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Research Article

Ultrasound-assisted emulsification microextraction for the determination of ephedrines in human urine by capillary electrophoresis with direct injection. Comparison with dispersive liquid–liquid microextraction

Ultrasound-assisted emulsification microextraction and dispersive liquid–liquid microextraction were compared for extraction of ephedrine, norephedrine, and pseudoephedrine from human urine samples prior to their determination by capillary electrophoresis. Formation of a microemulsion of the organic extract with an aqueous solution (at pH 3.2) containing 10% methanol facilitated the direct injection of the final extract into the capillary. Influential parameters affecting extraction efficiency were systematically studied and optimized. In order to enhance the sensitivity further, field-amplified sample injection was applied. Under optimum extraction and stacking conditions, enrichment factors of up to 140 and 1750 as compared to conventional capillary zone electrophoresis were obtained resulting in limits of detection of 12–33 μg/L and 1.0–2.8 μg/L with dispersive liquid–liquid microextraction and ultrasound-assisted emulsification microextraction when combined with field-amplified sample injection. Calibration graphs showed good linearity for urine samples by both methods with coefficients of determination higher than 0.9973 and percent relative standard deviations of the analyses in the range of 3.4–8.2% for ($n = 5$). The results showed that the use of ultrasound to assist microextraction provided higher extraction efficiencies than disperser solvents, regarding the hydrophilic nature of the investigated analytes.

Keywords: Dispersive liquid–liquid microextraction / Ephedrines / Field-amplified sample injection / Microemulsion / Ultrasound-assisted emulsification micro extraction
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1 Introduction

Stimulants are synthetic derivatives of the endogenous stimulant adrenaline and have similar pharmacological effect on mental function and behavior, producing excitement and euphoria and increase activity. One of the oldest therapeutically applied stimulants is the group of ephedrines, the major active compounds of which include ephedrine (Eph), norephedrine (Nor), and pseudoephedrine (Pse) [1]. Eph and Pse are a pair of diastereoisomeric sympathomimetic amines known to have central nervous system stimulating properties [2] and are used as active ingredients in medicine to cure asthma, cold, and hypersensitivity [3], whereas Nor is the metabolite of Eph in the body [4]. Use of Eph and Pse as doping agents in sports [5] and the health and social consequences led the World Anti Doping Agency (WADA) to include them in the 2012 prohibited list of pharmacological forbidden substances [6]. However, due to the frequent therapeutical use of ephedrines, their regulations are still complicated [1]. Recently, WADA has established a threshold value of 10 mg/L for Eph and 150 mg/L for Pse in urine; meanwhile Nor is not included in the list.

In antidoping analysis, ephedrines used to be quantified in urine mainly by gas chromatography (GC) [7, 8] and high-performance liquid chromatography (HPLC) [9, 10]. Lately, there has been an interest in the application of capillary electrophoresis (CE) for the determination of ephedrines due

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Abbreviations: 1-DO, 1-dodecanol; **1-UN**, 1-undecanol; **CZE**, capillary zone electrophoresis; **DLLME**, dispersive liquid–liquid microextraction; **DPE**, diphenyl ether; **Eph**, ephedrine; **FASI**, field-amplified sample injection; **Nor**, norephedrine; **Pse**, pseudoephedrine; **SDS**, sodium dodecyl sulfate; **USAEME**, ultrasound-assisted emulsification microextraction

to its extremely high separation efficiency, short analysis time, low operating cost, and wide application range [11, 12]. Nevertheless, because of the short detection optical length and low sample loading, CE has some limitations in terms of concentration sensitivity particularly with direct UV detection – the most widely used detection system in CE – which restricts its use for routine applications. On-line preconcentration techniques including sample stacking [13] and sweeping [14] have also been utilized to increase the sensitivity of CE for the determination of ephedrines. Although sample stacking and sweeping have enjoyed some degree of success in CE as efficient online sample preconcentration techniques, there is still a major problem when directly applied to biological samples (e.g. urine) without applying an appropriate sample pretreatment step as they suffer tremendously from matrix effects [15].

Since very small volumes are used in CE [i.e. from picoliter (pL) to nanoliter (nL)] liquid phase microextraction (LPME) has been shown to be a suitable sample pretreatment technique for CE through integrating it with single drop microextraction (SDME) [16], solvent bar microextraction (SBME) [17], hollow fiber-based liquid phase microextraction (HF-LPME) [18], and dispersive liquid–liquid microextraction (DLLME) [19]. DLLME was introduced by Assadi and coworkers in 2006 [20]. In this method, an appropriate mixture of an extraction solvent and a disperser solvent is used. The surface area between extraction solvent and sample solution is infinitely large because a cloudy solution can be formed. Therefore, extraction equilibrium can be reached quickly. This method has attracted much attention due to its advantages including fast analysis, low consumption of organic solvent, and simplicity [21]. Leong and Huang [22] proposed the use of less toxic organic solvents with lower density than water, low toxicity, and proper melting point near room temperature (in the range of 10–30°C). After extraction, the floated extraction solvent drop could be easily collected by solidifying it at low temperature. Hence, this method was termed as dispersive liquid–liquid microextraction based on solidification of floating organic drop (DLLME-SFO). The major disadvantage of DLLME and DLLME-SFO is that the addition of a relatively large volume of the organic disperser solvent (0.5–3 mL) leads to reduced extraction efficiency due to an increase in the solubility of the analytes in the sample solution [23].

Ultrasound-assisted emulsification microextraction (USAEME), proposed by Regueiro et al. in 2008 [24] is an effective technique since it avoids the use of organic disperser solvents. In USAEME, a microvolume of water-immiscible extraction solvent is dispersed into sample aqueous solutions by ultrasound-assisted emulsification without using disperser solvents, which results in higher extraction efficiency. After mass transfer, the two phases can be readily separated by centrifugation.

In this work, both DLLME and USAEME were applied and compared for extraction and preconcentration of the relatively hydrophilic compounds of Eph, Nor, and Pse in human urine. A novel formation of a microemulsion of the organic extract with an aqueous solution containing 10% (v/v) methanol facilitated the direct injection of the final extract into CE. Parameters affecting extraction efficiency, including pH and volume of sample and microemulsion solutions, type and volume of extraction and disperser solvents, time of ultrasound agitation, ionic strength as well as extraction time were systematically studied and optimized. In addition, the online preconcentration technique of field-amplified sample injection (FASI) was used to further enhance the sensitivity of CE.

2 Materials and methods

2.1 Reagents

Eph [99.06%, log*P* 1.05 (octanol–water partition coefficient), pK_a 9.38] was obtained from Santa Farma (Istanbul, Turkey); Pse (99.90%, log*P* 1.05, pK_a 9.38) and Nor (99.90%, log*P* 0.81, pK_a 8.47) [25] were kindly provided by Koçak Farma (Istanbul, Turkey). HPLC-grade methanol (Lab-Scan, Gliwice, Poland), acetonitrile (Sigma-Aldrich, St. Louis, MO, USA), and acetone (Merck, Darmstadt, Germany) were used. Sodium chloride was purchased from Merck. Diphenyl ether (DPE, 99.0%, log*P* 3.5), 1-undecanol (1-UN, 99.0%, log*P* 3.9), and 1-dodecanol (1-DO, 98.0%, log*P* 4.4) were obtained from Sigma-Aldrich (Steinheim, Germany). Chloroform (CF) (log*P* 1.8), toluene (TLN) (log*P* 2.5), and carbon tetrachloride (CTC) (log*P* 3.0) were purchased from Merck. Sodium tetraborate ($Na_2B_4O_7{\cdot}10\ H_2O$) was obtained from Sigma-Aldrich (Munich, Germany). Sodium dodecyl sulfate (SDS) was purchased from J. T. Baker (Deventer, Holland). NaOH and HCl were obtained from Merck. All reagents were at least of analytical grade unless otherwise indicated. Deionized water (18.2 MΩ cm) treated with Millipore (Simplicity, 185 water purification system) Milli-Q water purification apparatus was used for all aqueous solutions.

2.2 CE apparatus and conditions

The experiments were carried out on an HP^{3D} CE (Agilent Technologies, Waldbronn, Germany) equipped with an online UV diode-array detector (DAD) operated at a wavelength of 194 nm. Optimum wavelength for the target analyte was determined using 'Isoabsorbance' and '3-D' plots in the instrument's 'Data Analysis' software (Agilent Technologies). Conventional capillary zone electrophoresis (CZE) and FASI were performed using uncoated fused-silica capillaries (Postnova Analytics, Landsberg, Germany) of 75 μm i.d. and 64.5 cm length with effective length to the detector of 56 cm. Pressure injection in CZE and electrokinetic injection in FASI were employed. Injection was done at the anodic while detection was performed at the cathodic end of the capillary.

New capillaries were successively flushed with deionized water (10 min), 1.0 M NaOH (20 min), deionized water (20 min), and finally with the background electrolyte (BGE)

for 20 min. To assure reproducibility, the capillary was successively flushed with deionized water (1 min), 1.0 M NaOH (1 min), deionized water (2 min), and the BGE (2 min) at the end of each run.

In conventional CZE, the capillary was conditioned with a BGE (25 mM borate buffer containing 1.0 mM SDS, pH 9.3); the sample, prepared in this BGE, was injected for 5 s at 50 mbar and a positive voltage of 20 kV was applied. The analytes migrated in a homogeneous conductivity medium and were separated by the CZE mode.

In FASI, the capillary was conditioned with 25 mM borate buffer containing 1.0 mM SDS at pH 9.3; the sample present in a low-conductivity medium was electrokinetically injected for 20 s at 15 kV into a water plug previously injected for 5 s at 50 mbar; the analytes stacked at the boundary between the low-conductivity water plug and the high-conductivity BGE. The following separation occurred at 15 kV by the CZE mode.

2.3 Standard solutions and real samples

Individual stock solutions of Eph, Nor, and Pse at concentrations of 1000 mg/L were prepared in methanol and stored at 4°C. Fresh mixed standard solutions were daily prepared from these stock solutions by proper dilution with deionized water. All solutions and samples were degassed using a sonicator (Sonorex Bondelin Electronic, Walldorf, Germany) and filtered through 0.20 μm filters (Econofilters, Agilent Technologies, Darmstadt, Germany) before use.

Urine samples, obtained from a healthy male volunteer (33 years old), were spiked with prescribed concentrations of the analytes and frozen at –15°C. Samples were allowed to thaw at room temperature. The supernatant transparent solution was filtered through 0.20 μm filters, transferred into a glass test tube, and pH of this solution was adjusted to 11.2 using 0.1 mol/L NaOH solution and the extraction procedures described later were applied.

2.4 DLLME procedure

Four milliliters of the supernatant urine solution was mixed with acetonitrile at 2:1 (v/v) ratio and the ionic strength was increased by adding 1.0 g of NaCl in order to promote a salt-induced phase separation (salting-out extraction) between acetonitrile and the aqueous phase after the solution was vortex mixed for 1 min and centrifuged for 1 min at 4000 rpm. It is worthy to note that acetonitrile here served as the disperser solvent in the DLLME procedure. One milliliter of this acetonitrile was transferred into a glass test tube and mixed with 70 μL CF (as the extraction solvent). This mixture was rapidly injected into 4.0 mL of an aqueous solution, placed into a conical-bottomed glass test tube, using a micropipette. The tube was sealed and vortex mixed for 1 min; a cloudy suspension (consisting of water, acetonitrile, and CF) resulting from the dispersion of fine droplets of the extraction solvent in the aqueous solution formed. After centrifugation for 5 min at 5000 rpm, CF sedimented at the bottom of the test tube; the drop was carefully separated using a micropipette and was placed into a CE glass vial. Finally, 70 μL of an aqueous solution (at pH 3.2) containing 10% (v/v) methanol was added to the vial and the mixture was vortex-mixed for 20 s; a stable microemulsion formed and was directly injected into CE.

2.5 USAEME procedure

Two milliliters of the supernatant urine, obtained in Section 2.3, was transferred into a conical-bottomed glass test tube and 200 μL of DPE was delivered to this solution using a micropipette. The tube was sealed and agitated using ultrasonic bath for 25 min at a temperature of 40°C; the resulting cloudy solution was centrifuged for 5 min at a speed of 5000 rpm; the sedimented organic drop was solidified after 5 min at –15°C; the solidified drop was washed twice with cold water and placed into a CE glass vial; finally, 100 μL of an aqueous solution containing 10% methanol (pH 3.2) was added and the mixture was vortex-mixed for 20 s; a stable microemulsion formed and was directly injected into CE.

3 Results and discussion

3.1 Optimization of separation and FASI conditions

Influential separation parameters were systematically studied using a one-factor-at-a-time approach for finding out the optimum separation conditions for the three ephedrines. The evaluation was based on peak area, peak height, migration time, and resolution. The effect of borate buffer concentration in the BGE was evaluated at different concentration levels (15–60 mmol/L). Resolution was greatly increased by increasing buffer concentration from 15 to 50 mmol/L above which no noticeable improvement was obtained. Further increase in concentration increased the current and thus was avoided. Moreover, adding SDS (as an anionic surfactant) at different concentrations (0.5–5.0 mmol/L) to the BGE was also evaluated. Better resolution was obtained by adding 1.0 mmol/L SDS to the BGE (results not shown). Consequently, a borate buffer of 50 mmol/L containing 1.0 mmol/L SDS was selected, as this BGE provided the best separation and highest sensitivity for all analytes up to that point. In order to achieve a good separation among the target analytes in a short analysis time and to avoid Joule heating, a compromise separation voltage of 15 kV was chosen. Capillary temperature, studied in the range 25–32°C, had no noticeable effect on separation. A temperature of 30°C was chosen since this temperature was well above the freezing point of all tested extraction solvents.

FASI was performed by injecting the sample present in a low-conductivity matrix (microemulsion formed after the microextraction procedures) for a longer time (20 s) as

Table 1. Analytical performance of the DLLME-SFO-FASI-CE and DLLME-SFO-FASI-CE methods as compared to conventional CZE

Analyte	LOD				EF		
	Conventional CZE (mg/L)	FASI (mg/L)	DLLME-SFO-FASI (μg/L)	USAEME-FASI (μg/L)	FASI	DLLME-SFO-FASI	USAEME-FASI
Eph	2.1	0.10	15	1.2	21	140	1750
Nor	2.3	0.07	33	2.8	33	70	821
Pse	1.5	0.05	12	1.0	30	125	1500

compared to the conventional injection mode in CZE (50 mbar, 5 s) into a water plug previously injected into the capillary for 5 s at 50 mbar. Sample solutions were introduced at 15 kV for different intervals of time (15–30 s). A length corresponding to 20 s injection was chosen, since it generated the highest stacking efficiency without compromising resolution. Under optimum separation and stacking conditions, preconcentration factors of 21, 33, and 30 for Eph, Nor, and Pse, respectively, were obtained with FASI as compared to conventional CZE (Table 1).

3.2 Optimization of DLLME conditions

3.2.1 pH of sample solution

pH of the sample solution plays an important role in liquid–liquid extraction since extraction efficiency is greatly affected by the charge on the analytes. For basic drugs such as Eph, Nor, and Pse, aqueous sample solutions were commonly strongly alkalized to keep the analytes in their neutral form and consequently reduce their solubility in the urine matrix [5, 26]. The influence of sample pH on extraction efficiency was studied over the range 7.0–13.0. It was observed that increasing the pH increased extraction efficiency for the three analytes up to pH 11.2 after which extraction efficiency remained constant. This trend was consistent with pK_a values of the analytes. Therefore, pH 11.2 was set optimum for further experiments.

3.2.2 Type and volume of the extraction solvent

CTC, CF, TLN, 1-UN, 1-DO, and DPE were tested as the extraction solvents. The effect of extraction solvent on extraction efficiencies for the target analytes with the use of acetonitrile as the disperser solvent is shown in Fig. 1a. As can be seen, CF was found to give the highest extraction efficiency for all the ephedrines studied. Hence this solvent was selected as the extraction solvent for subsequent experiments.

By keeping other experimental conditions constant and 1.0 mL of acetonitrile containing different volumes of CF (50, 70, 100, and 150 μL) as the disperser and extraction solvent, respectively, the effect of extraction solvent volume on the extraction efficiency of the three ephedrines was studied. It was clear that the highest extraction efficiency was obtained

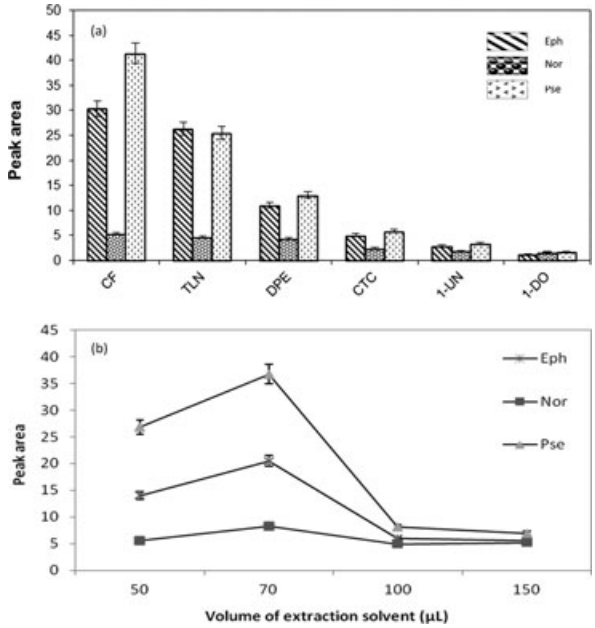

Figure 1. Effect of extraction solvent type (a) and volume (b) on DLLME. Conditions: sample volume: 4.0 mL; disperser solvent (acetonitrile) volume: 1.0 mL; extraction time: 20 s; extraction solvent volume in (a): 70 μL; extraction at room temperature and without salt addition; concentration of each ephedrine: 0.50 mg/L.

with 70 μL CF (Fig. 1b). Hence, this volume was selected as the optimized volume of the extraction solvent.

3.2.3 Type and volume of disperser solvent

Miscibility of the disperser solvent with the extraction solvent and sample solution was the most important criteria when selecting the disperser solvent in DLLME. Thereby, acetone, ethanol, acetonitrile, and methanol, which have this property, were suitable as disperser solvents. A series of sample solutions was extracted using 1.0 mL of each disperser solvent containing 70 μL CF (Fig. 2). Acetonitrile was found to give the highest extraction efficiency. Through investigations of the effect of disperser solvent volume on extraction efficiency, different volumes of acetonitrile in the range 0.5–2.0 mL were used. It was observed that increasing the volume from 0.5 to 1.0 mL resulted in a gradual increase in extraction efficiency, but beyond this point the extraction efficiency decreased steadily. This was probably due to the increase

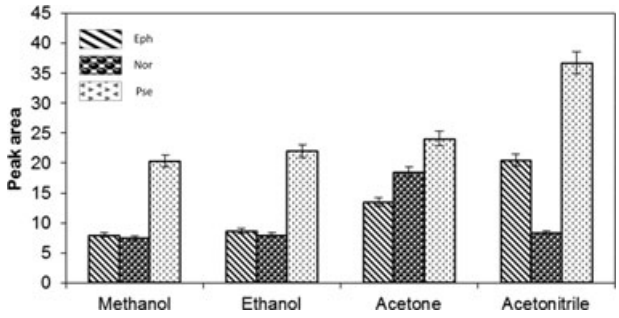

Figure 2. Effect of disperser solvent type on DLLME. Conditions—sample volume: 4.0 mL; disperser solvent volume: 1.0 mL; extraction time: 20 s; extraction solvent (chloroform) volume: 70 μL; extraction at room temperature and without salt addition; concentration of each ephedrine: 0.50 mg/L.

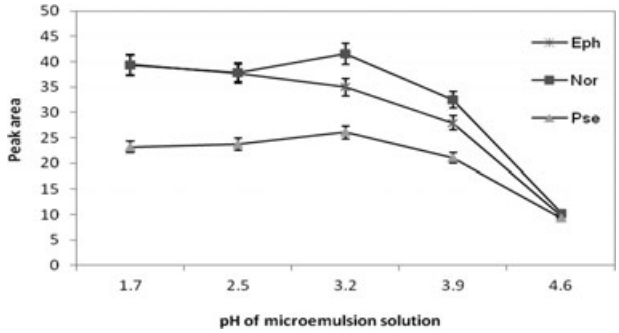

Figure 3. Effect of microemulsion solution pH on DLLME. Conditions, sample volume: 4.0 mL; disperser solvent (acetonitrile) volume: 1.0 mL; extraction time: 20 s; extraction solvent (chloroform) volume: 70 μL; extraction at room temperature and without salt addition; concentration of each ephedrine: 0.50 mg/L.

of solubility of the extraction solvent in the aqueous solution with the increase of the volume of acetonitrile. The optimized sensitivity was achieved when 1.0 mL acetonitrile was used.

3.2.4 pH and volume of microemulsion solution

Considering the compatibility of the final extract in LPME and the CE separation system, most work has been focused on three-phase (water-to-organic-to-water) [27,28], rather than two-phase (water-to-organic) LPME since, in the latter, the extract is organic and is therefore not suitable for direct CE analysis. Basheer et al. [17] have recently reported a strategy to couple LPME with nonaqueous capillary electrophoresis (NACE). However, since aqueous CE is much more widely applied, it would be useful to have a more direct approach to combine LPME and CE. The addition of an acidic aqueous solution to the organic extract not only protonated the analytes but also formed a microemulsion that was compatible with the separation system and therefore could be directly injected into the capillary without causing any current drop with pressure or electrokinetic injection. pH of the microemulsion solution was studied over the range 1.7–4.6. As can be seen from Fig. 3, increasing the pH from 1.7 to 3.2 resulted in increased extraction efficiency of Nor and Pse but slightly decreased extraction efficiency of Eph. Therefore, a compromised value of 3.2 was used for pH of the microemulsion solution. Moreover, it was observed that the addition of an optimized volume of 10% (v/v) of methanol to the emulsification solution followed by vortex mixing for 10 s dispersed the organic extraction solution into fine droplets and increased the stability of the microemulsion solution for more than 24 h.

In this two-phase LPME system, higher enrichment factors (EFs) can be achieved by increasing the volume ratio of the aqueous sample to the microemulsion solution. Therefore, it was necessary to optimize the volume of the microemulsion solution without compromising the overall extraction efficiency of the method. Volume of the microemulsion solution was increased from 10 to 100 μL at a constant volume of the extraction solvent (CF) of 70 μL. The results showed that the largest analytical response was obtained at a volume of 70 μL. Therefore, a volume of 70 μL of an aqueous solution (at pH 3.2) containing 10% (v/v) methanol was set optimal for further experiments.

3.2.5 Extraction time

The effect of extraction time (vortex mixing) on the extraction efficiency was examined in the range of 0–5 min under constant experimental conditions. The results obtained showed that the extraction time did not have any significant influence on the signal of the studied ephedrines. This was because in DLLME after formation of the cloudy solution, the surface area between the extraction solvent and the aqueous sample is infinitely large. Thereby, transition of the analyte from the aqueous sample into the extraction solvent is considerably fast. In fact, independence of extraction efficiency on time is the great advantage of DLLME. Based on the abovementioned, a vortex mixing time of 20 s was chosen as the extraction time.

3.3 Optimization of USAEME conditions

3.3.1 Type and volume of the extraction solvent

CF, CTC, TLN, 1-UN, 1-DO, and DPE were tested as the extraction solvents in USAEME. The highest extraction efficiency was obtained by using DPE as the extraction solvent. Therefore, DPE was selected for further experiments. It is worthy to note that although CF and TLN gave higher extraction efficiencies than DPE in DLLME (Fig. 1a), these two solvents resulted into turbid extracts at the end of the sonication period and less extraction efficiency, which were thought to be due to the cosedimentation of a white lipidic solid at the bottom of the conical test tube, probably consisting of carbamide and uric acid present in urine matrices. The effect of DPE volume on extraction efficiency was studied in the range 100–250 μL (Fig. 4). It was found that a volume of 200 μL of DPE resulted in the maximum extraction efficiency with

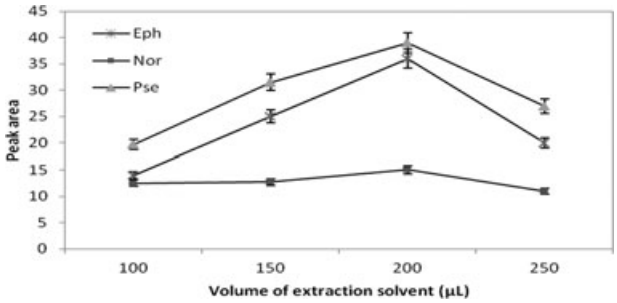

Figure 4. Effect of extraction solvent (DPE) volume on USAEME. Conditions, sample volume: 2.0 mL; extraction time: 25 min; extraction at 40°C and without salt addition; concentration of each ephedrine: 30.0 μg/L.

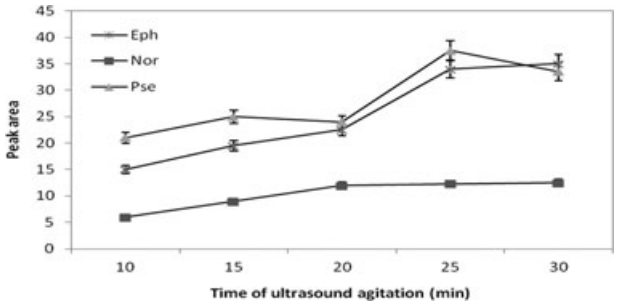

Figure 5. Effect of time of ultrasound agitation on USAEME. Conditions, sample volume: 2.0 mL; extraction at 40°C and without salt addition; concentration of each ephedrine: 30.0 μg/L.

other parameters kept constant. Therefore, this volume of DPE was used for further investigations.

3.3.2 Time of ultrasound agitation

Time of ultrasound agitation is one of the main factors in USAEME. The use of ultrasound accelerated the formation of a fine cloudy solution, which markedly increased extraction efficiency as shown in Fig. 5 and reduced equilibrium time. The effect of the ultrasound extraction time was studied over the time range 10–30 min. The results (Fig. 5) indicated that the extraction efficiencies increased by increasing the extraction time till equilibrium was achieved after 25 min. Therefore, a period of 25 min for ultrasound agitation was chosen for further experiments.

3.3.3 Salt addition

The effect of increasing the ionic strength of the sample solution on the extraction efficiency of Eph, Nor, and Pse in USAEME was evaluated by the addition of NaCl (0–2.5 mol/L) into the sample solution. It was observed that extraction efficiency decreased with increasing salt content. Similar results of salt addition were obtained in DLLME. Hence, further extractions were performed without salt addition.

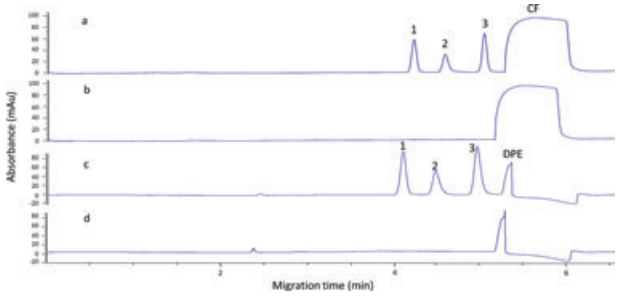

Figure 6. Electropherograms of blank and spiked urine samples obtained under optimum extraction and stacking conditions. (a) Urine sample spiked with the three ephedrines at 0.50 mg/L and extracted using DLLME; (b) blank urine sample extracted using DLLME; (c) urine sample spiked with the three ephedrines at 30.0 μg/L and extracted using USAEME; (d) blank urine sample extracted using USAEME. Peaks: 1, Eph; 2, Nor; 3, Pse.

3.4 Analytical performance of DLLME-CE-FASI and USAEME-CE-FASI

Performance of the DLLME-CE-FASI and USAEME-CE-FASI methods were evaluated in terms of precision, linearity, selectivity, limits of detection (LOD), and EFs as compared to conventional CZE. LODs and EFs, as compared to conventional CZE, with the two methods are summarized in Table 1.

Repeatability, expressed as relative standard deviations (RSDs) for five replicate analyses, ranged from 3.4 to 6.1% in DLLME-CE-FASI; while values ranged from 7.3 to 8.2% in USAEME-CE-FASI. Overall, repeatability of DLLME-CE-FASI was found to be better than that of USAEME-CE-FASI.

Linearity between peak areas and concentrations was investigated for both methods based on coefficients of determination (R^2). The results indicated that excellent linear relationships were attainable over wide concentration ranges with $R^2 \geq 0.9973$ in DLLME-CE-FASI and $R^2 \geq 0.9984$ in USAEME-CE-FASI.

Selectivity of the proposed methods was investigated by applying both to blank and spiked urine samples. Selectivity resided mainly in the use of CF in DLLME and DPE in USAEME as the extraction solvents which eliminated other hydrophilic constituents (e.g. salts, carbamide, and uric acid) followed by cation-selective FASI, which enhanced the introduction of the cationic analytes into the capillary. Figure 6 depicts typical electropherograms of blank and spiked urine samples obtained after DLLME and USAEME.

LOD, calculated for an S/N ratio of 3 (N: noise of the baseline calculated as the average of 11 noise peaks chosen at different places of the baseline void of analytical peaks), were 15, 33, and 12 μg/L with DLLME-CE-FASI and 1.2, 2.8, and 1.0 μg/L with USAEME-CE-FASI for Eph, Nor, and Pse, respectively (Table 1). From the above data, it was clear that the USAEME-CE-FASI is more sensitive than DLLME-CE-FASI, reflecting that applying USAEME as the extraction method enables higher enrichment of these analytes.

Table 2. Recovery of ephedrines in urine samples by DLLME-CE-FASI and USAEME-CE-FASI

Analyte	DLLME-CE-FASI			USAEME-CE-FASI		
	C_{added} (μg/L)	C_{found}[a] (μg/L)	RR (%)[b]	C_{added} (μg/L)	C_{found} (μg/L)	RR (%)
Eph	–	n.d.[c]	–	–	n.d.	–
	200	204 ± 11	102	15.0	14.7 ± 1.2	98.1
	1000	994 ± 30	99.4	100.0	103.3 ± 6.1	103.3
Nor	–	n.d.	–	–	n.d.	–
	400	382 ± 17	95.5	50.0	49.8 ± 2.4	99.6
	1000	945 ± 30	94.5	100.0	98.5 ± 3.6	98.5
Pse	–	n.d.	–	–	n.d.	–
	200	196 ± 7.2	98.0	15.0	14.8 ± 1.0	98.7
	1000	973 ± 27	97.3	100.0	99.3 ± 4.8	99.3

a) C_{found} (± standard deviation): average value of five replicate extractions.
b) RR: relative recovery, percentage value obtained considering extraction yields from matrix-matched calibration.
c) Not detected.

EFs were 140, 70, and 125 with DLLME-CE-FASI, and 1750, 821, and 1500 with USAEME-CE-FASI for Eph, Pse, and Nor, respectively (Table 1). Thus, EFs for the three target analytes obtained by USAEME-CE-FASI were higher than those obtained by DLLME-CE-FASI by approximately 12 times.

3.5 Analysis of urine samples by DLLME-CE-FASI and USAEME-CE-FASI

To evaluate their potentiality in real sample analysis and to study possible matrix effect on the analytes, the proposed methods were applied for the analysis of urine samples of a drug-free healthy male. Preliminary experiments showed that urine samples were free of the target analytes. Samples were therefore spiked with the analytes at different concentrations ranging from 200 to 1000 μg/L for DLLME-CE-FASI and 15.0 to 100.0 μg/L for USAEME-CE-FASI. Relative recoveries of the analytes (Table 2) were in the range of 94.5–102% and 98.1–103.3% with both methods, respectively.

3.6 Comparison with other preconcentration methods

A comparison of the proposed DLLME-CE-FASI and USAEME-CE-FASI methods in terms of LOD, linearity, and precision with different existing methods for extracting and determining Eph, Nor, and Pse in urine by CE is provided in Table 3. It can be seen that DLLME-CE-FASI gave higher LODs than the other methods which was thought to be due to the use of a disperser solvent which increased solubility of the analytes in the aqueous phase and led to lower extraction efficiency. The use of ultrasound to disperse the extraction solvent into the mixture is therefore a better alternative to organic disperser solvents for achieving higher EFs. Although extraction time in DLLME (20 s) was much shorter than it was in USAEME (25 min), the latter demonstrated higher sensitivities due to reduced solubility of the target analytes in the urine matrix in the absence of a disperser solvent. In comparison with other existing extraction methods used in combination with CE, the proposed two methods have some advantages including low consumption of organic solvents and reagents, simplicity of operation, and production of clear extracts for the analysis. In addition, since no specific holder is

Table 3. Comparison of the proposed methods with other reported methods for extraction of ephedrines in urine by CE

Method/analytical technique	Analyte	LOD (μg/L)	Linearity		RSD%	Ref.
			LDR (μg/L)	R^2		
SPME/CE-DAD[a]	Eph	3.00	20–5000	0.992	7.57	[29]
	Pse	5.00	20–5000	0.994	4.96	
PMME/CE-UV[b]	Eph	8.00	50–5000	0.998	4.0	[30]
	Pse	8.40	50–5000	0.998	3.8	
CME/OLBE-FASI[c]	Eph	0.15	5–200	0.9988	6.4	[15]
	Pse	0.20	5–200	0.9994	5.3	
PC-HFME-CE-NSM[d]	Nor	6	25–5000	0.995	6	[31]
DLLME-CE-FASI	Eph	15	50–1000	0.9992	4.2	This study
	Nor	33	110–1000	0.9998	3.4	
	Pse	12	40–1000	0.9973	6.1	
USAEME-CE-FASI	Eph	1.2	4.0–600	0.9984	7.3	This study
	Nor	2.8	9.0–600	0.9987	4.8	
	Pse	1.0	3.5–600	0.9985	8.2	

a) Solid–phase microextraction-capillary electrophoresis-photo diode array.
b) Poly monolith microextraction.
c) Centrifuge microextraction and on-line back-extraction field-amplified sample injection.
d) Polymer-coated hollow fiber microextraction-capillary electrophoresis-normal stacking mode.

required for supporting the organic extraction solvent like in hollow-fiber microextraction (HFME), DLLME and USAEME are both more robust and avoid the adsorption of hydrophobic substances present in urine matrices.

4 Conclusion

The main aims of this work were to couple DLLME and USAEME with FASI-CE and to compare their analytical performance for the extraction of Eph, Nor, and Pse as relatively polar drugs from human urine samples, and to present a novel approach for the direct injection of the resulting organic extracts into aqueous CE via the formation of a microemulsion with an aqueous solution at the desired pH containing 10% (v/v) methanol. The proposed methods were successfully developed for the extraction and analysis of the three ephedrines. Factors affecting separation, stacking efficiency of FASI, as well as microextraction efficiency in both methods were systematically investigated and optimized. Under optimum stacking and extraction conditions, LODs at the low microgram per liter (μg/L) level were obtained due to the high improvement factors achieved. DLLME-CE-FASI and USAEME-CE-FASI provided high sensitivities, with LODs lower than those obtained by CZE by 70–140 and 821–1750 times, respectively. The proposed methods demonstrated several advantages over other existing extraction methods such as robustness, high sample clean-up, low cost, and ease of applicability. Compared to DLLME-CE-FASI, USAEME-CE-FASI provided higher EFs (almost 12 times) within acceptable extraction times from urine samples. Since aqueous CE is much more widely used than NACE, introduction of the sample into the separation system as a microemulsion represents a potentially useful direct approach to combine DLLME and USAEME with aqueous CE.

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5 References

[1] Deventer, K., Pozo, O. J., Van Eenoo, P., Delbeke, F. T., *J. Chromatogr. B* 2009, *877*, 369–374.

[2] Konig, W. A., Ernst, K., *J. Chromatogr.* 1983, *280*, 135–141.

[3] Vandermerwe, P. J., Brown, L. W., Hendrikz, S. E., *J. Chromatogr. B* 1994, *661*, 357–361.

[4] Sorensen, L. K., *J. Chromatogr. B* 2011, *879*, 727–736.

[5] Zhang, Z. H., Zhang, C. G., Su, X. L., Ma, M., Chen, B., Yao, S., *Anal. Chim. Acta* 2008, *621*, 185–192.

[6] World Anti-Doping Agency, 2012 Prohibited List. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2012/WADA_Prohibited_List_2012_EN.pdf

[7] Van Eenoo, P., Delbeke, F. T., Roels, K., De Backer, P., *J. Chromatogr. B* 2001, *760*, 255–261.

[8] Spyridaki, M. H. E., Tsitsimpikou, C. J., Siskos, P. A., Georgakopoulos, C. G., *J. Chromatogr. B* 2001, *758*, 311–314.

[9] Imaz, C., Navajas, R., Carreras, D., Rodriguez, C., Rodriguez, A. F., *J. Chromatogr. A* 2000, *870*, 23–28.

[10] Fuh, M. R. S., Lu, K. T., *Talanta* 1999, *48*, 415–423.

[11] Sun, B. G., Macka, M., Haddad, P. R., *Electrophoresis* 2003, *24*, 2045–2053.

[12] Casiot, C., Alonso, M. C. B., Boisson, J., Donard, O. F. X., Potin-Gautier, M., *Analyst* 1998, *123*, 2887–2893.

[13] Zhu, H. D., Ren, C. L., Hu, S. Q., Zhou, X. M., Chen, H. L., Chen, X. G., *J. Chromatogr. A* 2011, *1218*, 733–738.

[14] Li, S. G., Li, H. Y., Cai, Z., Cheng, H., Li, L. J., *Chinese J. Anal. Chem.* 2009, *37*, 1137–1141.

[15] Fang, H. F., Zeng, Z. R., Liu, L., *Anal. Chem.* 2006, *78*, 6043–6049.

[16] Gao, W. H., Chen, G. P., Chen, T. F., Zhang, X. S., Chen, Y. W., Hu, Z. D., *Talanta* 2011, *83*, 1673–1679.

[17] Xu, L., Basheer, C., Lee, H. K., *J. Chromatogr. A* 2010, *1217*, 6036–6043.

[18] Li, P. J., Hu, B., *J. Chromatogr. A* 2011, *1218*, 4779–4787.

[19] Wen, Y. Y., Li, J. H., Zhang, W. W., Chen, L. X., *Electrophoresis* 2011, *32*, 2131–2138.

[20] Rezaee, M., Assadi, Y., Hosseinia, M. R. M., Aghaee, E., Ahmadi, F., Berijani, S., *J. Chromatogr. A* 2006, *1116*, 1–9.

[21] Birjandi, A. P., Bidari, A., Rezaei, F., Hosseini, M. R. M., Assadi, Y., *J. Chromatogr. A* 2008, *1193*, 19–25.

[22] Leong, M. I., Huang, S. D., *J. Chromatogr. A* 2008, *1211*, 8–12.

[23] Saraji, M., Bidgoli, A. A. H., *Anal. Bioanal. Chem.* 2010, *397*, 3107–3115.

[24] Regueiro, J., Llompart, M., Garcia-Jares, C., Garcia-Monteagudo, J. C., Cela, R., *J. Chromatogr. A* 2008, *1190*, 27–38.

[25] Hou, J. G., Zheng, J., Rizvi, S. A. A., Shamsi, S. A., *Electrophoresis* 2007, *28*, 1352–1363.

[26] Bagheri, H., Khallillian, F., Ahangar, L. E., *J. Sep. Sci.* 2008, *31*, 3212–3217.

[27] Zhu, Z. F., Zhou, X. M., Yan, N., Zhou, L., Chen, X. G., *J. Chromatogr. A* 2010, *1217*, 1856–1861.

[28] Liang, G. D., Choi, K., Chung, D. S., *Electrophoresis* 2009, *30*, 1953–1957.

[29] Fang, H. F., Liu, M. M., Zeng, Z. R., *Talanta* 2006, *68*, 979–986.

[30] Wei, F., Zhang, M., Feng, Y. Q., *J. Chromatogr. B* 2007, *850*, 38–44.

[31] Basheer, C., Wang, H. J., Jayaraman, A., Valiyaveettil, S., Lee, H. K., *J. Chromatogr. A* 2006, *1128*, 267–272.

