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Directly suspended droplet microextraction combined with single drop back-extraction as a new approach for sample preparation compatible with capillary electrophoresis

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ABSTRACT

A simple and novel method of directly suspended droplet microextraction (DSDME) combined with single drop back-extraction prior to capillary electrophoresis (CE) measurement is developed. In this technique, DSDME was firstly carried out under the maximum stirring rate for a desired time. Then, an aqueous droplet as back-extractive phase suspended at the needle tip was immersed in droplet of organic phase for back-extracted. After extraction, the aqueous droplet was transferred into a suitable vial and injected into CE for analysis. Three alkaloids were selected as model compounds for developing and evaluating the method performance. Under the optimum conditions, the enrichment factors ranged from 231 to 524. The relative standard deviations for five replicates were in the range of 4.8–8.1%. The calibration graph was linear in the range of 20–1000 ng mL^{-1} yielding correlation coefficients higher than 0.9983. The limit of detections varied from 8.1 to 14.1 ng mL^{-1}. Human urine samples were spiked with three alkaloids standard to assess the matrix effects and satisfactory results were obtained. The advantages of this method are simplicity of operation, rapid detection, low cost, high enrichment factor and little solvent consumption.

1. Introduction

In many analytical procedures, sample preparation is one of the most important steps. The objective of this challenging and critical step is to transfer the analyte into a form that is pre-purified, concentrated and compatible with the analytical system [1]. Liquid–liquid extraction (LLE) is probably one of the oldest accepted and widely used sample preparation procedures for pre-concentration and cleanup of aqueous samples [2]. However, it is time-consuming, tedious and requires large volume of high-purity solvents, which are often expensive and toxic, resulting in the production of hazardous laboratory waste [3]. In case of solid phase extraction (SPE), in comparison with LLE, although less time-consuming, it still requires toxic organic solvents for the elution step [4]. Besides, evaporation of solvent to dryness and the reconstitution of the dry residue in a suitable solvent for both techniques are unavoidable before CE analysis. Therefore, the simplification and miniaturization of the sample preparation methods are the noticeable trend.

Since its introduction in the early 1990s [5], solid-phase microextraction (SPME) has become popular for the analysis of organic compounds because it is solvent-free, relatively fast, portable and easy to use [6,7]. Nevertheless, only non-polar and slightly polar coatings are commercially available for SPME. Additionally, the SPME is plagued with the fragile nature of the fiber, limited range of coating selection and sample carry-over between runs [8], which are not overcome until now.

Liquid-phase microextraction (LPME), introduced by Jeannot and Cantwell [9], provides an alternative technique for sample preparation. In this method, the extracted analytes in the acceptor phase are directly injected into the analytical instruments. It is a novel sample preparation and preconcentration technique in which no toxic or harmful solvents are used in large quantities for the extraction. Besides, the capital equipment for the procedure is remarkably low cost and the procedures are simple and rapid in operation. It overcomes many of the disadvantages of LLE as well as some of those of SPME, such as independence of a commercial source and sample carryover. And the applications of this technique have been widely described in environmental and biological analysis [10,11].

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Recently, a new LPME method termed DSDME was proposed for extraction and determination of analytes [12]. In this method, a small volume of the organic solvent is delivered to the surface of an aqueous sample phase and then the mixture is stirred to extract the analytes. Under the proper stirring conditions, the motion of the vortex results in the formation of a larger single microdrop at the center of rotation and the analytes were extracted into the droplet. Besides, the rotations of the larger microdrop around a symmetrical axis cause internal recycling and intensify mass transfer inside the droplet [13]. The larger volumes of droplet is transferred into a conical vial and then injected into the GC and HPLC for analysis. However, as the general incompatible with the running buffer in CE, the extract is not directly analyzable by this technique.

Berberine (BBR), palmatine (PMT) and tetrahydropalmatine (THP), three representative active constituents of alkaloids isolated from *Rhizoma corydalis*, have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminths and chlamydia [14]. Furthermore, they also exhibit anti-cancer activities following evidence of antineoplastic properties [15,16]. Early publications have described techniques for the determination of alkaloids using micellar electrokinetic chromatography (MEKC) [17], HPLC [18–20], liquid chromatography–mass spectrometry (LC–MS) [21,22], ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) [23] and CE [24,25]. Among various types of methods, CE is widely employed due to low samples consumption, high separation efficiency and fast analysis speed. [24].

In the present technique, DSDME combined with single drop back-extraction as an extraction methodology for CE analysis is developed. The analytes were first extracted to a larger organic microdrop at the center of rotation by DSDME and then back-extracted into an aqueous back-extractive phase immersed in droplet of organic phase. We selected three alkaloids as model compound in development and evaluation of the procedure. The method was successfully applied to the determination of alkaloids in human urine samples prior to CE measurement.

2. Experimental

2.1. Reagents and materials

Berberine hydrochloride, palmatine hydrochloride, tetrahydropalmatine and strychnine (STN) (internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The 1 mg mL^{-1} individual stock solutions of the analytes and IS were prepared by dissolving of each standard in methanol. The 100 μg mL^{-1} of three alkaloids mixed standard solution and 200 μg mL^{-1} of IS standard solution were prepared in deionized water every week. Working solution was prepared everyday by spiking mixed standard solution and IS standard solution to NaOH solution during the optimization exercise. All the solutions were stored at 4 °C and filtered with 0.45 μm filters before use.

Sodium dihydrogen phosphate, sodium chloride, sodium hydroxide, hydrochloric acid, methanol, *n*-hexane and *n*-heptane were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Toluene was purchased from Jingu Business Industrial Co. Ltd. (Tianjin, China). Butyl acetate, *iso*-octanol and *n*-octanol were purchased from Guangzhou Chemical Reagent Plant (Guangzhou, China). All reagents are of analytical grade. The 0.45 μm filters was obtained from Xingya Purifying Materials Plant (Shanghai, China). Deionized water, obtained from a Milli-Q water purification system (Millipore, Bedford, USA), was used for preparing solutions throughout the experiment.

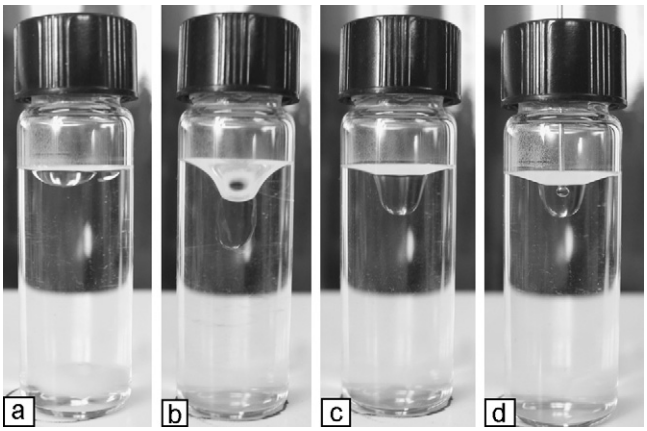

Fig. 1. Picture of the different steps for DSDME and single drop back-extraction: (a) addition of the organic extractive phase to the aqueous sample phase, (b) DSDME procedure at 1150 rpm, (c) stirring rate at 800 rpm and the larger droplet of organic phase kept steady, and (d) back-extraction procedure at 800 rpm.

2.2. Instruments

The extraction procedure was carried out in a 4.0 mL clear glass vial with screw top/silicon septa (lot: 162369H, Supelco, Bellefonte, PA, USA). Stirring of the solution was carried out by a Hot Plate Stirrer model PC-420D (Corning, USA) and a magnetic stirring bar (10 mm × 4 mm). A 10 μL flat-cut syringe (Hamilton-Bonaduz, Switzerland) was used to suspend the drop of acceptor phase during back-extraction.

A CL1030 Capillary electrophoresis system (Cailu, Beijing, China) equipped with a UV detector was used for the determinations. A fused silica separation capillary of 50 cm (41 cm effective length) × 50 μm I.D. ×375 μm O.D. (Yongnian, Hebei, China) was used throughout the study. The data acquisition was carried out with a HW-2000 Chromatography Workstation (Qianpu, Shanghai, China).

A PHS-3CA precision pH meter (Dapu, Shanghai, China) was used throughout the experiment.

2.3. Extraction procedure

The extraction procedures are shown in Fig. 1. Briefly, a 4.0 mL cylindrical vial with a stir bar was placed on a heating-magnetic stirrer. Then, a volume of 3.5 mL aqueous sample solution containing 200 ng mL^{-1} of three alkaloids (BBR, PMT and THP) and 400 ng mL^{-1} of IS (STN) was transferred into the vial as donor phase (200 mM NaOH, pH 13.3). A trifle of *n*-octanol (less than 60 μL) was dripped on the top of the aqueous sample (a), and the mixture was agitated for 8 min at 1150 rpm for DSDME (b). Afterwards, the stirring rate was adjusted at 800 rpm and the larger droplet of organic phase kept steady (c). Then, the microsyringe filled with 1 μL of 20 mM HCl was inserted into the vial by piercing the septa. The needle tip was fixed in the center of the larger droplet of organic extractive phase and the plunger of the syringe was depressed completely to suspend the droplet in it for back-extraction (d). After 10 min, the aqueous back-extractive phase was retracted into the syringe and transferred into a microvial. Then, the sample was introduced into CE. Finally, the capillary inlet was placed in a high potential buffer solution to carry out CE separation.

2.4. Electrophoresis conditions

When a new capillary was used, it was flushed with methanol for 30 min then with deionized water for 10 min, 1 M HCl for 30 min and 1 M NaOH for 30 min. At the beginning of each working day,

the capillary was consecutively rinsed with 0.2 M NaOH for 15 min, deionized water for 15 min and the running buffer for 15 min. Moreover, the capillary was flushed for 3 min between runs with running buffer. The support buffer was 50 mM NaH_2PO_4–20% methanol adjusted to pH 7.0. All solutions were filtered with 0.45 μm filters before use. The voltage during separations was 14 kV and UV detection at 225 nm was employed at the cathode end. Samples were introduced into the capillary by hydrodynamic injection, where the sample vial was raised by 15 cm for 10 s.

2.5. Calculation of enrichment factor

Enrichment factor (EF) was calculated by the following equation [26]:

$$EF = \frac{C_a}{C_d} \quad (1)$$

In this equation, C_a and C_d were the final and initial concentrations of the analytes in the aqueous back-extractive and aqueous sample phases, respectively. C_a was obtained from calibration graph of direct injections of standard solutions with concentrations in the range of 2–50 μg mL^{-1} under the optimized electrophoresis conditions mentioned above. And the curves, obtained by plotting the peak areas versus the concentrations of analytes, gave a high level of linearity with correlation coefficients (r^2) of 0.9964–0.9999.

3. Results and discussion

For the DSDME, as a special type of LLE, the concentration of analytes (C_o) in organic extractive phase can be represented as follows [12]:

$$C_o = \frac{\kappa C_d}{1+\kappa V_o/V_d} \quad (2)$$

where V_d and V_o were the volumes of the aqueous sample and organic extractive phase, respectively, C_d was initial concentrations of the analytes in the aqueous sample phase and κ was the distribution coefficient. As can be seen from this equation, C_o had a reverse correlation with V_o. The volume of organic extractive phase used for DSDME was relatively low, which was more appropriate to be chosen as the middle phase in the proposed technique. First, the deionized analytes in the aqueous sample phase were extracted into the organic extractive phase and achieve equilibrium in DSDME. When the aqueous back-extractive phase was immersed in the organic droplet, mass transfer of analytes occurred between them. With this mass transfer, the equilibrium in DSDME was broken. At the same time, a new equilibrium in DSDME would be established with the help of agitation of the aqueous acceptor phase. Therefore, the repeated procedures above ensured equilibrium was being repeatedly established and broken, which would result in the enrichment of the analytes in the aqueous acceptor phase.

3.1. Type and volume of the organic extractive phase

It was very important to select an appropriate organic solvent for obtaining a good selectivity and satisfactory extraction efficiency. As the organic extractant was discarded after back-extraction, its compatibility with an analytical instrument has low significance compared to earlier DSDME methods. In general, the organic solvent used as the extracting solvent in this method must have lower density than that of water; a very low solubility in water and high extraction capability for analytes. Apart from these requirements, the organic solvent should also have a high viscosity to form a well-settled phase and low volatility to prevent loss during extraction due to the low solvent consumption. Based on these criterions, several organic solvents (toluene, *n*-hexane, *n*-heptane, butyl acetate, *iso*-octanol, *n*-octanol) in differing characteristics were investigated (Table 1) [27–29]. In this study, 50 μL of the organic solvent mentioned above were tested at stirring rate of 1150 rpm for DSDME and 800 rpm for back-extraction, respectively. For toluene, *n*-hexane and *n*-heptane, due to the low viscosity and high volatility, the life time of the aqueous back-extractive phase cannot be too long because of dissolution and loss. Although butyl acetate had better extraction efficiency for BBR and THP, it was not a suitable solvent here due to the instability of the aqueous drop in long time. Therefore, *n*-octanol with high viscosity and better extraction efficiency was selected for further experiments.

The organic extractive phase volume was a key parameter affecting the extraction efficiency and the extraction kinetics. As shown in Eq. (2), lower volume of organic extractive phase was prerequisite for DSDME to achieve satisfactory extraction efficiency. Thus, the influence of the volume of the *n*-octanol was investigated in the range of 20–60 μL in 10 μL intervals. Although the use of lower volumes of organic extractive phase led to higher extraction efficiency, the repeatability values were poor when the volumes were lower than 50 μL. As a compromise, 50 μL of *n*-octanol was chosen in the following experiments.

3.2. Stirring rate in DSDME and back-extraction

The main purpose of microextraction techniques was to attain maximum extraction efficiency within a relatively short period of time [30,31]. The stirring aqueous sample phase could accelerate the kinetics of extraction by decreasing the thickness of the Nernst diffusion film around the interface between the aqueous sample and organic extractive phases, which would enhance diffusion of the analytes and shorten the extraction time to achieve equilibrium. Since the extraction of the analytes from the aqueous sample phase into the organic extractive phase was a slow equilibrium process [32], working solutions were extracted at maximum rate (1150 rpm) to accelerate extraction rate in DSDME procedure.

For back-extraction, 1 μL of aqueous back-extractive phase was immersed in the 50 μL of *n*-octanol and laid at the rotation axis of sample solution. In order to evaluate the effect of sample stirring, working solutions were extracted for 15 min with stirring rate varied in the range of 500–900 rpm after 3 min DSDME procedure. The results are shown in Fig. 2. It was observed that the extraction efficiency of analytes improved as the stirring rate increased to 800 rpm and then decreased with increasing of the stirring rate. The reason for the decrease of extraction efficiency was that the stirring rate above 800 rpm caused the instability and faster dissolution of the organic extractive phase which decreased the peak area. As a result, stirring rate at 800 rpm was suitable for back-extraction in this work.

3.3. pH of aqueous sample phase

The pH of aqueous sample phase was another important parameter which may affect the extraction efficiency in aqueous samples. It is known that the existence form of certain analytes will change with the change of solution pH and thereby affect their water-solubility and extractability. For basic drugs, the aqueous sample phases were commonly strongly alkalized to keep the analyte in its neutral form and consequently reduce their solubility within the samples. Thus, the extraction of the alkaloids were performed from the aqueous sample phase containing NaOH at the concentration range of 10–00 mM (pH in the range of 11–13.7), and the results are shown in Fig. 3. The results indicated that increase of NaOH concentrations provided significant enhancement of the extraction efficiency and the maximum extraction efficiency was achieved at 200 mM NaOH (pH 13.3). The increase in the ionic strength of the

Table 1
Characteristics of organic solvents and extraction efficiency.

Solvent	Density (g cm^{-3})	Solubility in water (g L^{-1})	Surface tension (dyn cm^{-1})	Viscosity (C.P.)	Peak area		
					BBR	PMT	THP
Toluene	0.87	0.53	28.5	0.59	–	–	–
n-Hexane	0.66	0.013	18.4	0.31	–	–	–
n-Heptane	0.68	0.003	19.8	0.41	–	–	–
Butyl acetate	0.88	0.83	24.8	0.63	6988	2955	81908
iso-Octanol	0.86	0.001	28.7	7.7	5311	3630	19803
n-Octanol	0.83	0.0003	27.5	10.64	6149	5688	19961

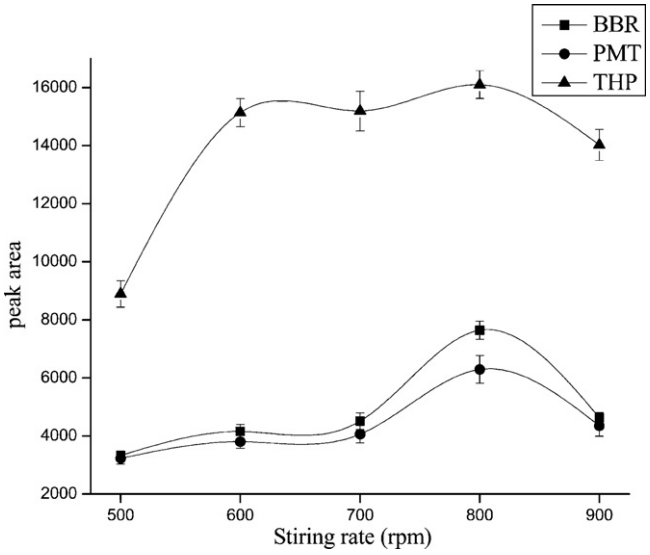

Fig. 2. Effect of stirring rate in back-extraction on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of 100 mM NaOH as aqueous sample phase; 3 min at 1150 rpm for DSDME; 15 min for back-extraction; 1 μL of 100 mM HCl as aqueous back-extractive phase; 25 °C and no salt addition.

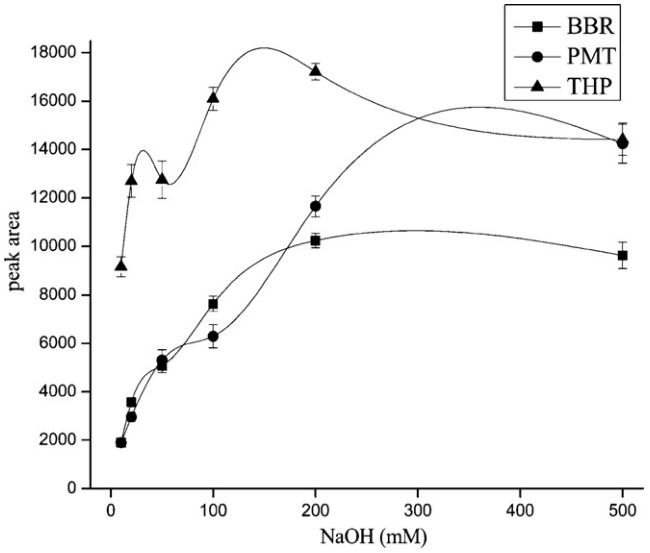

Fig. 3. Effect of pH of aqueous sample phase on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of aqueous sample phase; 3 min at 1150 rpm for DSDME; 15 min at 800 rpm for back-extraction; 1 μL of 100 mM HCl as aqueous back-extractive phase; 25 °C and no salt addition.

aqueous sample phase in the presence of higher concentrations of NaOH may cause the decrease of extraction efficiency [33]. Thus, 200 mM NaOH (pH 13.3) solution was chosen as donor phase in the subsequent extractions.

3.4. Extraction time in DSDME and back-extraction

Extraction is an equilibrium process, and the maximum extraction efficiency is obtained when the system is at equilibrium. Therefore, optimum time is required to reach equilibrium for both DSDME and back-extraction.

For the equilibrium of DSDME, extraction time was varied in the range of 1–10 min at stirring rate of 1150 rpm. The results are shown in Fig. 4. The extraction efficiency increased with increasing extraction time, until the equilibrium between aqueous sample phase and organic extractive phase was attained after extracting for 8 min. But after 8 min, the extraction efficiency showed a decline. For efficient extraction results, the optimized extraction time for the DSDME was 8 min.

The back-extraction was the mass transfer from the enriched organic extractive phase into the aqueous back-extractive phase and the extraction time was also investigated in the range of 5–20 min in 5 min intervals. Similar trend was observed during back-extraction as given in Fig. 5. As demonstrated in Fig. 5, the back-extraction equilibrium was established at 10 min. The reason for the loss in extraction efficiency with time might be due to miscibility or volatility of organic extractive phase. So a back-extraction time of 10 min was selected as the optimum in the following experiments.

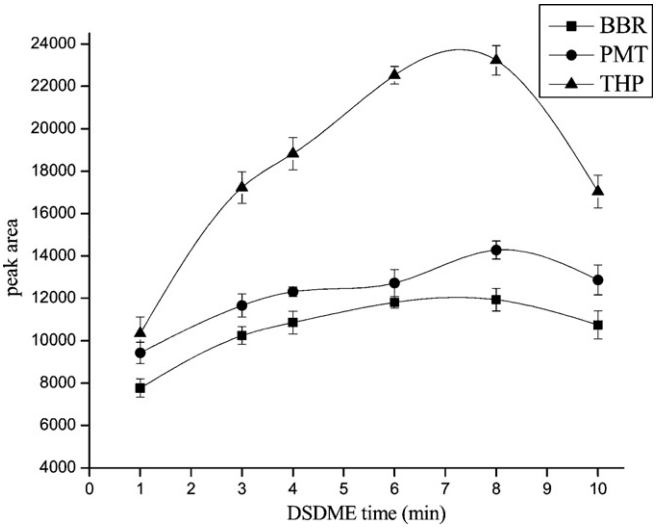

Fig. 4. Effect of DSDME time on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of 200 mM NaOH as aqueous sample phase; 1150 rpm for DSDME; 15 min at 800 rpm for back-extraction; 1 μL of 100 mM HCl as aqueous back-extractive phase; 25 °C and no salt addition.

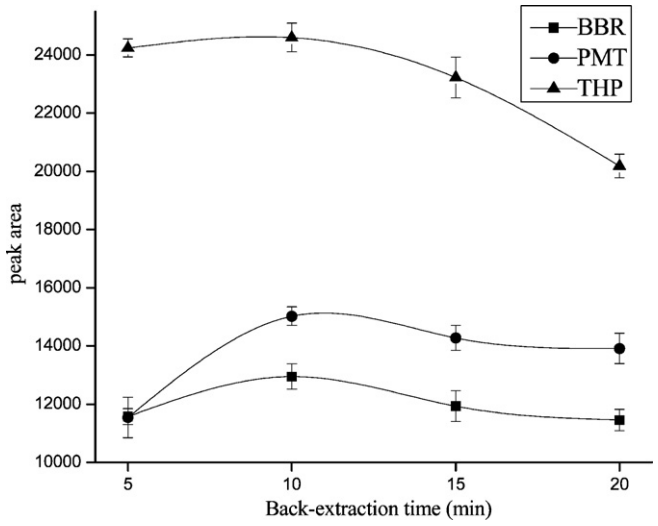

Fig. 5. Effect of back-extraction time on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of 200 mM NaOH as aqueous sample phase; 8 min at 1150 rpm for DSDME; 800 rpm for back-extraction; 1 μL of 100 mM HCl as aqueous back-extractive phase; 25 °C and no salt addition.

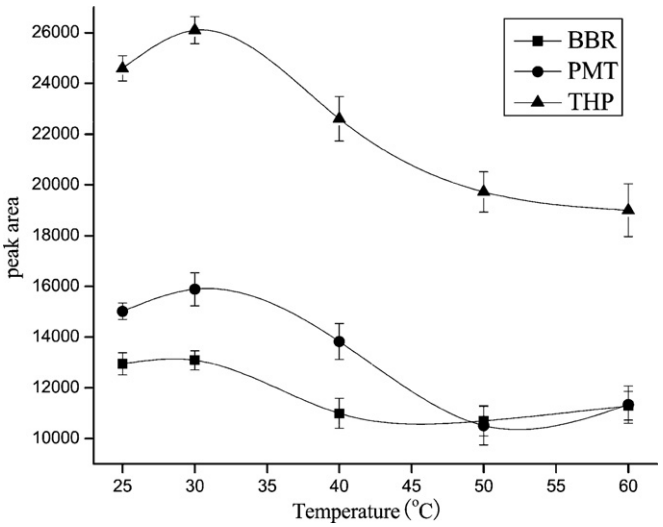

Fig. 7. Effect of temperature on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of 200 mM NaOH as aqueous sample phase; 8 min at 1150 rpm for DSDME; 800 rpm for back-extraction; 1 μL of 100 mM HCl as organic extractive phase and no salt addition.

3.5. NaCl concentration

Generally, addition of salt can decrease the solubility of analytes in the aqueous sample phase and enhance their partitioning into the aqueous back-extractive phase due to a salting-out effect [34], but some contradictory results have been reported [35–37]. For investigating the influence of salt addition on the extraction efficiency in this study, NaCl was added into the donor sample solution at a concentration between 0 and 20 (w/v, %) and the results are shown in Fig. 6. As seen from Fig. 6, the peak areas decreased with the increment of NaCl concentration in the studied range. The decrease in extraction efficiency with the increase of salt concentration may attributed to the reduction in diffusion rate of analytes from aqueous sample phase to the organic extractive phase since the addition of salt increased the viscosity of water solution [35,37] and changed in the charge state of the analyte molecules leading to decrease in the diffusion rate of analytes. On the basis of the above observations, no salt addition was selected since this quantity provided acceptable results for all analytes.

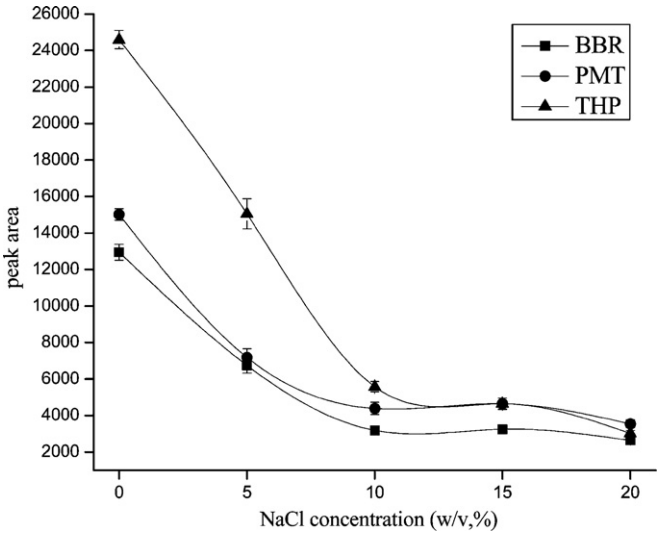

Fig. 6. Effect of NaCl concentration on the extraction efficiency. Extraction conditions: 200 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS; 50 μL of *n*-octanol as organic extractive phase; 3.5 mL of 200 mM NaOH as aqueous sample phase; 8 min at 1150 rpm for DSDME; 10 min at 800 rpm for back-extraction; 1 μL of 100 mM HCl as aqueous back-extractive phase; 25 °C.

3.6. Sample temperature

Temperature of the aqueous sample phase was another important parameter that should be well controlled. The higher temperature can enhance diffusion coefficient and partition coefficient of analytes between the aqueous sample phase and organic extractive phase in DSDME which would be expected to have an important effect on the extraction efficiency, the temperature should be well controlled. Thus, the effect of temperature on the extraction efficiency was investigated in the range of 25–60 °C. As seen in Fig. 7, the peak areas were enhanced by increasing the temperature from 25 to 30 °C, but slight decrease with the higher temperature. The reduction in the extraction efficiency might be due to the increased solubility of analytes in aqueous sample phases. Additionally, temperatures higher than 30 °C resulted in instability of the aqueous back-extractive phase. Therefore, the extraction temperature was chosen at 30 °C.

3.7. Selection of aqueous back-extractive phase

In general, there were two factors considered during selection of aqueous back-extractive phase: firstly, it must be compatible with the running buffer in CE system; secondly, it must ensure to provide appropriate extraction efficiency. Thus, HCl with different concentrations were tested. The effect of concentrations of HCl on the extraction efficiency was studied in the range of 5–100 mM. The results showed that the concentrations of HCl have an important impact on the extraction efficiency and the higher extraction efficiency was achieved at concentration of 20 mM. The addition of concentration of HCl increased the viscosity of aqueous back-extractive phase leading to decrease in the diffusion rate when the concentration of HCl was greater than 20 mM. Taking these into considerations, 20 mM HCl was selected as aqueous back-extractive phase.

Table 2
Performance of the proposed extraction procedure.

Analyte	Regression equation[a]	LR[b] (ng mL^{-1})	Correlation coefficient	RSD[c] (%)	LOD[d] (ng mL^{-1})	EF[e]
BBR	$Y = 0.00227X + 0.00292$	20–1000	0.9983	4.7	14.1	332
PMT	$Y = 0.00199X + 0.00714$	20–1000	0.9990	8.1	14.0	231
THP	$Y = 0.00599X - 0.0135$	20–1000	0.9993	6.3	8.1	524

[a] Calibration equation: the vertical coordinate of the standard curves showed the ratio of peak areas of analytes with IS, and the abscissa reflected the change of the concentration of analytes.
[b] LR: linear range.
[c] RSD: relative standard deviation; determined by analyzing standard solution at level of 200 ng mL^{-1} for five times.
[d] LOD: limit of detections for a S/N = 3.
[e] EF: enrichment factor.

3.8. Volume ratio of aqueous sample to aqueous back-extractive phase

In LPME, the extraction efficiency can be improved by increasing the volume ratio of aqueous sample and aqueous back-extractive phases. In the present work, the effect of the volume ratio on extraction efficiency was evaluated by changing the volume of the aqueous back-extractive phase from 1 μL to 3 μL while the volume of aqueous sample phase was kept constant at 3.5 mL. The results indicated that the highest extraction efficiency was obtained when 1 μL of HCl was chosen as aqueous back-extractive phase (i.e., donor/acceptor ratio of 3500:1), since the mass transfer took place more easily in a droplet with a smaller size [38]. The decrease in response after the volume higher than 1.0 μL resulted from the dilution of analytes in higher amount of aqueous back-extractive phase [39].

Over all, the optimized conditions of this method were: 3.5 mL sample solution containing 200 mM NaOH as aqueous sample phase; 50 μL of *n*-octanol as organic extractive phase in DSDME; 1 μL of 20 mM HCl as aqueous back-extractive phase in back-extraction; 8 min DSDME time with the stirring rate of 1150 rpm and 10 min back-extraction time with the stirring rate of 800 rpm at 30 °C and no salt addition.

3.9. Method validation

The method was validated in terms of its linearity, repeatability, detection of limits (LOD) and EF under the optimum conditions mentioned above. The results are shown in Table 2. The vertical coordinate of the standard curves showed the ratio of peak areas of analytes with IS, and the abscissa reflected the change of the concentration of analytes. As it is illustrated, good linearity was exhibited at concentration range of 20–1000 ng mL^{-1} with correlation coefficients (r^2) ranging from 0.9983 to 0.9993. The LOD of the proposed method varied from 8.1 to 14.1 ng mL^{-1} based on a signal-to-noise (S/N) of 3. The precision was determined by analyzing standard solution for five times and the relative standard deviations (RSD) were 4.8–8.1%. EF ranged from 231 to 524 by three times extraction of aqueous samples.

3.10. Application of the method

To evaluate the potentiality of the technique, the procedure was applied for the analysis of male and female urine samples. Initially, the samples were directly extracted after filtration. However, no three phase system was observed due to larger solubility of organic solvent in urine samples. Then, the urine samples were diluted five times with deionized water to reduce the matrix effect. The relative recoveries and reproducibility experiments were performed at three concentration levels of 50, 200 and 500 ng mL^{-1} for three alkaloids both in male and female urine samples. As shown in Table 3, relative recoveries of the extraction, calculated as the ratio of the response in urine samples and the deionized water samples [40], varied from 68.8 to 112% with the standard deviations lower than 10.8%. The representative chromatograms of the extract of urine samples from blank and spiking of alkaloids are shown in Fig. 8.

Table 3
Analytical results of urine samples.

Analyte	Concentration (ng mL^{-1})	Male urine		Female urine	
		RR[a] (%)	RSD[b] (%)	RR[a] (%)	RSD[b] (%)
BBR	50	83.2	1.3	82.4	1.6
	200	97.4	9.3	72.3	3.9
	500	103.9	9.8	95.5	3.5
PMT	50	75.7	2.0	74.9	5.9
	200	95.5	6.7	68.8	6.7
	500	107.6	4.1	112.0	10.8
THP	50	73.2	9.5	92.5	7.6
	200	104.3	6.4	90.6	4.9
	500	92.3	9.1	84.1	6.1

[a] RR: relative recoveries; calculated as the ratio of the response in real samples and the deionized water samples.
[b] RSD: relative standard deviations; $n = 3$.

3.11. Comparison with the single drop liquid–liquid–liquid microextraction

The major advantages of the present method were compared with the single drop liquid–liquid–liquid microextraction (SD-

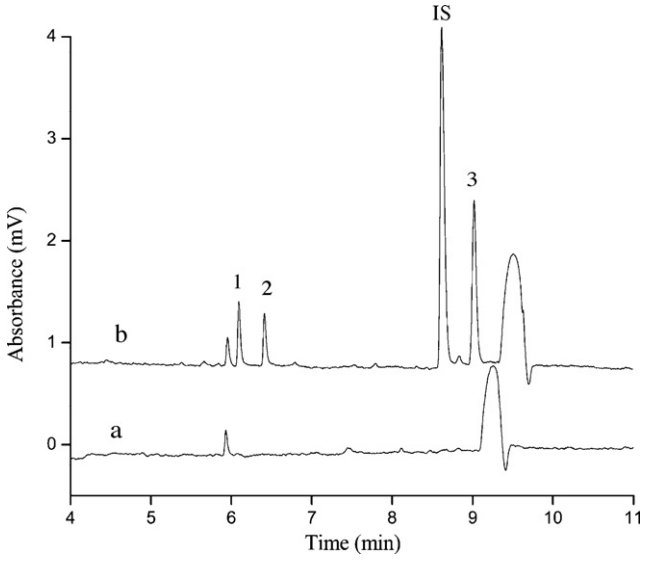

Fig. 8. Chromatogram of urine from blank (a) and after spiking at concentration level of 50 ng mL^{-1} of three alkaloids and 400 ng mL^{-1} of IS (b). CE conditions: running buffer, 50 mM NaH_2PO_4–20% methanol adjusted at pH 7.0; applied voltage, 14 kV; injection time, 10 s; UV detection 225 nm. Extraction temperature: 30 °C; other extraction conditions are same as Fig. 7. Peak identification: (1) BBR; (2) PMT; (3) THP.

Table 4
Comparison of present method with SD-LLLME[a].

Analyte	Present method	SD-LLLME[a]			
	Enrichment factor	Enrichment factor			
	10 min	10 min	20 min	30 min	40 min
BBR	332	86	86	183	71
PMT	231	64	79	115	109
THP	524	150	189	281	244

[a] Single drop liquid–liquid–liquid microextraction.

LLLME). For the SD-LLLME, the aqueous samples were "sealed" with layer of organic phase which would require hundreds microliter of organic solvents (about 400 μL). Therefore, the dilute extract in organic extractive phase would lead to the sharp rise in equilibrium extraction time [40,41]. But the present technique had very low solvent consumption, shorter extraction time and higher EF. Therefore, a comparison of two methods was performed on the extraction efficiency of model compounds. Identical conditions for both procedures were: 3.5 mL sample solution containing 200 mM NaOH as aqueous sample phase; 1 μL of 20 mM HCl as aqueous back-extractive phase; 8 min pre-extraction time with the stirring rate of 1150 rpm; 10 min back-extraction time with the stirring rate of 800 rpm at 30 °C and no salt addition. In SD-LLLME, 400 μL of *n*-octanol was placed at the surface of the sample solution as organic extractive phase in pre-extraction. But in the present method, 50 μL of *n*-octanol was chosen as organic extractive phase in DSDME. As shown in Table 4, higher enrichment factors were obtained by our new method. The extraction efficiency of SD-LLLME was further investigated by changing back-extraction time. The extraction efficiency of analytes improved as the back-extraction time increased to 30 min and then decreased with increasing of the time. According to the results in Table 4, the optimum enrichment factors of SD-LLLME were still lower than those of present method.

4. Conclusions

In this work, a newly designed approach of DSDME followed by single drop back-extraction is proposed. The potentiality of combination is made DSDME compatible with CE. Therefore, it would share the advantages of DSDME and CE methodologies. On the other hand, this method has higher selectivity, good relative recovery and reproducibility in the actual application. Despite this approach appears some shining point, it cannot be perfect due to its deficiency of limited extraction selection, which is also the weakness of common DSDME procedures. Future work will focus on this to make perfection. In conclusion, it is a promising and fast sample preparation method that can be employed in bio-analytical.

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