

An Improved Ionic Liquid-Based Headspace Single-Drop Microextraction–Liquid Chromatography Method for the Analysis of Camphor and Trans-Anethole in Compound Liquorice Tablets

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A simple, accurate and sensitive ionic liquid-based headspace single-drop microextraction procedure followed by high-performance liquid chromatography was developed and validated for the determination of camphor and trans-anethole in compound liquorice tablets. The volume of the ionic liquid microdrop was increased to 12 μ L by modifying the device of the suspended drop. The stability of the microdrop and the sensitivity of the method were improved. Under the optimum experimental conditions, the calculated calibration curves gave acceptable linearity for camphor and trans-anethole with correlation coefficients of 0.9990 and 0.9998, respectively. The repeatability of the proposed method, expressed as relative standard deviation, was below 4.5% ($n = 5$). The limits of detection for the two target analytes were found to be 9.77 and 1.95×10^{-2} μ g/mL, respectively. In this study, the separation, purification and enrichment were achieved in one step in an airtight system, which reduced the interferences caused by other complicated constituents, increased the signal-to-noise of the method and ensured the accuracy of the results because there was no loss of volatile components. It is expected to be widely applied for sample pretreatment of volatile components with high boiling points in samples with complicated matrices such as the extractions of plants or Chinese traditional drugs.

Introduction

Compound liquorice tablets (1) are commonly used as apophlegmatic and anti-inflammatory drugs that have excellent curative effects for tussis. They primarily contain liquorice-immersed ointment, opium, camphor, star anise oil and sodium benzoate. Several analytical methods (2, 3), including high-performance liquid chromatography (HPLC) and capillary electrophoresis, have been developed for the determination of glycyrrhizic acid, glycyrrhetic acid, morphine and sodium benzoate in compound liquorice tablets, which are essential for the quality control of these tablets.

Among the components of compound liquorice tablets, camphor and star anise oil can stimulate the bronchial mucosa and facilitate cough via enhancement of cough-reflex sensitivity. Camphor and the principal constituents of star anise oil, trans-anethole, are volatile, which might be lost during pharmaceutical preparation and storage. Thus, to ensure drug efficacy, it is necessary to monitor the contents of camphor and trans-anethole. However, no studies have been reported regarding the analysis of camphor and trans-anethole in compound liquorice tablets.

Because there are multiple constituents in compound liquorice tablets, sample preparation before instrumental analysis is usually necessary. Extraction procedures, such as the traditionally used liquid–liquid extraction (LLE) (4–8), or even the more recently introduced solid-phase extraction (SPE) (9–11) and solid-phase microextraction techniques (SPME) (12, 13) have been applied to the extraction of the volatile and semivolatile compounds in medicines. However, because there are many complicated constituents in compound liquorice tablets, LLE such as solvent extraction, hydrodistillation, ultrasonic-assisted extraction and microwave-assisted extraction always results in many interfering substances such as pigment, protein, carbohydrates and tannins in the extracts. These interfering substances may make the separation more difficult and decrease the lifetime of the column, because other constituents aside from the analytes remain on the column. In addition, these traditional extractions are mostly performed using the open system, which leads to a loss of volatile components and causes the results to be inaccurate. Furthermore, LLE are usually time-consuming and labor-intensive, which requires large amounts of toxic and environmentally unfriendly organic solvents. In SPE and SPME, the materials are relatively expensive and the carrying-over effects are usually inevitable. In addition, training is necessary to ensure the repeatability of the method. Some new extraction procedures have been recently developed for quantifying camphor or trans-anethole; for example, microwave-assisted extraction followed by headspace solid-phase microextraction (MAE-HS-SPME) (14), subcritical water extraction (SbCWE) (15) and ultrasonic nebulization extraction (UNE) (16). These methods are proved to be sensitive, novel and feasible; however, some complicated devices are usually needed and several steps are operated for a single extraction. As such, it is essential to develop a simple, fast, airtight and environmentally friendly analytical method for the determination of in compound liquorice tablets.

Currently, miniaturization of the traditional LLE attempts to eliminate or minimize these drawbacks. In this sense, single-drop microextraction (SDME) (17–23) and dispersive liquid–liquid microextraction (DLLME) (24–27) as miniaturization methods of LLE stand out because they are simple, fast, inexpensive, sensitive, airtight and environmentally friendly. However, DLLME has been primarily used for the extraction and preconcentration of organic compounds from water samples. These sample matrices are simple and can be used to direct LLE. However, LLE is not suitable for the sample

pretreatment of components in materials with complicated matrices; for example, the extractions of plants or Chinese traditional drugs. Headspace-SDME (HS-SDME) is usually selected for the extraction of volatile components, which reduces the interfering substances caused by other complicated constituents, because only volatile components are introduced into the headspace gas phase. This method presents much higher specificity and better purification efficiency. However, evaporation of the extracting solvent drop is an important drawback that deteriorates the method. In recent years, ionic liquids (ILs) (28–31), which are organic salts that are liquids at room temperature and have high boiling points, have been proposed for use in SDME (32–37). ILs have various advantages over traditional organic solvents, such as low vapor pressure, high stability, large viscosity, moderate dissolvability of organic compounds and adjustable miscibility and polarity. As such, longer sampling time and larger droplet volume can be achieved. In the meantime, the improvement of microdrop stability and method sensitivity is still the focus for the optimization of SDME. Several studies have reported improved SDME procedures by modifying the device of suspended microdrop (21, 35, 37) to increase the contact area of the microdrop and the bottom of the PTFE tube to the tip of the microsyringe needle. In the present work, the volume of the ionic liquid microdrop was increased to 12 μL by modifying the PTFE tube and circular flanges were made at the end of the suspended microdrop. The primary advantages of the improved device were the applicability of long-term extraction at high temperatures due to the unique properties of ILs, no interferences for the determination due to the compatibility of ILs with HPLC and no retention on the column. In addition, because the separation, purification and enrichment were achieved in one step, this method exhibited reduced interferences, simplified chromatographic separation, and increased signal-to-noise ratios. Furthermore, the extraction process was carried out in an airtight system, and thus the results were accurate because there was no loss of volatile components. Finally, by optimizing the variables affecting the HS-SDME process, 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF₆]) was demonstrated to be a suitable extraction solvent for HS-SDME of camphor and trans-anethole in compound liquorice tablets. The proposed IL-HS-SDME method is proved to be fast, simple, accurate, sensitive, inexpensive and environmental friendly, and is expected to be widely applied for sample pretreatment of volatile components with high boiling points in medicines with complicated matrices; for example, the extractions of plants and Chinese traditional drugs.

Experimental

Instrumentation

HPLC analyses were performed on a Shimadzu (Kyoto, Japan) LC-10A system consisting of an SCL-10A vp system controller, an LC-10AT vp pump, a CTO-10A vp column oven and an SPD-10A vp ultraviolet-visible (UV-Vis) detector. A personal computer equipped with a Shimadzu Class-VP Workstation for LC system was used to process all chromatographic data. Chromatographic separation was achieved on a Promosil C18 column (150 \times 4.6 mm i.d., 5 μm particle size) from Beijing

Agela Technologies Co. (Beijing, China) using methanol–water (70:30 *v/v*) as the mobile phase with a flow rate of 1 mL/min. The column temperature was set at 35°C and the elutes were detected by UV at 289 nm. DF-II temperature-controlled magnetic stirring apparatus (Jintan City Fu Hua Instrument Co., Jiangsu, China) was used for the control of the temperature of the water bath and the stirring rate of aqueous samples. A Shimadzu UV-2401PC UV-Vis recording spectrophotometer was used for UV-scanning to identify the detection wavelengths of camphor and trans-anethole.

Chemicals

Camphor was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China) and trans-anethole was obtained from Sigma-Aldrich (St. Louis, MO). Compound liquorice tablets were provided by Qinghai Pharmaceutical Factory Co. (Qinghai, China). The three ILs used in this paper, 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF₆]), 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM][BF₄]) and 1-hexyl-3-methylimidazolium tetrafluoroborate ([HMIM][BF₄]), were obtained from Shanghai Cheng Jie Chemical Co., Ltd. (Shanghai, China). Methanol (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride (North of Beijing Fine Chemical Co. Beijing, China) was used to adjust the ionic strength of the aqueous samples.

Preparation of standard and sample solutions

A standard stock solution containing both camphor and trans-anethole at 100 $\mu\text{g/mL}$ was prepared in methanol. Working solutions containing 0.156–10.0 $\mu\text{g/mL}$ of target compounds were prepared by serial dilutions of the mixed standard stock solution with water. Thirty milligrams of the compound liquorice tablet powder was transferred into a 100-mL volumetric flask and diluted to volume with water in the same manner as the sample solution. All solutions were stored in the dark at 4°C.

Extraction procedure

Five milliliters of each working standard solution or sample solution, containing 36% of sodium chloride (*w/v*), was placed in a 10-mL glass bottle containing a magnetic stirring bar (1.0 \times 0.5 cm) and stirred at the rate of 1,000 rpm. A 3-mm-long PTFE tube (0.6 mm i.d., 1 mm o.d.) was fitted to the blunt needle tip of a 25- μL Hamilton (Bonaduz, Switzerland) syringe (model 1702). Furthermore, to increase the contact area between the drop and the needle tip, the bottom of the PTFE tube was made to a shape of circular flanges (3.5 mm o.d.) by firing. In this way, the probability of drop detachment is decreased and a microdrop with volume up to 12 μL can be suspended at the tip of the syringe with good stability. Figure 1 shows the schematic diagram of the HS-SDME system. The microsyringe, typically containing 12 μL of the ionic liquid acceptor phase, was clamped above the bottle and its needle passed through the septum until its tip was 1 cm above the surface of the aqueous solution. The plunger was depressed and a microdrop of the ionic liquid phase was exposed to the headspace above the

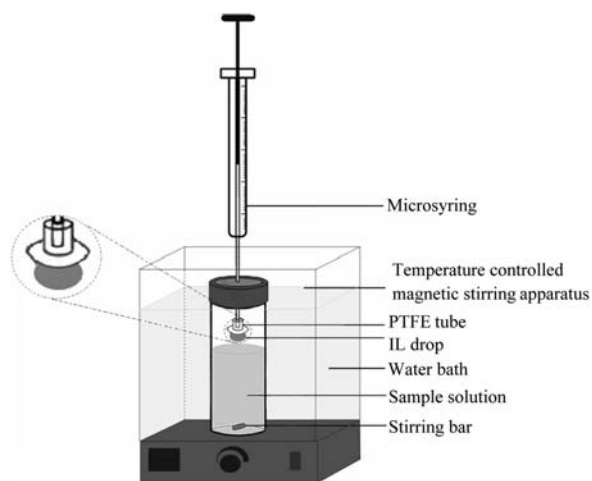


Figure 1. Schematic diagram of IL-HS-SDME system. Extraction conditions: extraction solvent, [BMIM][PF₆]; microdrop volume, 12 μ L; sample volume, 5.00 mL; concentration of NaCl (*w/v*), 36%; extraction temperature, 80°C; extraction time, 25 min; stirring rate, 1,000 rpm.

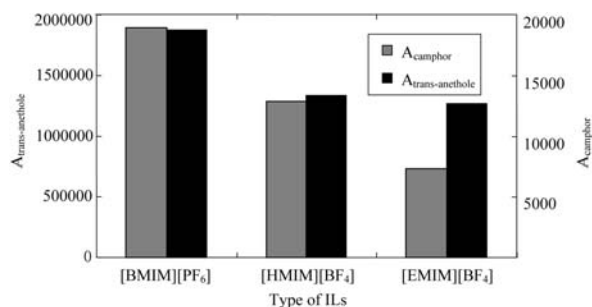


Figure 2. Effects of ILs on extraction efficiency. Extraction conditions: IL volume, 12 μ L; sample volume, 5.00 mL; concentration of NaCl (*w/v*), 36%; extraction temperature, 80°C; extraction time, 25 min; stirring rate, 1,000 rpm.

stirred solution at 80°C for 25 min. After extraction, the microdrop was retracted into the microsyringe, the tube was removed and the acceptor phase was directly injected into the HPLC system for analysis.

Results and Discussion

Optimization of HS-SDME procedure

All the variables, including type of ILs, microdrop volume, ionic strength, stirring speed, extraction temperature and time, were investigated to maximize the extraction yields of the two target analytes in the proposed procedure.

Selection of ILs

Three ILs ([BMIM][PF₆], [EMIM][BF₄] and [HMIM][BF₄]) were studied as extraction phases for HS-SDME. Briefly, 5 mL of sample solution containing 36% sodium chloride (*w/v*) was stirred at 1,000 rpm for 25 min using a 12- μ L droplet volume. The same trends ([BMIM][PF₆] > [HMIM][BF₄] > [EMIM][BF₄]) were observed for camphor and trans-anethole, as summarized in Figure 2. Therefore, to increase the sensitivity of the two

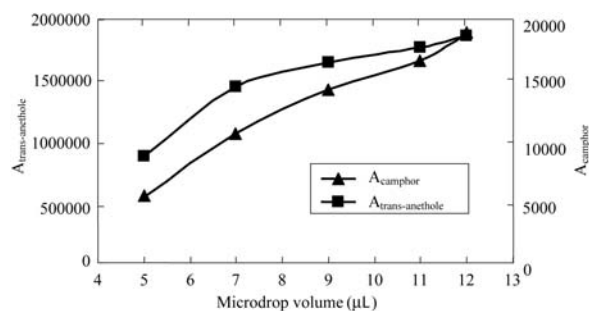


Figure 3. Effects of microdrop volume of ILs on extraction efficiency. Extraction conditions: extraction solvent, [BMIM][PF₆]; sample volume, 5.00 mL; concentration of NaCl (*w/v*), 36%; extraction temperature, 80°C; extraction time, 25 min; stirring rate, 1,000 rpm.

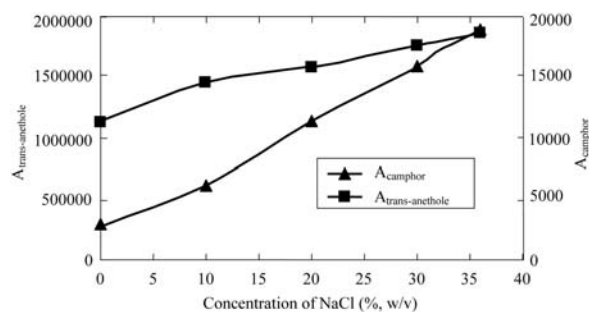


Figure 4. Effects of ionic strength on extraction efficiency. Extraction conditions: extraction solvent, [BMIM][PF₆]; microdrop volume, 12 μ L; sample volume, 5.00 mL; extraction temperature, 80°C; extraction time, 25 min; stirring rate, 1,000 rpm.

components, [BMIM][PF₆] was selected as the optimum extraction solvent.

Effect of microdrop volume

The volume of the IL microdrop is also an important variable in the extraction process. Different values (5, 7, 9, 11 and 12 μ L) were evaluated. As shown in Figure 3, the peak areas for camphor and trans-anethole were increased as the volume of [BMIM][PF₆] was increased. However, when a volume of 12 μ L was used, the microdrop was increased to 16 μ L with the attached vapor after extraction. It became unstable and was easily released from the tip of the syringe needle. Therefore, a volume larger than 12 μ L was not evaluated. As a compromise between the stability of the microdrop and higher extraction efficiency, a volume of 12 μ L was selected as the appropriate drop size.

Effect of ionic strength

It is well known that the ionic strength of LLE exhibits beneficial effects on a wide variety of analytes. In general, a salting-out effect has been observed because salt can decrease the solubilities of analytes in aqueous samples and enhance their partition into the headspace gas phase and then the acceptor phase. Therefore, the effect of ionic strength on the proposed procedure was evaluated by adding different amounts of sodium chloride [0 ~ 36% (*w/v*)] to the samples. As expected, ionic strength exhibited a positive effect on the extraction of

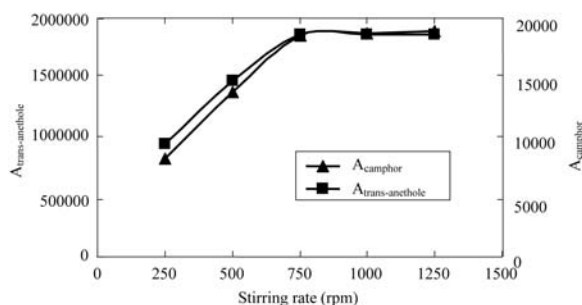


Figure 5. Effects of stirring rate on extraction efficiency. Extraction conditions: extraction solvent, [BMIM][PF₆]; microdrop volume, 12 μ L; sample volume, 5.00 mL; concentration of NaCl (w/v), 36%; extraction temperature, 80°C; extraction time, 25 min.

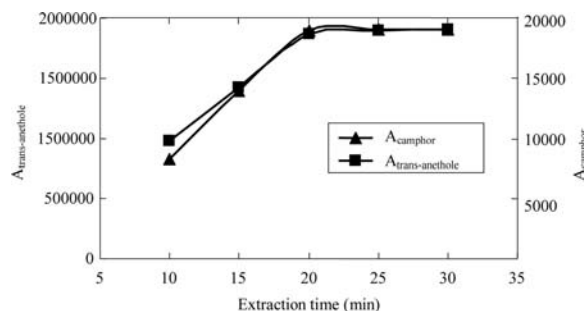


Figure 7. Effects of extraction time on extraction efficiency. Extraction conditions: extraction solvent, [BMIM][PF₆]; microdrop volume, 12 μ L; sample volume, 5.00 mL; concentration of NaCl (w/v), 36%; extraction temperature, 80°C; stirring rate, 1,000 rpm.

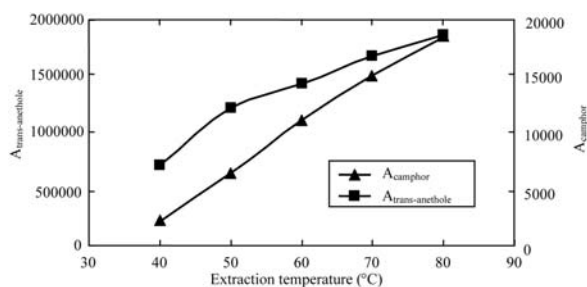


Figure 6. Effects of extraction temperature on extraction efficiency. Extraction conditions: extraction solvent, [BMIM][PF₆]; microdrop volume, 12 μ L; sample volume, 5.00 mL; concentration of NaCl (w/v), 36%; extraction time, 25 min; stirring rate, 1,000 rpm.

the two analytes (Figure 4). Therefore, an NaCl concentration of 36% was selected.

Effect of stirring rate

In HS-SDME, the agitation of the sample improves the mass transfer of the components in the aqueous phase and induces convection in the headspace, which means that the time required to reach the equilibrium is reduced. In the current experiments, the stirring rate was investigated between 250 and 1,250 rpm. As indicated in Figure 5, 750 rpm was enough to reach the highest extraction efficiency at the working conditions. Thus, the stirring rate of 1,000 rpm was selected to ensure repeatability of the method.

Effect of extraction temperature

For headspace analysis of volatile components with high boiling points, another important parameter is temperature. Temperature has a significant effect on both the kinetics and the thermodynamics of the extraction process. The effect of temperature on the extraction efficiency was investigated from 40 to 80°C. As shown in Figure 6, with increasing extraction temperature, the peak areas of camphor and trans-anethole were also increased. The results could be explained by the fact that an increase in temperature may lead to an improvement of the evaporation of the target components with high boiling points from the sample to the headspace, thus resulting in higher extraction efficiencies. However, when the sample solution is heated to a much higher temperature, some changes

may occur in the viscosity and volume of ILs. Additionally, high temperature can lead to high vapor pressure, which may cause leaking problems and other safety issues such as explosion of the glass vial. For these reasons, a temperature of 80°C was used for further work.

Effect of extraction time

In general, mass transfer is a time-dependent process and the maximum quantity of the target analytes are extracted into the ionic liquid phase when extraction equilibrium is reached. The effect of extraction time was studied by varying from 10 to 30 min. As shown in Figure 7, the extraction balance could be attained within 20 min, and extending extraction time had no significant effect on the extraction efficiency. To achieve higher extraction efficiency and obtain accurate and precise analysis, the extraction time of 25 min was selected.

Selection of the detection wavelength

Absorption spectra were recorded between the wavelengths of 200 and 400 nm at a 1-nm interval using a Shimadzu UV-2401PC UV-Vis recording spectrophotometer. The UV spectrum showed that camphor and trans-anethole had maximum absorbance at wavelengths of 289 and 257 nm, respectively. Due to the different chemical structures of camphor and trans-anethole, the peak area (absorption or response) of trans-anethole was over 100-fold higher than camphor at the same concentration. Therefore, to achieve higher signal-to-noise ratios for both analytes, the maximum absorbance wavelength of camphor, 289 nm, was selected as the detection wavelength.

Method validation

The specificity of the method was tested by comparing the standard solution, excipient placebo solutions and pharmaceutical solutions after extraction. The excipient placebo solutions (solutions without drugs) were prepared by the addition of common excipients to the pharmaceutical formulations at certain proportions. According to the HPLC chromatograms of standard solution, excipient solutions and pharmaceutical solutions, it was clear that no significant interference occurred for drug analysis (Figure 8).

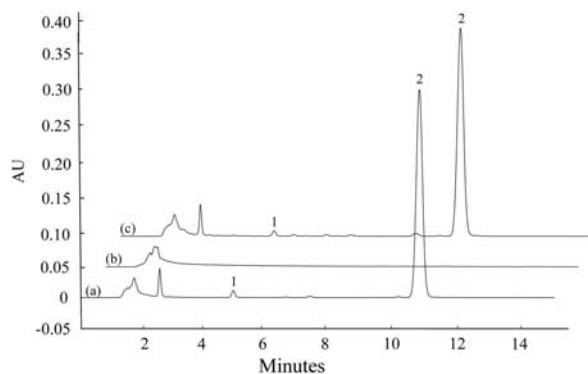


Figure 8. HPLC chromatograms of standard solution (a); excipient solutions (b); pharmaceutical solutions (c). Chromatographic separation was achieved on a Promosil C18 column (150 × 4.6 mm i.d., 5 μm particle size) using methanol–water (70:30 v/v) as the mobile phase at a flow rate of 1 mL/min. Detection wavelength, 289 nm; detection temperature, 35°C; peak 1, camphor; peak 2, trans-anethole.

Table I

Analytical Characteristics of the Proposed Method (*n* = 7)

Analytes	Camphor	Trans-anethole
Regression equation	$A = 1.33 \times 10^4 C + 1.59 \times 10^2$	$A = 1.45 \times 10^6 C + 2.53 \times 10^3$
Correlation coefficient (<i>r</i>)	0.9990	0.9998
Linearity range (μg/mL)	0.156–10.0	0.156–10.0
RSD (%)	2.7	3.4
Number of data points	7	7
LOQ (μg/L)	39.1	7.81×10^{-2}
LOD (μg/L)	9.77	1.95×10^{-2}

Table II

Intra-Day and Inter-Day RSD Values of Peak Areas for the Proposed Method (*n* = 5)

Analytes	Concentration spiked (μg/mL)	Intra-day RSD (%)	Inter-day RSD (%)
Camphor	1.00	2.7	4.5
	1.25	3.6	3.4
	1.50	2.4	2.9
Trans-anethole	1.00	1.2	3.2
	1.25	1.6	2.5
	1.50	2.3	1.8

To test the linearity of the proposed method, a nine-point standard curve was constructed with the concentration range from 0.156–10.0 μg/mL for the two analytes. The correlations of peak areas versus the concentrations of camphor and trans-anethole were obtained with a correlation coefficient (*r*) of 0.9990 and 0.9998, respectively. In Table I, the analytical characteristics are summarized for the proposed method. The limits of quantification (LOQ) and limits of detection (LOD), defined as signal-to-noise ratios (*S/N*) of 10 and 3, respectively, were determined separately by five-fold replicate analysis.

To assess the repeatability of the developed method, three different concentrations of the two analytes (within the linear range) were analyzed in five independent series on the same day (intra-day precision) and on six consecutive days (inter-day precision). The relative standard deviation (RSD) values of the peak areas for intra-day precision and inter-day precision were found to be from 1.2 to 3.6% and from 1.8 to 4.5%, respectively (Table II).

Table III

Percent Recovery for the Synthetic Mixture of Camphor and Trans-Anethole*

Analytes	Concentration spiked (μg/mL)	Recovery (%)	RSD (%)
Camphor	1.00	98.5	3.3
	1.25	100.3	2.6
	1.50	99.4	1.9
Trans-anethole	1.00	101.2	4.2
	1.25	100.6	2.2
	1.50	100.3	2.5

*The two analytes were added to the excipient placebo solutions.

Table IV

Determination of Camphor and Trans-Anethole in Three Batches of Compound Liquorice Tablets (*n* = 3) by the Proposed Method (IL-HS-SDME-HPLC) and UAE-HPLC

Method	Compound liquorice tablets	Camphor		Trans-anethole	
		Assay (mg/tablets)	RSD (%)	Assay (mg/tablets)	RSD (%)
Present work	Batch-1	0.676	2.6	0.590	4.7
	Batch-2	0.962	4.3	0.652	2.5
	Batch-3	0.825	1.8	0.509	3.2
UAE-HPLC	Batch-1	0.665	2.3	0.576	3.4
	Batch-2	0.947	2.9	0.650	2.1
	Batch-3	0.820	1.9	0.503	3.7

The accuracy of the proposed method was examined as percent recovery. Standard solutions with three different concentrations within the linearity range (80%, 100% and 120%) were added to a constant mixture of excipients in triplicate. The results showed excellent recovery, ranging from 98.5 to 101.2% with RSDs of less than 4.2% (Table III). Thus, it can be concluded that the proposed method is accurate and free from systematic errors.

Analysis of real samples

The applicability of the proposed method to the assay of camphor and trans-anethole in compound liquorice tablets was tested. Conventional ultrasonic-assisted extraction (UAE) (17) was carried out as a reference to compare the accuracy of the proposed method. Seventy-five milligrams of sample powders and 15 mL of *n*-hexane were added into the extraction vessel. After UAE for 5 min, the resulting suspension was filtered through a 0.45-μm micropore filter membrane and 0.3 μL of the extracts was injected into the gas chromatography (GC) system for analysis. The results obtained by the two methods are presented in Table IV. Although the contents of camphor trans-anethole in compound liquorice tablets by the present method were the same as those obtained by UAE, the sample solution extracted by UAE was difficult to separate and there were many interfering substances. Unlike UAE, the separation, purification and enrichment were achieved in one step in the present IL-HS-SDME method, which reduced the interferences caused by other complicated constituents and increased the *S/N* ratio of the method. Due to the high purification efficiency of this method, the chromatographic separation was very simple and completed within 12 min. Figure 8 shows that the HPLC chromatogram of the pharmaceutical solution is very clear with few interferences. Furthermore, only several microlitres of ILs were needed in this method, which are available in commercial production, and thus the cost was very low. In

addition, organic solvents were not used in this method. Taken together, the results demonstrated that the proposed method can be satisfactorily applied to the quantification of camphor trans-anethole in compound liquorice tablets with good accuracy and high sensitivity. In addition, there were some viabilities among different batches from 0.676 ~ 0.962 mg/tablets for camphor and 0.509 ~ 0.590 mg/tablets for trans-anethole with RSDs < 4.7%. This may be because they are volatile and their contents might be lost during pharmaceutical production and storage. To ensure the drug efficacy, it is essential to monitor their contents during the pharmaceutical production and storage process.

Conclusions

In this study, a new IL-HS-SDME method combined with HPLC was developed for the determination of camphor and trans-anethole in compound liquorice tablets. The room temperature liquid [BMIM][PF₆] was selected for the microextraction. The application of nonvolatile ionic liquid overcame many drawbacks that may occur when conventional organic solvents are used for extraction. Additionally, the modifying HS-SDME device also improved the stability and sensitivity of the method because it was suitable for a longer suspending procedure and the extraction volume was increased. In addition, the method was simple and rapid. The validation data showed that the method was selective, linear, precise and accurate for the measurement of camphor and trans-anethole in compound liquorice tablets. Furthermore, the proposed IL-HS-SDME method is expected to be widely applied, along with excellent sample cleanup, for the analysis of volatile components with high boiling points in medicines.

Acknowledgments

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