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Research Article

Dispersive liquid–liquid microextraction based on solidification of floating organic drop combined with field-amplified sample injection in capillary electrophoresis for the determination of beta(2)-agonists in bovine urine

Dispersive liquid–liquid microextraction based on solidification of floating organic drop (DLLME–SFO) was for the first time combined with field-amplified sample injection (FASI) in CE to determine four β_2-agonists (cimbuterol, clenbuterol, mabuterol, and mapenterol) in bovine urine. Optimum BGE consisted of 20 mM borate buffer and 0.1 mM SDS. Using salting-out extraction, β_2-agonists were extracted into ACN that was then used as the disperser solvent in DLLME–SFO. Optimum DLLME–SFO conditions were: 1.0 mL ACN, 50 μL 1-undecanol (extraction solvent), total extraction time 1.5 min, no salt addition. Back extraction into an aqueous solution (pH 2.0) facilitated direct injection of β_2-agonists into CE. Compared to conventional CZE, DLLME–SFO–FASI–CE achieved sensitivity enhancement factors of 41–1046 resulting in LODs in the range of 1.80–37.0 μg L^{-1}. Linear dynamic ranges of 0.15–10.0 mg L^{-1} for cimbuterol and 15–1000 μg L^{-1} for the other analytes were obtained with coefficients of determination (R^2) $\geq$ 0.9901 and RSD% $\leq$5.5 ($n = 5$). Finally, the applicability of the proposed method was successfully confirmed by determination of the four β_2-agonists in spiked bovine urine samples and accuracy higher than 96.0% was obtained.

Keywords:
Beta(2)-agonists / Bovine urine / Capillary electrophoresis / Dispersive liquid–liquid microextraction / Solidification of floating organic drop
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1 Introduction

The use of growth promoters in the production of animal products for human consumption has been an issue of scientific debate and public concern for many years [1]. Since the European ban on anabolic steroids was established in 1986, alternative substances such as β_2-agonists, which include cimbuterol (CIBT), clenbuterol (CBT), mabuterol (MBT), and mapenterol (MPT) (target analytes), have been introduced as growth-promoting agents [2]. These substances, in addition to their regular role in veterinary medicine, can reduce carcass fat, increase muscle mass, and improve growth rate, and feed conversion when fed to animals [3]. Nevertheless, the drug residues accumulated in animal tissues may pose a potential risk for acute human toxicity. Such toxic symptoms include: muscular tremors, vomiting, nervousness, and cardiac palpitations [4]. Illegal use of β_2-agonists as growth promoters for livestock is prohibited in the European Union and China [5].

Because of the presence of these compounds at trace levels and the complexity of the biological matrices analyzed, there is a continuous need for the development of rapid and easy sample pretreatment methods, followed by sensitive determination of several β_2-agonists within the same run [6]. Thus far, GC [7, 8] and LC [9, 10] have been the main techniques used for the determination of β_2-agonists in different matrices.

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Abbreviations: 1-UN, 1-undecanol; **BES**, back extraction solution; **CBT**, clenbuterol; **CIBT**, cimbuterol; **DLLME**, dispersive liquid–liquid microextraction; **DLLME–SFO**, DLLME based on solidification of floating organic drop; **DPE**, diphenyl ether; **EF**, enrichment factor; **FASI**, field-amplified sample injection; **HF–LPME**, hollow fiber-based LPME; **LDR**, linear dynamic range; **LPME**, liquid–phase microextraction; **MBT**, mabuterol; **m.p.**, melting point; **MPT**, mapenterol; **PMME**, polymer monolith microextraction; **SEF**, sensitivity enhancement factor; **SOE**, salting-out extraction; **SPME**, solid–phase microextraction

CE is a powerful complementary technique to LC and GC and has rapidly spread into a wide array of analytical areas as it is considered a green analytical technique, due to its low consumption of samples and reagents, extremely high separation efficiency, short analysis time, high versatility in terms of multiple separation modes, and excellent biocompatibility [11]. CE with UV detection [12], CE-LIF [13], and CE-MS [14] have been applied for the determination of some β_2-agonists and LODs of 2.0, 0.7, and 0.25 μg L^{-1}, respectively, were achieved. However, LIF detection required a laborious and time-consuming derivatization step. Mass spectrometer is expensive and is therefore not available in many laboratories. The most widely used detector for CE is UV but it suffers from low concentration sensitivity. It is therefore difficult to determine analytes in real samples directly at trace levels without a sample preconcentration step. To overcome this sensitivity problem, several on-line preconcentration strategies such as stacking [15] and sweeping [16] and/or offline ones have been developed. One of the simplest and most commonly used sample stacking techniques is field-amplified sample injection (FASI), in which ions enter the capillary by their own electrophoretic mobility as well as the EOF. More ions will be injected if their electrophoretic mobility is in the same direction as the EOF [17]. FASI has been applied to preconcentrate a variety of analytes including β_2-agonists [12, 18].

Since nanoliter volume is injected into CE, different modes of liquid–phase microextraction (LPME) such as single-drop microextraction [19], hollow fiber-based LPME (HF–LPME) [20], solvent-bar microextraction [21], and dispersive liquid–liquid microextraction (DLLME) [22] have been shown to be suitable sample pretreatment techniques for CE.

DLLME based on solidification of floating organic drop (DLLME–SFO) is a very simple, environmentally friendly and fast extraction technique introduced in 2008 by Huang et al. for the extraction and preconcentration of some halogenated organic compounds from water samples [23]. This technique was based on the rapid introduction of a suitable combination of a low-density extraction solvent and a disperser solvent into an aqueous sample solution resulting in the formation of a cloudy solution that was then centrifuged. In this system, extraction equilibrium was achieved very quickly due to the high surface contact between the sample and the extraction solvent droplets. After centrifugation, the floating organic drop was collected by solidifying it at low temperature, melted, and introduced into GC with electron-capture detection and GC–MS for analysis. Since then, DLLME–SFO has been coupled with HPLC [24], flame atomic absorption spectrometry [25], graphite furnace atomic absorption spectrometry [26], electrothermal atomic absorption spectrometry [27], and inductively coupled plasma-optical emission spectrometry [28]. To the best of our knowledge, DLLME–SFO has not yet been coupled with CE probably due to incompatibility of the final extract with BGEs used in CE. Therefore, it would be useful to extend the application scope of DLLME–SFO by coupling it with CE.

This study presents the first combination of DLLME–SFO and CE through the investigation of extraction and determination of four β_2-agonists (as model analytes) in bovine urine. In order to enhance the sensitivity of CE further, FASI was also carried out. β_2-Agonists were extracted into ACN that was then used as the disperser solvent in DLLME–SFO. After optimizing separation and FASI stacking conditions, the most influential parameters on DLLME–SFO such as sample pH, type and volume of the extraction and disperser solvents, ionic strength, and extraction time were investigated. In vial back extraction of the target analytes from the resulting organic drop into an aqueous phase facilitated their direct injection into CE.

2 Materials and methods

2.1 Reagents and solutions

All chemicals used were at least of analytical reagent grade. CIBT (pK_a = 9.40, log$P$ = 0.9), CBT (pK_a = 9.63, log$P$ = 2.3), MPT (pK_a = 9.78, log$P$ = 3.1), MBT (pK_a = 9.63, log$P$ = 2.6), and HPLC-grade ACN were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetone, sodium chloride, magnesium sulfate, calcium chloride, and propan-2-ol were acquired from Merck (Darmstadt, Germany). Diphenyl ether (DPE, 99.0%, log$P$ 3.5), 1-undecanol (1-UN, 99.0%, log$P$ 3.9), and 1-dodecanol (98.0%, log$P$ 4.4) were obtained from Sigma–Aldrich (Steinheim, Germany). SDS was from J. T. Baker (Deventer, Holland). DI water (18.2 MΩ·cm) was obtained using a Milli–Q water purification system (Millipore, Milford, MA, USA). Stock solutions of the target analytes were prepared by dissolving appropriate amounts in methanol to obtain 1000 mg L^{-1} solutions that were stored in the dark at –20°C. Aliquots of these solutions were daily diluted with DI water to prepare standard solutions of varying concentrations. Borate buffer was prepared from $Na_2B_4O_7.10H_2O$ obtained from Sigma–Aldrich (Steinheim, Germany). All BGEs and solutions were prepared in DI water and were stored in the dark at 4°C. When necessary, pH of the solutions was adjusted using 0.1 M NaOH (Merck, Darmstadt, Germany) and 0.1 M HCl (Sigma–Aldrich, Steinheim, Germany). BGE, standard solutions, and urine samples were degassed using a sonicator (Sonorex Bandelin Electronic, Walldorf, Germany) and filtered through 0.20 μm filters (Econofilters, Agilent Technologies, Waldronn, Germany) before use.

2.2 Instrumental

Experiments were carried out on an HP^{3D} CE (Agilent Technologies, Waldbronn, Germany). Conventional CZE and FASI were performed using uncoated fused-silica capillaries (Postnova Analytics, Landsberg, Germany) of 75 μm id and 64.5 cm length with effective length to the detector of 56 cm. Online UV detector (DAD) operated at a wavelength of

205 nm was used to monitor the analytes. Pressure and electrokinetic injection in conventional CZE and FASI, respectively, were employed throughout the experiments. A Thermo Orion, 720A pH meter (Beverly, MA, USA) equipped with a glass electrode was used for measuring the adjusted pH of all aqueous and buffer solutions.

New capillaries were successively flushed with DI water (10 min), 1.0 M NaOH (20 min), DI water (20 min), and finally with the BGE (20 min). To assure a good reproducibility, the capillary was successively flushed with DI water (1 min), 1.0 M NaOH (1 min), DI water (2 min), and the BGE (2 min) at the end of each run.

In conventional CZE, the capillary was conditioned with a BGE (20 mM borate buffer containing 0.1 mM SDS); the sample, prepared in this BGE, was injected for 5 s at 50 mbar and a positive voltage of 20 kV was applied.

In FASI, the capillary was conditioned with the same BGE; the analytes present in a low-conductivity medium were electrokinetically injected for 11 s at 20 kV into a water plug previously injected into the capillary at 50 mbar for 5 s.

2.3 Salting-out extraction procedure

Urine samples from healthy bovines were obtained from a slaughterhouse (Ankara, Turkey). β_2-Agonists-free samples were spiked with prescribed concentrations of the analytes and frozen at –20°C. The samples were allowed to thaw at room temperature prior to analysis. Four milliliters of the supernatant transparent solution were filtered through 0.20 μm filters, transferred into a glass test tube and pH of this solution was adjusted to 10.5 using 0.1 M NaOH solution. Next, the solution was mixed with ACN at 2:1 v/v ratio and the ionic strength was increased by adding 1.0 g of NaCl (0.25%, w/v) in order to promote a salt-induced phase separation between ACN and the aqueous phase after the solution was vortex mixed for 30 s and centrifuged for 1 min at 5000 rpm. One milliliter of the ACN layer was transferred into a glass test tube and the DLLME–SFO procedure was applied. ACN obtained here served as the disperser solvent in the subsequent DLLME–SFO procedure.

2.4 DLLME–SFO procedure

DLLME–SFO was performed as follows: 1.0 mL of the ACN resulting from the salting-out extraction (SOE) procedure described above (Section 2.3) was placed in a glass test tube and mixed with 50 μL 1-UN (as the extraction solvent). This 1-UN/ACN mixture was injected rapidly into 5.0 mL of DI water using a micropipette. The tube was sealed and vortex mixed for 30 s; a cloudy suspension formed in the test tube. After centrifugation for 5 min at 5000 rpm, the tube was placed at –20°C till the floating organic drop was solidified after 5 min. The drop was separated using a small medicine spatula.

2.5 Back extraction

The solidified organic drop was transferred into a glass insert inside a CE vial (Agilent Technologies, Waldbronn, Germany) where it melted rapidly at room temperature and the analytes were back extracted into 15 μL of an aqueous solution at pH 2.0 (back extraction solution, BES) after vortex mixing for 30 s and centrifugation at 4000 rpm for 1 min. Finally, the analytes were directly injected into CE through FASI (inlet at 4 mm offset) without the need to separate the floating organic phase from the vial.

3 Results and discussion

3.1 Optimization of separation and FASI conditions

Optimization of FASI conditions was performed with standard solutions prepared in BES. The evaluation was based on peak area, migration time, and resolution of the electrophoretic peaks. The effect of borate buffer (pH 9.3) at different concentration levels (10–30 mM) was studied. Although it had a little effect on peak areas, increasing buffer concentration within this range improved resolution. Further increase in concentration above 30 mM increased the current above 94.5 μA and was thus avoided. Moreover, adding SDS (as an anionic surfactant) at different concentrations (0–0.20 mM) to the BGE was tested. Resolution improved upon the addition of up to 0.10 mM SDS. Consequently, a borate buffer of 30 mM containing 0.10 mM SDS was selected, as this BGE provided the best separation and highest sensitivity. A separation voltage of 20 kV and a temperature of 32°C were found to give the lowest migration time while peak resolutions were higher than 2.

Although sensitivity increased with increasing electrokinetic injection time (in the range of 5–15 s), resolution between CBT and MBT decreased below 2 (Fig. 1) at injection times higher than 11 s. Hence, this value was chosen for further experiments.

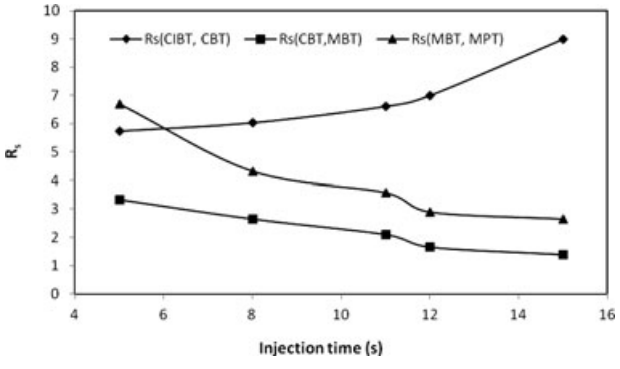

Figure 1. Effect of electrokinetic injection time on peak resolution.

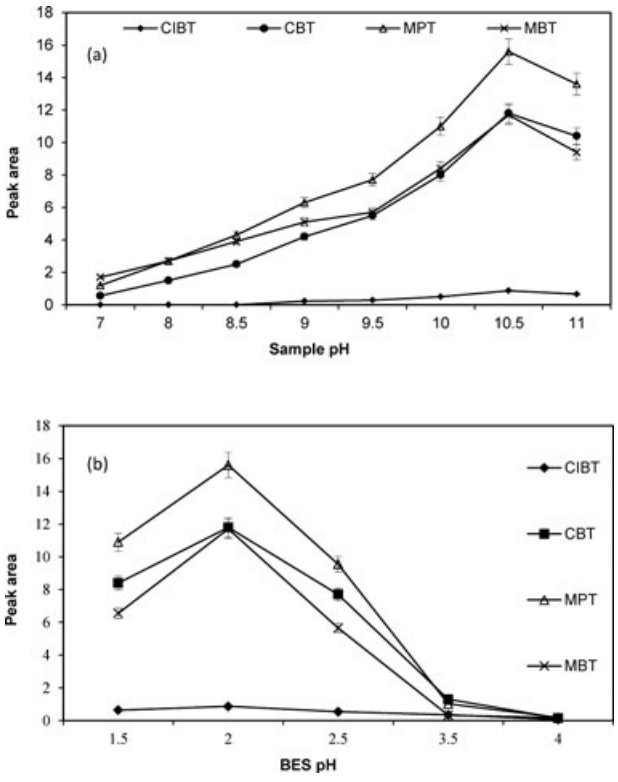

Figure 2. Effect of (A) sample and (B) BES pH on extraction efficiency. Samples spiked to 0.20 mg L^{-1} of the target analytes. Extraction conditions: urine sample volume 4 mL; aqueous sample volume 5 mL; analytes extracted into 50 μL 1-UN in 1.0 mL ACN at room temperature; no salt addition; back extraction: 15 μL BES at (A) pH 2.0 and (B) varying pH values.

3.2 Optimization of SOE conditions

Variables affecting extraction efficiency of SOE include pH of sample solution, type and volume of extraction solvent, type and amount of salt, and extraction time. Influence of sample pH was studied over the range 7.0–11.0 (Fig. 2A). The data indicated that extraction efficiency increased gradually as the pH of the sample solution was increased from 7.0 to 10.5, and then dropped off as the pH exceeded this value. This trend was consistent with pK_a values of the analytes (Section 2.1). Therefore, pH 10.5 was set optimum for further experiments.

As well as ACN, acetone and propan-2-ol were also assayed as the extraction solvents in SOE. ACN gave the highest extraction efficiency. Different volumes of ACN (1.0–6.0 mL) were investigated. The use of 2.0 mL ACN for 4.0 mL urine resulted in the salt-induced phase separation of ca. 1.1 mL ACN, 1.0 mL of which was used for further experiments and gave the highest extraction efficiency among the other volumes investigated (data not shown). The amounts of salts ($MgSO_4$, NaCl, and $CaCl_2$) and the corresponding signals obtained after extraction were compared. The largest peak areas for the four analytes were obtained with 1.0 g of NaCl,

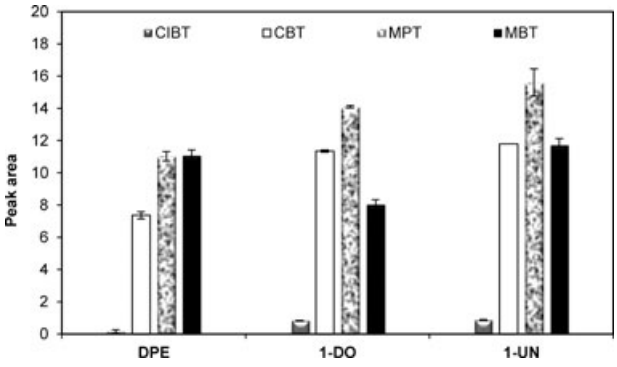

Figure 3. Effect of extraction solvent type on extraction efficiency. Samples spiked to 0.20 mg L^{-1} of the target analytes. Extraction conditions: urine sample volume 4 mL; aqueous sample volume 5 mL; analytes extracted into 50 μL of each extraction solvent in 1.0 mL ACN at room temperature; no salt addition; back extraction: 15 μL BES at pH 2.0.

such that this value was selected. Finally, extraction time of 30 s was chosen since no significant increase in the analytical signals occurred upon increasing the vortex-mixing time.

3.3 Optimization of DLLME–SFO and back extraction conditions

3.3.1 Selection of extraction solvent

Organic solvents that are appropriate for DLLME–SFO are selected according to the following characteristics: to have low volatility and low solubility in water for them to be stable during the extraction process; to have a high extraction efficiency for the analytes; to be separated from the analyte peaks in chromatographic/electrophoretic applications; and to have melting points (m.p.) near room temperature (preferably in the range 10–30°C). Accordingly, the most frequently used solvents of 1-UN (m.p.: 11°C; density: 0.830 g mL^{-1}) and 1-dodecanol (m.p.: 22–26°C; density: 0.833 g mL^{-1}) were investigated. In addition, DPE (m.p.: 25–27°C; density: 1.060 g mL^{-1}, solubility in water: 0.002 g in 100 mL of water at 25°C) was also tested as a new extraction solvent for DLLME–SFO applications. DPE is denser than water and sediments at the bottom of the extraction tube or floats at the surface depending on salt content in the sample solution due to proximity of its density to that of water. 1-UN gave the highest extraction efficiency among the other investigated solvents (Fig. 3).

3.3.2 Extraction solvent and BES volumes

Since the volumes of 1-UN and BES influence each other, their effect on extraction efficiency was studied simultaneously. It can be seen from Fig. 4 that 50 μL 1-UN and

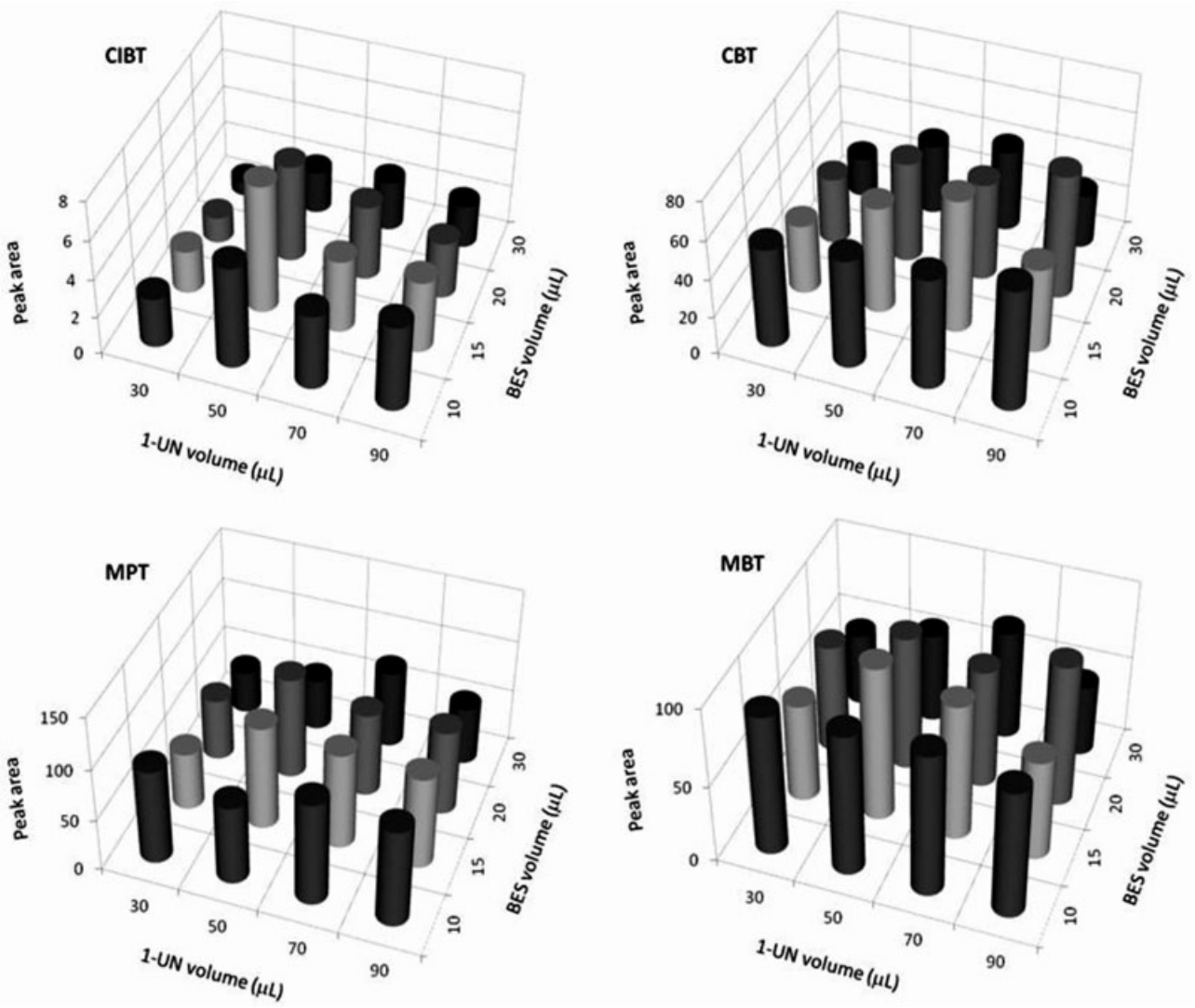

Figure 4. Interactive effect of extraction solvent and BES volumes on extraction efficiency. Samples spiked to 0.90 mg L^{-1} of the target analytes. Extraction conditions: urine sample volume 4 mL; aqueous sample volume 5 mL; analytes extracted into varying volumes of 1-UN (extraction solvent) in 1.0 mL ACN; no salt addition; back extraction: varying volumes of BES at pH 2.0.

15 μL BES were found optimum except for CBT that was found as 70 μL 1-UN and 15 μL BES (peak area was larger by 25%). Therefore, 50 μL 1-UN and 15 μL BES were set as optimum values. It is noteworthy that the collected volume of 1-UN after extraction was 47.6 ± 0.8 μL ($n = 5$).

3.3.3 BES pH

The effect of BES pH on extraction efficiency was investigated in the range of 1.5–4.0 (Fig. 2B). Variations in BES pH had no noticeable effect on the resolution of the studied analytes. The highest extraction efficiency was achieved at pH 2.0.

3.3.4 Extraction times

In DLLME–SFO, extraction time is defined as the time interval between the injection of the mixture of disperser and extraction solvents and the time at which the sample is centrifuged [29], which corresponded to the time of vortex mixing in this study. The effect of extraction time was examined in the range of 0–5 min with the other experimental conditions kept constant. The results showed that extraction time did not have any significant influence on the signals. In DLLME–SFO, formation of a cloudy solution infinitely increases the surface contact between the extraction solvent and the aqueous sample. Thereby, transition of the analytes from the aqueous sample into the extraction solvent was considerably fast. In fact, independence of extraction efficiency on extraction time is one of the great advantages of DLLME–SFO. Based

Table 1. Figures of merit of the proposed method

Analyte	Linearity (DLLME–SFO–FASI–CE)		RSD[a] (%) ($n = 5$)		LOD (μg L^{-1})			SEF
	LDR (μg L^{-1})	R^2	Intraday	Interday	CZE	FASI–CE	DLLME–SFO–FASI–CE	DLLME–SFO–FASI–CE[b]
CIBT	150–10 000	0.9901	3.4	4.8	1510	164.3	37.0	41
CBT	15–1000	0.9964	3.3	5.5	1890	84.5	1.90	996
MPT	15–1000	0.9937	2.5	4.3	1880	158.9	1.80	1046
MBT	15–1000	0.9971	2.9	4.2	1890	309.8	3.50	541

a) Data obtained by spiking urine with CIBT at 1.0 mg L^{-1} and CBT, MPT, MBT at 100 μg L^{-1} (each).
b) Ratio of LOD in conventional CZE to that with DLLME–SFO–FASI–CE.

Table 2. Accuracy of β_2–agonists from spiked bovine urine ($n = 5$)

Analyte	C_{added} (μg L^{-1})	C_{found} ($\pm$SD) (μg L^{-1})	Accuracy[a] (%)	C_{added} (μg L^{-1})	C_{found} ($\pm$SD) (μg L^{-1})	Accuracy[a] (%)
CIBT	—	n.d.[b]	—	1500	1530 $\pm$ 50	102.0
	500	490$\pm$20	98.0	5000	4960 $\pm$ 190	99.2
CBT	—	n.d.	—	150	146 $\pm$ 6.86	97.3
	50	48$\pm$2.3	96.0	500	491 $\pm$ 24.5	98.2
MPT	—	n.d.	—	150	145 $\pm$ 4.35	96.7
	50	49 $\pm$ 2.1	98.0	500	485 $\pm$ 16.9	97.0
MBT	—	n.d.	—	150	149 $\pm$ 5.66	99.3
	50	48.6 $\pm$ 2.0	97.2	500	498 $\pm$ 17.4	99.6

a) Percentage value obtained considering extraction yields from matrix-matched calibration.
b) Not detected.

on the above observations, an extraction time of 30 s was used in subsequent experiments.

Finally, the effect of extraction time on back extraction efficiency was also tested and was found to have no effect. This was thought to be due to the formation of a stable microemulsion in BES due to the presence of trace amount of ACN in 1-UN that greatly accelerated the mass transfer of the analytes back into the BES. Hence, a back extraction time of 30 s was chosen in further experiments. The total time of all the steps (e.g. centrifugation, solidification of the drop etc.) before injection is therefore approximately 15 min.

3.4 Quantitative aspects

Linearity of the proposed DLLME–SFO–FASI–CE method was evaluated using bovine urine samples spiked with the selected compounds at seven concentration levels ranging from 0.15 to 10 mg L^{-1} for CIBT and 15 to 1000 μg L^{-1} for the other three β_2-agonists. Table 1 lists the figures of merit of the proposed method in terms of linearity as related to linear dynamic ranges (LDR) and coefficients of determination (R^2), repeatability (intraday and interday precision), LOD, and sensitivity enhancement factor (SEF). Calibration graphs for β_2-agonists exhibited good linearity within the aforementioned LDRs with R^2 values ranging from 0.9901 to 0.9971. RSD% ($n = 5$), based on peak areas of five replicate runs of the extractions, ranged from 2.5 to 3.4% for intraday and from 4.2 to 5.5% for interday precision. SEF, defined as the ratio of LOD in conventional CZE to that with DLLME–SFO–FASI–CE, ranged between 41 for CIBT and 1046 for MPT. The low SEF for CIBT was probably due to the higher solubility of this analyte in the aqueous solution in the presence of ACN, taking into account its lowest log$P$ value among the other studied analytes (Section 2.1). High SEFs of the proposed method gave rise to LODs (calculated for an S/N ratio of 3; N: noise of the baseline calculated for 11 noise peaks chosen at different places of the baseline void of analytical peaks) in the range of 1.80 μg L^{-1} for MPT to 37.0 μg L^{-1} for CIBT.

3.5 Application of the method

In order to examine matrix effects and applicability to the analyses of field samples, the proposed method was used to determine β_2-agonists in real bovine urine samples. Preliminary experiments showed that the samples were expectedly free of the target analytes since illegal use of β_2-agonists is strictly controlled in the country. Therefore, they were spiked with the target analytes at three concentration levels of 500, 1500, 5000 μg L^{-1} for CIBT, and 50, 150, 500 μg L^{-1} for CBT, MPT, and MBT. Accuracy, expressed as percentage values obtained considering extraction yields from matrix-matched calibrations, were in the range of 96.0–102.0% (Table 2). Typical electropherograms of a blank and spiked urine samples after being subjected to the DLLME-SFO procedure are shown in Fig. 5.

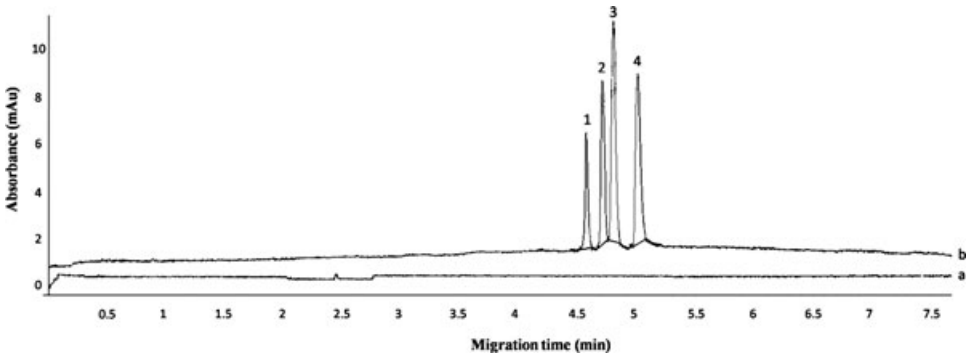

Figure 5. Electropherograms of (A) blank and (B) spiked bovine urine extracted with DLLME–SFO and analyzed under optimum FASI–CE. Peaks: (1) CIBT, (2) CBT, (3) MPT, (4) MBT, (1.0 mg L^{-1} CIBT and 100 μg L^{-1}, each of the other analytes).

Table 3. Comparison of the DLLME–SFO–FASI–CE method with other reported methods

Analyte	Sample	Extraction method/technique	Extraction time (min)	LOD (μg L^{-1})	LDR (μg L^{-1})	EF	Ref.
CBT	Human urine, serum	SPME–LC–UV[a)]	60	9, 5	10–500, 5–500	—	[33]
CBT	Water and human urine	HF–LPME–GC–MS[b)]	20	0.10	0.25–200	256	[30]
CBT	Human urine	PMME–HILIC–ESI–MS[c)]	5	0.08	0.4–400	—	[31]
CBT	Porcine tissues	SPE–USA–DLLME–HPLC–UV[d)]	2	0.07 (μg/kg)	0.19 –192 (μg/kg)	62	[32]
CIBT	Bovine urine	DLLME–SFO–FASI–CE	1.5	37.0	150–10 000 (CIBT)	41	
CBT				1.90		996	
MPT				1.80	15–1000 (CBT, MPT, MBT)	1046	
MBT				3.50		541	This study

a) Solid–phase microextraction–liquid chromatography–ultraviolet.
b) Hollow fiber–liquid phase microextraction–gas chromatography–mass spectrometry.
c) Polymer monolith microextraction–hydrophilic interaction chromatography–electrospray ionization-mass spectrometry.
d) Solid phase extraction–ultrasound assisted–dispersive liquid liquid microextraction–high performance liquid chromatography–ultraviolet.

3.6 Comparison with other preconcentration methods

The proposed DLLME–SFO–FASI–CE method was compared with previously reported microextraction methods (i.e. solid–phase microextraction (SPME), HF–LPME, PMME, and SPE–USA–DLLME) for the determination of β_2-agonists in different matrices, in the viewpoint of extraction time, LOD, LDR, and enrichment factor (EF) (Table 3). Our literature survey revealed the absence of microextraction methods for the simultaneous determination of the four studied β_2-agonists. DLLME–SFO–FASI–CE is most importantly the fastest among the other extraction methods (Table 3). With the exception of SPE–USA–DLLME that is also fast (2 min), extraction times ranged from 5 min with PMME to 60 min with SPME. DLLME–SFO–FASI–CE gave a lower LOD than SPME–LC–UV for CBT. Lower LODs for CBT were either obtained with highly sensitive detection systems [30, 31], yet much more expensive than CE–UV, or by combining more than one offline preconcentration techniques [32]. Since no specific holder is required for supporting the organic microdrop, the proposed method overcomes the limitation of small contact surface in HF–LPME and thus shortens the extraction times remarkably and eliminates the effect of air bubbles on the transport rate of the analytes. The relatively small surface area of polymer monoliths used in PMME is also considered as a significant disadvantage of this extraction method. Procedures based on SPE are laborious, time consuming, need large volumes of samples, and toxic organic solvents, and may suffer from "plugging" the small columns and disks employed [32].

4 Concluding remarks

In this study, a novel combination of DLLME–SFO and FASI in CE to extract, preconcentrate, and determine four β_2-agonists in bovine urine was successfully carried out. After optimization of separation, stacking and DLLME–SFO conditions, such as sample pH, type and volume of the extraction solvents, pH and volume of sample and back extraction solutions, ionic strength as well as extraction time, LODs for the target analytes were determined to be at the low μg L^{-1} levels. Compared to conventional CZE, the present method provided higher sensitivity, with LODs lower by 41–1046 times. The procedure was applied successfully to spiked bovine urine samples and accuracy higher than 96.0% was achieved. Low LODs, wide LDRs, acceptable reproducibilities, and interference-free electropherograms were obtained in the analysis of a reasonably complex matrix, indicating that the developed method has potential applicability in the determination of these target compounds in genuine samples using matrix-matched standards. This approach may be helpful to overcome the limitation of the low concentration sensitivity

of CE with direct UV detection to expand the applicability of this powerful technique to real-world analysis.

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