposed centrifugal force for particle separation goals. Li et al. have designed the systems based on capillary and centrifugal forces and particle deposition, separating plasma with 99% purity [21].

Maria et al. designed a microfluidic chip with a wettability gradient and a cylindrical chamber that separated blood plasma after 15 minutes with a purification efficiency of about 99.9%. Using this system, variation of contact angle on the inner surface, self-built-in filter, and sedimentation resulted in plasma separation. They developed another microfluidic system-based plasma separation method to measure TSH (thyroid-stimulating hormone) levels using physical barriers proportional to blood and plasma particles. Finally, Liu et al. developed microchannels having both hydrophilic and hydrophobic properties to extract plasma with a purity of 85% in less than 10 minutes [22].

In addition, the importance of microfluidic chip modelling is due to the improvement of experimental performance constraints, optimization of the processes, and achievement of exact results. For example, Zhang et al. considered

using a microfluidic chip with dielectric properties to classify blood cells based on the size to separate blood particles.
MATLAB and COMSOL software were used to calculate separation conditions, and in order to simulate the motion trajectory of cells in the microfluidic channel, the most effective parameters were selected [23].
Li et al. achieved 64% plasma separation by using a numerical technique to design a highly efficient microfluidic chip. Blood flow simulations were performed by a hybrid method of smoothed dissipative particle dynamics. The level of injected diluted blood and the speed of injecting blood in experiments have been declared by the immersed boundary method. The designed chip showed 40% efficiency in comparison with experimental plasma separation [24].
Shamloo et al. have presented a simple passive microfluidic device for blood plasma separation. Numerical studies and CFD simulation were used to solve the flow field, track the particles confined in it, and optimize channel dimensions and orientation angles. Utilizing optimization, they demonstrated that the performance of the device could be improved considerably, and an optimal design with a separation efficiency of 83% and a purity of 85% was achieved [25].
The purpose of the existing study was to extract the blood plasma for rapid diagnostic application at the point of care by designing a new surface-modified microfluidic chip through software simulation to predict and improve the performance of the device using the feedback from the experimental data. In this regard, for the recognition and prediction of the surface-modified microfluidic chip system from the CFD model, COMSOL metaphysics software was used. In addition, the CFD model designed based on fluid mechanics and mass transfer equations was validated by experimental data, and by using the validated model, the amount and purity of the extracted blood plasma were studied.

2. Research Methodology

2.1. Fabrication Procedure of Microchip

Standard soft lithography was utilized to fabricate the microfluidic devices as follows: a 180 μm thickness was obtained by spin coating SU8-2050 (MicroChem, USA) at 1000 rpm for 30 seconds on a silicon wafer diced at 7 cm by 4 cm. It was patterned by our designed mask under UV light to create a master mold to construct the bottom channel. The polydimethylsiloxane (PDMS) base and curing agent were mixed at a ratio of 10 : 1 (w/w), which was followed by degassing in a vacuum jar for 30 min. It was then poured on the silicon mold using a Petri dish and heat-treated at 70°C for 3 hrs. After detaching the PDMS from the mold, both the inlet opening and the cylindrical wall were punched with a biopsy punch. The bottom surface of the PDMS was cleaned with adhesive tape and then treated with air plasma (2 min at 1 mbar) along with a glass slide for permanent bonding. Similarly, a master mold was prepared using the SU8-2050 (1700 rpm for 30 s) to construct the top channel with 100 μm of thickness. After melding PDMS on the mold, the bottom surface of the top channel and the top surface of the bottom PDMS slab were exposed to air plasma. Then, they were bonded after aligning the top channel on the punched well. The whole device was finally cured at 100°C for 10 min. The change in different experiment parameters for the fabrication of microchips is shown in Table 1.

Table 1

The change of different experiment parameters for the microchip fabrication method.

No.	Subsurface					Top surface					Mold	
SU8 (rpm)	Basic baking (min)	Expose (s)	Later baking (min)	Hard baking (min)	SU8 (rpm)	Basic baking (min)	Expose (s)	Later baking (min)	Hard baking (min)	PDMS weight (gr)	Curing (cc)	1

1000	30	10	12	3	1600	20	10	10	5	21	0.097	2
1500	40	10	12	4	3000	20	10	10	4	21	0.18	3
1600	45	10	12	4	3000	25	10	11	4	21	0.17	4

Method number 2 was selected because of its better efficiency in blood plasma separation.

The cylindrical shaft part of the chip was fabricated of three sections: two hydrophilic upper and lower parts connected by one hydrophobic region in the middle. For this, the upper and lower segments were coated with Tween 80, while a small fraction of 1 mm at the top of the device (height) was covered by a rubber hose barrier (not coated). The plasma bonding was used for bonding the entire lower polymeric part to increase its hydrophobicity.

To measure the accuracy of blood volume, entering the inlet and also plasma volume, leaving the outlet, the whole blood was introduced into the device using a syringe pump at 0.8 μL/min, so the entrance volume was measurable. A pipette tip was inserted in the outlet to collect plasma. By connecting the pipette tip to a measurable micropipette (0.1–10 μL), it was possible to measure the plasma volume, easily by adjusting the gauge. Therefore, time, flow rate, and plasma volume were measurable using microsyringe device and micropipette accurately and precisely. In addition, the plasma purity was monitored by the H3 cell counter instrument.

The device's inlet diameter as well as its height inlet was adjusted to control the initial driving force. While a low amount of blood entered via the inlet region, the blood flow stopped due to the coagulation phenomena (Figure 1(a)). Meanwhile, when high blood quantity entered via the channel inlet region (Figure 1(b)), it passed through the cylindrical shaft with no separation. After several attempts, the optimized quantity of 10 μl blood was chosen as the appropriate amount of blood to be applied (3–5 droplets).

[figure(s) omitted; refer to PDF]

2.2. Tuning the Vertical Channel Height and the Duration of Bonding

The duration of plasma bonding was a very important factor when PDMS stuck to the silicon. The proper time for the operation was estimated to be 2 minutes. Furthermore, because the height of the cylindrical shaft depended on the height of the lower polymer block of the chip, the most appropriate size was determined to be between 4 and 5 mm, while exposure time was 2 min.

According to Table 2, by increasing the time of plasma bonding, the amount of hydrophobicity of the device was raised more than the desired limit for plasma separation (2 min), while the hydrophobic area in the middle of the cylindrical shaft was consumed at 1 mm. In optimal conditions (i.e., 2 min duration for plasma bonding and 4 mm of block height), a short distance was needed for blood to self-filtrate in the cylindrical shaft for the separation process.

Table 2

Different states of the plasma bonding device.

Raw	Polymer block height (mm)	Plasma bonding time (min)	Performance
1	3	4	Unfavorable
2	5	4	Unfavorable
3	**4**	**2**	**Favorable**

4	6	2	Nearly unfavorable
5	4	5	Unfavorable
6	**4.5**	**2**	**Favorable**
7	4.5	4	Unfavorable
8	6	4	Unfavorable

States 3 and 6 were selected because of better performance.

On the other hand, when the height of the polymer block was lower than the optimum size (4 mm), the plasma separation process became much more difficult due to its greater hydrophobicity. Conversely, when the polymer block height was more than the optimal size, the length of the hydrophilic increased, causing the whole blood to be removed. The separation section is shown in Figure 2(a).

[figure(s) omitted; refer to PDF]

2.3. Preparation of Blood Sample

The rheological properties of blood are affected by various environmental factors, such as temperature, pressure, and storage; blood should be freshly prepared before injection. Here, 10 μl of blood were poured into the inlet opening channel using a syringe pump for the separation process. Before introducing the blood into the chip, the number of cells of each blood sample was counted using a cell counter for future comparison. To facilitate the quantification of cells by the cell counter, adding the minimum amount of anticoagulant to the samples was necessary. The results of four blood determinations (WBC, RBC, HGB, and HCT) from cell counter data were compared to separate plasma exiting from the outlet opening chip.

The amount of blood needed to be optimized, i.e., the plasma separation process is actually affected by both capillary force and surface tensions induced by fabricated variables. If not, the greater volume of blood will show initial force movement, which can prevent plasma separation in the cylindrical shaft.

2.4. Plasma Separation Mechanism and Measurements

The basis of separation in the cylindrical well part based on the schematic below (which includes two hydrophilic parts and one hydrophobic part at the well part and the horizontal hydrophilic part of the microchip) is that when the blood sample reaches the first hydrophilic part of the cylindrical well part, due to the capillary force of the surface of the microchip and the hydrophilic nature of the plasma, the microchip separates the plasma from the other particles of the blood components, and this force passes the plasma through the middle nonhydrophilic region and directs it to the second hydrophilic part and the outlet of the chip. Also, the particles of blood that remain at the beginning of the hydrophobic part act like a filter for other blood particles, and thus separation is done. In this work, to improve separation, in addition to making two parts of the well hydrophilic, we also made the horizontal part of the microchip hydrophilic using Tween 80, which helped the performance of the chip in separation. Before measuring the outlet fluid flow from the chip, two parameters were evaluated: (a) plasma appearance and (b) plasma purity. Plasma purity was determined by the cell counter using the following:(1)$\text{Plasma Purity} = 1 - \frac{\text{number of blood cells in the output}}{\text{number of blood cells in input}}$.

Subsequently, to determine the volume of separated plasma from blood samples using a microfluidic chip at certain times, the COMSOL Multiphysics software was used.

2.5. Modelling

The 2D geometry arrangement of the plasma separator microchip as shown in Figure 3 was modelled in the form of the continuous flow at the microchannel in COMSOL Multiphysics (v.5.5). The main benefit of the suggested model is to display how the fluid flows into the microchannel and predict the amount of plasma separation and plasma concentration profile at each moment of the process. By using the suggested model, the behaviour of the system

can be studied with great accuracy. Finally, using equations (2) and (3), the dependent and average of the squares of the errors between the modelling and experimental data were determined.(2)R2=∑i=1nxi,exp−xavg2−∑i=1nxi,exp −xi,Model2∑i=1nxi,exp−xavg2,(3)MSE=∑i=1nxi,exp−xi,Model2N.

[figure(s) omitted; refer to PDF]

2.5.1. Governing Equations

The fluid flow in the microchannel was created under the influence of the capillary force of the microchannel hydrophilic part. The equations used for the modelling of the separation of blood plasma in the designed microchip are the continuity equation for the mass balance, the Navier–Stokes equations for the momentum balance, Fick's law equation for the mass transfer of the plasma, and finally, the Laplace equation for the dynamics of the mesh of the domain. The equations mentioned are listed in Table 3. In this table, μl, ρl, and v represent the sample's viscosity, density, and velocity vector inside the microchip, respectively. Also, DiT, Dif, ωi, Mi, Mn, and x, respectively, represent the thermal diffusion coefficient of the sample components, the mass diffusion coefficient of the sample components, the mass fraction of the sample components, the molecular weight of the sample components, the average molecular weight of the sample, and the separated plasma displacement vector. The following assumptions were also considered to solve these equations in the generated geometry:

(i) The viscosity and density of the blood sample were considered constant in the entire microchannel (at the ambient temperature and pressure)

(ii) The surface tension and contact angle of the blood sample plasma were considered constant in the hydrophilic area of the microchannel (at the ambient temperature and pressure)

(iii) The fluid flow inside the microchannel is considered stationary

(iv) The operating temperature of the solved model is assumed to be constant throughout the microchannel

(v) The diffusion coefficient of Fick's law equation for major components of the blood sample throughout the microchannel was supposed to be constant

Table 3

Governing and supplementary equations used in modelling.

Continuity equation	∇.ρlv=0
Momentum equation	∇.ρlvv=−∇p+∇.+ρlg
=μl∇v+∇vT	-
Mass transfer equation	∂ρlωi/∂t+∇•ji+ρv•∇ωi=0ji=−ρlDif∇ωi+ρωiDif∇Mn/Mn−jc.i+DiT∇T/T
Mn=∑iωi/Mi−1jc.i=ρlωi∑KMi/MnDKf∇ωK∇T=0	-
Dynamic mesh equation	∂2/∂X2∂x/∂t+∂2/∂Y2∂x/∂t=0

2.5.2. Boundary Conditions

All the boundary, initial, and volumetric conditions used to solve the partial differential equations mentioned in Figures 3(a)–3(c) are specified in full detail.

3. Results and Discussion

In this section, the results of microchip output as plasma separation efficiency were compared with the theoretical results obtained from modelling using the CFD method with COMSOL software. A batch system with a specific initial input value was used and designed to achieve plasma separation and microchip modelling.

3.1. Experimental Results

According to Table 1, method number 2 was selected because of its better efficiency in blood plasma separation. Therefore, this term formed the basis of simulation and manufacture.

3.1.1. Plasma Separation Efficiency

The amount and purity of the separated plasma were considered as criteria for separation efficiency. For diagnostic applications, the purity of outlet plasma is very important, whereas for plasma production purposes, the amount of plasma is the critical point.

3.1.2. Amount of Separated Plasma

In practice, after 12 minutes, the separated plasma was collected at the chip output by a syringe, and its volume was measured. However, to obtain the exact amount of separation based on time, COMSOL software was used. The output plasma volume at different times is reported in Table 4. At 12 minutes, the highest separation rate was recorded, which was 58.3% of the available plasma, equal to 3.5 microliters.

Table 4

Plasma separation efficiency for different time.

Time (min)	Outlet plasma amount (μl)
3	1
7	2.3
9	2.8
12	3.5

3.1.3. Purity of Separated Plasma

The results obtained from the cell counter device before and after separation are reported in Table 5. Values of four parameters, including RBC, WBC, HGB, and HCT, were compared. As recorded, the RBC count (before separation) was 4.42 million and that of white blood cells was 7,000 per 1 μl.

Table 5

Comparison of results between inlet fresh blood and outlet separated plasma by H3 cell counter.

Blood cells	Fresh blood	Separated plasma
WBC	7.11∗103/μl	7∗101/μl
RBC	4.42∗106/μl	4.34∗104/μl
HGB	12.2	0.006
HCT	39.9%	0.08%

Considering the analysed data for inlet and outlet of blood particles obtained by H3 cell counter measurement, as shown in Table 5, plasma purity calculated by (1) was obtained as 99%. This high purity demonstrates the excellent microchip performance of plasma separation in this study.

3.2. Modelling Results

3.2.1. Model Accuracy

The results of Figure 4(b) showed the value of mean squared error (MSE) and coefficient of determination (R^2) between the model and experimentally measured data. The mean squared error and the coefficient of determination were about 0.04585 and 0.9732, respectively, representing proper compatibility between the model and experimentally measured data. Therefore, the mathematical model of CFD can be utilized to predict the behaviour of the designed laboratory system, parametric study, and how blood plasma is separated.

[figure(s) omitted; refer to PDF]

To verify the CFD modelling with experimentally measured data, region size and boundary conditions were considered to be equal to the utilized one in the laboratory system. For comparison, the modelling data were compared to the laboratory data. As shown in Figure 4(a), the modelling outcomes were in high-grade accordance with the measurements gained experimentally, as demonstrated in Figure 4(b). Also, the difference between the values of experimental data and modelling results can be attributed to the error of measuring the data by the operator in the laboratory, the error due to the rapid coagulation of the blood sample in contact with the ambient air of the laboratory, and the error of numerical modelling, and simplifying assumptions.

3.2.2. Evaluation of Velocity Profile of Sample and Plasma Concentration and Displacement Rate of Separated Plasma Inside the Microchip

As mentioned in the previous sections, due to the excellent fitness between the laboratory and the modelling data, the steady-state velocity profile of the blood sample was obtained at different intervals of 3, 7, 9, and 12 minutes in Figures 5–7, respectively. As shown in Figure 5, the capillary force generated by the designed microchip causes suction of the plasma in the blood sample. This force creates convection mass transfer of the plasma and finally leads to movement of the plasma across the microchip towards the hydrophilic region and plasma separation. According to Figure 6, plasma concentrations throughout the microchip decreased over time due to convection and diffusion mass transfer into the hydrophilic region, and plasma concentrations in the hydrophilic region continuously increased. Finally, Figure 7 shows the displacement rate of separated plasma over time.

[figure(s) omitted; refer to PDF]

As seen, the height of the right side of the microchip increased over time, indicating the separation of plasma from the blood sample by the designed microchip. It should also be noted that the change in sample height at the entrance of the microchip was assumed to be negligible due to the large inlet diameter.

3.2.3. Prediction of the Amount of Plasma Separated Using CFD Modelling

As the CFD model was fitted to experimental data efficiently, the model was used to predict the end time of the complete plasma separation. Figure 8 shows the percentage of separated plasma volume over time based on the CFD model. It is clear that in about 88 minutes, a relatively complete separation percentage (99.9%) can be achieved by the designed microchip. However, this long time is due to the coagulation that occurs along the path and reduces the flow rate. However, the highest separation rate was recorded at 12 minutes, which is enough for the subsequent analysis.

[figure(s) omitted; refer to PDF]

Taken together, in the study, a software simulation was used to predict and improve the performance of the microdevice for plasma separation based on the feedback from the experimental data. In addition to acceptable correlation between experimental and software data, better separation efficiency and separation time were concluded in comparison with published data, introducing a simple, rapid, and high-throughput device for diagnostic application in resource-limited environments and point-of-care settings such as measuring biochemical components and blood biomarkers can be measured.

4. Conclusions

In this design, we fabricated a novel microfluidic system and modelled this system using transport phenomena in COMSOL Multiphysics software for plasma separation. Simulated aspects and feedback were applied to improve device fabrication in terms of flow rate, hydrophobicity, channel length, input load, and reaching a separation efficiency of 99%. An acceptable correlation was obtained between experimental data and data obtained from the software. The separation time of 20 minutes reported by Maria et al. [22] was improved to 12 min in our microchip.

Geometrically, the size of the lower part of the channel was decreased to get the desired separation condition through a cylindrical well. In comparison, the improved hydrophobicity of the horizontal channel increased blood flow to the cylindrical well, leading to decreased time of separation. Performing multiple tests to determine the system's performance is very time-consuming, costly, and has problems with regards to the preparation of blood samples and equipment. Hence, the process of separating plasma from blood samples was modelled using the CFD method based on transfer phenomena. The coefficient of determination ($R^2 = 0.9732$) of the CFD model with Fick's law, Navier–Stokes equations, and the Laplace equation showed the suitability of the model to estimate and predict the value of separated plasma. Consequently, the suggested model can be applied to other complex microchips in different operating conditions. The development of such novel microchips can be helpful for plasma production and postchip plasma analysis in clinics. This simple, fast, stable, high-throughput, and reliable microchip can benefit for providing plasma in point-of-care diagnostics.

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DETAILS

Subject:	Dielectric properties; Model accuracy; Finite element method; Software; Plasma; Research methodology; Microfluidics; Semiconductors; Erythrocytes; Plasma radiation; Photolithography; Hydrophobicity; Separation; Blood plasma; Design; Polydimethylsiloxane; Silicon wafers; Contact angle; Blood; Energy consumption; Laplace equation; Efficiency
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Green Spectrophotometric Determination of Organophosphate in Selected Fruits and Vegetables

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ABSTRACT (ENGLISH)

A simple, sensitive, precise, and environmentally safe spectrophotometry method was developed and validated for the determination of organophosphate in various fruits and vegetables using a UV-Visible spectrophotometer using a magnesia mixture. The volume of reagent used for analysis and the stability of the color complex were also optimized. The drug showed a stable white color complex at 420 nm. The greenness of the methods was estimated using an ecoscale (84), the Green Analytical Procedure Index, and AGREE (0.89), which were found to be excellent green method based on spectrophotometric determination. The method was validated using ICH guidelines and has acceptable values for linearity (0.5–2.5 mg/ml), accuracy (98.5–102.5%), precision, robustness, limit of detection (0.16 mg), and limit of quantification (0.486 mg). The concentration of the organophosphate in the analyzed sample was in the range of 0.003 to 2.45 mg. Altogether, the proposed green analytical method was found to be a simple, selective, sensitive, accurate, and ecofriendly method for the analysis of organophosphate in various fruits and vegetables.

FULL TEXT

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1. Introduction

Pesticides are chemical substances or mixture of chemicals meant for preventing, repelling, destroying, or controlling any pest like insects or other organisms. As per WHO 2008, pesticide poisoning is any pesticide-related injury or health effect including systemic and nonsystemic effect, resulting from suspected or confirmed exposure to a pesticide [1]. These are synthetic molecules aimed at being toxic towards fungi, plants, or animals that are detrimental to cultures. Fungicides, herbicides, and insecticides have been developed in order to control as specifically as possible these pests in order to protect cultures. Nevertheless, these pesticides can be toxic to human and wild fauna [2]. Vegetable cultivation attracts high rates of the application of pesticides and farmers in many developing countries use many acutely toxic insecticides on those crops. Apart from application of pesticides exposures can also occur for farmers involved in the harvesting process or who enter the sprayed field too soon after spraying [3]. Farmers, particularly in areas of commercial vegetable production, are primarily relying on chemical pest control methods. The frequency of pesticides used is 3 times per week. Around 97.6% of subjects used incomplete personal protective equipment [4].

Most of the Nepalese population lives in rural areas, and nearly 80% of the country's people are involved in agriculture which is an important factor for the national economy. Cultivation of vegetables in the agricultural lands of Nepal normally generates high earnings per unit area in comparison to the crops although it is cultivated in a wide area of Nepalese agricultural lands. The number of small-scale farmers growing vegetables for the domestic market in Nepal is increasing. Pesticides are widely used in Nepal to control various pests and disease in agriculture as well as livestock production. The increasing demands of food and vegetables have led to an increased use of pesticides. Among the pesticides used in Nepal, organophosphate compounds are the most commonly used [3].

In the 1930s, organophosphates were used as insecticides, but the German military developed these substances as neurotoxins in the Second World War [5]. Organophosphates (OP) are chemical substances produced by the process of esterification between phosphoric acid and alcohol. These chemicals are the main components of herbicides, pesticides, and insecticides [6]. Organophosphate poisoning can be acute or chronic. The symptoms of acute toxicity are hypersecretion, bronchoconstriction, myosis, diarrhea, bradycardia, central nervous system (CNS) depression, seizure, cyanosis, and coma [7]. The organophosphate poisoning was experienced by farmers through inhalation and dermal contact. The severity depends on pesticide type, dose, and duration of application and frequency of application. The intensity of organophosphate poisoning was influenced by the area of pesticide application, climate skill of the application, and personal [8]. The mechanism of organophosphate`s toxicity is via inhibiting the acetylcholinesterase (AchE), is an enzyme that degrades the neurotransmitter acetylcholine to choline and acetic acid. The inhibition of AchE will cause the increase in acetylcholine concentration in the synapse. This mechanism will cause some nicotinic and muscarinic symptoms and central and peripheral nervous system toxicity [9].

Due to the excess demand of numbers of fruits and vegetables, Nepal depends a lot in India which supplies these food stuffs from a long decade. There were few news stories in the newspaper, TV, and so on. related to use of excess pesticides in fruits and vegetables which are transported in border area of Nepal from India. Hence, this study will definitely provide the scientific statement regarding this issue. Among various fruits available in the markets, the selection of fruits i.e. mango and watermelon is to rationalize the study as these fruits are mostly available and consumed fruits in our Nepalese society. These are consumed in every family visit and devour by children, so studying these can make our research more relevant. The reason for choosing other fruits like kiwi is the popularity in the current context of Nepal. Kiwi and lemon are now famous as a source of Vitamin C, support heart health and the digestive system, protect anemia, reduce cancer risk, and to boost immune system. There is a rising demand for organic agricultural products due to consumer concern about the strong contamination of vegetables from the applied pesticides. Therefore, it is essential to identify the presence of pesticide residue in vegetables so that consumers could be protected. In this regard, this study is going to be initiated to assess the present status of organophosphate pesticide residue in eggplant, tomato, cucumber, cabbage, ladyfinger, cowpea, pointed gourd, and bean.

Various research studies have conducted for the determination of the organophosphate in foods using methods such as P-NMR [10], conductometry [11], and GC-MS/GC-NPD [12], where most of the methods use HPLC [13–16] or liquid-mass spectrometry [17]. Since these methods are highly sophisticated, expensive, and use a large amount of organic solvents along with a high amount of instrumental energy, so a developing country such as Nepal could not afford the availability of such machines in every local area. The use of less sophisticated analytical methods like UV-Visible spectrophotometer with more concern on greenness profile, if provides the similar results like above-mentioned methods, this will be an extremely economical method of detection. Hence, a simple, green economic UV-Visible spectrophotometric method was developed and validated for the analysis of fruits and vegetables.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

The fruits and vegetables (mango, kiwi, lemon, watermelon, tomato, cowpea, cucumber, cabbage, pointed gourd, ladyfinger, and eggplant) were collected from the commercial market of Raxaul, India, and local market from

Chitwan.

2.1.2. Pure Sample and Chemicals

Ethanol 95% (Changshu Hongsheng Fine Chemical Co. Ltd.), bromine water 99% (Nike Chemical India), disodium hydrogen orthophosphate 99.5% (Merck Life Science Pvt. Ltd.), ammonium chloride 99.7% (Sisco Research Laboratories Pvt. Ltd.), and magnesium chloride 99.0–101.0% (Fisher Scientific) were used for this research.

2.1.3. Equipment and Software Required

LT-2100 double beam UV-visual spectrophotometer with a 10 mm quartz cuvette was used to record the absorbance. The greenness of the method was established using GAPI chart version 1.0.0.

2.2. Methodology

2.2.1. Collection of Samples

The fruits and vegetables samples were collected from the local market (Chitwan) as well as from Raxaul, India, and proceed for extraction.

2.2.2. Extraction

A stainless steel knife was used to cut fruits and vegetable samples, sliced and diced them, and 1 gm sample was placed inside a glass test tube, as shown in the Figure 1. The extraction was carried as per Knowledge-Based Integrated Sustainable Agriculture and Nutrition (KISAN) II Project [18] using 2 ml of 95% alcohol (ethanol) and bromine water solution. Finally, the extracted solution was poured into a clean test tube prior to proceeding for test.

[figure(s) omitted; refer to PDF]

2.3. Method Development

Method development was done following the article published by Kumar Reddy et al. [19].

2.3.1. Selection of Suitable Wavelength

The wavelength maximum was selected by varying the wavelength from 200 to 800 nm.

2.3.2. Selection of Reagent Volume

The volume of optimization was carried out by varying the volume from 1 to 3 ml.

2.3.3. Mechanism of Color Complex Formation

Magnesia mixture was prepared by adding 2 gm magnesium chloride solution to 1 gm ammonium chloride and adding about 30 drops of ammonium hydroxide to above-mentioned solution after boiling and cooling it till the strong smell of ammonia is obtained. Thus, prepared solution is allowed to react with the sample extract to obtain white color of the magnesium phosphate and detected in UV-Visible spectrophotometer.

The intensity of the white precipitate depends on the amount of magnesium phosphate, which in turn depends on the phosphate present in fruit and vegetable samples. This is the basis of organophosphate pesticide analysis.

2.4. Sample Preparation

From each of the extracts, 1 ml sample was taken and added with the 2 ml of magnesia mixture in 10 ml of volumetric flask. The final volume is made up by distilled water for both Indian and local samples.

3. Results and Discussion

3.1. Selection of Wavelength Maxima

Disodium orthophosphate was taken as the standard for this analysis. It was allowed to react with the magnesia mixture to form a white color complex. The absorbance was observed at the maximum wavelength 420 nm after varying wavelength from 200 to 800 nm.

3.2. Optimization of Volume of Reagent

The optimization of the reagent was first established by varying volume of the reagent (1 ml to 3 ml), where the maximum absorbance at 2 ml was found. Hence, it was selected after optimizing volume of reagent, as shown in Table 1.

Table 1

Optimization of volume of reagent.

Volume of reagent (ml)	Absorbance
1	0.115
1.5	0.718
2	**0.836**
2.5	0.720
3	0.831

3.3. Stability of Colored Complex

After optimizing the volume of the reagent it was subjected for the stability test. The optimum time for completion of the reaction between disodium orthophosphate and magnesia mixture to obtain the white color was 1 min, and the complex was stable for 1 hour. Then the absorbance was measured, and it was quite stable with precise measurement which is shown in Table 2.

Table 2

Stability of colored complex.

Time (minute)	Absorbance
10	0.857
20	0.854
30	0.818
40	0.818
50	0.809
60	0.802
70	0.772

3.4. Preparation of Calibration Curve

0.5 gm of disodium orthophosphate was weighed and dissolved in distilled water and made up to 100 ml to prepare a 5 mg concentration of solution. The secondary stock solution was prepared at 0.5 mg, 1 mg, 1.5 mg, 2 mg, and 2.5 mg concentrations, respectively, after the addition of magnesia mixture and dilution with water. Finally, absorbance was observed in a UV spectrophotometer at a wavelength of 240 nm after obtaining it as the maximum wavelength.

3.5. Determination of Concentration of Organophosphate in Samples

3.5.1. Analysis in Vegetables

All randomly selected samples were collected and extraction of it was carried out. 1 ml of the extract was taken and diluted to 10 ml volumetric flask with 2 ml of magnesia mixture and 7 ml of water. The mixture was observed in a UV-Visible spectrophotometer and the absorbance obtained was recorded. Finally, the organophosphate concentration

on each vegetable was calculated and compared, as shown in Figure 2 and a statistical analysis was established (Table 3).

[figure(s) omitted; refer to PDF]

Table 3

Statistical analysis of the data.

Concentration of Indian sample in mg (X_1)	Concentration of local sample in mg (X_2)	X_1^2	X_2^2
1.976	0.473	3.904576	0.223729
0.024	0.003	0.000576	0.000009
1.992	0.007	3.968064	0.000049
2.144	0.029	4.596736	0.000841
1.992	0.007	3.968064	0.000049
1.353	0.013	1.830609	0.000169
$\sum X_1 = 9.481$	$\sum X_2 = 0.532$	$\sum X_1^2 =$ 18.26863	$\sum X_2^2 =$ 0.224846

$S_p^2 = 1/(n_1 + n_2 - 2)\ [\sum X_1^2 - (\sum X_{1)}^2/n_1 + \sum X_2^2 - (\sum X_2)^2/n_2]$. $S_p^2 = 0.346$. Again, mean of first sample $= \sum X_1/n_1 = 1.58$. Mean of second sample $= \sum X_2/n_2 = 0.00887$. Similarly, independent *t*-test (t) = (mean of 1st sample − mean of 2nd sample)/√ (S_p^2 /(1/n_1 + 1/n_2) = 4.391244267. Here, d.*f*= $n_1 + n_2 - 2 = 6 + 6 - 2 = 10$. The tabulated value of *t*-test at 5% level of significance at 10 d. *f.* is 2.228 (two tails). Now, p value for the *t*-test is between 0.002 − 0.001. Let's call it 0.0015. So, p value is lower than 0.05. Thus, calculated value > tabulated value so there is significant greater amount of organophosphate in Indian sample than local sample. Also, the p value shows significant difference in concentration.

3.5.2. Analysis in Fruits

Due to the unavailability of local seasonal fruit samples during the time of research, only Indian fruit samples were used for the determination of organophosphate, as shown in Figure 3.

[figure(s) omitted; refer to PDF]

3.6. Validation

The method was validated as per International Conference on Harmonization (ICH) Guidelines [20].

3.6.1. Linearity

The absorbance of complex was analyzed using UV-Visible spectrophotometer. The linearity graph is shown as per Figure 4.

[figure(s) omitted; refer to PDF]

3.6.2. Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) for the procedure were performed and the data were obtained as 0.160 mg and 0.486 mg, respectively.

3.6.3. Precision

The data for intraday and interday precision studies were obtained from three different concentrations. The percentage RSD was calculated and shown in the Table 4. The % RSD should be less than 2.5%.

Table 4

Precision result.

Drugs	Amount (mg)	Intraday ($n = 3$) (mean ± SD)	% RSD	Interday ($n = 3$) (mean ± SD)	% RSD	Acceptance criteria % RSD
Disodium orthophosphate	1	0.287 ± 0.003	1.066	0.286 ± 0.0030	1.066	<2.5%
1	0.177 ± 0.002	1.423	0.048 ± 0.001	2.0		1

3.6.4. Accuracy

The accuracy of the method was evaluated in triplicate at three concentration levels i.e. 80%, 100%, and 120%. The percentage of recoveries was calculated and shown in Table 5.

Table 5

Accuracy result.

Drug	Amount (mg)	Recovery level (%)	Amount added	Recovery amount	Amount recovered	% recovery	Acceptance criteria %
Disodium hydrogen orthophosphate	2	80	0.8	1.78	1.81	99.33	98–102
0.8	1.81	100.78	0.8	1.84	101.94	2	100
1	2.05	2.16	102.5	98–102	1	2.02	101
1	1.97	98.5	2	120	1.2	2.23	2.22
101.49	98–102	1.2	2.21	100.3	1.2	2.23	101.49

3.6.5. Robustness

(1) Variation of Wavelength. The robustness of the sample was carried out by the variation of wavelength at 239 and 241 nm (Table 6). The concentration of the sample selected was 1 mg/ml. The % RSD should be less than 2.5%, which showed that variation in the wavelength showed the method to be not robust.

Table 6

Variation of wavelength for robustness.

Wavelength (nm)	Absorbance			% RSD
*A*1	*A*2	*A*3	239	0.199
0.201	0.196	1.267	241	0.209

(2) Variation of Reagent Volume. The robustness of the sample was carried out by variation of reagent volume i.e. 1.8 ml and 2.2 ml, as shown in Table 7. The concentration of the sample selected was 1 mg/ml. The % RSD should be less than 2.5%, which showed that variation in the reagent volume showed the method to be not robust.

Table 7

Variation of reagent volume for robustness.

Volume of reagent (ml)	Absorbance			% RSD
*A*1	*A*2	*A*3	1.8	0.176
0.181	0.174	2.04	2.2	0.126

3.7. Greenness Profile Evaluation of the Proposed Spectrophotometric Method

3.7.1. Assessment Using Analytical Ecoscale

An excellent semiquantitative method applied to assess the greenness profile of the analytical methods is the analytical ecoscale [21, 22]. Based on the penalty points, the total score of the method is calculated. The ideal green analytical method is with an ecoscale score of 100.75 and 50 are the green methods which are named as excellent and fair green analytical methods, respectively. If the penalty point is less than 50, it is called the deficient green method. The ecoscale score of the proposed green analytical method is 84, as shown in Table 8.

Table 8

Penalty points for the determination of organophosphates by proposed spectrophotometric method.

Reagent/instruments	Penalty points
Proposed spectrophotometric method	Ethanol
4	Bromine water
4	Disodium hydrogen orthophosphate
0	Ammonium chloride
1	Magnesium chloride
1	Distilled water
0	Occupational hazards
3 (because of bromine water)	Waste
3	Instruments energy
0	Total penalty points
Σ100−16 = 84	Analytical ecoscale total score

84	Comment

3.7.2. Assessment Using Green Analytical Procedure Index (GAPI)

The qualitative method meant to measure the greenness is GAPI, which calculate greenness based on the stages involved in an analytical method [23, 24]. The two main stages of GAPI are sample preparation and instrumental assessment. A pictogram of five pentagrams is a visual output in GAPI and is used to evaluate and quantify the low, medium, and high environmental impact involved for each step of the methodology which can be analyzed by the green, yellow, or red color in each pentagram.

The application of GAPI in the proposed method is given in Table 9, and the pictogram is represented in Figure 5, which shows the method, has satisfied most of the criteria and confirms the proposed method as ecofriendly.

Table 9

Assessment of GAPI for the proposed method.

S. N		Category	Proposed method
I	*Sample preparation*		
1	Collection	UV	Green
2	Preservation	None	Green
3	Transport	None	Green
4	Storage	None	Green
5	Type of method: Direct or indirect	Simple procedures	Yellow
6	Scale of extraction	Simple extraction using ethanol	Green
7	Solvents/reagents used	Green solvents	Yellow
8	Additional treatments	None	Green
9	Reagent and solvents amount	<10 mL	Green
10	Health hazard	None	Green
11	Safety hazard	Bromine water was used very less so that flammability will be negligible	Green
–			

II	Instrumentation		
12	Energy	UV consumes ≤0.1 kWh per sample	Green
13	Occupational hazard (OH)	None	Green
14	Waste	Waste generated by the proposed method was 1–10 mL	Yellow
15	Waste treatment	Low degradation	Yellow

Additional mark: quantification ring in the middle of GAPI: procedure for quantification.

[figure(s) omitted; refer to PDF]

3.7.3. Assessment Using Analytical Greenness Metric (AGREE)

AGREE depends on 12 parameters equal to the 12 principles of Green Analytical Chemistry. Each principle or parameter contains a score range 0-1, which is calculated based on the hazardous to a particular principal of greenness. It looks like a classical clock shape consisting of numbers 1–12 on the edge of the circle, representing the philosophy of 12 principles [25]. As shown in Figure 6 with 0.89, the proposed method indicates the method was greenest in all aspects of green principles.

[figure(s) omitted; refer to PDF]

4. Conclusion

A novel green spectrophotometric method was designed using a magnesia mixture for the analysis of organophosphates in fruits and vegetables. Direct analysis of organophosphates was not possible due to a lack of chromophore which was resolved by a simple derivatization method using magnesia mixture. Validation of the proposed method was carried out as per ICH guidelines, allowing application of the proposed method in the determination of the fruits and vegetables samples. Ecoscale, GAPI, and AGREE assessment methods also affirmed the ecosafety of the developed spectrophotometric method and can be adapted to the established quality of other fruits and vegetables for organophosphate analysis.

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Discrimination of Traditional Chinese Medicine Syndromes in Type 2 Diabetic Patients Based on Metabolomics-Proteomics Profiles

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ABSTRACT (ENGLISH)

Aims of the Study. Traditional Chinese medicine (TCM) has thousand years of history, and syndrome differentiation is the foundation and essence of the TCM theory. As it has distinctive advantages in diagnosing and treating the type 2 diabetes mellitus (T2DM), the purpose of this research is to distinguish T2DM patients with or without damp-heat syndrome (DHS), as well as to discover biomarkers associated with syndrome employing the metabolomics-proteomics technique. *Materials and Methods*. The metabolomics-proteomics of sixty patients with T2DM were acquired by high-performance liquid chromatography (HPLC). In addition, some clinical features, containing total cholesterol (TC), triglycerides (TG), hemoglobin A1c (HbA1c), body mass index (BMI), and low-density lipoprotein (LDL) together with high-density lipoprotein (HDL), were determined via clinical detection strategies. Abundant metabolites and proteins, respectively, were identified with the analysis of liquid chromatography tandem mass spectrometry (LC-MS/MS). *Results*. 22 differentially abundant metabolites and 15 differentially abundant proteins were determined. The analysis of bioinformatics suggested that the differentially abundant proteins were commonly associated with the renin-angiotensin system, vitamin digestion and absorption, hypertrophic cardiomyopathy, and so on. Furthermore, differentially abundant metabolites were amino acids and were associated with the biosynthesis of CoA and pantothenate, together with the metabolisms of phenylalanine, beta-alanine, proline, and arginine. Combination analysis revealed that the vitamin metabolism pathway was predominantly affected. *Conclusions*. DHS

syndrome can be separated by certain metabolic-proteomic differences, and metabolism is particularly prominent, especially in vitamin digestion and absorption. From the molecular level, we provide preliminary data for the extensive application of TCM in the study of T2DM, and at the same time benefited in a sense diagnosis and treatment of T2DM.

FULL TEXT

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1. Introduction

Diabetes is a serious health problem in the world, and the management costs of the national health system and patients are high. The latent effect of diabetes on health care systems, health, and life expectancy along with financial costs will increase over the next few years [1]. The pathogenesis of T2DM is not entirely clear. It is the most common type of diabetes characterized through impaired β-cell function, insulin resistance (IR), chronic hyperglycemia, as well as comorbidities for instance cardiovascular disease and obesity [2]. Treatment of T2DM needs the sequence of latent measures to manage hyperlipidemia, hyperglycemia, and risk factors for a series of complications related to diabetes, as well as specific biomarkers.

Owing to the diversity of T2DM diseases, identifying biomarkers of T2DM has become a huge problem. They are employed to assess chemical characteristics, target validation, disease status, and the response of treatment [3]. With the progress of metabolic technology and proteomics, serum biomarkers of T2DM have existed. In addition, integrating various profiles for instance transcriptomics, proteomics, and metabolomics will better present the biological process and gene expression regulation of T2DM so as to formulate prevention strategies and reduce complications [4].

TCM is a medical system centered on medical care. It has more than 3000 years of continuous experience of practice and is improved via the treatment observation [5]. As a result, it has its own features and advantages in personalized treatment and early intervention. In the theory of TCM, syndrome differentiation (also known as pattern classification or Zheng differentiation) is the essence and basis [6]. Diagnosis is principally decided by the overall human symptoms observation, involving observation, touching, smelling, listening, and background research [7] instead of the test at the “micro” level. For the same disease, sometimes different treatment approaches are applied for the treatment of various pathological states. The relationship between these syndromes and the relevant treatment comes from practical experience and is improved through the investigations of long-term treatment. Nevertheless, due to the lack of technological and scientific means, TCM is facing serious challenges and lack of modern research [8]. Therefore, it is essential to explore the changes of compounds (containing fatty acids, proteins, metabolites, and so on) in several symptoms of a same disease in order to confirm these experiences and subsequently expand the disease understanding.

In accordance with the theory of TCM, T2DM is considered as *Xiaokezheng* having symptomatic polydipsia [8]. TCM in-depth classifies *Xiaokezheng* into distinct syndromes and has relevant clinical manifestations, containing damp-heat syndrome (DHS), *Qi Yin* deficiency, and *Qi* deficiency [9]. Based on different syndromes of TCM, TCM can provide more effective personalized treatment according to its pathological features. So far, more and more randomized clinical trials have focused on the advantages of TCM in the treatment of diabetes. In this research, we employed metabolomics-proteomics analysis to identify non-DHS and DHS syndromes of T2DM.

2. Materials and Methods

2.1. Plasma Sample Collection

The experiment was authorized through the ethics committee of the institute and complied with the principles of the Helsinki declaration. Moreover, from the patients, the informed consent to research protocol could be acquired. The plasma samples were harvested from the T2DM patients with and without the DHS syndrome (thirty in each group). In both experimental groups, all of the chose patients were diagnosed through syndrome differentiation of western medicine and TCM. They were offered via Nanjing Hospital of Traditional Chinese Medicine and adhered to the

guidance of the Hospital Human Subjects Committee. The values of fasting blood glucose (FPG) were more than 7.0 mmol/L, and some blood lipid parameters, for instance, TG, TC, LDL, and HDL, were acquired via clinical detection approaches. It was classified via two authentic TCM doctors on the basis of "Diabetes TCM Diagnostic Criteria" [9]. Table 1 shows the clinical features of TCM syndromes of T2DM.

Table 1

The clinical features of TCM syndromes of T2DM.

	Qi deficiency	*Qi* and *Yin* deficiency	Damp heat
Representative symptoms	Lethargy	Soreness of waist and knees	Thirsty
Constipation	Sleep hyperhidrosis	Diuresis	·
Holistic symptoms	Fatigue	Palpitations and insomnia	Hyperorexia
–			
Tongue appearance	Fat tongue	Reddish tongue	Red tongue with yellow fur
–			
Pulse pattern	Thin and weak pulse	Thin and rapid pulse	Stringy pulse

After fasting at night, venous blood was harvested as mentioned above. The buffy coat, plasma, and serum were isolated from whole blood and kept at a temperature of −80°C within four hours after collection. In order to repeat freeze-thaw cycles and maximize the longevity, serum and plasma samples were widely divided and placed at −80°C prior to the subsequent use. Levels of clinical features were determined by automated clinical laboratory methods using a diagnostic analyzer.

2.2. Data Processing and Metabolite Identification

The integrated and centroided data of UPLC-TOFMS were pretreated with XCMS software according to the manufacturer's recommendation [10], which was then normalized to the ionic strength of their respective internal standards in metabolomics analysis experiments [11]. The remarkably altered metabolites were identified via multivariate analysis. The Madison Metabolomics Consortium Database (MMCD) [12] as well as the Human Metabolome Database (HMDB) [13] were employed to determine the metabolites through accurate search based on mass. The identification of metabolite was demonstrated through the comparison of the retention time under identical chromatographic conditions and through the match of the cleavage mode of parent ions in biological samples with that of standard metabolites via applying tandem mass spectrometry (UPLC-TOFMS/MS).

2.3. TMT Proteomic Analysis

The UHPLC separation was carried out using a 1290 Infinity series UHPLC System (Agilent Technologies), equipped with a UPLC BEH Amide column (2.1 * 100 mm, 1.7 μm, Waters). The mobile phase consisted of 25 mmol/L of ammonium acetate and 25 mmol/L of ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The analysis was carried out with elution gradient as follows: 0~0.5 min, 95%B; 0.5~7.0 min, 95%~65% B; 7.0~8.0 min, 65%~40% B; 8.0~9.0 min, 40%B; 9.0~9.1 min, 40%~95%B; and 9.1~12.0 min, 95%B. The column temperature was25 °C. The auto-sampler temperature was 4°C, and the injection volume was 2 μL (pos) or 2 μL (neg), respectively. The TripleTOF 6600 mass spectrometry (AB Sciex) was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, the most intensive 12 precursor ions

with intensity above 100 were chosen for MS/MS at collision energy (CE) of 30 eV. The cycle time was 0.56 s. ESI source conditions were set as following: gas 1 as 60 psi, gas 2 as 60 psi, curtain gas as 35 psi, source temperature as 600°C, declustering potential as 60 V, ion spray voltage floating (ISVF) as 5000 V or −4000 V in positive or negative modes, respectively. One QC is inserted for every 8 samples, for a total of 7 QCs.

Protein Pilot software 3.0 (ABSCIEX) was used to quantify relative abundance and identify protein and peptide. MMTS was utilized as the fixed modification of the cysteine to analyze the data, and the database could be searched with the interval rate of confidence (95%) to identify the protein. For the target proteins, their high confidence peptides revealing abundant production spectrum were chose for the assay of multiple reaction monitoring (MRM). TargetLynx 2.0 was used to treat the data of MRM, and the Graph Pad Prism program v 5.0 was employed to conduct the statistical analysis and for the generation of the receiver operating features. Each peptide was compared with the Wilcoxon test.

2.4. Bioinformatics Analysis

The analysis of pathway enrichment and Gene Ontology (GO) was performed for the protein functional enrichment. In accordance with the presenting report, with the InterProScan database (v.5.14–53.0 https://www.ebi.ac.uk/interpro/), GO was annotated (containing the cellular component (CC), molecular function (MF), and the biological process (BP)). The exploration of pathway enrichment was implemented with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database [14]. The KEGG mapper (v.2.5, https://www.kegg.jp/kegg/mapper.html) along with KAAS (v.2.0, https://www.genome.jp/kaas-bin/kaas_main) were the major tools employed by the database of KEGG. The WoLF PSORT software (v.0.2, https://www.genscript.com/psort/wolf_psort.html) was applied to predict the subcellular localization. The heat map obtained via the function heatmap in R language package is applied to visualize the cluster members. For each annotation, the comparison of enrichment degree between all the identified proteins and differentially abundant proteins was performed with Fisher's exact test, and P less than 0.05 was regarded as significant.

2.5. Statistical Analyses

The biochemical and clinical data were presented with mean ± SD. The SPSS program for Windows (version 21 statistical software: Texas instruments, IL, USA) was employed for all of the statistical analyses. The Mann–Whitney/Wilcoxon and Student's *t*-test were exploited to carry out the differences between both TCM syndrome groups of T2DM when proper. The two-tailed p value is significant when FDR-adjusted$p<0.05$.

3. Results

3.1. Clinical Data of the Study Subjects

The biochemical and clinical data of the study subjects are reflected in Table 2. The DHS group had significantly higher values for TG ($P<0.001$), TC ($P<0.05$), fasting insulin (FINS, $P<0.05$), fasting c-peptide (FCP, $P<0.05$), and insulin resistance index (HOMA-IR, $P<0.05$). Furthermore, the DHS group showed a lower mean level of HDL and a higher level of LDL than the non-DHS group, although there was no statistical difference.

Table 2

Clinical and biochemical data of the study subjects.

Characteristics	T2DM with non-DHS ($n = 30$)	T2DM with DHS ($n = 30$)	p value
Age (years)	57.80 ± 11.82	52.97 ± 11.22	0.11
BMI (kg/m^2)	25.92 ± 3.28	26.70 ± 3.65	0.39
FBG (mmol/L)	9.48 ± 3.11	9.82 ± 2.68	0.65
PBG (mmol/L)	14.50 ± 4.12	15.00 ± 4.11	0.64

HbA1c (%)	9.16±1.70	9.42±1.59	0.54
TC (mM)	4.38±1.28	5.57±1.41	≤0.01
TG (mM)	1.58±1.10	3.10±1.79	≤0.01
LDL-C (mM)	2.79±0.83	3.14±1.14	0.18
HDL-C (mM)	1.13±0.22	1.00±0.29	0.06
FCP (pg/mL)	2.02±0.67	2.62±1.11	≤0.01
FINS (pmol/ml)	8.15±5.08	12.24±6.93	≤0.01
HOMA-IR	3.61±3.10	5.44±3.27	0.03

Data were presented as means±SD, and *t*-test was applied. The two-tailed p value was significant at <0.05. DHS: damp-heat syndrome; BMI: body mass index; FBG: fasting blood glucose; PBG: postprandial plasma glucose; HbA1c: glycosylated hemoglobin; TC: total cholesterol; TG: triglycerides; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; FCP: fasting c-peptide; FINS: fasting insulin; HOMA-IR: insulin resistance index.

3.2. Identification and Functional Enrichment Analysis of Differentially Abundant Proteins

Using the abovementioned analytical conditions, proteomic profiles from 30 T2DM patients with TCM syndrome with DHS and 30 without DHS were obtained by LC-MS/MS. There were 654 proteins available. After data management and normalization, there were 621 quantifiable proteins (Figure 1). In the end, fifteen proteins revealed obvious differences (P<0.05; FCa0

[figure(s) omitted; refer to PDF]

BP classification of the proteins suggested that various abnormal biological processes appeared in the syndrome of DHS, for instance positive regulation or regulation of the cholesterol esterification, sterol or steroid esterification, positive regulation of the steroid metabolism, and protein-lipid complex remodeling (Figure 1(c)). Remarkably, representative differentially abundant proteins including AGT and APOA4 participated in all the above process.

The classification of protein via CC indicated that most differentially expressed proteins are located in the extracellular regions, even in the extracellular organelles or vesicles. The classification of protein via MF indicated that more proteins were associated with iron binding, followed by cation binding, aminopeptidase activity, antioxidant activity, exopeptidase activity, and so on (Figure 1(c)). The following analysis of KEGG suggested that the most remarkable change pathways involved the renin-angiotensin system, vitamin digestion and absorption, hypertrophic cardiomyopathy, dilated cardiomyopathy, protein digestion and absorption, adrenergic signaling in cardiomyocytes, and metabolic pathways. In conclusion, extensive and comprehensive proteomic data revealed that many metabolic pathways of T2DM were abnormal in the syndrome of DHS.

3.3. Changes in Serum Metabolites Detected by Untargeted Metabolomics

Relative standard deviation denoising was conducted (individual peaks were filtered to remove noise, and the deviation value was filtered based on the relative standard deviation). 1895 peaks were determined. In addition, the approach of internal standard normalization was employed in analyzing the data. The data were scaled and then transformed logarithmically to minimize the influence of variable high variance and noise. After above transformations, the grouping and distribution of samples was visualized with principal component analysis (PCA). The confidence interval (95%) in the PCA score graph was employed as a threshold to determine latent outliers in the data set. The outcomes indicated that our present metabolomics data set has outstanding reproducibility and

stability (Figure 2(a)). Orthogonal projection with latent structures discriminate analysis (OPLS-DA) was performed to choose remarkably changed metabolites between non-DHS and DHS groups (Figure 2(b)). Subsequently, the calculation of the Q^2 and R^2 values was carried out with a 7-fold cross-validation, respectively, suggesting how well the variables were predicted and the changes in variables were explained. In order to test the prediction ability and robustness of the OPLS-DA model, 200 permutations were carried out in-depth, and subsequently, the intercept values of Q^2 and R^2 were acquired. Here, the Q^2 intercept value was the model reliability, the risk of over fitting, and the model robustness; the smaller the better (Figure 2(c)). In addition, the first principal component, the variable importance in the projection (VIP) value in the analysis of OPLS-DA, was acquired (Figure 2(d)). Metabolites with $P < 0.05$, $FC > 1$, and $VIP > 1$ (Student's *t*-test) were regarded to be remarkably changed metabolites. In the end, 22 metabolites which revealed significant differences in DHS ($FC > 1.2$ and $P < 0.05$) were determined. As shown in Figure 2(e), imidazole, L-pipecolic acid, L-citrulline, L-carnitine, and 3′-O-methylguanosine were decreased, while pantothenate, sphingomyelin, and thioetheramide-PC were increased. Furthermore, the subsequent metabolic pathways had significantly changed: the biosynthesis of CoA and pantothenate, the metabolisms of phenylalanine, beta-alanine, proline, and arginine (Figure 2(f)). These outcomes exhibited that vitamin and amino acid metabolism were changed in T2DM patients with the syndrome of DHS. These analyses were performed by using SIMCA (16.0.2) software.
[figure(s) omitted; refer to PDF]

3.4. Combination Analysis of Proteomics and Metabolomics Data

The outcomes of LC-MS/MS were analyzed with Paintomics3 (v.0.4.5, http://www.paintomics.org). We calculated the content information of all differentially expressed proteins and metabolites, compared the correlation between them, and used Spearman rank and rank correlation to analyze the different metabolites and proteins. We took the correlation coefficient *Q*-value <0.05 as the condition of significant correlation. The heatmap of proteomic-metabolomic correlation analysis for group DHS vs. non-DHS revealed that SLC8A3 and SERPINA10 had a positive relationship with pantothenate and negative with Thr-Tyr, and lysosomal pro-X carboxypeptidase (PRCP) had positive correlation with 3′-O-methylguanosine and Thr-Tyr and negative with pantothenate. DL-Norvaline was negatively related to oncoprotein-induced transcript 3 (OIT3), interferon-induced very large GTPase 1 (GVINP1), glutathione reductase (GSR), and endoplasmic reticulum aminopeptidase 1 (ERAP1). L-Pipecolic acid was negatively related to CBFA2T2, carbonic anhydrase 3 (CA3), and APOA4. Thioetheramide-PC was positively related with A-kinase anchor protein 12 (AKAP12) and negatively with biotinidase (BTD) (Figure 3(a)). Through the visual analysis of the KEGG diagram, it can be concluded that metabolites and protein were jointly regulated. The vitamin digestion and absorption pathway exhibited downregulation of APOA4 ($P < 0.05$, $FC = 0.60$), BTD ($P < 0.05$, $FC = 0.76$) and upregulation of pantothenate ($P < 0.05$, $FC = 1.20$) (Figure 3(b)). The integrated analysis outcomes indicated the potential mechanism of vitamin metabolism in T2DM patients with DHS syndrome.
[figure(s) omitted; refer to PDF]

4. Discussion

Omics are generally employed to elucidate the diabetes pathogenesis and screen latent phenotypic markers of T2DM. In comparison with single omics, integrated analysis of multiomics (the integrated analysis of metabolomics and proteomics [15] or metabolomics and transcriptomics [16], etc.) can more deeply reveal the molecular characteristics and physiological mechanism of this disease. In our work, DHS produced a lot of data on the proteomic analysis of T2DM patients' serum, reflecting the mechanisms of diabetes occurrence and development, for example the renin-angiotensin system, vitamin digestion and absorption, hypertrophic cardiomyopathy, dilated cardiomyopathy, protein digestion and absorption, adrenergic signaling in cardiomyocytes, and metabolic pathways. Serum metabolites can exhibit the body metabolic changes, which is helpful to the monitor of metabolic process in the state of disease [17]. With the aim of understanding the whole metabolic status of T2DM patients with DHS, we implemented a metabolomic analysis on the serum samples and acquired the metabolic profiles. Besides, we implemented an integrated metabolomic and proteomic analysis.

Long-term exposure to high blood glucose could enhance the risk of amputation, heart attacks, diabetic retinopathy

as well as strokes. Intuitively, T2DM could be classified into several cases without or with the complications. Nevertheless, so far, T2DM has not been classified in accordance with the clinical parameters. Interestingly, various syndromes of T2DM can be decided via the theory of TCM, despite they are on the basis of long-term experience of practical. More significantly, special treatment is generally carried out under the guidance of various syndrome types. As a result, with the aim of further understanding T2DM, it is essential to prove various syndromes through biological approaches, especially the analysis of omics with big data. In this study, two syndromes were explored applying the method of metabolomics-proteomics analysis, together with the clinical data including glucose measurements and four lipid parameters (TG, TC, and HDL together with LDL). PCA and OPLS-DA were carried out to, respectively, construct the discriminant models for the T2DM patients with two syndromes of TCM and next explore the correlation between syndromes and proteomics-metabolites. OPLS-DA is a complex and generally applied supervised clustering approach, which is employed to construct the best discriminant surface in order to isolate the best classification. The difference between the two groups was significant, and with Hotelling's *T*-square ellipse, the samples were basically within the confidence interval of 95%. Thus, the statistical analysis results indicated that the plasma metabolic profiles could exhibit some perturbations between DHS and non-DHS syndromes in TCM.

Many latent biomarkers of various syndromes have been found, suggesting that distinct disease pathological stages may be associated with the metabolic status closely. For both groups, DHS, vs. non-DHS, AGT, APOA4, SERPINA10, BTD, ERAP1, OIT3, PRCP, GSR, IGLV7-46, GVINP1, EXT2, AKAP12, CA3, and CBFA2T2 ($P<0.05$; FC <0.83 or >1.2) were the potential biomarkers. 6-Benzylaminopurine, alpha-N-phenylacetyl-L-glutamine, pantothenate, thioetheramide-PC, 1-stearoyl-2-oleoyl-sn-glycerol3-phosphocholine (SOPC), 1-palmitoylglycerol, 1H-indole-3-propanoic acid, imidazole, L-pipecolic acid, 3′-O-methylguanosine ($P<0.05$; FC <0.83 or >1.2, VIP >1.3) were the candidate metabolic biomarkers. These biomarkers are the major characteristics of this syndrome. The changes of their content are linked to the human symptoms of T2DM and can be employed to distinguish a variety of syndromes. According to Table 2, it is understandable why DHS patients with lower APOA4 exhibit significantly elevated TC and TG. The upregulated FCP, FINS, and HOMA-IR also reflect the worsening process of T2DM. It is an effective and innovative method to divide the disease into distinct stages in accordance with a variety of symptoms and then carry out the symptom-specific treatment. In addition, the observation of syndrome-related biomarkers is especially significant for the personalized treatment. Biomarkers related to syndrome of TCM can assist the clinical diagnosis, promote the modernization of TCM, and offer a reference for exhibiting the diabetes pathogenesis.

The proteomics analysis revealed that differentially abundant proteins were related to the vitamin digestion and absorption. After integrated study of the metabolomics and proteomics outcomes, it can be observed that both proteins (APOA4 and BTD) in the DHS group were downregulated as well as one amino acid metabolite (pantothenate) was upregulated in the serum, thus affecting the vitamin metabolism pathway. It has been reported that the levels of plasma vitamin C in patients with T2DM were relatively low, which made people pay growing attention to the preventive effect of vitamin C on T2DM and its related complications. The prospective cohort survey in seven countries exhibited that the glucose intolerance was negatively correlated with the intake of dietary vitamin C, reflecting that antioxidants, for example vitamin C, may possess a protective effect in the occurrence of T2DM and impaired glucose tolerance [18]. In addition, some clinical researches have confirmed that the supplementation of vitamin D can improve the principal metabolic parameters linked to the insulin resistance, containing HbA1c, TC, LDL, triglycerides, as well as HOMA-IR. The supplementation of vitamin D for three months in the elderly with the metabolic disorders can evidently increase the level of HDL and decrease the ratio of TG/HDL and HOMA-IR [19], which is also supported by our results. Similarly, the percentage of HbA1c in T2DM patients decreased by approximately 0.5% after the supplementation of vitamin D [20]. Pantetheine, also called vitamin B5, plays a crucial role in the CoA biosynthetic pathway. It presents good opportunity for drug discovery moving forward [21]. Since B vitamins are the cornerstone of all the cell repair, during the first two years of the supplementation of vitamin D, the increase of repair and the improvement of sleep ultimately leaded to the consumption of B5 reserves. Owing to the

lack of B5, the generation of coenzyme A in the brain was reduced, which may lead to the reduction of acetylcholine generation, resulting in sleep disorders. The decrease in CoA in the adrenal gland may lead to the reduction of the cortisol level (adrenal fatigue), resulting in the increase in arthritis, allergy, and inflammation [22]. As the DHS group showed lower APOA4 and higher pantothenic, it is understandable that non-DHS patients feature sleep hyperhidrosis, fatigue, as well as joint disorders. In the light of lots of articles [23–25] connecting vitamin D with the normal immune system function and the fact that B5 is essential for cortisol production, it can be speculated that the continuous deficiency of B5 and vitamin D may lead to the proinflammatory and abnormal state in patients with T2DM.

As the metabolomics was “untargeted” and the number of significant features in both the proteins and the metabolomics was not very many, further work and large follow-up studies are required to remedy the defect of this research.

5. Conclusion

To sum up, via implementing metabolomics and proteomics analysis of the serum of T2DM patients with or without DHS, we gave an initial understanding of the features of diabetes with two different TCM syndromes from a molecular perspective. Furthermore, we found that, in the DHS patients, the metabolic abnormalities were especially prominent, particularly vitamins metabolism. These results provided a fundamental data for the extensive application of TCM in the study of T2DM and at the same time benefited in a sense diagnosis and treatment of T2DM.

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A SiO_2 Hybrid Enzyme-Based Biosensor with Enhanced Electrochemical Stability for Accuracy Detection of Glucose

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ABSTRACT (ENGLISH)

A novel enzyme-based biosensor for glucose detection is successfully developed using layer-by-layer assembly technology. The introduction of commercially available SiO_2 was found to be a facile way to improve overall electrochemical stability. After 30 CV cycles, the proposed biosensor could retain 95% of its original current. The biosensor presents good detection stability and reproducibility with the detection concentration range of 1.96×10^{-9} to 7.24×10^{-7} M. This study demonstrated that the hybridization of cheap inorganic nanoparticles was a useful method in preparing high-performance biosensors with a much lower cost.

FULL TEXT

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1. Introduction

Diabetes mellitus is the most prevalent chronic disease, which always comes with abnormally high blood glucose levels [1]. On-time monitoring of the glucose level in the blood and body fluids could present useful information in the diabetes mellitus treatment. But the complex chemical and biological environment in the body fluids and blood presents a huge challenge for accurate direct detection of glucose. Since the first report of glucose biosensors by Clark and Lyons in 1962, enzyme-based biosensors have been widely studied and developed [2]. Electrochemical biosensors possess numerous advantages in the rapid and accurate detection. Also, the co-use of enzymatic reactions further improves the selectivity of the biosensor [3].

Glucose oxidase (GOx) is the most commonly used enzyme in glucose detection due to its high selectivity and rapid response. For GOx-based biosensor, the loading amount of GOx plays an important role in the detection accuracy and sensitivity. However, the good water solubility of GOx might lead to the leaching of GOx during electrochemical detection and further lead to poor performance in detection accuracy and reproducibility [4]. Thus, attempts have been made to achieve effective GOx immobilization by the introduction of an electrode matrix. Several materials have been explored for the efficient loading of the GOx, including carbon nanotubes (CNTs), polyaniline, graphene, and metal nanoparticles [5–8]. Among these, CNTs show great potential for enzyme loading due to their large surface area. However, despite the good conductivity of the CNTs themselves, the GOx immobilized electrode matrix still suffers from poor conductivity, which results in poor electrochemical properties [9]. Thus, materials, such as gold nanoparticles (AuNP) and the thionine (THi), are always doped into the electrode matrix for better conductivity [10].

Multicomponents electrode matrix presents great application potential, but the formation of a stable electrode matrix is still a challenge. Chemical crosslinking of different components in the electrode matrix could improve physical stability but raise costs and lower fabrication convenience. Also, the introduction of extra reaction reagents might alter the electrochemical properties [11]. Thus, a better way of constructing high-performance biosensors with high stability is still needed.

Here, a novel electrochemical glucose biosensor (SiO_2-CNTs/THi/AuNPs/GOx) with good stability was fabricated through noncovalent interaction with layer-by-layer assembly technology. CNTs were used as supporting materials, and SiO_2 hybrid was applied to improve the stability of the multi component electrode matrix. Thionine and AuNP were also introduced for enhanced conductivity. The role of the SiO_2 nanoparticles in the formation of the electrode matrix was studied, and enhanced stability was found by electrochemical analysis. The biosensor demonstrated that the SiO_2 hybrid was a novel and convenient method to produce high-performance glucose biosensors.

2. Materials and Methods

2.1. Chemicals

SiO_2 was purchased from Degussa AG, Germany. GO was purchased from Solarbio, Beijing. Glucose was from Jinshan Chemical Test, Chengdu. CNTs dispersion in N-methyl-2-pyrrolidone (NMP) was obtained from Chengdu Organic Chemicals co., Ltd. Chinese academy of sciences, Chengdu. Chloroauric acid (HAuCl4) was purchased from Sinopharm Chemical Reagent co., Ltd, Shanghai. Thionine was from Shanghai Yuanye Bio-technology Co., Ltd, Shanghai. All other reagents and solvents were of analytical grade and commercially available and used without further purification. Ultra-pure water (18.25 MΩ) was used throughout.

2.2. Preparation of SiO_2-CNTs/THi/AuNPs/GO Biosensors

Firstly, pretreat the glassy carbon electrode (GCE). GCE (φ3 mm) was first polished using Al_2O_3 (φ0.3 μm) and then with Al_2O_3 (φ0.05 μm) to be the mirror surface. Then, the electrode was washed with the ethanol and ultra-pure water for 3 times (10 minutes each time) in the sonicator. Then, a cyclic voltammetry (from −0.6 V to 1.0 V in 0.1 M·H_2SO_4 solution) was performed to activate the GCE.

Secondly, prepare the SiO_2-CNTs dispersion and AuNP dispersion. The SiO_2-CNTs dispersion in NMP was prepared by mixing SiO_2 with CNTs. The process can be done as follows: the SiO_2 powder (0.3 mg) was added to a CNTs solution in NMP (1.5 wt%, 7 μL) and then the ultra-pure water (1 mL) was added. The dispersion was further sonicated for 2 h to ensure the even distribution. The gold nanoparticle (AuNP) dispersion in water was prepared by reducing $HAuCl_4$ with sodium citrate, as reported before [12]. A typical procedure can be done as follows: $HAuCl_4$ (0.0100 g) was first dissolved in 100 mL ultra-pure water and then heated up to 100°C with vigorous shaking. The solution was then refluxed for 15 min, and a solution of sodium citrate (1.00 wt% in water) was added till the dispersion turned gray (approximately 0.8 mL was consumed). The solution was further refluxed till the color turned claret-red and then the dispersion was cooled to room temperature. The final AuNPs dispersion (approximately 0.01 wt%) was stored at 4°C.

Thirdly, fabricate the SiO_2-CNTs/THi/AuNPs/GO biosensor. The proposed electrode was prepared by repeatedly doping multicomponent onto the GCE in order (Figure 1). SiO_2-CNTs dispersion (4.00 μL), thionine solution (4.00 μL,

0.01 M in water) and AuNP dispersion (4.00 μL) were separately assembled onto the GCE. After each modification, the electrode was stored at 4°C for 4 h to vaporize the solvent. The modification of GO was accomplished with a saturated phosphate buffer solution (PBS) of GO with the same procedure.

[figure(s) omitted; refer to PDF]

2.3. Electrochemical Measurements

Electrochemical measurements were performed on a CHI 760E electrochemical workstation (Shanghai Chenhua Instruments Limited, China) with a conventional three-electrode system that consisted of a saturated calomel electrode (SCE) as the reference electrode, a platinum wire as the counter electrode, and a bare or modified GCE as the working electrode.

The detection of glucose via cyclic voltammetry (CV) was performed in PBS (5.00 mL, 0.05 M, pH 7.00) or PBS containing $K_3[Fe(CN)_6]$ (5.00 mL, 0.05 M, pH 7.00, concentration of $K_3[Fe(CN)_6]$ was 0.02 M) with a scanning rate of 100 mV/s (from −0.6 V to 1.0 V) at 25 ± 0.5°C (Figure 2). AC impedance was employed to characterize the modification process of the biosensor with a frequency range of 1 to 105 Hz and an amplitude of the AC potential of 5 mV.

[figure(s) omitted; refer to PDF]

For the detection of glucose, a concentration-current working curve was first acquired by adding a predetermined amount of glucose solution into the electrochemical cell. The concentration of glucose can be calculated from the current at oxidation peaks using the working curve.

2.4. Analysis and Characterization

The morphology of the samples was characterized with a Zeiss sigma 300 scanning electron microscope (SEM). Samples were firstly doped onto GCE as described above. Then the samples for each step were gently removed from GCE, collected, and used for SEM tests directly.

The FT-IR spectroscopy analysis was performed on a Nicolet Satellite infrared spectrometer in the range 400–4000 cm^{-1} with a resolution of 4 cm^{-1} using KBr pellet technique.

3. Results and Discussion

3.1. Biosensor Fabrication

The glucose-sensitive biosensor was formed by the composition of several components on GCE. The proposed biosensor utilizes GO to achieve specific glucose detection. The immobilization capacity of enzymes plays an important role in detection accuracy and reproducibility. To maintain the flexibility of biosensor production and enhance the reliability of enzyme loading, commercially available SiO_2 nanoparticles were introduced into the layer-by-layer assemble process. Also, the multicomponent electrode matrix, containing thionine and gold nanoparticles, was applied to the GCE to fulfil the requirements of sensitive and accurate glucose detection.

3.1.1. Layer-By-Layer Preparation of the Biosensor

The biosensor was prepared by doping the different composites onto GCE, and the successful modification of each step was confirmed by SEM (Figure 2) and FT-IR (Figure 3) technology. SiO_2-CNTs complex was first introduced onto GCE. As shown in Figure 2(a), no obvious self-aggregate of SiO_2 nanoparticles can be seen. On the contrary, SiO_2 nanoparticles were assembled onto CNTs. This phenomenon provides the possibility of SiO_2 acting as a crosslinker. The follow-up modification of thionine and gold nanoparticles leads to no obvious morphology changes (Figures 2(a)–2(c)), thus FT-IR and EDS were used to confirm the existence of thionine and gold nanoparticles, as shown in Figures 3 and 4. The appearance of a peak at 1610.4 cm^{-1} confirms the successful introduction of thionine molecular, while the yellow color of Au in EDS tests, as shown in Figure 4 confirms the existence of AuNPs in the final biosensor. After the addition of GO, large aggregates can be witnessed (Figure 2(d)). GO was introduced to achieve selective glucose detection.

[figure(s) omitted; refer to PDF]

The addition of SiO_2 was essential in this experiment. Pre-experiments (data not shown) found that without the addition of SiO_2, the modification of the GCE with the SiO_2-CNTs/THi/AuNPs/GO composites was not physically stable enough, and the composite might fall off from the GCE during the electrochemical experiments. Also, the

unstable formation of the electrode matrix might result in the loss of enzyme during the electrochemical test, which further leads to a poor electrochemical stability, as shown in Figure 5. The strong interaction between CNTs and SiO_2 nanoparticles made the modification of GCE through the dripping method much more effective and greatly helped to glue the composite onto GCE. By mixing SiO_2 nanoparticles with the NMP solution of CNTs, the SiO_2 was first absorbed onto the CNTs without obvious self-aggregation of SiO_2 (Figure 2(a)). Further introduction of different composites (Figures2(b), 4 (c), 4 (d)) happened on the SiO_2 nanoparticles, thus providing the “crosslink” effect of SiO_2.

[figure(s) omitted; refer to PDF]

3.2. Electrochemical Characterization

CV and AC impedance were also performed in PBS (0.05 M, pH = 7.00 with 0.02 M $K_3[Fe(CN)_6]$) to study the effects of modification on the electrochemical properties of the electrode. As shown in Figure 6, only the redox peak of $K_3[Fe(CN)_6]$) can be seen for SiO_2-CNTs (Figure 6(a)) electrode with a high impedance value (Figure 6(b)). After introducing thionine, the strong redox peaks of thionine can be found at −0.4 V and 0.1 V (Figure 6(b)), which confirms the existence of thionine. The introduction of thionine also decreased the impedance value (Figure 6(b)), due to the excellent electron transfer efficiency of thionine [13]. After the modification of gold nanoparticles (Figure 6(a)), the peak current decreased while the impedance value increased slightly. We suggested this phenomenon might be caused by the thiol-gold interaction formed between AuNP and -SH in thionine, which limits the electron transfer ability of thionine. Further modification of GOx leads to an obvious increase in impedance value (Figure 6(b)) since the GOx exhibits poor electroconductivity.

[figure(s) omitted; refer to PDF]

3.3. Optimization of Test Condition

The accurate detection of glucose heavily relies on the activity of the GOx modified on the electrode. Thus, to maximize the activity of the GOx and the sensitivity and accuracy of the detection, temperature and pH during the CV test were, respectively, optimized.

As shown in Figure 7, the current at 0.075 V reaches the maximum value (0.75×10^{-4} A) when the CV test is performed at 25°C, which indicates this temperature favors the activity of the GOx. This phenomenon was consistent with the literature report before. Lower temperatures inhibited the activity of GOx and led to a smaller current, while high temperatures caused irreversible damage to the enzyme. Thus, 25°C was selected as a favorable temperature for this biosensor in future experiments.

[figure(s) omitted; refer to PDF]

During the optimization of pH, different phenomena were found. According to Figure 8, the biosensor shows a similar sensitivity to glucose in a wide pH range from 4.50 to 7.00, indicating this biosensor might be suitable for a variety of samples from weak acids to neutrals. The peak current decreased dramatically when pH higher than 7.00, suggesting that the enzyme might be unstable in base. Thus, 7.00 was selected as favorable pH for this biosensor in future experiments.

[figure(s) omitted; refer to PDF]

3.4. Stability of the Biosensor

To confirm the effect of SiO_2 as a crosslinker, CV test of 30 CV cycles was conducted to study its influence on stability (Figure 5). For the biosensor without the SiO_2 hybrid, the current retention rate was about 60% after 30 CV cycles. While the addition of SiO_2 resulted in a 96% current retention after 30 cycles with a current loss of less than 5%. The much-improved long-term stability might result from the enhanced physical stability of the electrode matrix, as suggested from the SEM images above, in which SiO_2 acts as a crosslinker.

3.5. Working Curve and Determination of Glucose

Figure 9 shows the relation between the concentration of glucose and the peak current in which the concentration of glucose is between 1.96×10^{-9} and 7.24×10^{-7} M. The regression equation is $y = -2.86 \times 10^{-5} \log C - 6.14 \times 10^{-5}$ ($R^2 = 0.993$), which presents a good linear relationship between concentration and peak current.

[figure(s) omitted; refer to PDF]

The detection accuracy and precision of the proposed electrode were further analyzed through the detection of preweighed samples under the optimized condition. The accuracy was described as the mean relative error. [14] For a sample with a glucose concentration of 1.92×10^{-8} M, the biosensor suggests a concentration of 1.82×10^{-8} M, with an average detection recovery rate of 105% and accuracy of 5% ($n = 5$). This affirms that the proposed electrode could present an accurate detection toward glucose.

4. Conclusions

In summary, a novel glucose biosensor was successfully fabricated using layer-by-layer assembly technology. SiO_2 was introduced into the electrode matrix as the physical crosslinker. Electrochemical analyses were carried out to confirm the enhanced stability. The improved physical stability of the electrode matrix promoted GOx immobilization and further ensured accurate, sensitive, and selective glucose detection with a detection range from 1.96×10^{-9} to 7.24×10^{-7} M. This work demonstrated a novel way to fabricate an effective and efficient electrochemical biosensor through the hybridization of cheap inorganic components, which provides a new possible solution to bridging the gap between laboratory and practical applications of electrochemical analysis.

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A Novel, Simple, and Reliable Spectrophotometric Determination of Total Hexavalent Chromium by Complexation with a New Reagent of Thiazole Linked to 2H-Chromen-2-One

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ABSTRACT (ENGLISH)

Hexavalent chromium is a known environmental contaminant and carcinogen. In the current work, a simple, rapid, and reliable direct spectrophotometric method was used for the determination of total Cr (VI) in environmental samples. Acid-base equilibria and ionization constant (pK_a) of the new reagent 3-(2-(2-(4-(trifluoromethyl)benzylidene)hydrazineyl) thiazol-4-yl)-2H-chromen-2-one (thiazole linked to 2H-chromen-2-one, TFZ) were investigated. The value of pK_a for the reagent was found to be 7.6 which was initially reported. The reaction of the TFZ ligand with Cr (VI) was optimized to produce a highly absorbent complex at 370 nm and pH 7.0 within 1 min. With a correlation coefficient of 0.9994, the linear concentration range ranges from 2 to 20,000 ng·mL^{-1}. The detection limit and quantification limit were 0.73 and 2.43 ng·mL^{-1}, respectively. The method has high precision with relative standard deviations less than 1.0 and high accuracy with recovery of 100 ± 2%. A large excess of cations and anions did not interfere with the determination of Cr (VI). The proposed method was successfully applied to the determination of Cr (VI) in cement samples. The current method could be useful for the routine analysis of Cr (VI) in environmental labs.

FULL TEXT

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1. Introduction

Contamination of the environment with heavy metals is a growing concern due to their toxicity to humans, animals, including fish, and the ecosystem. In 1797, the French scientist Louis Vauquelin identified an element whose colour changed as it existed in different chemical forms. Because one of the compounds that included this element was green and “chroma,” meaning “colour,” the element was named chromium (Cr) [1]. Chromium has been identified as a poisonous and carcinogenic metal. Chromium is employed in steel manufacturing and polishing, alloying, electroplating, leather tanning and finishing, dyes and mordants, catalysts, oxidants, adhesives, paints, and wood preservatives [2]. In the environment, chromium can be found in two stable forms: Cr (VI) and Cr (III). Cr (III) is less toxic and less soluble than Cr (VI), whereas Cr (VI) is exceedingly toxic and extremely soluble [3]. Cr (VI) is roughly 100–300 times more hazardous than Cr (III) which has a high penetration capacity for biofilm, which can be transformed into a variety of reactive intermediates, resulting in DNA mutation and cancer [4, 5]. It also causes liver damage, lung congestion, inflammation, skin irritation, and ulcers [5]. According to the World Health Organization (WHO) and the Environmental Protection Agency (EPA), the maximum permissible level of chromium in drinking water is 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$, respectively [6, 7]. Directive 2003/53/EC of the European Union (EU) mandated the control and limitation of chromium levels in cement containing more than 2 $mg \cdot kg^{-1}$ of Cr (VI). Table 1 shows the recommended maximum permissible limit of chromium in fish, food, and animals’ meat.

Table 1

The recommended maximum permissible limit of chromium in fish, food, and animals’ meat.

Food matrix	Maximum permissible limit (MPL) (mg/kg)	Reference
Fish	0.65–4.35	[8]
Food	0.01–13	[9]
Animals’ meat	0.05	[10]

Several analytical techniques, such as electrothermal atomic absorption spectroscopy, are used to determine chromium [8], flame atomic absorption spectrometry (FAAS) [9], UV-Vis spectrophotometry [10], atomic absorption spectroscopy (AAS), and inductively coupled plasma optical emission spectroscopy/inductively coupled plasma mass spectrometry “(ICP-OES/ICP-MS) [2, 11]. AAS and ICP-OES/ICP-MS are both capable of detecting only total chromium without separating between different chromium species. As a result, various chromium species were separated using ion chromatography (IC) or high-performance liquid chromatography (HPLC) prior to detection using ICP-OES/ICP-MS and AAS [12, 13]. However, absorption spectrophotometry is more suitable and reliable for routine chemical analysis due to its widespread availability and relatively inexpensive cost of equipment, as well as the stability of its processes and the precision of its methodologies [14]. Moreover, spectrophotometry has opportunities not just for enhancing selectivity but also for improving sensitivity.

Thus, the aim of current work is to develop a new spectrophotometric method with a highly sensitive and wide linear range for Cr (VI) determination by complexation with the novel ligand 3-(2-(2-(4-(trifluoromethyl)benzylidene)hydrazineyl)thiazol-4-yl)-2H-chromen-2-one(3a) (thiazole linked to 2H-chromen-2-one, TFZ, Figure 1). Several experimental parameters which influence the formation of the Cr (VI)-THZ complex were investigated and optimized. Finally, the proposed method was applied to determine total soluble Cr (VI) in various cement, water, and soil samples.

[figure(s) omitted; refer to PDF]

2. Experimental Setup

2.1. Reagents and Chemicals

Potassium chromate was purchased as a yellow powder from Sigma Aldrich (USA). Iron (II) sulphate 7-hydrate, manganese sulphate monohydrate, copper sulphate, and cadmium nitrate hydrated were bought as powders from

TECHNO PHARMACHEM (India) and nickel sulphate extra pure from Loba. Chemi (India) were used as powders for foreign ions. Sodium phosphate dibasic dihydrate (Sigma Aldrich, USA) and sodium dihydrogen orthophosphate dihydrate (CDH, India) were used to prepare the solution in different pH levels. Ethanol was obtained from Sasol, KSA, which was used for the preparation of the reagent. Glassware and polyethylene containers were soaked in 10% nitric acid and washed with deionized water to eliminate the risk of contamination. Nitric acid was supplied by Sigma Aldrich (USA), while hydrochloric acid was obtained from Honeywell (USA) and hydrogen peroxide solution was purchased from Fluka (Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) and used without further purification. For the manufacture of aqueous solutions, double-distilled water was employed.

2.2. Instrumentation

The absorbance spectra were determined using a UV-visible spectrophotometer from PG Instruments, U.K., with a matched pair of 1 cm quartz cuvettes at a detecting wavelength between 250 and 700 nm. The buffer solutions' pH was measured with a pH meter from Eutech. Utilizing standard buffer solutions of pH 4.0, 7.0, and 10.0 at room temperature, the pH meter was calibrated.

2.3. Synthesis and Preparation of TFZ Ligand Solution

According to the publication of our research group, the TFZ ligand was produced [18].

2.4. Method for pK_a Determination

The pK_a of the TFZ ligand was determined using a method created by Pandey et al. [19]. A preliminary investigation was conducted to estimate the pK_a using the inflection approach. A graph of absorbance against pH was created. This curve inflection point was cited as a rough estimate of the pK_a value. The second approach consisted of determining the wavelengths of maximum absorption from the spectra of species with extreme pH values (pH = 2 and pH = 8 in this situation). The plot of the absorbance vs. pH at these wavelengths was applied. The pKa was determined by measuring the pH of the place where the two linear curves intersected at these wavelengths.

2.5. Preparation of Standard and Working Solutions of Cr (VI)

The Cr (VI) solution was prepared by dissolving 100 mg of potassium chromate sample in 100 mL distilled water. The working solutions of the Cr (VI) were prepared by dilution in the same solvent.

2.6. Procedure of Complex Formation

The formed coloured complex solution was simply and precisely prepared. A 0.1 mL of Cr (VI) was mixed with 9.8 mL of phosphate buffer (pH 7), and then 0.1 mL of ligand was added. The complex was rapidly formed after 1 min of mixing solutions.

2.7. Environmental Samples

Several samples were collected; three ground water samples from different places of Saudi Arabia were collected. Soil (surface) samples were collected from five different locations in Jeddah city, Saudi Arabia. Two cement samples were purchased from the local market.

2.8. Digestion of Soil and Cement Samples

The digestion of the soil and cement samples was performed according to the previously reported method with little modification [20, 21]. Typically, 0.5 g of samples were dissolved in a mixture of 15 mL nitric acid and 5 mL hydrochloric acid followed by the addition of 2 mL hydrogen peroxide and 2 mL water. The sample was placed in a suitable beaker and covered by a clock glass. The beaker was heated in an oil bath. The specified temperature profile included reaching 180 ± 5°C in less than 5.5 minutes and remaining at that temperature for 9.5 minutes to accomplish the desired reactions. After cooling, the beaker content was filtered and was ready for starting analysis.

3. Results and Discussion

3.1. Investigation of Acid-Base Equilibria of the TFZ Ligand

To accomplish this, the absorbance of the TFZ ligand was evaluated in buffers ranging in pH from 2.0 to 12.0. The buffer solutions were prepared by using 0.1 $mol{\cdot}L^{-1}$ sodium phosphate dibasic dihydrate (NaH_2PO_4) and sodium dihydrogen orthophosphate (Na_2HPO_4). Primary stock solution (1000 $\mu g{\cdot}mL^{-1}$) of the TFZ ligand was prepared in ethanol. All samples were analyzed after 10 $\mu g{\cdot}mL^{-1}$ working solutions were produced in corresponding buffers from primary stock solutions.

To calculate the ionization constant (pK_a) of TFZ ligand spectra, two graphical methods were applied. The simple first one was used to calculate a rough value of pK_a. Figure 2 shows the absorbance diagram of the TFZ ligand in buffer solutions of various pH values at a maximum wavelength of 400 nm. It is clear from the figure that the TFZ ligand exhibits pH dependent UV-absorption, and the high absorption was achieved at pH 8. Therefore, the pK_a value of 8 could be considered an approximate ionization constant for the TFZ ligand.

[figure(s) omitted; refer to PDF]

Another method was used to calculate the precise pK_a value of TFZ (L). The approach involved measuring the spectra of species with extreme pH levels (pH = 2 and pH = 8 in this instance) to identify the wavelengths of maximum absorption. As observed, the absorbance spectrum of the acidic solution exhibited a peak at 515 nm (HL^+), while the peak of the more basic solution occurred at 400 nm (L). The pKa was obtained by determining the pH of the point of intersection, and it was found to be 7.6.

3.2. Cr (VI) Complexation with the TFZ Ligand

3.2.1. Complexation Equilibria of Cr (VI) with TFZ

The pH study is essential since it can have a significant effect on the formation of metal-ligand complexes as well as the result of complex extraction. The effect of pH on the formation of the Cr (VI)-TFZ complex in the mixed water-ethanol medium was simply investigated by mixing equal volumes (0.1 mL) of each ligand and metal of 1000 $\mu g \cdot mL^{-1}$ and 9.8 mL of buffers with different pHs from acidic to basic media. After a waiting period of 1 min to complete the complex reaction, the absorbance was measured. It was found that the best pH for perfume complex formation and to get a maximum light response was at pH = 7 as shown in Figures 3 and 4. In the strong acidic medium, the complex's absorbance is low, and this may be due to ligand (Schiff base) instability.

[figure(s) omitted; refer to PDF]

A comparison between phosphate buffer blank solution, TFZ ligand solution, Cr (VI) solution, and TFZ-Cr (VI) complex solution under the optimized conditions of pH 7 is shown in Figure 5. It is clear that the maximum absorbance of the ligand (at 400 nm) and Cr (VI) (at 435 nm) was shifted to the maximum absorbance at 370 nm, proving the complex formation between Cr (VI) and TFZ. One can assume that the neutral form of the TFZ (L) is the prevalent ligand species in the pH of complexation with the liberation of one proton.

[figure(s) omitted; refer to PDF]

3.2.2. Effect of Sequence on Complex Formation

As shown in Table 2, the best sequence of solution addition (in complex formation) to achieve maximum absorbance is Cr (VI) (M) + phosphate buffer (B) + TFZ (L). This result is expected because buffered ligand solution is a suitable medium to form complexes with better colour intensity.

Table 2

Effect of sequence addition on complex colour intensity.

Sequence of addition	Absorbance
Metal (M) + buffer (B) + ligand (L)	0.335
Ligand (L) + buffer (B) + metal (M)	0.291

3.2.3. Effect of Time on Complex Formation

Five different times between 1 and 15 minutes were studied. The reaction was very fast, and the highest absorbance of the complex was obtained after 1 min of mixing metal with the ligand.

3.2.4. The Mole Ratio of the Complex

This method was performed by mixing different volumes of TFZ ligand solution with the same volume of Cr (VI) ions solution each time; the absorbance of resulted complex solutions was recorded after passing reaction time of 1 min. It is important to prepare each of metal ions and ligand solutions in equal concentrations. As shown in Figure 6, the

M : L mole ratio in complex equals to 1 : 1.
[figure(s) omitted; refer to PDF]

3.2.5. Calibration Characteristics

By mixing different concentrations of Cr (VI) with constant concentration of the ligand and leaving the solution mixture for 1 minute to make the reaction complete, the following analytical results were recorded, and figures of merit for the determination of Cr (VI) are shown in Table 3. By assessing a series of five solutions with a 10 ng·mL^{-1} Cr (VI) concentration, the reproducibility and accuracy of the procedure were determined. The relative standard deviation (RSD) was observed to be 0.975, and high accuracy with recovery values of 100 ± 2% was observed.

Table 3

Figures of merit for the determination of Cr (VI)-TFZ complexation.

Parameter	Value
Absorption wavelength for complexation (nm)	370
Detection limit (ng mL^{-1})	0.73
Quantification limit (ng mL^{-1})	2.43
Correlation coefficient (R)	0.9994
Linear working range (ng mL^{-1})	2–20000

3.2.6. Study of Interferences

To determine whether or not the proposed method is effective, the effects of a number of ions that are frequently found in environmental samples were investigated. The tolerance of the method to foreign ions was investigated with solutions containing 1000 μg·mL^{-1} of expected interfering cations and anions in the presence of 10 ng·mL^{-1} Cr (VI). The tolerance level for a given ion was the variation of absorbance readings from the predicted value by more than ± 2%. Experimental results showed that Cd, Cu, Cr (III), Fe, Mn, and Ni have no influence on the determination of Cr (VI).

3.2.7. Applications

The promise of TFZ as a reagent for direct spectrophotometric detection of total hexavalent chromium prompted us to examine the applicability of the method to the study of soluble hexavalent chromium in environmental samples at 370 nm. The determination of total hexavalent chromium was conducted in different environmental samples: three ground water samples, five soil (surface) samples, and two cement samples. The results of the analysis of water and soil samples showed that they were below the LOD value. The obtained values for Cr (VI) concentrations in cement samples using the proposed analytical method were found to be 4%. In the precision study, five analyses were performed for each sample. A good precision of the proposed method was obtained, which allow the application of the method to the routine analysis of cement.

To confirm the analytical characteristics of the current method compared to the previous published methods for Cr (VI) analysis in cement, all data which are very rare are collected in Table 4. The sensitivity of the current method is comparable to others.

Table 4

Methods for measuring Cr (VI) in cement using a variety of chromogenic agents: a comparison.

Method	Matrix	Reagent	LOD (mg/kg)	Reference

Spectrophotometric	Cement	Diphenylcarbazide	0.0010	[22]
Spectrophotometric	Cement	Variamine blue	0.0500	[22]
Spectrophotometric	Cement	1, 5-diphenylcarbazide	0.0013	[22]
Spectrophotometric	Cement	Thiazole linked to 2H-chromen-2-one	0.0007	This study

4. Conclusion

The current analytical method for the determination of Cr (VI) content in environmental samples has proved to be reliable, simple, and rapid. The method has the ability to be performed as a quick test for determining hexavalent chromium in cement. Numerous further advantages of the current method include the fact that the complexion reagent can be made on any scale with great purity and a specific chelation with Cr (VI) in neutral medium, there is no need for a pretreatment step to enhance the analyte concentration, and it is applicable to a wide range of analyte concentrations. Therefore, the current method could be used for selective trace determination of chromium in several matrices.

Authors' Contributions

Abdullah Akhdhar was responsible for the conceptualization, methodology, manuscript preparation, writing the original draft, and publication.

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DETAILS

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Polycyclic Aromatic Hydrocarbon (PAH) Contents of Four Species of Smoked Fish from Different Sites in Senegal

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ABSTRACT (ENGLISH)

Polycyclic aromatic hydrocarbons (PAHs) are compounds resulting from any incomplete combustion process. These are pollutants that have proven toxicity due to their carcinogenic nature and can contaminate food during traditional smoking methods. Their highly toxic effect on human health requires monitoring of their levels in food products and the development of appropriate analytical methods for their determination. Thus, this study was conducted to assess the level of PAHs contamination of four (4) species of smoked fish (*Arius heudelotii*, *Sardinella aurita*, *Ethmalosa fimbriata*, and *Sardinella maderensis*) which were sampled in seventeen (17) localities in Senegal. The compounds targeted in this study were benzo(a)pyrene (B(a)P), benzo(a)anthracene (B(a)A), benzo(b)fluoranthene (B(b)F), and chrysene (Chr). The QuEChERS method was used for the extraction of PAHs, and their contents were quantified by

gas chromatography (GC) coupled with mass spectroscopy (MS). The validation method was performed in accordance with the French standard NF V03-110 (2010). Satisfactory linearity (R^2>0.999), LOD (0.05–0.09 μg/kg), LOQ (0.19–0.24 μg/kg), and precision (1.33–3.13%) of the four PAHs were obtained. The results of analysis in the 17 localities showed that all samples are contaminated by the four (4) PAHs with great variability of the contents between the different species and their origin. The B(a)P and ∑4PAHS contents in the samples ranged from 1.7 to 33 μg/kg and from 4.8 to 1082.3 μg/kg, respectively. Twelve (12) samples showed high levels of B(a)P, ranging from 2.2 to 33 μg/kg, thus exceeding the maximum authorized level (2 μg/kg). Fourteen (14) samples showed an overall ∑4PAHS content varying from 14.8 to 1082.3 μg/kg, which is above the maximum authorized limit (12 μg/kg). The principal component analysis showed that *sardinella* (*Sardinella aurita* and *Sardinella maderensis*) have very low levels of B(a)P, B(b)F, B(a)A, and Chr contents. However, high ∑4PAHS contents characterize smoked fish of the Kong species (*Arius heudelotii*), from Cap Skiring, Diogne, Boudody, and Diaobé, and of the Cobo species (*Ethmalosa fimbriata*) from Djiffer. Thus, based on the authorized limits for PAHs in smoked fish, it appears that smoked fish of the *sardinella* species are less carcinogenic for human consumption.

FULL TEXT

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1. Introduction

Fishing is one of the most dynamic sectors in Senegal as it has experienced strong growth over the past three decades. Catches have increased considerably eight (8) times in thirty-two (32) years [1]. A source of jobs, income, and foreign currency for stakeholders and governments, fishing is a real center for economic and social development. In Senegal, the average of sea fishing landings over the period 2009–2018 was estimated at 421,000 tons, 89% of which was carried out by artisanal fishing. In 2018, the total national fish production reached more than 461,000 tons [2]. Most of the catches come from small-scale maritime fishing (94.7%) [3]. It should also be noted that fish is one of the main sources of animal protein for populations, thus contributing to more than 75% of protein intake [4–6]. However, the fish is known for its highly perishable nature [7]. Indeed, it can deteriorate very quickly and become unfit for consumption and even dangerous for health due to microbial proliferation, chemical modifications, and degradation by endogenous enzymes [4]. Thus, due to the large quantities produced, the perishable nature of the fish, and the lack of appropriate conservation equipment, large quantities of fish are processed using traditional methods.

Among these artisanal processing methods, smoking occupies a prominent place in developing countries. This technique is the main and sometimes the only means of supplying populations with fish, especially when they are far from fishing sites. Smoke not only gives fish a special taste and aroma but also improves preservation due to its dehydrating and bactericidal properties [8]. However, smoke, in particular that of wood, is a vector of compounds called polycyclic aromatic hydrocarbons (PAHs), known for several decades for their carcinogenic power in humans [9]. This artisanal processing technique is generally known to generate and increase the level of PAHs in food. Moreover, many studies have shown the presence of PAHs in smoked fish [10, 11]. Today, the presence of these PAHs in food products, especially smoked fish, is a subject of major concern.

It is in this context that this present study occurs, which had as its objective the determination of polycyclic aromatic hydrocarbons (PAHs) in four (4) species of smoked fish from different areas in Senegal. Thus, the contents of benzo(a)pyrene (B(a)P), benzo(a)anthracene (B(a)A), benzo(b)fluoranthene (B(b)F), and chrysene (Chr), enacted by European Commission (EC) Regulation No. 835/2011, were estimated. A principal component analysis (PCA) was done to assess the effect of fish species, source, and technology of processing on the quality of smoked fish.

2. Materials and Methods

2.1. Biological Material

The biological material consists of four (4) species of smoked fish that were chosen from the processors. These were Kong (*Arius heudelotii*), Round *sardinella* (*Sardinella aurita*), Cobo (*Ethmalosa fimbriata*), and Tass or Flat *sardinella* (*Sardinella maderensis*). The choice of these four species was justified by the fact that they are more

consumed in Senegal.

2.2. Sampling and Preservation

Smoked fish samples were collected between February and June 2021 in seventeen (17) fish-processing sites in Senegal. Figure 1 presents the geographical plan of the 17 smoked fish sampling sites. These sites are located on the Senegalese coast near the landing centers for artisanal fishing. Thus, for each sample, the fish were packed in bags and then stored at −4°C. Once in the laboratory, they were crushed, and then introduced into QuECherS (quick, easy, cheap, rugged, and safe) tubes and stored at −20°C in the freezer until the start of the analyses.

[figure(s) omitted; refer to PDF]

2.3. Chemicals

Chemicals and solvents used are of analytical reagent grade. The acetonitrile (99.9%) and the acetone (99.8%) were, respectively, obtained from Sigma-Aldrich and VWR Chemicals. QuEChERS extraction tubes were purchased from Thermo Fisher Scientific Inc. (USA) and consisted of 50 mL centrifuge tubes containing 4 g anhydrous magnesium sulfate and 1 g sodium acetate. The 4 standards PAHs (B(a)P (CAS No. 50-32-8), B(a)P (CAS No. 56-55-3), B(b)F (CAS No. 205-99-2), and Chr (CAS No. 218-01-9), manufactured by Supelco, were purchased as pure compounds.

2.4. Methods for the Extraction and Analysis of PAHs in Smoked Fish

The QuEChERS method was used for the extraction of PAHs [12]. The proposed method is fast and effective and can be successfully applied for PAHs determination in difficult matrices such as heat-treated food of animals [13]. For this, a test portion of 5 g of fish flesh was ground, homogenized, and introduced into a 50 mL QuECherS tube. Then, 20 mL of an acetonitrile-acetone mixture (60 : 40 v/v) is added thereto. The whole was stirred vigorously at 2000 rpm using a DLAB MX-S vortex (DLAB Scientific Inc.) for 30 seconds and then with an ultrasound bath for 5 minutes. After this step, centrifugation for 7 minutes at 3000 revolutions per minute was carried out, using a Medibas + centrifuge mod. 2741 (Auxilab). The supernatant was transferred to a 250 mL flask and then evaporated to dryness at 35°C using a rotary evaporator. Then, the concentrated residue was extracted with 10 mL of the acetonitrile-acetone mixture (60 : 40 v/v), and then the whole was transferred into another 50 mL QuEChERS tube and centrifuged at 2000 revolutions per minute for 1 minute. The supernatant was recovered and placed in a 100 mL flask and then evaporated at 35°C. The concentrated residue contained in the 100 mL flask was again recovered with 2 mL of ACN using a 1000 μL automatic pipette. Everything was transferred using a Pasteur pipette into a vial and then frozen for 24 hours to freeze the fat. Finally, the supernatant was collected using a Pasteur pipette into another vial. The latter was placed in a gas chromatograph (GC) of the Agilent 7890A type coupled to a mass spectrometer (MS) (Agilent 5975C). The apparatus was equipped with an automatic sampler. Separation of PAHs was conducted using a 5% phenyl-methylsilicone (HB-5MS) bonded-phase fused-silica capillary column (Hewlett-Packard, 30 m * 0.25 mm I.D., and film thickness 0.25 μm). Helium (99.999% purity) was used as the carrier gas with a flow rate of 1.2 mL/min. The injection port was adjusted in splitless mode, and the injection volume was 1.5 μL. The GC was programed as follows: initial temperature 70°C for 5 min and ramped at 25°C/min to 310°C for 13 min and allowed to stay for 10 min giving a total of run time of 28 min. The mass spectrometer (MS) was operated in the electron ionization mode, with an electron energy of 70 eV. The MS transfer line and ion source temperatures were adjusted at 310°C and 290°C, respectively.

Of the eight (8) PAH molecules recognized as carcinogenic when present in food, four (4) have been determined as benzo(a)pyrene (B(a)P), benzo(b)fluoranthene (B(b)F), benzo(a)anthracene (B(a)A), and chrysene (Chr).

In order to increase sensitivity, all the GC-MS measurements of the different samples were carried out in triplicate and the results recorded in the various tables represented the average.

2.5. Validation Method

The quantification method of PAHs in smoked fish samples was developed and validated according to the indications of the European Commission (no. 836/2011) and the French standard NF-V03-110 (2010). This procedure included successive steps and the determination of different parameters such as the linearity of the calibration interval, the detection and quantification limits, coefficients of variation for the repeatability, and

intermediate precision tests.

The identification of PAHs was verified by comparing the retention times of each standard solution between each test. For the linearity tests, the standard stock solutions were dissolved in acetonitrile and five working solutions (0.005, 0.1, 0.2, 0.5, and 1 ppm) were prepared to distinctly construct the calibration curves. These curves made it possible to establish an adequate correlation between the areas of the peaks and the concentrations of PAHs found in the samples tested. The limits of detection (LOD) and quantification (LOQ) were calculated from the standard PAH solution by separate 3 × 10 analysis according to the repository (NF-V03-110 2010). The values of these limits expressed in (μg/kg) are given by the following formulas:(1)LOD=mb+3σ,LOQ=mb+10σ,where **mb** is the average of each marker PAHs content in the standard solution and ***σ*** is the value of standard deviation for the norm for each PAH marker.

Besides, the analytical stability was assigned by the analysis of a standard solution of 1 ppm by calculating the relative standard deviation of the measurements. By repeating the analysis three times per day (intraday) for three consecutive days (interday), the precision (%) expressed as a coefficient of variation (CV) was obtained.(2)CV %=Standard deviationMean*100.

2.6. Statistical Analyses

All analyses were injected in triplicate to obtain a good precision. GC-MS data were acquired and analysed by Agilent Chemstation software for GC (G2070BA) and a Microsoft Excel version 2016 spreadsheet. A principal component analysis (PCA), which is the most widespread of the factorial methods [14], and a numerical classification were carried out on the results of analyses of the seventeen (17) zones in order to bring out the information carried by the four (4) PAHs contained in the samples. Thus, all analyses were performed with the software *R* (version 4.1.1, 2021).

3. Results

3.1. Validation of the PAHs Determination Method

The method validation results as the limit of detection (LOD), limit of quantification (LOQ), linearity, and retention times are shown in Table 1. B(b)F, Chr, B(a)A, and B(a)P were identified, respectively, at times 22.43, 23.66, 21.22, and 27.52 minutes. The determination of the correlation coefficients ranged from 0.9994 to 0.9999. The respective values of the detection limits were 0.05, 0.09, 0.07, and 0.08 μg/kg and those of quantification were 0.19, 0.24, 0.2, and 0.23 μg/kg for the B(b)F, B(a)A, Chr, and B(a)P. The LOD and LOQ values were within the acceptable limits of ≤0.3 μg/kg and ≤0.9 μg/kg, respectively, for each of the four PAHs (B(a)A, B(a)P, B(b)F, and Chr). The calculated coefficients of variation of the repeatability tests ranged from 1.33–2.25% to 1.95–3.13%, respectively, for intraday and interday. Corresponding results for the PAH4 are presented in Table 2. The tests of reproducibility corroborate the results' repeatability. Indeed, the coefficients of variation obtained were less than 15%. The results of these validation tests are in adequacy with the European Commission (EC) standard no. 836/2011. The data obtained from the validation of the method in this study confirm that GC-MS is satisfactory for the analysis and detection of PAH4 in smoked fish samples.

Table 1

The limit of detection (LOD), limit of quantification (LOQ), linearity, and retention times of the four standard PAHs.

PAHs	Calibration equations	Correlation coefficients (R^2)	Retention times in minutes	Limit of detection (LOD) (μg/kg)	Limit of quantification (LOQ) (μg/kg)
B(b)F	$y = 28.015x + 2.5925$	0.9994	24.43	0.05	0.19
B(a)A	$y = 24.994x - 0.1076$	0.9997	21.22	0.09	0.24

Chr	y=27.82x+ 1.5568	0.9995	22.66	0.07	0.20
Ba aP	y=24.535x+ 1.9721	0.9999	26.52	0.08	0.23

Table 2

Intraday and interday of precision of the four PAHs.

PAHs	CV (coefficient of variation in %)	
Intraday	Interday	B(b)F
2.25	3.13	B(a)A
2.17	2.88	Chr
3.33	3.74	BaaP

3.2. PAH Content in Smoked Fish Samples

The results from the analyses are presented in Table 3.

Table 3

PAH contents in seventeen (17) samples of smoked fish.

No.	Species	Origins			B(b)F (µg/kg)	B(a)A (µg/kg)	Chr (µg/kg)
B(a)P (µg/kg)	∑4PAH (µg/kg)	1	Kong	Boudody			357.8
107.2	142.6	28.4	636.0	2	Kong	Cap Skiring	
236.5	57.2	78.1	16.9	388.7	3	*Sardinella*	Cayar
1.7	1.2	1.3	0.6	4.8	4	Kong	Diaobé
822.3	121.6	133.2	5.2	1082.3	5	Kong	Diogue
249.5	59.5	76.2	13.2	398.4	6	*Sardinella*	Dionewar
3.3	1.3	1.5	2.3	8.4	7	Cobo	Djiffer
116.6	41.7	51.5	33.0	242.8	8	Kong	Fanda

46.4	8.9	23.9	2.2	81.4	9	*Sardinella*	Fass Boye
2.8	2.0	2.7	0.8	8.3	10	*Sardinella*	Joel
15.7	4.3	7.2	3.9	31.1	11	*Sardinella*	Kafoutine
11.4	2.4	6.7	1.4	21.9	12	*Sardinella*	Loumpoul
11.2	1.6	4.9	1.8	19.5	13	Tass	Mbour
10.5	2.4	2.6	2.3	17.8	14	*Sardinella*	Missirah
8.2	3.2	2.6	0.8	14.8	15	*Sardinella*	Niddior
37.1	8.8	16.3	6.8	69.0	16	*Sardinella*	Sedhiou
70.1	20.5	30.4	14.2	135.2	17	Kong	Thiaroye
40.1	5.6	14.3	2.3	62.3	Mean		

The results showed a great variability of the contents between the various species and according to their origin. The B(a)P molecule, representing 40% of the total carcinogenic risk attributed to PAHs [16], is present in all the samples analysed with sometimes levels that exceed the authorized limit (2 μg/kg). Thus, it appears from these analyses that the samples taken in the areas of Dionewar (2.3 μg/kg), Fanda (2.2 μg/kg), Joel (3.9 μg/kg), Mbour (2.3 μg/kg), Thiaroye (2.3 μg/kg), Boudody (28.4 μg/kg), Cap Skiring (16.9 μg/kg), Diaobé (5.2 μg/kg), Diogue (13.2 μg/kg), Djiffer (33 μg/Kg), Niddior (6.8 μg/kg) and Sedhiou (14.2 μg/kg) are not B(a)P compliant. The highest concentration of this molecule was observed in Djiffer with a value of 33 μg/kg. However, these values are lower than those obtained by [8] on smoked fish species from south Nigeria, the levels of which were estimated at 204 μg/kg for *Clarias gariepinus* and 288 μg/kg for the species *Ethmalosa fimbriata*. The mean value (8.0 μg/kg) of B(a)P observed in the samples is much higher than that found in [11] whose concentration was below the detection limit (0.01 μg/kg) but lower than that (52.7 μg/kg) obtained in [17] on smoked fish species.

The analysis of samples of smoked fish from various sites also revealed the presence of chrysene at variable concentration levels between 1.3 and 142.6 μg/kg. These were observed in samples from Cayar and Boudody, respectively. The average chrysene content is 35.1 μg/kg. The latter is higher than the mean values of B(a)A and B(a)P. Compared to the results in [18], a content of 69.7 μg/kg of chrysene was obtained in a species of smoked fish called *Thunnus albacares*.

Regarding B(b)F and B(a)A, the levels in the various samples analysed vary, respectively, from 1.7 to 822.3 μg/kg and from 1.2 to 121.6 μg/kg. The average contents of B(b)F and B(a)A, in the samples, were respectively evaluated at 120.1 and 26.4 μg/kg. Much lower levels were observed in [19] on several species of smoked fish, in the Beninese Coast. The average concentrations of B(a)A and B(b)F in these species were estimated, respectively, at 9.99 μg/kg and 5.79 μg/kg.

The total concentration of the four (4) molecules varies between 4.8 and 1082.3 μg/kg, respectively, observed in the samples from Cayar and Diaobé. Higher concentrations of the sum of PAHs, between 1295 and 2020 μg/kg, were obtained in [20]. The average content of ∑4PAHSs in the different samples studied is much higher than that obtained in [11] which was estimated at 12.47 μg/kg. The total PAH content recommended by the European Commission is 12 μg/kg. Thus, only the samples taken in the sites of Cayar (4.8 μg/kg), Dionewar (8.4 μg/kg), and

Fass Boye (8.3 μg/kg), with values below the standard, are therefore compliant.
It is clear that also samples from sites, such as Boudody, Dioabé, Diogue, Djiffer, Sedhiou, and Cap Skiring, have the highest PAH contents. The latter has been observed in species such as Kong (*Arius heudelotii*) and Cobo (*Ethmalosa fimbriata*).

3.3. Principal Component Analysis

Principal component analysis (PCA) was carried out to assess the effects of smoking, the nature of the fish species, and the place of processing on PAH levels. The first two dimensions (Dim 1 and Dim 2) express 98.90% of the total inertia (Table 4).

Table 4

Correlation between components and variables.

Variables	Main components	
Dimension 1	Dimension 2	B(b)F
0.818	0.163	B(a)A
0.986	0.011	Chr
0.981	0.001	B(a)P
0.457	**0.539**	Own value
3.24	0.71	Variance (%)
81.08	17.82	Cumulative variance (%)

Bold values represent the best correlation coefficients of each variable with respect to the dimensions (1 and 2).
The first dimension (Dim 1) contributes to 81.08% and the second (Dim 2) at 17.82%. They thus present the highest eigenvalues, respectively, equal to 3.24 and 0.71.
The variables B(a)A (0.986), B(b)F (0.818), and Chr (0.981) are positively well correlated with the first dimension (Dim 1). However, the second dimension is characterized by the variable B(a)P (0.539), which is positively correlated. Smoked fish have been grouped into three classes. Class 1 is made up of *sardinella* which have very low levels of B(a)P, B(b)F, B(a)A, and Chr. However, high levels of B(a)P and Chr characterize class 2 consisting of smoked fish of the Kong species, from Cap Skiring, Diogue, and Boudody, and of the Cobo species from Djiffer (Figures 2 and 3). The third class, which represents smoked fish of the Kong species from Diaobé, is characterized by B(b)F and ∑4PAH high. In short, based on the standards in terms of PAH content limits for smoked fish, it appears that smoked fish of the *sardinella* species are less carcinogenic for human consumption.
[figure(s) omitted; refer to PDF]

4. Discussion

The analysis of the results of this study shows that the B(a)P content and the sum of the ∑4PAHS contents in different zones and according to the species present a significant difference compared to the standard.
This trend could be attributed, on the one hand, to the differences in fat and moisture composition of each species as well as to the nature of the skin cover [21]. Indeed, according to [20], smoked fatty fish are more exposed to the development of PAHs than lean fish. Fat is associated with the pyro synthesis of PAHs. It would also be linked to the traditional methods of smoking carried out in these areas [22, 23]. Indeed, smoking is done exclusively by women,

and it is clear that they very often use wood lint or coconut shell as their main fuels. The pyrolysis of cellulose and lignin contained in the wood gives rise to the production of PAHs [24, 25]. Furthermore, the type of wood has a significant influence on the increase of PAH contents in smoked foods. Similarly, the use of cartons during the smoking process could modify the photosensitive properties of PAHs and cause their accumulation in foods [26]. In addition, this is the fact that the PAH content in smoked products varies and depends on environmental factors such as the presence or absence of light or oxygen. In addition, the long smoking times as in the species (*Arius heudelotii*), the temperature used and multiple smoking could lead to the high numbers obtained. Moreover, studies have shown that a smoking temperature above 450°C would allow the pyrolysis of the wood to generate more (PAH) [17, 27].

PAHs would also be present in fish products before smoking. Indeed, apart from spatiotemporal parameters, it has been revealed that the levels of PAHs would also be affected by several ecological factors. It has been demonstrated that vehicles, especially diesel engines, could also contribute significantly to the generation and release of PAHs into the environment [28]. In addition, studies have shown the presence of PAHs in marine sediments which could contaminate the fish before smoking [29]. The combined influence of all these variability parameters associated with possible environmental contamination would certainly constitute the source of the increase in PAH levels in these smoked fish. Thereby, the high levels of PAHs observed in certain areas of this study would probably be linked to ignorance of the existence of the production of PAHs during technological treatment and ignorance of the processes minimizing the production of PAHs. So, the adoption and use of appropriate facilities as studied in [30, 31] are required to considerably reduce the PAH content in smoked fish and to limit the contamination of finished products.

5. Conclusion

This study consisted of highlighting the presence of polycyclic aromatic hydrocarbons (PAHs) in traditionally smoked fish. Indeed, smoking, while helping to preserve fish, could also induce certain carcinogenic substances such as PAHs in processed fish. Sampling, carried out in seventeen (17) production areas in Senegal, revealed the presence of benzo(a)pyrene (B(a)P), benzo(a)anthracene (B(a)A), benzo(b)fluoranthene (B(b)F), and chrysene molecules (Chr). The results showed that twelve (12) or 71% and fourteen (14) sites or 82% present contents higher than the European Commission standards, respectively, for B(a)P and the sum of ∑4PAH. The highest PAH concentrations were observed in the Kong species (*Arius heudelotii*). These results, therefore, indicate that it is necessary to improve the smoking process in order to best limit the rate of contamination of the finished products with PAHs and ensure consumer safety. Moreover, it would be very important to carry out an analysis to assess the possible risks to human health associated with the consumption of these smoked fish in Senegal.

Authors' Contributions

All authors contributed to the realization of the study. El Hadji Moussa Diop, Bou Ndiaye, and Aliou Sow played a major role in data mining, results interpretation, and writing and reviewing the whole final manuscript. Mamadou Sall and Mamadou Saliou Thiam played a major role in sampling the smoked fish, preparing the extraction method, and analysing the samples. Falilou Mbacké Sambe critically revised the manuscript for significant intellectual content.

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Discrimination and Evaluation of Wild Paris Using UHPLC-QTOF-MS and FT-IR Spectroscopy in Combination with Multivariable Analysis

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ABSTRACT (ENGLISH)

Genus *Paris* has numerous bioactive constituents such as steroid saponins, flavonoids, and polysaccharose which are responsible for antitumor, hemostatic, and anthelmintic, etc. In this study, ultrahigh performance liquid chromatography coupled to time-of-flight mass spectrometer (UHPLC-QTOF-MS) and Fourier transform infrared (FT-IR) spectroscopy in combination with multivariable analysis were employed to discriminate the different species of *Paris* including *P. polyphylla* var. *yunnanensis* (PPY), *P. polyphylla* var. *alba*, *P. mairei* (PM), *P. vietnamensis*, and *P. polyphylla* var. *stenophylla*. Partial least square discriminate analysis based on UHPLC, FT-IR, and midlevel data fusion was used to distinguish 43 batches of Paris. Chemical constituents of different species *Paris* were determined by UHPLC-QTOF-MS. The result indicated that midlevel data fusion had a good performance in the classification compared to a single analytical technology. A total of 47 compounds were identified in different species *Paris*. The similar results indicated that PM could be treated as a proposal substitute of PPY.

FULL TEXT

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1. Introduction

Genus *Paris* (Lilaceae family) including 24 species, is mainly distributed in the southwest of China except for *Paris birmanica* and *P. japonica* [1]. As an ethnic medicine, it is recorded in Shennong Materia Medica which is one of the longest medical books in China for treatment of hyperspasmia and bite wound in the folk for a long history [2]. In addition, *P. polyphylla* var. *yunnanensis* (PPY) and *P. polyphylla* var. *chinensis* have been documented in Chinese Pharmacopoeia (ChP) named as Rhizome Paridis (RP) for treating in furunculosis, throat-swelling, traumatic injury, and so on [3]. Modern investigation suggests *Paris* has bioactive constituents such as steroid saponins, flavonoids, and polysaccharose which are responsible for antitumor, hemostatic, and anthelmintic, etc [4, 5]. The extraction of RP is material to make the Chinese patent drugs contain “Gongxuening capsule” and “Jidesheng Sheyao Tablet.” Numerous sources are gradually applied to the industrial production in decade years. Following price of commercial produce including PPY and PPC is higher than the past in the market. Up to now, *P. polyphylla* var. *stenophylla* (PPS), *P. mairei* (PM), and *P. vietnamensis* (PV) are barely investigated by isolation and identification of the major bioactive steroidal saponins [6–8]. At the same time, few research studies could not notice the *P. polyphylla* var. *alba* (PPA). The relationship between different species is hardly illuminated in previous study either.

In previous study, the research indicated an amount of active compounds was found in *Paris*, while only four bioactive markers were determined by ChP for the quality control of RP. It could not respond to multiconstituents and multitargets for traditional Chinese medicine. It was therefore necessary to develop an analytical method which could be responsible for the comprehensive chemical compounds. Fortunately, fingerprints analysis with the advantage of systematic and effective characteristics is used to evaluate the sample of different geographical origin [9, 10], parts [11], and species [12, 13]. As far as we know, chromatographic and spectrographic fingerprints were investigated by previous study. The former could provide the unambiguous and specific information of compounds based on the peak signal. At the same time, the later with the overall and convenient characteristics are used to illustrate the chemical profile. For evaluation of herbal medicine, ultrahigh performance liquid chromatography (UHPLC) as a fast and effective method could provide the targeted compounds of samples [14]. Meanwhile, Fourier transform infrared spectroscopy (FT-IR) with nondestructive and feasible character is a characteristic of integrated chemical information [15]. However, few research studies have been focused on the strategy which combined UHPLC and FT-IR with chemometric for evaluation of Genus *Paris*.

Compared to a single analytical technique, data fusion of different instrumental (spectroscopic and chromatographic) techniques together with multivariate chemometrics can increasethe model classification [16]. Three date fusion strategies (low-, mid-, and high level) can be carried out basically. Especially, mid-level fusion can be able to filter block noise and enables interpretation of the results, firstly extracts some relevant features from each data of instrument, and then merges them into a single array that is used for classification [17]. The aim of this investigation is to validate and develop an analytical strategy which can evaluate and discriminate different species of *Paris* with regard to PPY, PPA, PPS, PM, and PA. Firstly, chemical profiling of *Paris* was identified by UHPLC-QTOF-MS. Then, different species of *Paris* were discriminated through UHPLC, FT-IR, and data fusion coupled to chemometrics. It is expect that the study can provide a fundamental of genus *Paris* sources.

2. Experiment

2.1. Materials and Reagents

Forty-three samples of the fresh five species of Genus *Paris* were collected from different regions in Yunnan Province Southwestern China and were identified by Professor Jinyu Zhang in Yunnan Academy of Agricultural Sciences. The detailed information of samples was listed in Table 1. Voucher specimens were deposited in Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences. Standard compounds *Paris* saponin I (PSI, No: MUST-21110718) and *Paris* saponin II (PSII, No: MUST-21062804) were purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). *Paris* saponin VI (PSVI, No: PRF20080742) and *Paris* saponin VII

(PSVII, No: PRF20121241) were provided by Chengdu Biopurify Biotechnology Co., Ltd. The purity of all reference compounds was determined to be over 98% by UHPLC. The structures of standard constituents were displayed in Figure 1.

Table 1

Information of 43 bacthes six species of *Paris*.

Code	Number	Species	Site
PPA1-PPA9	9	*P. polyphylla* var. *alba*	Hani-Yi autonomous prefecture of Honghe of Yunnan province
PPY1-PPY10	10	*P. polyphylla* var. *yunnanensis*	Baoshan city of Yunnan province
PM1-PM10	10	*P. mairei*	Lijiang city of Yunnan province
PV1-PV10	10	*P. vietnamensis*	Pu’er city of Yunnan province
PPS1-PPS4	4	*P. polyphylla* var. *stenophylla*	Zhaotong city of Yunnan province

[figure(s) omitted; refer to PDF]

HPLC-grade acetonitrile and formic acid were provided by Dikmapure Co., Ltd. (Lake Forest, MA, USA), respectively. Distilled water was further purified by a Milli-Q system (Bedford, MA, USA). Analytical grade methanol was purchased from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Supersonic wave cleaning equipment (SY3200-T) was purchased from Hengda Ultrasonic Equipment Co., Ltd. (Zhejiang, China). Spectroscopic grade potassium bromide (KBr) was purchased from Fengchuan Fine Chemical Research Institute (Tianjin, China).

2.2. Apparatus

A UHPLC-UV system (Shimadzu, Kyoto, Japan) equipped with a degasser, binary gradient pumps, UV detector, a column oven, and an auto-sampler was utilized to obtain UHPLC fingerprints. System control and data analysis were conducted by LabSolution software (Shimadzu). The chromatographic separation was operated on Shim-pack XR-ODS III column (150 × 2.0 mm, 2.2 μm) with UV detector where the detection wavelength was set at 203 nm. The mobile phase was (A) 0.05% formic acid in water and (B) acetonitrile with a gradient program as follows: 17% B, 0–1.5 min; 17–23% B, 1.5–4.0 min; 23% B, 4.0–8.7 min; 23–38% B, 8.7–18 min; 38-60% B, 18-25.6 min; 60–17% B, 25.6–28 min; 28–32 min, 17%. In order to identify the chemical constituents of *Paris*, gradient program for UHPLC-QTOF-MS was 0–2 min, 17% B; 2–5 min, 17%–23% B; 5–10 min, 23%–40% B; 10–35.5 min, 40%–60% B; 35.5–38 min, 60%–95% B; 38–40 min, 95%; 40-41 min, 19% B. Other chromatographic parameters were as follows: The column temperature was maintained at 45°C. The flow rate was 0.45 mL/min. All samples were injected with 1 μL. Mass spectrometry was performed on a Triple-TOFTM 5600^{+} system mass spectrometer (AB SCIEX, Foster City, CA, USA). Data acquisition was conducted in the negative electrospray (ESI) ionization mode. The ESI-MS parameters were as follows: mass scan range for both TOF-MS and TOF-MS/MS: m/z 50–1200, ion spray voltage: 4500 V, atomizer temperature (TEM): 500°C; declustering potential (DP): 80 V; curtain air pressure (CUR): 40 psi; nebulize gas (Gas 1) and auxiliary gas (Gas 2) pressure: 50 psi. The collision energy was set at 50 eV. Instrument control, data acquisition, and analysis of data were Analyst TF 1.6 and PeakView 1.2.

For FT-IR, table press (Shanghai, China) was employed to press the samples into thin sample. FT-IR (Perkin-Elmer, Norwalk, CT, USA) was equipped with a deuterated triglycine sulfate detector. IR spectra were recorded from the

accumulation of 16 scans in 4000–400 cm^{-1} range with a resolution of 5 cm^{-1}.

2.3. Sample Preparation

For UHPLC, 0.1 g of sample powder after passing through a 60 mesh sieve was weighed accurately, transferring into 10 mL glass stopper tube and adding 2 mL of 80% methanol with ultrasonically extracted for 40 min at room temperature. The filtrates were collected by filter paper after sample solution cooling to the room temperature. All solution was stored at 4°C and passed through a 0.22 μm membrane filter before injection into the UHPLC system. The standard stock solution for qualitative analysis was dissolved all reference standards with methanol to final concentration of 1 mg/mL. For FT-IR, 1.5 mg of dried sample and 100 mg KBr was weighted accurately and mixed completely by the agate mortar and then a homogeneous tablet was pressed . The KBr pellet background of CO_2 and H_2O was deducted before experiment. The FT-IR spectra of sample were scanned 2 times and recorded after preheating 60 min under relative humidity 65% at the temperature 25°C.

2.4. Data Analysis

UHPLC chromatographic fingerprints were recorded by the sampling points from 2.5 to 29 min with the time interval of 0.0833 min. Owing to the disturbance of methanol signal noise, the sampling points were rejected before 2.5 min. Date of UHPLC was entered into *Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine* (Version 2004A, Chinese Pharmacopoeia Committee) for similarity analysis. For FT-IR, the data of spectra were entered into the software of OMNIC (Version 8.2, Thermo Fisher Scientific Inc, USA), which were used by noise reduction, baseline correction, and so on.

As a supervised method, PLS-DA is used to concentrate the multidimension data into two dimension, which can illustrate a sample whether it belong to a special class [18]. To evaluate and discriminate *Paris* of different species, PLS-DA based on UHPLC and FT-IR spectra was used to make the further study. All of data were imported into SIMCA-P^+ 10.0 (Umetrics AB, Umea, Sweden). To remove the baseline shifts and overlap peaks, the data were subjected to second derivative before analysis [19].

3. Results and Discussion

3.1. Method Validation

For UHPLC, steroid saponins including PSI, PSII, PSVI, and PSVII were used to develop and validate method for UHPLC. Precision was performed by the inter- and intraday variations to make the analytical method accurate. The intraday was accumulated by retention time and peak areas using six repetitive injection of mixed standards in a day and three consecutive days for the interday variation. The results implied that relative standard deviations (RSD) of intra- and interday were no more than 2.98% and 2.83%, respectively (Table 2).

Table 2

Intra- and inter-day of four standards.

	Intra-day (n=6)		Inter-day (n=3)	
t (%)	P (%)	t (%)	P (%)	PSI
0.06	1.36	0.11	2.57	PSII
0.06	2.98	0.12	2.83	PSVI
0.02	2.28	0.11	2.64	PSVII

"t" the retention time of common peak of standard; "P" the peak areas of common peak of standard.

For FT-IR, the precision was performed using five consecutive scan with a sample, and the results indicated that the correlation coefficient was ranged 0.9996–0.9999 with RSD = 0.05%. Stability Stability was calculated by scanning a sample every ten minutes and five consecutive times. The results suggested that correlation coefficient was

between 0.9995 and 0.9999, and RSD = 0.03%. Repeatability was carried out by five tablets with a sample for scanning, and the results implied that correlation coefficient was more than 0.9996, and RSD = 0.02%.

3.2. Similarity Analysis for UHPLC

UHPLC chromatographic fingerprint of 43 batches five species of *Paris* is shown in Figure 2. Peaks 1, 2, 3, and 4 in chromatographic pattern were assigned the bioactive compounds of PSVII, PSVI, PSII, and PSI based on the reference standards. As we can see, PSII and PSI changed significantly in each sample with even not being detected in PPA, whereas PSVI was found in the most of samples. It indicated that chemical constituents varied markedly for the *Paris* of different species. The result was similar to the previous research that the contents of chemical components have difference among collections of *Paris* of different geographical origins and species [20]. For similarity evaluation, the average chromatogram of 43 batches of samples was treated as the standard characteristic fingerprint. Similarity values were calculated by compared with each chromatogram of the *Paris* samples to the average chromatogram. As shown in Figure 3, the similarities by all of samples were no more than 0.66 (PM3). For the most of samples, the values were ranged from 0.55 to 0.65. The changed chemical constituents of sample may be discovered in PM9 (0.49) and PPS2 (0.38) with the value less than 0.5. However, it is difficult to clarify the relationship between different species of *Paris*.

[figure(s) omitted; refer to PDF]

3.3. Discrimination of *Paris* by UHPLC

As shown in Figure 4, a two-dimension score scatter plot with 95% confidence ellipses of PLS-DA was used to evaluate and discriminate the different species of *Paris*. It is significant among various sample PV from Simao City of Yunnan Province with four outliers PV1, PV2, PV3, and PV4. The collection sample with multiple soil, moisture, and surrounding environment may lead to vary in chemical components of each sample [9]. The phenomenon is necessary to work in the further study. It is obvious that the chemistry in each sample changed markedly for the similar species in the rhizome of PV. Moreover, group 1 including PPA, PPY, and PM was distributed in the positive principle compound (PC) 1; on the contrary, other species of *Paris* in the negative PC1 were assigned group 2. The previous morphological research reported that PPA, PPY, and PPS belong to the varieties of *P. polyphylla* Smith [1]. The similar profile was found according to the theory of macroscopical phenotype. Fortunately, it is associated with the result that is a close relationship between PPA and PPY compared to other species, especially PPA2, PPA6, PPA7, and PPA10 which are close to all of PPY species except for PPY2. However, the species of PPS had tight distance to PV without macroscopical theory. In group 1, *P. marirei* was closely related to *P. polyphylla* Smith, particularly in the species of PPY. As mentioned above, it is indicated that the chemical profile was usually related to the macroscopical characteristic with the different *Paris*. The similar report in the previous research shows that chemotaxonomic studies of nine *gentianaceae* indicate some secondary metabolites could be treated as potential chemotaxonomic markers to differentiate *gentianaceae* species. Moreover, the plots were weakly loose among the same species in the scatter plot which indicated that the chemical profile varied notably among the most of samples. As an official medical resource, PPY has been widely applied to industrial production and clinic. Due to long growth cycle and excessive excavation, the wild population of PPY is gradually decreased. Obviously, since they had the similar chemical profile, PPA and PM could be treated as a proposal substitute of PPY. UHPLC was used to evaluate and discriminate different species of *Paris* with the characteristic of rapid and accurate compared with other technology. However, the chromatographic pattern may not be responsible for the entire bioactive chemistry of sample. A convenient and nondestructive method by FT-IR is necessary for distinguishing *Paris*.

[figure(s) omitted; refer to PDF]

3.4. Discrimination of *Paris* by FT-IR

FT-IR is a technique based on the absorbance of light from 4000 to 400 cm^{-1}, which has been widely used for evaluation of herbal medicine and authentication of food products [21]. In this study, the samples of different species of *Paris* were analyzed by the FT-IR spectra shown in Figure 5. The similar position and shape of peak were found in the most of sample. The peaks were assigned as following: 3390, 2930, 1740, 1650, 1400, 1370, 1250, 1150, 1049, 930, 860, 765, and 700 cm^{-1}, which are characteristic common peaks of spectra. The wavelength at 3390 cm^{-1}

, generally the most prominent peak, was due to the stretching vibration peak of O-H in sugar moiety. The peak at 2930 cm^{-1} was assigned to the stretch of methylene group. Absorption band at around 1740 and 1650 cm^{-1} corresponding to bending C=O and C=C stretching, respectively, was potentially related to steroid saponins, flavonoids, and fatty acids. The peaks at 1400 and 1370 cm^{-1} were due to plane deviational vibrations of methylene or methyl group. A bond of 1250 cm^{-1} was responded to the vibration peak of C-O in alcohol hydroxyl group. In addition, according to the previous research that peaks at 930, 860, 1150, and 1049 cm^{-1}, these were due to steroidal saponins skeletal vibration [22]. As mentioned above, the similar reported study shows that the main constituents were steroids saponins in *Pairs*, others including flavonoids, fatty acids, and so on [5].

[figure(s) omitted; refer to PDF]

As shown in Figure 6, the two-dimension score plots were carried out by FT-IR in conjunction with PLS-DA for evaluation of different species of *Paris*. The result agreed with the analysis of UHPLC that PPY, PPA, and PM belonged to group 1 in positive PC2, and PV was attributed group 2 in negative PC2. In addition, the same phenomenon found that PPY was more closely related with PM than with PPA. However, the various results show that PPS was located in group 1 for this investigation. As the varieties of *P. polyphylla* Smith, PPS had the similar chemical constituents while the various contents were compared with UHPLC. It could draw a conclusion that the varieties of *P. polyphylla* including PPY and PPA were closely related with *P. marirei* no matter what analytical technology UHPLC or FT-IR held.

[figure(s) omitted; refer to PDF]

3.5. Discrimination of *Paris* by Midlevel Data Fusion

Midlevel data fusion for PLS-DA was used to discriminate *Paris* of different species. The combination of relevant features based on UHPLC and FT-IR was applied to establish a model which could be used for synthetic evaluation of *Paris*. As show in Figure 7, two dimension score PLS-DA of midlevel data fusion is displayed. Five species of *Paris* had a good performance for classification in the data fusion compared to a single instrumental data. Interestingly, it was similar to a single data model that PPY and PM had a close relation. It demonstrated that PM as a substitute of PPY with the further study was used to solve the resource shortage. In addition, all of PPA and PPS were located in the positive PC1 and PC2 except for PPS2. The chemical profile was related to macroscopical characteristic which was proved in this study.

[figure(s) omitted; refer to PDF]

3.6. Chemical Analysis of *Paris* Using UHPLC-QTOF-MS

The mix sample of different species *Paris* was determined by UHPLC-Q-TOF MS in the negative electrospray modes. The references PSI, PSII, PSVI, and PSVII were classified, and the cleavage patterns were summarized. In addition, the characteristic ionic fragments of compounds were summarized based on cleavage fragments and structures of *Paris*. Finally, the compounds were identified by molecular weight and secondary fragmentation. A total of 47 compounds were identified in different species *Paris*. As shown in Table 3, it included twenty-three isosproterenol saponins, five furostanol saponins, four cholestanol saponins, four flavonoids, three pentacyclic triterpenoids, two C21 steroids, two phytosterol saponins, and four others.

Table 3

Chemical constituents of PPA, PPY, PM, PV and PPS using UHPLC-QTOF-MS.

Peak	Compound name	Type	Observed m/z	$t_{(Rt)}$	Mass fragment

1	Parisyunnanoside G isomer	Isospirosteranol	1223.585 [M-H]$^-$	4.775	1077.5146 [M-H-Rha]$^-$, 931.4593 [M-H-2Rha]$^-$, 769.4178 [M-H-2Rha-Gal]$^-$, 641.3581 [M-H-2Rha-Gal-$C_5H_4O_4$]$^-$, 479.3058 [M-H-2Rha-Gal-Glc-$C_5H_4O_4$]$^-$
2	Paritriside A	Pentacyclic triterpenoids	765.1597 [M-H]$^-$	5.277	737.1350 [M-H-CO]$^-$, 633.1602 [M-H-CO-Ara]$^-$, 455.1043 [M-H-COO-Ara-Glc]$^-$
3	Pseudoproto-Pb	Furostanol	1079.4931 [M-H]$^-$	5.345	948.4591 [M-H-Xyl]$^-$, 623.1659 [M-H-Xyl-2Glc]$^-$
4	*β*-ecdysone	Cholestanol	479.3019 [M-H]$^-$	5.696	461.2961 [M-H_2O]$^-$, 319.1921 [M-$C_7H_{14}O_4$]$^-$, 301.1824[M-$C_7H_{14}O_4$-H_2O]$^-$, 283.1725 [M-$C_7H_{14}O_4$-2H_2O]$^-$
5	24-O-Gal-(23*S*,24*S*)-spirosta-5,25 (27)-diene-1*β*,3*β*,23,24-tetrol-1-O-Xyl (1-6)-Glc (1-3)[Rha (1-2)]-Glc isomer	Isospirosteranol	1223.5473 [M-H]$^-$	6.024	1091.5052 [M-H-Xyl]$^-$, 945.4456 [M-H-Xyl-Rha]$^-$
6	24-*O*-Gal-(23*S*,24*S*)-spirosta-5,25 (27)-diene-1*β*,3*β*,23,24-tetrol-1-O-Xyl (1-6)-Glc (1-3)[Rha (1-2)]-Glc	Isospirosteranol	1223.548 [M-H]$^-$	6.397	1091.5152 [M-H-Xyl]$^-$, 945.4583 [M-H-Xyl-Rha]$^-$
7	Parisyunnanoside G	Isospirosteranol	1269.5503 [M+COOH]$^-$	6.418	1091.5026 [M-H-Xyl]$^-$, 945.4307 [M-H-Xyl-Rha]$^-$, 799.3541 [M-H-Xyl-Rha-Fuc]$^-$, 637.3417 [M-H-Xyl-Rha-Fuc-Glc]$^-$
8	Kaempferol-3-O-Glc (1-4)-Glc	Flavonoids	610.2859 [M-H]$^-$	6.799	448.2910 [M-H-Glc]$^-$
9	7*β*-ol-sitosterol-3-O-Glc	Phytosterol	592.2584 [M-H]$^-$	7.117	564.2737, 548.2576, 515.1946, 119.0345,
10	Padelaoside B	Isospirosteranol	1355.59 [M-H]$^-$	6.857	1224.6083 [M-H-Xyl]$^-$, 1078.5370 [M-H-Xyl-Rha]$^-$, 897.4143 [M-H-Xyl-Rha-OGlc]$^-$
11	Parisyunnanoside G isomer	Isospirosteranol	1223.545 [M-H]$^-$	7.057	1091.4920 [M-H-Xyl]$^-$, 945.5193 [M-H-Xyl-Rha]$^-$

12	Parisyunnanoside J	C21 steroids	977.4307 [M-H]$^-$	7.619	845.3843 [M-H-Rha]$^-$, 797.3667 [M-H-Rha-C_2H_8O]$^-$, 665.3212 [M-H-2Xyl]$^-$, 519.2603 [M-H-2Xyl-Rha]$^-$
13	Parisyunnanoside H	Isospirosteranol	1061.4873 [M-H]$^-$	7.759	929.4440 [M-H-Xyl]$^-$, 783.3871 [M-H-Xyl-Rha]$^-$, 637.3271 [M-H-Xyl-Rha-Fuc]$^-$
14	Unkonwn 1	Others	479.5069 [M-H]$^-$	8.138	461.2921 [M-H_2O]$^-$, 319.1955 [M-$C_7H_{14}O_4$]$^-$, 301.1837 [M-$C_7H_{14}O_4$-H_2O]$^-$, 283.1706 [M-$C_7H_{14}O_4$-2H_2O]$^-$
15	Smilaxchinoside B	Furostanol	1195.535 [M-H]$^-$	8.64	1049.4931 [M-H-Rha]$^-$, 903.4696 [M-H-2Rha]$^-$, 741.4197 [M-H-2Rha-Glc]$^-$, 579.2568 [M-H-2Rha-2Glc]$^-$
16	Parisyunnanoside H isomer	Isospirosteranol	1061.4898 [M-H]$^-$	8.816	929.4470 [M-H-Xyl]$^-$, 765.3738 [M-H-Xyl-Gal]$^-$, 619.3119 [M-H-Xyl-Rha-Gal]$^-$, 601.3033 [M-H-Xyl-Gal-Rha-OH]$^-$, 439.2532 [M-H-Xyl-Rha-Gal-Glc-OH]$^-$
17	Parisyunnanoside H isomer	Isospirosteranol	1107.4977 [M-H]$^-$	8.846	929.4452 [M-H-Xyl]$^-$, 765.3761 [M-H-Xyl-Gal]$^-$, 619.3161 [M-H-Xyl-Rha-Gal]$^-$, 439.2543[M-H-Xyl-Rha-Gal-Glc-OH]$^-$
18	Polyphyllin H isomer	Furostanol	1109.5073 [M+COOH]$^-$	9.17	931.4595 [M-H-Xyl]$^-$, 785.4023 [M-H-Xyl-Rha]$^-$,623.3481 [M-H-Xyl-Rha-Glc]$^-$, 477.2890 [M-H-Xyl-2Rha-Glc]$^-$
19	Parisyunnanoside G isomer	Isospirosteranol	1223.5794 [M-H]$^-$	9.224	1139.5201 [M-H-C_5H_{14}]$^-$, 977.4672 [M-H-C_5H_{14}-Glc]$^-$
20	Polyphylloside IV	Isospirosteranol	1061.5255 [M-H]$^-$	9.451	916.4755 [M-H-Rha]$^-$, 770.4132 [M-H-Rha]$^-$
21	Chonglouoside SL-18	Isospirosteranol	931.4614 [M-H]$^-$	9.636	903.4320 [M-H-CO]$^-$, 757.3472 [M-H-CO-Rha]$^-$
22	Polyphyllin G	Furostanol	1049.524 [M-H]$^-$	11.445	903.4645 [M-H-Rha]$^-$, 757.3544 [M-H-2Rha]$^-$, 595.3110 [M-H-2Rha-Glc]$^-$, 433.1617 [M-H-2Rha-2Glc]$^-$

23	Polyphylloside IV isomer	Isospirosteranol	1061.5263 [M-H]⁻	14.781	929.4769 [M-H-Gal]⁻, 765.4073 [M-H-Gal-Rha]⁻, 619.0835 [M-H-Gal-2Rha]⁻, 439.1617 [M-H-Gal-2Rha-OGlc]⁻
24	Polyphylloside III/parisaponin I	Isospirosteranol	1093.5511 [M+COOH]⁻	14.953	901.4830 [M-H-Rha]⁻, 755.4256 [M-H-2Rha]⁻, 593.3341 [M-H-2Rha-Glc]⁻
25	Pennogenin-3-O-Glc-(1-5)-Ara (1-4) [Rha (1-2)]-Glc	Isospirosteranol	1033.5297 [M-H]⁻	15.231	901.4856 [M-H-Ara]⁻, 755.4224 [M-H-Ara-Rha]⁻, 593.3732 [M-H-Ara-Rha-Glc]⁻, 431.1308 [M-H-Ara-Rha-2Glc]⁻
26	Polyphyllin H	Furostanol	1063.5417 [M-H]⁻	15.551	917.4765 [M-H-Rha]⁻, 901.4845 [M-H-O-Rha]⁻, 755.4325 [M-H-O-2Rha]⁻, 593.3737 [M-H-O-2Rha-Glc]⁻, 431.3246 [M-H-O-2Rha-2Glc]⁻
27	Parispolyside E	Cholestanol	1073.5248 [M+COOH]⁻	16.493	893.4583 [M-H-Ara]⁻, 747.3036 [M-H-Ara-Rha]⁻, 585.3418 [M-H-Ara-Rha-Glc]⁻
28	Chonglouoside SL-3	Isospirosteranol	1063.539 [M-H]⁻	16.524	901.4854 [M-H-Glc]⁻, 755.4158 [M-Glc-Rha]⁻, 609.3327 [M-Glc-2Rha]⁻, 447.2354 [M-2Glc-2Rha]⁻
29	Chonglouoside SL-4	Isospirosteranol	1063.5428 [M-H]⁻	17.274	901.4424 [M-H-Glc]⁻, 755.3874 [M-Glc-Rha]⁻, 609.3722 [M-Glc-2Rha]⁻
30	Parispolyside E isomer	Cholestanol	1027.419 [M-H]⁻	17.72	929.4458 [M-H-$C_5H_{12}O$]⁻, 783.3883 [M-H-$C_5H_{12}O$-Rha]⁻, 637.3274 [M-H-$C_5H_{12}O$-2Rha]⁻,473.2534 [M-H-$C_5H_{12}O$-2Rha-Glc]⁻
31	3*β*-ol-oleane-12-en-28-oic acid- 3-O-Glc (1-2)-Glc	Pentacyclic triterpenoids	845.3762 [M+COOH]⁻	17.95	637.3279 [M-H-Glc]⁻, 475.3160 [M-H-2Glc]⁻
32	Parispseudoside B	Cholestanol	893.2173 [M-H]⁻	19.085	747.2188 [M-H-Rha]⁻, 585.1578 [M-H-Rha-Glc]⁻
33	Hypoglaucin H	C21 steroids	767.2189 [M-H]⁻	19.101	621.4030 [M-H-Rha]⁻

34	Glyceryl α-mono-palmitate	Others	329.2345 [M-H]⁻	19.303	247.2143, 229.1468, 211.1371, 171.1031, 139.1144
35	Parisaponin I	Isospirosteranol	1047.5331 [M-H]⁻	20.677	885.4764 [M-H-Glc]⁻, 739.4232 [M-H-Glc-Rha]⁻, 593.3678 [M-H-Glc-2Rha]⁻, 431.1245 [M-H-2Glc-2Rha]⁻
36	Parispseudoside A	Cholestanol	1229.6029 [M+COOH]⁻	20.876	1037.5500 [M-H-Rha]⁻, 891.4866 [M-H-2Rha]⁻, 745.4190 [M-H-3Rha]⁻
37	(8R,9R,10S,6Z)-triol-octadec- 6-enoic acid/glyceryl α-mono-palmitate	Others	329.2348 [M-H]⁻	22.429	
38	7β-ol-sitosterol-3-O-Glc	Phytosterol	541.3452 [M-H]⁻	23.046	379.2784 [M-H-Glc]⁻
39	Polyphyllin VII	Isospirosteranol	1029.5424 [M-H]⁻	25.37	883.4873 [M-H-Rha]⁻, 737.4273 [M-H-2Rha]⁻, 591.9858 [M-H-3Rha]⁻, 429.1363 [M-H-3Rha-Glc]⁻
40	Cussonoside B/pregna-5,16-diene-3B-ol-20-one-3-O-Rha (1-2)-Rha (1-2)-[Rha (1-4)]-Glc	Pentacyclic triterpenoids	915.4648 [M-H]⁻	25.898	737.4158 [M-H-O-Glc]⁻, 591.3557 [M-H-O-Glc-Rha]⁻
41	Myrincitrin	Flavonoids	313.241 [M-H]⁻	28.267	277.2196 [M-H-CH_2O]⁻
42	Polyphyllin II	Isospirosteranol	1013.5392 [M-H]⁻	29.057	867.4844 [M-H-Rha]⁻, 721.4232 [M-H-2Rha]⁻, 575.3578 [M-H-3Rha]⁻, 413.2257 [M-H-3Rha-Glc]⁻
43	Polyphyllin III	Isospirosteranol	883.4812 [M-H]⁻	29.411	737.4196 [M-H-Rha]⁻, 721.4225 [M-H-O-Rha]⁻, 575.3619 [M-H-2Rha]⁻, 413.1488 [M-H-2Rha-Glc]⁻
44	Polyphyllin I	Isospirosteranol	853.4645 [M-H]⁻	29.541	721.4166 [M-H-Ara]⁻, 575.3624 [M-H-Ara-Rha]⁻, 413.0833 [M-H-Ara-Rha-Glc]⁻
45	Polyphyllin V	Others	723.3857 [M-H]⁻	30.011	575.3649 [M-H-Rha]⁻, 397.1376 [M-H-Rha-Glc]⁻

46	7-O-Rha-kaempferol-3-O-Glc	Flavonoids	595.2918 [M-H]$^-$	30.165	415.2354 [M-H-Glc]$^-$
47	4,2′,4′-trihydroxy-chalcone	Flavonoids	255.2347 [M-H]$^-$	35.700	237.2208 [M-H-OH]$^-$

As the main type of Paris saponins, isoproterenol saponins included pennogenin and diosgenin type. Sixteen pennogenin saponin including peaks 1, 5, 6, 7, 10, 11, 13, 16, 17, 19, 21, 28, 29, 43, 44, and 45 were identified. We take peak 16 and 39 as an example for fragmentation pathway of pennogenin saponins. The parent ion at m/z 1107.4873 [M + COOH]-of peak 6 lost a xylose to generate m/z 929.4470, and the characteristic fragment ion of m/z 439.2541 was further generated through losing a rhamnose, a xylose, and a glucose. Peak 16 was unambiguously identified as parisyunnanoside H. Peak 39 exhibited deprotonated ion at m/z 1029.5424 losing three rhamnose and a glucose to generate m/z 429.1363. Peak 18 was identified as polyphyllin VII according to structure, molecular formula, fragment ions, and reference. Seven diosgenin saponins including peaks 20, 23, 24, 25, 35, 39, and 42 were identified. Peak 42 showed precursor ion [M-H]- at m/z 1013.5392 and product ion at m/z 867.4844 [M-H-Rha], m/z 721.4232 [M-H-2Rha], m/z 575.3578 [M-H-3Rha], and m/z 413.2257 [M-H-3Rha-Glc]. Peak 42 was identified as polyphyllin II according to structure, fragment ions, and reference. Peak 44 provides [M-H]-at m/z 853.4645 and lose a rhamnose, an arabinose, and a glucose to generate diagnostic ion of diosgenin saponin. It was identified as polyphyllin I.

Furostenol saponins was an F-ring opening, which always connected with glucose at C_3-OH and C_{26}-OH. We take peak 26 as an example for fragmentation pathway of furostenol saponins. It provides [M-H]-at m/z 1063.50417 and lose a rhamnose and a galactose to generate fragment ions m/z 917.4845 and m/z 755.4325, then lose two glucose at C-3 and C-26 to generate diagnostic ion at m/z 431.3246. Peak 26 was identified as polyphyllin H. In addition, cholestanol saponins, flavonoids, and other types were discovered in different species of *Paris*. In negative mode, myricetin (peak 41) was identified due to parent ion m/z 313.2410 losing a carbonyl group to generate m/z 285.2263. Peak 4 provides deprotonated ion at m/z 479.3019 which was dehydrated to generate fragment ion at m/z 461.2961 and lose $C_7H_{14}O_4$ to produce m/z 319.1921, then dehydrated finally to generate m/z 283.1725. It was identified as *β*-ecdysone.

In comparison of five species *Paris*, PPY, PM, and PV had the similar chemical constituents, especially for PM (Figure 8). The result agreed with the previous study UHPLC, FT-IR spectroscopy and mid-data fusion in combination with multivariable analysis. The common peaks of PPY and PM are peak 4 (*β*-ecdysone), peak 11 (Parisyunnanoside G isomer), peak 16 and 17 (Parisyunnanoside H isomer), peak 22 (Polyphyllin G), peak 25 (Pennogenin-3-O-Glc-(1-5)-Ara(1-4)[Rha(1-2)]-Glc), peak 26 (Polyphyllin H), peak 35 (Parisaponin I), peak 37 (glyceryl *α*-mono-palmitate), peak 39 (Polyphyllin VII), peak 40 (Cussonoside B), peak 41 (*Paris* saponins VI), peak 42 (Polyphyllin II), peak 43 (Polyphyllin III), peak 44 (Polyphyllin I), peak 45 (Polyphyllin V), peak 46 (7-O-Rha-kaempferol-3-O-Glc), and peak 47 (4,2′,4′-trihydroxy-chalcone).

[figure(s) omitted; refer to PDF]

4. Conclusions

To the best of our knowledge, the wild *Paris* was gradually decreasing due to the industrial production. The aim of this study was to distinguish Genus *Paris* including PPA, PPY, PM, PV, and PPS by using UHPLC, FT-IR and midlevel data fusion in combination with multivariate analysis. Data fusion could classify the sample better than a single data array. Chemical constituents of different species *Paris* were determined by UHPLC-Q-TOF MS. The similar results showed that chemical constituents of PM were similar to PYY. It is expected that PM as a substitute of PPY with the further study was used to solve the resource shortage.

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DETAILS

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Assessment of Selected Heavy Metals Concentration Level of Drinking Water in Gazer Town and Selected Kebele, South Ari District, Southern Ethiopia

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ABSTRACT (ENGLISH)

Drinking water quality is fundamental to human physiology and health. The aim of this study was to assess the quality of drinking water in Gazer Town and selected kebele, in south Ari district, South Omo zone, Southern Ethiopia. A total of four drinking water samples were collected from densely populated urban areas of the Gazer Town and one rural Kebele. All the collected samples were analyzed for eight heavy metals, (Cd, Co, Cu, Cr, Fe, Mn, Pb, and Zn) using standard procedures. The results were compared with other national and international standards. Among the analyzed samples, drinking water samples collected from selected kebele (Aynalem kebele), show mean concentrations heavy metals in (μg/L), (Mn (973 ± 10), Cu (1068 ± 1.5), Cr (2785 ± 25), Fe (4302 ± 15), Cd (1218 ± 18), Pb (720 ± 12), Co (1478 ± 3), and Zn (1790 ± 5)), and the results revel except, Co and Zn metals, all show concentrations higher than the national and international standards (such as USEPA (2008), WHO (2011), and New Zealand) recommended values. Among the eight heavy metals analyzed from drinking waters in Gazer Town, Cd and Cr were below the method detection than that of all sampling area. However, the concentration of Mn, Pb, Co, Cu, Fe, and Zn were ranged from mean values of 9 μg/L, 17.6 μg/L, 7.6 μg/L, 12 μg/L, 765 μg/L, and 494 μg/L, respectively. Except Pb metals, the analyzed metals in waters were below the currently recommended guidelines for drinking. Therefore, the government should adopt some treatment technologies such as sedimentation and aeration to minimize the concentration of zinc for safe drinking the water to the community of Gazer Town.

FULL TEXT

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1. Introduction

Water is extremely important nutrient for the human body and processing of various materials in industries. Even though, living organisms cannot exist without water and almost all industries require water to operate. As a result, water is important for life [1, 2]. In addition to that safe drinking water is a human birthright, as much a birthright as clean air. As a matter of fact, in most of the African and Asian countries, even in relatively advanced countries such as India, safe drinking water is not easily available because of contaminant issues. Currently, more than one billion peoples have lack of access to safe drinking water and in addition to that around 2.5 billion do not have access to adequate sanitation services [3]. This is due to smaller scale water quality assessments [4]. Therefore, assessing of safe drinking water is mandatory for human being to survive in life. Nowadays, drinking water is obtained from a variety of water sources such as wells, rivers, lakes, reservoirs, ponds, and ground water. Especially, more than half the world’s population depends on groundwater for survival because of it is an important source for drinking purpose and more reliable than the other water source [1, 5, 6]. However, water source is a great risk to human health once it is contaminated. When water becomes contaminated with toxic compounds, it must be treated before human consumption. Similarly, contaminated water can be dangerous to plants and animals when their metabolic processes are disrupted by drinking from these living things [7].

Contaminations of water occur because of a few key factors, i.e., industrial and sewage effluent discharge, agricultural industry, illegal garbage disposal, and leakage of leachate from landfills [8]. All these, water pollutant mainly consist of heavy metals, microorganisms, fertilizers, and thousands of toxic organic compounds [9]. Especially, disposal of heavy metals containing wastewater is a major environmental issue since contaminants can ultimately gain access to surface and groundwater [1] and also gain to human trough by using of these water sources for drinking purpose. Consequently, these cause a number of water borne diseases as the causes of health hazards [10]. Some heavy metals are essential for the health of living organisms. However, if the concentration level of metals is higher than that of recommended limits, their roles immediately are changed to a negative dimension and some of them are harmful even in small amount [10–12].

The level of seriousness of the problem is much worse in developing countries, especially in rural area. Mainly because of lack of technology, awareness and economic deficiency to treat metals from their drinking water, this results the maximum concentration limit (MCL) of metals from proposed WHO limits [13–15]. This enforces to take a great attention to protect the quality of drinking water. Therefore, monitoring a wider range of water quality parameters and other indicators related to drinking water services and their impacts are essential to provide a

nuanced understanding of the risk factors for contamination of different settings and drinking water services, including those used by vulnerable populations [4].

Currently, the level of heavy metal concentration in water sample has been determined by using advanced spectroscopic methods such as atomic absorption spectrometry (AAS), flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), microwave plasma atomic emission spectroscopy, and inductively coupled plasma spectroscopy (ICPS) [16].

Basically, studies and research findings are important to contribute some valid knowledge to the wellbeing of the society and for the advancements of society about the quality of water. The aim of this study was to determine the concentration level of some selected heavy metals of drinking water in Gazer Town and selected kebele South Ari district, South Omo Zone, Southern Ethiopia. According to reviews literatures, there were no further studies have been conducted in Gazer Town, on the level of heavy metal concentration regarding quality drinking water; and, the groundwater that are found on one selected kebele (such as Aynalem) is not used for drinking purposes because of unpleasant taste, odor, and color. Therefore, all this also might facilitate the researcher to conduct these studies regarding to concentration level of heavy metals in these areas from the point of view of water quality parameters. Also, the researcher believes that this small study will be a step stone and contribute its part for those people who are interested in the further study of this topic. Inspired by the motivations mentioned above, we used advanced spectroscopic techniques such as inductively coupled plasma optical emission spectroscopy (ICP-OES) to determine the concentration level of some heavy metals such as (Cd, Co, Cu, Cr, Fe, Mn, Pb, and Zn) of drinking water in Gazer Town and one selected kebele South Ari district, South Omo Zone, Southern Ethiopia and compare the results with other international standard agency. Results revel that, except Co and Zn metals the existence of high concentration of other metals compared with standard agency in selected kebele. In addition to that, some heavy metals concentration level is below detection limits like (Cd and Cr) in Gazer Town.

1.1. Description of the Study Area

Water samples were collected South Omo Zone, Southern Ethiopia specifically Gazer Town shown on the map (Figure 1). It is the nearest to the capital of the zone Town Jinka and 17 km away from it. Geographically, the area lies between 13° 40′ N and 14° 27′ N and between 36° 27′ E and 37° 32′ E. The traditional agro ecologies of the Woreda are Dega (30%), Woina-dega (65%), and Kola (5%) of the total areas. The Woreda has bimodal type of rain fall pattern and the mean annual rainfall ranges between 601–1600 mm. The mean annual temperature ranges between 10°C–1°C. Based on the 2007 national census conducted by the central statistical agency of Ethiopia, the Woreda has a total total population of 168,225, which is about 35% of the zone population [17].

[figure(s) omitted; refer to PDF]

2. Materials and Methods

2.1. Instrumentation and Apparatus

Polyethylene bottles and polyethylene bags were used to collect groundwater samples. Borosilicate Erlenmeyer flask and hot plate were used to digest the collected water samples. The pipettes (5 mL), 100 mL, 50 mL volumetric flask, 50 mL of Erlenmeyer flask and beakers were used to dilute the standards and samples solution. Inductively coupled plasma-optical emission spectrophotometer (ICP-OES), (Perkin Elmer MODEL Optima 8000, U.S.A.) equipped with Argon gas, with Plasma, auxiliary, Nebulizer, and RF Power, for the determination of Manganese, Iron, Cobalt, Zinc, Copper, Cadmium, Lead, and Chromium were used.

2.2. Chemicals and Standard Solutions

Chemicals that were used in the analysis are analytical grades. 35.4% of HCl (Loba, chemical Ltd, laboratory reagent, India) and 69% (Analytical *R*, IMO: Nitric acid solution BDH Laboratory supplies, England) of HNO_3 were used to digest the water samples. Stock standard solution of the metals Cu (1000 mg/L), Fe (1000 mg/L), Mn (1000 mg/L), Cd (1000 mg/L), Zn (1000 mg/L), Pb (1000 mg/L), Cr (1000 mg/L), and Co (1000 mg/L) prepared for inductively coupled plasma-optical emission spectrophotometer (ICP-OES), (Perkin Elmer MODEL Optima 8000, U.S.A.) were used for the preparation of calibration curves for the determination of metals in the sample. Distilled water was used for cleaning of glassware and dilution of sample solutions.

2.3. Water Sampling and Transportation

Representative samples of drinking water (spring and ground) were collected from Water samples were collected from three sampling sites Gazer town, on May 2019. Grab sampling technique was employed on selecting the site of the study area. Water samples were collected in polyethylene bottles from three different sampling sites (one from catchment tankers and two from along the three distribution network reservoirs) by using polyethylene glass [18]. The samples were transported to the laboratory and stored at 4°C until analysis.

2.4. Digestion Sample Water

Three replicates of 100 mL of water samples were taken into 50 mL of Erlenmeyer flask from each ground water and acidified with 3 : 1 HNO_3/HCl ratio. Then, the samples were heated on a hot plate to reduce the volume to a defined level (25 mL). Finally, the remaining part of the samples were cooled and (filtered through Whatman paper filter paper). Finally, samples were diluted to 100 mL volumetric flasks and ready for analysis by Inductively Coupled plasma-optical Emission spectrophotometer.

2.5. Calibration Standard Solutions

The calibration standard solutions were used to calibrate the instrument response with respect to the analyte concentration. Standard solutions of eight points were prepared for each analyte from their respective working standard solutions (100 mg/L) for metals. The calibration standard concentrations were within the working linear range of the instrument used for analysis. The prepared calibration standards: standard 1 (S_1), standard 2 (S_2), standard 3 (S_3), standard 4 (S_4), 5 (S_5), 6 (S_6), 7 (S_7), and standard 8 (S_3) for each analyte are given in Table 1.

Table 1

Series of working standards for determination of trace metals in water sample.

Analytes	Calibration standard solutions (mg/L)							
S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	Mn
0.5	1	2	4	6	8	10	12	Fe
0.5	1	2	4	6	8	10	12	Cr
0.5	1	2	4	6	8	10	12	Co
0.5	1	2	4	6	8	10	12	Zn
0.5	1	2	4	6	8	10	12	Ni
0.5	1	2	4	6	8	10	12	Cu
0.5	1	2	4	6	8	10	12	Pb
0.5	1	2	4	6	8	10	12	Cd

2.6. Instrumental Technique and Optimal Condition of ICP-OES

Concentrations of the heavy metals in all samples were measured using Inductively coupled plasma-optical emission spectrophotometer (ICP-OES). To analyze the sample on the ICP-OES, the instrument was adjusted in appropriate manner as shown in Table 2 such as, wavelength selector; plasma, auxiliary, Nebulizer, RF Power, gas, and optimal values of these parameters are those, which yield maximum absorbance value.

Table 2

Instrumental operating conditions used for flame atomic absorption spectrometer.

Elements	Wave length in (nm)	Plasma	Auxiliary	Nebulizer	RF power	Gas
Mn	279.5	10	0.3	0.7	1300	Ar
Zn	213.9	10	0.3	0.7	1300	Ar
Fe	248.3	10	0.3	0.7	1300	Ar
Cd	368.4	10	0.3	0.7	1300	Ar
Cu	324.7	10	0.3	0.7	1300	Ar
Pb	283.2	10	0.3	0.7	1300	Ar
Cr	357.5	10	0.3	0.7	1300	Ar
Co	228.6	10	0.3	0.7	1300	Ar

2.7. Data Analysis

The heavy metals were compared with WHO and Ethiopian Standard Agency (ESA) guidelines for drinking water which was reported in Table 3. The obtained data were also treated with one-way ANOVA to assess the variations of the parameters among the spring water samples and ground samples analyzed by using Origin pro 2019 version software.

Table 3

Drinking water contaminants and maximum admissible limit set by different national and international organizations (for health risk and aesthetic value) [3].

	Heavy metals (μg/L)									
As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	USEPA, 2008
10	5	100	100	1300	300	50	100	15	5000	EU, 1998
10	5	NM	50	2000	200	50	20	10	NM	WHO, 2008
10	3	NM	50	2000	NGL***	400	70	10	NGL**	Iranian, 1997

50	10	NM	50	1000	1000	500	NM	50	NM	Australi, 1996
7	2	NM	50[c]	2000	300[c]	500	20	10	3000[b]	Indian, 2005
50	10	NM	50[c]	1500	300	100	20	100	5000	New Zealand, 2008

*NM = not mentioned; **NGL = no guideline, because it occurs in drinking water at concentrations well below those at which toxic effects may occur; ***no guideline, because it is not of health concern at concentrations normally observed in drinking water but may affect the acceptability of water at concentration above 300 μg/L, [b]based on quality (aesthetic) not safety (health risk), [c]chromium as Cr^{+6} not total Cr.

3. Results and Discussion

3.1. Calibration Curve and the Determination Metal Concentrations

The quality of data resulted from ICP-OES measurements are affected by sample digestion, calibration, and standard solution preparation procedures. In this study, the instrument was calibrated by using eight series of working standard solutions, prepared from intermediate solutions for each metal under determination. The analytical calibration curves were plotted by using prepared standard solutions for every element (Cd, Cr, Pb, Zn, Cu, Fe, Co, and Mn). The value of correlation coefficients of calibration curves for each toxic and essential metal as well as the linearity of data on the calibration for Cr, Mn, Fe, Co, Pb, Zn, Cu, and Cd, are seen in Figures 2(a)–2(h).

[figure(s) omitted; refer to PDF]

3.2. The Concentration of Heavy Metal in Drinking Water

In this study, all analyzed heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Pb, and Zn) were detected in the water samples, and their concentrations (mean ± SD) are presented in Table 4.

In Figure 3, the lowest concentration of iron was detected in Gazer Town sample site while the highest concentration of iron was detected in Aynalem Kebele water site. All samples analyzed were below WHO permissible limits for iron in drinking water of 3,000 μgL^{-1} [19].

[figure(s) omitted; refer to PDF]

The lowest concentration of manganese was found in Gazer town water site while the highest concentration was observed in Aynalen Kebele water site as shown in (Figure 4). The water samples analyzed in Gazer town were below WHO permissible limits but the water samples analyzed at Aynalem Kebele were above WHO permissible limits for manganese in drinking of 400 μgL^{-1} (WHO 2017).

[figure(s) omitted; refer to PDF]

The highest concentration of copper was in Aynalem Kebele water sample and the lowest concentrations were observed at Gazer town sample site as shown in (Figure 5). All the water samples analyzed for copper were found below WHO permissible limits of 50 μgL^{-1} [19].

[figure(s) omitted; refer to PDF]

In Figure 6 shows the highest concentration of chromium was record in Aynalem kebele samples site and the lowest concentration were record in Gazer town kebele. The Aynalem kebele samples site of that chromium was detected are above the WHO maximum permissible limits and Gazer town sample site of that chromium was detected below WHO maximum permissible of 50 μgL^{-1} [19].

[figure(s) omitted; refer to PDF]

The highest concentration of cobalt was record at Aynalem Kebele water sample and the lowest concentrations

were observed at Gazer town sample site shown in (Figure 7). The Aynalem Kebele water samples analyzed for cobalt were found above WHO permissible limits and Gazer sample site were found below WHO permissible limits of 50 μgL^{-l} (WHO 2011) [20].
[figure(s) omitted; refer to PDF]
Also as shown in Figure 8, cadmium was detected only in Aynalem Kebele sample site. It was not detected in Gazer water sample site. The Cadmium detected in Aynalem Kebele sample site was above WHO permissible limits for in Cadmiuin drinking water of 50 μgL^{-l} [20].
[figure(s) omitted; refer to PDF]
The highest concentrations of lead in were obtained in Aynalem Kebele water samples however, the lowest concentrations were found at Gazer town water sample site in (Figure 9). Therefore, the lead detected in all sample sites is above WHO permissible limits in drinking water of 10 μgL^{-l} [20].
[figure(s) omitted; refer to PDF]
The least concentration of zinc was observed in Gazer Town water sample site while the highest concentration was recorded in Aynalem kebele water sample site shown in (Figure 10). All the water samples collected from Gazer Town contains zinc metals below the WHO maximum permissible limit of 5,000 μgL^{-l} (WHO 2017).
[figure(s) omitted; refer to PDF]

3.3. Lead Status

Lead occurs mostly in association with zinc and gets into water from corrosion of zinc coated ("galvanized") pipes [21] and leaching from water distribution pipes [22, 23]. The concentration of Pb obtained from Aynalem kebele and Gazer town site water sample site were around (720 ± 12 μg/L) and 13 ± 1.4 μg/L respectively shown in Table 4. Therefore, the obtained result shows that high concentration of Pb in both sampling area beyond the maximum permissible limit given by WHO (2004) and USEPA (2011). As results this may case health problem to the society unless strict measurement will be taken.

Table 4

Mean ± SD (in μg/L) of major heavy metals Pb, Cr, Mn, Cd, Co, Zn, and Cu drinking water samples.

Metals			Mn	Zn	Co	Cu	Cr	Fe	Cd	Pb
Places	Gazer town	S_1	4 ± 0.00	644* ± 1.9	1* ± 1	4* ± 0.2	BDL	677* ± 7	BDL	13 ± 1.4
S_2	BDL	BDL	20* ± 2	30* ± 2	BDL	BDL	BDL	20 ± 0.7	S_3	14 ± 0.0
344 ± 09	2 ± 0.1	2 ± 0.32	1 ± 0.2	427 ± 2.3	BDL	20 ± 0.2	Aynalem Kebele	S_4	973 ± 10	1790 ± 5

*ND = not detected.

3.4. Cadmium and Chromium

Mostly, high concentration of cadmium metal related with industrial and mining activities in the sampling areas [22, 23] and chromium metal related with Soil leaching. Especially, the high concentration of cadmium in drinking water is mostly related with corrosion of metal pipes, water tanks, and plumbing systems [24, 25]; this causes a disease of like short periods of time: nausea, vomiting, diarrhea, muscle cramps, salivation, sensory disturbances, liver injury, convulsions, shock, and renal failure as reported [26].
Our results reveal that the concentration of cadmium and chromium metals in Gazer town water sampling area was found to be below the detection limit. Therefore, no health problem related with these metals. However, high concentrations were observed in Aynalem kebele water sampling area regarding from maximum admissible limit of

international organizations like, WHO (2017) reported in (Figures 6 and 8) and in table.

3.5. Iron

Iron is the fourth most abundant element by mass in the earth's crust. In water, it occurs mainly in ferrous or ferric state. Iron in surface water generally present is ferric state [27]. As shown in Table 5, the concentration of Fe in the study area ranged from $4302 \pm 15\,\mu$g/L in Aynalem kebele sample water to $677 \pm 7\,\mu$g/L in Gazer Town sampling site. The results were found above the maximum permissible limit (0.3 mg/L) set by WHO (2004). This may cause health problem related with iron in the study area. But in the studied areas of Gazer town, iron content consistent with the desirable concentration limit of drinking water set by WHO (2011) and other international organizations, whereas high concentration was observed in the sampling area of Aynalem kebele. As reported in [24, 28], the prolonged consumption of drinking water with high concentration of iron may lead to liver disease called hemosiderosis.

Table 5

Compression of studied samples with different guidelines for drinking water quality (for health risk and aesthetic value) set by different national and international organizations.

Elements	Tested method	Standard requirement by different organization				Condition of studied samples Gazer town	Condition of studied samples Aynalem Kebele
Max (μg/L)				Ethiopia, 2011	USEPA, 2008	WHO, 2011	New Zealand, 2008
Cu	ICP-OES	2000	1300	2000	2000	Safe	Safe
Cd	ICP-OES	3	5	3	4	Safe	Not safe
Cr	ICP-OES	50	100	50	50	Safe	Not safe
Zn	ICP-OES	5000	5000	5000	1500	Safe	Safe

Mn	ICP-OES	500	50	400	400	Safe	Not safe
Fe	ICP-OES	0.3	300	300	200	Safe	Not safe
Co	ICP-OES	200	100	20	1000	Safe	Safe
Pb	ICP-OES	15	15	10	10	Not safe	Not safe

Source for international standards and Mebrahtuet al., (2011) [3].

3.6. Manganese

Manganese one of the most important elements used for the proper functioning of both humans and animals, as it is required for the functioning of many cellular enzymes. Nevertheless, high levels of manganese may harm brain development in infants and young children [25, 29]. Similarly as shown, the concentration of manganese was recorded 973 ± 10 μg/L at Aynalem kebele and 41 ± 0.00 μg/L in gazer town sample site. The result obtained from sites were below the permissible limit recommended by international water quality standards like WHO (0.1 μg/L) and USEPA (0.5 μg/L) guide lines. So, these sites were safe for domestic and irrigational use. In this study, manganese concentration which recorded a water sample from Gazer town is with complying the maximum admissible limit set by international agencies. But high concentration manganese was observed in the sampling site of Aynalem kebele.

3.7. Zinc

The dissolved concentration of zinc in the water samples from Aynalem kebele site recorded 1790 ± 5 μg/L and 644 ± 1.9 μg/L recorded at gazer town water sample. As shown in Table 5, the mean concentrations of zinc metal in the three sample sites were recorded below the permissible limit recommended by WHO and USEPA guideline range (3 mg/L) and (5 mg/L), respectively.

3.8. Copper

Copper is Exposure to high doses of copper can cause health problems. Short-term exposure to high levels of copper can cause gastrointestinal distress, nausea, vomiting, and diarrhea [26, 30]. Also, high concentration of cobalt in drinking water can cause interstitial lung disease. They are mostly present naturally in rock, soil, water, plants, animals, and air. In present study, the concentration of zinc and copper in both drinking water sampling sites of (Gazer town and Aynalem) were, below the WHO and different national guidelines for drinking water quality (Table 5). Therefore, from above-given points of view, no adverse effects concerning with this element in water for drinking purposes. Also, the concentration of Cobalt in all sampling area is within WHO (2017) maximum recommended range.

4. Conclusion and Remarks

The main goal of this paper was to assess the status of drinking water quality in Gazer town and selected kebele (Aynalem) areas regarding concentration level heavy metals. Most of the water samples are colorless and odorless. However, some water samples collected from Aynalem kebele are slightly colored due to muddiness. The current study results showed that the concentrations of most heavy metals in the water samples were within the permissible limit of the World Health Organization and Ethiopian Standard guidelines for drinking water quality, and except lead (Pb), all analyzed heavy metals in Gazer town were below the established guideline values. This high concentration of lead was observed due to corrosion of zinc coated ("galvanized") pipes or leaching from water distribution pipes and geogenic contaminants. Therefore, no health and aesthetic problems concern the analyzed heavy metals of the waters for drinking. But the results show that drinking water from Aynalem kebele is not good regarding these

selected heavy metal concentration levels, because it is higher than the MAL set by WHO, 2017 and different national guidelines for drinking water quality. This is an indication of weak drinking water treatment practices in these areas which, in turn, have implications on the health of the people.

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DETAILS

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Analytical Validation of Smartphone Spectroscopic Technic Used in an Educational Kinetic Study

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ABSTRACT (ENGLISH)

Use of smartphone-based spectroscopy is showing a constant growth since last year. It presents the advantage of being widely available for everyone. The most important thing is that it is still a low-cost method adapted to the education context. However, as all analytical methods, it should be validated to ensure the reliability of its results. In this study, we present the steps of the validation process with its statistical tests applied to the dosage of di-iode. Shapiro–Wilk test revealed that our method has a random character. Homogeneity of variance analyses using the Cochran test confirmed the precision of the method. The Fisher test revealed the linearity of the model of correlation between I_2 concentration and the response. The relation between response and concentration is $A = 1000\,C + 0.002$. From the parameters of the linear regression of the model, we deduced the limits of quantification and $X_{Lq} = 4 \cdot 10^{-5}$ mol·L^{-1} and $X_{Ld} = 1 \cdot 10^{-5}$ mol·L^{-1}. Thanks to tightness of the sample, the method of I_2 dosage was successfully applied in iodine quantification to monitor acetone iodination during time in the context of kinetic studies with minimum system trouble. Being low cost, this method can facilitate access to physical methods in educational laboratories.

FULL TEXT

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1. Introduction

Since the first edition of the EURACHEM Guide in 1998, a number of important developments in analytical quality have taken place. A growing interest is being accorded to measurement and analytical methods noticeably in relation with the development of new methods [1]. Validation of analytical methods is one of the topics requiring a sharing of practices in order to define of common guidelines for laboratories. This sharing ensures competence requirements for laboratories, proficiency testing providers, and reference material producers. As indicated in Figure 1, the life cycle of an analytical method evolves through the following:

(i) The selection of the method is a crucial step. Its selection affects directly the results.

(ii) The optimization of the method, an important step, that ensures the suitability of the method and the operation conditions of the routine.

(iii) The validation (internal or/and external) that ensures the verification of the results.

(iv) The routine use with a periodical control.

[figure(s) omitted; refer to PDF]

Smartphone-based spectroscopy is an emergent technic managed to quantify and describe physically human colour perception using a camera [2]. Nowadays, digitalizing images is becoming available for everyone [3]. Smartphone technology has now speared in every aspect of modern life. Since its first commercialization in 1990s, until now, smartphone use has widely expanded. In Tunisia, a North African country, in 2016, 70% of the Tunisian population possesses smartphones connected to either mobile or Wi-Fi connections according to a report of the consumer lab Ericson. The accessibility of these devices among high school students can encourage taking advantage of laboratory experiments and practical study. This type of spectroscopy is easy to handle and overcome technical problems related to material lack and damage [4–7]. It is important to be aware of the wide expansion of this type of spectroscopy which covers a lot of fields such as agriculture, biochemistry analyses, medical analyses, nanomaterial, and hazardous materials. This method can currently quantify copper [8], iron (III) [9], formaldehyde [10], water salinity [11], blood hematocrit [12], and acetazolamide [13].

The acceleration of this method spread out due to its facility and low-cost appeal to the necessity of an easy procedure establishment for its analytical validation [14]. In this study, we tried to implement a simple method using smartphone for the quantification of di-iode in order to quantify it in a kinetic lab. We describe the steps of the procedure for the analytical validation. As it is a non-normalized method, it has to be validated according to the EURACHEM Guide.

The acceleration of this method spread out due to its facility and low-cost appeals to the necessity of an easy procedure establishment for its analytical validation [14]. In this study, we tried to implement a simple method using smartphone for the quantification of di-iode in order to quantify it in a kinetic lab. Physical methods are preferred to chemical ones in reaction monitoring for kinetic studies. They are fast and they do not disturb progress. Sampling during the time is more accurate. UV-visible spectroscopy is the widely used technique mainly for coloured solutions [15]. But, this spectroscopic method is sophisticated and expensive. This apparatus cannot be afforded anywhere. Even it exists, it requires maintenance and spare parts. Use of smartphone overcomes this problem since it is affordable for all the students. So our method ensures availability at low-cost for educational institutions. However, the operation of photographing and transferring image to laptop for treatment by image *J* is still an awful operation in the method. To improve its accessibility and inclusiveness, it will be interesting to develop a smartphone application that treats images directly on the smartphone and not on a laptop. At this stage, we provide the method of smartphone use of a solution quantification, and we describe the steps of the procedure for its analytical validation. As it is a non-normalized method, it has to be validated according to the EURACHEM Guide [16]. This validation procedure has to be applied when developing smartphone new methods.

2. Materials and Methods

All reagents were handled while donning personal protection equipment (PPE), including a lab coat, gloves, and mainly eye protection under the hood. HCl is a strong acid, and skin contact should be avoided. Acetone and ethyl acetate are volatile organic solvents. They should be handled carefully. Iodine solutions must not be evacuated but stored to be correctly eliminated. Iodoacetone, a product of the reaction, is very powerful and harmful. All solutions containing this should be disposed of immediately after the experiment, and the apparatus was washed with plenty of water.

All used reagents are suitable for UV-visible spectroscopy. Acetone 99.5% and hydrogen chloride solution 1 $mol{\cdot}L^{-1}$ are delivered by Sigma-Aldrich. Iodine solution 0.5 $mol{\cdot}L^{-1}$ is provided by Merck.

In this study, samples of I_2 solutions were put in plates. This one was implanted in a carton-covered box with a small aperture. Camera was placed in front of the aperture to photograph the plate. Image acquisition was performed by a smartphone (Samsung Galaxy A31: android version 11, 48 megapixels back camera), used with flash.

Then, Beer–Lambert relation (1) allows the calculus of solutions concentration from the intensities of solutions color measured by RGB type 3 channels solution image treatment as follows:(1)$A=\log\frac{I_0}{I}$,where *I* represents measurement intensity corresponding to I_2 solution and represents measurement intensity corresponding to I_2 blank.

The chosen regions of interest (ROI) were squares of 400 pixels centred on the circles of each plate wells where

they were duplicated. The distribution of RGB values of every pixel was contained in histograms by applying the macro shared in the supporting information.

3. Results and Discussion

3.1. Method Validation

Reliable analytical data are a prerequisite for a correct interpretation of findings in the evaluation of scientific studies, as well as in daily routine work, analytical methods have to be validated [17]. According to the EURACHEM Guide recommendations, the required validation steps are random character, specificity, accuracy, and linearity [18]. In the case of our application, the determination of the limits of detection and quantification is also needed since the application aims to monitor the process of degradation until the total completion of the reagent. It is also important to express the results with their uncertainty. Therefore, we accomplished the process of validation by uncertainty determination of our method response.

The first parameter to evaluate in the validation process is the normal character or the random character of a series of responses to ensure the absence of bias in the results. Shapiro and Wilk [18, 19] proposed a statistic test verifying the hypothesis of normality for a random sample. Specificity traduces qualitatively the extent to which substances interfere with the determination of a substance according to a given procedure [20]. It is also an essential parameter to be verified in signal detection. It allows ensuring a negative response in the absence of measured species. Precision represents the closeness of agreement between independent test results using identical experimental procedure under stipulated conditions. It also proves the closeness between measured results and the true value of a standard sample [20]. Within a given range, the analytical responses may vary linearly with the concentration of the measured species. Linearity evaluation allows the determination of method sensitivity and method limits of detection and quantification.

Random character of the obtained measurements has to be checked and verified, it allows the confirmation of their independence and their normal distribution. Therefore, we consider 3 solutions of iodine (2.5×10^{-4} mol·L^{-1}) prepared separately from the commercial one. The dilutions and the measurements by our method were repeated for four days. Table 1 regroups the responses collected for 4 days.

Table 1

Random character of the responses of 3 iodine solutions for 4 days.

Day		Absorbance A_{ij}		
–				
1		0.255		0.230
0.238		2		0.255
0.219		0.252		3
0.244		0.241		0.250
4		0.246		0.251
0.239		-		
Median	R	Risk α	(p*n)/2	$R_{\alpha/2}$

$R_{1-(\alpha/2)}$	-			
0.245	8	5%	6	4
10	-			
Decision				
If $R R_{\alpha/2}$			Monotone derive	
If $R > R_{1-(\alpha/2)}$			Fast oscillation	
If $R_{\alpha/2} R R_{1-(\alpha/2)}$			Random distribution	
–				
Conclusion				
The distribution is random				

To evaluate the normal character of the collected data, we use the Shapiro–Wilk test resumed in Table 1 [18]. It consists of calculating the median of responses (0.245). The number of sequences $R=8$ is found by determining the number of values lower or higher than median. By referring to the values of $R_{\alpha/2}$ and $R_{1-(\alpha/2)}$ at the risk level $\alpha=5\%$ given by the table, we can see that the calculated value of R is ranged between $R_{\alpha/2}$ and $R_{1-(\alpha/2)}$. Thus, we conclude that the distribution of the measurements is normal [21, 22].

We developed our method to study the kinetic of a reaction where I_2 is a reactant. Therefore, to ensure the absence of interference between the dosed species and the matrix, we evaluate the specificity of the method. We dose I_2 in a mixture composed of acetone, HCl in aqueous medium, and ethyl acetate and I_2 in water. The different solutions served to fill the wells of the same plate. Figure 2 illustrates the responses of the two series.

[figure(s) omitted; refer to PDF]

It shows that the two series have the same concentrations with a relative difference inferior to 5%. Specificity test prove absence of interferences by the adjunction of the kinetic blocking mixture. Our method is consequently specific and does not present a risk of interference with matrices [23].

To evaluate homogeneity of variance analyses, the Cochran test permits verification of the precision method [24]. Absorbance of iodine solution (2.5×10^{-4} mol·L^{-1}) is measured 3 times in the same plate and during 4 different days. Table 2 regroups all responses [16, 18].

Table 2

Test of homogeneity of variance of iodine solution during 4 days.

Day (i)	A_{ij}			Sj2	
1	0.255	0.230	0.238	0.163	∑Sj2=0.619
2	0.255	0.252	0.219	0.399	Smax2=0.399

3	0.250	0.241	0.244	0.020	Ccal=Smax2/∑Sj2=
4	0.246	0.251	0.239	0.036	0.645
C_{Cri} (α=1%)=0.864				C_{Cri} (α=5%)=0.768	
Decision					
If $C_{Cal} C_{Cri}$ (α=1%)				Group Smax2 does not contain suspected measurements	
If $C_{Cal} C_{Cri}$ (α=5%)				Group Smax2 does not contain rejectable measurements	
Conclusion					
All the values are not suspected					

S_j = standard deviation of data series *J*.
We can perceive that the calculated constant C_{Cal} is less than the critical constant value at both risks of 5% and 1%. Therefore, using the Cochran test, we confirm that the variances are homogenous and there are no suspected measure [16, 18, 24]. Our method provides reliable responses with good precision.
To correlate the concentration of I_2 solutions with responses of our method, we use the Fisher test. This test allows the evaluation of relation linearity for iodine solutions in the range of concentrations: 0.5×10−4; 1.0×10−4; 2.0×10−4; 3.0×10−4; 4.0×10−4 mol·L^{-1} [25, 26]. Table 3 regroups all results.

Table 3

Fisher test for the linearity of the method.

Concentrations (mol·L^{-1})	*A*1	*A*2		*A*3
	0.00005	0.051	0.049	
0.059	Slope a_1 = 1000	0.00010	0.095	0.102
0.107	0.00020	0.201	0.191	
0.211	Intercept a_0 = 0.002	0.00030	0.302	0.310
0.298	0.00040	0.406	0.399	

0.401	Correlation coef. r^2 = 0.9999	F_{Tab} = 3.7	FCal=∑iYi¯−Yi^2/n−2/∑iYi−Yi¯2/np−1=0.03
Decision			
If FCal<FTab		The model is linear	
If FCal>FTab		The model is not linear	
Conclusion			
Calibration model is linear			

From Table 3, we conclude that the model of correlation between the concentration of I_2 solutions and responses is linear. The relation between the method response and the solution concentration in I_2 is given by the following equation:(2)A=1000C+0.002,with correlation coefficient r2=0.9999.

The detection limit is the smallest concentration that can be distinguished from the blank with a risk of 0.13%. In this case, the statistical test of comparison of the response at the value 0 becomes significant. The limit of quantification is determined with a risk of 0.05%. Their values are, respectively, calculated by equations (3) and (4) [16, 18, 24]:(3) XLD=a0+3Sa0a1,(4)XLQ=a0+10Sa0a1.With(5)Sa0=Se21n+X¯2∑iXi−X¯2,(6)Se2=∑ijyij−y2−a1∑iXi−X¯Yi¯−Yn−2.

Expression (3) gives $X_{LD} = 1 \cdot 10^{-5}$ mol·L^{-1} and expression (4) gives $X_{LQ} = 4 \cdot 10^{-5}$ mol·L^{-1}.

The statistical precision of a response is expressed by calculating the confidence interval, which indicates the margin of error when generalizing an estimate obtained to a population of *n* samples. The length of the interval centred on the mean value decreases as the sample size increases. We use the following formula to calculate the uncertainty *U* [27]:(7)U=z⋆σn,where *U*: uncertainty. *z* = value derived from the reduced centred normal distribution, equal to 1.96 if $\alpha = 0.05$ (degree of trust); σ: standard deviation. *n*: the number of I_2 solutions with a concentration of 2.5×10^{-4} mol·L^{-1}.

Application of equation (7) to the results found in Table 3 indicates the $U = 0.1\ 10^{-4}$ mol·L^{-1}.

This method provides a numerical result on continuous scale from the measurement of a signal directly related to the amount of analyte. Table 4 recapitulates the steps of the validation of this method.

Table 4

Recapitulation of validation steps.

Characteristic	Test	Description of the test
Random character	Shapiro–Wilk test conform	For the risk $\alpha = 5\%$, *R*, and 12 repetitions, the number of sequences *R* = 8 is between higher and lower values.
Specificity	Specific method	Response of solutions prepared in water and in reaction mixture are equal.
Precision	Cochran test conform	There is no aberrant responses in the 12 measurements; the fraction of higher variance to the sum of variances is less than Cochran critical vale at the risk $\alpha = 5\%$ and $\alpha = 5\%$.

Linearity	Fisher test conform	Fisher test showed that the fraction of calculated residuals and experimental ones is inferior to the tabulated Fisher value for 5 levels repeated 3 times.
Calibration function	$A = 1000\,C + 0.002$	Least square regression is involved to determinate the slop and the intercept of the calibration curve.
Correlation coefficient	0.9999	This value represents the fraction of the variation in one variable that may be explained by the other variable.
Limit of detection	1 10−5 $mol \cdot L^{-1}$	Statistical test of comparison of the response at the value 0 becomes significant. The limit of detection is determined with a risk of 0.05%.
Limit of quantification	4 10−5 $mol \cdot L^{-1}$	Statistical test of comparison of the response at the value 0 becomes significant. The limit of quantification is determined with a risk of 0.05%.
Uncertainty	±0.1 10−4 $mol \cdot L^{-1}$	Because of the difference between the real value and the measured one, a degree of uncertainty will pertain to measurement. Uncertainty is the absolute range in which measured value can be accepted.

3.2. Monitoring of Acetone Iodination by Smartphone

Di-iode is a yellow brownish species in aqueous solution [28]. Since it has marked colour, it can be easily adapted to smartphone spectroscopy quantification as described in the supporting information, and Figure 3 describes this procedure.

[figure(s) omitted; refer to PDF]

From the results mentioned above, we confirm the validation of the method used for the quantification of I_2. We used our validated method to monitor I_2 concentration evolution during the reaction of acetone iodination. This method of quantification by smartphone, being easy to implement, was used to verify the mechanism and study the kinetic of acetone iodination reaction by di-iode. The equation of the reaction [29] is as follows:(8)I2+CH3COCH3 H+ CH3CO CH2I+HI.

The acetone iodination mechanism in acid medium is complex. It was demonstrated that its law equation is as follows [29]:(9)r=kH+CH3COCH3.

Therefore, the reaction rate is of the first order toward H+ and CH3COCH3 and of order 0 toward I_2 [29–31].

At an ambient lab temperature of 298 K, we prepared 3 series of experiments carried out by mixing I_2 solution with acetone in acidified aqueous medium according to the composition detailed in Table 5.

Table 5

Composition of the experiments to evaluate the reaction order.

Experiment	V (HCl 1 M) (mL)	V (C_3H_6O) (mL)	V (I_2 0.05 M) (mL)
1	2.5	1.0	1.0
2	2.5	0.5	1.0

3	1.0	0.5	1.0

Figure 4 describes the evolution of I_2 concentration in three different initial conditions as a function of time. All variations are linear with a correlation coefficient up to 0.97, which confirms the order pseudo-zero-order to I_2. The equation of I_2 variation versus time for each experiment is regrouped in Table 6.

[figure(s) omitted; refer to PDF]

Table 6

Kinetic parameters of the reaction of iodination of acetone.

	Equation	Correlation coefficient
Exp (1)	$Y=-4.10^{-5}X+0.0005$	0.9764
Exp (2)	$Y=-2.10^{-5}X+0.0005$	0.9961
Exp (3)	$Y=-8.10^{-6}X+0.0005$	0.9967

Partial orders (a) and (b) toward acetone and acid can be deduced using different initial concentrations of the three experiments. The kinetic law is written for the experiments $i=1$; 2; 3 are as follows:(10)ri=kC3H6O0iaH+0ib.

From the initial rate, we calculate the partial orders $a=1$, $b=1$, and the rate constant $k=0.0035\,L^{2}\cdot mol^{-2}\cdot s^{-1}$. These results conform to those found before I_2 was quantified with UV-visible spectroscopy [29].

From these results, we demonstrate that using smartphone spectroscopy is reliable for the determination of I_2 concentration.

4. Conclusions

Use of smart technologies such as USB cameras or smartphones constitutes an available method that can be used for education. The quasi totality of students around the world processes such devices. Their use can facilitate the study of reaction evolution with a reduced cost. Because of the large spread of this method, we proposed in this study a procedure of validation with smartphone for I_2 quantification:

(i) We tested the random character of the method responses by the Shapiro–Wilk test.

(ii) We proved the specificity of the method. No difference was observed between the response of the method when I_2 is dissolved in water or in reaction mixture.

(iii) We verified that there is no suspected neither aberrant responses by the Cochran test.

(iv) We applied the Fisher test and we found that the method is linear. The equation of the calibration curve allowed us the determination of the limits of detection and quantification of the method.

(v) We calculated the uncertainty of the method.

The validated method of I_2 quantification is applied to the kinetic study of acetone iodation. This method can be more developed and used for other chemical reactions in laboratory. Our developed method serves the equity of studying kinetics with physical methods for the neediest institutions in sophisticated materials. Moreover, it ensures a considerable reduction of chemical quantities compared to the classical UV-visible spectrophotometer needing a minimum amount of solution to fulfil the cell. However, the operation of photographing and transferring image to laptop to be treated by image *J* is still an awful operation in the method. To improve its accessibility and inclusiveness, it will be interesting to develop a smartphone application that treats images directly on the smartphone and not on laptop.

Additional Points

Highlights. Validation means fit for purpose. Smartphone spectroscopy for quantification. Statistical test for analytical method validation.

Authors’ Contributions

Sahar Raissi is responsible for conceptualization, methodology, software, formal analysis, investigation, resources, and writing original paper. Fatma Fakhfakh is responsible for validation and reviewing.

Glossary

Abbreviations

A:Absorbance

I:Colour intensity of I_2 solution

I_0:Colour intensity of water

C:Concentration of I_2 solution

r^2:Correlation coefficient

A_{ij}:Absorbance of a I_2 solution number *i* during the day *j*

R:Number of sequences in the Shapiro–Wilk test

S_j:Standard deviation of data series *j*

S_{max}:Maximum standard deviation of data series

C_{Cal}:Cochran-calculated constant

C_{Cri}:Cochran critical constant

a_1:Slope of linear curve

a_0:Intercept of linear curve

F_{Cal}:Fisher test calculated value

F_{Tab}:Fisher test reference value

X_{Lq}:Limit of quantification

X_{Ld}:Limit of detection

U:Uncertainty

ri:Rate of the reaction

a:Partial order toward C_3H_6O

b:Partial order toward H^+

k:Constant rate.

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DETAILS

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Transcription Factor E2F1 Regulates the Expression of ADRB2

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ABSTRACT (ENGLISH)

Adrenergic beta-2-receptor (ADRB2) is highly expressed in various tissue cells, affecting the susceptibility, development, and drug efficacy of diseases such as bronchial asthma and malignant tumor. However, the transcriptional regulatory mechanism of the human ADRB2 gene remains unclear. This study aimed to clarify whether E2F transcription factor 1 (E2F1) was involved in the transcriptional regulation of the human ADRB2 gene. First, the 5′ flanking region of the human ADRB2 gene was cloned, and its activity was detected using A549 and BEAS-2B cells. Second, it was found that the overexpression of E2F1 could increase promoter activity by a dual-luciferase reporter gene assay. In contrast, treatment of knockdown of E2F1 significantly resulted in a decrease in its promoter activity. Moreover, mutation of the binding site of E2F1 greatly reduced the potential of human ADRB2 promoter transcriptional activity to be regulated by E2F1 overexpression and knockdown. Additionally, by real-time

quantitative PCR and Western blot analysis, we demonstrated that overexpression of E2F1 elevated the ADRB2 mRNA expression and protein levels while si-E2F1 reduced its expression. Finally, the consequence of the chromatin immunoprecipitation assay showed that E2F1 was able to bind to the promoter region of ADRB2 in vivo. These results confirmed that E2F1 upregulated the expression of the human ADRB2 gene.

FULL TEXT

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1. Introduction

ADRB2 gene is a 1200 bp intron-free gene located in the 5q31-q32 asthma susceptibility fragment [1, 2], encoding beta-2-adrenergic receptor (*β*2-AR), a member of the G protein-coupled transmembrane receptor superfamily, which can specifically bind catecholamine and other endogenous ligands and be activated by their agonists to produce a series of biological effects [3, 4].

ADRB2 is highly expressed in bronchial smooth muscle and has been proven to play an important role in the development of asthma. It mediated airway physiological activities such as bronchodilation, ciliated mucilage clearance [5, 6], inhibition of cholinergic nerve action [7], suppression of microexudation [8], and inhibition of mast cells and basophil releasing mediators [9, 10]. A growing number of studies have shown that the ADRB2 gene influenced the susceptibility to asthma severity, and the *β*2-AR agonist correlation of response. A meta-analysis demonstrated that ADRB2 haplotypes functioned as defensive factors for asthma [11]. Fu et al. [12] observed the dose-dependent relationship between ADRB2 5′-UTR methylation and risk for severe asthma. Recent evidence has shown that ADRB2 expression in patients with asthma is positively correlated with FEV1 and the response of salmeterol to asthma [13, 14]. In addition, *β*2-AR is highly expressed in most malignancies. Mounting evidence shows that activation of *β*2-AR signal pathway promotes the carceration, aggression, and metastasis of malignancies, which is related to the progression and treatment resistance of various malignant tumors, including breast cancer, gastric cancer, prostate cancer, and pancreatic cancer [15–19]. Moreover, recent research has shown that catecholamine significantly increased the proliferation, invasion, and viability of tumor cells in vivo, and this effect could be inhibited by an ADRB2 antagonist through suppression of the ERK1/2-JNK-MAPK pathway and transcription factors [20].

The E2F transcription factors (E2Fs) family is an important transcription factor family that regulates gene expression, which was discovered in 1986 by Kovesdi et al. [21]. Numerous studies have suggested that E2F1 can influence gene expression through the regulation of transcription and the half-life of RNA molecules [22, 23]. In fact, advances in high-throughput techniques have shown that E2F1 can bind to the promoters of a significant number of genes involved in most cellular pathways, influencing gene transcription and expression [24]. It was demonstrated that E2F1 regulated the transcriptional levels of target genes depending on the ERK1/2 pathway in advanced non-small cell lung cancer (NSCLC) [25]. Nevertheless, little is known about the transcriptional regulatory effects of E2F1 on the human ADRB2 gene.

In the present study, considering that ADRB2 is an asthma susceptibility gene and E2F1 is closely related to the occurrence of lung cancer and other diseases, we performed a dual-luciferase reporter gene assay in two different cell lines—A549 (human pulmonary adenocarcinoma cell) and BEAS-2B (transformed human bronchiolar epithelial cell), verifying that the transcription factor E2F1 served as a transcriptional activator of the human ADRB2 gene by binding to its promoter. Further, our results demonstrated that E2F1 increased the expression both mRNA expression and protein levels of the human ADRB2 gene. To the best of our knowledge, this study provides the first experimental evidence for the involvement of E2F1 in the transcriptional regulation of the ADRB2 gene.

2. Materials and Methods

2.1. Cell Lines and Culture

The A549 and BEAS-2B cell lines used here were purchased from the American Type Culture Collection. All cell lines were cultured at 37°C with 5% CO_2 in RPMI Medium 1640 (Gibco) with 10% fetal bovine serum (Gibco), 100

units/ml penicillin, 100 μg/ml streptomycin, and 100% humidity.

2.2. Plasmid Construction and Small Interfering RNA (siRNA)

Based on the ADRB2 gene (ENSG00000169252) sequences reported in the Ensembl database, we can find the sequence (−1,879 to 39) upstream of the transcription start site (TSS). The ADRB2 promoter fragment was obtained by polymerase chain reaction (PCR) using primers containing KpnI and HindIII restriction sites, which were designed with Primer 5.0 and synthesized by Sangon (Shanghai, China). The primer sequences were as follows: sense: 5′-CGGGGTACCCTCAGGCAGACCTGGGTCAAATCC-3′, and antisense: 5′-CCCAAGCTTAGTTCCAGCCCGTGC TCTGAAGAA-3′. The products of PCR and the luciferase reporter plasmid pGL3-basic (Invitrogen) were respectively digested using KpnI and HindIII, and then recombined them using T4 DNA ligase (Thermo). The products were used to transform *E. coli* DH5*α* competent cells, verified by Sanger sequencing and the resulting plasmid was termed pGL-1879/+39. According to the JASPAR (https://jaspar.genereg.net/), a series of the potential transcriptional binding sites of E2F1 were predicted (E2F1a-e). Moreover, according to the critical regulatory regions of the human ADRB2 gene promoter (−219~−1, Kobilka et al. [26]), the bioinformatics tools UCSC Genome Browser were used to evaluate the phylogenetic conservation of E2F1 binding sites in this section (E2F1Dd, E2F1e). Then, the binding site E2F1e (−123~−112), which was closest one to the TSS, was identified and suffered from mutation. The mutant plasmid named mut-E2F1 was synthesized based on the previous cloning of pGL-1879/+39. The mutated sequence of the E2F1 binding site (−123 GGGGAGGGAAA−112) was 5′-GGGGCAAGAAA-3′. Knockdown of the E2F1 was achieved by transfecting siRNA into A549 and BEAS-2B cells, respectively. The siRNA of E2F1 (si-E2F1) and the negative control (si-NC) were synthesized by Sangon (Shanghai, China). The following primers sequences (Stoleriu et al. [27]) were utilized in the research: sense: 5′-GACGUGUCAGGACCUUCGU-3′, and antisense: 5′-ACGAAGGUCCUGACACGUC-3′. The overexpression plasmid pcDNA-E2F1 and its corresponding negative control plasmid pcDNA3.1 were kept by our laboratory [28].

2.3. Transient Transfection and Dual-Luciferase Assay

Transient transfection of A549 and BEAS-2B cells was performed using Lipofectamine™ 3000 (L3000015, Invitrogen), according to the manufacturer's instructions. We used the RPMI Medium 1640 with 10% fetal bovine serum without any antibiotic to improve the effectiveness of transfection. The cells were seeded in 96-well plates (1.5×10^4/well) and cotransfected after 24 h with 100 ng of the promoter reporter plasmids (pGL3-basic, pGL-1879/+39, and mut-E2F1) together with the pRL-TK plasmid (used as an internal control; 4 ng; Promega). After another 24 h, the cells were harvested and assayed to detect the relative luciferase activities by the dual-luciferase reporter assay system (Promega). For overexpression or knockdown of E2F1 experiments, an additional 100 ng of pcDNA3.1, pcDNA-E2F1 or 100 nM of si-E2F1, si-NC (supplemental material for the experiments to verify that these were able to change the E2F1 protein levels as expected) were added to the transfection system as described above and cotransfected into cells with those reporter plasmids, respectively. At 24 h post-transfection, cells were lysed, and the luciferase activities were measured. All the experiments were performed independently in triplicate.

2.4. Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR, briefly, total RNA was isolated with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and then reverse-transcription was performed using HiScript III Reverse Transcriptase (Vazyme Biotech Co., Ltd.) to obtain cDNA. qPCR amplification was conducted using SYBR Green I Master Mix (Vazyme Biotech Co., Ltd.) with GAPDH being used as an internal control. The following primers of ADRB2 synthesized by Sangon (Shanghai, China) were as follows: sense: 5′-GTGATCATGGTCTTCGTCTACT-3′, antisense: 5′-CATGATGATGCC TAACGTCTTG-3′. Each reaction was performed in triplicate.

2.5. Western Blot Analysis

Total proteins of ADRB2 and E2F1 were extracted, respectively, in accordance with the instructions of the RIPA buffer (Solarbio) with 5x sample loading buffer (Beyotime) added and boiled at 100°C for 5 minutes. 10% SDS-PAGE gel was made using the SDS-PAGE Gel Kit (Solarbio), 75 V pre-electrophoresis, and 120 V constant pressure separation. The protein was transferred to PVDF membrane at a constant flow rate of 250 mA after 120 minutes at room temperature. After blocking with 5% skim milk powder in TBST saline (0.25 M Tris-HCl, 0.19 M NaCl and 0.1%

Tween 20) solution for 2 h, the primary antibody anti-ADRB2 (1 : 2000; Abcam; ab182136), anti-E2F1 (1 : 2000; Abcam; ab288369) and GAPDH Mouse McAb (1 : 2000; ProteinTech; cat. no. 60004-14-1-Ig) were utilized to treat the protein blots at 4°C overnight. Subsequently, the secondary antibody was incubated with anti-Rabbit IgG HRP (1 : 2000; Biosharp; BL003A) or HRP-conjugated goat anti-mouse IgG (1 : 2,000; ProteinTech; cat. no. SA00001-1) at room temperature for 2 h after washing with TBST. Next, the bands were visualized with the ECL reagent (L/N 7E410L0, Vazyme). Blot images were captured under the ChemiDoc XRS Image Lab System (1708265, BioRad) and further analyzed utilizing the Image J software. GAPDH were used as controls and were detected with mouse monoclonal anti-GAPDH antibody (Santa Cruz, CA). The experiment was repeated three times independently.

2.6. Chromatin Immunoprecipitation Assay (ChIP)

The ChIP assay was performed using the EZ-Magna ChIP A-Chromatin IP Kit (17–408, Millipore). BEAS-2B cells were cross-linked with 1% formaldehyde and stopped by the addition of 0.125 M glycine. The cells were cleaned in ice-cold PBS, scraped off, and collected into a 15 ml centrifuge tube. Protease inhibitor complexes were added to the cells lysed in cell lysis buffer. Sonication of cross-linked chromatin was performed at 200 watt with 30 rounds of 5 seconds pulses. The chromatin fragments were 150–200 bp in size, as verified by agarose gel electrophoresis. Diluted chromatin (1%) was collected and used as input. Subsequently, anti-E2F1 (3742S, CST), anti-acetyl histone H3 (positive control), and normal anti-Rabbit IgG (negative control) were added and incubated overnight at 4°C for immunoprecipitating the chromatin. Protein/antibody/chromatin complexes were pelleted through the magnetic beads. Beads were washed four times with ice-cold wash buffer (Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Baffer, Licl Immune Complex Wash, and TE Buffer). When the complexes were eluted by resuspension of the washed pellet in 1 M $NaHCO_3$ and 1% SDS for 30 minutes, proteinase K was added into and incubated at 62°C for 2°h and 95°C for 10 minutes to obtain separate DNA. The samples were then purified using mini-column centrifugation. The purified DNA was amplified by PCR and separated by gel electrophoresis on a 1% agarose gel. Enrichment of the DNA fragment was detected by qRT-PCR with SYBR Green I Master Mix (Vazyme). The specific primers used were as follows: sense: 5′-GGACACCACCTCCAGCTTTA-3′, antisense: 5′-GTGACGTAC GGGAACTTTCG-3′.

2.7. Statistical Analysis

The results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using unpaired t-test with SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

3. Result

3.1. The Predicted Promoter Fragment of Human ADRB2 Is Functional in A549 and BEAS-2B Cell Lines

After DNA sequencing verification, the luciferase assay was performed to determine the functionality of pGL-1879/+39 in A549 and BEAS-2B cells. The activity of pGL-1879/+39 was 491 and 140 times higher than that of the control plasmid pGL3-basic, suggesting that the recombinant plasmid was successfully constructed (Figures 1(a) and 1(b)).

[figure(s) omitted; refer to PDF]

3.2. E2F1 Is a Transcriptional Activator of the Human ADRB2 Gene

The presumptive transcription factor binding sites of E2F1 on the human ADRB2 gene promoter were predicted using the bioinformatics tool JASPAR (Figure 2(a)), indicating that E2F1 might have a potential function to regulate the expression of human ADRB2 gene. At the same time, phylogenetic conservation analysis was performed for the −230 to −1 sequences in the upstream TSS of the human ADRB2 gene. The results showed that the E2F1 binding sites in this sequence were subjected to partial variation in different species (Figure 2(b)). To investigate whether E2F1 regulated the activity of the ADRB2 promoter, the overexpression/knockdown of E2F1 plasmids and the negative control were respectively cotransfected into A549 and BEAS-2B cells. Then, the activities of the pGL3-basic empty vector with and without E2F1 overexpression/knockdown were measured to make sure that the empty vector does not respond to changes in E2F1 levels (the activity of the pGL3-basic empty vector in different samples was close to zero with no significant difference, as shown in Supplemental files Figure 1). As shown in Figures 2(d)

and 2(f), the overexpression of E2F1 significantly increased the luciferase activity of pGL-1879/+39 in A549 and BEAS-2B cells by 2.46 times and 1.73 times, respectively. On the contrary, knockdown of E2F1 led to a remarkable decrease in the promoter activity by 45.7% and 43.3% in A549 and BEAS-2B cells, respectively (Figures 2(e) and 2(g)). Moreover, mutation of the putative binding site E2F1e (GGGGAGGGAAA, Figure 2(c)) made it lose its potential to regulate the transcriptional activity of the human ADRB2 gene promoter by E2F1 overexpression and knockdown (Figures 2(h)–2(k)). These results indicated that E2F1 may play a role of positive regulation in the transcription of the promoter of the human ADRB2 gene by binding to the site predicted.

[figure(s) omitted; refer to PDF]

3.3. E2F1 Binds to the Promoter of Human ADRB2 Gene In Vivo

In the previous experiments, we speculated that E2F1 could regulate the ADRB2 promoter activity by binding to the upstream sequence of TSS. Therefore, chromatin immunoprecipitation assay was performed in untreated BEAS-2B cells to validate whether E2F1 interacted with the ADRB2 promoter in vivo. As shown in Figures 3(a) and 3(b), an enrichment of the ADRB2 promoter was monitored using anti-E2F1 antibody in BEAS-2B cells, which suggested that E2F1 was able to bind to the promoter region of human ADRB2 gene in vivo.

[figure(s) omitted; refer to PDF]

3.4. E2F1 Upregulates the Expression of Human ADRB2 Gene

In order to further examine the effect of E2F1 on the expression of human ADRB2 gene, the mRNA expression and protein levels assay of ADRB2 using qRT-PCR and Western blotting in BEAS-2B cells were carried out. Meanwhile, Western blotting was used to verify the efficiency of E2F1 overexpression and knockdown. BEAS-2B cells were transfected with siRNA or E2F1-overexpression plasmid. Then qRT-PCR using an equal amount of RNA was performed with specific primers to ADRB2 and GAPDH (control). Compared with the control vector, the overexpression of E2F1 enhanced the mRNA expression by 1.23 times. Oppositely, si-E2F1 resulted in downregulation of its expression by 36% (Figures 4(a) and 4(b)). Correspondingly, cells were treated with the same condition as described previously. After 48 h, the lysates were obtained, and Western blotting was performed to analyze the expression of E2F1, ADRB2 and GAPDH in the same sample. As shown in Figures 4(c), overexpression of E2F1 could effectively improve the protein levels of ADRB2 and E2F1 by 46% and 67.6%, respectively. In contrast, knockdown E2F1 was able to efficaciously reduce its levels by 42.1% and 58%. In addition, Western blotting analyses were performed on the lysates extracted from BEAS-2B cells transfected with equivalent amounts of ADRB2 and E2F1, and the result showed that there was no statistical significance between their control group, which further proved that the change in E2F1 was able to regulate the expression of ADRB2. These results suggested that E2F1 could positively regulate the expression of the human ADRB2 gene at mRNA and protein levels.

[figure(s) omitted; refer to PDF]

4. Discussion

The present study focused on the basal transcription regulation of the human ADRB2 promoter. Jiang and Kunos [29] demonstrated that transcriptional enhancement of the rat ADRB2 promoter may be mediated by the element resided in the 500 bp fragment. Jaeger et al. [30], constructing a stepwise deletion of the 5′ flanking sequence of the promoter region (−1324/+33~−269/+33), revealed that the minimal promoter of porcine ADRB2 was located in the −307~−209 region. Compared with the pGL3-basic reporter vector, promoter activity of pGL-307/+33 was 3-fold increased in COS-7, 7-fold increased in HepG2, 5-fold increased in C2C12, and 2-fold increased in 3T3-L1. Also, this study has identified the importance of −400~−209 region in the regulation of basal expression of porcine ADRB2 and the lack of the presence of key regulatory element in the 5′ flanking −882~−709 region. Johnatty et al. [31] showed that the majority of promoter activity resides within a 549 bp fragment immediately 5′ to the start of translation and identified four naturally occurring polymorphisms (−468C G, −367T C, −47T C, −20T C). These variants led to highly significant alteration of the luciferase activity of the human ADRB2 promoter. The pCTTC and pCCTC constructs led to an increase in promoter expression of 30% compared with the normalized construct pCTTT. Two other constructs, pCTCT and pGCCT showed reduction of three times. In our present study, a 1918 bp

fragment of the human ADRB2 promoter was cloned into a reporter plasmid and a dual-luciferase reporter gene assay was performed. The luciferase assayed revealed a 491-fold increase in ADRB2 promoter activity in A549 and a 140-fold increase in BEAS-2B from pGL-1879/+39 compared with the empty pGL3-basic reporter vector, indicating a functional promoter in the pGL-1879/+39 region of ADRB2.

E2F1 is generally considered to be “activating E2F” and mainly binds the retinoblastoma protein (pRB) in a cycle-dependent manner, that is, regulates cell G1/S process through the nucleus RB/E2F pathway [32, 33]. It is supposed to relate to the occurrence and development of proliferative diseases. Compared with normal bronchial epithelial cells, E2F1 expression in the lung tissues of asthma patients was different and regulated by c-Myc that regulated cell proliferation and apoptosis [34]. The latest research showed that E2F1 affected TGF-β1-induced pulmonary fibrosis and epithelial-mesenchymal transition (EMT) in BEAS-2B cells through the miR-106b-5p/E2F1/SIX1 signaling pathway [35]. In addition, mutations in retinoblastoma tumor suppressor genes (RB1) or components that regulate the CDK/RB/E2F pathway are present in almost every human malignancy. In breast cancer, there is often an EMT associated with RB-E2F1 pathway [36]. Meng et al. [37] illustrated that the proliferation, migration, and EMT of non-small cell lung cancer (NSCLC) cells were promoted through E2F1 bound to the promoter of long intergenic nonprotein coding RNA 461 (LINC00461), a molecule with oncogenic potential in several cancers. Moreover, overexpression of E2F1 has been reported to be related to tumor growth and cell survival in prostate cancer (PCa), whereas knockdown treatment of E2F1 inhibited cell cycle progression, invasion, and migration of PCa cell lines in vitro, as well as tumor growth and EMT in vivo [38].

Previous studies have shown that EZH2 was able to bind to the human ADRB2 promoter and repressed ADRB2 expression, which promoted metastatic prostate cancer [39]. Boulay et al. [40] have confirmed that ADRB2 is a new direct target gene of HIC1, an inhibitor for tumor invasion. Treatment of HIC1 with small interference was able to downregulate the expression of ADRB2, thereby reducing the migration and invasion of MDA-MB-231 cells which are highly malignant breast cancer cells. In present study, we found that human ADRB2 promoter contained several E2F1 binding sites by bioinformatics analyses. Overexpression of E2F1 enhanced the promoter activity of ADRB2, on the contrary, knockdown of E2F1 using siRNA decreased its activity. The mutation of an E2F1 putative binding site in the key regulatory region of the ADRB2 promoter revealed that it lost its potential to regulate the transcriptional activity of the human ADRB2 gene promoter, although this site was not conserved consistently in different species. We also demonstrated that E2F1 was able to bind to the promoter of ADRB2 in vivo by the ChIP experiment. Moreover, overexpression of E2F1 upregulated both mRNA expression and protein levels of the human ADRB2 gene, as well as knockdown of E2F1 led to a significant reduction in its expression. Accordingly, we speculate that E2F1 may affect the progression and prognosis of various diseases such as asthma, malignant tumor through basal transcriptional regulation of human ADRB2 gene, and that E2F1 may become a new therapeutic target and prognostic marker. But the specific regulation mechanism still needs further study.

To sum up, in this study, we confirmed that E2F1 was able to bind to the ADRB2 promoter in vivo. Meanwhile, E2F1 can upregulate the promoter activity and expression of ADRB2 at the basal transcriptional level. These results may provide novel ideas for further understanding the molecular regulatory mechanism of the human ADRB2 gene.

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Separation of Amino Acids, Dyes, and Pigments Using Novel Pressurized Circular TLC Assembly for Secure Medical Imaging Applications

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ABSTRACT (ENGLISH)

A novel pressurized flow system for circular thin-layer chromatography (PC-TLC) has been successfully established and employed for the separation of amino acids, dyes, and pigments for safe medical imaging applications. In this system, the mobile phase is applied to a regular TLC plate through the tube and needle of an intravenous infusion set. The needle was fused in a hole underneath the center of the plate, while the second side end of the tube was connected to a microburette containing the solvent. This new assembly proved itself better in terms of separation time (within 5 minutes) and controlled flow of the solvent and horizontal movement of analyte components over chromatograms with better separation and R_f values (glutamine: 0.26, valine: 0.44, phenylalanine: 0.60, chlorophyll a: 0.52, chlorophyll b: 0.43, xanthophyll: 0.18, carotenoid: 0.97, and pheophytin: 0.60) when a number of samples of amino acids, dyes, and pigments were separated by the developed apparatus and the conventional TLC procedure. The developed method was found distinctly rapid, precise, and eco-friendly (less solvent consuming) as compared to traditional ascending TLC.

FULL TEXT

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1. Introduction

Thin-layer chromatography is one of the common classical chromatographic techniques used by analytical chemists to separate and identify different components of a complex sample. TLC has also been employed to evaluate the purity of a compound and monitor the progress of a reaction. High sensitivity, low cost, little separation time, and a wide range of applications are the factors which make this technique worldwide popular. It is a simple nonhazardous separation method which does not require any sophisticated instrumentation. Separation occurs due to the relative solubility of sample components in the mobile phase and the relative adsorption on the stationary phase, which is usually uniformly coated on a glass, plastic, or aluminum sheet. The most common adsorbents used for TLC are silica, alumina, and cellulose. A mobile phase is usually a single or mixture of two or more miscible solvents depending on the nature of the sample [1]. In ascending TLC, the liquid mobile phase is drawn up through the stationary phase by capillary action when, after loading the sample and standards, the adsorbent coated plate is vertically placed in a chromatographic tank. The mobile phase drags the sample components to different heights while travelling on the stationary phase. The separation of different components takes place on the basis of their retardation due to a stationary phase and solubility in the mobile phase [2]. Because of the importance of the separation of amino acids in TLC, different approaches have been reported for prediction of these retardation tendencies of such samples [3] like quantifiable structure-R_f connection of amino acids in various solvents for RP-TLC [4], structure-retardation factor relationship of amino acids in different mobile phases [5], and prediction of R_f of amino acids in RP-TLC with ethanol-sodium azide mixture as a mobile phase [6]. Different solvents were reported for variations in separation of colored components of leaf extracts [7].

A number of advancements in the classical TLC methodology [8] have been introduced since the pioneering work of Stahl in the early 1950s [9]. Some of the innovations improved the reproducibility, speed, and resolution of this technique as well as made it simpler and more cost-effective [10]. Improvements have also been made in the use of

higher quality adsorbents along with the overpressure mobile phase. In this regard, reverse phase TLC and high-performance TLC are two examples [11]. Another is separating dyes with circular TLC [12]. One of the new ideas in TLC is to use a pressurized flow of the mobile phase over the adsorbent [5]. TLC with pressurized solvent flow, also known as “overpressure thin-layer chromatography” can be especially useful in high-performance TLC [13] where the plates are coated with finer particle adsorbents and the mobile phase travels more slowly to improve resolution [14]. The systems that are still used to apply the solvent under pressure are complex in design, expensive to purchase, cumbersome to organize, and difficult to operate. To get reproducible results from such systems is not an easy job for a nontechnical person. Our group already introduced a number of simple TLC activities for undergraduate students [15]. In the described procedure, the solvent is introduced in the center of the plate from a fine, controlled side burette through a thin plastic pipe. The solvent flows under gravity pressure and spreads on the adsorbent horizontally [16]. This system saved significant time and gave better resolution than classical TLC. The present system of feeding solvent to the plate is much simpler and faster than classical ascending TLC, making it perfect for high-performance TLC [17].

2. Experimental

2.1. Apparatus Assembly

Commercially available 10 × 10 inch TLC silica gel 60 F254 plates were used. A TLC plate was fixed on the same size glass plate and placed on the wooden blocks, as shown in Figure 1. A small hole (1 mm) was bored in the center of both plates. The needle and the pipe of an intravenous infusion set (model: Mediset, SF-35) was employed to deliver the solvent to the TLC plate. The needle was gently inserted in the central hole of the glass plate till its end reached the TLC plate, and the plastic pipe was connected to the outlet of the burette (model: Pyrex, 3B 846B) containing the solvent.

[figure(s) omitted; refer to PDF]

2.2. Chemicals Required

TLC silica gel 60 F254 plates (Merck KGaA), samples of amino acids (glutamine, valine, and phenylalanine), ninhydrin, inks (fountain pen ink and ballpoint ink), and ethanol and spinach leaves for leaf extracts were used in this study. All chemicals were of AnalR grade and obtained from Merck (Germany). Leaf extracts were prepared by crushing fresh leaves of spinach plants and extracting liquid from ethanol using Soxhlet apparatus [18].

2.3. PC-TLC Operating Setup

With the help of a 5 μL micropipette, a sample spot was applied on a circle of 1 inch diameter on the midpoint of the plate. In order to saturate the system with solvent vapors, the spots were covered with a 10-inch Petri dish. After sampling, the flow-control valve of the burette was slowly opened to allow the solvent to flow over the plate. For the establishment of equilibrium, the solvent was not allowed to flow more than 0.2 mm/min over TLC. The flow of the mobile phase was organized primarily with the stopper at the end of the burette and second with the drop-falling valve mechanism of the infusion set. After adequate development, the plate was removed from the assembly, dried in an oven, and R_f values of colored bands were measured.

For the sake of comparison, samples of amino acids, inks, dyes, leaf extracts, and pigments were also analyzed by using classical ascending TLC. Strips (3 × 6 inches) of the same commercial plates and solvent composition were employed in the classical procedure. The separation and compound identification of leaf extracts have already been performed with the help of classical ascending TLC [19]. In this work, only the R_f values obtained for sample constituents, time consumed for separation, and resolution were compared by both methods.

2.4. Separation of Amino Acids

Amino acids such as phenylalanine, tryptophan, and valine [20] were separated by the described procedure of PC-TLC as well as by classical ascending TLC [21]. The solvent system used for the separation of amino acids consists of *n*-butanol, water, and acetic acid in a ratio of 60 : 25 : 15. Ninhydrin spray was employed to detect amino acids on the TLC plate [22].

2.5. Separation of Inks and Dyes

Inks used in fountain pens and ballpoints are usually the mixtures of various colored dyes and can be separated

easily by TLC [1]. Both types of inks were used as samples in the present work. The solvent system used for the separation of ink ingredients consists of *n*-butanol, water, and acetic acid in a ratio of 60 : 25 : 15.

2.6. Separation of Pigments from Spinach Extracts

In this set of experiment, extracts of spinach [20] leaves were used as samples. The extracts were obtained from acetone by applying standard procedures [7] and analyzed by the described and classical TLC procedures. The mobile phase used for the separation of spinach extract ingredients was a mixture of hexane and acetone in a ratio of 7 : 3.

3. Results and Discussion

Pressurized circular TLC (PC-TLC) is an advanced form of circular TLC, which is also known as multidimensional TLC in the literature [16]. This PC-TLC assembly has been generally found to be simpler, faster, and more environmentally friendly than conventional ascending TLC. The new technique has numerous advantages over classical ascending TLC [12]. PC-TLC could be successfully employed for the separation of all types of colored samples with better resolution. The horizontal spreading of solvent in the case of PC-TLC takes less than half time for separation, giving better resolution. In addition, the solvent flowrate can be controlled through a stopper and valve system provided in the assembly. As compared to classical TLC, far less volume of solvent is required for achieving satisfactory separation. The use of relatively less volume of solvent makes the technique environmentally friendly. Another advantage which the newly described method offers is the provision of gradient elution which is not possible in classical TLC.

3.1. Amino Acids

A synthetic mixture of three amino acids, phenylalanine, tryptophan, and valine, was analyzed by the newly described procedure and classical ascending TLC. The results obtained in both the techniques have been presented in Table 1, and separation is shown in Figure 2.

Table 1

Separation of amino acids by classical and pressurized TLC.

Systems used for separation	Time consumed (min)	R_f values obtained for different amino acids		
Glutamine	Valine	Phenylalanine	Ascending TLC	19
0.25	0.43	0.61	PC-TLC	5

[figure(s) omitted; refer to PDF]

3.2. Inks and Dyes

Fountain pen ink and ballpoint ink are usually mixtures of various dyes which can be easily separated with the help of TLC. Both ink sample components were separated by classical and pressuized TLC. The R_f values and time consumed for separation of various components have been compared in Tables 2 and 3, and resolution is shown in Figures 3 and 4.

Table 2

Separation of fountain pen ink by classical and pressurized TLC.

Systems used for separation	Time consumed (min)	R_f values obtained for different components

Red	Blue	Green	Ascending TLC	20
0.77	0.84	0.79	PC-TLC	5

Table 3

Separation of ballpoint ink by classical and pressurized TLC.

Systems used for separation	Time consumed (min)	R_f values obtained for different components		
Red	Blue	Green	Ascending TLC	20
0.55	0.24	0.67	PC-TLC	5

[figure(s) omitted; refer to PDF]

3.3. Pigments in Leaf Extracts

Green leaves of plants contain several components which can be easily separated by TLC [20]. In this work, the extracts of spinach leaves have been analyzed by the described procedure of PC-TLC as well as by classical ascending TLC as used for beet root extract analysis [23]. The results of chromatographic analysis of the spinach extract are given in Table 4, and comparison of resolution of both method R_f has been shown in Figure 5, which indicated that almost the same R_f values resulted with PC-TLC within 10 minutes as compared to conventional methods.

Table 4

Separation of pigments from the extract of spinach leaves by both methods.

Systems used for separation	Time taken (min)	R_f values obtained for different pigments present in the spinach extract				
Chlorophyll a	Chlorophyll b	Xanthophyll	Carotenoid	Pheophytin	Classical TLC	18
0.52	0.42	0.17	0.98	0.59	PC-TLC	5

[figure(s) omitted; refer to PDF]

4. Conclusion

Pressurized circular TLC (PC-TLC) is a relatively simple, fast, and cost-effective technique of TLC, which can yield

better resolution of all types of samples which can be analyzed by classical ascending thin-layer chromatography for medical imaging applications. As compared to normal TLC, the new method consumes almost half the time (within 5 minutes) for obtaining even better results. This technique needs far less volume of solvent for separation and hence can be claimed as cost-effective as well as environmentally friendly. In addition to other advantages, gradient elution can conveniently be incorporated in the described procedure, whereas this facility is not possible in normal ascending TLC.

Disclosure

This work is part of the University of the Punjab, Pakistan.

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Flavonoids as Strong Inhibitors of MAPK3: A Computational Drug Discovery Approach

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ABSTRACT (ENGLISH)

Background. Mitogen-activated protein kinase 3 (MAPK3) mediates the onset, progression, metastasis, drug resistance, and poor prognosis in various malignancies, including glioma, liver, ovarian, thyroid, lung, breast, gastric, and oral cancers. Negative regulation of MAPK3 expression using miRNAs has led to therapeutic effects in cancer. *Objectives*. The present study performed molecular docking and dynamics simulation to identify potential MAPK3 inhibitors from natural flavonoids, possibly leading to drug development in cancer therapy. *Methods*. A computational drug discovery approach was performed using the AutoDock tool to identify potential MAPK3 inhibitors from 46 plant-based flavonoids. A cross-validation study was executed using the Schrödinger Maestro docking tool. Molecular dynamics (MD) was executed to evaluate the stability of docked poses between the top-ranked compounds and the MAPK3 catalytic domain. Interactions among the most potent MAPK3 inhibitors and residues within the receptor's active site were studied using the BIOVIA Discovery Studio Visualizer before and after 100 ns MD simulations. *Results*. Kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and

vicenin-2 exhibited a magnificent binding affinity to the receptor's active site. In addition, the stability of the docked poses of these compounds seemed to be stable after ~45 ns computer simulations. *Conclusion*. The present study suggests that kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and vicenin-2 could strongly bind to the MAPK3 catalytic site and could be assigned as a potent inhibitor for MAPK3. These findings may be helpful in the treatment of various cancers. However, further validation experiments are needed.

FULL TEXT

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1. Introduction

Mitogen-activated protein kinase 3 (MAPK3), called extracellular signal-regulated kinase 1 (ERK1), is a critical ERK/MAPK pathway cell signaling molecule. It mediates the transmission of signals from a cell's exterior to its interior. The ERK/MAPK pathway regulates apoptosis, cell proliferation, and migration [1, 2]. MAPK3 phosphorylates its downstream cytoplasmic protein, activating several nuclear transcription factors (e.g., c-Jun and c-fos) and participating in apoptosis and cell proliferation [3, 4]. The overexpression and/or hyperactivity of MAPK3 has been linked to the initiation, development, cancer cell migration, and drug resistance in various carcinomas, including liver, thyroid, lung, and gastric cancers [5]. Previously, Du et al. [6] reported that miR-143 upregulation reduced breast cancer cell proliferation by targeting MAPK3. Additionally, Cao et al. [5] demonstrated that the reduced miR-129 expression and MAPK3 overexpression were associated with cisplatin resistance in gastric cancer cells. MiR-129 overexpression also diminished the cell proliferation via downregulating MAPK3 leading to enhanced cell apoptosis. These results confirm the significant role of MAPK3 in tumorigenesis and drug resistance in different cancers.

Molecular docking analysis is a structural bioinformatics approach commonly used for drug discovery, identifying potential inhibitors for a given biological target. Molecular docking also unravels interaction modes between macromolecules and drug candidates [7, 8]. Molecular docking analysis includes three steps: macromolecule structure, preparation of small molecule structure, and evaluation of binding affinity between ligand and receptor [9]. Postdocking studies such as molecular dynamics (MD) analysis are frequently used to understand better ligands' dynamic manner and stability within the receptors' binding site (e.g., proteins' active site). Moreover, MD enables assessing the flexibility of residues inside the catalytic site of proteins [10–12].

Flavonoids are secondary metabolites predominantly found in the plant kingdom, playing a significant role in plant development [13]. The basic structure of flavonoids has been shown in our previous study [14]. Generally, they comprise a C6-C3-C6 skeleton, in which the C6 rings are aromatic, and the C3 is a bridge linker [15]. Due to their high binding affinity to enzymes [16], flavonoids have demonstrated a wide range of pharmacological behaviors in medicine, including anticancer, antioxidant, anti-inflammatory, antiallergic, antibacterial, and antiviral properties [17, 18]. There is growing evidence suggesting the curative potential of flavonoids in different cancers such as breast [19], colorectal [20], oral [21], and lung cancers [22], as well as hepatocellular carcinoma [19].

The present study suggested that flavonoids may act as potential inhibitors of MAPK3 activity, leading to downregulating its downstream signaling pathways and reducing cell proliferation and migration. Therefore, we performed a molecular docking analysis to evaluate the binding affinity of several flavonoids to the MAPK3 active site. According to estimated inhibition constant values (K_i) between studied ligands and the protein active site, top-ranked MAPK3 inhibitors were introduced, interaction modes among top-ranked flavonoids and residues inside the MAPK3 catalytic site were analyzed, and the stability of the docked pose of the best MAPK3 inhibitor was studied by executing MD simulation. The present results might be beneficial in cancer treatment.

2. Materials and Methods

2.1. Structural Preparation of MAPK3 and Flavonoids

The three-dimensional structure of MAPK3 was downloaded at 1.4 Å X-ray resolution from the RCSB database [23], which is available at https://www.rcsb.org (PDB ID: 4QTB) [24]. The 4QTB file included two polypeptide chains: A and B. The total number of residues in each chain was checked using the Notepad++ tool. Accordingly, chains A

and B included 351 and 348 residues, respectively. Therefore, chain A was selected for further analysis. Critical amino acids within the active site were identified by analyzing the two structure interactions among the 38Z (positive control inhibitor of the protein with a PDB ID of 24866313) and residues inside the active site of the protein using the BIOVIA Discovery Studio Visualizer version 19.1.0.18287, as well as reviewing the study by Chaikuad et al. [25]. Next, the 38Z molecule was eliminated from the PDB file, and energy optimization was executed via the Swiss-pdbViewer version 4.1.0, available at https://spdbv.unil.ch [26].

Previous studies have reported about 6000 flavonoids contributing to the colorful pigments of fruits, vegetables, and medicinal herbs [27]. The present study selected 46 flavonoids mainly found in commonly used fruits and vegetables, including onions, lettuce, kale, apples, tomatoes, berries, grapes, red grapes, red grapes, raspberries, strawberries, bilberries, merlot grapes, blueberries, and blackberries [27]. Therefore, 46 natural flavonoids were considered for identifying possible MAPK3 inhibitors, and the binding affinity of a standard drug (name: Purvalanol; PubChem ID: 448991; DrugBank ID: DB02733) to the active site of MAPK3 was regarded as a positive control inhibitor in this study. Structural preparation and energy minimization of flavonoids were explained in our previous study [28].

2.2. Molecular Docking

AutoDock 4.0 was used as a semiflexible docking tool [29, 30]. The software was installed in a windows-based computer system with the following features: installed memory: 32GB; processor: Intel Core i7; system type: 64-bit. Twenty-six residues were observed inside the active site of the receptor, including Tyr36, Ile48, Ala52, Tyr53, Lys54, Val56, Ser57, Tyr64, Ala69, Lys71, Ile73, Tyr81, Arg84, Thr85, Gln105, Asp106, Leu107, Lys114, Asp123, Met125, Asp128, Lys131, Cys166, Asp167, Leu173, and Asp184. To avoid missing any atoms related to the residues cooperated in the MAPK3 active site, the grid box was set to 84, 60, and 70, pointing in *x*, *y*, and *z* coordinates, respectively, with a spacing of 0.375 Å. The grid box centers were 33.335 Å, 55.015 Å, and 49.3 Å for *X*, *Y*, and *Y* directions, respectively. Fifty conformations were constructed for each flavonoid using the Lamarckian genetic algorithm [31] and ranked based on their estimated binding energies.

2.3. Cross-Validation Study

The most potent MAPK3 inhibitors achieved from the AutoDock tool were selected for cross-validation study. In this regard, the Schrödinger Maestro docking tool version 10.2 was used to calculate the docking scores [32, 33]. The lowest dock score (Glide score) was assigned as the best-docked model for each component. Furthermore, the prime MM-GBSA approach was utilized to indicate the relative binding energies [34].

2.4. Molecular Dynamics

MD was executed in 100 ns (1,00,000 ps) simulations by Discovery Studio Client software version 16.1.0.15350 to evaluate the stability of the docked poses between the top-ranked flavonoids, based on the AutoDock tool and Schrodinger Maestro docking software and MAPK3 active site. Advanced settings for MD simulation are mentioned in our previous report [28]. Furthermore, the root mean square fluctuation (RMSF) of MAPK3 residues and the time evolution of root mean square deviation (RMSD) of the receptor backbone atoms complexed with the top-ranked flavonoids were analyzed. BIOVIA Discovery Studio Visualizer 19.1.0.18287 was used to unravel interactions between top-ranked flavonoids and residues inside the MAPK3 active site and to illustrate two- and three-dimensional views of their docked poses.

2.5. Pharmacokinetic and Toxicology Assessment

The SwissADME online web server, available at https://www.swissadme.ch/ [35], evaluated the selected flavonoids' absorption, distribution, metabolism, and excretion (ADME). Furthermore, the ligands' Lethal Dose 50 (LD50) was predicted using the ProTox-IIweb server available from https://tox.charite.de/protox_II/ [36].

3. Results

3.1. Binding Affinity Assessment Using AutoDock

According to the virtual screening analysis achieved by AutoDock 4.0, four and 32 compounds demonstrated Ki values at the micromolar (uM) and nanomolar (nM) scales, respectively. Besides, it was estimated that nine compounds, including orientin, kaempferol 3-rutinoside-7-sophoroside, rutin, isoquercitrin, vicenin-2, amentoflavone,

quercetin-3-rhamnoside, nicotiflorin, and sophoraflavanone G, could potentially bind to the MAPK3 active site at the picomolar (pM) scale. Also, a salient binding affinity was observed between kaempferol 3-rutinoside-4′-glucoside (PubChem ID: 44258844) and MAPK3 catalytic site with the *K*i and $\Delta G_{binding}$ values of 731.68 femtomolar (fM) and −16.65 kcal/mol, respectively. Therefore, the present study calculated the *K*i value for ten compounds at either pM or fM concentrations. These flavonoids were considered top-ranked MAPK3 inhibitors among the studied flavonoids based on the AutoDock tool. Figure 1 demonstrates the chemical structures of these flavonoids and purvalanol. The $\Delta G_{binding}$ value between purvalanol and MAPK3 active site was estimated as −8.53 kcal/mol. Accordingly, 39 flavonoids demonstrated a higher binding affinity to the MAPK3 catalytic site than the positive control inhibitor. Figure 2 presents $\Delta G_{binding}$ values between top-ranked flavonoids achieved from the AutoDock tool, the standard drug, and MAPK3 active site. The estimated binding energies and *K*i values for 46 flavonoids and the control inhibitor in this study are presented in Table 1. Furthermore, the details of energies between top-ranked inhibitors and MAPK3 catalytic domain are shown in Table 2.

[figure(s) omitted; refer to PDF]

Table 1

Binding energy and inhibition constant values for 46 flavonoids and a positive control inhibitor docked to the MAPK3 active site, achieved from the AutoDock tool.

PubChem ID	Ligand name	Inhibition constant (*k*i)	Binding energy (kcal/mol)
44258844	Kaempferol 3-rutinoside-4′-glucoside	731.68 fM	−16.56
5281675	Orientin	1.92 pM	−15.98
44258853	Kaempferol 3-rutinoside-7-sophoroside	4.41 pM	−15.49
5280805	Rutin	19.54 pM	−14.61
5280804	Isoquercitrin	60.05 pM	−13.94
442664	Vicenin-2	168.28 pM	−13.33
5281600	Amentoflavone	212.47 pM	−13.20
5318767	Nicotiflorin	343.03 pM	−12.91
5353915	Quercetin-3-rhamnoside	343 pM	−12.91
72936	Sophoraflavanone G	608.24 pM	−12.57
5280441	Vitexin	1.16 nM	−12.19
5280459	Quercitrin	1.86 nM	−11.91
10095180	Kaempferol 7-O-glucoside	1.98 nM	−11.87

5282102	Astragalin	2.39 nM	−11.76
9911508	Astragarin	4.69 nM	−11.36
5281672	Myricetin	20.66 nM	−10.48
439533	Taxifolin	25.43 nM	−10.36
5280704	Apigenin-7-glucoside	27.61 nM	−10.31
5280343	Quercetin	32.01 nM	−10.22
5316673	Afzelin	36.07 nM	−10.15
5280637	Cynaroside	39.83 nM	−10.10
5280681	3-O-methylquercetin	41.41 nM	−10.07
1203	Epicatechin	50.71 nM	−9.95
471	Dihydroquercetin	53.59 nM	−9.92
5280544	Herbacetin	62.60 nM	−9.83
5280445	Luteolin	65.25 nM	−9.80
5281654	Isorhamnetin	85.22 nM	−9.64
5281614	Fisetin	91.83 nM	−9.60
9064	Catechin	152.68 nM	−9.30
638278	Isoliquiritigenin	177.83 nM	−9.21
5318998	Licochalcone A	225.53 nM	−9.07
14309735	Xanthogalenol	280.13 nM	−8.94
5281670	Morin	298.46 nM	−8.90
629440	Hemileiocarpin	319.53 nM	−8.86
443639	Epiafzelechin	326.71 nM	−8.85
5281612	Diosmetin	385.31 nM	−8.75

5280443	Apigenin	433.98 nM	−8.68
124052	Glabridin	455.60 nM	−8.65
5317435	Fustin	480.39 nM	−8.62
5280863	Kaempferol	590.49 nM	−8.50
5281607	Chrysin	732.28 nM	−8.37
72281	Hesperetin	996.15 nM	−8.19
25201019	Ponciretin	1.01 μM	−8.18
639665	XanthohuMol	3.20 μM	−7.50
5280378	Formononetin	4.06 μM	−7.36
10680	Flavone	1.74 μM	−7.17
448991	Purvalanol (ctrl)	559.25 nM	−8.53

MAPK3, mitogen-activated protein kinase 3; ctrl, control.

Table 2

Details of energies between top-ranked flavonoids, positive control inhibitor, and MAPK3 catalytic site, achieved from the AutoDock tool.

Ligand name	Final intermolecular energy (kcal/mol)	Final total internal energy (kcal/mol)	Torsional free energy (kcal/mol)	Unbound system's energy (kcal/mol)	Estimated binding energy (kcal/mol)
Kaempferol 3-rutinoside-4′-glucoside	−9.04	−16.62	5.97	−3.14	−16.56
Orientin	−9.61	−10.86	3.58	−0.91	−15.98
Kaempferol 3-rutinoside-7-sophoroside	−6.54	−21.39	7.46	−4.98	−15.49
Rutin	−10.12	−12.91	5.07	−3.35	−14.61
Isoquercitrin	−10.82	−9.24	3.88	−2.24	−13.94

Vicenin-2	−11.49	−9.34	5.07	−2.43	−13.33
Amentoflavone	−9.89	−7.5	3.28	−0.92	−13.20
Quercetin-3-rhamnoside	−12.08	−6.52	3.28	−2.4	−12.91
Nicotiflorin	−11.48	−9.06	4.77	−2.86	−12.91
Sophoraflavanone G	−12.81	−3.1	2.68	−0.65	−12.57
Purvalanol (ctrl)	−9.26	−3.36	2.98	−1.1	−8.53

MAPK3, mitogen-activated protein kinase 3; ctrl, control.

3.2. Cross-Validation Study Using Schrödinger Maestro Docking Tool

Cross-validation analysis was performed for kaempferol 3-rutinoside-4′-glucoside, orientin, kaempferol 3-rutinoside-7-sophoroside, rutin, isoquercitrin, vicenin-2, amentoflavone, quercetin-3-rhamnoside, nicotiflorin, sophoraflavanone G, and purvalanol. According to the results achieved from the Schrödinger Maestro docking tool, four metabolites, including kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and vicenin-2, demonstrated docking scores −10 kcal/mol. These components assigned the most potent MAPK3 inhibitors based on the AutoDock 4.0 and Schrödinger Maestro docking tool version 10.2. Therefore, MD was executed to evaluate the strength of their docked poses in 100 ns computer simulation. Table 3 presents the docking scores and MM-GBSA results calculated by the Schrödinger Maestro docking tool. The prime MM-GBSA analysis calculates the $\Delta G_{binding}$ value between ligands and macromolecules.

Table 3

Schrödinger Maestro docking scores and relative binding free energies (kcal/mol) of top-ranked flavonoids (based on the AutoDock software) against MAPK3 active site (PDB ID: 4QTB; chain A).

Ligand name	Docking score (kcal/mol)	MMGBSA result (kcal/mol)
Kaempferol 3-rutinoside-4′-glucoside	−11.20	−42.65
Kaempferol 3-rutinoside-7-sophoroside	−11.03	−74.64
Vicenin-2	−10.32	−47.35
Rutin	−10.22	−25.88
Isoquercitrin	−9.25	−59.06
Nicotiflorin	−8.90	−44.55
Quercetin-3-rhamnoside	−7.34	−53.38

Sophoraflavanone G	−7.23	−55.43
Orientin	−7.09	−58.66
Amentoflavone	−7.04	−51.23
Purvalanol	−6.49	−47.01

3.3. Stability of the Docked Poses

Regarding MD analysis, the docked poses between kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, vicenin-2, and MAPK3 active site were stable after ~45 ns computer simulations. Figure 3 demonstrates RMSF and RMSD for MAPK3 backbone atoms complexed with top-ranked flavonoids in this study and the standard drug. Figure 4 illustrates the superimposed structures of top-ranked complexes before and after MD simulations.

[figure(s) omitted; refer to PDF]

BIOVIA Discovery Studio Visualizer 19.1.0.18287 unraveled hydrogen bonds and hydrophobic interactions between top-ranked flavonoids (based on the AutoDock tool and Schrodinger Maestro docking software), purvalanol, and the enzyme’s active site (Figure 5 and Table 4). The number of H bonds was increased after 100 ns computer simulations for kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and vicenin-2.

[figure(s) omitted; refer to PDF]

Table 4

Interaction modes between top-ranked flavonoids and residues within the MAPK3 active site.

PubChem ID	Ligand name	Hydrogen bond (distance Å)	Hydrophobic interaction (distance Å)
44258844	Kaempferol 3-rutinoside-4′-glucoside (before MD)	Glu50 (2.97); Ile48 (4.64); Lys131 (4.99); Asp184 (4.58); Ala52 (3.91); Ser170 (4.47)	NA
44258844	Kaempferol 3-rutinoside-4′-glucoside (after MD)	Arg84 (4.39); Asp166 (4.15, 4.86); Asn171 (3.85); Lys168 (4.49, 4.67); Ser170 (3.90, 3.11, 3.27, 4.05); Asp128 (4.21, 3.57); Ile48 (4.33); Ala52 (3.80, 4.16)	Tyr53 (7.06)
44258853	Kaempferol 3-rutinoside-7-sophoroside (before MD)	Ala52 (3.44); Asp184 (4.80); Asp166 (4.68); Arg84 (4.63); Gly49 (3.42)	NA
44258853	Kaempferol 3-rutinoside-7-sophoroside (after MD)	Asp166 (4.77); Asp184 (4.49, 4.05); Gly51 (3.80); Asp128 (4.72); Lys131 (4.82); Tyr47 (4.75); Glu50 (4.02, 4.62); Arg84 (4.85)	NA

5280805	Rutin (before MD)	Asp184 (4.51, 3.06, 3.07, 3.89, 4.67)	Tyr53 (6.38); Cys183 (6.02); Lys71 (6.10); Val56 (5.22, 5.58); Leu173 (5.11); Ile48 (6.14); Tyr130 (4.19); Lys131 (4.00)
5280805	Rutin (after MD)	Thr127 (4.37); Lys131 (4.65); Asp128 (3.96); Asp184 (3.88, 3.75); Ser170 (4.42); Asn171 (3.40); Gly51 (4.34); Glu50 (4.79); Lys71 (3.49); Gln122 (4.30, 4.25)	Ile48 (5.95); Val56 (5.87)
442664	Vicenin-2 (before MD)	Asp128 (3.69, 4.23); Lys131 (4.93); Tyr53 (4.82); Asp184 (2.82); Asn171 (3.78, 3.11)	Val56 (5.10, 4.67); Leu173 (5.43); Ala69 (6.33); Cys183 (7.41)
442664	Vicenin-2 (after MD)	Glu50 (3.85); Lys131 (4.69); Asp128 (3.23); Thr127 (4.04); Asp123 (4.66); Gln122 (3.36); Cys183 (3.94); Lys71 (4.90); Ser170 (4.67, 3.55)	Ala69 (6.31); Leu124 (5.70); Leu173 (5.67, 5.61)
24866313	Purvalanol (before MD)	Glu50 (3.98, 4.07); Asp128 (3.55)	Val56 (6.09, 4.60); Ile48 (5.76, 5.13, 5.56); Leu173 (5.51, 5.41); Ala69 (5.12); Met125 (4.50); Cys183 (5.54)
24866313	Purvalanol (after MD)	Asp128 (4.02); Gln122 (4.69)	Val56 (5.40); Ile48 (5.23, 5.43, 5.63); Tyr53 (5.06); Cys183 (5.13)

MAPK3, mitogen-activated protein kinase 3; ctrl, control.

3.4. ADMET Assessment

SwissADME provides valuable information related to the pharmacokinetic features of compounds. The following ADME was predicted for 46 flavonoids studies in the present study: gastrointestinal (GI) and blood–brain barrier (BBB) permeability, P-gp (P-glycoprotein) substrate, cytochrome P-450 inhibition, and skin permeation coefficient (kp). Rutin and vicenin-2 revealed more appropriate ADME among top-ranked flavonoids. Besides, none of the compounds demonstrated considerable toxicity. Table 5 lists the results of ADME and the toxicity of the compounds.

Table 5

Predicted ADMET of 46flavonoids in this study.

Ligand name	GI abs	BBB permeant	P-gpsubstrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log kp	LD50 (mg/kg)
Kaempferol 3-rutinoside-4′-glucoside	Low	No	Yes	No	No	No	No	No	−12.85	5000
Orientin	Low	No	No	No	No	No	No	No	−9.14	1213

Kaempferol 3-rutinoside-7-sophoroside	Low	No	Yes	No	No	No	No	No	−14.98	5000
Rutin	Low	No	No	No	No	No	No	No	−11.66	5000
Isoquercitrin	Low	No	No	No	No	No	No	No	−9.6	1190
Vicenin-2	Low	No	No	No	No	No	No	No	−11.53	536
Amentoflavone	Low	No	No	No	No	No	No	No	−6.01	3919
Nicotiflorin	Low	No	No	No	No	No	No	No	−10.93	5000
Quercetin-3-rhamnoside	Low	No	Yes	No	No	No	No	No	−9.15	5000
Sophoraflavanone G	High	No	No	No	No	Yes	No	Yes	−4.79	2000
Vitexin	Low	No	No	No	No	No	No	No	−8.79	1190
Quercetin	High	No	No	Yes	No	No	Yes	Yes	−7.05	159
Kaempferol 7-O-glucoside	Low	No	No	No	No	No	No	No	−8.52	5000
Astragalin	Low	No	No	No	No	No	No	No	−8.52	5000
Astragarin	Low	No	No	No	No	No	No	No	−8.52	5000
Myricetin	Low	No	No	Yes	No	No	No	Yes	−7.4	159

Taxifolin	High	No	No	No	No	No	No	No	−7.48	159
Apigenin-7-glucoside	Low	No	Yes	No	No	No	No	No	−7.65	5000
Quercetin	High	No	No	Yes	No	No	Yes	Yes	−7.05	159
Afzelin	Low	No	No	No	No	No	No	No	−8.07	5000
Cynaroside	Low	No	Yes	No	No	No	No	No	−8	5000
3-O-methylquercetin	High	No	No	Yes	No	No	Yes	Yes	−6.31	5000
Epicatechin	High	No	Yes	No	No	No	No	No	−7.82	10000
Dihydroquercetin	High	No	No	No	No	No	No	No	−7.48	2000
Herbacetin	High	No	No	Yes	No	No	Yes	Yes	−6.6	3919
Luteolin	High	No	No	Yes	No	No	Yes	Yes	−6.25	3919
Isorhamnetin	High	No	No	Yes	No	No	Yes	Yes	−6.9	5000
Fisetin	High	No	No	Yes	No	No	Yes	Yes	−6.65	159
Catechine	High	No	Yes	No	No	No	No	No	−7.82	10000
Isoliquiritigenin	High	Yes	No	Yes	No	Yes	No	Yes	−5.61	1048
Licochalcone A	High	Yes	No	Yes	Yes	Yes	No	Yes	−4.89	1000

Xanthogalenol	High	No	No	Yes	No	Yes	No	Yes	−4.86	1000
Morin	High	No	No	Yes	No	No	Yes	Yes	−7.05	3919
Hemileiocarpin	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	−5.59	500
Epiafzelechin	High	No	Yes	No	No	No	No	No	−7.46	2500
Diosmetin	High	No	No	Yes	No	Yes	Yes	Yes	−5.93	3919
Apigenin	High	No	No	Yes	No	No	Yes	Yes	−5.8	2500
Glabridin	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	−5.52	500
Fustin	High	No	No	No	No	No	No	No	−7.44	2000
Kaempferol	High	No	No	Yes	No	No	Yes	Yes	−6.7	3919
Chrysin	High	Yes	No	Yes	No	No	Yes	Yes	−5.35	2500
Hesperetin	High	No	Yes	Yes	No	No	No	Yes	−6.3	2000
Ponciretin	High	Yes	Yes	Yes	Yes	No	No	Yes	−6.02	2000
Xanthohumol	High	No	No	Yes	No	Yes	No	Yes	−4.86	3800
Formononetin	High	Yes	No	Yes	No	No	Yes	Yes	−5.95	2500
Flavone	High	Yes	No	Yes	Yes	No	No	No	−5.13	2500

Purvalanol (standard drug)	High	No	No	No	No	Yes	No	Yes	−6.24	465

GI, gastrointestinal; abs, absorption; BBB, blood–brain barrier; P-gp, p-glycoprotein; CYP, cytochrome p-450; Kp, skin permeation coefficient; LD50, lethal dose 50%.

4. Discussion

MAPK3 is a serine/threonine kinase involved in the phosphorylation and translocation of several cytosolic proteins into the nucleus, leading to the dysregulation of several vital pathways and biological processes associated with apoptosis and cell proliferation [37]. Elevated expression and/or activity of MAPK3 is linked to the onset, development, drug resistance, and metastasis of various carcinomas such as ovarian cancer [38], glioma [39], lung cancer [40], and breast cancer [41]. Therefore, the present study executed a computational drug discovery approach to identify potential MAPK3 inhibitors from natural flavonoids, which have widely exhibited anticanter effects [42]. According to the AutoDock results, kaempferol 3-rutinoside-4′-glucoside demonstrated the highest binding affinity to the MAPK3 active site ($\Delta G_{binding}$ = −16.56 kcal/mol; *K*i = 731.68 fM) followed by orientin, kaempferol 3-rutinoside-7-sophoroside, rutin, isoquercitrin, vicenin-2, amentoflavone, nicotiflorin, quercetin-3-rhamnoside, and sophoraflavanone G. The cross-validation study also confirmed the high affinity of binding between kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, vicenin-2, and MAPK3 active site. Therefore, these metabolites were considered the most potent MAPK3 inhibitors from the studied flavonoids.

Kaempferol 3-rutinoside-4′-glucoside is isolated from the fruits of Lyciumruthenicum Murr [43] and the leaves of Agave sisalana Perrine ex Engelm [44]. This compound demonstrated six H-bonds with Ile48, Glu50, Ala52, Lys131, Ser170, and Asp184 within the MAPK3 catalytic site before MD simulation. This flavonoid also exhibited 15 hydrogen and one hydrophobic interaction with Ala52, Tyr53, Ile48, Arg84, Asp128, Asp166, Lys168, Ser170, and Asn171, after 100 ns MD simulation. In addition, salient binding affinities were estimated between kaempferol 3-rutinoside-7-sophoroside, kaempferol 7-O-glucoside, and MAPK3 active site with the $\Delta G_{binding}$ value of −15.49 and −11.87 kcal/mol, respectively.

Kaempferol 3-rutinoside-7-sophoroside formed five hydrogen bonds with the Gly49, Ala52, Arg84, Asp166, and Asp184 located in the MAPK3 active site before MD simulation. This compound formed ten H bonds with the Tyr47, Glu50, Gly51, Arg84, Asp128, Lys131, Asp166, and Asp184, after 100 ns MD simulations. Besides, kaempferol showed a lower binding affinity to the MAPK3 catalytic site compared to that of its glycosylated forms with a $\Delta G_{binding}$ value of −8.5 kcal/mol, indicating a positive correlation between binding sugar moieties to the rings A, B, and C and enhancing binding affinity between kaempferol and MAPK3 active site. According to a previous report [14], kaempferol exhibited a considerable binding affinity to the matrix metalloproteinase 8 (MMP8) catalytic domain with a $\Delta G_{binding}$ value of −10.88 kcal/mol. Due to the critical role of MMP8 [45] and MAPK3 in cancer development and metastasis, kaempferol and its glycosylated forms could be considered drug candidates for cancer therapy.

Kaempferol is a yellow flavonoid [46] mainly found in apples, tomatoes, grapes, pine, green tea, and angelica [47]. Antioxidant and anti-inflammatory properties of kaempferol [46] may lead to therapeutic effects in various cancers by regulating cell cycle, apoptosis, metastasis, and angiogenesis [48]. Fouzder et al. [49] demonstrated that kaempferol diminished Nrf2 at mRNA and protein levels in nonsmall cell lung cancer cells, leading to the downregulation of Nrf2 downstream genes, including GST, AKR1C1, HO1, and NQO1, resulting in cancer cells sensitive to apoptosis. Pan et al. [50] executed an experimental study to examine the effects of kaempferol on rheumatoid arthritis (RA) *in vitro* and *in vivo*. The authors examined the cell migration and invasion using scratch assays and the Boyden chamber approaches, respectively. The cytoskeletal reorganization of RA fibroblast-like synoviocytes was tested using immunofluorescence staining. Real-time PCR and western blotting assays were used to examine the MMP expression levels. Pan et al. [50] reported that kaempferol diminished cell migration and invasion by downregulating the MAPK pathway, reduced the MMP expression, and inhibited the actin reorganization in RA FLSs. Huang et al. [51] demonstrated that kaempferol significantly inhibited the expression of several genes mediating inflammatory response, including interleukin (IL)-1*β*, cyclooxygenase-2, and nitric oxide synthase. Furthermore, kaempferol

diminished collagen II degradations by inhibiting MMP1, MMP3, and MMP13 expression. In addition, kaempferol downregulated the p38 MAPK pathway. Taken together, Huang et al. [51] introduced kaempferol as a compound with therapeutic effects in osteoarthritis.

Orientin was this study's second leading potential MAPK3 inhibitor with the $\Delta G_{binding}$ and *K*i values of −15.98 kcal/mol and 1.92 pM, respectively. This flavonoid exhibited seven hydrogen and two hydrophobic interactions with the Gly49, Tyr53, Glu50, Val56, Asp128, Ser170, and Asn171 inside the MAPK3 catalytic site. Khamverdi et al. [52] also reported a considerable binding affinity between orientin and another protein kinase named glycogen synthase kinase 3 beta (GSK3B) with the criteria of $\Delta G_{binding}$ and *K*i values of −9.43 kcal/mol and 123.19 nM, respectively. Moreover, orientin has demonstrated a high affinity of binding to the MMP8 ($\Delta G_{binding} = -10.56$ kcal/mol) [14] and MMP13 ($\Delta G_{binding} = -10.5$ kcal/mol) [53] catalytic sites. The role of MMP13 in cancer progression [54] and metastasis [55] is evident. Therefore, orientin could be assigned as a potential drug candidate for cancer treatment with inhibitory effects against proteins involved in cancer onset, development, and metastasis.

Orientin is predominantly found in dayflower, millet, passion fruit, and pigeon pea leaves [56]. Several pharmacological features have been reported for orientin, such as antioxidant, anti-inflammatory, antimicrobial, and radio-protective effects [57]. Kim et al. [58] demonstrated that orientin inhibited the invasive behavior of breast cancer cells via downregulating MMP9 and interleukin (IL-8) expression. Furthermore, Tian et al. [59] reported that orientin reduced cell proliferation and enhanced the apoptosis process in T24 human transitional cell bladder carcinoma cells in vitro by inhibiting nuclear factor-kappaB (NF-κB).

Wu et al. [60] demonstrated that mulberry ethanol extract (MBE) and rutin (the most abundant phenolic component in MBE) significantly reduced the expression of several genes mediating the MAPK pathway, including ERK, JNK, p38, and caspase-3, leading to reduced oxidative stress in gastric mucosal epithelial cells. This was done using the quantitative PCR method. Rutin demonstrated five hydrogen and nine hydrophobic interactions with the Ile48, Tyr53, Lys71, Tyr130, Lys131, Val56, Leu173, Cys183, and Asp184 before MD simulation. Besides, rutin exhibited 12 hydrogen and two hydrophobic interactions with the Ile48, Glu50, Gly51, Val56, Lys71, Glu122, Thr127, Asp128, Lys131, Ser170, Asn171, and Asp184 within the MAPK3 active site after 100 ns MD simulations.

Chen et al. [61] reported that vicenin-3 considerably reduced the expression levels of nitric oxide, prostaglandin E2, MMP1, MMP3, MMP13, and A disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS). Moreover, vicenin-3 inhibited the p38 MAPK signaling pathway; the results were similar to that of SB203580 (a well-known p38 MAPK inhibitor). The present study evaluated the binding affinity between vicenin-2 and MAPK3 active site. Vicenin-2 showed seven hydrogen and five hydrophobic interactions with the Tyr53, Val56, Ala69, Ala128, Lys131, Asn171, Leu173, Cys183, and Asp184 before MD analysis. Furthermore, this compound illustrated ten hydrogen and four hydrophobic interactions with the Glu50, Lys71, Gln122, Asp123, Thr127, Asp128, Lys131, Ser170, and Cys183 after 100 ns computer simulations.

By analyzing the binding affinities between top-ranked MAPK3 inhibitors achieved from the AutoDock tool in this study and comparing the results with their corresponding structures, the following notes are suggested:

(1) By comparing the results of kaempferol with its glycosylated forms, it might be suggested that binding a sugar moiety (or sugar moieties) to the basic structure of flavonoids elevates the binding affinity of the compound to MAPK3

(2) By analyzing the $\Delta G_{binding}$ values between kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, vicenin-2, and MAPK3 catalytic site, it could be hypothesized that binding a disaccharide to the ring C or two monosaccharides to the ring A considerably elevates the binding affinity of the compound to the MAPK3

5. Conclusion

In conclusion, the binding affinity of 46 flavonoids to the MAPK3 catalytic domain was estimated using the AutoDock tool. Thirty-nine flavonoids exhibited a higher binding affinity to the MAPK3 active site than the standard drug. A cross-validation study was executed on top-ranked flavonoids based on the AutoDock software. Kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and vicenin-2 exhibited excellent binding

affinity using AutoDock 4.0 and Schrödinger Maestro docking tool. Also, these flavonoids' docked poses seemed stable after ~45 ns MD simulations. The present study suggests kaempferol 3-rutinoside-4′-glucoside, kaempferol 3-rutinoside-7-sophoroside, rutin, and vicenin-2 as potent inhibitors of MAPK3. These findings may be helpful in the treatment of various cancers. However, *in vitro*, *in vivo*, and clinical trial studies are needed.

Ethical Approval

The present study was approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (ethics no.IR.UMSHA.REC.1401.259).

Authors' Contributions

AT, ZB, and LS designed the study. Docking operations with the AutoDock tool were conducted by PK. ZA executed the cross-validation analysis. MD simulations were performed by AT. The results were analyzed and discussed by AT, ZB, and LS. AT wrote the manuscript. ZB and LS edited the manuscript. All authors read and approved the final version of the manuscript.

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Glossary

Abbreviations

MAPK3:Mitogen-activated protein kinase 3

ERK1:Extracellular signal-regulated kinase 1

Ki:Inhibition constant

RMSF:Root mean square fluctuation

RMSD:Root mean square deviation

MD:Molecular dynamics

μM:Micromolar

nM:Nanomolar

pM:Picomolar

ns:Nanosecond.

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DETAILS

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The Determination of Polymyxin B in Critically Ill Patients by the HPLC-MS/MS Method

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ABSTRACT (ENGLISH)

Polymyxin B (PB) is a dose-dependent drug used to treat multidrug-resistantgram-negative bacteria, for which a suitable method is needed to determine clinical samples. A simple, economical, and efficient high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) method was developed and validated for polymyxin B1 (PB1), polymyxin B1-Ile (PB1-I), polymyxin B2 (PB2), and polymyxin B3 (PB3) in human plasma. Chromatographic column was Waters BEH C18 column (2.1 × 50 mm, 1.7 μm). Phase A was water with 0.2% formic acid (FA), and phase B was acetonitrile containing 0.2% FA. The elution method is gradient elutio. The total analysis time was 5 min. The pretreatment method involved protein precipitation using acetonitrile containing 0.2% trifluoroacetic acid and 0.1% FA as the precipitant. The recovery rate was 92–99%. The total quantity of PB1 and PB1-I was measured in the linear range of 100–8000 ng/mL. Simultaneously, the total amounts of PB2 and PB3 were measured in the linear range of 11.9–948.5 ng/mL. This validated method was successfully applied to the pharmacokinetics of PB in critically ill patients.

FULL TEXT

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1. Introduction

Polymyxin B (PB) is a lipopeptide antibiotic extracted from the fermentation products of *Bacillus polymyxa* [1]. In clinical practice, considered the “last line of defense,” it is primarily used for infections caused by gram-negative bacteria with multidrug resistance, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* [2, 3]. PB is a dose-dependent drug discovered in the 1950s and has significant adverse effects on the kidney, which has impact on critically ill patients [3–6]. Therefore, it is important to maintain the drug concentration within the therapeutic window. Therefore, therapeutic drug monitoring (TDM) of PB is necessary for clinical practice. The major components of PB are polymyxinB1 (PB1), B1-Ile (PB1-I), B2 (PB2), and B3 (PB3) [7–9]. Most studies have measured the concentrations of PB1 and PB2 [10–12]. However, in several pharmacopeias, the total content of PB1, PB1-I, PB2, and PB3 in the dried product should not be less than 80% [13–15]. Therefore, it was necessary to measure the sum of the four components. In previous studies, thin-layer chromatography, high-performance liquid chromatography (HPLC), and high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) have been used for the determination of PB1 and PB2 in plasma [11, 16–19]. HPLC-MS/MS has been the most commonly used method in the past decade owing to its convenience and accuracy in quantitative analysis [20]. Several studies have used HPLC-MS/MS to detect the PB content in human plasma, but only one has measured four components [10, 12, 19, 21, 22]. Hee KH [19] described a method for the determination of PB1, PB1-I, PB2, and PB3, but the run time was long (7.5 min) and the recovery rate using the protein precipitation method (PPE) was low (53–76%).

Pretreatment is necessary to quantify plasma samples using HPLC-MS/MS. Among the pretreatment methods, PB, PPE, and solid-phase extraction (SPE) are commonly used. Several studies measured PB1, PB1-I, and PB2 using SPE, and the recovery rate was ~60% [12, 21, 22]. However, SPE is expensive and complex. Covelli et al. [10] extracted PB1 and PB2 from human plasma with acetonitrile (ACN) containing 2.0% trifluoroacetic acid (TFA) and yielded higher recovery (93.5–101.2%) with a run time as long as 20 min. However, the pretreatment process was complex, including the processes of extraction, nitrogen blowing, and re-dissolution, and the run time was long. A simple, economical, and efficient HPLC-MS/MS method is required to measure the concentrations of PB1, PB1-I, PB2, and PB3.

In this study, PPE was used to pretreat human plasma. ACN with 0.2% TFA and 0.1% formic acid (FA) as the

extraction solution, the content of PB1, PB1-I, PB2, and PB3 was determined, which was more suitable for TDM.

2. Materials and Methods

2.1. Chemicals and Reagents

Polymyxin B sulfate (purity: 88.5%, lot 1116937) and polymyxin E (PE) sulfate (purity: 93.7%, lot 137369) were purchased from *Dr.Ehrenstorfer GmbH* (Augsburg, Germany). For the standard PB sulfate, the sulfate content was 14.9%. The mixture of PB1, PB1-I, PB2, and PB3 was 81.7, 7.7, 9.3, and 1.3%, respectively. LC-MS-grade ACN was purchased from *Thermo Fisher Scientific* (Massachusetts, USA). Formic acid was obtained from *Sigma-Aldrich* (St. Louis, MO, USA). HPLC-grade trifluoroacetic acid was obtained from *MACKLIN* (Shanghai, China).

2.2. Equipment

The LC-MS/MS system consisted of an Agilent 1260 HPLC system equipped with a cooled autosampler (4°C) and an Agilent 6460 electrospray ionization-triple quadrupole mass spectrometer (Agilent Technologies, USA). The chromatographic column was BEH C18 (Waters, 2.1 × 50 mm, 1.7 μm) (Waters Corporation, USA). Ultrapure water was prepared using the Milli-Q Direct 8 (E. Merck, Darmstadt, Germany) water purification system. A Heraeus Multifuge X1R (Thermo Fisher Scientific, USA) high-speed refrigerated centrifuge was used for the centrifugation. SHIMADZU-AUW120D (Shimadzu Corporation, Japan) was used for the weighting.

2.3. HPLC-MS/MS Conditions

2.3.1. HPLC Conditions

A Waters BEH C18 column (2.1 × 50 mm, 1.7 μm) was used for the separation of PB. The column temperature was maintained at 35°C. Mobile phase A was water with 0.2% FA (v/v). Mobile phase B was ACN containing 0.2% FA (v/v). Gradient elution was adopted in the experiment: 0.0–0.5 min, 5% B; 0.5–1.0 min, 5–60% B; 1.0–1.5 min, 60% B; 1.5–2.0 min, 60–90% B; 2.0–2.5 min, 90% B; and 2.5–3.2 min, 90–5% B. The posttime was 1.8 min to reach equilibrium. The flow rate was 0.4 mL/min. The injection volume was 5 μL.

2.3.2. Mass Spectrometric Conditions

The MS was based on the multiple reaction monitoring mode (MRM) and positive ionization mode. The precursor ion was $[M+2H]^{2+}$ for PB and PE2 (internal standard, IS). Quantification ion pairs were PB1/PB1-I: 602.7/101.1; PB2/PB3: 595.7/101.1; and PE2: 578.7/101.1. Dwell was 50 for all components. For PB1/PB1-I, the fragmentor was 196, and collision energy (CE) was 37 volts (V); for PB2/PB3, they were 181 and 37 V; for PE2, they were 130 and 35 V.

2.4. Preparation of Stock and Working Solution

Standard and IS stock solutions were prepared in Milli-Q water containing 1% FA (v/v) at 1 mg/mL (with all substances) and subpacked in EP tubes. Working solutions were diluted from the stock solution. The concentrations of PB1 in the PB1-I's working solution were 160, 120, 100, 48, 24, 12, 6, and 2 μg/mL. The quality control (QC) working solutions were 140, 80, 20, and 4 μg/mL. The IS solution, polymyxin E2, was diluted in 1% FA water to 7.5 μg/mL. All solutions were stored at −80°C.

2.5. Preparation of Calibration Samples and QC Samples

Blank plasma (190 μL) and 10 μL working solution were mixed to prepare calibration curves of 8000, 6000, 5000, 2400, 1200, 600, 300, and 100 ng/ml for PB1/PB1-I, and 948.5, 711.4, 592.8, 284.6, 142.3, 71.1, 35.6, and 11.9 ng/mL for PB2/PB3. Similar to the QC standards, for PB1/PB1-I, the low QC (QCL), medium QC 1 (QCM1), medium QC 2 (QCM2), and high QC (QCH) were 200, 1000, 4000, and 7000 ng/ml, respectively. For PB2/PB3, QC standards were 23.7, 118.6, 474.3, and 830.0 ng/ml. First, a 100 μL calibration sample or QC sample was removed; 10 μL IS solution, 200 μL ACN with 0.1% FA (v/v), and 0.2% TFA (v/v/v) were added successively. The samples were vortexed for 2 min to make precipate the protein fully, then centrifugated at 18800 g at 4 ℃ for 15 min. About 200 μL of the supernatant was sucked and transferred into 96-well plates for analysis.

2.6. Extraction Using PPE

Volume of 200 μL ACN with 0.2% TFA and 0.1% FA was added to a 100 μL plasma sample containing 10 μL IS solution, vortexed for 2 min, and centrifugated at 18800 g at 4°C for 15 min. The supernatant was then transferred to a 96-well plate for analysis.

2.7. Method Validation

Method validation was based on the Chinese Pharmacopoeia (2020) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

2.7.1. Selectivity and Matrix Effect

Six blank matrices obtained from different individual sources, one high-fat matrix obtained from volunteers with abnormally high triglyceride levels, and the hemolytic matrix prepared by adding hemolytic whole blood (2%, v/v) to the blank matrix were selected to comprehensively evaluate the selectivity and matrix effect of this method. For selectivity, the lower limit of quantification (LLOQ) and blank-level samples were used for evaluation. Eight blank samples were prepared in the above matrices by adding 15 μL solvent and 200 μL precipitant to 95 μL of different matrices. LLOQ samples were prepared with working solutions of LLOQ, IS solution, and matrices. The responses attributable to interfering components in the retention time should not be more than 20% of PB and not more than 5% of IS in the LLOQ sample of each matrix. The matrix effect was evaluated by analyzing at least three replicates at low and high QC concentrations. The QCL and QCH of plasma samples were prepared using their working solutions with the eight matrices in QC sample preparation. QCL and QCH of the solvent samples were prepared using 5 μL working solution, 95 μL solvent, 10 μL IS solution, and 200 μL precipitant. The ratio of analyte and IS in the matrices and solvent, respectively, is calculated as the matrix factor (MF) of the analyte and IS. The IS-normalized MF was then calculated to evaluate the matrix effect. The coefficient of variation (CV) of the IS-normalized MF of the eight matrices should not be greater than 15%.(1)MF of analyte=peak area of analyte in the matrixpeak area of analyte in solvent,MF of IS=peak area of IS in the matrixpeak area of IS in solvent,IS-normalized MF=MF of the analyteMF of IS.

2.7.2. Extraction Recovery

The extraction recovery was calculated by the peak area ratios of samples recovered from plasma, extracted blank plasma, and IS spiked with the same concentrations of PB. Samples at four concentrations were analyzed in triplicates.(2)extraction recovery=peak area of the analyte added before extraction/peak area of ISpeak area of the analyte added to the extracted supernatant/peak area of IS*100%.

2.7.3. Calibration Curve and Carry-Over

Eight calibration-level samples, a blank sample, and a zero sample were used to establish the curve. The preparation was the same as that described in Section 2.6. The upper limit of quantification (ULOQ) and LLOQ are shown in the curve. The LLOQ is the lowest point of the curve, whereas the ULOQ is the highest. Fitting the curve by the least square regression analysis, the accuracy of the LLOQ should be within ±20%; other calibration samples should be within ±15%. Carry-over was assessed using blank samples after calibration at the ULOQ. Compared with the LLOQ, the area of the analyte should not be greater than 20 or 5% for the IS.

2.7.4. Accuracy and Precision

Accuracy and precision were determined using five QCs: LLOQ, QCL, QCM1, QCM2, and QCH. Five replicates at each concentration level were paralleled for each run. The between-run accuracy and precision were evaluated in three runs over two days. The accuracy of the LLOQ should be within ±20% and that of other QCs should be within ±15% overall. The precision of the LLOQ should not exceed 20%; the other QCs should not exceed 15% overall.

2.7.5. Dilution Integrity

During the investigation of dilution integrity, the dilution QC concentration was 10000 ng/mL, which was diluted with blank plasma. The two dilution factors investigated were two and four, respectively. There were five replicates for each dilution factor. The mean accuracy of the dilution QC samples should be within ±15%; the precision should not exceed 15%.

2.7.6. Stability

QC samples (QCL, QCM1, QCM2, and QCH) were used to investigate the stability of the stock solutions, working solutions, and samples. Each QC was set with three parallels. The stability of plasma samples stored at room temperature (25°C) for 4 h, at 4°C in a refrigerator for 24 h, at −20°C for 37 days, and at −80°C for 97 days was investigated. The freeze-thaw stability of −20°C and −80°C freeze-thaw cycles at three times each was investigated. The stability of the stock and working solutions stored at −80°C for 97 days was investigated. The accuracy of the

quality control sample should be within ±15% of the nominal concentration; the precision should be within 15%.

2.8. Application to the TDM and Population Pharmacokinetics (PPK) Study

Plasma samples were collected from critically ill patients who had received at least a third dose of polymyxin B in EDTA-K2 blood collection tubes. Blood samples were collected at seven time points: 10 min before drug administration, 5 min, 1, 2, 4, and 8 h after infusion, and 10 min before the next drug administration. Blood samples were centrifuged at 996 g at 4°C for 10 min, separated into EP tubes, and frozen at −80°C. This study was approved by the institutional review board of Guangdong Provincial People's Hospital. A total of 350 clinical plasma samples were collected. The PB content was measured using the method developed in this study. The results were used to study the population pharmacokinetics (PPK). Alternatively, this method could be used for clinical drug concentration monitoring. Plasma samples of the peak and valley concentrations of PB were collected and measured using HPLC-MS/MS after validation.

3. Results and Discussion

3.1. LC-MS/MS Method Development

PB standards include PB1, PB1-I, PB2, and PB3. In this method, PB1 and PB1-I were not separated by chromatography, similar to PB2 and PB3. PB's structural formula is shown in Figure 1. PB1 and PB1-I are isomers, and the quantitative ion pair was the same at 602.7/101.1. Based on previous reports [19, 20], it was considered that they could be determined as one peak. Similarly, PB2 and PB3 were isomers. The quantitative ion pair was 595.7/101.1, suggesting that they could be determined as one peak too. The PB content is the sum of the four components. The precursor ion of PB1/PB1-I was $[M+2H]^{2+}$ 602.7. The iron product is shown in Figure 2. Iron 101.0 was selected, which was common to PB1 and PB1-I, and had the highest response. The precursor ion of PB2/PB3 was 595.7, a form of $[M+2H]^{2+}$. The product scan of iron is shown in Figure 2; 101.0 was selected for the same reasons. The product scan of IS is shown in Figure 2; the iron pair was 578.7/101.1. The typical peak shapes of the blank, plasma standard of LLOQ and ULOQ, IS, and clinical samples are shown in Figure 3. Overall, it is feasible to measure the PB components of the same mass together as the peak shape of each concentration is good, meeting the quantitative requirements.

[figure(s) omitted; refer to PDF]

For the measurement of clinical samples, PPE is simple, more convenient, and more economical than SPE. Therefore, PPE was adopted in this method. Comparing the extraction recovery and the peak area of ACN, ACNe containing 0.1% FA, ACN with 0.2% TFA and 0.1% FA, and ACN with 0.1–2% TFA, we found that the addition of TFA can increase the response of polymyxin, which was consistent with what has been reported [19]. When the extraction solution was pure ACN, the response and extraction recovery were low, which did not meet the quantitative requirements. Considering that TFA has ionic inhibition on MS and corrosivity, it should only be added to the extraction, and the concentration should not be high. After comparison and screening, ACN containing 0.2% TFA and 0.1% FA was selected as the extraction solution. The response and extraction recovery of PB met the analytical requirements. The extraction recovery rate was 92–99%; CV <5% (Table 1). The TFA concentration in the final analysis sample was 1.3%. TFA was only added to the sample and not to the mobile phase, which had no impact on the MS.

Table 1

Extraction recovery and matrix effect of PB.

Component	Extraction recovery	Matrix effect

Nominal concentration (ng/mL)	Recovery		QC level	Mean of normalized MF	CV (%)	Mean (%)
CV (%)	PB1/PB1-I	200	91.9	4.3	QCL	1.03
12.7	1000	97.0	1.1	4000	94.3	2.7
QCH	1.10	6.4	7000	92.1	1.9	·
PB2/PB3	23.7	98.4	3.6	QCL	1.03	8.9
118.6	99.1	0.7	474.3	97.6	0.9	QCH

During the experiment, the Waters HSS T3 column (2.1 × 100 mm, 3.5 μm) [20], Agilent poroshell 120 SB-C18 (2.1 × 50 mm, 2.7 μm), and Waters BEH C18 (2.1 × 50 mm, 1.7 μm) were evaluated. The results showed that the carry-over of Waters BEH C18 was the smallest. Therefore, this column was selected for analysis.

The entire time was 5 min, including an analysis time of 3.2 min and a posttime of 1.8 min. The retention time of PB1/PB1-I was 2.06 min; PB2/PB3 was 2.04 min. At 0–0.5 min, the aqueous phase was the main phase for keeping PB on the column and eluting substances with large polarity in the sample. In the first minute, they were almost salt. Therefore, no MS was conducted. Subsequently, the organic phase ratio was increased to elute analytes. A high organic phase (90% phase B) was used to elute impurities with low polarity. Then, returned to the initial proportion and postrun for 1.8 min to stabilize the pressure.

3.2. Method Validation

3.2.1. Linearity and Carry-Over

Each validation or sample measurement was performed simultaneously using a standard curve. Eight points were selected to construct the standard curve, and $1/X^2$ was the weight factor. The correlation coefficient R^2 was greater than 0.99. For PB1/PB1-I, the linearity was $y = 1.2477x - 0.0214$; for PB2/PB3, the linearity was $y = 2.0285x + 0.0050$ (Table 2). The linear range was determined by referring to the reported range of PB, the residual effect, and the actual sample concentration distribution. The concentrations of 35 samples from five patients were 100–5000 ng/mL. Therefore, the LLOQ was set at 100 ng/ml; the ULOQ was increased to 8000 ng/mL. Compared with previous methods [12, 19, 21, 22], the number of diluted clinical samples can be reduced.

Table 2

The calibration curve of PB.

	Batch	Slope	Intercept	R^2
PB1/1-I	1	1.3627	−0.0193	0.997
2	1.1022	−0.0184	0.996	3
1.2782	−0.0264	0.998	-	
Average		1.2477	−0.0214	0.997

–				
PB2/3	1	2.1410	0.0021	0.996
2	1.8879	0.0072	0.995	3
2.0566	0.0055	0.996	-	
Average		2.0285	0.0050	0.996

3.2.2. Precision and Accuracy

The intrabatch and interbatch accuracy and precision of the three batches were verified within two days. Five QC samples were selected (LLOQ, QCL, QCM1, QCM2, and QCH). The accuracy of the three intrabatch analyses was within ±15%; the CV was within 10% (Table 3). The interassay accuracy was within ±15%; the CV was within 10%, as shown in Table 3. This implies that the method is accurate and precise.

Table 3

Intrabatch and interbatch precision and accuracy for PB in plasma.

Component	Nominal conc. (ng/mL)	Intraday (n=5)			Interbatch (n=3)		
Found conc. (ng/mL) mean (SD)	Acc. (%)	CV (%)	Found conc. (ng/mL) mean (SD)	Acc. (%)	CV (%)	*PB1/PB1-I*	
LLOQ	100	100 (0.005)	99.9	5.4	94 (0.005)	94.0	5.0
QCL	200	188 (0.007)	94.2	3.5	190 (0.012)	94.8	6.3
QCM1	1000	934 (0.039)	93.4	4.1	953 (0.059)	95.3	6.2
QCM2	4000	3972 (0.082)	99.3	2.1	4128 (0.274)	103.2	6.6
QCH	7000	7036 (0.040)	100.5	0.6	7105 (0.328)	101.5	4.6
–							
PB2/PB3							
LLOQ	11.9	12.7 (0.001)	106.8	6.6	12.7 (0.001)	107.0	6.4
QCL	23.7	24.0 (0.002)	101.3	6.6	24.7 (0.002)	104.3	7.2

QCM1	118.6	113.8 (0.002)	96.0	2.1	114.6 (0.005)	96.6	4.1
QCM2	474.3	452.4 (0.016)	95.4	3.4	466.0 (0.022)	98.3	4.7
QCH	830.0	801.0 (0.006)	96.5	0.8	793.7 (0.033)	95.6	4.2

*accuracy = (found concentration)/(nominal concentration) *100%; *precision = (standard deviation)/(average of found concentration) *100%. *conc. is concentration; acc. is accuracy; SD is standard deviation.

3.2.3. Selectivity and Matrix Effect

The area of the analyte at the retention time in the blank sample was lower than 20% in the LLOQ. The IS area was lower than 5%, indicating high selectivity. As showed in Table 1, the matrix effect was investigated using QCH and QCL. For PB1/PB1-I, the IS-normalized MF of QCL was 1.03 and of QCH was 1.10. For PB2/PB3, it was 1.03 and 1.05. The CV did not exceed 15% of each level, which implies that there was no matrix effect among six different batches of the normal, hemolytic, and high-fat matrix.

3.2.4. Integrity of Dilution

The investigation of the two dilution factors, two and four, is presented in Table 4. The accuracy was within ±15%; the precision was within 5%. For PB1/PB1-I, the mean accuracies were 109.1 and 108.2%; the CV was 2.0 and 3.7%, respectively. For PB2/PB3, the mean accuracies were 100.6 and 101.2%; CV was 1.0 and 2.3%, respectively. Therefore, samples higher than 8000 ng/mL can be determined using dilution. The highest concentration that can be measured by this method is 32000 ng/mL.

Table 4

Integrity of dilution of PB.

Component	PB1				PB2			
Nominal conc. (ng/mL)	Mean of found conc. (ng/mL)	Mean of Acc. (%)	CV (%)	Nominal conc. (ng/mL)	Mean of found conc. (ng/mL)	Mean of Acc. (%)	CV (%)	Dilution factor 2
10000	10905	109.1	2.0	1185.7	1192.4	100.6	1.0	Dilution factor 4

3.2.5. Stability

Stability was investigated for short-term, freeze-thaw, and long-term stability. Table 5 shows that the sample was stable at room temperature for 4 h, at 4°C for 24 h, at −20°C, and at −80°C for three freeze-thaw cycles. The extracted supernatant was stable in an automatic sampler for 24 h. The samples were also stable when stored at −20°C for 37 days and −80°C for 97 days. The working solution was stable when stored at −20°C for 18 days. The working and stock solutions were stable at −80°C for 97 days. The stability of PB was based on the requirements of

the experiments, which met the experimental requirements.

Table 5

Stability of PBs.

QC level	Accuracy of PB1/PB1-I				Accuracy of PB2/PB3			
Low (%)	Medium 1 (%)	Medium 2 (%)	High (%)	Low (%)	Medium 1 (%)	Medium 2 (%)	High (%)	QC sample, room temp, 4 h
100.3	96.1	90.8	97.6	90.5	97.2	88.6	95.0	QC sample, 4°C, 24 h
104.8	107.8	111.2	106.9	107.6	108.4	109.0	103.4	Autosampler, 4°C, 24 h
106.8	104.6	101.0	98.9	110.4	105.3	112.0	105.9	Freeze–thaw, −20 °C, 3 times
88.8	85.7	87.4	88.6	96.8	91.0	92.3	92.3	Freeze–thaw, −80 °C, 3 times

106.5	90.9	97.1	101.4	105.4	101.1	96.2	103.3	QC sample, −20 °C, 37 days
113.9	104.3	105.0	113.1	103.6	96.8	99.3	104.6	QC sample, −80 °C, 94 days
102.5	105.0	105.4	107.3	109.2	106.2	104.7	105.2	Working solution, −20 °C, 18 days
110.2	105.0	105.6	110.6	95.8	101.3	99.7	101.5	Working solution, −80 °C, 94 days
91.6	96.3	103.3	99.3	98.5	95.2	101.0	96.9	Store solution, −80 °C, 94 days

3.3. Clinical Application

The established HPLC-MS/MS method was used to measure more than 300 samples collected clinically. A steady-state metabolic curve of polymyxin B was obtained, as shown in Figure 4. This also suggests that the method can be applied to TDM and PPK.

[figure(s) omitted; refer to PDF]

4. Conclusions

In this study, a precise, accurate, and convenient method for the determination of PB was developed and validated with a linearity range of 100–8000 ng/mL for PB1/PB1-I and 11.9–948.5 ng/mL for PB2/PB3 within 5 min. To our knowledge, this is the first study to measure PB1, PB1-I, PB2, and PB3 together, which is convenient. The method was successfully applied to a PPK study of 350 samples. Therefore, it was also suitable for TDM.

Consent

Written informed consent was obtained from each patient or their legal representatives.

Authors' Contributions

Yirong Wang, Jingchun Chen, and Jinpan Du contributed equally to this work. XPW and CBC equally contributed to the design of the research and interpretation of the data. YRW, JCC, and JPD contributed to the conception and design of the research, as well as interpretation of the data, and critically revised the manuscript. YRW, JCC, and JPD performed the research and collected data. YRW experimented and analyzed the data. All authors contributed to the acquisition and analysis of the data, drafted the manuscript, and agreed to be fully accountable for ensuring the integrity and accuracy of the work. All authors read and approved the final manuscript.

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Comparative Study on Chemical Constituents of Ginseng Flowers with Four Consecutive Cultivation Age

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ABSTRACT (ENGLISH)

The harvest period of cultivated ginseng is generally 4–6 years. Ginseng flowers (GFs), the nonmedicinal parts, are usually removed every autumn, in which components are generally believed to stay unchanged with the increasing cultivation age. Recently, few documents were reported on the variation of volatile organic compounds (VOCs) and other components about ginseng flowers. This study had an insight into the variation of the chemical constituents with the cultivation ages through the comparison of the volatile organic compounds, gross ginsenosides, crude polysaccharide, and gross proteins of ginseng flowers from 3-, 4-, 5-, and 6-yr-old (GF3, GF4, GF5, and GF6) which were conducted by headspace solid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry (HS-SPME-GC-QQQ/MS) and spectroscopic analysis combined with multivariate statistical analysis, including one-way ANOVA analysis and *T* test. The results indicated that the crude polysaccharide contents raised significantly depending on cultivation age except 6-yr-old, whereas the gross ginsenosides and the gross protein content were indistinctive. According to the peak intensity of determined VOCs, the contents of most differential compounds arranged in an order from high to low are GF3, GF4, GF5, and GF6, such as the compounds **2**–**15**, **17**–

19, **22**, and **25**–**26**, therefore, they can be inferred that they are important markers to identify the age of GFs. 461 common differential compounds were gained and 26 common volatile organic compounds were identified with RSI >800 and RI and RIx no more than 30, including alcohols (such as **11**, **12**, and **15**), sesquiterpenes (such as **2**, **3**, and **4**), esters (such as **1** and **26**), naphthalene and naphthol (such as **7** and **20**), which had potential effects on curing Alzheimer's disease, inflammatory diseases, and prostate cancer based on network pharmacology analysis. This paper firstly revealed the variation rules of constitutions of GFs, which may provide a reference for the harvest and making rational application.

FULL TEXT

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1. Introduction

Ginseng (*Panax ginseng* C. A. Mey.) is a kind of herb belonging to the Araliaceae family with the reputation of being "The King of Herbs" that has a long international history [1]. The root and rhizome, the stem, leaf, flower bud, and berry of the ginseng are rich in ginsenosides [2–4]. Recently, interest in the flower buds, as a component of ginseng, has grown because they have valuable medicinal potential with antifatigue and immunity-enhancing functions [5], although ginseng flowers (GFs) buds are not recorded in the Chinese Pharmacopoeia (2020 edition) [6]. It has been reported that ginsenosides and polysaccharide are the major bioactive constituents of GFs with gross ginsenoside contents approximately five times than those in the ginseng root [7–9].

The quality of traditional Chinese medicinal (TCM) materials is usually defined by their texture and color but their VOCs have rarely been considered. VOCs acting as sensory indicators or a marker play a key role in production or evaluating product quality because of the potent efficacy of VOCs [10]. They represent about 1% of plant secondary metabolites with around 1700 substances, mediate plant semi chemistry, and are involved in abiotic stress responses [11]. Extraction is a crucial procedure for analyzing VOCs with many methods being reported, such as hydro-distillation, microwave-assisted extractions, and carbon dioxide supercritical fluid extraction [12, 13]. However, these methods are time- and labor-consuming. Recently, headspace solid-phase microextraction (HS-SPME) has been increasingly used for analyzing VOCs, researchers can perform reliable analysis of VOCs of different kind of samples, especially aldehydes, ketones, alcohols, esters, phenolic compounds, and some key terpenic hydrocarbons, such as foodstuffs, plants, and their metabolites, because the technique is generally solvent-free, quite rapid, require minimum sample preparation, and minimize the matrix constituents [14–16].

Chemometrics is a discipline related to the application of mathematics, statistics, and computer science. The main missions of the chemometrics are manipulating and analyzing the chemical data, dealt with complex data sets to obtain the chemical information to the maximum extent [17-18]. At present, an increasing number of applications of chemometric pattern recognition in the analysis of food or agricultural products, such as wine aging studies, authenticity identification of TCM, and evaluation of the extraction method of artichoke [19–21].

The present study will use physicochemical methods to determine the content of gross ginsenosides, crude polysaccharide, and gross protein in GFs from 3-, 4-, 5-, and 6-yr-old ginseng plants (GF3, GF4, GF5, and GF6), which will be extracted and analyzed by the headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-QQQ/MS). Pattern recognition approaches, such as principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), will be used to discover potential chemical markers. The results will be useful for clearly discriminating between GFs of various ages, and thus improve the quality control of GFs both as a raw drug material and for their use in TCM formulations.

2. Materials and Methods

2.1. Sample Collection, Chemicals, and Reagents

GFs of various ages were collected in June 2019 from Fusong (Jilin) and authenticated by Professor Changbao Chen (Changchun University of Chinese Medicine). After steaming at 40°C for 48 h, then crushed through 20-mesh sieves.

Ginsenoside Re (purity >98%) was obtained from the Department of Organic Chemistry, Jilin University; bovine serum albumin (BSA) was purchased from the Chinese Academy of Metrology; the glucose standard was purchased from Beijing Putian Tongchuang Biotechnology Co., Ltd. (Beijing, China); n-alkanes (C_8-C_{30}) was purchased from Alfa Aesar (Tewksbury, MA, USA); methanol, anhydrous ethanol, concentrated sulfuric acid, and glacial acetic acid (all analytically pure) were purchased from Beijing Chemical Plant (Beijing, China); polydimethylsiloxane-divinylbenzene solid-phase microextraction head (PDMS-DVB 65 μm) and 12 mL headspace vials were purchased from Supelco (Bellefonte, PA, USA).

2.2. Contents of Gross Ginsenosides in GFs of Various Ages

The gross ginsenosides content was determined by using the reference method of the State Standard of the People's Republic of China (GB/T 19506-2009): GF powder (passed through a 60-mesh sieve) was accurately weighed (about 1.0 g) then packaged in a neutral filter paper. The powder was extracted with ether using a Soxhlet extractor for 1 h. The sample package was then dried to evaporate the ether solvent. Methanol was added to the extractor to soak overnight. The next day, the appropriate amount of methanol was added to repeat the extraction for 6 times. The methanol extracts were combined and recovered, steaming a small amount of methanol extracts in the water bath and then dissolving them in water. 30 mL water extract was extracted for 4 times with 30 mL of water-saturated n-butanol. The upper liquid was steamed, then dissolved in methanol and made up to 10 mL. Finally, the end product was used as the ginsenosides sample solutions for further analysis.

The total ginsenoside content was determined using the reference method of the product of geographical indication-Jilin Changbaishan ginseng (GB/T 19506-2009), Ginsenoside Re (National Institutes for Food and Drug Control, Beijing, China)was used as the standard to calculate the content of the gross ginsenoside. In order to prepare the ginsenoside Re standard solution, 10 mg ginsenoside Re was put into a 10 mL volumetric flask, dilute to scale with methanol as solvent and mixed. 10 μL, 20 μL, 30 μL, 40 μL, 60 μL, 80 μL, and 100 μL of the standard solution and 30 μL sample solution were transferred to 10 mL tubes and dried (60°C water bath). Then, 0.5 ml 8% vanillin-ethanol and 5 ml 72% concentrated sulfuric acid were added to the prepared tubes. After fully shaking and mixing, the solution was heated in a 60°C water bath for 10 min and then cooled down in an ice-water bath for 10 min immediately. The mixed reagent without ginsenoside was used as a reference. Finally, both were determined at 544 nm using an enzyme calibration (Infinite M200 PRO, Tecan, Switzerland). To reduce the error in determining the gross ginsenosides content, three parallel extracts were obtained from the same origin of GF raw materials. The changes in gross ginsenosides content in GFs of various ages were then compared. The following equation was used to calculate the gross ginsenoside content:

Equation (1) of Cui et al. [22] was used to calculate the gross ginsenosides content:(1)X%=m1*A2/A1m2*100%, where X is the gross ginsenosides content; m_1 represents the weight of ginsenoside Re, m_2 is the weight of GF powder, and A_1 and A_2 are the absorbances of the ginsenoside Re standard solution and sample solution, respectively.

2.3. Contents of Crude Polysaccharide in GFs of Various Ages

The crude polysaccharide content of the GFs was determined by a phenol-sulfuric acid method [23]. GF powder (2.00 g) was accurately weighed and added with 70 mL of water and extracted using a 100 mL volumetric flask for 30 min by ultrasonic extraction. The extract solution was placed in a 100°C water bath for extraction for 4 h and diluted to a 100 mL after cooled. The 5 mL solution was mixed with 15 mL, 5 mL, and 5 mL of ethanol solution and centrifuged at a speed of 10000 r/min for 10 min, respectively. The supernatant was discarded and the residue was dissolved in 100 mL volumetric flask with water. Take 2 mL solution in 25 mL graduated test tube with stopper and shake well after added 5% phenol solution 1.0 mL. Add 5 mL sulfuric acid solution quickly and also shake for 5 min. Placing it in boiling water bath for 20 min and then cooled to room temperature. The glucose standard solution 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mL was accurately removed and the standard curve was made by the previous method. The mixed reagent without glucose was used as a reference. Finally, both were determined at 486 nm using an enzyme calibration. Three parallel extracts were obtained from the same origin of GF raw materials.

2.4. Contents of Gross Protein in GFs of Various Ages

Different volumes (0, 10, 20, 30, 40, 50, and 60 μL) of the BSA standard solution (1 mg/mL) were transferred to an Eppendorf tube, then PBS buffer was added to make up to a volume of 150 μL. After taking out 15 μL and placing in another tube in turn, 285 μL of Coomassie brilliant blue solution was added, mixed well then left at room temperature for 5–10 min. The solution was then transferred to a 96-well plate to measure the absorbance at 595 nm by an enzyme calibration. The standard curve was drawn with protein concentration (mg/15 μL) as the abscissa and optical density as the ordinate. The reagent without BSA was used as a blank control. 15 μL of GF extracts were used to determine the gross protein content of GFs in the above method. The content values were gained by three parallel samples ($n = 3$).

2.5. Analysis of VOCs in GFs of Various Ages

2.5.1. Sample Pretreatment

The VOCs from the GFs were extracted by using HS-SPME. A sample of GFs about 100 mg was accurately weighed and transferred to a 12 mL headspace vial and then placed in a water bath at 70°C for 30 min. Thereafter, the VOCs were collected using a PDMS-DVB SPME fiber by exposing the fiber to the headspace of 2 cm above the sample for another 45 min under the same conditions. The fibers were activated before sampling according to the instructions. After this step, the SPME fiber was inserted directly into the injection port of the GC system for thermal desorption (30 s) in a splitless mode.

2.5.2. Preparation of Quality Control and Blank Samples

The quality control (QC) samples were obtained by mixing the same amount of all the analyzed samples. The QC samples were processed as described previously. To ensure the stability and reproducibility of the experiment, QC samples were injected once every 6 to 8 GF samples.

To eliminate interference between the experimental environment and different samples, the blank sample was injected before the GF samples of various ages, that is, the above method was used without any powder. Blank sample were injected before each QC sample.

2.5.3. Gas Chromatograph-Mass Spectrometer Conditions

The GC-QQQ/MS system comprised of a TRACE 1310 system and TSQ ENDURA mass spectrometer with electron impact (EI) ion source and Xcalibur data processing system and was purchased from Thermo Scientific (Waltham, MA, USA). The GC-QQQ/MS instrument was used to analyze the VOCs of the GFs with a DB-5MS silica capillary column (30 m × 0.25 mm, 0.25 μm). The temperature of the injector was set at 250°C. Helium (99.999% purity) was used as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1 : 30. The temperature sequence of the column was programmed as follows: an initial temperature of 50°C held for 2 min, increased at 10°C/min to 200°C then increased to 280°C at 5°C/min and held there for 10 min. The mass detector was equipped with EI, the ionization voltage was set at 70 eV in the 50–550 m/z scan range for the mass spectra collection, and the temperatures of the ion source and mass spectrum transmission line were 280°C.

2.5.4. Qualitative and Quantitative Analysis

The VOCs of the GFs were identified as described by Choi et al. [24]. GC-QQQ/MS was used to determine the total ion chromatogram (TIC) of the VOCs of the GFs. The chromatogram was processed by automatic mass spectral deconvolution and identification system (AMDIS) software (https://www.amdis.net/) to eliminate interference and increase the accuracy of matching results, overlapping peaks were separated to purify the mass spectra. Finally, the detection results were qualitatively analyzed using the NIST spectrometry library combined with the temperature-programmed retention indices (PTRI). The mixture of a homologous series of n-alkane standards (C_8–C_{30}) dissolved in n-hexane, employed for auxiliary qualitative analysis, was analyzed under the same conditions as the samples. The RI of the samples was calculated by a linear heating formula, equation (2), with the chemical structure with the closest similarity to the mass spectrum and RI value being selected as the best identification result.

$$RI = 100n + 100\frac{t_x - t_n}{t_{n+1} - t_n} \quad (2)$$

where n represents the number of carbon atoms, t_x represents the retention time of the analyzed compound, and t_n and t_{n+1} represent the retention times of the outflow peaks of the n-alkanes with carbon numbers of n and $n+1$, respectively. Otherwise, $t_n < t_x < t_{n+1}$.

The quantitative method adopted in this experiment is the peak area normalization method.

2.5.5. Statistical Analysis

The GC-MS data were collected by Xcalibur software (version 2.2). XCMS Online (https://xcmsonline.scripps.edu/) was used to preprocess the GC-QQQ/MS data, using peak matching, peak alignment, and peak area normalization, leading to the data table containing the sample name, retention time, mass-to-charge ratio, and peak intensity. The data table was then imported into SIMCA-p software (version 17.0, Umetrics AB, Umeå, Sweden) for processing and a discriminant model for distinguishing between GFs of various ages was established using PCA and OPLS-DA. The data for the different VOCs were presented as means ± standard errors. In combination with the *T* test differential compounds were screened out based on the variable importance in projection (VIP) > 1 and $p<0.05$. Based on the literature combined with the NIST 11 library, only differential compounds with a relative strength index (RSI) greater than 800 were recorded, at the same time, the experimental RIx, which do not differ from the reported RI values by more than 30, are considered an important indicator for identification. The figures were drawn using Origin 2021 (OriginLab Corp., Northampton, MA, USA), GraphPad Prism 7 (Graph Pad Software, San Diego, CA, USA), and Draw Venn diagram online (https://bioinformatics.psb.ugent.be/webtools/Venn/).

3. Results and Discussion

3.1. Gross Ginsenoside Content

The calibration curve and the gross ginsenosides content (external standard method) in GFs for each age were summarized in Tables 1 and 2, the method showed good linearity ($R^2 > 0.999$). Figure 1(a) shows that the gross ginsenoside content did not increase linearly. The GFs of a 4-yr-old exhibited the highest average content (16.68 ± 0.98%), and the 5-yr-old is the lowest (14.55 ± 0.20%), but the subsequent pairwise comparison models showed no significant differences $p>0.05$.

Table 1

The correlation coefficients between gross ginsenosides, crude polysaccharide, and gross protein contents of ginseng flowers (GFs).

Components	Calibration curves	R^2
Gross ginsenosides	$y=0.0019x-0.0059$	0.9991
Crude polysaccharide	$y=0.0040x+0.0008$	0.9988
Gross protein	$y=0.0058x+0.0857$	0.9978

Table 2

The contents of gross ginsenosides, crude polysaccharide, and gross protein in GFs of various ages ($n=3$).

Components	GF3 (%)	GF4 (%)	GF5 (%)	GF6 (%)
Gross ginsenosides	16.51 ± 0.97	16.68 ± 0.98	14.55 ± 0.20	15.59 ± 1.20
Crude polysaccharide	3.96 ± 0.25	4.46 ± 0.33	5.60 ± 0.25	3.78 ± 0.14
Gross protein	10.30 ± 0.48	10.03 ± 0.67	10.28 ± 0.67	11.40 ± 0.73

[figure(s) omitted; refer to PDF]

3.2. Crude Polysaccharide Content

The correlation coefficients between the content of crude polysaccharide in GFs are shown in Table 1. The specific values of the gross crude polysaccharide content (external standard method) in GFs for each age are summarized in

Table 2, the method showed good linearity ($R^2 > 0.99$). Figure 1(b) shows that the crude polysaccharide content did not increase linearly, but tended to increase as the age increased from 3- to 5-yr-old and decrease as the age of 6-yr-old. The GF5 sample exhibited the highest average content (5.60 ± 0.25%) and the GF6 sample showed the lowest value (3.78 ± 0.14%), the pairwise comparison model showed differences $p<0.05$. Compared with GFs from 3-yr-olds, the crude polysaccharide content increased significantly at the 4-yr-old $p<0.05$ and extremely significant at the 5-yr-old $p<0.01$. Compared with those from 4-yr-olds, the content of GFs from 5-yr-olds increased extremely significant $p<0.01$ but 6-yr-old GFs decreased significantly $p<0.05$. Compared with GFs from 5-yr-olds, the crude polysaccharide content of GFs from 6-yr-olds had decreased extremely significant $p<0.01$.

3.3. Gross Protein Content

The calibration curve and the gross protein content (external standard method) in GFs for each age are summarized in Tables 1 and 2, the method showed good linearity ($R^2 > 0.99$). Figure 1(c) shows that the gross protein content did not increase linearly. The GF6 samples exhibited the highest average content (11.40 ± 0.73%) and the GF4 samples showed the lowest value (10.03 ± 0.67%), but the subsequent pairwise comparison models showed no significant differences $p>0.05$.

3.4. VOCs

3.4.1. Multivariate Statistical Analysis of VOCs in GFs of Various Ages

Although the VOCs in the GFs of various ages can be measured by qualitative and quantitative analyses, these methods have little help in determining the characteristic VOCs in the samples. PCA is a multivariate statistical analysis method that can reduce the dimensionality of the data while information on the original data still retained by using several variables to select a smaller number of important variables by linearly transforming the data. Thus, PCA can reflect the trends in the data such as clusters and groupings with chemical similarities or differences [25–28], so this method can be used to determine the characteristic VOCs of GFs of various ages.

In the scatterplot of the PCA score (Figure S1), QC samples were clustered closely, indicating that the method was stable and reproducible. However, among the four groups of GFs of various ages, except that GF5-5 had poor clustering patterns in PCA models, the distribution effect of other samples was perfect.

3.4.2. Discovering and Identifying Chemical Markers of VOCs in GFs of Various Ages

Supervised OPLS-DA was performed based on the above PCA model to define the most significant contributors towards discrimination. OPLS-DA not only extends PCA regression, but also provides better discriminatory ability than PCA on the larger divergence of intraclass for samples [29]. In the present study, the GF3 and GF4 samples (GF3 vs. GF4), GF3 and GF5 samples (GF3 vs. GF5), and GF3 and GF6 samples (GF3 vs. GF6) were distinctly separated in the PCA and OPLS-DA score plot (Figures 2(a)–2(f)), suggesting that the various ages contributed to the distinctive VOCs of the GFs. For example, the parameters value of R^2X for the GF3 vs. GF4 of OPLS-DA model was 0.753, indicating that 75.3% of the variation in the dataset could be modeled by the selected components. The value of R^2Y was 1, indicating that the model fitted the data very well. The value of Q^2 was 0.795, indicating a good predictive ability. The other two groups of pairwise comparison models also exhibited perfect fitness and predictivity. Overall, establishing the OPLS-DA model was effective for discriminating between the GFs.

[figure(s) omitted; refer to PDF]

Combined with the *T* test, the common differential compounds in GF3, GF4, GF5, and GF6 were screened out by the threshold of VIP > 1 and $p<0.05$. 674 differential compounds were screened out between GF3 vs. GF4, 866 and 971 differential compounds were screened out between GF3 vs. GF5 and GF3 vs. GF6, respectively. Figure 3 shows that 461 compounds with common symbolic differences in GFs from 4 ages were screened out. The TIC of the blank and 26 common differential compounds identified by NIST 11 and the literature are shown in Figure 4. It is not difficult to see that the blank had little effect on the sample. The preliminary list of components is shown in Table 3. The values of fold change describe the extent of the change from the initial to the final value. For the calculation of the fold change value of GFs of two ages, it was obtained by using the peak area of a compound of GF of the older age to that of the younger one. For example, the value of fold change was 2.3692, indicating that the peak intensity had increased by 2.3692 times between GFs from 3-yr-old to 4-yr-old. Figure 5 summarizes the trend in the 26

identified common differential compounds of GFs and can also be visualized intuitively from the heatmap and dendrogram of HCA (Figure 6). For GFs of various ages, the colors of the heatmap and dendrogram varied dramatically in the GF3 and GF4 columns but varied more subtly in the GF5 and GF6 columns. This phenomenon indicated that depending on the age of the ginseng, the intensity of several peaks varied, thus demonstrating that different growth periods/ages change the metabolic composition of ginseng, the contents of GF3 and GF4 changed significantly, but over the next two years changed inapparently. Regarding specific compounds, it can be seen that the content of differential compound **1** was highest in GF4, and the contents of differential compounds **2**–**26** were highest in GF3. According to the peak intensity of determined VOCs, the contents of most differential compounds arranged in an order from high to low are GF3, GF4, GF5, and GF6, such as the compounds **2**–**15**, **17**–**19**, **22**, and **25**–**26**; therefore, they can be inferred that they are important markers to identify the age of GFs. But other differential compounds arranged in an order from high to low are GF3, GF4, GF6, and GF5, such as the differential compounds **16**, **20**–**21**, and **24**. It is notable that the lowest content of differential compound **23** was in GF4, followed by those from GF5, GF6, and GF3, showing a various trend from the other differential compounds.
[figure(s) omitted; refer to PDF]

Table 3

Identification of common differential compounds in GFs from 3-, 4-, 5-, and 6-yr-old.

Peaks	*t*/min	RI_x	RI	RSI	Identification	GF4 vs. 3		GF5 vs. 3		GF6 vs. 3		CAS
$\|log_2$ fold change$\|$	Trends	$\|log_2$ fold change$\|$	Trends	$\|log_2$ fold change$\|$	Trends	Esters						
1	11.04	1229	1225	845	3-Hexenyl-3-methylbutanoate	2.3692	Up	1.3944	Up	1.3045	Up	10032-11-8
26	20.08	1919	1919	821	Hexadecanoic acid, methyl ester	0.8165	Down	0.7553	Down	0.829	Down	112-39-0
–												
Terpenes												
2	13.34	1393	1393	927	Cyclohexane,1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1α,2β,4β)]-	0.6242	Down	0.2964	Down	0.2598	Down	515-13-9
3	13.73	1422	1423	897	Tricyclo [2.2.1.0 (2,6)] heptane,1,7-dimethyl-7-(4-methyl-3-pentenyl)-, (-)-	0.733	Down	0.3609	Down	0.2925	Down	512-61-8

4	14.08	1450	1450	924	(E)-β-Famesene	0.6548	Down	0.39	Down	0.3472	Down	18794-84-8
5	14.59	1489	1485	941	1,6-Cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E)]-	0.6304	Down	0.3595	Down	0.2948	Down	23986-74-5
6	14.69	1497	1497	896	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2α,4aα,8aβ)]-	0.676	Down	0.4982	Down	0.4338	Down	473-13-2
8	14.84	1509	1509	870	β-Bisabolene	0.8151	Down	0.5243	Down	0.4408	Down	495-61-4
9	14.93	1516	1513	836	(+)-Valencene	0.5445	Down	0.3795	Down	0.3467	Down	4630/7/3
10	15.01	1523	1531	822	trans-Z-α-bisabolene epoxide	0.7683	Down	0.5707	Down	0.4441	Down	-
14	16.07	1610	1612	845	Caryophyllene oxide	0.7711	Down	0.5562	Down	0.4656	Down	1139-30-6
16	16.58	1654	1646	801	Ledene oxide-(II)	0.7556	Down	0.4812	Down	0.522	Down	-
18	16.78	1671	1672	857	Aromadendrene oxide-(1)	0.7213	Down	0.576	Down	0.5022	Down	94020-95-8

19	16.88	1679	1678	839	Aromadendrene oxide-(2)	0.7956	Down	0.6549	Down	0.5982	Down	85710-39-0
–												
Alcohols												
11	15.47	1561	1559	826	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	0.7274	Down	0.5609	Down	0.4569	Down	7212-44-4
12	15.78	1586	1587	825	1H-cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1a*π*4*π*4a*π*7*π*7a*π*7b*π*]-	0.4204	Down	0.3405	Down	0.2994	Down	552-02-3
13	15.93	1598	1595	836	Ledol	0.7974	Down	0.5245	Down	0.4864	Down	577-27-5
15	16.2	1621	1619	933	(−)-Spathulenol	0.8015	Down	0.5889	Down	0.5577	Down	77171-55-2
17	16.74	1668	1668	882	*β*-santalol	0.705	Down	0.5284	Down	0.5149	Down	77-42-9
21	17.3	1714	1714	851	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	0.8203	Down	0.6263	Down	0.6352	Down	726134-57-2
22	17.52	1732	1732	820	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol	0.7628	Down	0.6464	Down	0.5929	Down	-
24	17.83	1757	1753	809	Lanceol, cis	0.7969	Down	0.5485	Down	0.6216	Down	10067-28-4

25	18.31	1795	1794	888	2-Hexadecanol	0.1308	Down	0.1057	Down	0.0787	Down	14852-31-4
–												
Naphthalene and naphthol												
7	14.77	1503	1502	871	Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	0.6686	Down	0.4835	Down	0.4011	Down	483-75-0
20	17.03	1692	1693	830	1-Naphthalenol,decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1α,4aβ,8aα)]-	0.7796	Down	0.5554	Down	0.5667	Down	473-04-1
–												
Others												
23	17.55	1734	1739	828	Murolan-3,9(11)-diene-10-peroxy	0.2798	Down	0.4756	Down	0.6198	Down	-

[figure(s) omitted; refer to PDF]

3.5. Network Analysis between the Active Variable Metabolites and Diseases

Recently, an increasingly interest of medical usage of GF which is leading to a rapid progress of research into their pharmacological effects on many related diseases and they are now considered to be a trustworthy plant with medicinal potential. With the increase of cultivation age, the main efficacy of GFs will change to a certain extent, which may be related to these 26 identified common differential compounds of VOCs. Accordingly, the 26 identified common differential compounds of VOCs are researched for the related diseases. The CAS, chemical name, or InChIKey of the 26 identified differential compounds were used to search in the TCMSP database. Figure 7 shows that 95 diseases were related to 16 differential compounds retrieved from the TCMSP database, which were uploaded to Cytoscape software (version 3.8.2) for network mapping. Among them, 16 identified common differential compounds of VOCs found to be related to diseases are mainly sesquiterpenes and alcohols, including **2**, **3**, **4**, **5**, **6**, **7**, **8**, **11**, **12**, **13**, **14**, **15**, **17**, **19**, **20**, and **26**. For these diseases, Alzheimer’s disease, inflammatory diseases, prostate cancer, breast cancer, anxiety disorders, insomnia, and lung cancer were the major diseases. By searching for common differential compounds and related diseases, we can predict the therapeutic effect of GFs of different ages on related diseases.

[figure(s) omitted; refer to PDF]

Polysaccharides and proteins, the basic substances concerned with plant growth and development, are involved in a series of physiological activities such as seed germination and development, root and leaf differentiation, fruit ripening, embryo formation, and senescence. The present study found that the crude polysaccharide content of GFs increased significantly at the age of 3 to 5, but decreased apparently at the age of 6, but there was no significant

difference in the content between 3- and 6-yr-old, the results showed that the accumulation of crude polysaccharide of GFs was not positively correlated with the age of GFs. The gross ginsenosides and gross protein content of GFs changed slightly with the increase of age, but the changes were unobvious, indicating that the growth period of GFs had little effect on the gross ginsenosides and gross protein content.

The accumulation of chemical components is directly related to the activity of secondary metabolism-related regulatory enzymes and the expression of related genes. With the continuous growth and development of plants, the related enzyme activities and the expression level of regulatory genes will also change, resulting in the production and accumulation of chemical components in the process of plant growth and development are affected, therefore, the content of chemical components in different growth periods is different [30, 31]. Previous studies had shown that the content of volatile oil in flowers is affected by the distribution density of oil cells and the accumulation degree of volatile oil in oil cells [32]. Therefore, the content of VOCs in GFs may be closely related to the growth and development of oil cells. It can be inferred that ginseng blossoms after the 3-yr-old and the accumulation of early substances is deep, which can provide more energy and nutrition for the growth and development of oil cells in the buds of GFs. Therefore, the content of most VOCs is the highest in the 3-yr-old.

Sesquiterpenes and alcohols are the main identified common differential compounds [33]. Sesquiterpenes are an abundant group belonging to the terpenoid family, with a C15 structure comprise of three isoprene units. At least 300 types of sesquiterpenes have been discovered yet and numerous sesquiterpenes are an essential oil constituent which usually are released from the flowers and leaves as chemical messenger in plant signaling, particularly in the defense mechanism against biotic and abiotic stresses, such as to increase the plant tolerance, to inhibit the pathogen growth, and to attract pollinators or dispel predatory and parasitic insects [34]. Previous studies have shown that sesquiterpenes have anti-inflammatory and antioxidant effects, and the anti-inflammatory effect might mediate by the NF-κB and MAPKs signaling pathways [35]. The results of the present study have shown that the age of the GF had an important influence on the chemical constituents and biological activities of GFs. For instance, if crude polysaccharide is the focus of research interest, it is recommended that GF5 should be harvested; if the target is on gross ginsenosides, gross proteins, or VOCs, it is advocated to harvest GF3.

4. Conclusions

GF is a potential medicinal part of ginseng and has been increasingly appreciated by consumers and growers worldwide. Elucidating the differences in the contents of GFs of various ages, the gross ginsenosides, crude polysaccharide, and gross protein were achieved using a physicochemical method. An accurate and feasible analytical method based on HS-SPME-GC-QQQ/MS combined with multivariate statistical analyses was then developed to allow a comprehensive comparison of the VOCs of GFs of various ages, such as alcohols, terpenes, esters, and so on, leading to establishing a discriminant model. To our knowledge, this is the first report that systematically compares the differences of the contents of 26 identified differential compounds in GFs of various ages. The method used in this investigation comprehensively analyzed the chemical composition of GFs, which can contribute to a reference for the collection and rational application of GFs of various ages, as well as can bring new methods and ideas for the identification and quality assessment of GFs of various ages and other Chinese medicinal material. However, this method still has some limitations, such as the nondestructive detection of ginseng age cannot be achieved. In the future, we hope to develop more techniques for nondestructive identification of ginseng age, which can provide theoretical guidance for future production practice and contribute to the development of ginseng industry.

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DETAILS

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Ascorbic Acid Content and Antioxidant Activities of White and Brown Teff [Eragrostic tef (Zucc.)Trotter] Grains and Injera

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ABSTRACT (ENGLISH)

Teff [*Eragrostis tef* (Zuccagni) Trotter] is a cereal grain originating in Ethiopia as a staple food for millions of people. Its grain is a gluten-free superfood and got acceptance as a medicinal ingredient. Therefore, it is worthwhile to determine the antioxidative activities and L-ascorbic acid contents of teff grain and its baked food (injera). This study aimed to determine the ascorbic acid contents and antioxidant activities in the aqueous extract of the white and brown teff grains and their injera samples using iodimetric titration and UV-Vis spectrophotometric methods, respectively. The ascorbic acid contents in the white and brown teff ranged from 67.9–112.6 mg/100 g and 69.2–117.2 mg/100 g, respectively, and those in injera of the selected teff samples ranged from 30.5–32.9 mg/100 g and 37.3–43.0 mg/100 g, respectively. The antioxidant activities ranged from 1.26–7.04 μmol AAE/g for the white teff grains, 1.44–6.29 μmol AAE/g for the brown teff grains, 1.81–2.47 μmol AAE/g for white teff injera, and 3.89–4.86 μmol AAE/g for the brown teff injera samples. Findings of the present study have revealed that white teff and brown teff grains and their injera were found to have a higher content of ascorbic acid than commonly consumed grains and vegetables. No significant difference ($\alpha = 0.05$) has been observed between the two varieties of teff grains with

respect to the ascorbic acid content and antioxidant activities. However, there was a statistically significant difference ($\alpha = 0.05$) in the ascorbic acid content and antioxidant activities between the teff grains and their injera samples. Therefore, this study indicated that teff grains and injera are rich in ascorbic acid content and antioxidant activities as compared to other cereal grains and are very crucial for human nutrition and health.

FULL TEXT

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1. Introduction

The need for food security and Ethiopia's diverse climatological and ecological conditions has driven most subsistence farmers to grow various crops [1]. Cereal crops are the staples and the most important source of nutrients for mankind [2]. Teff [*Eragrostis tef* (Zuccagni) Trotter] is a cereal grain originating in Ethiopia as a staple food to millions of people [3]. Only five cereals (rice, wheat, maize, sorghum, and millet) account for more than half of global bread consumption. Excessive use of these selected cereals has the potential to result in genetic losses and difficulty meeting future agricultural demands [4]. Teff offers significantly higher nutritional values than most other cereal grains [5]. It is commercially classified as white and brown cultivars [6]. Teff cultivars outperform all cereal grains in terms of nutritional values, as they contain high levels of carbohydrate, protein, fat, dietary fibers, starch, essential minerals, essential amino acids, vitamins [7–9], polyphenols, volatiles such as aldehydes, ketones, and alcohols, and fatty acids [3]. Teff's global demand is currently increasing due to its healthier nutritional quality, and when compared to other grains, it plays an important role in food security [5, 10]. Furthermore, teff grain is a gluten-free superfood that may benefit people with celiac disease and is gaining acceptance as a medicinal ingredient [3, 5, 9]. Its antioxidative activities, for example, can help prevent malaria and hemoglobin levels in the human body; it can also help prevent diabetes. Furthermore, teff bread has a longer shelf life and a slower aging rate than rice, wheat, maize, sorghum, and barley [9, 11]. Teff can also be used as a fat substitute in producing low-calorie foods due to its high viscosity and low gelling ability [4]. Due to this reason, teff is increasingly studied under the lens of local and international research to support its cultivation and commercialization [12].

Vitamins are essential micronutrients that can be fat-soluble (A, D, E, and K) or water-soluble (B and C) [13, 14]. They are beneficial for the prevention and treatment of various diseases, including heart disease, high cholesterol levels, eye disorders, and skin disorders [7, 11]. Furthermore, vitamins are essential for growth, metabolism, reproduction, and overall health. Dietary vitamin intake is critical except for vitamins D and B1, which the human body cannot synthesize [13].

Niacin, vitamin B6, thiamin, riboflavin, vitamin K (phylloquinone), vitamin A, α-tocopherol [7, 15], and vitamin C [4, 7] are all abundant in teff grains. Vitamin C (L-ascorbic acid) has antioxidant properties and anti-inflammatory and antiapoptotic properties. It can also improve the immune system in humans by lowering the body's susceptibility to viral infections. It is one of the most commonly used health supplements to boost immunity and alleviate symptoms caused by COVID-19 infections [16, 17].

Most of plants and animals produce ascorbic acid from D-glucose or D-galactose [18]. L-ascorbic acid, as an antioxidant, lowers the risk of arteriosclerosis, cardiovascular disease, infectious diseases, asthma, cataract, diabetes mellitus, and some types of cancer [19]. It helps relieve common cold symptoms and plays an important role in wound healing. It also prevents free radical oxidation, preventing cell damage, and is commonly used as a food additive [20, 21]. L-ascorbic acid is also necessary to prevent scurvy and maintain healthy skin, gums, and blood vessels. It aids in the formation of collagen, the absorption of inorganic iron, the reduction of plasma cholesterol levels, the inhibition of nitrosoamine formation [21, 22], and the metabolism of tyrosine, folic acid, and tryptophan [21].

L-ascorbic acid less than 300 mg in the body results in scurvy and other disease symptoms. The maximum ascorbic acid in the body is limited to about 2 g for normal health. With high doses of L-ascorbic acid (over 2 g), unabsorbed ascorbate is degraded in the intestine, causing diarrhea. Furthermore, excessive L-ascorbic acid consumption

resulted in renal problems, nausea, and gastric irritation [23]. Despite the importance of teff grains for human food and nutritional security, there needs to be more qualitative and quantitative information on vitamins in general, and ascorbic acid in particular in the literature.

The inclusion of antioxidant foods in daily diet is critical to deactivating free radicals [24]. Teff grains have shown better antioxidant potential than other cereals [9]. This makes injera (ready-to-eat food) to be healthy food. Among the two teff varieties, brown teff injera had shown superior antioxidant potentials compared to white teff injera [25]. Various analytical methods for determining ascorbic acid content in fruits and vegetables have been reported in the literature, including potentiometric and reductometric methods [18], high-performance liquid chromatography coupled with ultraviolet spectrophotometry (HPLC-UV) [20, 26], and volumetric and spectrophotometric methods [27, 28]. However, most of these methods are time-consuming, involve multiple chromatographic steps, and require highly skilled technicians, making them problematic. To the best of the researcher's knowledge, no analytical methods for determining L-ascorbic acid contents in cereal grains, specifically teff grain and injera samples, have been reported. As a result, this study aimed to (1) determine the L-ascorbic acid contents in the aqueous extract of the white and brown teff grains and their injera using redox titration with a standardized solution of iodine, (2) evaluate the antioxidant activities of the white and brown teff grains and their injera using the DPPH assay, and (3) correlate the L-ascorbic acid contents and antioxidant activities of the white and brown teff grains.

2. Materials and Methods

2.1. Apparatus and Instrument

The experiment was carried out using an electronic balance (model: PW254, China) with a precision of 0.0001 g, a grinder (high-speed multifunctional grinder, Shanghai, China), a centrifuge (model: 80-2, China), and a burette set up (10 mL). The absorbance of the prepared standards and sample extracts was measured using a double beam UV-VIS-NIR spectrometer (Lambda 950, Perkin Elmer, UK) with a 1 cm path length quartz cuvette.

2.2. Chemicals and Reagents

This study used chemicals such as KIO_3, KI, L-ascorbic acid (Riede-de Haen, Germany), starch indicators, and 96% H_2SO_4 (Carlo Erba, Italy). Throughout the experiment, distilled and deionized water was used.

2.3. Sample Collection and Pretreatment

The sample collection and pretreatment were described elsewhere [3, 9].

Injera was made to compare the ascorbic acid content of white and brown teff grains. The traditional fermentation of teff flour was used to prepare injera. Teff flour was combined with the previous batch's water and ersho (without yeast additives). For primary fermentation, the mixture was fermented for 42 hours. A portion of the batter was mixed and boiled after primary fermentation to produce absit (gelatinization process). The prepared absit mixes were added to the primary fermented batter and allowed to ferment for 4 hours.

Finally, the batter was made to injera using Mitad. The prepared injera was dried for four days at room temperature before being ground to mesh size with an electronic grinder, and it was prepared for extraction.

2.4. Extraction of Ascorbic Acid in the White and Brown Teff Grains and Injera Samples

Yisak et al. [9] described the adopted method to extract the ascorbic acid content of white and brown teff grains and injera samples. In brief, 0.25 g of ground white teff, brown teff, and injera samples was soaked for 10 min in 20 mL of the extraction solvent (deionized water). Handshaking extracted the ascorbic acid from the wetted samples for 20 minutes, and the mixture was centrifuged for 15 minute at 3000 rpm. Finally, the supernatant was filtered through Whatman filter paper and prepared for analysis.

2.5. Preparation of Reagents and Standards

The preparation of 3 M H_2SO_4, 0.5% starch indicators and preparation of iodine solution for the titration method were described elsewhere [29].

2.5.1. Preparation of the Ascorbic Acid Standard (100 mg/L) Solution for Standardization in the Titration Method

A 100 mg/L-ascorbic acid standard solution was made by dissolving 0.005 g ascorbic acid (L-ascorbic acid) in a 50 mL volumetric flask with distilled water and then filling the flask to the mark with the solvent.

2.5.2. Standardizing Iodine Solutions for the Titration Method

A 100 mL Erlenmeyer flask was filled with 20 mL of 100 mg/L-ascorbic acid standard solution, and 1 mL of 0.5% starch solution was added. This solution was titrated with a small volume of iodine solution (1.95 mL) until the endpoint was reached. The endpoint was discovered when the first sign of the purple-blue color appeared during the titration process. The initial and final volumes of the iodine solution were then measured. The average concentration of the iodine solution was calculated by repeating titration three times and averaging the three results.

2.6. Determination of Ascorbic Acid Content by Iodimetric Titration

Many researchers reported titration as a preferable method for ascorbic acid determination due to its simplicity, low cost, and speed. The ascorbic acid determination method was adopted from Belete et al. [29] and Satpathy et al. [30] with some modifications. The oxidation-reduction reaction was carried out based on iodimetric titration of the sample. To determine the amount of ascorbic acid, 20 mL of the sample extract was taken and 1 mL of 0.5% starch solution was added to each extract.

The solution was titrated against the prepared iodine solution while shaking continuously, and the endpoint for each sample was recorded. The titration was performed in triplicate, and the results were presented as a mean ± SD on a dry basis from triplicate measurements.

2.6.1. Determination of Antioxidant Activities of the White and Brown Teff Grains and Their Injera Samples

The antioxidant activity of the white and brown teff grains and their injera extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity method described by Yisak et al. [9] with some modifications. In a 200 mL volumetric flask, 0.02 g of DPPH was dissolved with a small amount of methanol. After DPPH was completely dissolved, the flask was filled to the mark with methanol to achieve a solution concentration of 253.60 μmol/L. 3 mL of methanol and 2 mL of DPPH solution were used as controls. Furthermore, an ascorbic acid stock solution (1135.6 μmol/L) was prepared by dissolving 0.02 g of ascorbic acid in 100 mL of the volumetric flask with methanol. A calibration curve was established by preparing different concentrations of the stock solution (136.3, 102.2, 68.14, 17.74, and 6.036 μmol/L). A volume of 1 mL of each standard ascorbic acid solution was transferred into five different 25 mL volumetric flasks, and each flask received 3 mL of methanol and 2 mL of DPPH solution before being incubated in the dark at room temperature for 60 min. Finally, the absorbance at 517 nm was measured. A 1 mL portion of the extract was mixed with 3 mL of methanol and 2 mL of DPPH solution for the samples. For 60 min, the mixture was kept in the dark at room temperature. Using a calibration curve, the results were expressed as micromoles of ascorbic acid equivalent/g on a dry basis (μmol AAE/g). Each sample was scanned three times.

2.7. Statistical Analysis

All analyses were performed in triplicate. On a dry matter basis, the results were reported as a mean ± SD. The differences in mean values between teff grain varieties and injera were determined using one-way ANOVA, followed by Tukey's honestly significant difference (HSD) multiple rank test (α = 0.05) [31]. Minitab 17 software was used for all statistical analyses.

3. Results and Discussion

3.1. Determination of Ascorbic Acid Content in Teff

Tables 1 and 2 show the determined ascorbic acid contents of white teff grains and brown teff grains and their injera samples in milligrams per 100 g of flour on a dry basis (mg/100 g dry basis). The ascorbic acid contents of the white and brown teff aqueous extracts were 67.9–112.6 mg/100 g and 69.2–117.2 mg/100 g, respectively, and those in injera of the selected teff samples ranged from 30.5–32.9 mg/100 g and 37.3–43.0 mg/100 g, respectively. As shown in Table 1, the brown teff grain sample from Were Ilu district (South Wollo zone) contained the highest quantity of ascorbic acid. In contrast, the white teff grain sample from Minjar Shenkora district (North Shewa zone) contained the least quantity of all the teff grains analyzed. The ascorbic acid content of teff grains is higher than that of the selected teff injera samples. Because ascorbic acid is susceptible to food-processing procedures and radiation due to its high solubility in water, cooking can affect its content. However, significant losses do not occur with the typical household cooking method [21]. Furthermore, Otemuyiwa et al. [32] described that a combination of leaching and chemical destruction causes vitamin loss during cooking.

Table 1

Ascorbic acid content (mean ± SD) of the white and brown teff [*Eragrostis tef* (Zuccagni) Trotter] grains determined by iodimetric titration.

Region	Administrative zone	District	Variety of teff	Sample ID	Concentration (mg/100 g)
Amhara	North Shewa	Minjar Shenkora	White teff	AW-1	67.9 ± 3.2
Brown teff	AB-19	88.0 ± 2.3	South Wollo	Dessie Zuria	White teff
AW-5	72.6 ± 2.8	Brown teff	AB-23	81.2 ± 5.5	Were Ilu
White teff	AW-6	112.6 ± 2.0	Brown teff	AB-24	117.2 ± 5.9
East Gojjam	Goncha Siso Enese	White teff	AW-8	75.9 ± 1.6	Brown teff
AB-26	75.0 ± 3.2	-			
Oromia	Arsi	Jeju	White teff	OW-11	74.0 ± 5.6
Brown teff	OB-29	77.5 ± 1.9	East Shewa	Ada’a	White teff
OW-13	81.0 ± 3.0	Brown teff	OB-31	106.0 ± 4.5	Bishoftu
White teff	OW-14	71.2 ± 2.9	Brown teff	OB-32	69.2 ± 2.30
–					
SNNPR	Hadiya	Soro	White teff	SW-17	76.9 ± 2.9
Brown teff	SB-35	78.1 ± 1.6	Gomibora	White teff	SW-18

Table 2
Comparison of the ascorbic acid content (mean ± SD) in the selected white and brown teff [*Eragrostis tef* (Zuccagni) Trotter)] grains and injera samples.

Sample ID	Variety of teff	Concentration (mg/100 g)	
Grain	Injera	AW-5	White teff
72.6 ± 2.8[b]	30.5 ± 1.8[d]	AB-23	Brown teff
81.2 ± 5.5[b]	37.3 ± 2.9[cd]	OW-13	White teff
81.0 ± 3.0[b]	32.9 ± 1.5[d]	OB-31	Brown teff

Note. Values (means ± SD, $n = 3$) within a row and column with different superscript letters are significantly different ($\alpha = 0.05$).

Gebremariam et al. [7] reported an ascorbic acid content in teff of 88 mg/100 g, which is within the range of the teff grains and higher than the content in the current study's injera samples. However, the authors did not provide any information about the methods used to determine ascorbic acid in the teff grain samples. In comparison to this study, the ascorbic acid content of raw rice (0.3–1.1 mg/100 g), cooked rice (0.04–0.56 mg/100 g) [32], and barley grains (0.35–0.38 mg/100 g is very low [33]. Furthermore, Satpathy et al. [30] reported ascorbic acid concentrations in some vegetables, including garlic (40.95 mg/100 g), onion (30.79 mg/100 g), potato (33.65 mg/100 g), tomato (16.47 mg/100 g), pea (50.84 mg/100 g), common bean (41.28 mg/100 g), pumpkin (36.30 mg/100 g), and cucumber (24.23 mg/100 g), which are in agreement with the content of ascorbic acid in the selected teff injera samples and lower than the teff grain samples of the present study. Therefore, it is evident from the present findings that one can get enough ascorbic acid in a given teff injera food.

One-way ANOVA ($\alpha = 0.05$) was used to test for the presence of significant differences in the mean concentration of ascorbic acid in white and brown teff grains (Table 1) and their injera samples (Table 2). The ANOVA test revealed no statistically significant differences ($\alpha = 0.05$) between the two teff grain varieties. Still, there was a significant difference ($\alpha = 0.05$) in the mean concentration of ascorbic acid between the teff grains and their injera samples.

3.2. Precision and Recovery of the Method

The precision (% RSD) and accuracy (% recovery) of the titration method for determining ascorbic acid content were assessed. As a result, the method's repeatability was evaluated by calculating the relative standard deviation (RSD) of triplicate measurements, which yielded 1.8–7.6%, indicating that the method is precise. The reproducibility (recovery) test was carried out by adding a known amount of ascorbic acid to injera, white, and brown teff extracts. The spiked solution was analyzed three times to obtain the average recovery (R), which was calculated using the formula: $[(C_S - C)/C_A] \times 100 = \%R$, where C_S represents the concentration of the spiked sample extract, C represents the concentration of the unspiked sample extract, and C_A represents the concentration of the spiked ascorbic acid. The percent recovery results (98.6–104%) in Table 3 demonstrate that the method is reproducible for determining ascorbic acid in teff grain and injera extracts.

Table 3

Recovery results of ascorbic acid by the redox titration method.

Variety of sample	Amount of ascorbic acid in the sample before spiking (mg)	Amount of ascorbic acid spiked (mg)	Amount of ascorbic acid found after spiking (mg)	Recovery (%) ($n = 3$)
White teff grain	0.238	0.0952	0.332	98.7 ± 4.3
Brown teff grain	0.218	0.0872	0.304	98.6 ± 5.3
White teff injera	0.088	0.0348	0.124	104 ± 4.9
Brown teff injera	0.083	0.0332	0.117	102 ± 4.3

3.3. Determination of Antioxidant Activities of the White and Brown Teff Grains and Their Injera Samples

The antioxidant capacity of cereal grains can be determined using a variety of assays. The DPPH assay was used in this study to assess the antioxidant activities of the ascorbic acid in white and brown teff grains and their respective

injera samples (Table 4). Figure 1 shows the calibration curve ($y = -0.0051x + 0.94686$) of ascorbic acid standard DPPH scavenging activities determined by its regression coefficient ($R^2 = 0.9995$). Figure 2 depicts the UV–Vis overlay spectra of ascorbic acid standard DPPH scavenging activities.

Table 4

DPPH radical scavenging activities in the white and brown teff grains varieties.

Sample region	Variety of teff	Sample ID	Antioxidant activities (μmol AAE/g)
Amhara	White teff	AW-1	3.35 ± 0.36
AW-5	3.05 ± 0.14	AW-6	3.65 ± 0.39
AW-8	1.44 ± 0.21	Brown teff	AB-19
5.67 ± 0.21	AB-23	4.65 ± 0.11	AB-24
7.04 ± 0.36	AB-26	4.93 ± 0.14	-
Oromia	White teff	OW-11	1.49 ± 0.25
OW-13	2.99 ± 0.21	OW-14	1.26 ± 0.18
Brown teff	OB-29	4.31 ± 0.15	OB-31
6.06 ± 0.20	OB-32	4.31 ± 0.14	-
SNNPR	White teff	SW-17	2.90 ± 0.25
SW-18	3.95 ± 0.14	Brown teff	SB-35

ID = identification; AAE = ascorbic acid equivalent.

[figure(s) omitted; refer to PDF]

The antioxidant activity levels in white and brown teff grains were 1.26–7.04 μmol AAE/g and 1.44–6.29 μmol AAE/g, respectively. The lowest and highest values of DPPH radical scavenging activities in white teff grain samples were determined for Ada'a district (East Shewa zone) and Goncha Siso Enese district (East Gojjam zone), respectively, while those of brown teff grain samples were determined for Dessie Zuria district (South Wollo zone) and Gomibora district (Hadiya zone). Furthermore, the DPPH scavenging activities of white and brown teff injera were found to be 1.81–2.47 μmol AAE/g and 3.89–4.86 μmol AAE/g, respectively.

This study found that the antioxidant activities of white and brown teff grains are comparable to those of the literature reports of other cereal grains presented as μmol of trolox equivalent antioxidant capacities per g of the sample (μ mol TE/g sample) like rice (1.39–10 μmol TE/g) [34], wheat (7–10 μmol TE/g) [35], white teff (4.32–6.36 μmol TE/g), and brown teff (6.54–7.16 μmol TE/g) [36], but they are higher than the antioxidant activities of the white teff (2.10–2.14 μmol TE/g) and brown teff grains (2.14–4.30 μmol TE/g) reported by Kotaskova et al. [37]. One-way ANOVA indicated that there was no significant differences ($\alpha = 0.05$) in the mean antioxidant activity content between the white and brown teff grains. The low correlation ($r = 0.14937$) between ascorbic acid contents and in vitro antioxidant activities (Figure 3) in the teff grains may suggest that the major antioxidant compounds in the sample

might be bioactive compounds other than ascorbic acid.

[figure(s) omitted; refer to PDF]

The antioxidant activities of some selected white and brown teff injera prepared in 42 hours of fermentation were evaluated using the DPPH assay, as shown in Table 5. As a result, the antioxidant activity content of the injera samples was lower than that of the teff grains using the DPPH method. This is because bioactive compounds such as ascorbic acid, which acts as an antioxidant, are susceptible to cooking and radiation, resulting in a loss of DPPH scavenging activity in the injera samples. The one-way ANOVA test revealed significant differences ($\alpha = 0.05$) between the white and brown teff grains and their injera samples (Table 5).

Table 5

Comparison of the antioxidant activities in the aqueous extract of white and brown teff grains and their injera samples.

Sample ID	Antioxidant activity (μmol AAE/g)	
Grain	Injera	AW-5
3.05 ± 0.14^{d}	2.47 ± 0.28^{de}	AB-23
4.65 ± 0.11^{b}	3.89 ± 0.13^{c}	OW-13
2.99 ± 0.21^{d}	1.81 ± 0.33^{e}	OB-31

ID = identification; AAE = ascorbic acid equivalent. Values (means ± SD, $n = 3$) within a row and column with different superscript letters are significantly different ($\alpha = 0.05$).

4. Conclusion

The teff's ascorbic acid content is important for human health and is used as a quality indicator parameter. As a result, the study reported ascorbic acid contents and antioxidant activities in aqueous extracts of white teff grains and brown teff grains and their injera samples. The mean ascorbic acid content and antioxidant activities determined in the white and brown teff grains have shown no significant differences ($\alpha = 0.05$). However, the mean concentration of ascorbic acid and antioxidant activities of the selected teff grains and their injera samples differed significantly ($\alpha = 0.05$). According to this study, teff grains and their corresponding injera have high ascorbic acid content and antioxidant activity. Furthermore, the ascorbic acid content of teff grain and injera was higher than that of commonly consumed grains and vegetables. Were Ilu district of the South Wollo zone had the highest ascorbic acid content in white and brown teff grains. To the best of our knowledge, this is the first study to determine the ascorbic acid content of teff grains and injera. As a result, this finding can serve as a foundation for future research on using teff grains in functional foods.

5. Recommendations

It is fact that teff grain is typically used in fermented foods. The endogenous and microbial enzymes derived from teff grain flour initiate injera dough fermentation which could be an interesting area of future research. Besides, establishing standards for the quality and quantity of starter culture, fermentation conditions, and other ingredients can let researchers investigate biological activities and prospective applications.

Authors' Contributions

BSC, MR, and EEY were responsible for conceptualization. HY and AB were responsible for experimental data collection and analysis. HY was responsible for writing of the original draft. BSC were responsible for writing, reviewing, and editing the manuscript. BSC, MR, and EEY were responsible for supervision. All authors have read and agreed to the published version of the manuscript.

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DETAILS

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Evaluation of Antibacterial and Anticancer Characteristics of Silver Nanoparticles Synthesized from Plant Extracts of Wrightia tinctoria and Acacia chundra

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ABSTRACT (ENGLISH)

The study showed the ability to synthesize environmentally friendly silver nanoparticles (AgNPs) using extracts from *Wrightia tinctoria* seeds and *Acacia chundra* stems. Surface plasmon resonance peaks in the UV-Vis absorption spectra of both plant extracts verified AgNP synthesis. The structural and morphological properties of the AgNPs were investigated using analytical techniques such as XRD, FTIR, TEM, and EDAX. The AgNPs have an FCC crystalline structure, according to XRD study, and their sizes range from 20 to 40 nm, according to TEM images. Based on the results, these plant extracts have been identified as suitable bioresources for AgNP production. The study also showed that both AgNPs had significant levels of antibacterial activity when tested on four different microbial strains using the agar-well diffusion method. The bacteria tested included two Gram-positive strains (*Staphylococcus aureus* and *Micrococcus luteus*) and two Gram-negative strains (*Proteus vulgaris* and *Escherichia coli*). Furthermore, the AgNPs were found to have a significant anticancer effect on MCF-7 cell lines, suggesting that they may be useful in therapeutic applications. Overall, this research highlights the potential of the plant extracts considered as a source for synthesizing eco-friendly AgNPs with potential applications in medicine and other fields.

FULL TEXT

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1. Introduction

Nanotechnology enables the production of materials with a wide range of morphologies, dimensions, and chemical compositions, all of which fall within the range of 1–100 nanometers. Nanoparticles have a wide range of uses in electronics, biology, and medicine due to their optical, antibacterial, therapeutic, and catalytic capabilities [1, 2]. The higher the ratio of surface area to volume of the nanoparticles is, the more efficient they are in biological systems. Traditional methods of chemical and physical synthesis of nanomaterials require more time and involve the use of more hazardous reducing or stabilizing chemicals [1–3]. Plants contain bioactive components such as phenols, flavonoids, saponins, glycosides, tannins, alkaloids, polysaccharides, proteins, terpenoids, amines, ketones, and aldehydes that act as reducing, stabilizing, and capping agents in the conversion of metal ions to metal nanoparticles. Hence, the use of plant resources will eliminate the need for toxic solvents and chemicals [1–8]. The aforementioned issues can be addressed, and biocompatibility with nanoparticles can be obtained, by using plant extracts as reducers and stabilizers in the synthesis of ecologically friendly metallic nanoparticles [9, 10]. Pharmaceuticals, coatings, biological labeling, and packaging are just few of the industries where nanoparticles might be useful [5–9]. Silver nanoparticles (AgNPs) have attracted a lot of attention from researchers. In spite of the fact that higher concentrations of silver are toxic, a number of studies [8–10] demonstrate that lower concentrations of $AgNO_3$ have superior chemical stability and catalytic activity, biocompatibility, and intrinsic therapeutic potential. Researchers from every corner of the globe are interested in silver nanoparticles because of the remarkable antibacterial qualities that they possess. Tribal plants are revered in India as the ecosphere's equivalent of a botanical garden because they are used to treat a wide range of illnesses and physical distress and are also used as medicines. The bulk of the country's rural population practices traditional Indian medicine, such as Ayurveda [11]. The employment of nanoparticles in conjunction with well-known and extensively distributed traditional plants has thrown fresh light on the study of natural science. There have been a lot of studies conducted on how to make silver nanoparticles with potential anticancer and antibacterial action. Some examples include *Arctium lappa* fruit extract [3], *Solanum melongena* leaves [3], *Taraxacum mongolicum* leaves [3], red currants berries [4], alcoholic flower extracts [5], *Pisum sativum* L extracts [6], *Mangifera indica* seed extracts [7], berry extracts [9], *Cucumis prophetarum* leaf extracts [10], and *Alpina katsumadai* seed extract.

The *Wrightia tinctoria* (Wt) tree is an *Apocynaceae* family member that is found in several places in India [12, 13]. It is a deciduous tree of modest to medium size. The seeds and the bark of this plant are both employed in the practice of traditional Indian medicine as a means of curing gastrointestinal conditions including diarrhea and dysentery. This plant's bark has been demonstrated to be useful in treating a range of diseases, including abdominal pain, skin infections, and wounds; it can even reverse the effects of snake venom [14]. Furthermore, the seeds of this plant have been shown to have aphrodisiac properties [12–15]. *Wrightia tinctoria* is mentioned in several traditional medicinal systems used in South Asia as a useful cure for the treatment of heart palpitations and excessive blood pressure. Previous studies on the *Wrightia tinctoria* plant discovered important chemical components such as alkaloids, triterpenoids, steroids, and flavonoids [14]. *Acacia catechu* (Ac), commonly known as Katha or Karangali, is a well-known medicinal shrub in India [14–17]. Catechins, flavanol glycosides, flavonal dimers, caffeine, and rhamnetin are some of the plant's helpful components [18]. The antibacterial and wound-healing abilities of this plant have been studied. It is used alone or in combination with cinnamon or opium to treat chronic diarrhea. *Acacia catechu* water decoctions are commonly drunk as health beverages, particularly in the Indian state of Kerala and neighboring states in India's southern region [18, 19]. The water decoction is supposed to enhance skin complexion, cleanse the blood of pollutants, and boost the immune system. The anti-inflammatory qualities of stem bark extract are one of its pharmacological capabilities. When tested against the MCF-7 cell line, the methanolic extract of *Acacia catechu* stem bark was found to be effective as both an antioxidant and an anticancer

agent [20]. This discovery was made possible because methanolic extraction is more stable than water extraction. Our goal in this study is to develop an easy green synthesis of AgNPs using *Wrightia tinctoria* seeds and *Acacia chundra* stems. The generated AgNPs were analyzed by UV-Vis spectroscopy, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), and energy dispersive X-ray spectroscopy (EDAX). All of the AgNPs' characteristics, including their crystalline nature, size, and surface features, were investigated. An investigation of the biological activities of biosynthesized AgNPs was conducted. We used the well diffusion experiment to test the antibacterial activity of the AgNPs against both Gram-positive (*Micrococcus luteus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Proteus vulgaris*). Furthermore, the anticancer potential of the produced AgNPs has been investigated. Figure 1 presents the schematic representation of the experimental process.

[figure(s) omitted; refer to PDF]

2. Materials and Methods

All the reagents in this study were of analytical grade, used without further purification. The *Wrightia tinctoria* (Wt) seeds were obtained in the SP. Kovil, Kancheepuram, and *Acacia catechu* (Ac) stem bark were collected from Chennai district.

2.1. Preparation of Extract Using Seeds of *Wrightia tinctoria* (Wt)

The seeds of the Wrightia tinctoria plant were thorough washings, first with water from the running faucet, then with distilled water twice, to remove any dirt or other organic particles that may have contaminated them. They are left to air-dry in the shade for a period of 20 days before being ground into a powder using a standard blender. The powder is kept in a container that is hermetically sealed and placed in the refrigerator. A 250 ml conical flask was filled with 10 g of powder and 200 ml of distilled water. The solution was brought to a temperature of 60°C and then boiled for two hrs. After being allowed to cool, the extract solution was passed through a Whatman 41 filter to remove any particles that were still present. In the end, the Wt extract solution was retained for use after being stored in a refrigerator at a temperature of 40°C.

2.2. Preparation of Extract Using Stem of *Acacia catechu* (Ac)

Following a comprehensive cleaning, the stems of *Acacia catechu* were allowed to dry at room temperature. The stem was cut into very small pieces, and then it was boiled for 20 min in a round-bottom flask containing 250 ml of distilled water. For the purpose of filtering the extract, Whatman No. 1 filter paper was utilized. The produced extract was placed in storage for use in further studies.

2.3. Synthesis of AgNPs

To convert the Ag^+ ions, 25 ml of the plant extract sample is combined with 100 ml of an aqueous solution that contains 1 nM of $AgNO_3$ [21]. After 24 hrs, the reaction mixture was settled to its final state. Silver nanoparticle production was inferred from the change in reaction mixture colour. To further separate the reaction mixture, it was centrifuged at 2,000 rpm for 10 min. After that, the AgNPs were separated from the sample by centrifuging it for 15 min at a speed of 16,000 rpm. The AgNPs were collected and dispersed in acetone before being stored.

2.4. AgNP Characterization

The identification of silver nanoparticles was confirmed, and their physio-chemical properties were studied through several characterization methods. The AgNP formation was confirmed by using a Shimadzu UV-1800 spectrophotometer. The spectral data were collected in the 200–800 nm range. The X-ray diffraction measurements were taken with a Bruker diffractometer outfitted with Cu radiation (=1.5406). For FTIR study, a Perkin Elmer spectrometer was used, and spectra were gathered in the 500–4000 cm^{-1} region. The TEM study was performed using the JEOL JEM-2100 microscope. The nanoparticle solution was dried on a copper matrix to prepare the samples for analysis.

2.5. Antibacterial Activity

In this research, four bacterial strains were chosen: two Gram-positive (*Micrococcus luteus* and *Staphylococcus aureus*) and two Gram-negative (*Proteus vulgaris* and *Escherichia coli*) to assess the antibacterial effectiveness of the synthesized AgNPs using the Agar-well diffusion method. These bacterium isolates were acquired from the

National Chemical Laboratory in Pune, India, and were kept at 4°C until further use. These microorganisms are clinically important because they can induce a variety of diseases. The AgNPs were synthesized in a 100% DMSO solution at four distinct concentrations (25, 50, 75, and 100 μg/ml). Each well received 100 μl of an enriched AgNP sample. To assess antibacterial effectiveness, the radius of the zone of inhibition around each well was determined. Three different experiments were performed, and the average of the data was used to determine AgNPs' antibacterial efficacy.

2.6. Anticancer Activity

Cancer is a hereditary illness that quickly disseminates throughout the body, causing irreparable damage to human tissue [18]. Breast cancer, which is the growth of malignant cells in the breast, is a significant cause of high female cancer mortality rates. In this work, the in vitro anticancer effects of SeWt-AgNPs and StAc-AgNPs were investigated on MCF-7 cell lines at varying dosages using the MTT test. The National Centre for Cell Science in Pune, India, provided the MCF-7 breast cancer cell line that was used in this study. Cells were planted at a density of 1×10^4/well in 96-well plates and allowed to grow for 24 hrs at 37°C in a humidified atmosphere. After that, they were given an AgNP treatment at concentrations of 1.5, 6.25, 12.5, 50, and 100 μg/ml and placed in an incubator for 24 hrs. The MTT test was used in accordance with the procedure to determine the level of viability shown by cells that had been treated with these compounds as well as those that had been left untreated (serving as a control). The expression was what was used to determine whether or not the cells were viable.(1)% viability=mean absorbance of treated group mean absorbance of control group × 100.

3. Results and Discussion

3.1. Characterization Studies of the Silver Nanoparticles

3.1.1. UV-Vis Analysis

The formation of AgNPs is typically indicated by a change in the colour of the reaction mixture, which changed from colourless to yellowish-brown after 24 hrs in this research, showing that the plant extracts used were successful at reducing silver nitrate. A UV-Vis spectrometer was used to examine the surface plasmon resonance (SPR) region during AgNP production. The amount of peaks in the SPR band, their occurrence, and their changes over time provide crucial information for describing the nanoparticles' properties. The UV-Vis spectra of SeWt-AgNPs and StAc-AgNPs revealed absorption peaks at 443 nm and 440 nm, respectively (as shown in Figures 2(a) and 2(b)). Furthermore, no typical peaks were detected, suggesting that the solution did not contain an overabundance of starting materials. These findings support earlier research by demonstrating the effective synthesis of AgNPs using plant extracts [21–24]. Rabaa Algotiml et al. [1] synthesized biogenic AgNPs from extracts of three distinct types of marine algae (green, brown, and red algae), and the absorption maxima were found to be at 424, 409, and 415 nm, respectively. Similarly, peaks at 420 nm were detected when the surface plasmon resonance (SPR) band of silver nanoparticles prepared using Calotropis procera latex was observed by a UV-Vis spectrophotometer [23]. Previous research and the current results show the possibility of using plant extracts to make eco-friendly AgNPs with a variety of absorption spectra that can be used in a variety of uses, including medicine and technology.

[figure(s) omitted; refer to PDF]

3.1.2. XRD Studies

The X-ray diffraction (XRD) is widely used to study the structural and crystalline properties of materials. The structural properties of SeWt-AgNPs and StAc-AgNPs were investigated using XRD analysis in this study. The XRD patterns found for both samples verified the existence of a crystalline structure and matched the elemental silver face-centered cubic (FCC) lattice structure. The appearance of four different peaks in the XRD spectra of SeWt-AgNPs (Figure 3(a)) at 27.78°, 32.19°, 46.2°, and 76.5° and StAc-AgNPs at 27.7°, 32.2°, 45.8°, and 76.5° (Figure 3(b)) showed the FCC crystal structure of the synthesized AgNPs. Some minor peaks in the XRD spectra were also detected, which could be ascribed to surface reactions of either biological or chemical origin. The findings of a number of other researchers were similar to the conclusion reached in the present investigation [3–8].

[figure(s) omitted; refer to PDF]

3.1.3. TEM Analysis

Transmission electron microscopy (TEM) was utilized for the purpose of determining the morphology, shape, and size of nanoparticles. Figures 4(a) and 4(b) show the TEM micrograph of SeWt-AgNPs and StAc-AgNPs, respectively. It was discovered that the majority of the created AgNPs were in the shape of spheres. The TEM pictures were processed with the image J program, which resulted in the creation of a histogram displaying the distribution of particle sizes. It has been determined that the diameters of SeWt-AgNPs and StAc-AgNPs are between 20 and 40 nm, with the former having a value that is considerably greater than the latter. SeWt-AgNPs had a median particle size of 39.75 nm (Figure 5(a)) whereas the StAc-AgNPs had a median particle size of 27.79 nm (Figure 5(b)). In their study using seeds from the *Mangifera indica* plant, Donga and Chanda [7] found spherical AgNPs of varied sizes from 9 to 61 nm. The extract of *Alpinia katsumadai* seed produced quasispherical AgNPs with an average particle size of 12 nm [12].

[figure(s) omitted; refer to PDF]

3.1.4. EDAX Spectra Analysis

Energy dispersive X-ray spectroscopy, often known as EDAX, is a method that may be utilized to ascertain the elemental components that are present in the specimen. It was determined that surface Plasmon resonance was the cause of the absorption peak that was observed in the EDAX spectra (Figures 6(a) and 6(b)), which was located at around 2.5 keV. This provides more evidence that silver ions can be broken down into elemental silver [25].

[figure(s) omitted; refer to PDF]

3.1.5. FTIR Spectral Analysis

The FTIR studies reveal the peaks in the produced SeWt-AgNPs at 1069 cm^{-1}, 1545 cm^{-1}, 1653 cm^{-1}, 2858 cm^{-1}, 2924 cm^{-1}, and 3408 cm^{-1}, respectively (Figure 7(a)). In the FTIR spectra of SeWt (Figure 7(b)), bands could be seen at 1066 cm^{-1}, 1537 cm^{-1}, 1646 cm^{-1}, 2855 cm^{-1}, 2923 cm^{-1}, and 3366 cm^{-1}. The peaks at 1066 cm^{-1} belong to the C–H bending vibration, the bands at 1537 cm^{-1} relate to the N–O stretching vibrations, 1646 cm^{-1} corresponds to the diketone stretching vibrations, and 3366 cm^{-1} refers to the stretching vibration of –OH groups. The shift in the peak values slightly, from 1066 cm^{-1} to 1069 cm^{-1} and from 1537 cm^{-1} to 1545 cm^{-1}, in case of SeWt-AgNPs corresponds to the phytochemicals that were involved in the reduction and stabilization of AgNPs. The FTIR spectra of AgNPs (Figure 7(d)) revealed the existence of bands at the following wavelengths: 1041 cm^{-1}, 1288 cm^{-1}, 1512 cm^{-1}, 2700 cm^{-1}, 2931 cm^{-1}, and 3394 cm^{-1}. The peaks at 1066 cm^{-1} correspond to C–H bending vibrations, the bands at 1288 cm^{-1} correspond to –OH stretching vibrations, 2700 cm^{-1} and 2923 cm^{-1} correspond to asymmetric –CH2–, symmetric –CH3, and –CH2 stretching, and 3394 cm^{-1} corresponds to the stretching vibration of bonded and nonbonded O–H groups, with minor shifts after the formation of the StAc-AgNPs (Figure 7(c)).

[figure(s) omitted; refer to PDF]

3.2. Antibacterial Activity

Against a diverse collection of microorganisms, the antibacterial efficacy of the AgNPs was evaluated at four distinct concentrations: 25, 50, 75, and 100 μg/ml (Figures 8 and 9). When compared to StAc-AgNPs, it has been demonstrated that SeWt-AgNPs possess antibacterial activity that is considerably less potent. At each concentration, the SeWt-AgNPs demonstrated their antibacterial activity. When Gram-negative bacteria were exposed to SeWt-AgNPs, they displayed an antibacterial activity that was remarkably similar to antibacterial activity of Gram-positive bacteria. When compared to their antibacterial activity against other bacteria, StAc-AgNPs have a level of effectiveness that is marginally higher against *E. coli* and *B. subtilis*. The antibacterial activity varies based on the concentration of the nanoparticles as well as the type of bacteria being studied. All of the sensitive bacteria exhibit growth-restriction zones with a diameter that ranges from 11 mm to 20 mm. Silver nanoparticles are among the most significant metal nanoparticles because of their robust antibacterial capabilities. Because of these features, silver nanoparticles are among the most significant types of metal nanoparticles. The AgNPs, which were produced from an aqueous extract of the outer peel of Pisum sativum, were shown to be bactericidal against four human pathogenic bacteria (with inhibition zones ranging from 8.70 to 11.10 mm on agar plates) [6]. It has been discovered that green silver nanoparticles (AgNPs) produced from an aqueous extract of *Mangifera indica* seeds are more efficient than traditional antibacterial treatments against Gram-negative bacteria [7]. Table 1 contains a discussion of

the findings of microbial efficacy of AgNPs produced from different plant extracts.
[figure(s) omitted; refer to PDF]

Table 1

Comparative study of various natural sources used for the preparation of AgNPs and their antibacterial activity.

S. No.	Source	Antibacterial activity against	Application	Citation
1	*Tinospora cordifolia* (stem)	*P. aeruginosa*	Resistance to multidrug resistant bacteria	[26]
2	*Cucumis sativus*	*Mycobacterium tuberculosis*	Healing of wounds	[27]
3	*Calotropis procera* (leaves)	*Vibrio cholera*	Treatment of waterborne diseases	[28]
4	*Acalypha indica* (leaf)	*E. coli* and *Vibrio cholera*	Reduction of microbial loading in drinking water	[29]
5	*Taraxacum officinale* (leaf)	*P. syringae*	Disease management	[30]
6	*Boerhaavia diffusa*	*F. branchiophilum*	Protection of fishery livestock	[31]
7	*Wrightia tinctoria* (seeds)	*B. subtilis, S. aureus, E. coli,* and *P. vulgaris*	Inhibition of bacterial infection in target organism	Current study

3.3. Anticancer Activity

AgNP cell viability was measured using the MTT test. When delivered at concentrations between 1.5 and 100 *μ*g/ml, both SeWt-AgNPs and StAc-AgNPs show significant cytotoxicity (Figure 10). It was shown that both types of AgNPs were able to prevent the formation of cancer cells by the fact that the proliferation of the MCF cell line was dramatically decreased across the board. Light microscopy was used to examine the morphology of MCF-7 cancer cells after they had been incubated with AgNPs for 24 hrs at doses of 1.5, 6.25, 12.5, 50, and 100 *μ*g/ml (Figures 11 and 12). At concentrations of 1.5 *μ*g/ml of AgNPs, there are no discernible alterations in the morphology of the photos. However, at 6.25, 12.5, 50, and 100 *μ*g/ml, AgNPs inhibited cell proliferation at a dose-dependent rate. There was a noticeable shift in cell shape at both 50 and 100 *μ*g/ml. The results demonstrated a significant reduction in MCF-7 cell viability as compared to the plant extract. We can attribute this decline to the presence of AgNPs. Table 2 shows the findings of a study contrasting the antineoplastic activity of the various natural sources used to produce AgNPs.
[figure(s) omitted; refer to PDF]

Table 2

Comparative study of various natural sources used for the preparation of AgNPs and their antineoplastic activity.

S. No.	Source	Anticancer activity against	Application	Citation

1	*Gymnema sylvestre*	HT29	Inhibition of growth of the human colon carcinoma	[32]
2	*Hibiscus sabdariffa*	U87	Inhibition of growth of the human brain carcinoma	[33]
3	*Moringa oleifera*	A549 and SNO	Inhibition of growth of the human lung and esophageal carcinoma	[34]
4	*Podophyllum hexandrum*	HeLa	Inhibition of growth of the human lung and cervical carcinoma	[35]
5	*Rhus chinensis*	MKN-28, Hep3B, and MG-63	Inhibition of growth of the human liver and stomach carcinoma	[36]
6	*Wrightia tinctoria* (seeds)	MCF-7	Inhibition of growth of the breast carcinoma	Current study

4. Conclusions

The *Wrightia tinctoria* seed extracts and the *Acacia catechu* stem extracts were both utilized during the course of this experiment to facilitate the synthesis of the AgNPs. UV-Vis spectrophotometry confirms the formation of AgNPs, and the XRD analysis reveals the FCC crystalline structure of the AgNPs. The TEM micrographs demonstrated that the AgNPs had a spherical shape and that their diameters ranged from 20 to 40 nm. The AgNPs were found to be uniformly distributed. Overall, the study has shown that plant extracts can be used as eco-friendly and cost-effective alternatives to traditional chemical methods for the synthesis of AgNPs. The synthesized AgNPs showed promising antibacterial and anticancer activities, indicating their potential use in various biomedical applications. Further research is needed to explore the mechanisms of action and toxicity of these AgNPs and to optimize their synthesis parameters to enhance their properties for specific applications.

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DETAILS

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Correlation between SMADs and Colorectal Cancer Expression, Prognosis, and Immune Infiltrates

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ABSTRACT (ENGLISH)

Background. In recent years, the incidence and mortality of colorectal cancer (CRC) are increasing, and the 5-year survival rate of advanced metastatic CRC is poor. Small mothers against decapentaplegic (SMAD) superfamily are intracellular signal transduction proteins associated with the development and prognosis of a variety of tumors. At present, no study has systematically analysed the relationship between SMADs and CRC. *Methods*. Here, R3.6.3 was used to analyse the expression of SMADs in pan-cancer and CRC. Protein expression of SMADs were analysed by Human Protein Atlas (HPA). Gene expression profiling interactive analysis (GEPIA) was used to evaluate the correlation between SMADs and tumor stage in CRC. The effect of R language and GEPIA on prognosis was analysed. Mutation rates of SMADs in CRC were determined by cBioPortal, and potentially related genes were predicted using GeneMANIA. R analysis was used to correlate immune cell infiltration in CRC. *Results*. Both SMAD1 and SMAD2 were found to be weakly expressed in CRC and correlated with the immune invasion level. SMAD1 was correlated with patient prognosis, and SMAD2 was correlated with tumor stage. SMAD3, SMAD4, and SMAD7 were all expressed at low levels in CRC and associated with a variety of immune cells. SMAD3 and SMAD4 proteins were also expressed at low levels, and SMAD4 had the highest mutation rate. SMAD5 and SMAD6 were overexpressed in CRC, and SMAD6 was also associated with patient overall survival (OS) and CD8+ T cells,

macrophages, and neutrophils. *Conclusions*. Our results reveal innovative and strong evidence that SMADs can be used as biomarkers for the treatment and prognosis of CRC.

FULL TEXT

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1. Introduction

Colorectal cancer (CRC) is widely known as one of the most pervasive malignancies due to its third highest morbidity (10.0%) and second highest mortality (9.4%) among all cancers worldwide, and its morbidity and mortality are on the rise year by year [1]. The 5-year survival rate for advanced metastatic colorectal cancer is less than 20% [2]. The main treatment methods for CRC are surgery, radiotherapy, and chemotherapy, which are good for early colorectal cancer but poor for advanced and metastatic CRC [3]. There is no good treatment for advanced metastatic colorectal cancer. To eliminate the high incidence and mortality of CRC, further exploration of meaningful biomarkers is urgently needed to strengthen its therapeutic efficacy.

There are eight small mothers against decapentaplegic (SMAD) codes in the human genome [4]. SMAD proteins are a family of signal transduction molecules involved in the transforming growth factor β (TGF-β) ligand pathway. SMADs belong to the intracellular protein family with a total length of 500 amino acids, among which SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8 act as TGF-β receptors in mammals, of which SMAD8 is generally expressed as SMAD9. SMAD4 is a common pathway mediator, and SMAD6 and SMAD7 inhibit SMAD [5]. The main function of SMADs is to control the gene program, transcriptional regulation, and signal transduction, which can mediate TGF-β/SMAD, Notch, ERK (extracellular regulated protein kinases)/MAPK (mitogen-activated protein kinase), Hippo, JAK (janus kinase)/STAT (signal transducer and activator of transcription), Hedgehog, BMP (bone morphogenetic protein)/SMAD, and so on [6]. SMADs have been implicated in cell proliferation, migration, apoptosis, and immune regulation of cancer cells [7–9]. SMADs are associated with lung, pancreas, liver, gastrointestinal tumors, and so on [10]. However, there are few comprehensive studies on the expression, prognosis, and immune infiltration of the SMAD superfamily as a whole and colorectal cancer.

With the wide application of big data sets, the collection in the field of biomedicine is called omics, including various genomics, transcriptomics, proteomics, and metabolomics, from this perspective, many new and better ways of disease diagnosis and treatment and mechanism research have been found [11]. Omics methods have been applied in the screening and diagnosis of various tumors, including CRC. The application of various omics methods is of great value in understanding the pathological process of CRC, identifying CRC markers and predicting prognosis [12].

In this study, we used public databases and R language for in-depth analysis of the correlation between SMADs and the occurrence and development of CRC, as well as prognostic analysis and immune infiltration analysis of CRC patients to demonstrate the value of different SMADs in the occurrence, prognosis, and immune infiltration of colorectal cancer.

2. Materials and Methods

2.1. The Human Protein Atlas (HPA)

The Human Protein Atlas (HPA) (http://www.proteinatlas.org/pathology) maps human proteins by analysing the effects of clinical results on various omics, primarily based on the relationship between the genome-wide transcriptome of protein-coding genes of 17 cancer types and clinical results [13]. In this study, we used this database to investigate the relationship between SMAD proteins and CRC.

2.2. The Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA (http://gepia.cancer-pku.cn/) is an online web address based on The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression database (GTEx) consisting of thousands of tumor and healthy tissue sample data using standard processing pipelines, providing key interactive and customizable functionality [14]. In this study, GEPIA was used to analyse the correlation between SMADs and the pathological stage of CRC, and its prognostic

value was analysed by this method.

2.3. cBioPortal

cBioPortal (http://cbioportal.org) is a free open platform for multidimensional cancer genome analysis, detection, and visualization at the deoxyribonucleic acid (DNA) level [15]. In this study, cBioPortal was used to predict mutation rates of the SMAD gene family in CRC.

2.4. GeneMANIA

GeneMANIA (http://www.genemania.org) is a rich and friendly website for hypothesis of gene function, analysis of gene lists, identification of functionally similar genes, biofunctional genomics, and more [16]. In this study, we explored the SMAD interaction network and associated genes through the GeneMANIA database.

2.5. Statistical Analysis

All statistical analyses were performed using R (V3.6.3). The differences were visualized using the ggplot2 software package. Paired *t* tests and Mann–Whitney *U* tests were used to detect differences between colorectal cancer tissues and adjacent normal tissues. The R package survminer was used for visualization of prognostic value, and the survival software package was used for statistical analysis of survival data. The single sample gene enrichment analysis (ssGSEA) package of gene set variation analysis (GSVA) [17] was used for immune infiltration analysis, and the Shapiro–Wilk normality test and Spearman correlation coefficient calculation were used to detect the correlation of immune infiltration.

3. Results

3.1. Differential Expression of SMADs in Pan-Cancer and CRC

The expression differences of SMADs across cancers were detected by the ggplot2 software package, as shown in Figure 1. Then, the same package was used to detect the differential expression of SMADs in 51 normal samples and 647 colorectal cancer samples (Figure 2), and the results showed that the expression levels of SMAD1-4, SMAD7, and SMAD9 were significantly downregulated, while the expression levels of SMAD5 and SMAD6 were significantly upregulated. The specific situation was analysed as follows.

[figure(s) omitted; refer to PDF]

Unpaired sample analysis showed that the expression of SMAD1 in CRC was significantly lower than that in adjacent colorectal normal tissues (Figure 2(a), 3.207 ± 0.539 vs. 3.654 ± 0.236, ppp$=0.002$), the expression of SMAD4 in CRC was absolutely lower than that in adjacent colorectal normal tissues (Figure 2(d), 3.440 ± 0.644 vs. 3.908 ± 0.288, pppp

[figure(s) omitted; refer to PDF]

3.2. Correlation between SMADs and CRC Tumor Stage

By evaluating the correlation between SMAD expression and tumor stage in CRC patients, the results are shown in Figure 3. The analysis results showed that the SMAD2 and SMAD7 groups had noticeable differences (Figures 3(b) and 3(g), all pp>0.05).

[figure(s) omitted; refer to PDF]

3.3. Protein Expression of SMADs in CRC

Protein expression of SMADs in normal intestine and CRC tissues was analysed by HPA, as shown in Figure 4. The results showed that the protein expression levels of SMAD1 and SMAD2 were significantly increased in CRC tissues (Figures 4(a) and 4(b)), the protein expression levels of SMAD3, SMAD4, and SMAD5 were significantly decreased in CRC tissues (Figures 4(c)–4(e)), and the protein expression levels of SMAD7 was not significantly different (Figure 4(f)).

[figure(s) omitted; refer to PDF]

3.4. Prognostic Value of SMADs in Colorectal Cancer

R package survminer and survival were used to analyse overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI) indicators of survival prognosis of CRC patients by SMADs, as shown in Figure 5. The results showed that SMAD1 was significantly correlated with DSS ($p=0.037$) and PFI ($p=0.02$) in CRC patients (Figure 5(a)). SMAD9 was significantly correlated with OS ($p=0.038$) and DSS ($p=0.035$) in CRC patients (Figure

5(h)), while other results showed no significant differences.
[figure(s) omitted; refer to PDF]
GEPIA was used to analyse OS and disease-free survival (RFS) indicators of the prognostic value of SMADs for CRC patients, as shown in Figure 6. Analysis showed that SMAD6 and SMAD9 were significantly correlated with OS in CRC patients (Figures 6(f) and 6(h)), while no significant correlations were found in others.
[figure(s) omitted; refer to PDF]

3.5. Analysis of SMAD Gene Mutation and Interaction Expression in CRC

The frequency of SMAD changes in CRC was detected by cBioPortal. The results showed that in 881 CRC patients, the mutations of SMAD1 and SMAD6 were 1.9%, SMAD2 was 7%, SMAD3 and SMAD5 were 5%, SMAD4 was 18%, and the mutation rate was 4% for SMAD7 and 2.8% for SMAD9. The OncoPrints contained in-frame mutations, missense mutations, splice mutations, truncating mutations, structural variants, amplifications, deep deletions, and no alterations (Figure 7(a)). Through the GeneMANIA database, twenty genes associated with the interaction network with SMADs were analysed (Figure 7(b)).
[figure(s) omitted; refer to PDF]

3.6. Correlation with Immune Infiltration

The ssGSEA package of GSVA was used to comprehensively analyse the relationship between SMADs and immune cell infiltration, as shown in Figure 8 and Table 1. The results showed that the expression of SMAD1, SMAD4, and SMAD7 was positively correlated with the infiltration of B cells, CD8+ T cells, dendritic cells (DCs), eosinophil macrophages, and neutrophils (Figures 8(a), 8(d), and 8(g)). SMAD2 expression was positively correlated with CD8+ T cells, macrophages, and neutrophils (Figure 8(b)). SMAD3 expression was positively correlated with B cells, CD8+ T cells, eosinophils, and macrophages (Figure 8(c)). SMAD5 expression was positively correlated with macrophage infiltration, while SMAD5 expression was negatively correlated with DC infiltration (Figure 8(e)). SMAD6 expression was positively correlated with DC infiltration, and SMAD6 expression was negatively correlated with CD8+ T cell, macrophage, and neutrophil infiltration (Figure 8(f)). The expression of SMAD9 was positively correlated with eosinophil infiltration, and the expression of SMAD9 was negatively correlated with neutrophil infiltration (Figure 8(h)).
[figure(s) omitted; refer to PDF]

Table 1

Correlation between immune cell infiltration and SMADs in CRC.

Genes	Spearman	*Cells*					
B cells	CD8+ T cells	DC	Eosinophils	Macrophages	Neutrophils	SMAD1	r
0.230	0.190	0.160	0.190	0.280	0.220	p	<0.001
<0.001	<0.001	<0.001	<0.001	<0.001	-		
SMAD2	r	0.049	0.210	0.037	0.037	0.290	0.130
p	0.217	<0.001	0.347	0.352	<0.001	0.001	·
SMAD3	r	0.085	0.120	0.054	0.200	0.086	0.046
p	0.030	0.002	0.170	<0.001	0.029	0.246	·

SMAD4	r	0.130	0.230	0.130	0.084	0.340	0.220
p	0.001	<0.001	0.001	0.032	<0.001	<0.001	·
SMAD5	r	−0.062	0.050	−0.150	−0.041	0.110	−0.031
p	0.116	0.205	<0.001	0.297	0.005	0.436	·
SMAD6	r	0.004	−0.097	0.089	0.032	−0.310	−0.170
p	0.910	0.014	0.024	0.417	<0.001	<0.001	·
SMAD7	r	0.260	0.170	0.160	0.260	0.130	0.078
p	<0.001	<0.001	<0.001	<0.001	0.001	0.048	·
SMAD9	r	0.047	0.009	0.071	0.100	−0.033	−0.110

4. Discussion

Studies have shown that SMADs are involved in the development, metastasis, prognosis, and immune microenvironment of many tumors. Immune infiltrating cells are related to the tumor microenvironment and influence tumor growth and metastasis. The high expression of SMAD1, SMAD2, and SMAD4 in gastric cancer tissues is significantly correlated with the prognosis of patients [18]. Studies related to lung cancer have found that the expressions of SMAD6, SMAD7, and SMAD9 in SMADs are downregulated in lung cancer and significantly correlated with the prognosis of patients [19]. However, studies related to SMADs and the occurrence, development, prognosis, and immunity of CRC have not been fully clarified.

SMAD1 is the activation type of SMAD receptor, which is involved in modifying cell growth, differentiation, apoptosis, and other processes and plays an important role in the body's immune system. Current studies on SMAD1 in CRC have shown that high expression of SMAD1 can induce apoptosis of CRC [20]. SMAD1 can promote the occurrence of CRC tumors and induce migration and autophagy processes [21]. This study claimed that low expression of SMAD1 in colorectal cancer was related to prognosis and immune cell infiltration, but SMAD1 protein was significantly increased in colorectal cancer tissues. These results suggest that high SMAD1 expression can be used as a diagnostic marker for CRC and as a marker associated with poor prognosis and immunoinfiltration when SMAD1 begins to be low expressed in CRC.

SMAD2 plays different roles in different stages of cancer by regulating various biological processes [22]. In colorectal cancer, the tumor suppressor gene NIT1 is realized by activating the SMAD2/3 signaling pathway [23]. SMAD2 can promote the development of CRC by regulating the polarization of tumor macrophages [24]. In this study, SMAD2 expression in CRC was low, which was significantly different from colorectal cancer tumor stage, associated with CD8+ T cells, macrophages, and neutrophils, and had a high mutation rate. The results of this study are consistent with those of other studies, suggesting that low expression of SMAD2 is correlated with clinical malignancy and affects tumor immune microenvironment.

SMAD3 plays the dual role of oncogene and tumor suppressor gene in tumor formation, and can be used as a prognostic marker for tumors [22]. SMAD4 is a tumor suppressor gene that plays a central role in TGF-β signaling pathway transduction [25]. In CRC, SMAD3 reduces its expression through miR-4429 and ultimately inhibits the occurrence, development, and metastasis of cancer cells [26]. A meta-analysis showed that a high mutation rate of SMAD4 in CRC patients was associated with poor prognosis but not with clinical stage [27]. This study showed that SMAD3, SMAD4, and their proteins were significantly underexpressed in colorectal cancer. However, there was no

significant correlation between tumor stage and prognosis. The maximum mutation rate of SMAD4 in CRC was 18%. Studies on immune infiltration have shown that SMAD3 and SMAD4 are associated with a variety of immune cells. Our results are generally consistent with previous reports, suggesting that SMAD3 and SMAD4 can act as tumor suppressor genes of CRC and influence patient immune status. However, whether SMAD4 can be used as a prognostic indicator needs further validation.

SMAD5 mediates TGF-β superfamily ligand signaling pathways as oncogenic genes [28]. SMAD6 can also regulate TGF-β signaling pathway, which is conducive to tumor growth, spread, and metastasis [29]. Overexpression of miR-186-5p in CRC can significantly reduce SMAD6, ultimately inhibiting the proliferation and migration of CRC cells and increasing the apoptosis of CRC cells [30]. This study found that SMAD5 and SMAD6 were significantly overexpressed in colorectal cancer. SMAD6 was significantly correlated with OS. These results are consistent with our study of SMAD5 and SMAD6. These results demonstrated that SMAD5 and SMAD6 could be used as oncogenes of CRC, and SMAD6 could also be used as a prognostic biomolecule.

SMAD7 is an inhibitor of TGF-β signaling pathway and antagonizes TGF-β-mediated diseases. SMAD7 plays a dual role in different tumor stages. As a tumor suppressor gene in the early stage and a tumor promoter gene in the late stage, SmAD7 is positively correlated with the degree of malignancy [31]. In CRC, SMAD7 can upregulate miR-424 by silencing circTBL1XR1, thus promoting the proliferation, invasion, and metastasis of CRC [32]. miR-4775 overexpression in CRC promotes invasion, metastasis, and epithelial-mesenchymal transition (EMT) processes of cancer cells by activating SMAD7 [33]. In this study, SMAD7 expression was significantly reduced in CRC and was associated with a variety of immune cells. Our study is consistent with the current relevant experimental verification, and the current literature suggests that there is a difference in colorectal-related expression between this study and SMAD7. Considering the dual role of SMAD7, CRC tissues may be in different stages, which is consistent with the actual situation. SMAD7 is both an oncogene and a tumor suppressor gene in CRC and can be used as a marker to evaluate the state of the immune microenvironment.

However, there are only eight members of the SMAD family from 1 to 8. However, some databases SMAD8 is directly named SMAD9, and some databases have both SMAD8 and SMAD9, so it is impossible to perform specific analysis, so further analysis will not be conducted here.

Our study has some shortcomings. First, this study was mainly obtained through database analysis without relevant experimental verification. To better study the relationship between CRC and SMADs, experimental verification is needed to further verify the results and make the results more convincing. Second, due to the ambiguity between SMAD8 and SMAD9 in different databases, specific analysis is not possible. Therefore, our team needs to continue to carry out relevant experimental verification in cell, animal, and clinical aspects.

5. Conclusions

In conclusion, this study used R language and several different database systems to analyse the differential expression, mutation rate, prognostic analysis, and immune infiltration of SMAD family members in CRC. The results showed that SMAD1, SMAD2, SMAD3, SMAD4, and SMAD7 were significantly downregulated in CRC, while SMAD5 and SMAD were significantly upregulated in CRC. SMAD1 and SMAD2 proteins were significantly increased in CRC, SMAD3, SMAD4, and SMAD5 proteins were significantly decreased in CRC, and SMAD7 and SMAD9 protein expression was not significantly different. Only SMAD2 was associated with tumor stage of CRC. In terms of prognostic analysis, only SMAD1 was significantly correlated with DSS and PFI, while SMAD6 was significantly correlated with OS. SMAD4 had the highest mutation rate. In immune infiltration, SMAD1, SMAD2, SMAD3, SMAD4, and SMAD7 were positively correlated with a variety of immune cells. By studying the relationship between SMADs family and CRC, in clinical practice, patients with high expression of SMAD1 and SMAD2 and low expression of SMAD3, SMAD4, and SMAD5 in tissue specimens can be identified as CRC, which can be used as diagnostic markers. In order to understand the stage of the tumor, the increase of SMAD2 value can be detected. Based on the correlation between the expression level of a large number of patients and the stage, the interval range can be formulated to further determine the malignant degree of CRC in clinic. The high expression of SMAD1 and low expression of SMAD6 can be detected to determine the prognosis of patients. In order to understand the immune

microenvironment of CRC and develop immunotherapy methods, SMAD1, SMAD2, SMAD3, SMAD4, and SMAD7 of patients are of guiding significance. Through the above systematic discussion, the diagnosis, treatment, and survival prognosis of CRC patients can be evaluated clinically by detecting the expression level of SMADs family, which is convenient and has guiding value.

Consent

Not applicable.

Disclosure

A preprint has previously been published [34].

Authors' Contributions

Ning Ding designed the main research scheme. Tao Zhang and Yanru Yao conducted data analysis. The first draft was drafted by Ning Ding and Hongbiao Luo and revised by Yongheng He and Tianshu Peng. The authors have read and approved the final manuscript.

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Glossary

Nomenclature

CRC:Colorectal cancer

SMAD:Small mothers against decapentaplegic

TGF-β:Transforming growth factor β

ERK:Extracellular regulated protein kinases

MAPK:Mitogen-activated protein kinase

JAK:Janus kinase

STAT:Signal transducer and activator of transcription

BMP:Bone morphogenetic protein

HPA:Human Protein Atlas

GEPIA:Gene expression profiling interactive analysis

TCGA:The Cancer Genome Atlas

GTEx:The Genotype-Tissue Expression database

ssGSEA:Single sample gene enrichment analysis

GSVA:Gene set variation analysis

OS:Overall survival

DSS:Disease-specific survival

PFI:Progression-free interval

DCs:Dendritic cells

HCC:Hepatocellular carcinoma

EMT:Epithelial-mesenchymal transition.

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DETAILS

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Neonicotinoid Analysis in Sunflower (Helianthus annuus) Honey Samples Collected around Tekirdag in Turkey

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ABSTRACT (ENGLISH)

In recent years, the widespread use of neonicotinoids in agricultural areas has caused environmental pollution due to its lower toxicity to mammals. Honey bees, which are considered as biological indicators of environmental pollution, can carry these pollutants to the hives. Forager bees returning from sunflower crops that have been treated with neonicotinoids treated sunflower fields cause residue accumulation in the hives, which reason colony-level adverse effects. This study analyses neonicotinoid residues in sunflower (*Helianthus annuus*) honey sampled by beekeepers from Tekirdag province. Honey samples have been subjected to liquid-liquid extraction methods before liquid chromatography-mass spectrometry (LC-MS/MS). The method validation was carried out to fulfill all the necessary requirements of procedures SANCO/12571/2013. Accuracy was in the range of 93.63–108.56%, for recovery in the range of 63.04–103.19%, and for precision in the range 6.03–12.77%. Detection and quantification limits were determined according to the maximum residue limits of each analyte. No neonicotinoid residues were found above the maximum residue limit in the sunflower honey samples analysed.

FULL TEXT

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1. Introduction

Neonicotinoids are insecticides that show nicotine-like stimulating effects by binding to nicotinic acetylcholine receptors in the CNS. Neonicotinoids are more toxic to insects because they bind more tightly and irreversibly to nicotinic acetylcholine receptors in insects than in mammals [1, 2].

Neonicotinoid-type plant protection products are widely used in the agricultural field, especially for preserving seeds due to their less toxic effect on mammals; they also have unintended consequences on nontarget organisms like agrarian pollinators [3]. Pollution is also detected in the fields where neonicotinoid-treated seeds are planted and in the application area’s surface or public drinking water sources [4].

Commercial beekeepers move millions of honeybee colonies to sunflower fields to get sunflower honey during the blooming sessions. During these visits, honey bees carry possible pesticide residues in the environment to the hives by means of nectar and pollen. Neonicotinoids can affect the bee’s ability to fly for feeding purposes, such as learning and remembering ways to reach food sources. Therefore, it can be considered as part of the important role in colony collapse disorder [5].

The neonicotinoids have been restricted in the first Europe due to the adverse environmental effects resulting from a severe decrease in honey bees, insect populations, and bird species and numbers. The European Union

established maximum residue limits (MRLs) for acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam in the range of 10–200 ng·k^{-1} [6].

The importance of sunflower honey in the global honey trade is different; its flavour is not strong dominantly. Therefore, it blends well with other honey types, and it is one of the most suitable and economic honey for commercial blending. Honey, which must comply with EU standard norms, must be reliable regarding food safety and public health. High pesticide concentrations can cause high mortality in bees, loss of colonies, and honey production unsuitable for food safety [7].

For public health, pesticides in honey and other food have become a severe health and safety checkpoint worldwide, and demands for detecting chemicals that may pose an environmental risk have increased in recent years. Successful results have been obtained in analysing multiple residues of antibiotics and pesticides in honey using liquid chromatography-mass spectrometry.

Different extraction methods and devices have been used to determine neonicotinoid residues in sunflower honey. Liquid-liquid extraction (LLE) [8], solid-phase extraction (SPE) [9, 10], QuEChERS extraction [11–13], and dispersive liquid-liquid microextraction (DLLME) [14] can be counted among the effective extraction methods used. A high-pressure liquid chromatography diode array detector (HPLC/DAD) [15], a gas chromatography-mass spectrometer (GC-MS/MS) [13], and liquid chromatography-mass spectrometry (LC-MS/MS) are used for neonicotinoid analyses [12, 16].

This study aims to determine neonicotinoid residues in sunflower honey collected from 33 different beekeepers around Tekirdag province. According to the results obtained, a risk assessment will be made in terms of public health. The study is the first to investigate neonicotinoid pesticide residues in honey around Tekirdag. It is thought that it will importantly contribute to databases in this regard.

2. Materials and Methods

2.1. Chemicals and Reagents

The following standards for neonicotinoids were used: imidacloprid (99.9%), acetamiprid (99.9%), clothianidin (99.9%), nitenpyram (99.9%), thiacloprid (99.9%), dinotefuran (98.8%), and thiamethoxam (99.6%). The purity of all compounds was greater than 98%. The internal standard of clothianidin-d3 (97%) was obtained from Sigma-Aldrich. Acetonitrile for HPLC was obtained from Fluka. Dichloromethane and glacial acetic acid for analysis were obtained from Merck.

2.2. Standards and Solutions

2.2.1. Standard Stock Solution (1 mg·mL^{-1} Each)

10 mg of each reference standard was weighed into a 10 mL Class A graduated flask. Sufficient methanol was added up to the mark. Stock solutions were protected at −18°C.

2.2.2. S_2-Working Standard Solution (10 ng·mL^{-1} Each)

0.1 mL of each reference standard stock solution was taken and placed in a 10 mL measuring balloon. Sufficient methanol was added up to the mark. The prepared working solutions were stored at 4–6°C.

2.2.3. S_4-Standard Working Solution (100 ng·mL^{-1} Each)

0.1 mL of each S_2-standard working solution was taken and placed in a 10 ml measuring flask. Sufficient methanol was added up to the mark. All standard working solutions were stored at 4–6°C. This prepared solution was used to define reference standards in the MS detector.

2.2.4. Reference Standard Solution Mix for the Spike

Spiking solutions were prepared at 10 ng·mL^{-1} for clothianidin, dinotefuran, nitenpyram, and thiamethoxam, 50 ng·mL^{-1} for acetamiprid and imidacloprid, and 200 ng·mL^{-1} for thiacloprid, according to the levels of maximum residue limit (MRL) in the honey, respectively (Table 1). One mL of clothianidin, dinotefuran, nitenpyram, and thiamethoxam S_4 working solution, 5 mL of acetamiprid and imidacloprid S_4 working solution, and 0.2 mL of thiacloprid S_2 working solution were put into a 10 mL measuring balloon and filled with methanol up to the mark.

Table 1

Summary of LOD, LOQ, CC*α*, and CC*β*.

Analyte	Calibration range (μg kg^{-1})	Linearity (r^2)	Limit of detection (LOD)	Limit of quantification (LOQ)	Decision limit (CCα)	Detection capability (CCβ)	MRL (EU) (μg kg^{-1})
Dinotefuran	5–20	0.9908	6.25	9.45	10.74	11.50	10
Nitenpyram	5–20	0.9984	6.15	9.77	11.08	12.17	10
Thiamethoxam	5–20	0.9963	6.42	9.72	11.48	12.94	10
Clothianidin	5–20	0.9910	5.42	8.70	10.94	11.88	10
Imidacloprid	25–100	0.9956	26.63	36.94	57.18	64.37	50
Acetamiprid	25–100	0.9954	29.18	43.20	56.86	63.73	50
Thiacloprid	100–400	0.9922	102.67	155.34	221.11	242.22	200

2.2.5. Internal Standard Solution

Clothianidin-d_3 was used as an internal standard at a 10 ng·mL^{-1}.

2.2.6. Mobile Phase *A*

Acetonitrile was used as a mobile phase *A*.

2.2.7. Mobile Phase *B*

2 mL of acetic acid were placed in a 1 L flask and the reagent diluted with water to the marked line. The mobile phases were degassed in an ultrasonic bath for 15 min.

2.3. Collection of Samples

During sunflower honey harvest, honey samples were collected from supers at each of the 33 stationary apiaries in July/August of 2015 from 10 different district centres of Tekirdag (40°58′41″N, 27°30′42″E). All samples were confirmed to be sunflower honey by pollen analysis.

2.4. Extraction of Honey Samples

The extraction method [14] was used for the honey samples. Briefly, two grams of each honey sample were weighed into 15 mL polypropylene centrifuge tubes and an internal standard solution (100 μL) was added to the tubes. The mixed standard spiking solutions were added (50, 100, 150, and 200 μL) to control the quality of the samples. 0.5 mL of acetonitrile and 2.0 mL of dichloromethane were placed in each tube. The tubes were mixed by vortex for 1 minute, incubated in an ultrasonic bath for 10 minutes, returned to the vortex for 1 minute, and centrifuged at 2,500g for 5 minutes, 6 mL of supernatant was then removed using a pipette and transferred into glass tubes. The organic fraction was evaporated to dryness in a stream of nitrogen at 40°C within a water bath. Two mL of mobile phase was added onto the dry residue and mixed by vortex for two minutes. The result was filtered into an autosampler vial using a 0.2 μm syringe filter.

2.5. Instrumentation

Analyses were performed on AB Sciex 3200 QTRAP brand/model high-pressure liquid chromatography-mass spectrometry equipment controlled by Analyst 1.6.1 software. An Agilent Poroshell 120 SB: C18 2.7 μm 100 × 3.0 mm column was used for chromatographic separation. Acetonitrile (A) and water acidified with 0.2% acetic acid were used as the mobile phase. The linear gradient mobile phase; 0-1 min 80% *A*, 1–3.3 min 50% *A*, and 3.4–6 min 80% *A* , and flow rate of 0.3 mL/min. The injection volume was 10 μL, and the column temperature was 40°C.

2.6. Mass Spectrometry

The MS/MS detector parameters and precursor-product ions of each referenced standard substance are shown in Table 2. A capillary voltage of 5500 V, nebulizer gas of 7 psi, curtain gas of 30 psi, heater gas of 50 psi, and collision gas of 50 psi were set. The temperature of the TurboIonSprey module was set at 400°C.

Table 2

MS/MS detector parameters and retention times (RT).

Analytes	RT	Precursor ion (*m*/z)	Product ion (*m*/z)	DP (volts)	EP (volts)	CEP (volts)	CE (volts)	CXP (volts)
Dinotefuran	2.4	203.08	129.1*	36	8	18	15	4
114.1	36	8	18	17	4		-	
Nitenpyram	2.8	271.12	225.20*	36	5.5	16	15	4
56.00	36	5.5	16	49	4	Thiameth oxam	3.0	292.00
211.10*	31	11.5	16	15	4	132.10	31	11.5
16	25	4			-			
Clothianidin	3.2	250.07	132.00*	41	7.5	14	19	4
169.10	41	7.5	14	15	4		-	
Clothianidin-d3 (IS)	3.2	253.01	132.00	41	8	14	23	4
–								
Imidacloprid	3.3	256.10	290.10*	36	9	14	19	4
175.10	36	9	14	21	4		-	
Acetamiprid	3.4	223.07	126.20	41	9	12	27	4
99.20	41	9	12	47	4		-	

Thiacloprid	3.7	253.06	126.20	46	12	14	29	4

*Confirmative ion. DP: declustering potential, EP: entrance potential, CEP: cell exit potential, CE: collision energy, CXP: collision cell exit potential, IS: internal standard.

Ionization was performed in positive ion mode using the electrospray ionization (ESI) module.

3. Results and Discussion

3.1. Method Validation

The selectivity/sensitivity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), decision limit (CCα), detection capability (CCβ), accuracy, and recovery parameters were calculated for the method validation.

3.1.1. Specificity/Selectivity

Blank samples were analysed by loading different standard substances; no interference was observed in the retention times. It was concluded that the analysis method was suitable for selectivity/sensitivity. The chromatogram obtained from loading at the MRL level is shown in Figure 1.

[figure(s) omitted; refer to PDF]

3.1.2. Linearity

To determine the linearity of the method, six parallel analyses were performed using four different concentration points at 0.5, 1, 1.5, and 2 MRL levels in accordance with the MRL level in honey. Calibration curves for each standard substance were created. The r^2 value in the calibration curve of each standard item was found to be between 0.9908 and 0.9984 (Table 1).

3.1.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

To determine the limit of detection and the limit of quantification, 10 parallel analyses were performed at 0.5 MRL. The results obtained are shown in Table 1.

3.1.4. Decision Limit (CC$_\alpha$) and Detection Capability (CC$_\beta$)

The decision limit (CC$_\alpha$) and detection capability (CC$_\beta$) were calculated using the results obtained from the study linearity and are shown in Table 1.

3.1.5. Accuracy

The accuracy was calculated using the study linearity and recovery results shown in Table 3.

Table 3

Accuracy and recovery of neonicotinoids in honey samples.

	Added amount (μg kg^{-1})	Mean amount calculated (μg kg^{-1})	RSD (%)	Accuracy (%)	Recovery (%)
Dinotefuran	5	5.25	11.56	105.00	84.08
10	10.86	7.11	108.56	82.57	15
15.79	6.16	105.29	82.52	20	18.72
6.92	93.63	78.94	-		
Nitenpyram	5	5.13	7.98	102.75	87.92
10	10.42	7.27	104.20	92.75	15
15.71	12.77	104.80	74.75	20	19.08

8.20	95.30	63.71	-		
Thiamethoxa m	5	4.98	8.27	99.69	71.36
10	10.03	9.75	100.25	84.50	15
15.25	7.27	101.59	78.63	20	19.79
8.21	98.74	73.02	-		
Clothianidin	5	5.06	11.94	101.34	63.04
10	10.13	10.94	101.31	73.65	15
15.18	10.09	101.23	70.72	20	19.75
6.03	98.81	86.06	-		
Imidacloprid	25	23.55	10.92	94.10	74.68
50	51.59	6.48	103.21	74.98	75
77.64	7.26	103.63	77.57	100	96.70
6.61	96.70	71.26	-		
Acetamiprid	25	23.61	10.34	94.50	103.19
50	52.36	8.42	104.70	95.83	75
79.23	9.37	105.53	94.94	100	94.70
8.56	94.70	93.64	-		
Thiacloprid	100	100.23	7.13	100.23	94.12
200	209.00	9.30	104.45	92.00	300
318.00	10.60	105.99	92.21	400	377.88

3.1.6. Recovery

To determine recovery, analysis was performed according to the blank fortified sample at levels 0.5, 1, 1.5, and 2 MRL shown in Table 3.

According to the sunflower honey samples analysis, the MRL value was not detected for any neonicotinoid residue. The data under the maximum residue levels have not been evaluated. The results of the analysis of the honey

samples are shown in Table 4.

Table 4

The results of analysis honey samples.

No.	Dinotefuran (ug·kg^{-1})	Nitenpyram (ug·kg^{-1})	Thiamethoxa m (ug·kg^{-1})	Clothianidin (ug·kg^{-1})	Imidacloprid (ug·kg^{-1})	Acetamiprid (ug·kg^{-1})	Thiacloprid (ug·kg^{-1})
1	0.64	<0	<0	0.21	1.23	1.95	0.65
2	0.53	<0	<0	0.15	1.4	1.97	0.53
3	0.47	<0	<0	0.57	1.21	2.07	0.58
4	0.40	<0	<0	0.15	1.22	1.95	0.50
5	0.48	<0	<0	0.67	1.26	1.95	0.64
6	0.52	<0	<0	0.32	1.21	1.93	0.51
7	0.61	<0	<0	0.15	1.33	1.94	0.50
8	0.42	<0	<0	<0	1.23	1.94	0.51
9	0.60	<0	<0	<0	1.22	1.95	0.51
10	0.55	<0	<0	0.46	1.26	1.97	0.51
11	0.73	<0	<0	0.21	1.43	2.00	0.54
12	1.03	<0	<0	1.39	1.33	1.99	0.52
13	0.96	<0	<0	<0	1.24	3.64	0.54
14	0.51	<0	<0	0.28	1.27	1.98	0.52
15	0.58	<0	<0	0.23	1.25	1.94	0.52
16	1.12	<0	<0	0.17	1.30	1.95	0.53
17	0.87	<0	<0	0.12	1.22	1.95	0.51
18	0.71	<0	<0	1.49	1.24	1.96	0.54
19	0.53	<0	<0	0.23	1.22	1.96	0.52

20	0.60	<0	<0	0.36	1.25	1.99	0.55
21	0.43	<0	<0	0.13	1.24	1.95	0.56
22	0.98	<0	0.07	1.58	1.46	2.06	0.52
23	0.88	<0	<0	<0	1.24	2.16	0.51
24	1.18	<0	<0	0.36	1.33	1.98	0.58
25	1.02	<0	<0	0.57	1.48	1.99	0.65
26	1.35	<0	<0	0.50	1.33	1.98	0.56
27	0.95	<0	<0	<0	1.27	1.96	0.54
28	1.56	<0	<0	0.37	1.46	2.06	0.53
29	0.64	<0	<0	0.64	1.23	1.93	0.51
30	0.89	<0	<0	0.11	1.25	3.39	0.52
31	0.55	<0	<0	0.68	1.44	1.95	0.51
32	0.81	<0	1	0.81	1.78	2.01	0.53
33	0.48	<0	<0	0.51	1.24	2.46	0.51

3.2. Discussion

The analytical method was validated in conformity to the SANCO 12571/2013, results are presented in Tables 1 and 3. The matrix-matched curves showed good linearity ($r^2 > 0.99$) for all the analytes. The concentrations of the analytes were obtained directly from the matrix calibration curve with the use of internal standards. The selectivity of the method was found to be gratifying with no interference peaks from endogenous compounds in the retention time of the target analytes in honey samples. Precision, expressed as the repeatability, gave the RSD values in agreement with the SANCO criteria of RSD ≤20%. The RSD, were in the range of 6.3–12.77% for honey samples. Satisfactory average recoveries were calculated used of the internal standards. The average recovery result ranged 63.04–103.19% for honey samples, and is in accordance with the SANCO validation guideline of recovery, which should be in the range of 60–140%.

The results of analysis of honey samples: no neonicotinoid pesticide residues were detected above the maximum residue limits in honey samples collected from Tekirdag province and its surroundings. Among the possible reasons, a neonicotinoid pesticide type drug is not used in and around Tekirdag. This may have been caused by agricultural producers' avoidance of the use of neonicotinoid pesticides, as some countries in Europe have banned or restricted the use of neonicotinoid pesticides.

Previous studies published about the confirmation method and validation of the residues of neonicotinoids in honey are summarised below.

In a study by Kavanagh et al. in Irish honey samples, imidacloprid was found to be the most common neonicotinoid

(found in 13.43% of honey samples), followed by clothianidin (12.40%) and thiacloprid (11.37%). They concluded that the frequency of imidacloprid in honey samples may not be limited to its use in the agricultural field but may also occur due to its presence in a range of commercial products used in sports and recreational lawn care products, herb care homes, home gardens, and locally public parks [17].

In Austria, acetonitrile extraction and dispersive solid-phase extraction (QuEChERS type) were used in Tanner and Czerwenka's analytical method to detect neonicotinoid residues in honey. Residues of acetamiprid, thiacloprid, and thiamethoxam were detected in Austrian honey samples; however, no sample exceeded the maximum residue limits. Flower honey samples contained more neonicotinoid residues than forest honey samples [18]. It is seen that the level of neonicotinoid pesticide residues in honeys of Austria detected in this study are below the maximum residue limits in line with the results obtained from our study.

Ligor et al. developed a method using QuEChERS extraction and UHPLC/UV to determine neonicotinoid residues in honey samples. The method was applied to honey collected from Poland and other countries. 53 honey samples were analysed, and neonicotinoids were detected at concentrations higher than the LOQ in 19 honey samples from Australia (3 samples), Brazil (1 sample), Italy (1 sample), and Poland (12 samples). No neonicotinoid residues were detected in the Turkish honey sample [19]. The absence of neonicotinoid residues in the analysis of honey samples from Turkey seems to be in line with the result of our study.

In the study by Woodcock, we evaluated the effectiveness of this policy in reducing the risk of exposure to honeybees by collecting 130 honey samples from beekeepers in the UK before (2014: $N = 21$) and after (2015: $N = 109$) the enactment of the moratorium. Neonicotinoids were present in approximately half of the honey samples taken before the moratorium and in more than one-fifth of the honey samples taken after the moratorium. Clothianidin was the most frequently detected neonicotinoid [20].

A 3-year field study was conducted in France from 2002 to 2005 to examine pesticide residues found in colonies and honeybee (*Apis mellifera L.*) colony health by Chauzat et al. No pesticide residues were detected in 12.7% of the sampling periods. It was reported that no statistical relationship was found between colony mortality and pesticide residues. Imidacloprid residues were frequently detected in pollen, honey, and honeybee samples [21].

Mrzlikar et al. developed a reliable analytical method using two extraction techniques (SPE, QuEChERS) and LC-MS/MS (SRM) for five neonicotinoids in 51 honey samples collected between 2014 and 2016. Despite being banned in the country in 2011, residues of acetamiprid and thiacloprid were detected in low contamination [12].

An average of 8.2 $ng \cdot g^{-1}$ clothianidin and 17.2 $ng \cdot g^{-1}$ thiamethoxam were detected in 68% and 75% of honey samples, respectively, from hives located 30 km from Saskatchewan City in Canada. Moreover, clothianidin was found in >50% of bee and pollen samples. Imidacloprid was detected in ~30% of honey samples [11].

In a study by Han et al., a total of 94 honey samples were selected from the Chinese market, based on the production region and sales volume in 2020. Neonicotinoids and their metabolites were detected in 97.9% of honey samples. Acetamiprid, thiamethoxam, and imidacloprid were the top three neonicotinoids in honey with detection frequencies of 92.6%, 90.4%, and 73.4%, respectively [22].

A study conducted in North America from 2007 to 2008 examined the effects of pesticides on the health of bee colonies. 1% of 208 wax samples, 17.7% of 350 pollen samples, and 0.0% of 140 honey samples were detected as having imidacloprid residues [23].

Residues of neonicotinoids were investigated in honey, pollen, and bee samples sampled in Greece between 2011 and 2013, while any residue did not detect in the honey samples. However, 0.7–14.7 $ng \cdot g^{-1}$ clothianidin in bee samples in 2011, 6.1–69.04 $ng \cdot g^{-1}$ in pollen samples, and 2.7–39.9 $ng \cdot g^{-1}$ was detected in 2012 bee samples and 308.3–1273 $ng \cdot g^{-1}$ clothianidin in pollen samples [24]. The absence of neonicotinoid residues in the analysis of honey samples from Turkey seems to be in line with the result of our study.

Residues of neonicotinoid products restricted in the European Union were not found in honey samples from Tekirdag on the European side of Turkey. According to the results of the study, we can say that the use of neonicotinoid products has decreased in our country.

4. Conclusions

According to the analysis results of 33 sunflower honey samples collected from around and Tekirdag province are free of any neonicotinoid residues exceeding the maximum residue limits were detected. It was understood that in further studies, more honey samples should be analysed as well as other hive products.

Disclosure

An earlier version of the manuscript has been presented as a preprint in the following link: https://www.researchsquare.com/article/rs-1683983/v1 [25]. 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.

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DETAILS

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Change in Serum Uric Acid is a Useful Predictor of All-Cause Mortality among Community-Dwelling Persons

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ABSTRACT (ENGLISH)

There is limited research on the association between longitudinal variability in serum uric acid (SUA) and all-cause mortality in the general population, although recent studies have suggested that changes in SUA are associated with all-cause mortality in adults. This study aims to examine the association between percentage change in SUA (%dSUA = 100 × (cohort 2 SUA − cohort 1 SUA)/(time × cohort 1 SUA) and all-cause mortality. This study is based on 1,301 participants, of whom 543 were male (63 ± 11 years) and 758 were female (63 ± 9 years). We obtained adjusted relative risk estimates for all-cause mortality and used a Cox proportional hazards model, adjusted for possible confounders, to determine the hazard ratio (HR) and 95% confidence interval (CI) of %dSUA. Of all the participants, 79 (6.1%) were deceased, and of these, 45 were male (8.3%) and 34 were female (4.5%). The multivariable-adjusted HRs (95% CI) for all-cause mortality for the first, second to fourth (reference), and fifth %dSUA quintiles were 3.79 (1.67–8.48), 1.00, and 0.87 (0.29–2.61) for male participants and 4.00 (1.43–11.2), 1.00, and 1.19 (0.46–3.05) for female participants, respectively. Participants with a body mass index of <22 kg/m^2 had a significantly higher HR, forming a *U*-shaped curve for the first (HR, 7.59; 95% CI, 2.13–27.0) and fifth quintiles (HR, 2.93; 95% CI, 1.05–8.18) relative to the reference. Percentage change in SUA is independently and significantly associated with future all-cause mortality among community-dwelling persons.

FULL TEXT

DETAILS

Subject:	Triglycerides; Diabetes; Uric acid; Body mass index; Mortality; Kidney diseases; Gender; High density lipoprotein; Females; Cholesterol; Metabolism; Statistical analysis; Medical prognosis; Age; Confidence intervals; Blood pressure; Hypertension; Males; Body size; Cardiovascular disease; Antihypertensives; Statistical models
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Comparative Pharmacokinetic Study of 5 Active Ingredients after Oral Administration of Zuogui Pill in Osteoporotic Rats with Different Syndrome Types

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ABSTRACT (ENGLISH)

Zuogui Pill is a kidney-yin-tonifying formula in traditional Chinese medicine that is widely used to manage osteoporosis with kidney-yin-deficiency in China. Herein, an efficient and accurate high-performance liquid chromatography-tandem mass spectrometry method was developed to determine the concentrations of 5 bioactive compounds in rat plasma following oral administration of Zuogui Pill. Because drug absorption and distribution differ under physiological and pathological conditions, the established method was used to quantify blood components and dynamic change in osteoporotic rats with different syndrome types. Moreover, integrated pharmacokinetic study was conducted to describe the overall pharmacokinetic characteristics of traditional Chinese medicine. The results showed that the absorption, distribution, and metabolism of Zuogui Pill varied widely under different states. The bioavailability of most active components showed significant advantages in osteoporotic rats with kidney-yin-deficiency, which corresponds to the opinion that Zuogui Pill has the effect of nourishing kidney-yin. It is hoped that this finding could interpret the pharmacodynamic substances and mechanism of Zuogui Pill in the treatment of osteoporosis with kidney-yin-deficiency.

FULL TEXT

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1. Introduction

Osteoporosis is a progressive bone remolding disease characterized by osteopenia and bone structure deterioration. The essence of osteoporosis is an imbalance between bone formation and bone resorption, which can cause skeletal fragility, increased fracture risk, and post-fracture mortality [1]. Recent evidence shows that the incidence of osteoporosis has been increasing year by year. Global osteoporosis-induced hip fracture patients are expected to increase by 550% by 2050 [2, 3]. Hormone replacement therapy (HRT) is currently proven to be effective in preventing bone loss and lowering the risk of fractures in osteoporotic patients [4, 5]. Nonetheless, long-term HRT has been linked to an increased risk of a variety of diseases [6, 7]. Concerns about the adverse effects of HRT have heightened interest in developing alternative strategies for osteoporosis treatment, and traditional Chinese medicine (TCM) has attracted considerable interest due to its superior curative effect and lower side effects. The TCM theory points out that the prevalence of osteoporosis is basically kidney deficiency; clinically, it mainly manifests as kidney-yang-deficiency and kidney-yin-deficiency syndrome [8–10]. As a result, kidney-tonifying

is a common and traditional strategy for treating bone diseases such as osteoporosis with TCM herbal formulas that have kidney-tonifying activity.

Zuogui Pill is a classic kidney-yin-tonifying formula which was developed by a famous TCM doctor Jingyue Zhang during the Ming dynasty (1563–1640 AD). The formula consists of Rehmanniae Radix Praeparata, Dioscoreae Rhizoma, Lycii Fructus, Corni Fructus, Cyathulae Radix, Cuscutae Semen, Cervi Cornus Colla, and Testudinis Carapacis et Plastri Colla. The majority of TCM in this formula have similar and synergistic kidney-yin-tonifying functions and thus have the potential to benefit bone health [11, 12]. Many bioactive ingredients in Zuogui Pill, including aucubin, loganin, chlorogenic acid, and morroniside can promote osteoblast differentiation, reduce calcitonin levels, and inhibit osteoblast apoptosis and osteoclast proliferation [13–17]. Meanwhile, protocatechuic aldehyde has been shown to be effective in the prevention and treatment of osteoporosis [18, 19]. Therefore, we selected the 5 bioactive components of Zuogui Pill (Figure 1) for further study.

[figure(s) omitted; refer to PDF]

Mounting evidence shows that differences in body conditions can influence drug absorption and distribution [20, 21]. From the perspectives of serum pharmacochemistry research ideas, only the components that can be absorbed are important for clinical effects [22]. Thus, studying pharmacokinetic properties in rats under pathological and normal conditions may reveal the mechanism of Zuogui Pill in the treatment of osteoporosis. Up to now, the methods for qualitative and quantitative detection of chlorogenic acid, loganin, and morroniside in Zuogui Pill have been established [23, 24]. There is no pharmacokinetic study on the active ingredients of Zuogui Pill in rat plasma. In this study, we developed an efficient and rapid HPLC-MS/MS method for detecting 5 components in plasma. Following that, the developed method was successfully used to compare the pharmacokinetic characteristics of Zuogui Pill after oral administration in normal rats, osteoporotic rats, and osteoporotic rats with kidney-yang-deficiency and kidney-yin-deficiency syndrome. Due to the complex composition of TCM compound, any single component poses limitations in describing the overall pharmacokinetic characteristics of TCM. Consequently, a study of integrated pharmacokinetics in each syndrome was conducted for systematic evaluation [25].

2. Materials and Methods

2.1. Chemicals and Reagents

Rehmanniae Radix Praeparata, Dioscoreae Rhizoma, Lycii Fructus, Corni Fructus, Cyathulae Radix, Cuscutae Semen, Cervi Cornus Colla, and Testudinis Carapacis were purchased from Huadong Pharmaceutical (Hangzhou, China) and confirmed by Professor Rusong Zhang (School of Pharmaceutical Science, Zhejiang Chinese Medical University).

Aucubin, loganin, protocatechuic aldehyde, chlorogenic acid, and tinidazole (internal standard, IS) (purity > 98%) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Morroniside (purity > 98%) was procured from Beijing Yanxinlv Biological Technology (Beijing, China). Methanol, acetonitrile, ammonium formate, and formic acid (HPLC-grade) were obtained from Merck (Darmstadt, Germany). Hydrocortisone injections were provided by Jinyao Amino Acid Co. Ltd. (Tianjin, China). Thyroxine tablets were supplied by Great Wall Pharmaceutical Co. Ltd. (Shanghai, China). Ultrapure water was purchased from Wahaha Co. Ltd. (Hangzhou, China).

2.2. Preparation of Zuogui Pill Extract

The Zuogui Pill extract was prepared using a previously described method [26]. The raw materials, which included Rehmanniae Radix Praeparata (24 g), Dioscoreae Rhizoma (12 g), Lycii Fructus (12 g), Corni Fructus (12 g), Cyathulae Radix (9 g), Cuscutae Semen (12 g), Cervi Cornus Colla (12 g), and Testudinis Carapacis (12 g), were sliced and soaked in a 10-fold volume of 50% ethanol for 60 min. After that, a 30 min ultrasonic extraction was performed twice at 45°C. The extract solution was filtered and condensed to a 4 g/ml concentration.

2.3. Animals and Establishment of Osteoporosis Models

Female Sprague-Dawley rats (180–220 g) were purchased from Shanghai Laboratory Animal Centre (Shanghai, China). The rats were maintained in a standard environment (22 ± 1°C, 60 ± 10% relative humidity, and a 12 h light/dark cycle) for one week to acclimate and fed water and food ad libitum. Twenty-four rats were divided

randomly into three groups and treated through ovary enucleation to establish the model of osteoporosis [27]. Ten weeks after operation, osteoporosis models of kidney-yang-deficiency and kidney-yin-deficiency were established by intramuscular injection of hydrocortisone (25 mg/kg) and intragastric administration of thyroxine suspension (160 mg/kg) for 10 days, respectively. The femur of two rats from each group was then stained with hematoxylin and eosin (H &E) to assess histopathological change. Also, the validation of osteoporosis kidney-yang-deficiency and kidney-yin-deficiency models was judged by detecting the level of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in rat plasma [28]. Laboratory Animal Management and Ethics Committee of Zhejiang Chinese Medical University approved all animal treatments (ZSLL-2014-48).

2.4. Preparation of Standards and Quality Controls

The standards of 5 active compounds and IS were weighed and solubilized in methanol to 1.0 mg/mL. To make working solutions, stock solutions were diluted with methanol, and the IS working solutions were diluted in a methanol : acetonitrile ratio of 1 : 1 at a final concentration of 50 ng/mL.

To obtain calibration standards, 10 μL working solutions were added in 90 μL blank plasma. The final concentrations were 1–1000 ng/mL for chlorogenic acid, 20–20000 ng/mL for loganin, 4–4000 ng/mL for morroniside, 0.5–500 ng/mL for aucubin, and 0.05–50 ng/mL for protocatechuic aldehyde.

Quality control (QC) samples were prepared at the following concentrations: 2, 40, and 800 ng/mL for chlorogenic acid, 40, 800, and 16000 ng/mL for loganin, 8, 160, and 3200 ng/mL for morroniside, 1, 20, and 400 ng/mL for aucubin, and 0.1, 2, and 40 ng/mL for protocatechuic aldehyde.

2.5. Sample Preparation

One hundred microliters of plasma was mixed with 100 μL of the IS solution, followed by 700 μL of methanol:acetonitrile (1 : 1). After 2 min of vortex mixing, the mixture was spun for 15 min at 8000 rpm and 4°C. The supernatants were collected and dried in a high-purity N_2 environment. The residues were redissolved in 100 μL methanol and centrifuged for 15 min at 13000 rpm and 4°C. Finally, an aliquot of 10 μL supernatants was used to conduct the analysis.

2.6. Instrument and Analytical Conditions

An Agilent 1290 HPLC system (Agilent, California, USA) was used in conjunction with an AB Sciex Q-TRAP 5500 MS/MS system (AB Sciex, Foster City, USA) equipped with an ESI source to measure the 5 components.

The LC separation was performed on a Waters Atlantis C18 column (3.0 mm × 150 mm, 3 μm) with a mobile phase of 5 mmol/L ammonium formate in 0.05% formic acid aqueous solution (A) and acetonitrile (B) flowing at a rate of 0.3 mL/min. The gradient elution program was performed as follows: 0-1 min, 90% A; 1–5 min, 90–20% A; 5–10 min, 20% A; 10–10.1 min, 20–90% A; and 10.1–12 min, 90% A. The column temperature was 20°C.

The mass spectrometer was set to detect in a negative mode under the following conditions: ion spray voltage, 4500 V; curtain gas, 40 psi; source temperature, 650°C; and collision gas, medium. The analytes were quantified using multiple reaction monitoring, and the optimized parameters are listed in Table 1.

Table 1

Mass spectrometric parameters of 5 analytes and IS.

Analytes	*Q*1 (m/z)	*Q*3 (m/z)	DP (V)	EP (V)	CE (eV)	CXP (V)
Chlorogenic acid	353.1	191.0	−68	−10	−24	−17
Loganin	389.1	227.0	−78	−7	−36	−15
Morroniside	451.1	243.0	−50	−7	−23	−22
Aucubin	345.0	183.0	−50	−6	−21	−15

Protocatechuic aldehyde	137.0	107.9	−37	−10	−30	−10
IS	292.0	126.1	−52	−11	−17	−12

2.7. Method Validation

The methods were validated in accordance with US Food and Drug Administration guidelines for bioanalytical method validation [29].

2.7.1. Specificity

Chromatograms of blank biological samples from six rats, plasma samples containing standards, and plasma samples after administration of Zuogui Pill extract were compared to assess selectivity.

2.7.2. Linearity and Sensitivity

Calibration curves were constructed by drawing peak area ratios of 5 compounds to IS against nominal concentrations using least squares regression (weighted factor: $1/x^2$). The lower limit of quantification (LLOQ) was defined as the lowest level on calibration curves with a signal-to-noise ratio (S/N) = 10 used to demonstrate method sensitivity.

2.7.3. Accuracy and Precision

Six batches of QC samples at three levels and LLOQ were used to determine accuracy and precision. To determine intra-day accuracy and precision, samples were analysed on a single assay day, while inter-day accuracy and precision were determined over three consecutive days.

2.7.4. Extraction Recovery and Matrix Effect

To determine extraction recovery, peak areas of analytes in plasma samples were compared to post-extraction plasma containing pure analyte standards at corresponding concentrations. The matrix effect was calculated by comparing the peak areas of analytes added to blank plasma after extraction to those of standards liquefied in methanol at comparable concentrations.

2.7.5. Stability

QC samples at three levels using five replicates were used to evaluate the stability under the following conditions: 4 h at room temperature (25°C), 24 h in autosampler (4°C), and three freeze-thaw cycles (−20°C to 25°C).

2.8. Pharmacokinetic Study and Statistical Analysis

Four groups of rats were orally administered with Zuogui Pill extract at a dose of 1.5 mL/100 g. Then, 0.3 mL blood samples were collected from orbital veins at 0, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after administration, placed in an anticoagulant tube, and centrifuged for 15 min at 5000 rpm and 4°C. The plasma was prepared and stored at −20°C until analysis.

Phoenix WinNonlin 6.4 software was used to estimate pharmacokinetic parameters using noncompartmental analysis. Furthermore, SPSS 26 software was used to compute comparisons of the pharmacokinetic data among the four groups according to a one-way ANOVA. The results are presented as mean ± SD.

2.9. Integrated Pharmacokinetic Study

Integrated pharmacokinetic study was conducted based on the area under the concentration-time curve from zero to infinity ($AUC_{0-\infty}$) of each component [30, 31]. The ratio of $AUC_{0-\infty}$ of 5 components to total $AUC_{0-\infty}$ was defined as the weight coefficient (ω) in the integrated concentration using equations (1) and (2). Formula (3) was used for obtaining the integrated concentration of the 5 components, where C1–C5 represent the concentration of the five components, and the integrated pharmacokinetic parameters were further calculated.(1)$\omega_j = \frac{AUC_{0-\infty j}}{\sum_1^5 AUC_{0-\infty}}$,(2)$\sum_1^5 AUC_{0-\infty} = AUC_{0-\infty 1} + AUC_{0-\infty 2} + AUC_{0-\infty 3} + AUC_{0-\infty 4} + AUC_{0-\infty 5}$,(3)$C_T = \omega_1 \times C_1 + \omega_2 \times C_2 + \omega_3 \times C_3 + \omega_4 \times C_4 + \omega_5 \times C_5$.

3. Results and Discussion

3.1. Evaluation of Osteoporosis Models

H &E staining revealed that the subchondral bone and trabecula were intact and arranged regularly in the normal rats. Osteocytes in trabecular bone were clearly visible, and the nuclei were centrally located and large. The bone marrow was rich in hematopoietic cells, with relatively few adipocytes in the medullary cavity and normal

morphology, while the trabecular bone area and the number of hematopoietic cells were significantly reduced in the osteoporosis group. In addition, the volume of adipocytes increased and even fused into vesicles (Figure 2), both of which confirmed the successful establishment of the osteoporosis model.

[figure(s) omitted; refer to PDF]

The contents of cAMP and cGMP in rat plasma are covered in Table 2. After modelling, the plasma content of cAMP in the osteoporotic rats with kidney-yang-deficiency significantly decreased ($P<0.01$), while that of cGMP increased ($P<0.01$) compared with the osteoporosis group. Also, the levels of plasma cAMP and cGMP showed the opposite trend in kidney-yin-deficiency rats. The increase or decrease of the cAMP/cGMP ratio indicated that the two types of osteoporosis models were successfully established.

Table 2

Plasma concentrations of cAMP and cGMP in rats ($n=6$).

Groups	cAMP (nmol/L)	cGMP (nmol/L)	cAMP/cGMP
Normal	101.7 ± 5.971	25.62 ± 3.176	3.901 ± 0.327
Osteoporosis	100.4 ± 3.302	26.46 ± 1.498	3.805 ± 0.233
Kidney-yang-deficiency	85.30 ± 5.061**	35.84 ± 3.170**	2.393 ± 0.234**
Kidney-yin-deficiency	119.1 ± 9.349*	23.20 ± 1.357*	5.129 ± 0.509**

*$p<0.05$ and **$p<0.01$, according to *t*-test, versus osteoporosis rats.

3.2. Operational Condition Optimization

Considering the low drug concentration in plasma due to its first-pass effect, QTOF-MS was used for analysis in the pre-experiment. However, the sensitivity of QTOF-MS cannot meet the quantitative requirements; therefore, Q-TRAP MS was employed for detection in the end.

Different types of columns were tested, including Agilent ZORJBAX Extend C_{18} column (4.6 mm × 150 mm, 5 μm), Agilent ZORJBAX SB C_{18} column (4.6 mm × 150 mm, 5 μm) and Waters Atlantis C_{18} column (3.0 mm × 150 mm, 3 μm), and it turned out that Waters Atlantis C_{18} column (3.0 mm × 150 mm, 3 μm) could present a better peak shape and isolation. Acetonitrile, methanol, and several volatile acid-base additives in the aqueous phase such as formic acid, ammonia, ammonium formate, and ammonium acetate were evaluated to optimize the chromatography conditions. Finally, acetonitrile and 5 mmol/L ammonium formate in 0.05% formic acid aqueous solution were chosen as the mobile-phase system.

Because the response value of analytes to be measured was higher in the negative ion ionization state than in the positive mode, the former was used for the assay. Figure 3 depicts the ion mass spectrums.

[figure(s) omitted; refer to PDF]

3.3. Concentrations of the 5 Compounds in Zuogui Pill

The contents of 5 analytes were evaluated according to the method described in Section 2.6. The contents of chlorogenic acid, loganin, morroniside, aucubin, and protocatechuic aldehyde in Zuogui Pill were 33.2, 479, 411, 16.2, and 5.61 μg/mL, respectively.

3.4. Method Validation

3.4.1. Specificity

Figure 4 shows chromatograms of different plasma, including blank plasma, blank plasma with standards, and plasma sample from a rat 30 min after oral administration. The results demonstrated that endogenous plasma components did not influence the test substance or internal standard. Also, the IS response in the blank sample was no more than 5% of the average IS responses of the calibrators and QCs.

[figure(s) omitted; refer to PDF]

3.4.2. Linearity and Sensitivity

Analyte calibration curves were linear over their respective ranges, with correlation coefficients (*R*) greater than 0.9990 for all sequences. The data in Table 3 demonstrated that the linearity and LLOQ were suitable for assay with acceptable precision and accuracy.

Table 3

Calibration curves, correlation coefficients, linear ranges, and LLOQs of 5 compounds.

Compounds	Calibration curves	Correlation coefficients (*r*)	Linear range (ng/mL)	LLOQ (ng/mL)
Chlorogenic acid	$y=0.0226x-0.0985$	0.9997	1–1000	1
Loganin	$y=0.0023x+0.0297$	0.9996	20–20000	20
Morroniside	$y=0.0114x+0.1404$	0.9993	4–4000	4
Aucubin	$y=0.0016x-0.0175$	0.9990	0.5–500	0.5
Protocatechuic aldehyde	$y=0.1696x+2.2261$	0.9992	0.05–50	0.05

3.4.3. Accuracy and Precision

Table 4 shows the results of the inter- and intra-day accuracy and precision of the 5 components. The precision was less than 15%, while the accuracy was 2.3% to 18.6% for intra-day assay and 2.5% to 18.0% for inter-day assay. All the data showed that the method was reliable and accurate.

Table 4

Accuracy and precision of 5 analytes in rat plasma ($n=6$).

Analytes	Concentration (ng/mL)	Accuracy (%)		Precision (%)	
Intra-day	Inter-day	Intra-day	Inter-day	Chlorogenic acid	1
18.6	18.0	12.4	9.5	2	12.1
14.3	10.0	6.1	40	10.2	11.0
10.3	7.6	800	6.7	8.1	3.9
8.2	-				
Loganin	20	17.4	14.2	10.6	13.7

40	13.8	12.1	7.1	10.9	800
7.5	5.6	6.4	5.8	16000	2.3
2.5	3.9	4.1	-		
Morroniside	4	13.3	16.6	10.6	12.0
8	11.1	9.4	8.7	8.7	160
6.6	9.2	7.3	2.9	3200	3.4
8.6	5.9	3.1	-		
Aucubin	0.5	13.0	14.7	10.4	10.5
1	12.5	9.0	7.5	8.0	20
3.7	7.1	5.8	5.3	400	4.0
5.3	3.7	4.9	-		
Protocatechuic aldehyde	0.05	11.4	14.6	10.2	13.8
0.1	8.0	13.2	9.6	9.1	2
7.7	13.2	7.4	8.4	40	6.2

3.4.4. Extraction Recovery and Matrix Effect

The extraction recovery of the analytes at different selected levels ranged from 65.0% to 91.9%, and the average matrix effect at the same concentrations ranged from 76.3% to 94.4% (Table 5). The absence of matrix components in plasma results in a striking variation in the mass spectrum response to analytes and IS, which is consistent with the analytical requirements of the biological sample.

Table 5

Recover and matrix effect of 5 analytes in rat plasma (*n*=6).

Analytes	Concentration (ng/mL)	Recovery (%)		Matrix effect (%)	
Mean	RSD	Mean	RSD	Chlorogenic acid	2
65.0	8.5	85.3	6.1	40	88.2
4.6	90.7	4.4	800	78.0	10.3

86.8	4.7	-			
Loganin	40	81.3	3.3	76.3	14.9
800	87.6	9.7	93.7	5.9	16000
91.9	7.0	86.5	3.7	·	
Morroniside	8	80.3	1.8	92.0	8.3
160	82.2	6.6	76.7	5.4	3200
84.1	10.7	85.5	1.9	·	
Aucubin	1	76.7	4.3	80.7	6.1
20	77.7	3.3	94.4	14.4	400
88.0	2.6	91.8	8.9	·	
Protocatechuic aldehyde	0.1	85.4	2.7	81.5	6.1
2	85.8	9.6	79.4	6.0	40

3.4.5. Stability

The stability analysis of the 5 compounds revealed no obvious degradation of components detected in rat plasma under the above storage conditions with RSD below 15% (Table 6).

Table 6

Stability of 5 analytes in rat plasma under various conditions (n=5).

Analytes	Concentration (ng/mL)	Room temperature for 4 h (%)		Autosampler at 4°C for 24 h (%)		Three freeze-thaw cycles (%)	
Mean	RSD	Mean	RSD	Mean	RSD	Chlorogenic acid	2
86.5	6.0	85.1	4.6	89.1	5.1	40	85.9

2.2	93.7	3.8	89.3	7.7	800	85.7	4.2
87.3	6.2	80.9	10.9	-			
Loganin	40	88.3	9.5	94.6	5.1	86.4	12.4
800	87.4	6.1	85.8	7.0	89.5	6.9	16000
85.6	11.3	84.4	3.9	92.4	3.3	·	
Morroniside	8	90.8	9.1	89.6	5.8	93.0	2.7
160	86.1	7.5	87.6	4.6	91.5	6.3	3200
98.3	3.4	95.0	2.1	89.8	7.7	·	
Aucubin	1	89.8	2.8	88.6	4.7	84.2	9.0
20	91.3	8.0	92.5	7.4	86.9	8.3	400
92.8	6.6	90.2	5.8	89.1	6.1	·	
Protocatechuic aldehyde	0.1	86.8	2.5	88.4	8.9	90.8	6.5
2	90.9	3.3	89.3	6.5	83.4	5.7	40

3.5. Pharmacokinetic Analysis

The validated method was used for pharmacokinetic assays of the 5 active compounds in plasma following oral administration of Zuogui Pill. Figure 5shows the mean plasma concentrations versus time profiles of 5 compounds and integration under various syndrome types.Table 7 displays the pharmacokinetic variables of the 5 compounds as well as the integrated pharmacokinetic variables.

[figure(s) omitted; refer to PDF]

Table 7

Pharmacokinetic parameters of the 5 compounds in rats after oral administration of Zuogui Pill extract and integrated pharmacokinetic parameters (*n*=6).

Compounds	Groups	$t_{1/2}$(h)	T_{max}(h)	C_{max}(ng/mL)	AUC_{0-t}(h*ng/mL)	$AUC_{0-\infty}$(h*ng/mL)	CL_F_obs (mL/h/kg)	MRT_{0-t}(h)	$MRT_{0-\infty}$(h)
Chlorogenic acid	Normal	8.17±1.79	0.25±0.00	346.14±63.99	497.98±34.72*	546.26±38.80	914.49±64.88	5.46±0.48*	8.17±1.46
Osteoporosis	6.38±1.96	0.26±0.02	279.27±56.34	435.76±59.78	489.67±77.69	1036.09±154.41	6.78±0.68	9.72±2.61	Yang-deficiency
5.41±1.84	0.25±0.00	430.29±98.55**	263.13±40.25**	284.31±33.34**	1767.90±187.68**	4.52±1.46	6.56±2.64*	Yin-deficiency	6.61±3.84
0.25±0.00	353.83±83.80	218.55±23.29**	225.89±25.03**	2223.36±229.72**	3.03±0.38**	4.10±1.02**	·		
Loganin	Normal	4.51±0.45**	0.25±0.00	13508.61±2029.85**	20134.29±3958.79	20523.97±3947.05	361.54±73.02	4.58±0.47	5.10±0.41
Osteoporosis	2.74±0.96	0.25±0.00	8381.77±1123.47	17912.12±6898.16	17998.56±6945.75	460.13±196.92	4.91±1.29	5.02±1.34	Yang-deficiency
5.62±1.63**	0.21±0.05	14.72±1.38**	27.46±4.83**	28.61±4.91**	257023.69±42243.40**	5.50±0.67	6.62±0.49	Yin-deficiency	3.75±0.70
0.25±0.00	16025.83±2787.58**	41028.61±6356.26**	41727.10±6203.34**	176.22±32.93	6.51±0.91**	6.92±0.98	·		
Morroniside	Normal	3.66±1.18 *	0.29±0.10	1719.66±410.02**	2151.00±498.88	2171.33±509.45	2966.14±684.64*	3.41±0.33**	3.66±0.48*
Osteoporosis	2.40±0.51	0.26±0.02	1109.11±163.31	1724.77±149.05	1741.55±136.08	3553.71±268.04	4.37±0.43	4.51±0.38	Yang-deficiency
3.83±0.64**	0.19±0.04*	1450.08±337.06	1726.42±217.11	1750.35±215.20	3562.14±427.53	4.68±0.24	5.03±0.33	Yin-deficiency	2.90±0.65

0.24±0.03	1752.12± 321.11**	3764.53± 842.38**	3782.41± 832.52**	1700.88± 406.17**	5.08±0.74*	5.21±0.78*	·		
Aucubin	Normal	5.97± 2.89	0.38± 0.14**	84.43± 32.22	170.50± 64.82	204.69± 68.61	1358.04± 638.52	5.38± 2.01	8.58± 2.71
Osteoporosis	3.67± 0.78	0.17± 0.00	102.55± 23.62	116.40± 11.00	155.64± 24.54	1592.72± 276.68	3.06± 0.12	5.62± 1.09	Yang-deficiency
7.40±1.68**	0.25± 0.00*	95.99± 24.19	48.33± 14.05**	111.33± 29.94	2328.79± 701.23*	2.95±0.38	11.69± 1.31**	Yin-deficiency	8.68± 2.92**
0.25±0.00*	181.24± 30.66**	201.40± 32.43**	272.67± 70.63**	959.16± 279.31*	7.32±1.92*	14.14±5.85	·		
Protocatechuic aldehyde	Normal	10.87± 4.48	0.45±0.10	18.86± 3.62**	64.15± 10.30**	119.88± 32.93**	750.08± 184.83*	5.14± 0.17*	15.85 ±6.12
Osteoporosis	12.01± 4.69	0.30± 0.00	10.10± 1.99	34.92± 2.37	72.28± 21.64	1279.06± 400.34	5.52± 0.21	17.76± 6.26	Yang-deficiency
2.09±0.89*	0.25± 0.07	11.38± 1.31	24.70± 1.69**	28.68± 6.49**	3031.25± 535.72**	2.55±0.18**	3.50± 1.38*	Yin-deficiency	2.20± 0.83*
0.17±0.07	8.81± 1.61	27.48± 3.10**	28.87± 2.69**	2933.07± 265.58**	3.24± 0.37**	3.79±0.79*	·		
Integrated	Normal	4.51± 0.44**	0.25±0.00	11927.33± 1782.67**	17749.62± 3510.27	18091.33± 3477.17	390.40± 78.84	4.57± 0.47	5.09± 0.40
Osteoporosis	2.74± 0.95	0.25± 0.00	7474.98± 1002.61	16439.99± 6012.37	16516.89± 6056.71	472.07± 192.41	4.77± 1.27	4.87± 1.32	Yang-deficiency
3.91±0.65**	0.19± 0.04*	1210.34± 269.75**	1486.54± 179.09**	1507.82± 177.63**	3452.60± 389.82**	4.51±0.23	4.87± 0.33	Yin-deficiency	3.79± 0.64*

*p<0.05 and **p<0.01, according to a one-way ANOVA, versus osteoporosis rats.

Although the dosage of Zuogui Pill in each group was the same, the pharmacokinetic parameters of the four groups

were dramatically different. The elimination half-life ($t_{1/2}$) of chlorogenic acid, loganin, morroniside, and aucubin in the osteoporosis group was shorter than that in the normal group, indicating that they were eliminated faster in the osteoporosis state. Clearance (CL) for the 5 compounds was increased in osteoporotic rats. All the components were absorbed and reached their peak plasma concentration in 1 h. Aside from aucubin, the maximum plasma concentration (C_{max}) of the remaining four components was higher in normal rats than in osteoporotic rats. Furthermore, in the osteoporosis group, the $AUC_{0\text{–}t}$ and $AUC_{0\text{–}\infty}$ of all ingredients were reduced, particularly in protocatechuic aldehyde ($P<0.05$). By comparing the pharmacokinetic parameters in normal and osteoporosis groups, we found that the bioavailability of the 5 components in normal rats was higher than that in osteoporotic rats, while the removal rates of the 5 components in normal rats were lower.

The clearance of all analytes was statistically increased ($P<0.05$) in kidney-yang-deficiency rats, while the $AUC_{0\text{–}t}$ and $AUC_{0\text{–}\infty}$ of chlorogenic acid, loganin, aucubin, and protocatechuic aldehyde were significantly decreased ($P<0.01$), suggesting that the above components were poorly absorbed and rapidly metabolized in this state. Except for protocatechuic aldehyde, C_{max} of other compounds increased remarkably ($P<0.05$) in the kidney-yin-deficiency group when compared with the osteoporosis group. Increases in the value of mean residence time (MRT), $AUC_{0\text{–}t}$, and $AUC_{0\text{–}\infty}$ of loganin, morroniside, and aucubin were observed ($P<0.05$) in the kidney-yin-deficiency group. The results demonstrated that the three components had better absorption and a long duration, which may lead to a significant accumulation of active components in vivo. Thus, loganin, morroniside, and aucubin are likely to be the medicinal compositions in the treatment of osteoporosis with kidney-yin-deficiency in Zuogui Pill extract as reported in the literature [32].

The above changes in pharmacokinetic behaviours could be due to the fact that different types of osteoporosis potentially influence the absorption, distribution, and metabolism of drugs. One of the factors influencing the rate and quantity of drug absorption in osteoporosis is the change in gut microbiota and metabolite [33, 34]. The phenomenon that all the components showed double or multiple peaks in the mean plasma concentration-time profiles could be attributed to Zuogui Pill reabsorption in plasma and enterohepatic circulation following oral administration [35, 36].

3.6. Integrated Pharmacokinetic Analysis

TCM compounds are formulated in accordance with the composition theory of a TCM prescription, which contains complex chemical components that potentially interact with one another to influence their pharmacokinetic behaviours [37]. The results of our assay revealed significant differences in the pharmacokinetic parameters of 5 compounds, demonstrating that any single ingredient poses limitations in describing pharmacokinetic behaviour in rats. The integrated pharmacokinetic parameters, on the other hand, were more practical and reasonable. According to Figure 5 and Table 6, it was obvious that the bioavailability and effective drug duration of integrated ingredient showed significant advantages in the state of kidney-yin-deficiency by comparison with other groups, which was consistent with the TCM theory that Zuogui Pill has the effect of nourishing kidney-yin [38].

4. Conclusions

The presently developed HPLC-MS/MS method is efficient and effective in detecting the 5 analytes in plasma. Therefore, it was used to compare the pharmacokinetics of 5 components in different syndrome types of osteoporosis. The evaluation of the pharmacokinetic behaviours of the research objects may aid in better understanding the mechanism of the pharmacological action and provide references on the clinical application of Zuogui Pill.

Authors' Contributions

Jiawei Qiu and Yaoyao Zhu contributed equally to this study.

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DETAILS

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Comparative Study of the Flavonoid Content in Radix Scutellaria from Different Cultivation Areas in China

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ABSTRACT (ENGLISH)

Scutellariabaicalensis Georgi, an important perennial herb, is widely distributed and used all over the world. The root of *S. baicalensis* (*Radix Scutellaria*) is rich in flavonoids with a variety of bioactive effects and is widely used in clinic. The different geographical and climatic conditions of different cultivated areas of *S. baicalensis* lead to the differences of the main components in *Radix Scutellaria.* The main objective of this study was to evaluate the difference of flavonoid content in *Radix Scutellaria* from different cultivated areas in China. The mobile phase system, elution gradient, detection wavelength, and other chromatographic conditions for high-performance liquid chromatography-diode array detection (HPLC-DAD) determination of 8 flavonoids in *Radix Scutellaria* were optimized. The contents of flavonoids in 38 samples of *Radix Scutellaria* collected from seven main genuine cultivated areas were determined, and the correlation between the content, cultivated area, and the biological activities of *Radix Scutellaria* was compared. The results implied that baicalin, wogonoside, and baicalein were the

three main flavonoids with the highest contents in *Radix Scutellaria*. The content of flavonoids in different cultivated areas was very different, which had significant regionality and was closely related to the natural conditions of various places. The antioxidant and antitumor activities of the extract of *Radix Scutellaria* were closely related to the content of flavonoids, and high contents of baicalin, wogonoside, and baicalein positively improved biological activities.

FULL TEXT

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1. Introduction

Scutellaria baicalensis Georgi (Chinese skullcap) is a perennial herb belonging to genus *Scutellaria* (Labiatae). The traditional Chinese medicine *Radix Scutellaria* refers to the dry root of *S. baicalensis* and is one of the bulk medicinal materials commonly used all over the world [1, 2]. *Radix Scutellaria* had the effects of clearing away heat and dampness, purging fire and detoxifying, stopping blood gas, and calming fetus and had been used clinically for more than 2000 years in China [1–5]. *Radix Scutellariae* had also played an important role in *Qingfei Paidu Decoction* in the *National Diagnosis Treatment Protocol for COVID-19 in China* [6, 7].

Radix Scutellaria is rich in a variety of active ingredients, including flavonoids, glycosides, terpenoids, micronutrient, enzymes, sterols, and organic acids [1, 3, 8]. Flavonoids, typically baicalin, baicalein, wogonin, and wogonoside, are the main active components and the material basis for the pharmacological activities of *Radix Scutellaria* [9–12]. Modern research showed that flavonoids in *Radix Scutellaria* had antioxidant, antibacterial, antiviral, antitumor, anticancer, anti-inflammatory, and other pharmacological effects [1–5, 13–17] and could also improve the levels of monoamine transmitters and brain neurotrophic factors in the brain [18–20].

S. baicalensis has strong adaptability to the environment, is widely distributed and commonly used throughout the world, and is also the traditional herbal medicine in China, Mongolia, North Korea, and Japan [2–4]. *S. baicalensis* is geographically widespread in almost all provinces and regions of China, mainly in Hebei, Henan, Gansu, Inner Mongolia, Shaanxi, Shanxi, Shandong, and other places [11, 12, 21–23]. *S. baicalensis* mostly grows in the warm, cool, semi-humid, and semi-arid environment of middle and high mountains or plateau grassland with good sunshine. The diversity of growth environment and the different geographical and climatic conditions of different cultivated areas lead to the differences of the main components in *Radix Scutellaria* [12, 24, 25]. As an important industrial crop, *S. baicalensis* is widely cultivated in China, among which Gansu, Hebei, Henan, Shandong, Shaanxi, Shanxi, and Gansu are the genuine producing areas of *S. baicalensis* [11, 12, 23].

HPLC is a very widely used analytical method with the advantages of simple operation, high efficiency, and high sensitivity and is widely used in a variety of natural active substance content determination [26–28]. In this study, a method for simultaneous determination of 8 main flavonoids in *Radix Scutellaria* was established to evaluate the content differences of main flavonoids in *Radix Scutellaria* collected from different production areas and different planting environments. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were employed to select the key indexes affecting the quality of *Radix Scutellaria*, and the quality evaluation system of *Radix Scutellaria* was preliminarily established. The study provided research reference for the utilization of *Radix Scutellaria* and the evaluation of the quality of genuine medicinal materials and reference basis for the quality control and evaluation of *S. baicalensis* and related products.

2. Materials and Methods

2.1. Materials

The *Radix Scutellaria* material (Table 1), collected from different regions of China, was identified as the dry root of the medicinal plant *Scutellaria baicalensis* Georgi by Professor Dingxu Li, School of Agriculture, Henan University of Science and Technology. The geographical and climatic information of different cultivated areas is shown in Table S1.

Table 1

Collection information and numbers of *Radix Scutellaria* samples from different origins.

Samples	Cultivated areas	Location
HQ-1	Gansu Province (A)	Min County
HQ-2	Weiyuan County	HQ-3
Zhang County	HQ-4	Hexi District
HQ-5	Tanchang County	·
HQ-6	Inner Mongolia Autonomous Region (B)	Hohhot
HQ-7	Ningcheng County	HQ-8
Songshan District	HQ-9	Songshan District
–		
HQ-10	Shandong Province (C)	Juancheng County
HQ-11	Juancheng County	HQ-12
Laiwu District	HQ-13	Yishui County
HQ-14	Hedong District	HQ-15
Ju County	HQ-16	Ju County
HQ-17	Yinan County	HQ-18
Wulian County	HQ-19	Zhucheng city
–		
HQ-20	Shanxi Province (D)	Quwo County
HQ-21	Quwo County	HQ-22
Jiang County	HQ-23	Xiangfen County
HQ-24	Wenxi County	HQ-25
Wenxi County	HQ-26	Xinjiang County
–		

HQ-27		Yichuan County
HQ-28	Henan Province (E)	Yichuan County
HQ-29	Yiyang County	HQ-30
Yiyang County	HQ-31	Hui County
HQ-32	Heibei Province (F)	Anguo city
HQ-33	Anguo city	HQ-34
Weichang County	–	
HQ-35	Shaanxi Province (G)	Heyang County
HQ-36	Heyang County	HQ-37
Shangzhou District	HQ-38	Tongguan County

Reference substances (Figure 1), scutellarin (batch number: ST03110120), baicalin (ST01860120), scutellarein (ST06340120), wogonoside (ST08350120), baicalein (ST01870120), wogonin (ST01710120), chrysin (ST00270120), and oroxylin A (ST23660120), were obtained from Shanghai Standard Biotechnology Co. Ltd. with the purity over 98%.
[figure(s) omitted; refer to PDF]

2.2. Preparation of the Samples

The samples of *Radix Scutellaria* from different cultivated areas were dried in an oven at 80°C to constant weight and then were crushed with a high-speed pulverizer and sieved with 80 meshes. All samples were kept in sealed bags at room temperature for further research.

2.3. Preparation of Working Standard Solutions

The reference flavonoids of scutellarin, baicalin, scutellarein, wogonoside, baicalein, wogonin, chrysin, and oroxylin A were accurately weighed (8–10 mg) and dissolved in an appropriate amount of methanol, and the mixed reference stock solution was prepared with the mass concentration of 0.19 mg·mL^{-1}, 0.23 mg·mL^{-1}, 0.21 mg·mL^{-1}, 0.21 mg·mL^{-1}, 0.2 mg·mL^{-1}, 0.22 mg·mL^{-1}, 0.18 mg·mL^{-1}, and 0.19 mg·mL^{-1}, respectively. The reference standard solutions were obtained by diluting reference stock solution into six concentration gradients, comprising scutellarin of 19–209 μg·mL^{-1}, baicalin of 23–253 μg·mL^{-1}, scutellarein of 21–231 μg·mL^{-1}, wogonoside of 21–231 μg·mL^{-1}, baicalein of 20–220 μg·mL^{-1}, wogonin of 22–242 μg·mL^{-1}, chrysin of 18–198 μg·mL^{-1}, and oroxylin A of 19–209 μg·mL^{-1}. The reference standard solutions were filtered with 0.45 μm organic microporous filter membrane, and the filtrate was collected for HPLC analysis.

2.4. Preparation of Sample Solution

The sample powder (4.0 g) of *Radix Scutellaria* was accurately weighed and was placed in a 250 mL Soxhlet reflux device and extracted with 100 mL methanol solution in a constant temperature water bath at 85°C for 4 hours under reflux. The extract solution was cooled to room temperature, and then fixed the volume in a 100 mL volumetric flask with methanol. The solution was filtered by 0.45 μm organic microporous filter membrane, and then the filtrate was collected for HPLC analysis.

2.5. Apparatus and Chromatographic Conditions

An agilent 1100 high performance liquid chromatograph series system equipped with a quaternionic pump (G1311A), a diode array detector (DAD, G1315A/B), an online vacuum degassing device (G1322A), a column temperature box (G1316A), and an automatic sampler (G1313A) was employed in the determination. The chromatographic column was Agilent Zorbax Eclipse SB-AQ-C18 (250 mm × 4.6 mm, 5 μm). Mobile phase was acetonitrile (A)-0.1% phosphoric acid (B) with the gradient elution of 0 min (A, 30%) 5 min (A, 35%) 10 min (A, 40%) 15 min (A, 50%) 22 min (A, 50%) 35 min (A, 30%). The flow rate was 1.0 mL·min^{-1} with the detection wavelength of 280 nm, the column temperature of 30°C, and the injection volume of 10 μL.

The separated compounds were identified by comparing the retention time of the test sample with the reference sample. Based on the peak area corresponding to each component, the identified flavonoids were quantitatively analyzed by the external standard method according to the standard correction curve.(1)Y%=CR×Ax/AR×D×Vm× 100%,where *Y* is the content of sample; A_x is the peak area of test article; A_R is the peak area of control article; *D* is the dilution multiple of test article; C_R is the concentration of control sample (mg·mL^{-1}); *m* is the amount of test sample (g); and *V* is the volume of test sample (mL).

2.6. Method Validation Parameters

The contents of 8 flavonoids in *Radix Scutellaria* were determined by HPLC, and the linear relationship and linear range, stability, precision, repeatability, and sample recovery of the established method were evaluated. The calibration curve of each compound was constructed with the mixed reference standard solution containing 8 flavonoids, and the mixed reference standard solution was analyzed with the increase of gradient concentration of the reference standard solutions to investigate the linear range and calculate the regression parameters. The *least squares method* was used to calculate the regression equation between the chromatographic peak area (*y*) and the concentration (*x*) of 8 flavonoids, and the correlation coefficient (R^2) was used to reflect the correlation degree between the peak area and the concentration [12]. The methodological investigation of the determination method was further carried out through the determination of repeatability, stability, precision, and sample recovery according to the method of literature [9, 12, 29]. In order to verify the repeatability of the determination method, six different sample solutions prepared in parallel of *Radix Scutellaria* sample from the same origin (HQ-1) were analyzed, and the contents and relative standard deviation (RSD) values of eight flavonoids were calculated. The tested solution prepared above was stored at room temperature for 0, 2, 4, 8, 12, 16, and 24 hours, the samples were determined, and the contents and RSD values of eight flavonoids were calculated to test the stability of the method. The tested solution prepared above was continuously determined for six times, and contents and RSD values were obtained to test the precision of the method. The accuracy of the method was evaluated through the recovery rate experiment with 0.8 mL, 1.0 mL, and 1.2 mL of mixed control solution containing scutellarin (0.677 mg·mL^{-1}), baicalin (1.214 mg·mL^{-1}), scutellarein (0.261 mg·mL^{-1}), wogonoside (0.704 mg·mL^{-1}), baicalein (0.603 mg·mL^{-1}), wogonin (0.232 mg·mL^{-1}), chrysin (0.107 mg·mL^{-1}), and oroxylin A (0.077 mg·mL^{-1}), which were added to the known content of *Radix Scutellaria* samples, respectively. The flavonoids were extracted, the contents of the 8 flavonoids were determined under the chromatographic conditions above, and recovery and RSD values were calculated. The limit of detection (LOD) and limit of quantification (LOQ) were determined by continuously diluting the reference standard solution until the signal-to-noise ratio (S/N) was about 3 or 10, respectively.

2.7. Determination of Total Flavonoids

The contents of total flavonoids (TFs) in *Radix Scutellaria* from different location of cultivated areas were determined by UV spectrophotometry reported in literature [21]. The samples of *Radix Scutellaria* were extracted by the method described in Section 2.4, and the powder of *Radix Scutellaria* extract was obtained by vacuum concentration and weighed. The dried powder of *Radix Scutellaria* extract (5 mg) was weighed accurately and dissolved in methanol and then transferred into a 25 mL volumetric flask, and then methanol was added to obtain the test solution with a mass concentration of 0.2 mg·mL^{-1}. 0.8 ml of 0.1 mol·L^{-1} aluminum trichloride solution and 1 mL of 1 mol·L^{-1} potassium acetate solution were added into the test solution and then mixed well. After bathing in water at 20°C for 20 min, the absorbance of prepared test solution was measured at the wavelength of 415 nm, and methanol was

used as the blank control.

The rutin solution of 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 mg·mL^{-1} was used as abscissa, and the corresponding absorbance was used as ordinate to determine the regression curve. The regression equation was obtained as $y = 0.06553x + 0.00031$ (where x is absorbance and y is the concentration of rutin standard solution, in mg·mL^{-1}) with R^2 of 0.9997. The total flavonoid content was calculated according to the calibration curve and the method reported in literature [21].

2.8. The Bioactive Activities

The antioxidant activities of the total flavonoids extract were evaluated through the inhibitory effects on 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals according to the method described in literature [30, 31]. The antitumor activity of total flavonoids on tumor cells was determined using the Thiazolyl Blue Tetrazolium Bromide (MTT) method [13].

2.9. Data Analysis

In the study, PCA and HCA were employed to select the key indexes affecting the quality of *Radix Scutellaria*, and the quality evaluation system of *Radix Scutellaria* was preliminarily established [32–35]. The study provided research reference for the utilization of *Radix Scutellaria* and the evaluation of the quality of genuine medicinal materials. Three parallel experiments were conducted in each experimental group, and the experimental results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the data between the treatments.

3. Results and Discussion

3.1. Optimization of Extraction Conditions

As conventional organic solvents, methanol and ethanol were widely used as the solvents in the extraction of flavonoids due to the high polarity of flavonoids in *Radix Scutellaria* [9, 12]. The effects of ultrasonic extraction, Soxhlet extraction, and reflux extraction on the yields of total flavonoids from *S. baicalensis* were compared with those using methanol and ethanol as extraction solvent. The results revealed that the extraction yield of total flavonoids by methanol (10.75%) was significantly higher than that of ethanol (6.88%) under the ultrasonic extraction. The methanol thus was more suitable for the extraction of flavonoids from *Radix Scutellaria* in this study. The yield of total flavonoids by Soxhlet extraction (13.06%) was higher than that by reflux extraction (11.73%) and ultrasonic extraction (10.75%) with methanol used as extraction solvent. It may be that the solubility of flavonoids in solvent has a certain saturation in the extraction process, and the extraction rate of ultrasonic extraction and reflux extraction will not be improved when reaching saturation. While Soxhlet extraction method makes use of the principle of solvent reflux and siphon, the flavonoids can be extracted by pure solvent every time, so the extraction efficiency is higher. The results are consistent with those reported in the literature [36, 37]. Therefore, the Soxhlet extraction method with methanol as the extraction solvent was used to extract flavonoids from samples of *Radix Scutellaria*.

3.2. Optimization of Chromatographic Conditions

The mobile phase systems, water-acetonitrile-methanol-phosphoric acid (60 : 38 : 30 : 1, V/V/V/V) [9], acetonitrile (A)-0.02% acetic acid (B) [26], methanol (A)-acetonitrile (B)-0.1% formic acid (C) [12], methanol (A)-0.05% formic acid (B) [38], methanol-1% acetic acid (50 : 50 : 1) [39], and acetonitrile (A)-0.1% formic acid (B) [40], had been used formerly for the determination of flavonoids in *Radix Scutellaria*. On the basis of previous research and the multiple comparative experimental studies, the acetonitrile (A)-0.1% phosphoric acid (B) mobile phase system was employed to determine 8 flavonoids at the same time for the effective separation for the determined compounds (Figure 2). Acetonitrile (A)-0.1% phosphoric acid (B) was selected as the mobile phase system, and the optimized gradient elution condition of 0 min (A, 30%) 5 min (A, 35%) 10 min (A, 40%) 15 min (A, 50%) 22 min (A, 50%) 35 min (A, 30%) was obtained in the study. These chromatographic conditions led to a good separation efficiency with good peak shape and appropriate retention time, and the flavonoids in the sample reached the baseline separation (Figure 2).

[figure(s) omitted; refer to PDF]

In the experiment, the maximum absorption wavelengths of 8 flavonoids were scanned in the wavelength range of

200–400 nm. Among them, scutellarin and scutellarein were well absorbed at 335 nm, and baicalin, baicalein, wogonoside, wogonin, and oroxylin A had the maximum absorption at 280 nm, and the maximum absorption wavelength of chrysin was 250 nm. The results of the selected absorption wavelength were close to the detection wavelengths of 262 nm, 278 nm, and 340 nm reported in the literature [9–12]. The HPLC chromatograms at the wavelengths of 335 nm, 280 nm, and 250 nm were combined, and wavelength of 280 nm was selected in the study for the stable baseline and good resolution (Figure 2).

3.3. HPLC Method Validation

The determination method was evaluated through the verification of linearity, precision, repeatability, stability, and sample recovery. The linear range, regression equation, and R^2 of 8 flavonoids were obtained as shown in Table 2. The results implied that the method had a good linear relationship and a wide linear range and could be used for the quantitative analysis of flavonoids in *Radix Scutellaria*. Under the same conditions, the same sample was evaluated for 6 times, and the values of RSD of scutellarin, baicalin, scutellarein, baicalein, wogonoside, wogonin, chrysin, and oroxylin A were calculated to be 1.53%, 0.21%, 0.31%, 0.30%, 0.25%, 0.22%, 0.23%, and 0.24%, respectively, indicating that the precision of the instrument was good. The RSD values of the eight components measured after being placed at room temperature for 0, 2, 4, 8, 12, 16, and 24 hours were 1.54%, 0.84%, 0.77%, 0.73%, 0.62%, 0.93%, 0.98%, and 0.92%, respectively, which revealed that the prepared solution has good stability within 24 hours. Six sample solutions were extracted in parallel under the same conditions for determination, and the RSD values were 1.41%, 0.65%, 0.77%, 0.75%, 0.71%, 0.76%, 0.96%, and 0.99%, respectively, and the results implied that the method has good repeatability. The average recovery of the 8 compounds was 99.066–100.433% with the RSD of 0.619–1.763%, which indicated that the method had good sample recovery (Table S2). The LOD and LOQ were determined, and the results are shown in Table 2.

Table 2

Validation parameters of the developed RP-HPLC/DAD method.

Compounds	Regressive equation	R^2	Linear range ($\mu g \cdot mL^{-1}$)	LOD ($\mu g \cdot mL^{-1}$)	LOQ ($\mu g \cdot mL^{-1}$)	Recovery (%) (RSD %)
Scutellarin	$y = 12.131x + 173.310$	0.9994	19.00–209.00	0.558	1.859	99.1 (1.34)
Baicalin	$y = 22.262x + 3.827$	0.9998	23.00–253.00	0.795	2.650	99.5 (0.62)
Scutellarein	$y = 40.394x - 13.768$	0.9999	21.00–231.00	0.614	2.048	99.1 (1.50)
Wogonoside	$y = 21.478x - 8.961$	0.9996	21.00–231.00	0.656	2.187	100.0 (1.34)
Baicalein	$y = 24.628x - 18.258$	0.9998	20.00–220.00	0.581	1.937	100.4 (1.19)
Wogonin	$y = 39.549x - 10.583$	0.9997	22.00–242.00	0.646	2.154	99.2 (1.76)

Chrysin	$y = 49.741x - 11.652$	0.9999	18.00–198.00	0.529	1.765	99.2 (1.44)
Oroxylin A	$y = 62.627x - 15.032$	0.9995	19.00–209.00	0.508	1.692	99.7 (1.26)

The results suggested that the precision, stability, repeatability, and sample recovery of this method met the analysis requirements, and the method had the characteristics of fast, accurate, reliable, and strong specificity. Therefore, the method was suitable for the content determination of flavonoids in *Radix Scutellaria* and could also provide a scientific basis for the quality evaluation and effective quality control of *Radix Scutellaria*.

3.4. Method Application

An HPLC method was established for the simultaneous determination of 8 main flavonoids in the extracts of 38 *Radix Scutellaria* samples from seven cultivated areas. The chromatograms of the mixed control and representative sample at the detection wavelength of 280 nm are shown in Figure 2. The results revealed that the eight flavonoids were completely separated from each other and the baseline was stable. The retention time of all the compounds to be analyzed was within 25 min, and the test time was much smaller than that (30–50 minutes) reported in the literature [9–12], indicating that this method can significantly shorten the test time. The monomer contents of 8 flavonoids in samples from different cultivated areas are shown in Table 3. The flavonoids were confirmed according to the comparison of retention time results between the samples and the reference substance.

Table 3

Content of main flavonoids ($mg \cdot g^{-1}$) and the total flavonoids (TFs, %) in the sample.

Sample	Scutellarin	Baicalin	Scutellarein	Wogonoside	Baicalein	Wogonin	Chrysin	Oroxylin A	TCEF*	TF
HQ-1	6.038 ± 0.082	160.114 ± 1.093	2.653 ± 0.231	9.420 ± 0.730	15.001 ± 1.095	4.574 ± 0.420	2.291 ± 0.187	1.904 ± 0.173	201.995	9.386
HQ-2	6.897 ± 1.063	150.800 ± 0.568	2.943 ± 0.411	9.714 ± 1.271	15.590 ± 0.874	4.840 ± 0.536	2.388 ± 0.046	1.792 ± 0.025	194.963	10.963
HQ-3	6.280 ± 0.392	147.888 ± 0.485	2.753 ± 0.177	9.068 ± 0.449	15.226 ± 0.289	4.337 ± 0.238	2.420 ± 0.111	1.807 ± 0.093	189.778	9.314
HQ-4	6.979 ± 0.416	166.238 ± 0.640	3.257 ± 0.034	10.047 ± 0.381	14.820 ± 0.469	4.446 ± 0.269	1.961 ± 0.131	1.828 ± 0.109	209.575	11.285
HQ-5	7.281 ± 0.048	194.956 ± 0.941	3.752 ± 0.041	11.82 ± 0.107	16.609 ± 0.078	4.993 ± 0.053	2.641 ± 0.058	2.041 ± 0.031	244.094	12.952
HQ-6	7.856 ± 0.016	159.858 ± 0.661	3.723 ± 0.051	9.038 ± 0.243	6.168 ± 0.033	1.444 ± 0.011	1.973 ± 0.016	1.071 ± 0.012	191.129	7.229
HQ-7	2.882 ± 0.037	108.092 ± 0.396	2.498 ± 0.056	5.244 ± 0.073	4.609 ± 0.043	1.011 ± 0.018	1.601 ± 0.002	0.799 ± 0.007	126.737	4.052

HQ-8	6.029± 0.330	114.696± 0.283	2.480± 0.200	5.332± 0.503	4.758± 0.169	1.694± 0.070	1.260± 0.040	1.310± 0.038	137.559	4.366
HQ-9	6.713± 0.336	141.735± 0.409	3.132± 0.105	6.027± 0.189	7.452± 0.269	3.054± 0.134	1.397± 0.049	1.424± 0.056	170.933	5.733
HQ-10	2.867± 0.054	171.291± 0.943	2.766± 0.143	11.572± 0.752	2.992± 0.039	0.822± 0.028	0.700± 0.001	0.227± 0.003	193.237	4.527
HQ-11	5.140± 0.065	141.157± 0.097	2.152± 0.093	7.869± 0.640	10.809± 0.285	3.449± 0.076	1.113± 0.083	0.816± 0.075	172.505	6.305
HQ-12	8.582± 0.064	162.409± 0.180	4.270± 0.095	9.756± 0.232	6.632± 0.168	1.337± 0.009	2.276± 0.064	1.047± 0.011	196.310	7.804
HQ-13	4.193± 0.457	160.339± 1.190	3.479± 0.020	8.447± 0.532	5.854± 0.380	1.164± 0.052	2.380± 0.133	0.987± 0.066	186.843	5.648
HQ-14	6.677± 0.267	144.045± 0.458	3.898± 0.036	8.876± 0.205	3.888± 0.112	0.621± 0.108	1.865± 0.214	0.540± 0.006	170.409	5.155
HQ-15	3.293± 0.320	84.129± 0.490	1.398± 0.096	4.640± 0.168	2.584± 0.026	0.648± 0.043	1.017± 0.247	0.378± 0.131	98.088	2.867
HQ-16	8.405± 0.079	180.185± 1.196	3.910± 0.254	10.991± 0.623	10.794± 0.345	3.509± 0.215	2.091± 0.123	1.274± 0.077	221.159	9.735
HQ-17	6.731± 0.266	133.749± 0.250	3.133± 0.028	7.381± 0.030	5.300± 0.080	1.168± 0.043	2.228± 0.081	0.974± 0.030	160.665	5.134
HQ-18	6.977± 0.268	161.894± 0.555	3.325± 0.304	9.798± 1.416	16.066± 0.195	5.012± 0.551	1.991± 0.213	1.745± 0.189	206.808	12.159
HQ-19	7.246± 0.180	154.120± 1.237	3.102± 0.207	7.965± 0.127	15.030± 0.004	5.103± 0.073	2.506± 0.028	1.319± 0.021	196.391	9.318
HQ-20	1.057± 0.292	46.972± 0.062	1.221± 0.052	2.378± 0.128	0.993± 0.077	0.245± 0.007	0.438± 0.031	0.027± 0.016	53.330	1.988
HQ-21	5.414± 0.246	138.679± 1.45	3.436± 0.640	6.880± 0.742	9.930± 0.846	3.018± 0.132	1.398± 0.080	1.197± 0.078	169.951	6.119
HQ-22	6.756± 0.101	160.256± 0.959	3.517± 0.190	9.118± 0.401	6.594± 0.365	2.005± 0.090	1.095± 0.004	0.941± 0.023	190.282	6.619

HQ-23	5.804±0.187	155.457±0.818	3.183±0.060	6.757±0.352	8.428±0.170	2.409±0.052	1.430±0.024	1.298±0.028	184.765	6.044
HQ-24	3.395±0.061	105.854±0.184	2.178±0.048	5.708±0.228	12.924±0.999	3.382±0.066	1.712±0.172	1.356±0.157	136.509	5.491
HQ-25	8.065±0.104	160.150±1.229	1.334±0.108	9.383±1.036	13.494±0.609	4.253±0.118	2.029±0.051	1.775±0.047	200.481	8.279
HQ-26	5.707±0.281	157.037±0.820	3.311±0.101	7.220±0.178	10.635±0.487	3.438±0.223	1.570±0.091	1.327±0.080	190.245	7.686
HQ-27	4.148±0.060	178.953±0.874	2.608±0.047	11.332±0.141	6.150±0.396	2.478±0.019	1.476±0.028	0.531±0.003	207.676	6.744
HQ-28	5.985±0.496	164.129±1.142	4.048±0.199	8.404±0.262	10.872±0.347	3.214±0.223	1.210±0.181	1.441±0.095	199.303	8.168
HQ-29	5.674±0.336	121.349±1.648	2.123±0.031	7.705±1.002	6.798±0.559	2.427±0.162	1.950±0.093	0.777±0.051	148.803	4.727
HQ-30	6.894±0.396	161.141±1.124	4.967±0.467	9.310±0.778	11.823±0.368	3.982±0.303	1.637±0.111	2.176±0.158	201.929	9.321
HQ-31	3.801±0.071	168.433±1.873	4.563±0.713	9.364±1.608	5.525±0.629	1.977±0.139	2.026±0.215	1.473±0.170	197.163	6.340
HQ-32	3.812±0.214	89.595±0.874	1.748±0.240	4.121±0.616	6.055±0.390	1.913±0.208	1.609±0.286	0.960±0.086	109.813	3.991
HQ-33	4.604±0.257	181.392±1.143	2.803±0.021	9.321±1.822	8.744±0.769	3.201±0.314	1.119±0.096	0.843±0.076	212.026	7.329
HQ-34	7.278±0.724	126.826±0.803	2.786±0.252	7.065±0.050	6.408±0.531	2.495±0.239	1.141±0.094	0.825±0.073	154.824	5.073
HQ-35	1.886±0.063	52.686±0.230	1.337±0.059	2.761±0.163	1.531±0.096	0.118±0.074	0.667±0.038	0.145±0.004	61.132	2.288
HQ-36	6.013±0.813	151.883±0.593	2.980±0.039	7.361±0.050	7.965±0.222	2.764±0.020	1.645±0.035	1.194±0.067	181.806	5.955
HQ-37	11.949±0.271	164.368±0.447	3.043±0.157	7.343±0.064	4.234±0.026	0.560±0.033	2.555±0.028	0.526±0.093	194.577	6.822

HQ-38	8.417 ± 0.020	156.616 ± 0.386	4.173 ± 0.307	7.789 ± 0.232	15.992 ± 0.798	5.181 ± 0.241	2.576 ± 0.101	2.652 ± 0.123	203.397	11.395

*TCEF: total content of the eight flavonoids.

The results implied that 8 flavonoids were generally found in 38 samples from different cultivated areas. There were significant differences in the contents of 8 flavonoids in *Radix Scutellaria* from different cultivated areas (P<0.01). Baicalin was the highest among the eight flavonoids in the samples with the content from 46.972 mg·g^{-1} to 194.956 mg·g^{-1}, accounting for over 77% of the total content of the eight flavonoids (TCEF) in the samples. Compounds wogonoside and baicalein were another two flavonoids with high content in *Radix Scutellaria* with the contents of 2.378–11.82 mg·g^{-1} and 0.993–16.609 mg·g^{-1}, respectively. Compounds chrysin (0.438–2.641 mg·g^{-1}) and oroxylin A (0.027–2.652 mg·g^{-1}) were the two flavonoids with relatively low content in *Radix Scutellaria*. Scutellarin, scutellarein, and wogonin were three flavonoids with medium contents of 1.057–11.949 mg·g^{-1}, 1.221–4.967 mg·g^{-1}, and 0.118–5.181 mg·g^{-1} in different cultivated areas, respectively. The total content of the TCEF in the samples ranged from 53.330 mg·g^{-1} to 244.094 mg·g^{-1}.

Among all the samples, HQ-5 (194.956 mg·g^{-1}), HQ-33 (181.392 mg·g^{-1}), and HQ-16 (180.185 mg·g^{-1}) had the highest content of baicalin, while HQ-20 (46.972 mg·g^{-1}) and HQ-35 (52.686 mg·g^{-1}) had the lowest baicalin content. The content of baicalin in HQ-5 sample was over 4 times than that of HQ-20 sample, indicating that the content of baicalin in samples from different cultivated areas varied greatly. In addition to baicalin, the contents of scutellarin, scutellarein, wogonoside, baicalein, chrysin, oroxylin A, and TCFF in HQ-20 sample were the lowest in all samples, only with the contents of 1.057 mg·g^{-1}, 1.221 mg·g^{-1}, 2.378 mg·g^{-1}, 0.993 mg·g^{-1}, 0.438 mg·g^{-1}, 0.027 mg·g^{-1}, and 53.330 mg·g^{-1}, respectively, which revealed that the sample might be the worst quality. The content of scutellarin in HQ-37 sample (11.949 mg·g^{-1}) was the highest in all samples, while the contents of wogonin and oroxylin A were at low level only with the content of 0.560 mg·g^{-1} and 0.526 mg·g^{-1}, respectively. In HQ-30 sample, the content of scutellarein (4.967 mg·g^{-1}) was the highest in all the samples. Among the samples from all cultivated areas, baicalin, wogonoside, baicalein, and TCFF in HQ-5 sample were the highest with contents of 19.496 mg·g^{-1}, 11.82 mg·g^{-1}, 16.609 mg·g^{-1}, and 244.094 mg·g^{-1}, respectively. The contents of scutellarin (7.281 mg·g^{-1}), scutellarein (3.752 mg·g^{-1}), wogonin (4.993 mg·g^{-1}), chrysin (2.641 mg·g^{-1}), and oroxylin A (2.041 mg·g^{-1}) were all at high level, which implied that the quality of the sample from this cultivated area was the best.

The above results implied that the main active components of flavonoids in *Radix Scutellaria* were baicalin, wogonoside, baicalein, wogonin, and scutellarin, which were consistent with the research results reported in the literature [10, 12]. The contents of these active components could be used to evaluate the quality of medicinal plant *S. baicalensis*. There were significant differences in the content of flavonoids in different cultivated areas, which revealed that the growth environment had an important impact on the content of flavonoids in *Radix Scutellaria* [11, 24, 25].

Baicalin, wogonoside, and baicalein had good antibacterial property [41–43], which was consistent with the results that *Radix Scutellariae* had been used to treat antibacterial diseases in clinic [44, 45]. Baicalin and baicalein also had potential as therapeutic or supplementary agents for the treatment of breast cancer [46], and the anticancer properties of *Radix Scutellariae* could be attributed to its high content of wogonin, baicalein, and baicalin [3]. A study conducted by Zhang also revealed that baicalin might be one of the main components of *Radix Scutellariae* in the treatment of fetal irritability [47]. It was found that baicalin and baicalein were also the main active substances of traditional Chinese medicine *Radix Scutellariae* with good liver protection and inhibition of liver injury [48]. Baicalein and baicalin were also considered the main material basis of *Radix Scutellariae* for the treatment of the infection of the upper respiratory tract and to cure hyperactivity cough [49–51]. The above studies had confirmed that baicalin and baicalein were the main active substances of *Radix Scutellariae* with a variety of bioactive effects.

The average content of the eight flavonoids in different planting areas is shown in Table 4. It could be seen from the Table 4 that the contents of flavonoids in the samples from seven main genuine producing areas were significantly different (P<0.01). The average contents of baicalin (163.999 mg·g^{-1}), wogonoside (10.014 mg·g^{-1}), baicalein (15.449

mg·g^{-1}), wogonin (4.638 mg·g^{-1}), chrysin (2.340 mg·g^{-1}), and oroxylin A (1.875 mg·g^{-1}) in Gansu cultivated area were all higher than those in other six genuine producing areas, and the content of scutellarin (6.695 mg·g^{-1}) and scutellarein (3.072 mg·g^{-1}) was at a high level, which resulted in the highest content of TCEF (208.081 mg·g^{-1}) and TF (10.778%). The results implied that the quality of *S. baicalensis* in Gansu planting cultivated area was the best as the content of main flavonoids was significantly higher than that in other planting cultivated areas. The cultivated areas of Shaanxi were characterized by the highest content of scutellarin (7.066 mg·g^{-1}) and the lowest content of wogonoside (6.314 mg·g^{-1}). Compared with that in other planting areas, the samples of Henan cultivated area had the highest content of scutellarein (3.662 mg·g^{-1}), and the content of baicalin (158.801 mg·g^{-1}), wogonoside (9.223 mg·g^{-1}), wogonin (2.816 mg·g^{-1}), and oroxylin A (1.280 mg·g^{-1}) in Henan cultivated area was only lower than that in Gansu cultivated area, while it was higher than that in the other five cultivated areas, which led to the content of TCEF (190.975 mg·g^{-1}) only lower than that in Gansu and higher than that in the samples from other cultivated areas. The cultivated area in Inner Mongolia was characterized by the lowest content of baicalin (131.090 mg·g^{-1}), baicalein (5.747 mg·g^{-1}), and wogonin (1.801 mg·g^{-1}), and the content of wogonoside (6.410 mg·g^{-1}) was also at a low level, which resulted in the lowest content of TCEF (156.590 mg·g^{-1}) in the samples of cultivated area in Inner Mongolia. The contents of chrysin (1.289 mg·g^{-1}), scutellarein (2.446 mg·g^{-1}), and oroxylin A (0.876 mg·g^{-1}) in the samples from Hebei cultivated areas were the lowest among all cultivated areas. At the same time, the contents of scutellarin, wogonoside, and baicalein were also at a low level, which was the main reason that TCEF and TF were at a low level in the samples of Hebei cultivated areas.

Table 4

Average content of main flavonoid components in different growing areas (mg·g^{-1}).

Flavonoids	Gansu	Inner Mongolia	Shandong	Shanxi	Henan	Hebei	Shaanxi
Scutellarin	6.695 ± 0.408	5.870 ± 0.186	6.011 ± 0.090	5.171 ± 0.145	5.300 ± 0.465	5.231 ± 0.148	7.066 ± 0.017
Baicalin	163.999 ± 0.26	131.090 ± 0.393	149.332 ± 0.654	132.058 ± 0.371	158.801 ± 0.453	132.604 ± 0.687	131.389 ± 0.142
Scutellarein	3.072 ± 0.156	2.958 ± 0.083	3.140 ± 0.299	2.592 ± 0.068	3.662 ± 0.161	2.446 ± 0.278	2.883 ± 0.093
Wogonoside	10.014 ± 0.442	6.410 ± 0.171	8.729 ± 0.874	6.778 ± 0.160	9.223 ± 0.511	6.836 ± 0.588	6.314 ± 0.261
Baicalein	15.449 ± 0.418	5.747 ± 0.149	7.995 ± 0.319	9.000 ± 0.378	8.234 ± 0.365	7.069 ± 0.231	7.431 ± 0.364
Wogonin	4.638 ± 0.184	1.801 ± 0.040	2.283 ± 0.123	2.678 ± 0.102	2.816 ± 0.224	2.536 ± 0.074	2.156 ± 0.176
Chrysin	2.340 ± 0.057	1.558 ± 0.091	1.817 ± 0.085	1.382 ± 0.069	1.660 ± 0.035	1.289 ± 0.072	1.861 ± 0.085

Oroxylin A	1.875 ± 0.061	1.151 ± 0.027	0.931 ± 0.073	1.131 ± 0.063	1.280 ± 0.027	0.876 ± 0.062	1.129 ± 0.076
TCEF	208.081	156.590	180.241	160.795	190.975	158.888	160.228
TF (%)	10.778	5.755	6.883	5.980	6.365	4.916	6.437

It was traditionally believed that the genuine producing areas of *S. baicalensis* were in Shandong, Henan, Gansu, Shanxi, Hebei, Inner Mongolia, and other places [23]. In this study, the evaluation content of flavonoids in Shanxi, Hebei, and Inner Mongolia Autonomous Region was not high, which was consistent with the research results reported in the literature [11, 22] but different from the research which believed that the content of active components in *Radix Scutellariae* in Hebei and Inner Mongolia was the highest [21], which might be due to different sampling ranges and other reasons. From the experimental results, the quality of *Radix Scutellariae* in the traditional genuine producing areas was not necessarily the best. The quality of *Radix Scutellariae* from different cultivated areas could be improved by improving germplasm resources and changing cultivation methods.

The studies of the effects of different elevations and different lighting conditions (shade slope and sunny slope) on the contents of flavonoids revealed that high altitude and sunny slope were conducive to increasing the accumulation of total flavonoids in *Radix Scutellariae* [24]. The effects of environmental factors on photosynthetic physiology and flavonoid constituent of Radix Scutellariae also suggested that photosynthetic active radiation and soil water content were important environmental factors impacting on photosynthesis of S. baicalensis, and soil water content, relative humidity, and atmospheric CO2 concentration were important environmental factors impacting on baicalin content, and photosynthetic active radiation, atmospheric pressure, and atmospheric temperature were important environmental factors impacting on baicalin content [25]. The high average altitude, mild and humid climate, and strong light intensity might be the reason for the high content of flavonoids in *Radix Scutellariae* in Tanchang County.

As a bulk medicinal material and a widely used traditional Chinese medicine, *S. baicalensis* had a large planting scale in China, with the characteristics of large planting area and wide range. The difference of climate, geographical environment, and other external factors would affect the active components in *Radix Scutellariae*. Therefore, the systematic determination of the content of main flavonoids will provide a guarantee for the quality evaluation of medicinal materials in different cultivated areas.

3.5. Principal Component Analysis

Based on the above content determination results, scutellarin ($X1$), baicalin ($X2$), wogonoside ($X3$), baicalein ($X4$), and total flavonoids ($X5$) were selected as indicators for PCA. One-way ANOVA was used to compare the data between the treatments, and Bartlett's test of sphericity was used to detect the correlation between variables [32, 35]. All statistical analyses were performed at 95% confidence level using SPSS 26.0 software. From the analysis results (Table 5), it could be seen that the value of the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy (0.587) in this example was greater than 0.5, indicating that PCA could be used to evaluate the results [32, 35]. The significance of Bartlett's test of sphericity (0.001) was less than 0.01, which revealed that the correlation between variables was very significant, and factor analysis could be carried out in the study [32, 35].

Table 5

The detection results of KMO and Bartlett.

Index		Value
KMO measure of sampling adequacy		0.587

Bartlett's test of sphericity	Approximate chi-square	30.447
Freedom	10.000	Significance

The results of PCA (Table 6) of the above five components (*X*1–*X*5) showed that the characteristic values of the first two principal components (*X*1 and *X*2) were greater than 1, and the contribution rate of cumulative variance was 93.028%, and the characteristic value of the first principal component was 3.574 and the contribution rate was 71.477%, representing 71.477% of all information, which implied that scutellarin was a main factor closely related to the origin of *Radix Scutellariae*. The above results implied that these two factors have a high degree of explanation for the whole result [33]. The two principal components could be used to replace the above five specific contents to evaluate the quality of *Radix Scutellariae* from different planting areas.

Table 6

Results of PCA of flavonoids.

Component	Initial eigenvalue			Extraction sum of squared loading			
Sum	Variance (%)	Cumulative (%)	Sum	Variance (%)	Cumulative (%)	X_1
3.574	71.477	71.477	3.574	71.477	71.477	X_2
1.078	21.552	93.028	1.078	21.552	93.028	X_3
0.323	6.453	99.482				X_4
0.019	0.375	99.857				X_5

The principal component matrix was employed for the selection of the principal components, the original data were normalized and the variables were saved, and the PCA model according to the principal component matrix was constructed (Table 7). The principal component model was calculated as follows:(2)F=0.768F1+0.232F2,F1=0.228X1+0.479X2+0.478X3+0.484X4+0.506X5,F2=0.459X1−0.182X2−0.203X3+0.034X4+0.124X5.

Table 7

Principal component matrix of flavonoids.

Component	Initial factor load		Score coefficient	
1	2	1	2	X_1
0.432	0.868	0.121	0.806	X_2
0.906	−0.345	0.254	−0.320	X_3
0.903	−0.383	0.253	−0.355	X_4

0.915	0.065	0.256	0.060	X_5

According to the principal component model, the principal component scores of seven planting areas were obtained (Table 8). The results implied that the top three scores in the comprehensive evaluation were 2.963 in Gansu, 0.341 in Shandong, and 0.307 in Henan, indicating that the quality of *Radix Scutellariae* in Gansu planting area was the best and could be used as genuine medicinal material, followed by Shandong and Henan.

Table 8

Principal component scores of *Scutellaria baicalensis* from different origins.

Cultivated areas	F_1 value	F_2 value	F value	Ranking
Gansu	3.780	0.253	2.963	1
Inner Mongolia	−1.539	0.209	−1.135	6
Shandong	0.488	−0.146	0.341	2
Shanxi	−1.045	−0.232	−0.857	5
Henan	0.640	−0.801	0.307	3
Hebei	−1.572	−0.300	−1.277	7
Shaanxi	−0.752	1.017	−0.342	4

3.6. Hierarchical Cluster Analysis of the Samples

Taking the content of 8 flavonoids and total flavonoids in *Radix Scutellariae* as indicators, the clustering analysis of *Radix Scutellariae* from different cultivated areas was carried out by the clustering method of square Euclidean distance coefficient and centroid clustering (Figure 3). The results revealed that the seven cultivated areas could be divided into three groups: group I was Shandong and Henan; group II was Inner Mongolia, Hebei, Shaanxi, and Shanxi; and group III was Gansu. There were similarities and differences in the content of flavonoids in *Radix Scutellariae* from different cultivated areas. The contents of baicalin, wogonoside, and baicalein in group III were significantly higher than those in group I and group II. The contents of scutellarin, scutellarein, and chrysin in group I were higher than those in group II. Due to the high content of baicalin, wogonoside, and baicalein, the quality of *Radix Scutellariae* in Gansu Province was the best. Shandong and Henan were clustered together earlier, which might be related to the difference of their growth environment and the small differentiation of population diversity, and results were consistent with the research results reported in the literature [11].

[figure(s) omitted; refer to PDF]

The cluster analysis method reflected the diversity differentiation of *S. baicalensis* in different cultivated areas, and the different cultivated areas would make the chemical composition and content of active components of *Radix Scutellariae* different. It also revealed that the quality of *Radix Scutellariae* was affected by geographical location and environmental factors, which might be related to the superposition of precipitation, atmospheric temperature, sunshine duration, and soil properties [11, 24, 25].

3.7. The Bioactive Activities

The antioxidant activities of the total flavonoids extract were evaluated through the inhibitory effects on ABTS and DPPH radicals, and the antitumor activity of total flavonoids on HepG2 was also determined using the MTT method, and the IC_{50} values are shown in Table 9. The IC_{50} values of oroxylin A ($67.389 \pm 3.14\,\mu g \cdot mL^{-1}$), scutellarin (65.913 ±

4.22 μg·mL^{-1}), wogonin (89.005 ± 5.62 μg·mL^{-1}), wogonoside (133.469 ± 8.24 μg·mL^{-1}), baicalein (69.608 ± 4.01 μg·mL^{-1}), chrysin (123.324 ± 7.11 μg·mL^{-1}), and baicalin (70.902 ± 4.09 μg·mL^{-1}) on HepG2 were also determined in the study.

Table 9

Mean IC_{50} values for two free radicals and HepG2 from different growing areas.

Cultivated areas	IC_{50} (μg/mL)		
DPPH	ABTS	HepG2	Gansu
102.804 ± 0.202	35.540 ± 0.246	346.852 ± 3.612	Inner Mongolia
134.192 ± 0.084	55.600 ± 0.130	415.080 ± 4.183	Shandong
178.275 ± 0.056	89.140 ± 0.069	477.990 ± 4.362	Shanxi
211.619 ± 0.157	59.883 ± 0.120	408.828 ± 3.118	Henan
124.980 ± 0.310	57.850 ± 0.393	389.814 ± 2.119	Hebei
125.602 ± 0.539	39.022 ± 0.276	420.717 ± 4.478	Shaanxi

The results suggested that the flavonoid extracts of *Radix Scutellariae* from different cultivated areas had certain effects of scavenging free radicals and inhibitory activity on cancer cells, but the activity varied greatly in different cultivated areas. The sample from Gansu Province had the strongest scavenging effect on DPPH and ABTS free radicals, as well as the inhibitory effect on cancer cell HepG2, which was closely related to the high content of flavonoids in the samples. From the inhibitory activity of flavonoids on cancer cells, although the compounds scutellarin (65.913 ± 4.22 μg·mL^{-1}) and oroxylin A (67.389 ± 3.14 μg·mL^{-1}) had greater inhibitory effect on cancer cell HepG2 than that of other compounds, the effects of the two compounds on the anticancer activity of the samples from corresponding places were weak because of the relatively low contents in the samples. Baicalein and baicalin were the main flavonoids in *Radix Scutellariae*, and the high antitumor activities of baicalein (69.608 ± 4.01 μg·mL^{-1}) and baicalin (70.902 ± 4.09 μg·mL^{-1}) along with the high content led to strong anticancer activity in Henan and Shanxi samples. From the content determination results, baicalin, baicalein, wogonoside, and wogonin were the main flavonoids with high content in *Radix Scutellariae*. It was found that the order of free radical elimination activity of these four flavonoids was baicalin > baicalein > wogonin > wogonoside [52]. The higher content of baicalin was main reason that Henan samples had strong inhibitory effects on DPPH free radicals. Although the content of flavonoids was not high, Hebei sample had a strong inhibitory effect on DPPH free radical with the low IC_{50} values (39.022 ± 0.276 of ABTS and 125.602 ± 0.539 of DPPH), which implied that there might be other flavonoids with high antioxidant activity in the samples.

However, based on the results of activity determination, the activities of flavonoids extract did not have a good positive correlation with the total content of flavonoids in *Radix Scutellariae*, and higher total flavonoid content did not mean a higher bioactivity. It implied that the bioactivities of *Radix Scutellariae* were related to the content of active flavonoids and the bioactivity of the flavonoids.

4. Conclusions

In this study, a method for the simultaneous determination of 8 main flavonoids in *Radix Scutellariae* from different genuine producing areas was established for the first time, and the chromatographic conditions were optimized. The chromatographic conditions with good resolution and appropriate retention time were obtained. The results

suggested that flavonoids in *Radix Scutellariae* from different cultivated areas varied greatly, and baicalin, wogonoside, baicalein, wogonin, and scutellarin were the main flavonoids in *Radix Scutellariae*, and the content of baicalin was the highest, accounting for more than 77% of the total flavonoids. The content of flavonoids in *Radix Scutellariae* planted in Gansu cultivated areas was the highest with the content of baicalein, baicalin, wogonin, chrysin, wogonoside, and oroxylin A, and TCEF and TF were much higher than those in other cultivated areas. It implied that the content of flavonoids in *Radix Scutellariae* of Gansu was high in the cultivated areas, which implied that the quality of *Radix Scutellariae* might be good. In addition, the content of flavonoids in *Radix Scutellariae* in Henan and Shandong cultivated areas was also at high level. The results of PCA revealed that Gansu, Shandong, and Henan were the three producing areas with the highest content of flavonoids and the best quality. The results of HCA also confirmed that *S. baicalensis* planted in Gansu cultivated areas had higher content of flavonoids than those of other cultivated areas. The antioxidant and anticancer activities of the extract of *Radix Scutellaria* were closely related to the content of flavonoids, and high contents of baicalin, wogonoside, and baicalein were helpful to improve biological activities. The results implied that there were great differences in the content of flavonoids in samples from different genuine producing areas of *S. baicalensis*. This study had good guiding significance for the quality evaluation of *Radix Scutellariae*.

Authors' Contributions

Yiying Zheng and Shengnan Zhou contributed equally to this work.

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Application of Carbon Nanofiber-Modified Concrete in Industrial Building Design

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ABSTRACT (ENGLISH)

In order to explore the influence law and action mechanism of carbon nanofibers on the basic mechanical properties of concrete, the author proposes the mechanical properties and microscopic mechanism of carbon nanofiber-modified concrete. Concrete was prepared with different dosages of carbon nanofibers, and the compressive strength, flexural strength, and splitting strength of carbon nanofiber-modified concrete were tested, and the modification mechanism was explored. Experimental results show that an appropriate amount of carbon nanofibers can improve the mechanical properties of concrete. When the dosage is 0.3%, the mechanical properties of carbon nanofiber-modified concrete are the best, and its compressive strength, flexural strength, and split tensile strength are increased by 9.2%, 13.2%, and 17.5%, respectively, compared with plain concrete. Carbon nanofibers can form a three-dimensional network structure inside the concrete, which can improve the microscopic morphology of the concrete, enhance the toughness and integrity of the concrete, fill the pore defects inside the concrete, refine the pore size distribution, and consume part of the fracture failure energy when the concrete is damaged.

FULL TEXT

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1. Introduction

As we all know, the three major building materials widely used in civil engineering are cement, steel, and wood [1]. Concrete is composed of cement and sand aggregates and has compressive strength, as well as resistant to water, fire, and corrosion. In recent years, there has been a lot of research and development in the process of housing planning. It would also be the first choice of people in the home appliance industry in the 20th century. However, because concrete is a hard material with poor tensile strength, reinforced concrete using steel as a supporting material greatly improves the tensile and flexural strengths of concrete. Steel bars are not corrosion-resistant, and in a harsh environment, corrosion is strong and the strength of concrete is lost, so the structure cannot achieve the design model design. In the development of building materials, new problems are constantly arising, and people are constantly seeking new reinforcing materials to replace steel bars. The main research objects are carbon fiber (CFRP), aramid fiber (AFRP), and glass fiber (GFRP) [2]. Carbon fiber has become the focus of research on concrete reinforcement due to its advantages of electrical conductivity, light weight, high strength, large modulus, corrosion resistance, and high temperature resistance.

Carbon fiber is a high-strength, high-modulus, corrosion-resistant, electrically and thermally conductive fibrous carbon material developed in the 1960s [3]. Carbon fiber reinforcement not only improves the flexural and tensile strengths of cement composite materials but also increases the toughness of cement materials, giving traditional cement building materials new properties (light weight, high strength, impact resistance, shrinkage resistance, electrical conductivity, and so on). This makes carbon a very ideal building material. Carbon fiber-reinforced cement concrete (CFRC) is a composite material that was researched and developed in the 1970s. Studies have shown that carbon fiber cement-based composites overcome the shortcomings of cement-based materials such as low tensile strength and flexural strength and large drying shrinkage and have high tensile strength, good impermeability, and a good inhibitory effect on concrete cracks caused by temperature stress, shrinkage, and creep, and the impact resistance and fatigue resistance are greatly improved.

Also, the dispersibility and electrical stability of carbon fiber in cement matrix are still outstanding issues, which directly affect the mechanical properties and pressure sensitivity of carbon fiber cement-based composites. Due to the difficulty in dispersing carbon fiber, the mechanical and electrical properties of its cement base and concrete are not stable enough. Regarding the dispersion of carbon fiber, relevant scholars have done in-depth research, but the problem has not been fundamentally solved. It is still difficult to apply a large number of projects in engineering, and

the high price of carbon fiber also limits its wide application. Therefore, we need to seek conductive materials that have good compatibility with cement-based materials, do not affect their mechanical properties, and have good pressure sensitivity. The emergence of nanomaterials makes us see the light of day. Nanocarbon black is one of the cheaper nanomaterials; it has excellent electrical conductivity and high diffusivity, small size, large specific surface area, and excellent interface properties. It can be spread evenly in the cement matrix without dispersant, which not only reduces the artificial strength and high sensitivity of the cementitious and rock pressure-sensitive materials but also improves their stability and makes them less expensive. The sensitivity of nanocarbon black stone materials is good. It is a good business and shows a promising future of use.

2. Literature Review

Among the carbon nanofiber-reinforced composite materials, the most studied matrix materials are mainly metal-based, polymer-based, and so on. There are few studies on composite materials with cement-based materials as the matrix, and they are still in their infancy. The research focus mainly includes the dispersion of carbon nanofibers in the matrix and the mechanical properties and durability of carbon nanofiber cement-based composites. Due to the high ratio of carbon nanofibers and strong van der Waals force, carbon nanofibers are prone to agglomeration and entanglement, so it is difficult to achieve a uniform dispersion state in cementitious materials, paper, which cannot improve the effect. In addition, agglomerated carbon nanofibers, like impurities in cement materials, inhibit the hydration of the cement and ultimately affect its microscopic morphology. Foreign researchers have carried out detailed research on the dispersion of carbon nanofibers and carbon nanofiber cement-based composites and often use a combination of ultrasonic treatment and dispersion to uniformly disperse carbon nanofibers in cementitious materials.

Setiawan et al. used a polycarboxylic acid superplasticizer as a dispersant, and with the ultrasonic process, the carbon nanofibers were uniformly dispersed in the aqueous solution. The dosages of 0.1% and 0.2%, respectively, were applied to the cement-based materials, and the water-cement ratio was 0.4. The mechanical properties of carbon nanofiber cement-based composites were studied at 7 days, 14 days, and 28 days, respectively, including flexural strength, fracture deformation, ultimate strain, and toughness. The dispersion of carbon nanofibers in the matrix is also discussed. The results of the study showed that at 7 and 14 days of age, carbon nanofibers had no obvious enhancement effect on cement-based materials, but at 28 days, the mechanical properties (fracture deformation, flexural strength, ultimate strain, and toughness) of carbon nanofiber cement-based composites were all higher than those of blank samples [4]. Abregú et al. used polycytic acid superplasticizer as dispersant and firstly prepared carbon nanofiber dispersion suspension by the ultrasonic method. Then, the prepared suspension is applied to cement concrete to prepare nanocarbon fiber cement-based composite material [5]. The results show that the dispersion state of carbon nanofibers is regional. The carbon nanofibers are not uniformly dispersed on the fracture surface, so the dispersion effect is not very good. At the same time, the relationship between the particle size of cement and the dispersion of carbon nanofibers was discussed. When a large amount of carbon nanofibers is added, the larger the cement particles are, the more unfavorable their dispersion is in the cement matrix.

Rajeshwari et al. applied silica fume to carbon nanofiber cement-based composites, and the content of carbon nanofibers was 2 wt%. The research results show that the addition of silica fume is beneficial to the dispersion effect of carbon nanofibers in the cement-based matrix. The bonding strength of carbon nanofibers to a cement-based matrix is also enhanced. At the same time, the addition of carbon nanofibers can effectively improve the pore structure of the composite material, make the body more compact, and cause the pores change to a smaller size [6].

Maleki et al. studied the mechanical properties of carbon nanofiber cement-based composites, and the content of carbon nanofibers was 0.048 wt%. In order to improve the dispersibility of carbon nanofibers in the cement-based matrix, dispersant and ultrasonic treatment were firstly applied to prepare a uniformly dispersed aqueous solution of carbon nanofibers. When the ultrasonic capacity is 2800 kJ/1 and the ratio of dispersant to carbon nanofibers is 4 : 1, the best dispersion suspension can be obtained [7].

The author intends to prepare nanocarbon fiber reinforced cement concrete specimens, starting from the strength indicators such as compressive strength, flexural strength, and splitting strength, and explore the influence law and

mechanism of carbon nanofibers on the basic mechanical properties of concrete; the modification effect of carbon nanofibers on concrete is explored from the microscopic level in order to provide a theoretical basis and application basis for the research and application of high-durability protective materials and to make up for the deficiencies in the existing research on carbon nanofiber modified concrete.

3. Research Methods

3.1. Test

3.1.1. Preparation of Raw Materials and Test Pieces

The raw materials used are cement: P·O 42.5R grade cement; sand: medium sand with a fineness modulus of 2.9, an apparent density of 2620 kg/m^3, and a mud content of 1.1%; limestone crushed stone: large crushed stone (apparent density 2730 kg/m^3) and small crushed stone (apparent density 2644 kg/m^3) are mixed in a mass ratio of 7 : 3; water reducing agent: polycarboxylate high-performance water reducing agent (see Table 1 for performance); defoaming agent: tributyl phosphate defoaming agent, content 99.6% and density of 0.974~0.980 g/cm^3; fiber: carbon nanofiber (see Table 2 for technical parameters).

Table 1

Performance index of polycarboxylate superplasticizer.

Water reduction rate (%)	Bleeding rate (%)	Gas content (%)	Coagulation time (min)		Compressive strength ratio		Shrinkage ratio (%)
Initial setting	Final coagulation	3 d	7 d	≥26	≤65	≤3.5	−65

Table 2

Technical parameters of carbon nanofibers.

Diameter (nm)	Length (μm)	Resistivity (Ω·cm)	Thermal conductivity [W/(m·°C)]	Specific surface area (m^2/g)	Thermal expansion coefficient (1/°C)	Bulk density (g/cm^3)	True density (g/cm^3)
150~200	10~20	<0.012	2000	300	1	0.18	2

A total of two types of specimens were prepared for the test: a cube specimen of 100 mm × 100 mm × 100 mm and a prismatic specimen of 100 mm × 100 mm × 400 mm [8, 9]. The preparation steps are as follows: ① preparation of carbon nanofiber dispersion: mix the water reducing agent into the water and stir evenly, add carbon nanofibers and half-part defoamer, stir at high speed for 2 minutes, add the remaining half-part defoamer, and manually stir at low speed for 5 minutes until there are no obvious bubbles in the dispersion; ② preparation of the concrete mixture: mix cement, sand, and stone evenly by the dry mixing method, then add nanocarbon fiber dispersion while stirring, and finally stir for 2 minutes; and ③ pouring and curing: after pouring and forming, cure under standard conditions for 28 d. The test mix ratios are shown in Table 3.

Table 3

Mixing ratio of test pieces kg/m^3.

Numbering	Cement	Water	Sand	Gravel	Carbon nanofiber	Water reducer	Defoamer
PC	495	180	672	1008	0	5	3
CNFC01	495	180	672	1008	0.18	7.5	4.5
CNFC02	495	180	672	1008	0.37	10	6
CNFC03	495	180	672	1008	0.55	12.5	7.5
CNFC04	495	180	672	1008	0.71	15	9
CNFC05	495	180	672	1008	0.9	5	3

3.2. Test Method

In accordance with the methods in GB 50081-2002, “Standards for Mechanical Properties of Ordinary Concrete,” the basic mechanical properties of concrete specimens were tested. Among them, the compressive strength test uses an electrohydraulic servo compressive testing machine to pressurize the cube specimen; when the specimen is close to failure, stop adjusting the throttle until it breaks, record its load-displacement curve, and multiply the test result by a conversion factor of 0.95. The flexural strength test uses a flexural testing machine to test the prismatic specimen [10]. The test result needs to be multiplied by a conversion factor of 0.85. The splitting strength test uses the electrohydraulic servo test system and the split-pull test device to conduct the split-pull test on the cube specimen, and the test results need to be multiplied by the conversion factor of 0.85.

4. Analysis of Results

4.1. Compressive Strength

Figure 1 is a graph showing the effect of carbon nanofiber content on the compressive strength of concrete. It can be seen from Figure 1 that ① when the content of carbon nanofibers is 0.1%, 0.2%, 0.3%, and 0.4%, the compressive strength of concrete is increased by 2.5%, 6.1%, 9.2%, and 6.8%, respectively; compared with ordinary concrete, it shows that an appropriate amount of carbon nanofibers can effectively improve the compressive strength of concrete, and the improvement effect is the best when the dosage is 0.3%; ② when the content of carbon nanofibers is 0.5%, the compressive strength is reduced by 1% compared with ordinary concrete; and ③ with the increase of the content of carbon nanofibers, the compressive strength of concrete increases first and then decreases, indicating that carbon nanofibers cannot be added to the concrete blindly; too much carbon nanofibers will not only reduce the improvement effect but also cause waste of resources [11]. The following is the formula for calculating the compressive strength:(1)fcu=FmaxA.

[figure(s) omitted; refer to PDF]

In the formula, fcu is the compressive strength of concrete cube (MPa); Fmax is the maximum load (N); and *A* is the cross-sectional area of specimen under compression (mm^2).

4.2. Flexural Strength

Figure 2 shows the effect of carbon nanofiber content on the flexural strength of concrete. It can be seen from Figure 2 that ① when the content of carbon nanofibers is less than 0.3%, the flexural strength of carbon nanofiber-reinforced cement concrete increases with the increase of the content. After the dosage exceeds 0.3%, the flexural strength of concrete decreases sharply with the increase of the dosage [12]. When the content of carbon nanofibers is 0.1%, 0.2%, 0.3%, and 0.4%, the flexural strength of carbon nanofiber-reinforced cement concrete is increased by 3.7%, 8.9%, 13.2%, and 7.3%, respectively, compared with ordinary concrete. The improvement effect is the best when the amount is 0.3%; ② when the dosage is 0.5%, the flexural strength of carbon nanofiber-reinforced cement concrete is 5.2% lower than that of ordinary concrete, indicating that adding too much carbon nanofibers will not only

not improve the flexural strength of concrete but even deteriorate its flexural strength [13, 14].

[figure(s) omitted; refer to PDF]

4.3. Split Tensile Strength

Figure 3 shows the effect of carbon nanofiber content on the splitting tensile strength of concrete. It can be seen from Figure 3 that ① when the content of nanocarbon fiber is less than 0.3%, with the increase of the content, the splitting tensile strength of nanocarbon fiber-reinforced cement concrete increases continuously, but when the content exceeds 0.3%, the splitting tensile strength decreases sharply [15]. When the content of carbon nanofibers is 0.1%, 0.2%, 0.3%, and 0.4%, the splitting tensile strength of carbon nanofiber-reinforced cement concrete is increased by 2.8%, 10.8%, 17.5%, and 9.5%, respectively, compared with ordinary concrete. The improvement effect is more obvious when the amount is 0.2%~0.3%; ② when the amount is 0.5%, the split tensile strength of carbon nanofiber-reinforced cement concrete is 6.3% lower than that of plain concrete, indicating that the increase of carbon nanofibers deteriorates the tensile strength of concrete [16].

[figure(s) omitted; refer to PDF]

4.4. Modification Mechanism

4.4.1. Distribution of Carbon Nanofibers in Concrete

There are a lot of holes in ordinary concrete, the hydration products are in a loose state, and the integrity is poor. When the dosage is 0.1%, the carbon nanofibers are sparsely distributed in the concrete, and only a few sporadically interspersed in the gel material can be seen under the scanning electron microscope, so the modification of concrete is of little significance. Continuing to increase the dosage, the carbon nanofibers are distributed more and more widely in the concrete, and the hydration products are interwoven vertically and horizontally, overlapping each other into a three-dimensional network structure, and the material integrity is gradually strengthened. The crystal form of the hydration product is smaller than that of ordinary concrete, and when the dosage is 0.3%, the distribution and modification effect of carbon nanofibers in concrete are better [17]. Carbon nanofiber particles have high surface activity, which can accelerate the hydration of cement when incorporated into concrete. Due to the nucleation and adsorption of nanomaterials, the hydration product gradually forms a network structure with nanoparticles as the core, which inhibits the formation of large crystals and reduces the degree of crystal orientation; thereby, the interface structure between cement stone and aggregate is improved, and the strength of concrete is improved. When the dosage is 0.4%, the strong van der Waals force makes carbon nanofibers agglomerate in a small area. When the dosage is 0.5%, the agglomeration phenomenon is more obvious, the distribution of carbon nanofibers in the hydration products around the agglomerates is significantly reduced, and the carbon nanofibers are difficult to play their modification role. It will even cause a weak area inside the concrete, resulting in a concrete strength lower than that of plain concrete without carbon nanofibers, which will adversely affect the mechanical properties and durability of the concrete [18].

4.5. Molecular Chain Effect of Carbon Nanofibers

Carbon nanofiber monofilaments are wrapped by C-S-H gel particles, which connect the gel particles together like molecular chains, thereby enhancing the toughness and integrity of the gel.

4.6. Filling Effect of Carbon Nanofibers

Carbon nanofibers are very small in size and can have a small size effect when mixed into concrete, filling part of the pore defects of the concrete, effectively reducing the content of macropores in concrete and improving the particle gradation of cementitious materials. The fine pores are filled in the structure, the pore structure is refined inside the concrete, and the strength, compactness, and impermeability of the concrete are improved [19].

4.7. Bridging Effect of Carbon Nanofibers and Pull-Out and Fracture Effects When Concrete Is Damaged

The fibrous structure of carbon nanofibers bridges the micropores and microcracks inside the concrete structure, effectively preventing the further development of microcracks. At the same time, the molecular chain effect of carbon nanofibers strengthens the connection between the components, enhances the integrity of the concrete, and then improves the strength of the concrete. With the continuous destruction of concrete under the external action, the microcracks gradually expand, and the tensile stress received by the carbon nanofibers in the process of crack

development gradually increases and finally pulls out of the cement slurry or directly breaks [20]. In the process of pulling out and breaking, when the carbon nanofibers break free from the bondage of the cement slurry or destroy themselves, part of the breaking energy is consumed, thereby inhibiting the development of microcracks in concrete.

5. Conclusion

(1) Adding an appropriate amount of carbon nanofibers can improve the mechanical properties of concrete. When the dosage is 0.3%, the basic mechanical strength indicators of the material are the best, and the compressive strength, flexural strength, and split tensile strength are better than those of plain concrete; they are improved by 9.2%, 13.2%, and 17.5%, respectively.

(2) An appropriate amount of carbon nanofibers is well dispersed in concrete, which can form a three-dimensional network structure and reduce the crystal form of hydration products.

(3) The carbon nanofibers in the modified concrete connect the gel particles together like molecular chains, which enhances the toughness and integrity of the gel.

(4) The size of carbon nanofibers is extremely small, which can fill the pore defects in concrete to a certain extent.

(5) The carbon nanofibers are bridged between the microcracks of the modified concrete, which can consume part of the fracture failure energy when the concrete is damaged.

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DETAILS

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Health Risk Assessment of Pesticide Residues in Drinking Water of Upper Jhelum Region in Kashmir Valley-India by GC-MS/MS

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ABSTRACT (ENGLISH)

Globally growing demand for agricultural and farm foods has more or less become dependent on chemical pesticides to maintain the supply chain, which undoubtedly boosts agricultural production. However, pesticides not only impact the target pests but cause hazard to human health. Pesticides are ubiquitous and can be found in almost every component of the environment. They can therefore impair human and biota health when present over the threshold level. The present study assessed the concentration of commonly used pesticides for agricultural purposes but get mixed in different sources of water, as such fifteen sampling sites along the upper Jhelum basin of Kashmir valley were chosen. For the analysis, 60 water samples were obtained from different water sources. Gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) was used to determine pesticide residues in water samples. Pesticide residues from 10 of the 26 commonly used pesticides were detected in water samples. Difenoconazole had the highest concentration among the pesticides detected, with a mean concentration of $0.412 \pm 0.424\,\mu$g/L ranging from $0.0\,\mu$g/L to $0.8196\,\mu$g/L. The target hazards quotient (THQ) was used to quantify the possible noncarcinogenic health risks associated with drinking pesticide-contaminated water. Only chlorpyrifos and quinalphos were detected >1 in RWS3 (1.6571), RWS4 (1.0285), RWS14 (1.2571), and RWS15 (1.2000) sample sites, implying that the drinking water poses a health risk to humans. Hence, pesticide hazards should be mitigated and rigorous monitoring is needed to reduce pesticide residues in drinking water.

FULL TEXT

DETAILS

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Fast and Sensitive Detection of SARS-CoV-2 Nucleic Acid Using a Rapid Detection System Free of RNA Extraction

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ABSTRACT (ENGLISH)

Objectives. To establish and evaluate the analytical and clinical performance of the Flash20 SARS-CoV-2 nucleic acid rapid detection system free of RNA extraction. *Methods*. The limit of detection (LoD) was determined using a negative nasopharyngeal swab matrix spiked with different concentrations of SARS-CoV-2 virus; a total of 734,337 reference sequences of viral genomes from GenBank were used for the in-silico analysis to assess the inclusivity of the assay. The specificity of the system was evaluated by testing 27 medically relevant organisms. A total of 115 clinical specimens were collected and tested on the Flash20 SARS-CoV-2 detection system and with an FDA-approved comparator test to assess the clinical performance of the system. *Results*. The LoD of the Flash20 SARS-CoV-2 detection system is 250 copies/mL with a positive rate ≥90% ($n = 20$); alignments results showed that over 99% identity of the primer and probe of the Flash20 SARS-CoV-2 nucleic acid rapid detection system to the available SARS-CoV-2 sequences; the omicron samples tested 100% positive. None of the 27 organisms showed cross-reactivity with the Flash20 SARS-CoV-2 nucleic acid rapid detection system. Among all the 215 clinical samples, the Flash20 SARS-CoV-2 nucleic acid rapid detection system exhibits a high sensitivity of 99.24% (131/132) and 100% (83/83) specificity. *Conclusion*. The nucleic acid rapid detection system provides sensitive and accurate detection of SARS-CoV-2 free of RNA extraction. The high sensitivity and short time to results of approximately 35 minutes may impact earlier infection control and disease management.

FULL TEXT

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1. Introduction

In December 2019, a cluster of patients with pneumonia of unknown cause was found in Wuhan, Hubei province in China. A novel betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified as the causative agent and caused coronavirus disease 2019 (COVID-19) [1]. SARS-CoV-2 can be person-to-person transmitted effectively through aerosols or fomites [2, 3]. With transmission capabilities before symptom onset [4], this disease has rapidly spread across the world [5]. On 7th September, 2021, over 220 million of confirmed cases of COVID-19 had been reported globally and the cumulative number of deaths over 4.5 million [6]. The global public health issue caused serious economic losses worldwide. The World Health Organization (WHO) recommends a combination of measures to control the spread of COVID-19, including rapid diagnosis and immediate isolation of cases, rigorous tracking, and precautionary self-isolation of close contacts [7]. A fast, reliable, and accurate diagnostic test would play an extremely important role in SARS-CoV-2 infection prevention and control.

Nucleic acid amplification tests (NAATs), such as real-timereverse-transcription polymerase chain reaction (RT-qPCR), are the gold standard for diagnosing SARS-CoV-2 infection [8–10]. The National Medical Products Administration (NMPA) in China has approved RT-qPCR technology as detection method for COVID-19 [11]. Conventional RT-PCR is a multistep process that involves the isolation and purification of nucleic acids from a clinical sample and detects the viral RNA. It is tedious, time-consuming, labor-intensive, and requires highly skilled

technicians. In this case, the SARS-CoV-2 tests have required turnaround times of nearly 6–8 hours or more [12, 13]. The use of testing with a rapid turnaround may allow for an earlier detection isolation of confirmed cases, facilitate earlier infection control, and disease management [13, 14].

In this study, we developed a real-time RT-PCR-based SARS-CoV-2 POCT (Point of Care Test) (Coyote Bioscience Co., Ltd., Beijing, China) that can be performed without the need for RNA extraction and uses one-step, real-time RT-PCR directly on a fast real-time PCR cycler (Figure 1). The entire process of sample to result can be completed within 35 minutes, which is much quicker than the 3-4 hours required for conventional real-time RT-PCR. The rapid SARS-CoV-2 detection system is evaluated for the analytical performance, including the limit of detection (LoD), inclusivity, cross-reactivity, and interference. The clinical performance of the system was assessed by testing 115 clinical samples and compared with the DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit, which is FDA EUA-authorized.

[figure(s) omitted; refer to PDF]

2. Materials and Methods

2.1. Reagent and Instrument

Flash20 SARS-CoV-2 nucleic acid rapid detection system is developed based on the technical of molecular parallel reaction. The nucleic acid of the virus is released by the lysis agent prior to or during the process of reverse transcription amplification in parallel with deoxyribonucleic acid (DNA) amplification. The system is RNA extraction-free, includes inhibition-resistant reverse transcriptase and DNA polymerase, and includes the addition of a PCR enhancer, which improves amplification efficiency and leads to the overall higher sensitivity of the assay. Briefly, a 15 μL oropharyngeal or nasopharyngeal swab sample in Coyote's VTM is treated by 15 μL of respiratory sample buffer. The treated sample can be tested by adding to RT-PCR reaction directly. The thermal cycles are as follows: 42°C for 3 min, 94°C for 10 s (94°C for 3 sec and 55°C for 10 sec)$*$15 cycles, (94°C for 3 sec, 55°C for 10 sec, and reading)$*$30 cycles. The entire testing time from sample to answer is about 35 min.

DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit was used as a reference reagent in the clinical evaluation. DiaCarta kit is conducted on ABI7500 real-time PCR instrument.

2.2. Limit of Detection (LoD)

The LoD was determined by evaluating different concentrations of SARS-CoV-2 virus from BEI Resources spiked into SARS-CoV-2 negative nasopharyngeal matrix. The negative nasopharyngeal matrix is mixed with 10 negative nasopharyngeal swabs eluted with Coyote viral transport medium. A total of 6 concentrations, including 2000 copies/mL, 1000 copies/mL, 500 copies/mL, 400 copies/mL, 300 copies/mL, and 200 copies/mL were tested. The lowest concentration at which all 4 replications were positive was designated as the tentative LoD. A panel of 5 concentrations around the tentative LoD, including 300 copies/mL, 250 copies/mL, 200 copies/mL, 150 copies/mL, and 100 copies/mL were further tested in replicates of 20 to determine the LoD. The LoD is the lowest concentration that can be reproducibly detected ≥90% of the time.

2.3. Inclusivity Analysis

Evaluation of the reactivity of the Flash20 SARS-CoV-2 nucleic acid rapid detection system was performed by sequence alignment of the assay oligonucleotide primer and probe sequences with all publicly available nucleic acid sequences for SARS-CoV-2 in GenBank as of March 29, 2021, to demonstrate the predicted inclusivity of the Flash20 SARS-CoV-2 nucleic acid rapid detection system. The genomes enrolled for in-silico analysis met the following criteria: (1) genome length > 29,000 bp; (2) N proportion (not specific A, T, C, and G) <5% of the genome; (3) isolated from a human source.

The cultured Omicron variant was validated for inclusivity by spiking the variant into negative nasopharyngeal matrix to the concentration of 750 copies/mL. The Omicron variant samples were tested by the Flash20 SARS-CoV-2 nucleic acid rapid detection system for three replicates.

2.4. Specificity Analysis

A total of 27 organisms were obtained and tested to evaluate the specificity of the kit. Human coronavirus 229E, human coronavirus NL63, MERS-coronavirus (irradiated), adenovirus, human metapneumovirus (hMPV), human

parainfluenza virus 1, human parainfluenza virus 2, human parainfluenza virus 3, human parainfluenza virus 4a, human parainfluenza virus 4b, influenza B, enterovirus, and respiratory syncytial virus A were from BEI resource, and human coronavirus OC43, influenza A, Rhinovirus, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, pooled human nasal wash, *Bordetella pertussis*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pnuemophila*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Candida albicans* were obtained from ATCC. Three replicates of each organism stock were tested.

2.5. Interference Study

SARS-CoV-2 negative nasopharyngeal swabs were collected in triplicate and spiked with potential interferent, including nasal secretion, dexamethasone, zanamivir, tobramycin, adrenalin, menthol, and blood (human). Additional negative nasopharyngeal swabs were collected and spiked with SARS-CoV-2 inactive virus (CoA NR-52287 BEI resource, lot number 70039068) at 3X LoD in addition to the potential interfering substances. Three concentration levels of the interference substances were tested in triplicates.

2.6. Clinical Evaluation

The clinical performance study was conducted with 179 leftover samples and 36 fresh samples collected and tested by PacGenomic CLIA Lab (Part I), DiaCarta CLIA Lab (Part II), and the China-Japan Friendship Hospital. A consent form was signed by the patients of these samples that agreed their sample can be used for investigational studies. Among these patients, 108 were male and 105 were female and the gender of the other 2 was unknown. The number of patients aged between 25 and 64 were 139 in total. 115 samples were collected at PacGenomic lab and DiaCarta CLIA Lab and tested for their SARS-CoV-2 infection status with the TaqPath RT-PCR assay. The samples were kept frozen for up to 8 weeks. 100 samples were collected at the China-Japan Friendship Hospital. 64 of these samples were kept frozen for up to 6 weeks, and 36 of these samples were fresh sample. The samples were chosen randomly and blinded to the operators to be tested on the new system. These leftover samples were split into two parts, one part was tested on the Flash20 FlashDetect™ SARS-CoV-2 Detection system and second on the comparator test (DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit, which is FDA-authorized EUA with the LoD of 600 NDU/mL. The sample information is provided in Tables 1 and 2.

Table 1

Patients' age distribution.

Patient age groups	All subjects (*n* = 215)
<14 years of age	29
14–24 years of age	38
25–64 years of age	139
≥65 years of age	9

Table 2

Genders of the patients.

Sex	All subjects (*n* = 215)
Male	108
Female	105

Unknown	2

2.7. Statistical Analysis

A two-by-two table was constructed to assess the agreement between the Flash20 SARS-CoV-2 nucleic acid rapid detection system and the DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit. The level of agreement between assays was determined by Cohen's kappa, the sensitivity (positive percent agreement) and specificity (negative percent agreement) with two-side 95% confidence interval (CI).

3. Results

3.1. The LoD for Nasopharyngeal/Oropharyngeal Swabs

LoD studies were performed to determine the lowest detectable concentration of SARS-CoV-2 at which approximately 95% of all (true positive) replicates are tested positive. The detection rate of the system for virus samples no less than 300 copies/ml is 100%. When the virus concentration is more than 250 copies/ml, the detection rate of the Flash20 SARS-CoV-2 nucleic acid rapid detection system for positive samples is more than 95% (95%-ORF1ab, 90%-N). The results of the LoD confirmation study are shown in Table 3. The Ct values of the LoD estimation are provided in Appendix-1. These results demonstrate that the LoD of the Flash20 SARS-CoV-2 nucleic acid rapid detection system is 250 copies/mL for nasopharyngeal/oropharyngeal swabs, which is 1.875 copies/reaction. The amplification curves for ORF1ab and N genes of SARS-CoV-2 with a concentration of 250 copies/mL are shown in Figure 2.

Table 3

LoD determination study results.

Titer SARS-CoV-2 virus (copies/mL)	Replicates	ORF1ab gene positive result (%)	N gene positive result (%)	Percentage of positive rate (%)
2000	4	100 (4/4)	100 (4/4)	100 (4/4)
1000	4	100 (4/4)	100 (4/4)	100 (4/4)
500	4	100 (4/4)	100 (4/4)	100 (4/4)
400	4	100 (4/4)	100 (4/4)	100 (4/4)
300	4	100 (4/4)	100 (4/4)	100 (4/4)
200	4	75 (3/4)	75 (3/4)	75 (3/4)
300	20	100 (20/20)	100 (20/20)	100 (20/20)
250	20	95 (19/20)	90 (18/20)	95 (19/20)
200	20	80 (16/20)	85 (17/20)	95 (19/20)
150	20	80 (16/20)	80 (16/20)	90 (18/20)
100	20	50 (10/20)	60 (12/20)	70 (14/20)

[figure(s) omitted; refer to PDF]

3.2. Inclusivity Evaluation Results

A total of 4,086,522 isolates that met enrollment criteria were enrolled. The 4,086,522 viral genomes were aligned against the primer/probe sets used.

All of the alignments showed over 99% identity of the Flash20 SARS-CoV-2 nucleic acid rapid detection system to the available SARS-CoV-2 sequences. The sequences containing more than 2 mismatches in primer and probe and more than 1 mismatch in the 3′ end of primer and probe are listed in Figure 3.

[figure(s) omitted; refer to PDF]

The Omicron variant sample with the concentration of 750 copies/mL was tested 100% positive. The amplification curve for ORF1ab and N gene of the Omicron variant sample is listed in Figure 4.

[figure(s) omitted; refer to PDF]

3.3. Specificity Analysis

All 27 organisms evaluated gave negative results for the detection of the SARS-CoV-2 virus, which demonstrates that the Flash20 SARS-CoV-2 nucleic acid rapid detection system assay design does not react with related pathogens or other highly prevalent disease agents. The results of the specificity analysis are shown in Table 4.

Table 4

Cross-reactivity analysis.

Organism	Titer	Cross-reaction
Human coronavirus 229E	1.4×10^5 TCID50/mL	No
Human coronavirus OC43	1.4×10^5 TCID50/mL	No
Human coronavirus NL63	1.4×10^5 TCID50/mL	No
MERS-coronavirus (irradiated)	1.4×10^5 TCID50/mL	No
Adenovirus	1.4×10^5 TCID50/mL	No
Human metapneumovirus (hMPV)	1.4×10^5 TCID50/mL	No
Human parainfluenza virus 1	1.4×10^5 TCID50/mL	No
Human parainfluenza virus 2	1.4×10^5 TCID50/mL	No
Human parainfluenza virus 3	1.4×10^5 TCID50/mL	No
Human parainfluenza virus 4 a	1.4×10^5 TCID50/mL	No
Human parainfluenza virus 4 b	1.4×10^5 TCID50/mL	No
Influenza A	1.4×10^5 TCID50/mL	No
Influenza B	1.4×10^5 TCID50/mL	No

Enterovirus	1.4×10^{5} TCID50/mL	No
Respiratory syncytial virus A	1.4×10^{5} TCID50/mL	No
Rhinovirus	1.4×10^{5} TCID50/mL	No
Haemophilus influenzae	1.0×10^{6} cells/mL	No
Streptococcus pneumoniae	1.0×10^{6} cfu/mL	No
Streptococcus pyogenes	1.0×10^{6} org/mL	No
Pooled human nasal wash	N/A	No
Bordetella pertussis	1.0×10^{6} cells/mL	No
Mycoplasma pneumoniae	1.0×10^{6} cfu/mL	No
Chlamydia pneumoniae	1.0×10^{6} IFU/mL	No
Legionella pnuemophila	1.0×10^{6} cfu/mL	No
Staphylococcus aureus	$>10^{4}$ cfu/vial	No
Staphylococcus epidermidis	1.0×10^{6} cfu/mL	No
Candida albicans	1.0×10^{6} cfu/mL	No

3.4. Interference Study Results

None of these potential interferences were found to inhibit the performance of the assay, as all swabs, with and without the potential interferences were found to have the expected results. The results of the interference study results are shown in Table 5.

Table 5

Potential interfering testing results.

Potential interfering substance	Concentration	Positive results (detected X/3)	Negative results (detected X/3)
Nasal secretion	2.5%	3/3	0/3
5%	3/3	0/3	10%
3/3	0/3	-	
Dexamethasone	0.05 mg/L	3/3	0/3

0.1 mg/L	3/3	0/3	0.15 mg/L
3/3	0/3	-	
Zanamivir	2.5 mg/L	3/3	0/3
5 mg/L	3/3	0/3	10 mg/L
3/3	0/3	-	
Tobramycin	50 mg/L	3/3	0/3
100 mg/L	3/3	0/3	150 mg/L
3/3	0/3	-	
Adrenalin	0.1 mg/L	3/3	0/3
0.2 mg/L	3/3	0/3	0.25 mg/L
3/3	0/3	-	
Menthol	20 mg/L	3/3	0/3
25 mg/L	3/3	0/3	50 mg/L
3/3	0/3	-	
Blood (human)	0.5%	3/3	0/3
1%	3/3	0/3	1.5%

3.5. High Sensitivity and Specificity for Clinical Samples

Among the total of 115 clinical samples, 63 were tested at the PacGenomic site, 52 were tested at DiaCarta site, and the rests were tested at the China-Japan Friendship Hospital. The positive percent agreement and negative percent agreement between the FDA Emergency Use Authorized RT-PCR test and the Flash20 SARS-CoV-2 nucleic acid rapid detection system were calculated using all the valid results. As indicated in Table 6, the Flash20 SARS-CoV-2 nucleic acid rapid detection system exhibits a high sensitivity and specificity of 99.24% (131/132) and 100% (83/83), respectively. Individual sample results on the Flash20 FlashDetect™ SARS-CoV-2 Detection kit and the DiaCarta QuantiVirus™ SARS-CoV-2 are provided in Appendix-2.

Table 6

Test results compared to DiaCarta QuantiVirus™ SARS-CoV-2.

Flash20 SARS-CoV-2 nucleic acid rapid detection system	DiaCarta QuantiVirus™ SARS-CoV-2

Positive	Negative	Total	Positive
131	0	131	Negative
1	83	84	Total
132	83	215	Sensitivity
99.24% (95% CI: 95.85% to 99.98%)			Specificity

3.6. Comparison with Other COVID-19 Nucleic Acid System

The performance of Flash20 SARS-CoV-2 detection system was compared with three SARS-CoV-2 detection systems approved by the U.S. Food and Drug Administration (FDA), and the information is listed in Table 7.

Table 7

Comparison with other COVID-19 nucleic acid system[1).]

Names	Flash20 SARS-CoV-2 detection system	Xpert Xpress SARS-CoV-2 test	Visby medical COVID-19 point of care test	DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit
Test type	POCT	POCT	POCT	Conventional PCR
Method	RT-PCR	RT-PCR	RT-PCR	RT-PCR
Sample type	Nasopharyngeal and oropharyngeal	Nasopharyngeal, oropharyngeal, nasal, mid-turbinate swab, and nasal wash/aspirate specimens	Nasopharyngeal, anterior nasal, and mid-turbinate swabs	Upper respiratory specimen sputum
Time from sample to answer	30 min	45 min	30 min	>2 hours
LoD	250 copies/mL	0.0200 PFU/mL	435 copies/swab	200 copies/mL or 100 copies/mL[1)]
Sensitivity	98%	97.80%	100%	100%
Specificity	100%	95.60%	95%	100%
Target gene	ORF1ab and N	N2 and E	N	ORF1ab, N, and E

[1)]The LoD of DiaCarta QuantiVirus™ SARS-CoV-2 Test Kit is different in detecting instrument, 200 copies/mL for ABI QuantStudio 5 and ABI 7500 Fast Dx, and 100 copies/mL for Bio-Rad CFX384.

4. Discussion

In this study, we showed a rapid detection system for nucleic acid from the SARS-CoV-2 virus, with high sensitivity and specificity. The Flash20 SARS-CoV-2 system is an easy-to-use, on-demand format that generates results in about 35 min.

Current routine PCR products for diagnostics for SARS-CoV-2 can perform high-throughput processing, but the turnaround time limits their usability in patient management and infection control. The use of rapid diagnostic tests with sensitivity and specificity comparable with current standard molecular diagnostic because of the reduced time spent by uninfected individuals in health-care settings where they may be at increased risk of infection. Lessons learned from the recent Ebola virus and Zika virus epidemics are that delay in developing the right diagnostic for the right population at the right time has been a costly barrier to disease control and prevention [15]. If rapid tests had been available throughout the Ebola epidemic, one study estimate, for Sierra Leone, fast detection testing might have reduced the scale of the epidemic by over a third [16].

Key advantages of rapid molecular diagnostic products are simple operation procedures and rapid detection, which is significantly faster than the seven hours currently required by traditional molecular testing. High-speed detection reduces turnaround time (TAT) for the diagnosis of COVID-19, thus allowing prompt decision making regarding the isolation of infected patients [17]. Multiple rapid molecular diagnostic products have received FDA emergency use authorization (EUA), including Cepheid Xpert Xpress SARS-CoV-2 (Xpert Xpress) and Abbott ID NOW COVID-19 (ID NOW) [18]. Xpert Xpress is easy to use and the run-time is 45 min, which include loading the sample and the cartridge. ID NOW has the sample-to-answer time at about 17 min to result. Marie et al. compared Xpert Xpress and ID NOW to the Roche Cobas SARS-CoV-2 assay for samples with low, medium, and high viral concentrations [19]. The Xpert Xpress showed a very high level of agreement with the cobas assay, but ID NOW did not detect most specimens with Ct value ≥30. These findings confirm those published by Hogan et al. [20–22]. In this study, we assess the analytical and clinical performance of Flash20 SARS-CoV-2 system. Flash20 SARS-CoV-2 system is a rapid, sensitive, and accurate platforms with results available in 35 min, and hands on time about 1-2 min. Flash20 SARS-CoV-2 nucleic acid rapid detection system is developed based on direct RT-qPCR with simple sample treatment. Proper specimen collection and storage are critical to the performance of this test. PCR inhibitors should be avoided in RT-qPCR reactions. Synthetic fiber swabs with thin plastic or wire shafts were recommended by the Centers for Disease Control and Prevention (CDC). Calcium alginate swabs or swabs should not be used with wooden shafts, as they may contain substances that inactivate some viruses and may inhibit molecular tests. As guanidine-contained VTM is incompatible with RT-qPCR, VTM without guanidine should be used with the Flash20 SARS-CoV-2 nucleic acid rapid detection system. Coyote's VTM is validated and recommended to be use with the system. Insert a swab into the nostril, parallel to the palate, and leave the swab in place for a few seconds to absorb secretions. Place swab immediately into a sterile tube containing sample storage solution.

The LoD of the Flash SARS-CoV-2 system, established with SARS-CoV-2 virus in the nasopharyngeal matrix was 250 copies/mL. Fifty-four out of 55 positive samples detected by the reference kit (DiaCarta QuantiVirus™ SARS-CoV-2) were confirmed with the Flash20 SARS-CoV-2 nucleic acid rapid detection system, with a diagnostic sensitivity of 98.18%. The Flash20 SARS-CoV-2 system detected 18 low-level positive samples with Ct value >30, with the highest of Ct value equal 36.32. But it did not detect 1 low-level positive sample with Ct value of 34.03. The Flash SARS-CoV-2 system is for use on nasopharyngeal swabs in Coyote viral transport medium. The current evaluation on clinical samples was done using both DiaCarta and Coyote VTM, as choice of transport medium is limited during the current pandemic. Moreover, a 100% of analytical and clinical specificity was observed against other viruses, such as human coronavirus 229E, human coronavirus OC43, human coronavirus NL63, and MERS-coronavirus. The number of specimens included in the clinical trial is only 115, but these specimens were chosen to span the positivity range of clinical specimens, including those specimens with a low viral load.

The limitation of the Flash20 SARS-CoV-2 system is the small number of testing ports per instrument, since each

instrument can support 4 positions. This limitation can be offset by the rapidity of the assay, and/or adding modules/bays for more capacity.

Authors' Contributions

Liang Ma, Yongtong Cao, and Yong Cui conceptualized the study. Liang Ma, Peng Gao, Yanyan Fan, Xiaomu Kong, Yongwei Jiang, Meimei Zhao, Yi Liu, and Hong Huang performed data curation. Liang Ma provided funding acquisition. Peng Gao, Yanyan Fan, Yongwei Jiang, Meimei Zhao, Yi Liu, and Hong Huang performed data detection. Xiaomu Kong provided statistics. Liang Ma, Yongtong Cao, and Yong Cui wrote the manuscript.

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DETAILS

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The Value of Peripheral Blood Leukocyte Parameters in the Early Diagnosis and Clinical Prognosis of Sepsis

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ABSTRACT (ENGLISH)

Background. Early diagnosis of sepsis is the key to timely, targeted treatment. Cell population data (CPD) has been widely used in many diseases, but its predictive value for early diagnosis and the clinical outcome of sepsis remains unclear. Therefore, this paper discusses whether peripheral blood leukocyte parameters can be used as predictive indicators for early diagnosis and the clinical outcome of sepsis. *Methods*. A retrospective study of 45 patients with sepsis, 53 patients with nonseptic infections, and 86 healthy check-ups admitted to Gansu Provincial Hospital from January 2021 to June 2022 was done using a hematology analyzer. *Results*. The results of LYMPH#, HFLC#, IG#, NE-WX, LY-WX, LY-WY, and MO-WX showed better diagnostic efficiency in the sepsis group and nonseptic infection group. When the seven differential leukocyte parameters were used to establish diagnostic models, the sensitivity and specificity were 82.20% and 77.40%, respectively. Correlation analysis showed that LYMPH# and HFLC# were positively correlated with PCT (P<0.05). The clinical outcome of sepsis showed that the leukocyte parameters of discharged WBC and LY-X had better predictive efficacy. When the two differential leukocyte parameters were used to establish diagnostic models, the sensitivity and specificity were 90.90% and 100.00%. Cox regression analysis showed that leukocyte parameters of discharged WBC and LY-X were independent predictors of clinical outcomes (P<0.05). *Conclusion*. Leucocyte parameters HFLC#, IG#, NE-WX, LY-WX, LY-WY, and MO-WX had a certain auxiliary effect on the early diagnosis of sepsis leukocyte parameters of discharged WBC and LY-X were independent predictors of clinical outcomes in patients with sepsis. Therefore, peripheral blood leukocyte parameters may have predictive value for early diagnosis and the clinical outcome of sepsis, but large-scale retrospective studies are still needed to prove our preliminary results.

FULL TEXT

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1. Introduction

Sepsis is a life-threatening systemic inflammatory response syndrome with organ dysfunction caused by the dysregulated host response to infection [1]. Sepsis has a high morbidity and mortality rate and a very poor prognosis, and the incidence tends to increase year by year, causing a serious social burden. Therefore, sepsis has become an important global public health problem [2–4]. In the past few decades, a large number of serum (plasma) experimental tests have been conducted on sepsis patients, and the molecular markers of sepsis have been found to include C-reactive protein (CRP), procalcitonin (PCT), presepsin, interleukin-6 (IL-6), and neutrophil CD64 [5–7]. During the study, it was found that PCT and presepsin may be the most effective detection means for early

diagnosis, prognostic monitoring, and clinical treatment of sepsis [8, 9]. However, it has not been fully verified that these biomarkers can help clinicians identify sepsis as early as possible and accurately, carry out treatment, and predict prognosis [10, 11]. As a result, interest in identifying new, low-cost, routinely available indicators of infection has been stimulated. Studies have shown [12–14] that changes in the response of cell population data (CPD) to various stimuli (such as infection) can rapidly provide information on leukocyte activation, such as the cell complexity, fluorescence intensity, cell size, and distribution width of neutrophils, monocytes, and lymphocytes, which can quantitatively analyze cell morphology and function. At the same time, the method of acquisition and operation is simple, convenient, and rapid, which provides a new method to improve the early diagnosis of sepsis. Notably, the new generation of hematology analyzers can automatically obtain CPD parameters during standard cell count analysis, significantly reducing the need for additional blood tests and costs [15]. The aim of this study was to evaluate the clinical relevance of leukocyte parameters as early diagnostic parameters of sepsis or septic shock and to test the predictive role of leukocyte parameters in the prognosis of sepsis.

2. Materials and Methods

2.1. General Information

A total of 45 patients with sepsis and 53 patients with nonseptic infections who visited Gansu Provincial Hospital from January 2021 to June 2022 were selected as research subjects. Another 86 healthy subjects underwent physical examination in the same period and were selected as the healthy control group. Laboratory and auxiliary examination results and clinical outcomes of patients with sepsis were collected.

2.1.1. Inclusion Criteria

In line with the definition of sepsis in the “Save Sepsis Movement: Guidelines for the International Management of Sepsis and Septic Shock (2016)” jointly developed by the American Society of Critical Care Medicine and the European Society of Critical Care Medicine in 2016 [1].

2.1.2. Exclusion Criteria

(1) Do not actively cooperate or give up halfway; (2) incomplete case data after admission; (3) admission time <24 hours; (4) severe liver and kidney diseases, advanced malignant tumors, hematological diseases, serious heart diseases, and acute cerebrovascular diseases.

2.2. Study Groups

(1) According to the definition of sepsis [1], the patients were divided into the sepsis group, nonseptic infection group, and healthy control group.

(2) According to the clinical outcome, the patients were divided into an unhealed group and a cured group.

2.3. Study Methods

Blood culture and bacterial identification were performed using the BacT/Alert3D blood culture instrument and the VitEK-2 automatic bacterial identification system. Blood samples were collected for peripheral blood cell analysis using a hematology analyzer (Sysmex XN9000®) and matching reagents. 26 leukocyte parameters were recorded in the sepsis group, the nonseptic infection group, the healthy control group at admission ($t0$), and the sepsis group at discharge.

2.4. Primary Outcome

We evaluate the predictive value of peripheral blood leukocyte parameters for early diagnosis and the clinical outcome of sepsis.

2.5. Secondary Outcomes

We investigate whether there is a correlation between peripheral blood leukocyte parameters and PCT in the early stage of sepsis and the diagnostic value of PCT in the clinical outcome analysis of sepsis patients.

2.6. Ethics

The study was approved by the Institutional Ethics Committee of Gansu Provincial Hospital. Written informed consent was obtained from each participant or their family members.

2.7. Statistical Analysis

PASS11 software was used to estimate the sample size: this study was a randomized controlled trial designed in

parallel, and the two groups were the sepsis and nonseptic infection groups, respectively. The peripheral blood leukocyte parameter values of the study subjects were the main observational outcome index. According to previous literature reports (or pretest results), NA = 37 cases and NB = 37 cases were calculated. Assuming that the loss of follow-up rate of the subjects is 10%, sample size NA = 37 ÷ 0.9 = 41 cases, NB = 37 ÷ 0.9 = 41 cases. Finally, 41 cases were included in the sepsis group, and 41 cases were included in the nonseptic infection group, for a total of at least 82 cases.

Statistical analysis was conducted using IBM SPSS STATISTICS (version 26.0). Normal distribution measurement data were expressed as $x \pm s$, analysis of variance was compared between groups, non-normal distribution measurement data were expressed as *M* (P25, P75), and a nonparametric rank sum test wad compared between groups. Pearson's correlation analysis was performed between PCT and leukocyte parameters. Multivariate Cox regression analysis was used to determine the risk factors. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were drawn to evaluate the differential diagnostic efficacy of leukocyte parameters and the predictive value of clinical outcome. $P<0.05$ indicated statistical significance.

3. Results

(3.1) Brief description of leukocyte parameters [16]. Please see Table 1

(3.2) Diagnostic value of peripheral blood leukocyte parameters in early sepsis

3.1. Comparison of General Clinical Data between the Sepsis Group and Nonseptic Group

There were no significant differences in gender, age, underlying diseases, and multiple site infection between the sepsis and nonseptic groups ($P>0.05$). The pulse and maximum body temperature of the sepsis group were higher than those of the nonseptic group, and the systolic blood pressure and diastolic pressure were significantly lower than those of the nonseptic group ($P<0.05$), Table 1, as shown in Table 2.

Table 1

Brief description of leukocyte parameters.

Leukocyte parameters	Cell type description and instructions
WBC	White blood cell count
NEUT#	Neutrophil absolute concentration
LYMPH#	Lymphocyte absolute concentration
MONO#	Monocyte absolute concentration
EO#	Eosinophil absolute concentration
BASO#	Basophil absolute concentration
HFLC#	High fluorescence large cell absolute concentration
IG#	Immature granulocyte absolute concentration
NE-SSC	Mean side scattered light distribution width of the neutrophil
NE-SFL	Mean fluorescent light distribution width of the neutrophil

NE-FSC	Mean forward scattered light distribution width of the neutrophil
LY-X	Mean side scattered light intensity of the lymphocyte
LY-Y	Mean fluorescent light intensity of the lymphocyte
LY-Z	Mean forward scattered light intensity of the lymphocyte
MO-X	Mean side scattered light intensity of the monocyte
MO-Y	Mean fluorescent light intensity of the monocyte
MO-Z	Mean forward scattered light intensity of the monocyte
NE-WX	Side scattered light distribution width of the neutrophil
NE-WY	Fluorescent light distribution width of the neutrophil
NE-WZ	Forward scattered light distribution width of the neutrophil
LY-WX	Side scattered light intensity of the lymphocyte
LY-WY	Forward scattered light intensity of the lymphocyte
LY-WZ	Forward scattered light intensity of the lymphocyte
MO-WX	Side scattered light distribution width of the monocyte
MO-WY	Fluorescent light distribution width of the monocyte
MO-WZ	Forward scattered light distribution width of the monocyte

Table 2

Comparison of general clinical data between sepsis and nonseptic infection groups.

Clinical features	Sepsis group (*n*=45)	Nonseptic infection (*n*=53)	*t*-value	P value
Gender (male/female)	23/22	32/21	61.99	0.357
Age	57.5±12.68	52.79±10.59	3.47	0.601
Underlying diseases				
Hypertension	11.00 (24.44)	9.00 (16.98)	78.38	0.503

Diabetes	6.00 (13.33)	5.00 (9.43)	80.79	0.234
Maximum body temperature (°C)	37.05 ± 0.35	36.90 ± 0.19	2.58	0.012
Multiple site infection	8.00 (17.78)	6.00 (11.32)	71.90	0.001
Pulse (times/min)	99.11 ± 10.26	89.49 ± 5.31	5.68	0.286
Systolic blood pressure (mm Hg)	118.58 ± 9.42	122.13 ± 8.16	−2.00	0.048
Diastolic blood pressure (mm Hg)	66.13 ± 5.76	74.47 ± 4.34	−7.98	0.001

Abbreviations: 1 mm Hg = 0.133 kPa.

3.2. Comparison of Leukocyte Parameters between the Sepsis Group, Nonseptic Infection Group, and Healthy Control Group

WBC, NEUT#, LYMPH#, MONO#, EO#, HFLC#, IG#, NE-SFL, LY-Y, LY-Z, MO-X, MO-Y, MO-Z, NE-WY, NE-WZ, LY-WX, LY-WZ, MO-WX, MO-WY of sepsis group and the nonseptic infection group were compared with the healthy control group, and the difference was statistically significant (P<0.05). LYMPH#, BASO#, HFLC#, IG#, NE-WX, LY-WX, LY-WY, MO-WX of the sepsis group were higher than those of the nonseptic infection group, and NE-FSC of the sepsis group was lower than that of the nonseptic infection group, and the differences were statistically significant (P<0.05). Please see Table 3.

Table 3

Leukocyte parameter test results of sepsis group, nonseptic infection group, and healthy physical examination group.

Leukocyte parameters	Sepsis group (*n* = 45)	Non-septic infection group (*n* = 45)	Healthy control group (*n* = 86)	P value
WBC ($\times 10^9$/L)	9.62 (7.13, 14.42)	8.80 (6.88, 10.80)	5.90 (4.95, 6.80)	<0.05[bc]
NEUT# ($\times 10^9$/L)	7.56 (5.51, 11.94)	7.12 (5.37, 9.21)	3.22 (2.54, 4.01)	<0.05[bc]
LYMPH# ($\times 10^9$/L)	1.49 ± 0.95	0.96 ± 0.71	2.03 ± 0.47	<0.05[abc]
MONO# ($\times 10^9$/L)	0.58 (0.46, 0.75)	0.53 (0.35, 0.70)	0.39 (0.33, 0.47)	<0.05[bc]
EO# ($\times 10^9$/L)	0.02 (0.00, 0.10)	0.01 (0.00, 0.04)	0.09 (0.06, 0.15)	<0.05[bc]
BASO# ($\times 10^9$/L)	0.02 (0.01, 0.02)	0.01 (0.00, 0.01)	0.02 (0.02, 0.03)	<0.05[ac]
HFLC# ($\times 10^9$/L)	0.03 (0.01, 0.06)	0.00 (0.00, 0.01)	0.00 (0.00, 0.00)	<0.05[abc]
IG# ($\times 10^9$/L)	0.11 (0.55, 0.335)	0.05 (0.03, 0.08)	0.01 (0.01, 0.02)	<0.05[abc]
NE-SSC	147.33 ± 8.56	150.76 ± 4.91	150.15 ± 3.00	—

NE-SFL	50.90 (47.90, 58.85)	49.90 (45.8, 53.75)	41.40 (40.18, 43.13)	<0.05[bc]
NE-FSC	82.34±8.44	89.03±4.8	85.00±2.68	<0.05[ac]
LY-X	77.91±4.51	77.23±4.01	76.08±1.29	—
LY-Y	67.30 (62.85, 72.50)	65.10 (60.95, 70.15)	57.70 (56.30, 59.43)	<0.05[bc]
LY-Z	58.00 (55.85, 60.30)	58.80 (57.10, 59.90)	54.25 (53.80, 55.00)	<0.05[bc]
MO-X	119.50 (117.45, 122.35)	118.20 (114.70, 121.20)	113.85 (113.20, 114.70)	<0.05[bc]
MO-Y	115.40 (106.45, 124.15)	113.20 (105.4, 118.1)	97.65 (94.8, 101.23)	<0.05
MO-Z	66.94±3.86	67.86±3.17	63.51±1.79	<0.05[bc]
NE-WX	337 (316.5, 361.5)	316 (304.50, 325)	308.50 (298, 318)	<0.05[ab]
NE-WY	671 (619, 791.5)	698 (662.5, 773.5)	611.50 (598, 628)	<0.05[bc]
NE-WZ	738 (706.5, 770)	703 (680.5, 730.5)	638.5 (621.75, 661)	<0.05[bc]
LY-WX	554 (510, 630.5)	501 (457, 567)	461 (440, 489.5)	<0.05[abc]
LY-WY	1010 (913.5, 1102.5)	902 (814, 996)	870.5 (832.25, 919.5)	<0.05[ab]
LY-WZ	624 (574.5, 716.5)	622 (576.5, 658.5)	572 (559, 593.75)	<0.05[bc]
MO-WX	277 (247, 296)	253 (234, 279)	243 (230.75, 253)	<0.05[abc]
MO-WY	727 (668.5, 799.5)	707 (643, 773)	649.5 (597.25, 708.25)	<0.05[bc]
MO-WZ	665.56±150.17	630.55±85.34	617.20±68.85	—

Note: the letters in superscript indicate the results of post hoc tests: [a]significant difference between the septic and nonseptic infection groups in post hoc comparison; [b]significant difference between the septic and healthy control groups in post hoc comparison; [c]significant difference between the nonseptic infection and healthy control groups in post hoc comparison.

3.3. Efficacy Evaluation of Leukocyte Parameters in Differential Diagnosis between Septic and Nonseptic Infection Groups

LYMPH#, HFLC#, IG#, NE-FSC, NE-WX, LY-WX, LY-WY, MO-WX with statistically significant leukocyte parameters were selected to make ROC curves for differential diagnosis of sepsis and nonseptic infection, and LYMPH#, HFLC#, IG#, NE-WX, LY-WX, LY-WY, MO-WX, and the area under the curve >0.60, have a better differential diagnosis performance in early sepsis, as shown in Figure 1. Seven differential leukocyte parameters were used to

establish diagnostic models, as shown in Figure 2. Leukocyte parameters such as AUC, cut-off, sensitivity, specificity, positive predictive value, and negative predictive value are as shown in Table 4.

[figure(s) omitted; refer to PDF]

Table 4

Efficacy evaluation of leukocyte parameters in differential diagnosis between sepsis and nonseptic infection.

Leukocyte parameters	AUC (95%CI)	Cut-off	Se (%)	Sp (%)	PPV (%)	NPV (%)
LYMPH#	0.686 (0.580, 0.791)	0.84	75.60	56.60	59.67	73.21
HFLC#	0.751 (0.652, 0.849)	0.03	53.30	88.70	80.02	69.11
NE-WX	0.722 (0.617, 0.827)	331.50	57.80	86.80	78.80	70.78
LY-WX	0.677 (0.571, 0.784)	506.50	77.80	54.70	59.32	74.37
LY-WY	0.713 (0.612, 0.814)	954.00	64.40	69.80	64.42	69.78
MO-WX	0.657 (0.549, 0.765)	276.50	55.60	71.70	62.52	65.54
IG#	0.724 (0.619, 0.829)	0.09	64.40	79.20	72.44	72.38
LYMPH#+HFLC#+NE-WX+LY-WX+LY-WY+MO-WX+IG#	0.829 (0.739, 0.913)	0.62	82.20	77.40	75.54	83.66

Abbreviations. AUC, area under the receiver operating characteristic curve; CI, confidence interval; Se, sensitivity, Sp, specificity; PPV, positive pretest value; NPV, negative pretest value.

3.4. The Correlation between Leukocyte Parameter Results and PCT in Sepsis and Non-Septic Infection

LYMPH#, HFLC#, and PCT were positively correlated ($P<0.05$) as shown in Table 5. The correlation analysis between LYMPH#, HFLC#, and PCT is shown in Figure 3.

Table 5

Correlation between leukocyte parameter and PCT (*R*).

Indicators	LYMPH#	HFLC#	IG#	NE-WX	LY-WX	LY-WY	MO-WX
PCT	0.515	0.339	0.083	0.006	−0.043	0.037	−0.097

[figure(s) omitted; refer to PDF]

3.5. Predictive Value of Peripheral Blood Leukocyte Parameters for Clinical Outcomes of Patients with Sepsis

3.5.1. Comparison of Leukocyte Parameters between Unhealed and Cured Groups

The results showed that three of the 26 leukocyte parameters were statistically significant in the clinical outcome analysis of sepsis patients (P<0.05), which were WBC, NEUT#, and LY-X as shown in Table 6.

Table 6

Detection results of leukocyte parameters in admission group and discharge group.

Leukocyte parameters	Unhealed group (*N*=45)	Cured group (*N*=45)	*F*/*t*	P value
Leukocyte parameters on admission				
WBC ($\times 10^9$/L)	19.0 (15.4, 21.0)	8.7 (6.9, 11.8)	−3.6	<0.05
NEUT# ($\times 10^9$/L)	16.2 (13.1, 19.3)	6.9 (5.0, 7.9)	−3.6	<0.05
LY-X	75.4±5.4	78.7±4.0	2.1	<0.05
Leukocyte parameters on discharged				
WBC ($\times 10^9$/L)	11.3 (10.9, 11.9)	5.5 (4.0, 7.9)	−3.8	<0.05
NEUT# ($\times 10^9$/L)	9.1 (9.0, 9.6)	3.8 (3.0, 5.1)	−3.8	<0.05
LY-X	77.1 (74.5, 78.6)	78.6 (76.3, 81.0)	−2.3	<0.05

3.5.2. Influence of Leukocyte Parameters on Clinical Outcomes of Patients with Sepsis

Univariate Cox regression analysis showed that WBC, NEUT#, and LY-X during admission and discharge were predictive factors of clinical outcome in patients with sepsis (P<0.05). Multivariate Cox regression analysis showed that WBC and LY-X at discharge were still independent predictors of clinical outcome in patients with sepsis (P<0.05), as shown in Table 7.

Table 7

Cox regression analysis of leukocyte parameters on clinical outcome of sepsis patients.

Leukocyte parameters	Univariate cox regression		Multivariate cox regression analysis	
HR	95% CI	HR	95% CI	Leukocyte parameters on admission
WBC	1.171	1.0178–1.273*	—	—
NEUT#	1.168	1.074–1.271*	—	—
LY-X	0.803	0.696–0.928*	—	—

Leukocyte parameters on discharged				
WBC	1.541	1.240–1.194*	1.721	1.236–2.396*
NEUT#	1.597	1.253–2.035*	—	—
LY-X	1.259	1.025–1.546*	1.476	1.028–2.118*

Abbreviations: HR, hazard ratio; CI, confidence interval; *P<0.05.

3.5.3. Predictive Value of Leukocyte Parameters and PCT for Clinical Outcome in Patients with Sepsis

The results of ROC analysis showed that the discharge leukocyte parameters WBC and LY-X had better performance in predicting the clinical outcome of patients with sepsis. Combined diagnosis of discharged leukocyte parameters WBC and LY-X, as shown in Figure 4. Leukocyte parameters such as AUC, cut-off, sensitivity, specificity, positive predictive value, and negative predictive value are as shown in Table 8.

[figure(s) omitted; refer to PDF]

Table 8

Predictive value of leukocyte parameters for clinical outcome in patients with sepsis.

Leukocyte parameters	AUC (95% CI)	Cut-off	Se (%)	Sq (%)	PPV (%)	NPV (%)
WBC on discharged	0.900 (0.714–1.000)	10.26	90.00	100.00	100.00	90.91
LY-X on discharged	0.743 (0.579–0.907)	81.10	80.00	77.10	77.75	79.40
PCT on admission	0.581 (0.392–0.771)	0.59	91.00	78.50	80.89	89.71
PCT discharged	0.693 (0.452–0.933)	0.25	70.00	74.30	73.15	71.24
WBC + LY-X on discharged	0.900 (0.714–1.000)	0.580	90.00	100.00	100.00	90.91

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; Se, sensitivity, Sp, specificity; PPV, positive pretest value; NPV, negative pretest value.

4. Discussion

Each year millions of patients died of sepsis, mortality rate close to 30%, which caused serious damage to human health, so the early recognition and appropriate treatment is crucial for improving the prognosis of patients with sepsis, but the general blood culture and drug sensitive test need 3–5 d out as a result, so early identification of bacterial infections or suspicions is the first step toward sepsis treatment [17]. Therefore, it is particularly important to provide clinically objective, rapid, and accurate experimental detection indicators for the diagnosis and symptomatic treatment of sepsis patients. In this study, we analyzed the parameters of peripheral blood leukocytes in patients with sepsis to explore their diagnostic value for sepsis. Related literature [18–20] has shown that when the body is infected, the changes of peripheral blood cells are not only the single occurrence of WBC and the proportional change of various classification counts, but also the generation of rod-shaped nuclei and other immature granulocytes and the morphological changes of numerous cells, including the appearance of neutrophils toxic particles, vacuoles, and dule bodies. The cytoplasmic particles of lymphocytes increased, and their volume increased. Monocytes migrate and deform, and their volume and morphology change to some extent. At the same

time, through the analysis of the indicators reflecting the left shift in granulocyte, monocyte, and lymphocyte morphology and the change in intracytoplasmic structure complexity, it was found that they have a certain value in predicting and differentiating infection.

It was found that the number of lymphatic markers HFLC#, IG#, NE-WX, LY-WX, LY-WY, and MO-WX was significantly changed in the differential diagnosis of the sepsis group and the nonseptic infection group. Lymphatic #, BASO#, HFLC#, IG#, NE-WX, LY-WX, LY-WY, and MO-WX were higher than those of the nonseptic infection group and could be correlated with the increase of peripheral blood mononuclear cells and neutrophil and lymphocyte cytoplasmic particles caused by the activation of the mononuclear macrophage system after infection, while NE-FSC was lower than that of the sepsis group. In the nonseptic infection group, immunosuppression may be associated with the immune imbalance in the body when severe bloodstream infection occurs, which further confirmed the changes in the size and internal structure of neutrophils and lymphocytes in the development of sepsis patients [13, 14, 21], and the combined diagnosis of these indicators was more effective. Its sensitivity and negative predictive value were higher than those of a single test. HFLC# is a new quantitative parameter provided by a blood cell analyzer based on the principle of flow cytometry combined with nucleic acid fluorescence staining technology. It can detect the quantitative indicators of lymphocyte qualitative change from different angles and does not depend on the quantity change. The results of this study showed that the specificity and positive predictive value of HFLC# in the sepsis group were higher than other monitoring indicators, and the value (reference range of HFLC#: 0-0.01 Gpt/l) was higher than that of the nonseptic infection group and the healthy body test group, which was consistent with the research results of Arneth et al. [22]. Related literature also shows that HFLC# has been intensively studied as a potential marker of sepsis [23, 24].

In the clinical outcome analysis of patients with sepsis, the leukocyte parameters WBC and LY-X in discharge were independent risk factors for predicting the clinical outcome of patients with sepsis. When the body is infected and tissue is damaged, WBC will rapidly increase its accumulation and phagocytose the invading pathogens [25]. As a routine clinical examination indicator, WBC plays a certain guiding role in the body infection. However, due to the large individual differences and the fact that WBC is easily affected by mental, emotional, sports, and surrounding environment factors, its normal range value is relatively wide, which has certain limitations in the diagnosis of sepsis patients [26]. Therefore, it is often necessary to combine it with other indicators to make a more accurate judgment of the patient's condition. Therefore, the sensitivity, specificity, positive predictive value, and negative predictive value of the combined diagnosis of the discharged leukocyte parameters WBC and LY-X have better predictive value for the clinical outcome of patients with sepsis.

As a precursor of calcitonin, PCT is produced by the thyroid gland when the body is not infected. When the body has a severe systemic infection, the lung, liver, kidney, brain, and pancreas are the main sources of PCT [27]. The value of procalcitonin is positively correlated with the severity of infection and is one of the most commonly used inflammatory indicators in clinical practice [28]. The value of procalcitonin can increase rapidly in 2~4 h after infection, and reaches a peak in 24~48 h, and can increase to 1000 times of the normal value in severe infection. A number of studies have shown that CRP, IL-6, and other markers have more diagnostic value in sepsis [29, 30]. There was a correlation between LYMPH# and HFLC# and the inflammatory index PCT in the differential diagnosis of sepsis group and nonseptic infection group, but the correlation was weak. In addition, in the clinical outcome analysis of patients with sepsis, the predictive ability of PCT was weaker than that of the combined diagnosis of WBC and LY-X in discharge. The main reasons may be two aspects: First, the variation of serum PCT levels in sepsis patients is large, and PCT is often difficult to accurately reflect the occurrence and progression of sepsis at the early stage of the disease. Secondly, in different stages of disease, differences in detection methods, size of infected organs, types of pathogenic bacteria, and the immune inflammatory state of the body make it difficult to uniformly define the critical value of PCT [31]. Traumatic stress and surgery can also cause an increase of the serum PCT level [32]. However, the results of this study enhance the predictive value of peripheral blood leukocyte parameters for sepsis, so clinicians can consider it an auxiliary indicator. However, the results of this study point out the predictive value of peripheral blood leukocyte parameters for sepsis, so clinicians can consider it an auxiliary

indicator.

In summary, peripheral blood leukocyte parameters may be helpful for clinicians to predict early diagnosis and the clinical outcome of sepsis. However, this study has certain limitations: (1) The included study is a retrospective clinical study, and selection bias is inevitable. We will conduct a larger prospective study in the future; (2) the sample size of some included studies is relatively small, which may lead to the bias of the analysis results; and (3) patients with sepsis were not graded for severity. Despite these limitations, our study provides new insights into the value of peripheral blood leukocyte parameters in the early diagnosis and clinical outcome of sepsis.

Disclosure

Yuandan He and Qianqian Liu are the co-first authors.

Authors' Contributions

Yuandan He and Qianqian Liu are responsible for the design and conception of the paper, the collection and collation of data, and the writing of the paper and the article. Lianhua Wei is responsible for the quality control and proofreading of the paper, the overall responsibility for the article, and the supervision and management. Keke Li and Zhipeng Sun are responsible for reviewing and revising the papers. Wenjuan Li, Fangmin Geng, Zhangping Lu, and Hongwei Zhang collated documents/data.

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DETAILS

Subject:	Infections; Neutrophils; Blood; Blood pressure; Parameter sensitivity; Health care; Sepsis; Normal distribution; Lymphocytes; Body temperature; Diagnosis; Diagnostic systems; Compounds; Mathematical models; Regression analysis; Light; Leukocytes; Critical care; Hematology; Clinical outcomes; Statistical analysis; Lymph; Correlation analysis
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Comparison between the Chemical Composition of Essential Oil from Commercial Products and Biocultivated *Lavandula angustifolia* Mill.

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ABSTRACT (ENGLISH)

The main aim of this study was to assess the differences in the chemical composition of essential oil from biocultivated *Lavandula angustifolia* in the Thracian Lowland floristic region, Bulgaria, and commercially available products from Bulgarian markets. Following the analytical results conducted with gas chromatography-mass

spectrometry, we have established some differences in the chemical composition of the tested samples. The essential oil of biocultivated lavender contained 35 compounds, which represent 94.13% of the total oil. Samples from commercial products contained 28–42 compounds that represent 93.03–98.69% of the total oil. All the examined samples were rich in monoterpene hydrocarbons (1.68–12.77%), oxygenated monoterpenes (70.42–87.96%), sesquiterpene hydrocarbons (4.03–13.78%), and oxygenated sesquiterpenes (0.14–0.76%). The dominant components in all examined samples were linalool (20.0–45.0%) and linalyl acetate (20.79–39.91%). All the examined commercial samples contained linalool and linalyl acetate as was described in the European Pharmacopoeia, but in one of the samples, the quality of linalyl acetate is lower than that recommended in the European Pharmacopoeia.

FULL TEXT

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1. Introduction

Lavender (*Lavandula angustifolia Mill.*) is one of the most popular aromatic plants in the Lamiaceae family with origin in the Mediterranean region and is cultivated worldwide for medicinal and commercial purposes [1, 2]. *Lavandula angustifolia* (*L. angustifolia*) has great economic values as an essential oil-producing plant. Because of its characteristic and pleasant aroma, as well as its therapeutic properties, the essential oil of lavender is of considerable importance in pharmaceutical, cosmetics, perfume, food, and flavor industries [3–5]. The floral essential oil of lavender is documented to have therapeutic effects such as antibacterial, antioxidant, antifungal, carminative, sedative, antidepressive, analgesic, and anti-inflammatory [3, 6, 7]. In addition, according to Basch et al., the aroma of lavender is one of the most widely utilized in aromatherapy, considered to be relaxing, with anxiolytic effects [8]. The multiple therapeutic applications of *L. angustifolia* are attributed mainly to the presence of volatile bioactive substances contained in the essential oil [4].

Lavender essential oil is usually produced by steam or hydrodistillation from flowering tops, and other used methods for oil extraction are supercritical CO_2 fluid extraction, microwave, ultrasound, and turbohydrodistillation [9]. The chemical composition of the EO differs according to the extraction technique [5, 10]. However, essential oil from *L. angustifolia* is composed of various constituents, including esters: linalyl acetate, lavandulyl acetate, and geranyl acetate; alcohols: linalool, *α*-terpineol, and terpinen-4-ol; sesquiterpenes: *β*-caryophyllene; and monoterpene: cis-*β*-ocimene [2, 4, 10–13]. The greater proportion is linalyl acetate and linalool which are considered active constituents, but linalool is considered one of the most examined odorant molecules [14]. Both components are responsible for therapeutic effects. Moreover, all constituents contribute to the synergism of the total therapeutic effect. Today, lavender is cultivated around the world and enjoys continuing popularity for various therapeutic and cosmetic purposes [3, 12].

According to Stanev et al., Bulgaria has a long tradition of lavender cultivation and essential oil production dating since the 1900s [15]. Moreover, according to Stanev et al., Bulgarian lavender populations are characterized by high adaptability to the geographic, climate, and soil conditions of the country and consequently high essential oil content and quality [16]. This placed Bulgaria as one of the main lavender-growing and essential oil-producing countries, along with France [1, 13, 17]. Due to the economic value of lavender essential oil, interest in the development of new commercial products has increased. This study compares the chemical composition of essential oils from biocultivated *L. angustifolia* and commercial products in Bulgaria.

2. Materials and Methods

2.1. Chemicals and Reagents

For determination of retention indices (RIs), heptane (99%), octane (≥99%), nonane (99%), decane (≥99%), undecane (≥99%), dodecane (99%), tridecane (≥99%), tetradecane (≥99%), hexadecane (≥99%), heptadecane (99%), octadecane (99%), nonadecane (99%), and eicosane (99%) purchased from Merck KGaA (Darmstadt, Germany) were used. For diluting essential oils, hexane purchased from Thermo Fisher Scientific GmbH (Bremen,

Germany) was used.

2.2. Plant Material and Oil Extraction

L. angustifolia was cultivated in the area of Belashtitsa, Thracian Lowland floristic region, Bulgaria. The cultivated plant has grown in a continental climate with an average annual temperature of 12.3°C and rendzina soil. The essential oil was obtained from the air-dried flowering tops of lavender by hydrodistillation using the Clevenger apparatus for 3 h according to the standard procedure described in the European Pharmacopoeia 9 (07/2018:1534) [18]. After completion of distillation, the collected oil was dried over anhydrous sodium sulfate and stored in dark glass vials at 4°C until GC-MS analysis.

2.3. Chromatographic Conditions

Gas chromatography-mass spectrometry (GC-MS) was used for the analysis. GC-MS analyses were carried out using Bruker Scion 436-GC SQ MS, Bremen, Germany. The column used was a Bruker BR-5ms fused silica capillary (0.25 *µ*m film thickness and 15 m × 0.25 mm i.d.). The oven temperature was initially held at 45°C for 1 min and then increased to 140 at 3°C/min, and after that, it was increased to 250°C at 17°C/min and then held for 1 min. The flow rate of helium (carrier gas) was 1 mL/min. The injector split ratio was 1 : 50, and the injection volume was 1 *µ* L. The range of *m*/*z* was 50–350 in the full-scan mode. To compare the spectral data and retention indices of compounds, the Wiley NIST11 Mass Spectral Library (NIST11/2011/EPA/NIH) and the literature were used. Retention index values were calculated and compared with reported values for a C7–C20 series of *n*-alkane standards.

3. Results and Discussion

The distilled lavender EO had a strong odour of linalool and linalyl acetate. Its colour was yellow. The extracted essential oil and commercial essential oils were diluted with hexane and analyzed with GC-MS. Figure 1 shows the chromatogram of the EO of the cultivated lavender.

[figure(s) omitted; refer to PDF]

The essential oil of the cultivated lavender contained 35 compounds, which represent 94.13% of the total oil. Samples from commercial products contained 28–42, which represent 93.03–98.69% of the total oil. Table 1 shows the chemical composition found in essential oils from biocultivated *L. angustifolia* (CLA) and those from commercial products (CP 1–7).

Table 1

The GC data for essential oil components identified in cultivated *L. angustifolia* and commercial products, where tr indicates traces, less than 0.05%.

No.	Compound	RI	Class	CLA	CP 1	CP 2	CP 3	CP 4	CP 5	CP 6	CP 7
1	*α*-Pinene	937	MH	0.14	0.35	0.14	0.21	0.11	0.22	0.55	0.31
2	Camphene	947	MH	0.2	tr	0.17	0.17	tr	0.19	0.39	0.19
3	*β*-Pinene	967	MH	tr	—	—	tr	0.11	0.11	0.38	tr
4	1-Octen-3-ol	973	O	0.10	tr	0.33	0.16	0.21	0.26	0.15	0.20
5	3-Octanone	976	O	0.16	0.13	1.75	0.29	0.20	0.44	tr	1.38
6	*β*-Myrcene	980	MH	0.74	0.11	0.48	0.43	0.55	0.34	0.42	0.65

7	3-Octanol	988	O	—	—	0.66	—	—	—	tr	0.43
8	3-Carene	993	MH	—	—	tr	0.13	—	tr	—	0.13
9	Acetic acid, hexyl ester	1001	O	0.1	1.86	0.73	0.34	0.13	0.26	0.16	0.69
10	*p*-Cymene	1003	MH	tr	0.54	—	tr	—	0.25	0.14	tr
11	o-Cymene	1007	MH	0.13	—	0.24	0.18	tr	0.10	—	0.20
12	Limonene	1010	MH	0.33	—	1.21	0.55	0.33	0.40	0.74	0.93
13	Eucalyptol	1013	MO	0.42	5.38	1.58	0.86	2.17	0.79	3.57	0.90
14	***Trans-β-* ocimene**	**1022**	**MH**	**6.70**	**0.29**	**1.21**	**7.19**	**4.93**	**3.87**	**1.46**	**5.81**
15	**cis-*β*-ocimene**	**1031**	**MH**	**2.89**	**0.15**	**1.21**	**2.47**	**1.86**	**3.84**	**0.37**	**4.06**
16	*γ*-Terpinene	1039	MH	tr	0.11	0.13	0.15	tr	0.11	0.14	0.30
17	Linalool oxide	1051	MO	1.10	—	0.21	0.16	tr	0.33	0.19	0.23
18	*α*-Terpinolene	1064	MH	0.20	0.14	tr	tr	0.11	tr	0.29	0.14
19	***β*-Linalool**	**1087**	**MO**	**23.13**	**34.04**	**35.99**	**24.34**	**35.32**	**26.60**	**24.68**	**26.92**
20	1-Octen-3-yl acetate	1095	O	1.01	—	0.46	0.68	0.36	0.76	0.25	0.82
21	Camphor	1123	MO	0.34	2.78	0.47	0.29	0.13	0.46	8.29	0.28
22	Isoborneol	1140	MO	—	0.64	—	—	—	—	—	—
23	Lavandulol	1150	MO	—	—	2.34	—	1.64	—	—	1.68
24	Endo-borneol	1151	MO	1.68	1.62	—	1.44	—	2.44	2.82	—
25	**(-)-Terpinen-4-ol**	**1161**	**MO**	**2.28**	**2.15**	**2.64**	**3.97**	**1.54**	**3.36**	**3.48**	**5.34**
26	Cryptone	1166	MO	0.37	—	0.35	0.28	—	—	—	0.22
27	***α*-Terpineol**	**1179**	**MO**	**3.95**	**0.66**	**1.29**	**0.98**	**—**	**0.75**	**0.64**	**1.15**
28	Cis-geraniol	1214	MO	0.17	—	0.16	tr	0.21	0.10	—	0.13

29	Cuminal	1215	MO	—	—	0.20	0.15	—	tr	—	—
30	**Linalyl acetate**	**1249**	**MO**	**31.46**	**39.91**	**20.79**	**36.97**	**35.39**	**36.03**	**36.47**	**30.79**
31	Bornyl acetate	1273	MO	0.57	0.26	—	0.22	0.13	0.14	tr	0.15
32	**Lavandulyl acetate**	**1282**	**MO**	**4.21**	**0.41**	**3.57**	**3.87**	**2.88**	**3.67**	**2.40**	**3.53**
33	Nerol acetate	1359	MO	1.01	tr	0.32	0.31	0.43	0.12	0.14	0.28
34	Geranyl acetate	1380	MO	1.85	0.11	0.51	0.51	0.81	0.44	0.23	0.45
35	Zingiberene	1440	SH	—	—	0.18	tr	tr	tr	0.11	tr
36	**Caryophyllene**	**1407**	**SH**	**2.87**	**4.64**	**5.15**	**5.72**	**3.85**	**6.09**	**1.96**	**4.06**
37	*α*- Santalene	1412	SH	0.87	tr	—	0.69	0.44	0.78	0.33	0.50
38	Trans-*α*-bergamotene	1427	SH	0.18	—	0.17	0.20	0.12	0.20	—	0.13
39	Humulene	1442	SH	Tr	0.28	0.13	0.14	tr	0.20	tr	tr
40	**Cis-*β*-farnesene**	**1454**	**SH**	**3.99**	**0.23**	**tr**	**3.26**	**2.77**	**—**	**—**	**3.78**
41	Trans-*β*-farnesene	1455	SH	—	—	6.85	0.14	—	2.39	1.37	—
42	Gemacrene D	1468	SH	—	—	0.89	0.61	0.59	—	tr	0.69
43	*β*-Copaene	1469	SH	0.56	tr	—	—	—	—	tr	—
44	*γ*-Cadinene	1490	SH	—	—	0.26	0.29	tr	0.25	0.26	0.19
45	*β*-Sesquiphellandrene	1510	SH	—	—	0.15	—	tr	0.14	—	tr
46	Caryophyllene oxide	1563	SO	—	0.15	0.50	0.34	0.14	0.65	0.10	0.26
47	Tau-cadinol	1607	SO	tr	—	0.13	tr	—	0.11	0.17	tr
48	*α*-Bisabolol	1615	SO	—	tr	—	—	—	—	0.38	—
–											
Terpene classes											

	Monoterpene hydrocarbons (MHs)			11.33	1.68	4.79	1148	8.00	9.43	4.88	12.77
	Oxygenated monoterpenes (MOs)			72.94	87.96	70.42	74.35	80.45	75.23	82.91	72.05
	Sesquiterpene hydrocarbons (SHs)			8.47	5.15	13.78	11.05	7.77	10.05	4.03	9.35
	Oxygenated sesquiterpenes (SOs)			—	0.15	0.63	0.34	0.14	0.76	0.65	0.26
	Others (O)			1.37	1.99	3.93	1.47	0.90	1.72	0.56	3.52
	Total identified			94.13	96.93	93.55	98.69	97.26	97.19	93.03	97.95

The results show the mean values of the three independent samples of each sample, CLA and CP 1–7. The standard error of the mean does not exceed 2% of it and has been removed to simplify reporting.

The essential oil obtained from the biocultivated lavender was characterized by monoterpene hydrocarbons (11.33%), oxygenated monoterpenes (72.94%), and sesquiterpenes hydrocarbons (8.47%). Among the monoterpene hydrocarbons, the main components were trans-*β*- ocimene (6.70%) and cis-*β*-ocimene (2.89%). Oxygenated monoterpenes were detected in higher amounts. The prevailing components from oxygenated monoterpenes were linalyl acetate (31.46%), *β*-linalool (23.13%), lavandulyl acetate (4.21%), *α*-terpineol (3.95%), and (-)-terpinen-4-ol (2.28%). The presence of linalool and linalyl acetate in lavender essential oil determined its anti-inflammatory, cytotoxic, antimicrobial, repellent effect, sedative, local anaesthetic, analgesic, antioxidant, antimicrobial, and other activities [14, 19–22]. The main volatile components of sesquiterpene hydrocarbons were caryophyllene (2.87%) and cis-*β*-farnesene (3.99%).

All analyzed commercial essential oils were rich in monoterpene hydrocarbons (1.68–12.77%), oxygenated monoterpenes (70.42–87.96%), sesquiterpenes hydrocarbons (4.03–13.78%), and oxygenated sesquiterpenes (0.14–0.76%). The representatives of the monoterpenes with the highest content were trans-*β*- ocimene (0.29–7.19%) with higher amounts in CP 3 and cis-*β*-ocimene (0.15–4.06%) with higher amounts in CP 7. Oxygenated monoterpenes, especially *β*-linalool (24.34–35.99%) and linalyl acetate (20.79–39.91%), were in higher amounts in all of the commercial products. They were followed by (-)- terpinen-4-ol (1.54–5.34%), lavandulyl acetate (0.41–3.87%), and eucalyptol (0.79–5.38%). The sesquiterpene hydrocarbons caryophyllene (1.96–6.09%), cis-*β*-farnesene (0.08–3.78%) and trans-*β*-farnesene (0.14–6.85%) were also isolated. Oxygenated sesquiterpenes (0.14–0.76%) were in the lower concentration in all of the analyzed commercial products.

According to the European Pharmacopoeia, the relative content of lavender oil compounds should be in the following ranges: limonene (maximum 1.0%), 1,8-cineole (maximum 2.5%), 3-octanone (0.1–5.0%), camphor (maximum 1.2%), linalool (20.0–45.0%), linalyl acetate (25.0–47.0%), terpinen-4-ol (0.1–8.0%), lavandulyl acetate (minimum 0.2%), lavandulol (minimum 0.1%), and *α*-terpineol (maximum 2.0%) [18]. Only one sample contained more than 1% lemonene, and the rest corresponded to the requirements of the European Pharmacopoeia. In our results, only in two of the commercial products, the amount of 1,8-cineole was higher than 2.5%. According to the requirements of the European Pharmacopoeia, isolated 3-octanone was in the described ranges in all of the examined samples, but in two of the examined samples (CP 2 and CP 6), camphor did not correspond to the requirements. All the samples contained the recommended amounts of linalool and terpinen-4-ol. Only sample 2 contained less than 25% linalyl acetate. Not all the samples corresponded to the requirements for lavandulyl

acetate, lavandulol, and α-terpineol.
The obtained results can be compared with these by other researchers. In research conducted with Ukrainian cultivars, Pokajewicz et. al. found that linalool (11.42–44.05%), (-)-terpinen-4-ol (1.17–11.25%), and linalyl acetate (15.79–35.27%) as the main chemical compounds [23]. Moreover, Dong et al., researchers from China, reported linalool (19.71%), linalyl acetate (26.61%), α-terpineol (3.61%), and lavandulyl acetate (12.68%) as the main compounds [24]. In addition, Adaszynska et al., researchers from Poland, reported that linalool (15.85–23.88%) and linalyl anthranilate (1.58–12.78%) are the main ingredients together with geraniol acetate (2.37–10.61%), caryophyllene (2.78–6.24%), 1-terpinen-4-ol (5.53–9.73%), and p-menth-1-en-8-ol (3.98–7.94%) [25].
The difference in the composition of lavender essential oils may be due to influence of cultivation methods and different geographic regions on the accumulation of chemical compounds [26–28]. According to Bara, some of the exogenous factors such as light and soil (pH and constituents) may increase the concentration of terpenes [29]. It is considered that many enzymes of secondary pathways are UV-B-dependent [29, 30]. Hassiotis et al. reported that temperature and the flowering stage have a positive influence on the EO composition, but rainfall during the flowering period has a negative influence on EO content [31]. The linalool content is influenced by temperature, flower development, and rainfalls, and rainfalls during the harvest period decrease linalool production [31]. Also, the addition of synthetic compounds would affect the differences in the chemical composition and its concentrations [6, 32]. Moreover, according to Filly et. al., the different isolation methods of essential oil may also lead to differences in composition [33]. For further studies, it is recommended conducting a survey on adding synthetic and part synthetic compounds to commercial products containing essential oils. The method used for analyses of the essential oil chemical profile is also important, and the GC-MS analysis represents well separation and identification of volatile compounds [34]. The method described above could also be used for further analysis.

4. Conclusions

A total of 50 volatile compounds were found in lavender biocultivated essential oil, which represents 93.17% of the total oil. The following terpene classes were found in the essential oil from lavender biocultivated essential oil: monoterpene hydrocarbons (11.33%), oxygenated monoterpenes (72.94%), and sesquiterpene hydrocarbons (8.47%). Oxygenated monoterpenes were detected in higher amounts. The prevailing components from them were linalool (23.13%) and linalyl acetate (31.46%). Volatile compounds found in the commercial products were 28–42, which represent 93.55–98.69% of the total oil. Commercial products were rich in oxygenated monoterpenes, and especially, linalool (24.34–35.32%) and linalyl acetate (20.79–39.91%) were in higher amounts. The results of this study indicate that the essential oil content and quality of the analyzed commercial products corresponded to the recommendations given in the European Pharmacopoeia.

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DETAILS

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Abebe, A. W., Sbhatu, D. B., Goitom, G. B., Kiros, H. A., & Gebreyohannes, G. (2023). Phytochemical constituents of adansonia digitata L. (baobab) fruit pulp from tekeze valley, tigrai, ethiopia. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/5591059

Baobab (Adansonia digitata L) is a large tree species growing in semiarid and arid lowlands of Ethiopia and other places. The plant is valued by natives for its contributions as a cash crop and livelihood tree. Previous studies using samples from different countries have documented their phytochemical profiles and nutritional and health benefits. This study explored the phytochemical constituents and biological activities of fruit pulp extracts of baobab collected from Tekeze Valley, Tigrai, Ethiopia. To this end, qualitative phytochemical screening tests, quantitative phytochemical analyses, and gas chromatography-mass spectrometry (GC-MS) analysis were carried out using aqueous extract. Analyses of antioxidant activities were also conducted with aqueous- and methanol-extracts using of 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), and hydroxyl (OH) radical scavenging activity assays. The qualitative screening tests showed the presence of flavonoids, phenols, saponins, tannins, and terpenoids. Quantitative analyses of these phytochemicals at 25, 50, and 100 g/mL aqueous extract resulted in 0.0252 to 0.1000% yields. Yields of flavonoids, phenols, and saponins were higher at 50 g/mL extract, while that of tannins and terpenoids were higher at 100 g/mL. GC-MS analysis resulted in 15 predominant compounds including (1,2bis(trimethylsilyl)benzene (13.17%), 2-methyl-7-phenylindole (11.75%), 2-ethylacridine (10.11%), and benzb]-1,4-oxazepine-4(5H)-thione,2,3-dihydro-2,8-dimethyl (10.11%). Aqueous and methanol extracts showed concentration-dependent antioxidant activities. In all the assays and concentrations, the antioxidant activities of both extracts were lower than that of the ascorbic acid standard. At equal extract concentrations (e.g., 100 and 250 µg/mL), methanol extract had higher antioxidant activities than aqueous extract. The findings can encourage future initiatives towards large-scale research for compiling a complete phytochemical profile of the fruit pulp of the Ethiopian baobab.

Abera, S., Yohannes, W., & Bhagwan, S. C. (2023). Effect of processing methods on antinutritional factors (oxalate, phytate, and tannin) and their interaction with minerals (calcium, iron, and zinc) in red, white, and black kidney beans. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6762027

The purpose of this study was to assess how different processing techniques affected mineral compositions, antinutritional factors, and their interactions in red, white, and black kidney beans consumed in Ethiopia. Mineral contents were found to be 41–44, 58–78, and 112–126 mg Ca/100 g in the raw, soaked, and cooked samples, respectively. Iron content in the raw, soaked and cooked samples were found to be 2.77–2.97, 1.94–2.20 and 2.87–3.28 mg Fe/100 g, respectively, showing 26–30% loss on soaking followed by 33–48% increase on cooking. While Zn content in the raw, soaked and cooked samples were found to be 2.47–3.26, 3.34–4.68 and 2.83–3.31 mg Zn/100 g, respectively, showing 35–43% increase on soaking followed by 15–29% decrease on cooking. In the case of antinutrients, both treatments showed incredible decrements. Phytate in the raw samples was 178-179 mg/100 g and showed a 12–16% decrement on soaking and a 37-38% decrement up on cooking, oxalate was 1.5–1.8 mg/100 g in the raw samples and showed a 4.4–13% decrement during treatments, and tannin in the raw samples was 102–160 mg/100 g and showed a 23–30% decrement on soaking, followed by 21–41% during cooking. Phytate : Ca and oxalate : Ca molar ratios in soaked and cooked samples were within the critical values in the raw samples. In contrast, phytate : Zn and Ca × phytate : Zn in all treatments were found to be within the critical value, confirming the good bioavailability of zinc in all the samples, while phytate : Fe was found over the critical value, showing its poor availability.

Chemistry International Journal, o. A. (2023). Retracted: Oxidative potential and nanoantioxidant activity of flavonoids and phenolic acids in sophora flavescens. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9786262

Zhang, D., Wang, W., Zhao, H., Wang, S., Yu, M., Zhang, D., . . . Chen, D. (2023). Structural identification of impurities in pioglitazone hydrochloride preparations by 2D-UHPLC-Q-exactive orbitrap HRMS and their toxicity

prediction. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/2096521

Pharmaceutical companies and regulatory agencies have more and more concerns for impurities in pharmaceuticals and their toxicity. In this work, heart-cutting two-dimensional ultrahigh-performance liquid chromatography (2D-UHPLC) in combination with high-resolution mass spectrometry (HRMS) was used, setting HRMS as positive mode of electrospray ionization to identify five impurities in pioglitazone hydrochloride preparations. With the heart-cutting 2D-UHPLC and online desalting technique, the structures of five impurities were deduced in an analysis of MSn data. And three of them, Impurity-2, Impurity-3, and Impurity-5, have never been reported before. The fragmentation patterns of five impurities were proposed on a basis of accurate mass and fragment ions in this study. Since the toxicity of impurities is relevant to their structures, toxicology of all five impurities was predicted by three software tools, and the result showed that these compounds have good safety profile.

Xiang-Pu, Z., Zhang, S., Chun-Yan, X., Wei-Wei, L., Hai-Bo Ling, Luo, Y., . . . Yi, C. (2023). Liquid chromatography-tandem mass spectrometry detection of human and veterinary drugs and pesticides in surface water. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6350669

Antibiotics and pesticides are widespread in most rivers and lakes due to the overuse of antibiotics and pesticides, but there are few methods for simultaneous analysis of antibiotics and pesticides in aquatic environments. To address this knowledge gap, a concise and sensitive analytical method is proposed in which three classes of human and veterinary drugs (sulfonamides, macrolides, and hormones) and two classes of pesticides (organophosphorus and neonicotinoids) are simultaneously extracted and determined in surface water. The solid-phase extraction column with Cleanert PEP-2 was preconditioned sequentially with 6 mL of methanol, ultrapure water, and citric acid buffer (pH 3.0) each for simultaneous extraction and further purification. The forty-seven target analytes were analysed by LC-MS/MS in positive and negative ion modes. The LC separation was performed using a Sigma-Aldrich C18 column with 0.1% formic acid in water and acetonitrile as a gradient eluting mobile phase in positive ion mode. The internal standard method was used to overcome the inevitable matrix effects in LC-MS/MS analysis. The matrix effects of most target analytes were in the range of 27–151%. The recoveries of forty analytes in the three concentrations (10, 50, and 100 $ng\ L^{-1}$) of surface water spiked samples ranged from 41 to 127%. The method quantitative limits of the analytes were in the range of 0.40–5.49 $ng\ L^{-1}$. Application of the method to analyze samples in the eight runoff outlets of the Pearl River Delta showed that some antibiotics and pesticides were detected, and the concentration of parathion was as high as 154 $ng\ L^{-1}$. A powerful tool for quickly and efficiently screening for contaminants in surface water has been presented.

Xia, B., Li, Y., Liu, Y., Sun, W., Chen, J., Li, L., . . . Cheng, H. (2023). Rapid separation of asiatic acid, quercetin, and kaempferol from traditional chinese medicine centella asiatica (L.) urban using HSCCC-semi-prep-HPLC and the assessment of their potential as fatty acid synthase inhibitors. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/7769368

The main objective of this study was to rapidly separate asiatic acid (AA), quercetin (QCN), and kaempferol (KPL) from Centella asiatica (L.) Urban using high-speed counter-current chromatography (HSCCC) in tandem with the UV detector of semipreparative high-performance liquid chromatography (Semi-Prep-HPLC) and to evaluate their potential as inhibitors of fatty acid synthetase (FAS). To efficiently prepare large amounts of AA, QCN, and KPL from Centella asiatica (L.) Urban, rapid and simple methods by HSCCC were established respectively based on the partition coefficients (K values) of crude samples. The conditions of HSCCC-Semi-Prep-HPLC for the large-scale separation of AA, QCN, and KPL from Centella asiatica (L.) Urban were established and optimized. This included selecting the solvent system, flow rate, rotation speed, and so on. HSCCC-Semi-Prep-HPLC was successfully applied to separate and purify AA, QCN, and KPL, with n-hexane-n-butanol-methanol-water (3 : 1 : 3 : 3, V : V : V : V) as the solvent system for AA, which was detected at a wavelength of 210 nm with the stationary phase retention of 70%, and with n-hexane-ethyl acetate-methanol-water (0.8 : 0.9 : 1.2 : 1, V : V : V : V) as the solvent system for the co-separation of QCN and KPL, which was detected at a wavelength of 254 nm with the stationary phase retention of 65%. AA could be isolated at a large scale with high purity (>91.0%) in only one-step HSCCC-Semi-Prep-HPLC separation (within 150 min) under the optimized conditions. Meanwhile, QCN and KPL could be simultaneously

isolated at a large scale with high purity (>99.1%) by another one-step HSCCC-Semi-Prep-HPLC separation (within 240 min) under the optimized conditions. The assessment of inhibition potential revealed that AA exhibited the strongest inhibitory effect on FAS, with an IC50 of 9.52 ± 0.76 μg/mL. Madecassic acid (MA) followed closely with IC50 values of 10.84 ± 0.92 μg/mL. QCN and KPL showed similar and relatively weaker inhibitory effects on FAS, with IC50 values of 43.09 ± 2.98 μg/mL and 36.90 ± 1.83 μg/mL, respectively. Overall, the HSCCC-Semi-Prep-HPLC method proved to be a highly efficient and reliable technique for separating AA, QCN, and KPL from Centella asiatica (L.) Urban, and the isolated compounds showed potential as FAS inhibitors.

Yang, J., Chi, L., & Li, S. (2023). Measurement of magnesium, zinc, and copper in human serum by using isotope dilution inductively coupled plasma mass spectrometry (ID ICP-MS). International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6612672

In order to evaluate the reliability of the ID ICP-MS method for the measurement of magnesium, zinc, and copper in human serum, we investigated the traceability, precision, trueness, and uncertainty of the method. This method traces the contents of magnesium, zinc, and copper in human serum to the standard materials NIST SRM3131a, SRM3168a, and SRM3114 respectively, thus completing the traceability to SI unit. The repeatability of this method for measuring magnesium, zinc, and copper in the human serum reference material GBW09152 was found to be 0.2%, 0.7%, and 0.6% (n = 9), respectively. The measurement, when employed to measure the magnesium, zinc, and copper in standard materials, had caused a maximum deviation of less than 0.88%, 1.35%, and 1.15%, respectively. The measurement results are within the stated uncertainty range of standard materials. The expanded uncertainties were 0.2 mg·kg−1, 0.04 mg·kg−1, and 0.08 mg·kg−1 (K = 2) for magnesium, zinc, and copper, respectively. Therefore, this method has high trueness, good reproducibility, and simple operation and is suitable for tracing the values of magnesium, zinc, and copper in human serum.

Soleimani, N., Dehghani, S., Mohammad, H. A., Mohammadzadeh, S., Fard, E. A., Atefeh, Z. S., . . . Ebrahimi, M. (2023). Comparing jaffe and enzymatic methods for creatinine measurement at various icterus levels and their impacts on liver transplant allocation. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9804533

The Model for End-Stage Liver Disease (MELD) scoring system is used to prioritize liver transplantations and assess disease severity. This includes the international normalized ratio (INR), creatinine, and total bilirubin. Since there are several ways to measure creatinine, MELD scores can produce inconsistent results. The objectives of this study were to define a valid cut-off for bilirubin interference in creatinine measurement and to assess the effects of various icteric levels on creatinine measurement and liver transplant allocation. A total of 400 serum samples were categorized into four groups based on their icteric indices and total bilirubin levels, including non-, mild, moderate, and severe icteric samples. Both chemical Jaffe and enzymatic techniques were used to determine the creatinine levels in all four groups, and the findings were compared. In parallel, serum samples from 83 liver transplant candidate patients were divided into three groups depending on their bilirubin levels and then similarly evaluated and interpreted. The MELD scores were then computed for each group and compared. In icteric samples, the enzymatic method produced higher results for the creatinine concentrations than the Jaffe method did, and the mean creatinine difference rose from 0.08 in nonicteric group to 1.95 in groups with severe icterus. In addition, the enzymatic approach yielded higher findings for creatinine and subsequently for MELD scores in patients who were liver transplant candidates. When the bilirubin concentration was above the 4 mg/dL threshold, there were differences between the approaches for both the creatinine and the MELD score (p values: 0.0001 and 0.027, respectively). The chemical Jaffe is a readily available and considerably cost-effective method for measuring creatinine. However, it is influenced by a variety of known and unknown interfering substances, and it should be applied cautiously when working with icteric samples. Alternate techniques such as the enzymatic method should be considered when the bilirubin level exceeds 4 mg/dL. Though this cut-off is instrument and kit-dependent, each laboratory is advised to have its cut-off for bilirubin interference.

Chemistry International Journal, o. A. (2023). Retracted: Application of isotope tracer in cross-well nanometre tracer testing. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9893685

Chemistry International Journal, o. A. (2023). Retracted: Influence of nanosemiconductor materials on thermal stability of solar cells. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9812606

Chemistry International Journal, o. A. (2023). Retracted: Preparation and optical properties of compound nanopowder art ceramics. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9854036

Chemistry International Journal, o. A. (2023). Retracted: Multiobjective optimization design of green building energy consumption based on inorganic thermal insulation nanomaterials. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9782540

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Chemistry International Journal, o. A. (2023). Retracted: Broadband design of midinfrared chiral metamaterials based on the indium tin oxide conical helix. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9856434

Chemistry International Journal, o. A. (2023). Retracted: Application of carbon nanofiber-modified concrete in industrial building design. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9862328

Chemistry International Journal, o. A. (2023). Retracted: Preparation and synergistic anti-tumor effect of iridium oxide nanocomposites under microscope. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9763513

Chemistry International Journal, o. A. (2023). Retracted: Human health risk prediction method of regional atmospheric environmental pollution sources based on PMF and PCA analysis under artificial intelligence cloud model. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9845298

Ingida, M., Bedane, G., Adugna, F., Nigusu, D., Hussen, M., & Chala, H. S. (2023). Wastewater treatment using wood ash and cement as chemical coagulant. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/8274687

Water is essential for daily activities and maintaining human well-being. However, in many less-developed countries, including Ethiopia, the lack of a well-developed wastewater treatment system leads to contaminated surface water. This poses significant risks to human health. To address this problem, wastewater can be treated using locally available materials such as wood ash and cement as chemical coagulants. The objective of this study was to treat wastewater using these materials. The study involved analyzing a 20-liter sample of wastewater from the Awetu River in Jimma City, Ethiopia. The materials used for the treatment included wood ash, cement, and lemon. Various doses of cement and wood ash were prepared and added to the wastewater. The results showed that 5 g was the optimum dosage for effectively treating the wastewater. The treated water at the optimum dosage exhibited significant improvements in turbidity, total dissolved solids, conductivity, and color, meeting drinking water criteria. Overall, the study concludes that locally available materials such as wood ash and cement can be successfully utilized as chemical coagulants for wastewater treatment. This approach offers a viable solution for improving water quality and reducing the risk of waterborne diseases.

Bekele, H., Yohannes, W., & Megersa, N. (2023). A highly selective analytical method based on salt-assisted liquid-liquid extraction for trace-level enrichment of multiclass pesticide residues in cow milk for quantitative liquid chromatographic analysis. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/1754956

In this study, a simple, inexpensive, selective, and fast salting-out assisted liquid-liquid extraction (SALLE) technique coupled with high-pressure liquid chromatography-diode array detection (HPLC-DAD) was developed for the extraction, preconcentration, and analysis of trace level seven multiclass pesticide residues in pasteurized and raw cow milk samples. The significant factors that affect the extent to which the target analytes are extracted, such as the type of extraction solvent and its volume, the type and concentration of salting-out salts, the pH of the solution, and the extraction time, have been investigated. Under optimum conditions, the correlation coefficient (r2) was obtained within a range of 0.9982–0.9997 for a broad linear range concentration of 2–1500 ng·mL−1. Reliable sensitivity was achieved with limits of detection (LODs) and limits of quantification (LOQs) ranging from 0.58–2.56 ng·mL−1 and 1.95–8.51 ng·mL−1, respectively. While precision with interday and intraday in terms of relative standard deviations (RSDs) was observed in the range of 1.97– 7.88% and 4.52– 8.04%, respectively. The results of the precision studies reveal that good repeatability and reproducibility (RSDs <9) were achieved, thus showing a low variability extraction of the developed method. Finally, the proposed and validated approach was effectively used to extract and determine pesticide residues in real milk matrices; however, the target analytes were not detected in all samples.

Yao, L., Meng, X., Luo, Y., Huang, X., Luo, S., & Wang, J. (2023). Next-generation sequencing analysis of 3 uterine adenosarcomas with heterogeneously differentiated genomic mutations. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/7436368

Uterine adenosarcoma (UA) is an uncommon mixed tumor containing a benign to at most mildly atypical epithelial component and a sarcoma-like stroma, usually a low-grade, stromal component, with rare heterogeneous elements. Currently, tumor etiology is largely unknown. To better understand the gene mutations in UA, next-generation sequencing (NGS) technology analysis was performed. This study showed that two low-grade UAs with heterologous components had ATRX gene frameshift mutation, and one patient had a MED12 missense mutation. Copy number amplification genes were mainly observed on chromosome 12q13–15. In this study, PIK3/AKT/PTEN pathway mutations were found to be common in adenosarcoma. In addition, a rare BCORL1-PRR14L fusion mutation was also identified. These findings provide a basis for future research into these molecular changes in tumorigenesis and targeted therapy.

Parveen, S., Saeed, F., Fozia, B. F., Parveen, N., Idrees, N., Nasir, S., & Fanja, R. (2023). QSPR modeling of fungicides using topological descriptors. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9625588

A topological index is a real number that is obtained from a chemical graph’s structure. Determining the physiochemical and biological characteristics of a variety of medications is useful since it more accurately represents the theoretical characteristics of organic molecules. This is accomplished using degree-based topological indices. The QSPR research has improved the structural understanding of the physiochemical properties of fungicides. Thirteen fungicides are examined for some of their physiochemical properties, and a QSPR model is built using nine of the drugs’ topological indices. Here, we examine the degree to which the topological indices and physiochemical attributes are connected. To do this, we create networks connecting each of the topological indices to the properties of fungicides and computationally construct topological indices of the drugs mentioned above. According to this QSPR model, the melting point, boiling point, flash point, complexity, surface tension, etc. of fungicides are strongly connected. It was discovered that the topological indices (TIs) applied to the fungicides more accurately represent their theoretical features and show a strong correlation with their physical attributes.

Chen, F., Ma, Y., Cui, Y., Wang, W., Mei, C., Nie, J., . . . Zhou, X. (2023). Determination of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma by UPLC-MS/MS and their pharmacokinetics. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/4747771

An ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method was developed for the determination of tenacissoside G, tenacissoside H, and tenacissoside I in rat plasma. The rat plasma was treated with liquid-liquid extraction using ethyl acetate. The determination was performed on the UPLC HSS T3

column (50 mm × 2.1 mm, 1.8 μm) with a mobile phase consisting of acetonitrile-water (containing 0.1% formic acid) and gradient elution at a flow rate of 0.4 mL/min. Electrospray (ESI) positive ion mode detection and multireaction monitoring (MRM) quantitative analysis were performed. A total of 36 rats were given tenacissoside G, tenacissoside H, and tenacissoside I, respectively, orally (5 mg/kg) and intravenously (1 mg/kg), with 6 rats in each group, to evaluate the pharmacokinetic difference of tenacissoside G, tenacissoside H, and tenacissoside I in rats. The calibration curves showed good linearity in the range of 5–2000 ng/mL, where r was greater than 0.99. The results of precision, accuracy, recovery, matrix effect, and stability met the requirements of biological sample detection methods. The established UPLC-MS/MS method was successfully applied to pharmacokinetic studies of tenacissoside G, tenacissoside H, and tenacissoside I, and the bioavailability was 22.9%, 89.8%, and 9.4%, respectively.

Chemistry International Journal, o. A. (2023). Retracted: Prediction of dissolved oxygen concentration in sewage treatment process based on data recognition algorithm. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9807109

Gebreyohannes, G., & Sbhatu, D. B. (2023). Wild mushrooms: A hidden treasure of novel bioactive compounds. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6694961

Secondary metabolites are hidden gems in mushrooms. Understanding these secondary metabolites' biological and pharmacological effects can be aided by identifying them. The purpose of this work was to profile the mycochemical components of the extracts of Auricularia auricula judae, Microporus xanthopus, Termitomyces umkowaani, Trametes elegans, and Trametes versicolor to comprehend their biological and pharmacological capabilities. Mushroom samples were collected from Kenya's Arabuko–Sokoke and Kakamega National Reserved Forests and identified using morphological and molecular techniques. Chloroform, 70% ethanol, and hot water solvents were used to extract the mycochemical components. Gas chromatography mass spectrometry (GC-MS) was used to analyze the chloroform, 70% ethanol, and hot water extracts of all the species examined. A total of 51 compounds were isolated from all extracts and classified as carboxylic acids, esters, phenols, fatty acids, alcohol, epoxides, aldehydes, fatty aldehydes, isoprenoid lipids, and steroids. Tetracosamethyl-cyclododecasiloxane (18.90%), oleic acid (72.90%), phenol, 2, 6-bis (1, 1-dimethylethyl)-4-methyl-, and methylcarbamate (26.56%) were all found in high concentrations in A. auricular judae, M. xanthopus, T. umkowaani, T. elegans, and T. versicolor, respectively. Fatty acids make up the majority of the compounds isolated from the T. elegans chloroform extract and the T. umkowaani 70% ethanol extract, respectively. Particularly, these fatty acids play crucial roles in the anti-inflammatory, hypocholesterolemic, anticancer, and antibiofilm formation activities. These bioactive elements indicate that the extracts of five wild mushrooms may be reliable sources of secondary metabolites for therapeutic development. Therefore, additional research is required to comprehend the usefulness of these chemicals in many functional areas and to improve the present understanding of macrofungi.

Wu, S., Abdu Ahmed Abdullah Al-Maskri, Li, Q., Liu, J., & Cai, S. (2023). A novel miRNA detection method using loop-mediated isothermal amplification. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6624884

A novel ligation-based loop-mediated isothermal amplification has been developed for miRNA detection. Two stem-loop structure DNA linker A/B probes which hybridized with miRNA were designed to establish a rapid and ultrasensitive miRNA-LAMP system for miRNA detection. Target miR-200a was used to template the ligation of Linker A/B probes with SplintR Ligase and used as a dumbbell-shaped amplicon. By adding BIP/FIP and Bst 2.0 DNA polymerase, the LAMP reaction was carried out, which brought greatly improved amplification efficiency. The double-stranded DNA fluorescent dye EvaGreen was added for the detection of amplification product to achieve the quantification of the target miRNA. This method can detect miRNA in a linear range of seven orders of magnitude, with a detection limit of 100 fM. Therefore, this ultrasensitive miRNA-LAMP assay provides a new path for the highly sensitive quantitative analysis of miRNA, thereby bringing convenience to clinical diagnosis and prognostic research.

Nguyen, Q. T., & Le, V. T. (2023). Determination of methanol, acetaldehyde, and ethyl acetate in thousand folds of ethanol sample by headspace gas chromatography with mass spectrometry. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/8851265

Alcohol beverages have been widely consumed in several parts of the world. In this study, volatile organic compounds in alcoholic beverages including acetaldehyde, ethyl acetate, methanol, and higher ethanol were investigated and evaluated using a headspace gas chromatograph equipped with a mass spectrometer. This study evaluated the suitability of the chromatographic system, linearity, limit of detection, and limit of quantification, accuracy, and precision of the single and simultaneous determination of acetaldehyde, ethyl acetate, and methanol in thousand folds of ethanol. Results showed that the acetaldehyde concentration in local beer samples and local manual product liqueur samples ranged from 4.65 to 13.22 mg/L and from 5.55 to 75.96 mg/L, respectively, but in local industrial product liqueur samples, acetaldehyde was not detected. Methanol was only detected in a few local beer samples and locally manually produced liqueur samples within low concentrations. Ethyl acetate was only detected in all local beer samples, but it was not present in local industrial product liqueur samples.

Chemistry International Journal, o. A. (2023). Retracted: Mo-si-B alloy formed by optional laser melting process. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9837685

Chemistry International Journal, o. A. (2023). Retracted: Application of particle swarm algorithm in nanoscale damage detection and identification of steel structure. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9815124

Chemistry International Journal, o. A. (2023). Retracted: Stability analysis and construction parameter optimization of tunnels in the fractured zone of faults. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9841604

Chemistry International Journal, o. A. (2023). Retracted: Application of nanometer heavy-duty coating in the optimization of process parameters for power generation machinery. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9871454

Chemistry International Journal, o. A. (2023). Retracted: Blasting law of liquid CO2 phase change in coal mine based on numerical simulation. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9897515

Chemistry International Journal, o. A. (2023). Retracted: A systematic study on the extraction and image reproduction of ceramic sculpture artworks. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9836308

Chemistry International Journal, o. A. (2023). Retracted: Application of carbon fiber cement-based composites in improving construction durability. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9861854

Chemistry International Journal, o. A. (2023). Retracted: Effect of nano titanium oxide with different surface treatments on color stability of red-tinted silicone rubber. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9832684

Mengstie, Y. A., Desta, W. M., & Alemayehu, E. (2023). Assessment of drinking water quality in urban water supply systems: The case of hawassa city, ethiopia. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/8880601

In many developing countries, such as Ethiopia, water quality and the risk of water-related diseases are serious public health issues. The present study goal was to assess the drinking water quality from source to household tap water. To characterize and analyze drinking water quality parameters, 21 water samples were collected, of which 11 water samples were collected from sources (spring, borehole, and river), 4 from service reservoirs, and 6 from tap

water. The mean values of the parameters were as follows: total dissolved solids (TDS) (142.79 mg/L), temperature (22.08°C), turbidity (9.49 NTU), electrical conductivity (EC) (250.14°µS/cm), pH (7.45 mg/L), fluoride (1.15 mg/L), nitrate (NO_3^-) (2.91 mg/L), total hardness (TH) (57.45 mg/L), calcium (41.7 6 mg/l), magnesium (10.74 mg/L), phosphate (0.44 mg/L), sulfate (3.99 mg/L), residual chlorine (1.53 mg/L), alkalinity (196.39 mg/L), and microbiological (total coliform and coliform/CFU) which were the main physiochemical parameters analyzed for the study. The findings revealed that the majority of the water quality parameters tested were within the WHO and National Drinking Water Quality Standards (NDWQS). However, some of the parameters such as temperature, turbidity, fluoride, and residual chlorine did not meet the standards. The mean temperatures at the source, reservoir, and tap water were 22.01°C 22.5°C,and 21.83°C, respectively. Turbidity levels in source samples ranged from 10 to 45 NTU, with a mean of 24.5 NTU, exceeding the WHO's recommendation of less than 5 NTU. The Boko Alamura well had a high fluoride content (3.9 mg/l), which was above the WHO and NDWQS permissible limits. There was no free residual chlorine in the tap water sample. The results show that the Hawassa drinking water supply did not contain total or fecal coliform in any of the samples tested. The overall WQI for the water source, reservoir, and tap water was also determined to be 89, 71, and 69.7 points, respectively. Therefore, based on the WQI result, Hawassa drinking water quality is good for the source, reservoir, and tap water.

Chemistry International Journal, o. A. (2023). Retracted: Electrochemical preparation of nanocatalysts and their application in electrocatalysis. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9867946

Chemistry International Journal, o. A. (2023). Retracted: Residential environment pollution monitoring system based on cloud computing and internet of things. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9858523

Chemistry International Journal, o. A. (2023). Retracted: Clinical observation of MRI scanning combined with clinical nursing for surgical breast cancer patients. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9820248

Chemistry International Journal, o. A. (2023). Retracted: Influence of nano-cutting fluid in new cutting and forming processes on heat transfer performance of mechanical engineering. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9769210

Chemistry International Journal, o. A. (2023). Retracted: Effect of free formaldehyde on chemical structure and thermal properties of nano-titanium dioxide resin. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9813219

Chemistry International Journal, o. A. (2023). Retracted: 3D modeling of sculpture nano-ceramics under sparse image sequence. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9847539

Chemistry International Journal, o. A. (2023). Retracted: Application of realistic 3D model in building prefabricated nanomaterial structure. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9823812

Chemistry International Journal, o. A. (2023). Retracted: Calculation and analysis of nonlinear algorithm for stability of nanosilica powder soft soil pile foundation. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9851287

Chemistry International Journal, o. A. (2023). Retracted: Load test analysis of a long-span prestressed nano-concrete highway bridge. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9853014

Chemistry International Journal, o. A. (2023). Retracted: Effect of aeolian sand powder addition on frost resistance of concrete pavement. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9852609

Chemistry International Journal, o. A. (2023). Retracted: Test and detection of antifreezing and anticorrosion performance of carbon nanofiber bridge concrete. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9857634

Chemistry International Journal, o. A. (2023). Retracted: Application of cement-based composite nanomaterials in prefabricated thin-wall light steel structure composite wall. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9816598

Chemistry International Journal, o. A. (2023). Retracted: Electrochemical intelligent recognition of mineral materials based on superpixel image segmentation. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9754785

Chemistry International Journal, o. A. (2023). Retracted: Mining geological environment monitoring and real-time transmission based on internet of things technology. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9769415

Chemistry International Journal, o. A. (2023). Retracted: Mechanical performance test and numerical simulation analysis of building steel plate and concrete composite structure. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9834902

Feng-Shuo Yang, Hei-Hwa, L., Li-Ping, T., Yung-Hung, L., Yung-Sheng, L., Yi-Cheng, L., . . . Yi-Ching, L. (2023). Simultaneous determination and stability analysis of ten new psychoactive substances including synthetic cathinones, phenethylamines, and ketamine substitutes in urine using liquid chromatography-tandem mass spectrometry. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/9895595

Knowing the stability of drugs is important to ensure accurate and reliable results of drug concentrations. This study evaluated the stability of ten new psychoactive substances (NPSs) in urine and methanol/water at different storage temperatures. Quantitative analyses were performed using liquid chromatography-tandem mass spectrometry. Three replicates of each storage condition were analyzed at day 0 and after 7, 14-, 30-, 60-, and 90 days with storage at +25°C, +4°C, and −20°C. For each analyte, the percent difference at each time interval from day 0 was calculated for each storage condition. Para-methoxyamphetamine (PMA), para-methoxymethamphetamine (PMMA), deschloroketamine (DCK), and 2-fluorodeschloroketamine (2-FDCK) were stable in urine, even when stored for 90-day periods at various temperatures. For synthetic cathinones, the concentrations declined over time at room temperature (+25°C) in urine but were relatively stable in methanol solvent with 0.1% formic acid. The significant degradation was found at +25°C, and the most excellent stability was shown by samples stored at −20°C. Phenethylamines (PMA and PMMA) and ketamine substitutes (DCK and 2-FDCK) were relatively more stable than synthetic cathinones (mephedrone, butylone, pentylone, ephylone, 4-MEAPP, and eutylone).

Qian, Z., Huang, D., He, Z., He, Q., Tan, G., Huang, Q., . . . Li, W. (2023). Rapid determination of three organic acids in polygonum vivipari rhizoma via one marker by HPLC-UV at equal absorption wavelength and effervescence-assisted matrix solid-phase dispersion. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/5546053

A rapid HPLC-UV method for the determination of three organic acids (neochlorogenic acid, chlorogenic acid, and cryptochlorogenic acid) in Polygoni Vivipari Rhizoma (PVR) by one marker was developed. The sample was prepared by effervescence-assisted matrix solid-phase dispersion (EA-MSPD). The separation of compounds was performed on a Poroshell column. The equal absorption wavelength was set as follows: 292 nm (0~7 min) and 324 nm (7~10 min). The analytical time including sample extraction and HPLC separation time was 12 min. The analytical method validation such as accuracy (recoveries 99.85%–106.29% and RSD < 2.9%), precision (RSD < 1.3%), reproducibility (RSD < 1.7%), and stability tests (RSD < 0.7% in 24 h) proved that the established HPLC method was suitable for determination of three organic acids in PVR. The contents of three analytes obtained by the external standard method with three markers and the equal absorption wavelength method with one marker were similar (RSD ≤ 2.0%). The developed method, which is rapid and reference compound saving, is an improved quality

evaluation method of PVR.

Getahun, M., Rahel, B. A., Ashenafi, K. S., Alem, E. W., & Kasahun, A. E. (2023). Evaluation of antibiotics residues in milk and meat using different analytical methods. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/4380261

Veterinary drugs are pharmacologically and biologically active chemical agents. At present, veterinary drugs are extensively used to prevent and treat animal diseases, to promote animal growth, and to improve the conversion rate of feed. However, the use of veterinary drugs in food-producing animals may leave residues of the parent compounds and/or their metabolites in food products resulting in harmful effects on humans. To ensure food safety, sensitive and effective analytical methods have been developing rapidly. This review describes sample extraction and cleanup methods, and different analytical techniques are used for the determination of veterinary drug residues in milk and meat. Sample extraction methods, such as solvent extraction, liquid-liquid extraction, and cleanup methods such as dispersive solid-phase extraction and immunoaffinity chromatography, were summarized. Different types of analytical methods such as microbial, immunological, biosensor, thin layer chromatography, high-performance liquid chromatography, and liquid chromatography–tandem mass spectrometry were discussed for the analysis of veterinary drug residues in animal-derived foods. Liquid chromatography–tandem mass spectrometry is the most widely used analytical technique for the determination of antibiotic drug residues. This is due to the powerful separation of LC and accurate identification of MS, and LC-MS/MS is more popular in the analysis of veterinary drug residues.

Amini, H., Sokhansanj, A., Akrami, M., & Haririan, I. (2023). Design and fabrication of a high performance microfluidic chip for blood plasma separation: Modelling and prediction of system behaviour via CFD method. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/3648247

This paper presents a single-step microfluidic system designed for passive separation of human fresh blood plasma using direct capillary forces. Our microfluidic system is composed of a cylindrical well between upper and lower channel pairs produced by soft photolithography. The microchip was fabricated based on hydrophobicity differences upon suitable cylindrical surfaces using gravitational and capillary forces and lateral migration of plasma and red blood cells. The plasma radiation was applied to attach the polymeric segment (polydimethylsiloxane (PDMS)) to the glass. Meanwhile, Tween 80 was used as a surfactant to increase the hydrophobicity of the lateral channel surfaces. This led to the higher movement of whole blood, including plasma. Fick's law of diffusion was validated for this diffusion transfer, the Navier–Stokes equation was used for the momentum balance, and the Laplace equation was utilized for the dynamics of the mesh. A model with high accuracy using the COMSOL Multiphysics software was created to predict the capillary forces and chip model validation. RBCs (red blood cells) were measured by the H3 cell counter instrument, by which 99% plasma purity was achieved. Practically, 58.3% of the plasma was separated from the blood within 12 min. Correlation between plasma separation results obtained from software and experimental data showed a coefficient of determination equal to 0.9732. This simple, rapid, stable, and reliable microchip can be considered as a promising candidate for providing plasma in point-of-care diagnostics.

Sharma, H., Hari, P. S., Paudel, K., Raila, A., Kandel, S., Chaudhary, P., & Bhattarai, K. (2023). Green spectrophotometric determination of organophosphate in selected fruits and vegetables. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6691659

A simple, sensitive, precise, and environmentally safe spectrophotometry method was developed and validated for the determination of organophosphate in various fruits and vegetables using a UV-Visible spectrophotometer using a magnesia mixture. The volume of reagent used for analysis and the stability of the color complex were also optimized. The drug showed a stable white color complex at 420 nm. The greenness of the methods was estimated using an ecoscale (84), the Green Analytical Procedure Index, and AGREE (0.89), which were found to be excellent green method based on spectrophotometric determination. The method was validated using ICH guidelines and has acceptable values for linearity (0.5–2.5 mg/ml), accuracy (98.5–102.5%), precision, robustness, limit of detection (0.16 mg), and limit of quantification (0.486 mg). The concentration of the organophosphate in the analyzed sample

was in the range of 0.003 to 2.45 mg. Altogether, the proposed green analytical method was found to be a simple, selective, sensitive, accurate, and ecofriendly method for the analysis of organophosphate in various fruits and vegetables.

Shao, X., Hu, G., Lu, Y., Li, M., Shen, B., Kong, W., . . . Ran, Y. (2023). Discrimination of traditional chinese medicine syndromes in type 2 diabetic patients based on metabolomics-proteomics profiles. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/5722131

Aims of the Study. Traditional Chinese medicine (TCM) has thousand years of history, and syndrome differentiation is the foundation and essence of the TCM theory. As it has distinctive advantages in diagnosing and treating the type 2 diabetes mellitus (T2DM), the purpose of this research is to distinguish T2DM patients with or without damp-heat syndrome (DHS), as well as to discover biomarkers associated with syndrome employing the metabolomics-proteomics technique. Materials and Methods. The metabolomics-proteomics of sixty patients with T2DM were acquired by high-performance liquid chromatography (HPLC). In addition, some clinical features, containing total cholesterol (TC), triglycerides (TG), hemoglobin A1c (HbA1c), body mass index (BMI), and low-density lipoprotein (LDL) together with high-density lipoprotein (HDL), were determined via clinical detection strategies. Abundant metabolites and proteins, respectively, were identified with the analysis of liquid chromatography tandem mass spectrometry (LC-MS/MS). Results. 22 differentially abundant metabolites and 15 differentially abundant proteins were determined. The analysis of bioinformatics suggested that the differentially abundant proteins were commonly associated with the renin-angiotensin system, vitamin digestion and absorption, hypertrophic cardiomyopathy, and so on. Furthermore, differentially abundant metabolites were amino acids and were associated with the biosynthesis of CoA and pantothenate, together with the metabolisms of phenylalanine, beta-alanine, proline, and arginine. Combination analysis revealed that the vitamin metabolism pathway was predominantly affected. Conclusions. DHS syndrome can be separated by certain metabolic-proteomic differences, and metabolism is particularly prominent, especially in vitamin digestion and absorption. From the molecular level, we provide preliminary data for the extensive application of TCM in the study of T2DM, and at the same time benefited in a sense diagnosis and treatment of T2DM.

Li, M., Yang, Y., Li, J., Shang, S., & Fu, X. (2023). A SiO2 hybrid enzyme-based biosensor with enhanced electrochemical stability for accuracy detection of glucose. International Journal of Analytical Chemistry, 2023 doi:https://doi.org/10.1155/2023/6620613

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