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Rapid determination of panaxynol in a traditional Chinese medicine of *Saposhnikovia divaricata* by pressurized hot water extraction followed by liquid-phase microextraction and gas chromatography–mass spectrometry

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Abstract

Panaxynol is a bioactive component in traditional Chinese medicines (TCMs), such as *Saposhnikovia divaricata* and *Panax ginseng*. In the work, two solvent-free sample techniques of pressurized hot water extraction (PHWE) and headspace liquid-phase microextraction (HS-LPME) were combined and developed for the determination of panaxynol in a TCM of *S. divaricata*. Panaxynol in the TCM samples from different growing areas was extracted by PHWE in dynamic mode, followed by extraction and concentration with HS-LPME and analysis with gas chromatography–mass spectrometry (GC–MS). The PHWE and HS-LPME parameters were optimized and the method validations were studied. Panaxynol in *S. divaricata* from four different growing areas was quantitatively analyzed by internal standard method. These results have shown that PHWE-LPME-GC–MS is a simple, rapid, efficient and low-cost method for the determination of panaxynol in TCMs and is a potential tool for TCM quality assessment.

Keywords: Panaxynol; Pressurized hot water extraction; Headspace liquid-phase microextraction; Extraction method; Traditional Chinese medicine; *Saposhnikovia divaricata*

1. Introduction

Traditional Chinese medicines (TCMs) are gaining more and more attention in modern pharmaceutical institutes as they provide important resource for drug development. *Saposhnikovia divaricata* (Turcz.) Schischk (Fangfeng in Chinese) is one of the most widely used TCMs. Its dry roots have been applied to the treatment of rheumatism, rheum and cancer [1]. Panaxynol (Fig. 1), an important bioactive compound, has been isolated from the essential oils of *S. divaricata* and *Panax ginseng* [2,3]. It can inhibit the aggregation, release reaction and thromboxane formation in rabbit platelets [2,3]. Panaxynol was regarded as an active principle on the inhibition of nitrite production by inducible nitric oxide synthase [4]. Recently, Kuo et al. found that panaxynol had high-inhibitory activity on various tumor cells proliferation [5].

In China, *S. divaricata* is cultured in many areas, such as Neimeng, Ganshu and Sichuan. Different natural conditions including sun, soil and climate lead to discrepancy in the TCM quality. According to clinical experiment, *S. divaricata* from Neimeng has better drug effect than those from other growing areas [1]. It is very important to compare the qualities of TCM from different areas. Due to that panaxynol playing a key role in the course of disease treatment, it is possible to evaluate the TCM quality by determination of panaxynol in the TCM. This demands the development of a simple, rapid, low-cost method for determination of panaxyol in the TCM samples.

Steam distillation (SD) and supercritical CO_2 extraction followed by gas chromatography–mass spectrometry (GC–MS) were applied to determination of panaxynol in *S. divaricata* [6–9]. Recently, in our laboratory, a novel

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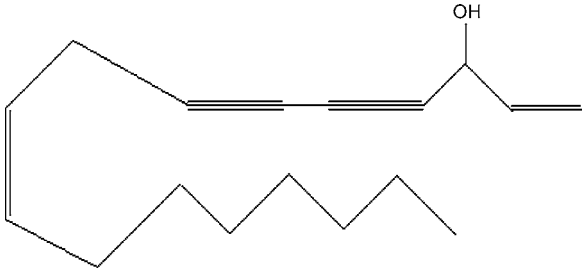

Fig. 1. The chemical structure of panaxyol.

sample technique, solid-phase microextraction (SPME) with simplicity, rapidness and no need of solvent, was developed for determination of TCM essential oils [10–12]. However, only semi-quantitative analysis of the volatile compounds in plant materials can be obtained by direct SPME.

More recently, pressurized hot water extraction (PHWE), a sample extraction technique based on the used of water as an extractant in dynamic mode, at temperatures between 100 and 374 °C (critical point of water, 221 bar and 374 °C) and a pressure high enough to maintain the liquid state, is emerging as a powerful alternative for solid sample extraction [13–15]. The technique has proven to be a powerful tool for extraction of essential oils in plant materials [16–20]. Since PHWE is a dynamic extraction method, the aqueous extract (about 10–50 ml) has to be extracted and concentrated before analysis. Liquid–liquid extraction (LLE) [21], solid-phase extraction (SPE) [22,23] and SPME [24,25] were developed for this purpose. PHWE combined with SPME has been shown to be a better approach to analysis of volatile compounds in solid samples [24–26]. However, the disadvantages are that SPME fiber is still relatively expensive and polymer coating is fragile and easily broken. Furthermore, sample carryover is sometimes difficult or impossible to be eliminated. Therefore, a new sample technique with low-cost, simplicity and rapidness was desirable.

In 1996, Jeannot et al. introduced a new sample extraction and concentration technique, liquid-phase microextraction (LPME) [27–29]. In 2001, Jeannot and co-workers developed microdrop headspace liquid-phase microextraction (MD-HS-LPME) [30]. In the MD-HS-LPME mode, extraction solvent with high-boiling point and low-vapor pressure was required. In order to overcome this limit, Lee developed organic solvent film (OSF) HS-LPME mode, where the thin OSF formed in a microsyringe barrel through the movement of the plunger was used as an extraction interface [31]. It is shown that HS-LPME is a simple, rapid and low-cost method for analysis of chlorobenzenes in soil [31].

In this work, the two sample techniques of PHWE and HS-LPME were combined and developed for determination of panaxynol in *S. divaricata* (Turcz.) Schischk. The PHWE and HS-LPME conditions were optimized and the method validations were studied.

2. Materials and methods

2.1. PHWE instruments

PHWE was performed using the following assembly: a Shimadzu LC10AD pump was used to propel the water used as extractant through the system. An extractor (a prototype designed and patented by Salvador and Merchan [32]), consisting of a stainless steel cylindrical extraction chamber (8 cm × 3 mm i.d.), closed with screws at either end that permit the circulation of the leaching fluid through them was used. The screw caps also contain stainless steel filter plates (2 μm in thickness and 1/4 in. i.d.; 1 in. = 2.54 cm) to ensure that the plant material remains in the extraction chamber. This chamber, together with a stainless steel preheater, is located in an oven, designed to work up to 300 °C and controlled using a Toho TC-22 temperature controller. A cooler system, (consisting of a coil coupled to an Ultraterm 6000383 P-Selecta recirculation bath) was used to cool the fluid from the oven to a constant temperature close to 25 °C, thus avoiding the losses of volatiles caused by the hot water. The outlet of this coil was coupled to a stainless steel variable restrictor that was used to control the pressure in the system in order to maintain the extractant water in liquid state.

2.2. Materials

The dried roots of *S. divaricata* (Turcz.) Schischk from four growing areas (Ganshu, Neimeng, Yunnan and Sichuan, China) were purchased from Leiyungshang Company, Shanghai, China. The TCM samples were ground to fine powder. A 0.200 g of each sample was used for pressurized hot water extraction. A 65-μm polydimethylsiloxane (PDMS)/divinylbenzene (DVB) was purchased from Supelco, Bellefonte, PA, USA.

Panaxyol and menthol (IS) standards were all provided by the National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China. Toluene, 1-octanol and cyclohexane (HPLC grade) are from Chemical Regent Company, Shanghai, China.

Distilled water purified through a Milli-Q deionizing unit (Millipore, Milwaukee, USA) was used as extractant. Ten microliters of GC microsyringe was purchased from Anpu Company, Shanghai, China. The magnetic stirrer was purchased from ShiLe Company, Shanghai, China.

2.3. Calibration solution preparation

Panaxynol stock solution (3.0 mg/ml) was prepared in methanol and stored at −4 °C. For quantitative analysis of panaxynol in *S. divaricata*, working standard solutions with the concentrations of 0.3, 1.5, 3.0, 6.0 and 30 μg/ml were prepared by dilution of the stock solution with distilled water.

2.4. GC–MS analysis

HP 5890 GC system, coupled with a HP MD5973 quadrupole mass spectrometer was used in the study. A fused-silica capillary HP-5MS column with 30 m long, 0.25 mm i.d. and 0.25 μm film was from Agilent, USA, which was used for separation. The carrier gas was helium with flow rate of 1.0 ml/min. Splitless modes were used. The injector temperature was set as 250 °C. The column temperature was programmed to rise from an initial temperature of 50–280 °C at 10 °C/min, hold for 5.0 min. The quadrupole temperature and transfer line temperature were 150 and 280 °C, respectively. To avoid saturation of the MS detector, solvent delay time of 9.0 min was used in the quantitative analysis.

2.5. Pressurized hot water extraction and headspace liquid-phase microextraction

At first, extraction of panaxynol in *S. divaricata* was performed using the PHWE assembly described above. A 0.2 g of each sample was extracted by water at these conditions: a constant flow rate of 2 ml/min, extraction time of 10 min, chamber temperature of 175 °C and pressure of 40 bar. Sequentially, 4.0 ml aqueous extract was introduced into an 8 ml headspace vial with a 1-cm stir bar. Before HS-LPME, it was preheated at 80 °C for 5 min, with a stirring ratio of 1100 rpm. HS-LPME was performed at 80 °C by using a 10 μl GC microsyringe (Fig. 2). According to Shen and Lee's method [31], HS-LPME was carried out: (1) withdraw 2.0 μl of cyclohexane (containing 20 μg/l menthol) into the microsyringe. (2) Insert the microsyringe needle into headspace vial and keep the needle suspended over aqueous extract. (3) Withdraw 5.0 μl of gaseous sample at 1.0 μl/s, then depress the plunger back the original mark immediately and hold for 5.0 s. The same process was repeated 30 times. Finally, the microsyringe needle was removed from the headspace vial and injected into GC–MS for analysis.

To obtain the calibration curve for panaxynol, the working standard solutions (4.0 ml) ranged from 0.3 to 30.0 μg/ml were introduced into 8-ml headspace vials. HS-LPME was performed under the same conditions described above.

2.6. Pressurized hot water extraction and headspace solid-phase microextraction

According to our previous method [26], *S. divaricata* (Turcz.) Schischk sample from Neimeng, was extracted by pressurized hot water extraction at the conditions described above. Sequentially, 4.0 ml aqueous extract was introduced into an 8 ml headspace vial with a 1-cm stir bar, and headspace extracted by using a PDMS-DVB fiber at 25 °C for 10 min. Finally, the extracted analytes were analyzed by GC–MS.

2.7. Relative recovery, repeatability, detection limit and relative accuracy

The relative recovery of the method was investigated by adding 0.1 ml standard stock solution (3.0 mg/ml) to a *S. divaricata* sample (0.2 g) containing known amounts of panaxynol. Triplicate measurements were performed by PHWE-HS-LPME-GC–MS.

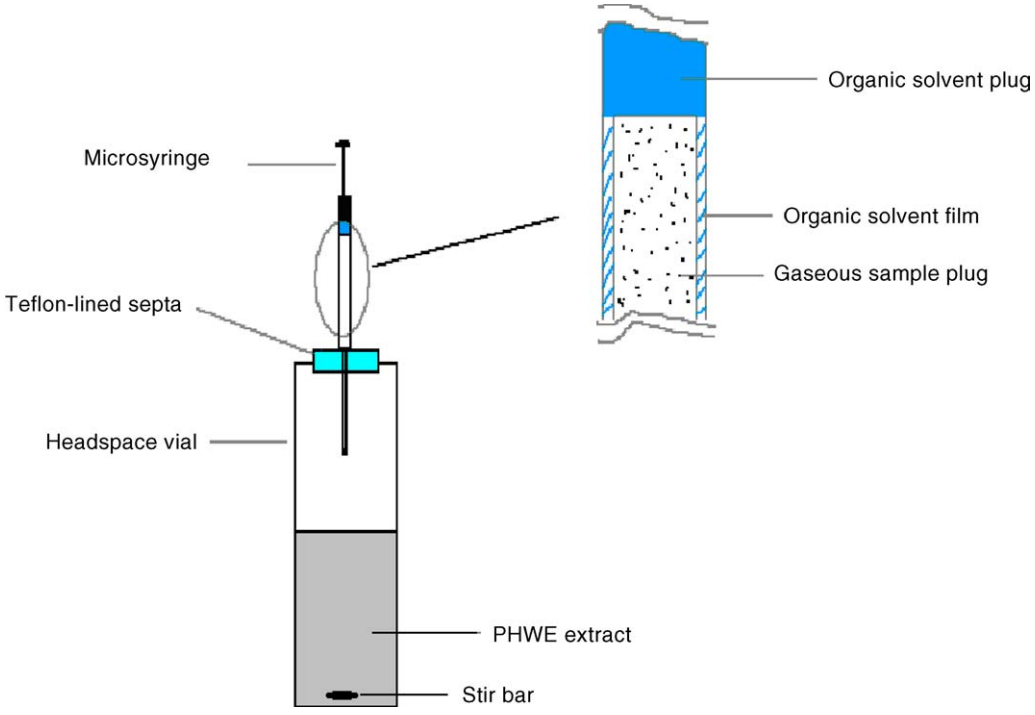

Fig. 2. Diagram of headspace liquid-phase microextraction of panaxynol in PHWE extract.

The repeatability was studied by four replicate analyses of panaxynol in *S. divaricata* from Neimeng by PHWE and LPME at the optimum conditions.

Four replicate analyses of the working solution with the concentration of 0.3 μg/ml were performed. On basis of S/N = 3, the detection limit for panaxynol was estimated.

To obtain the relative accuracy, steam distillation (SD) of the TCM was performed. Fifty grams of *S. divaricata* (Turcz.) Schischk sample from Neimeng was put into a 1000 ml distillation flask. Five hundred milliliters of distilled water were added and volatile oil distillation apparatus was set according to the Chinese pharmacopoeia (Chinese pharmacopoeia committee publishing house of people's Health, 2000, Part I: Appendix 64). The mixture was distilled for 6 h. Oil was collected from the condenser, dried over anhydrous sodium sulfate. The obtained essential oil was introduced into 5.0 ml volumetric flask, and the final volume of the extract was adjusted to 5.0 ml with *n*-hexane.

3. Results and discussion

3.1. Optimization of PHWE and HS-LPME conditions

PHWE has been demonstrated to be a powerful tool for extraction volatile compounds in solid samples [13–20]. The aqueous extracts by PHWE had to further extraction and concentration, prior to GC–MS analysis. As we know, OSF-HS-LPME is a simple, rapid and low-cost extraction and concentration technique. In the work, the two sampling techniques were combined and developed for determination of panaxynol. At first, optimization of PHWE and HS-LPME conditions was performed.

3.1.1. PHWE conditions

In the course of PHWE, temperature and pressure can affect the extraction efficiency by changing water polarity. *S. divaricata* (Turcz.) Schischk sample (0.2 g) from Neimeng, China was extracted by water at four temperatures of 125, 150, 175 and 200 °C and three pressures of 20, 40 and 60 bar. Extraction time of 10 min and a constant flow rate of 2.0 ml/min were used. After PHWE, a volume of 4.0 ml aqueous extract and 1-cm stir bar were introduced into an 8 ml headspace vial. The aqueous sample was preheated at 80 °C for 5 min, with stirring ratio of 1100 rpm, prior to HS-LPME. HS-LPME was performed at the conditions: Extraction temperature of 80 °C, extraction solvent of 2.0 μl 1-octanol, withdraw gaseous sample volume of 5.0 μl, plunger movement speed of 1.0 μl/s and extraction cycles of 30 times were used. The peak area ratios of panaxynol to menthol (IS) at different PHWE conditions are shown in Table 1. Little effect of pressure on extraction efficiency is observed. An intermediate value of 40 bar was used. The temperature is the key variable, for panaxynol, the best extraction efficiency was obtained at 175 °C (Table 1). Hence, 175 °C was selected as the PHWE temperature. Sequentially, extraction time and flow rate were studied. Extraction time from 5 to 20 min and flow rate from 0.5 to 4.0 ml/min were tested. It was observed from experimental that the maximum extraction efficiency of panaxyol was achieved at extraction time of 10 min and flow rate of 2.0 ml/min.

Table 1
Peak area ratio of panaxynol to IS obtained at the different PHWE temperatures and pressures

Temperature (°C)	Peak area ratio of panaxynol to IS		
	At 20 bar	At 40 bar	At 60 bar
125	0.87	0.91	0.86
150	1.49	1.52	1.51
175	2.46	2.51	2.48
200	1.89	1.94	1.96

3.1.2. HS-LPME conditions

Some factors, such as solvent, sampling volume, withdrawal rate, extraction cycles, the ratio between sample and headspace volume, and ion strength of aqueous solution can affect LPME extraction results. In the work, optimization of HS-LPME was performed.

At first, the selection of the extraction solvent should be carefully considered. According to Shen and Lee's principles [31], three solvents of cyclohexane, toluene and 1-octanol were selected as the extraction solvents of panaxynol. A volume of 4.0 ml aqueous extract obtained under the optimal PHWE conditions was used. HS-LPME was performed at the conditions: extraction temperature of 80 °C, a volume of 2.0 μl solvent, the plunger movement speed of 1.0 μl/s with 30 extraction cycles. The peak area ratio of panaxynol to menthol (IS) by using 1-octanol, cyclohexane and toluene are 2.48, 1.26 and 1.13, respectively. This shows that 1-octanol had better extraction efficiency than the other two solvents, due to the compatibility between panaxynol with the solvent (principle of like attracts like). Therefore, 1-octanol is selected as the extraction solvent of HS-LPME.

The sampling volume refers to the volume of the gaseous sample withdrawn into the syringe barrel. Fig. 3 is the effect of sampling volume on extraction amount of panaxynol. It is shown that the amount of the analyte increases linearly in the sampling volume of 2–5 μl and linearity is very poor when the sample volume more than 5 μl. Therefore, 5 μl was used as the sampling volume in the future work.

The effect of plunger withdrawal rate (0.5–5.0 μl/s) on the extraction efficiency was investigated. The peak area ratio of panaxynol to IS at the different plunger withdrawal rates (0.5, 1, 2, 4 and 5 μl/s) were 1.42, 2.51, 2.42, 1.63 and 1.21, respectively. The low-extraction efficiency was obtained at 0.5 μl/s, this may be due to the OSF was partly lost by solvent evaporation because of the slow plunger movement. However, with fast plunger movement, such as 4 and 5 μl/s, the extraction efficiencies were also low, probably because that the fast movement made the OSF thicker and extraction time shorter, and a heterogeneous OSF might have been formed, which

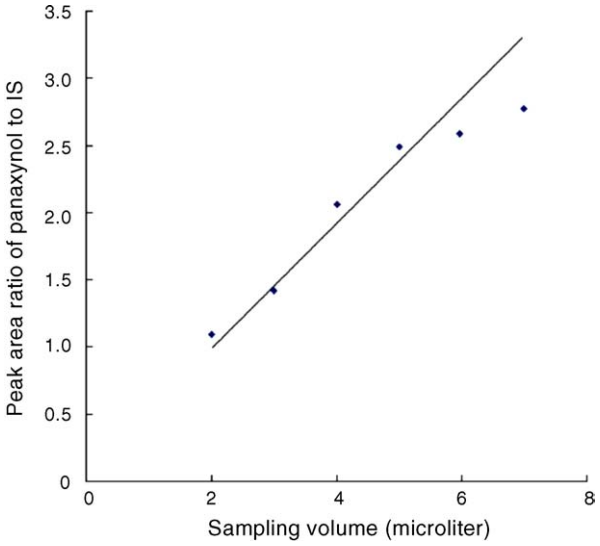

Fig. 3. Effect of LPME sampling volume on the extraction amount of panaxynol.

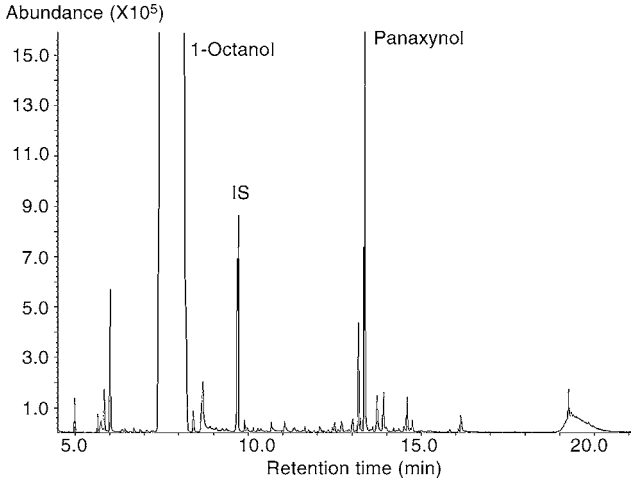

Fig. 4. Total ion chromatogram of essential oil in *S. divaricata* (Turcz.) Schischk from Neimeng by PHWE-HS-LPME-GC–MS.

would affect the extract efficiencies. Consequently, 1.0 μl/s was chosen as the optimal withdrawal rate since the best extraction was obtained at the rate.

The number of extraction cycles was also studied. The linear relationship between the extraction cycles (from 10 to 50) and the analyte amount enriched in the extraction solvent was observed. The cycle number of 30 times was employed. More extraction cycles would lead to poorer precision since the procedure was manually controlled, although higher sensitivity would result.

The ratio between sample and headspace volumes was studied. Three different volumes (2, 4 and 6 ml) of aqueous extract obtained under the optimal PHWE conditions were introduced into 8-ml headspace vials, respectively. We found the maximum amount of panaxynol was obtained at the ratio between sample and headspace volumes of 1:1. Finally, ionic strength was studied. A 0.5, 1.0 and 1.5 g to 4 ml NaCl was added into the aqueous solutions by PHWE, respectively. The extracted solutions with different ionic strength was obtained and extracted by HS-LPME. Little effect of ionic strength on extraction efficiency was observed.

3.2. Determination of panaxynol in S. divaricata (Turcz.) Schischk by PHWE-HS-LPME

Fig. 4 is the GC–MS total ion chromatogram of the essential oil in *S. divaricata* (Turcz.) Schischk from Neimeng by PHWE-HS-LPME at the optimal conditions. Panaxynol in the TCM was identified by Wiley Mass Spectra Library and verified by the standard. Its retention time is 13.42 min. To quantify panaxynol, HS-LPME of the working standard solutions was performed. Three replicate measurements were carried out and the calibration curve was obtained. The quantitative equation for panaxynol is $Y = 0.71X + 0.43$ ($R^2 = 0.994$) (Y, peak area ratio of panaxynol peak area to IS peak area; X, panaxynol concentration, mg/g). Panaxynol concentrations in the TCM samples from four different areas were determined by internal standard method. The panaxynol concentrations in the TCM samples from Ganshu, Sichuan, Yunnan and Neimeng are 0.89, 1.36, 0.69 and 2.87 mg/g, respectively. High content of panaxynol was found in the TCM from Neimeng, China. On basis of the amount of the active compound, panaxynol in *S. divaricata* from Neimeng was regarded to be the best. This is consistent with that obtained according to the TCM experiment [1]. The results show that quality assessment can be performed by quantitative analysis of panaxynol in *S. divaricata*.

3.3. Relative recovery, repeatability, detection limit and relative accuracy

The method validation including relative recovery, repeatability, detection limit and relative accuracy was studied. The relative recovery was 82%, which was obtained by comparison of the real value of the added-panaxynol with calculation value. The method repeatability was expressed by using relative standard deviation (R.S.D.) value. Four replicate analyses of panaxynol in the TCM from Neimeng were carried out and the R.S.D. value was 12.8%. This shows that the proposed method has acceptance repeatability. Limit of detection (LD) was estimated by replicate analyses of 0.3 μg/ml working solution. The LD value for panaxynol was about 2 μg/g, which is much less than the panaxynol concentration in *S. divaricata*. To obtain the relative accuracy, comparison of the panaxynol concentration by PHWE-HS-LPME with that by SD was performed. The concentration rate of 98:100 shows that PHWE-HS-LPME has a good relative accuracy.

In our previous study [26], PHWE-SPME was successfully combined and applied to analysis of essential oil in TCM. However, its disadvantages are that SPME fibers are expensive, fragile and easily broken. In the work, HS-LPME was used to overcome the shortcomings. To compare

headspace extraction efficiency of HS-LPME with that of HS-SPME, the aqueous solutions from the TCM was extracted by the two headspace modes. The peak areas of panaxynol by HS-SPME and HS-LPME were 3.64×10^7 and 4.26×10^8, respectively. Obviously, HS-SPME has a better extraction efficiency. Because the panaxynol in these TCM samples is very high (more than 0.8 mg/g), HS-LPME efficiency was enough for extraction of panaxynol in TCM samples. In addition, in the course of HS-LPME, fresh solvent was used for each headspace extraction, thus sample carryover did not occur.

4. Conclusions

In present work, two sample extraction techniques were successfully combined and applied to extraction of panaxynol in TCMs. Because PHWE equipment is homemade, and very little solvent (only 2 μl) and little extraction time were adopted, the method was simple, inexpensive and fast. Moreover, little sample mass (0.2 g) is needed. HS-LPME has the potential of being automated, which should ensure better repeatability and sensitivity than achievable by the current manually operated system. The proposed method provided a simple, rapid and low-cost approach for determination of panaxynol in TCMs. It is a potential tool for the quality evaluation of *S. divaricata*.

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