Journal of Chromatography A, 1217 (2010) 1856–1861

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

On-line combination of single-drop liquid–liquid–liquid microextraction with capillary electrophoresis for sample cleanup and preconcentration: A simple and efficient approach to determining trace analyte in real matrices

Zaifang Zhu[a,b], Ximin Zhou[a,b], Na Yan[a,b], Lei Zhou[a,b], Xingguo Chen[a,b,*]

[a] National Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, China
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, China

ARTICLE INFO

Article history:
Received 16 September 2009
Received in revised form
22 December 2009
Accepted 7 January 2010
Available online 14 January 2010

Keywords:
On-line combination
Adenine
Capillary electrophoresis
Single-drop liquid–liquid–liquid microextraction
Sample cleanup
Preconcentration

ABSTRACT

In order to improve the sensitivity of capillary electrophoresis (CE) and overcome the deficiency of commercial CE instruments in handling complex matrices directly, we proposed a novel technique which combined single-drop liquid–liquid–liquid microextraction (SD-LLLME) with CE on-line. In this technique, SD-LLLME was realized using a commercial CE instrument and, to further concentrate the target analyte, large-volume sample stacking combined sweeping without polarity switching was utilized. Even though without agitating the donor phase in the extraction process, the model compound, adenine was enriched 550-fold in only 10 min. The enrichment factors were 760 and 1030 when the extraction time was extended to 30 and 60 min, respectively. The relative standard deviations (RSDs) of adenine were 5.24% and 2.29% for peak area and migration time, respectively, which indicated that this method was much more reproducible compared to the existing methods that combined sample-preparation strategies with CE. In addition, this approach was selective while cleaning up target analyte. These mentioned advantages allowed the developed method to be an attractive approach to determining trace target compounds in complex real samples.

1. Introduction

Poor sensitivity is considered to be one of the major limitations of capillary electrophoresis (CE), particularly when compared with traditional chromatographic techniques [1]. To address this issue, a number of novel and innovative methods have been developed [2–6]. These methods overcame the drawbacks of CE in analyzing trace targets and expanded its application. However, they usually cannot handle complex sample matrices directly and, as a result, a sample-preparation step is commonly required. In many analytical procedures, sample preparation is the time- and cost-determining step, and consequently its simplification, miniaturization and automation are desirable when developing new analytical methods.

Extraction, as a classical sample pretreatment approach, not only can concentrate but also is able to cleanup the analytes of interest while rendering them in a form that is compatible with the analytical system. Since its introduction in 1990 [7], solid-phase microextraction (SPME) has been successfully coupled with CE off-line or in-line [8–10]. However, the sample carry-over and robustness of the extraction fiber can be problematic. Additionally, automated SPME systems are expensive and normally out of the reach of most laboratories. Liquid-phase extraction (LPE) is the most widely used sample-preparation technique for liquid samples, prescribed in many standard analytical methods [11]. Although it offers high reproducibility and high sample capacity, it is considered to be a time-consuming, tedious, multistage operation, where problems of emulsion formation obstruct automation. Liquid-phase microextraction (LPME) [12–15], as the miniaturized mode of the traditional LPE method, overcomes many of the disadvantages of LPE as well as some of those of SPME [16]. It is simple, fast and is characterized by its affordability, and reliance on widely available apparatus. However, due to the general incompatibility of the extracting organic phase in conventional LPME with the normal running buffer in CE, the combination of LPME with CE is not routine and poses some operational difficulties. In 2004, Choi et al. realized directly the combination of single-drop microextraction (SDME), a popular liquid–liquid microextraction (LLME) technique, with CE [17]. A drop of basic aqueous phase hanging at the capillary inlet tip was covered with *n*-octanol as a thin organic film. When the two-phase droplet was placed into an acidic aqueous sample solution, acidic analytes were extracted

* Corresponding author at: Department of Chemistry, Lanzhou University, South Tianshui Road #222, Gansu, Lanzhou 730000, China. Tel.: +86 931 891 2763; fax: +86 931 891 2582.
E-mail address: chenxg@lzu.edu.cn (X. Chen).

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doi:10.1016/j.chroma.2010.01.013

into the aqueous basic phase through the organic film. Although an interesting approach, the operation seems cumbersome, and appears inconvenient to be accomplished on a commercial CE instrument. In addition, the uncertainties in the manual control of the droplet formation resulted in poor reproducibility and the relative standard deviations (RSDs) for corrected peak area with 15-min extraction were ~30% [17]. With a longer extraction time, the reproducibility became poorer and the drop tended to became detached from the capillary tip. In 2009, SDME was realized with commercial CE instruments of varying sophistication by the same group [18]. Although this process was proved to be more accurate and reliable, the RSDs of the extraction results were still relatively large when internal standardization was not utilized. Moreover, in order to enhance extraction efficiency and improve reproducibility, a laboratory-made microstirrer retrofit was installed in the sample vial. This usually poses some technical challenges for commercial CE instruments and goes against modern trends toward simplification and automation of chemical analysis.

In the past ten years, single-drop liquid–liquid–liquid microextraction (SD-LLLME) had been proved to be a very effective sample clean-up method [5,19–22]. It was usually utilized to extract ionizable and chargeable analytes in complex environmental and biological samples. The three liquid phases are aqueous donor phase (sample), organic phase and aqueous acceptor phase, which is a single drop. Analytes were firstly extracted from the donor phase into the organic phase and then back-extracted into the acceptor phase from the organic phase [23]. In this work, we for the first time adopted SD-LLLME as an on-line sample-preparation technique prior to CE. This approach allowed commercial CE instruments to directly handle complex sample matrices while sample preconcentration was realized. As neither the CE instrument nor the capillary was required to be modified, the influence of the operator's manual manipulation on the performance and reliability of the developed method was reduced.

In order to further improve the sensitivity, we combined large-volume sample stacking combined with sweeping without polarity switching in CE (LVSS-sweeping). Sweeping is a simple and convenient on-line sample concentration way for either charged or neutral analytes [2,24–26]. It is the picking and accumulation of analyte molecules by the pseudostationary phase that penetrates the sample zone. This causes a unique focusing effect which relies on how the pseudostationary phase enters the sample solution (nonmicelle buffer) and sweeps the analytes. The higher the affinity of analytes toward the pseudostationary phase, the greater the concentration effect. In 2008, our group for the first time combined sweeping with large-volume sample stacking on-line [27]. It can be utilized to enrich anionic, neutral and cationic analytes [27,28]. Even though without agitating the donor solution when the extraction was processed, the model compound, adenine was enriched 550-fold in 10 min. When the extraction time was prolonged to 60 min, 1030-fold enrichment was accomplished. Apart from being simple, fast and selective, this technique enjoyed better reproducibility compared with the existing methods [17,18,29,30] and was environment-friendly since nontoxic ethyl acetate was utilized as the organic phase of SD-LLLME.

2. Experimental

2.1. Reagents

Adenine, caffeine and theophylline were obtained from National Institute for Control of Pharmaceutical and Bio-products (Beijing, China). Sodium dodecyl sulfate (SDS), ethylene glycol, tetraborate, ethyl ether and ethyl acetate were products of Tianjin Chemical Reagent Factory (Tianjin, China). *n*-Octanol and toluene were from Shanghai Chemical Plant (Shanghai, China). Green Tea was purchased from a local supermarket. All solvents and reagents were of analytical grade and used without further purification. Distilled water was used throughout.

2.2. Apparatus

All capillary electropherograms were recorded from a Beckman P/ACE MDQ system (Fullerton, CA, USA), equipped with a diode-array UV detector (190–600 nm). Data acquisition and instrument control were carried out using 32 Karat software (Version 7.0). Electrophoresis was performed in fused silica capillaries of 50 μm i.d. and 365 μm o.d. obtained from Yongnian Ruifeng Chromatogram Equipment (Yongnian, China). All capillaries were 50.2 cm long having an effective length of 40 cm and were thermostated at 25 °C.

2.3. Electrophoretic conditions

The running buffer was 10 mM tetraborate buffer containing 30% (v/v) ethylene glycol and 50 mM SDS without adjusting pH (measured pH value was 7.8). Prior to its first use, the capillary was conditioned by rinsing with 0.5 M NaOH (10 min, 20 psi), distilled water (10 min, 20 psi), followed by 1.0 M HCl (20 min, 20 psi), distilled water (10 min, 20 psi), and finally the running buffer (10 min, 20 psi). To assure a good reproducibility, the capillary was rinsed sequentially with distilled water (3 min, 20 psi), 0.5 M NaOH (5 min, 20 psi), distilled water (3 min, 20 psi) and running buffer (2 min, 20 psi) at the beginning of each experimental session. Between two runs, a rinse-cycle of 0.5 M NaOH (2 min, 20 psi), distilled water (3 min, 20 psi) and running buffer (2 min, 20 psi) was employed. A constant voltage of 25 kV was applied during analysis, and analyte were detected at 256 nm.

2.4. Standard and Green Tea sample preparation

Standard stock solution of adenine with the concentration of 500 μg/mL was prepared by dissolving the appropriate amount in distilled water and, when in use, diluted to the required concentration with distilled water or the Green Tea sample solution. Green Tea was finely powdered and then 3 g of it was accurately weighed. The weighed sample was extracted with 10 mL distilled water for 20 min in an ultrasonic bath and followed by centrifugation at 1500 × *g* for 5 min. The extracting procedure was repeated two times. The total volume of extracts were filtered through a 0.45 μm pore size cellulose acetate membrane and diluted with distilled water to final volume of 25 mL for direct analysis.

2.5. SD-LLLME

The aqueous sample solution (donor phase), sealed with an organic solvent plug (organic phase) in a buffer vial of Beckman P/ACE MDQ system, was used for SD-LLLME and the acceptor phase was acidic aqueous solution. Schematics of the extraction procedure were shown in Fig. 1. SD-LLLME in this work consists of the following four steps: (a) the acceptor solution was injected into the capillary, which was firstly filled with the running buffer; (b) the drop formation was realized as a backward pressure was applied, and a photographic image was shown in Fig. 2. In this step, it was crucial to keep the inlet end of the capillary in the organic phase; (c) the sample in the donor phase was extracted into the organic phase and subsequently back-extracted into the acceptor phase; (d) the enriched extractant was injected into the capillary with a forward pressure. As volumes of the solution injected into or pushed out of capillary were proportional to the applying time of pressure, they

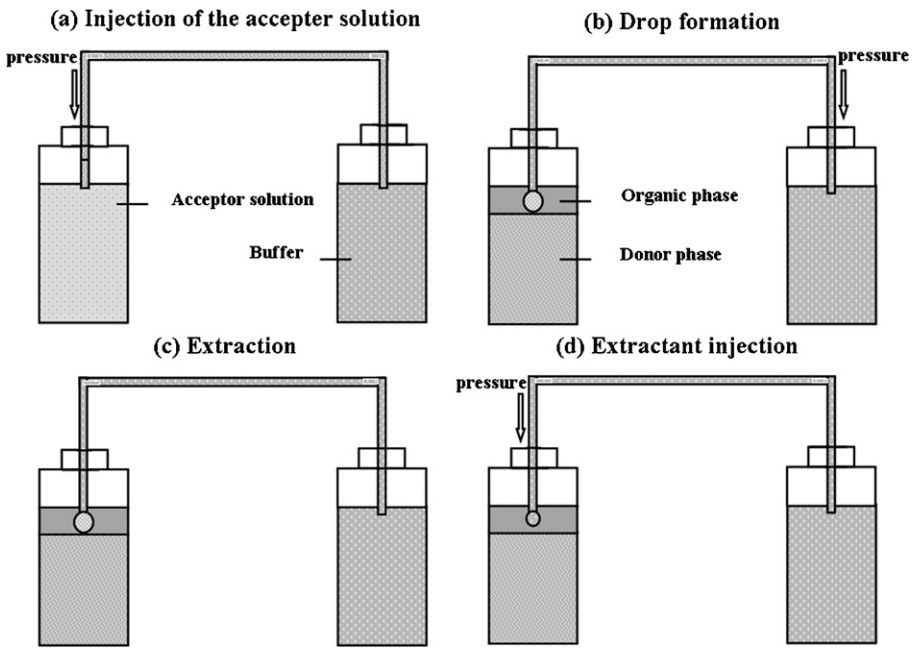

Fig. 1. Procedures of SD-LLLME. (a) Injection of the acceptor solution, (b) drop formation, (c) extraction of the sample into the acceptor phase from the organic phase, and (d) injection of the extractant.

can be given by

$$V_{a,i} = k_{a,i} t_{a,i} \quad (1)$$

$$V_{drop} = k_{drop} t_{drop} \quad (2)$$

$$V_{ee,i} = k_{ee,i} t_{ee,i} \quad (3)$$

where $V_{a,i}$, V_{drop} and $V_{ee,i}$ represent the volumes of the acceptor solution injected into capillary, the droplet and the enriched extractant injected into capillary. $t_{a,i}$, t_{drop} and $t_{ee,i}$ are the applying time of pressure in step a, b and d. The constants, $k_{a,i}$, k_{drop} and $k_{ee,i}$, are respective proportional coefficients. In this experiment, as the applying pressure in SD-LLLME was kept invariable at 0.5 psi, we can assume $k_{a,i}$, = k_{drop} = $k_{ee,i}$ = k. Under this assumption, Eqs. (1)–(3) can be written as

$$\frac{V_{a,i}}{t_{a,i}} = \frac{V_{drop}}{t_{drop}} = \frac{V_{ee,i}}{t_{ee,i}} = k \quad (4)$$

This equation indicates that volumes of the solution injected into or pushed out of capillary can be controlled by only varying the applying time of constant pressure in SD-LLLME.

3. Results and discussion

3.1. Basic principle of SD-LLLME

SD-LLLME involves a series of reversible extractions. The analyte in the donor phase is first extracted into the organic phase and then back-extracted into the acceptor phase. For analyte i, the extraction equation can be written as follows:

$$i_d \leftrightharpoons i_{org} \leftrightharpoons i_a \quad (5)$$

where the subscript d represents the donor phase, org the organic phase, and a the acceptor phase. At equilibrium, the enrichment factor (EF), which is defined as the ratio of the equilibrium concentration of analyte in the acceptor phase ($c_{d,i}$) to the initial concentration of analyte in the donor phase ($c_{a,eq}$), can be represented by [17,19,20]

$$EF = \frac{c_{a,eq}}{c_{d,i}} = \frac{1}{K_{org/a}/K_{org/d} + K_{org/a}(V_{org}/V_d) + V_a/V_d} \quad (6)$$

in which the V represents the volume of the phase denoted by the subscript; $K_{org/a}$ is the equilibrium distribution coefficient between the organic phase and the acceptor phase; $K_{org/d}$ is the equilibrium distribution coefficient between the organic phase and the donor

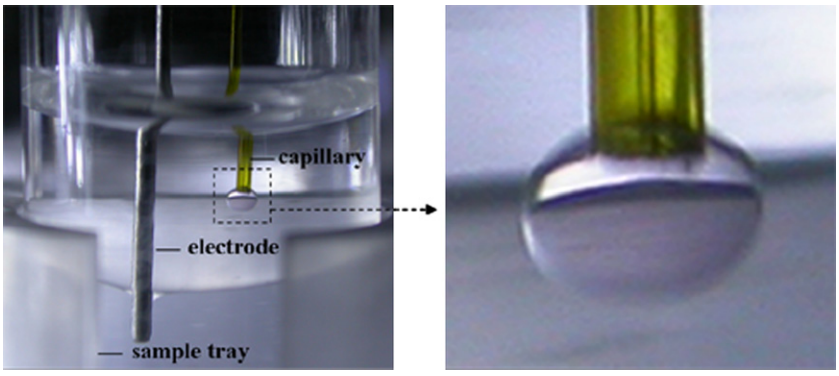

Fig. 2. Image of a droplet in the organic phase.

phase. $K_{org/a}$ and $K_{org/d}$ depend on the characteristics of acceptor phase, organic phase and donor phase. The variety of V_d, V_{org} and V_a can be simply realized in the experiment process. In order to get the optimal extraction conditions and high extraction efficiency, the peak area of adenine was utilized as the CE response to evaluate the extraction efficiency under various conditions.

3.2. Selection of the organic phase and optimization of its volume

The type of organic solvent utilized in SD-LLLME was an essential consideration for the success of the extraction. Based on comparison of selectivity, extraction efficiency, incidence of drop loss, rate of drop dissolution and level of toxicity, ethyl acetate, ethyl ether, *n*-octanol and toluene were evaluated in this work. When ethyl ether or toluene was utilized, the enrichment was less than 40 under optimum conditions. *n*-Octanol provided relatively higher enrichment efficiency compared with ethyl ether and toluene. But, concerning the fact that ethyl acetate provided the highest enrichment factor among all the investigated organic solvents and it was environment-friendly, we selected ethyl acetate as the organic phase. However, its utilization also led drop dissolution to be serious and the droplet disappeared at an extended extraction time. In order to settle this problem, ethyl acetate saturated with water was utilized. This approach allowed the extraction to be processed without drop dissolution even though the extraction time was extended to 90 min. In addition, the loss rate of the droplet was negligible (no loss if the CE instrument was not shaken by accident).

The effect of the ethyl acetate volume on extraction efficiency was also investigated. The extraction efficiency decreased as the volume of ethyl acetate increased from 200 to 400 μL, which indicated that the thin organic membrane benefited the transport of the analyte to the acceptor phase. When the volume was smaller than 200 μL, drop formation in the organic plug became difficult to operate. So ethyl acetate of 200 μL was used in the experiment.

3.3. Adjustment of the composition of the acceptor phase and the donor phase

In SD-LLLME, the adjustment of the composition of the acceptor phase and the donor phase is critical, since it could change the ionization form of target analyte and could thereby affect their water solubility and extractability. In addition, the composition of the acceptor phase would also affect the enrichment process in CE. In an attempt to improve the enrichment efficiency, HCl was investigated as the acceptor phase by varying its concentration from 0.001 to 0.1 M, and the results were shown in Fig. 3. It was observed that there was no CE response of adenine when HCl concentration was low. This could be ascribed to the fact that target analyte was not able to be finely ionized if the acceptor phase was weak acid. As the HCl concentration increased from 0.005 to 0.05 M, peak height increased, but further increase above 0.05 M was followed by decrease of peak height and widening of peak shape. So 0.05 M HCl was selected as the acceptor phase for further experiment.

Theoretically, when pH of the donor phase was the pI of adenine, the concentration of neutral analyte in the donor phase was highest and the analyte was easiest to be extracted from the donor phase into the organic phase. In this work, as the pI of the model compound, adenine, was 7.0, the extraction efficiency was supposed to be the highest when pH of the donor phase was 7.0. So we optimized pH of the donor phase around 7.0 and results indicated that as long as there was no much difference between pH of the donor phase and the pI of adenine, analyte can be enriched efficiently and the enrichment factor was satisfactory. In the rest of the study, standard and Green Tea sample solutions were directly analyzed without adjustment (measured pH values were 6.8 and 4.3, respectively).

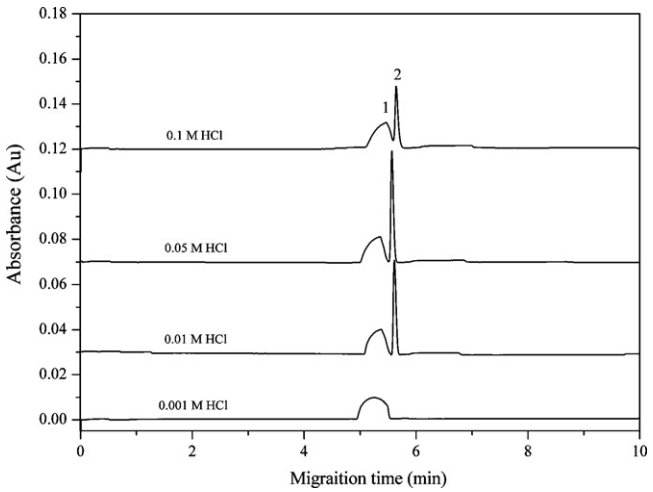

Fig. 3. Effect of HCl concentration on enrichment. (1, system peak, was caused by the composition difference between the buffer and the sample solution. It broadened as the injection time increased. 2, adenine peak.) The concentration of adenine was 1 μg/mL, enriched by 10-min SD-LLLME in which $t_{a,i} = t_{drop} = 5.0$ min, $t_{ee,i} = 1.5$ min; running buffer, 10 mM tetraborate buffer containing 30% (v/v) ethylene glycol and 50 mM SDS without adjusting pH (measured pH value was 7.8); uncoated fused silica capillary, 50.2 cm (40 cm to the detector) × 50 μm i.d.; applied voltage, 25 kV; cartridge temperature, 25 °C.

3.4. Drop formation

Drop formation is a crucial step in the method development of SD-LLLME. Initially, all the acceptor solution injected into the capillary was justly backward pushed out to form the droplet. Based on Eq. (4), this could be realized through keeping $t_{a,i} = t_{drop}$. The effect of the applying time was investigated in the range of 1.0–5.5 min while $t_{ee,i}$ was less than 1.5 min. This meant that if $t_{a,i} = t_{drop} < 1.5$ min, $t_{ee,i} = t_{a,i} = t_{drop}$. Otherwise, $t_{ee,i}$ was 1.5 min. We operated in this way because peak widened seriously if $t_{ee,i}$ was larger than 1.5 min while keeping $t_{a,i} = t_{drop}$. The highest enrichment factor of 110 was obtained when 5.0 min was selected.

In the further experiment, the enrichment efficiency was found to be greatly enhanced when t_{drop} was larger than $t_{a,i}$ (shown in Fig. 4). Based on this unexpected phenomenon, we investigated the effect of $t_{a,i}$ in the range of 1.0–4.5 min while keeping $t_{drop} = t_{ee,i} = 5.0$ min. The highest enrichment factor was obtained

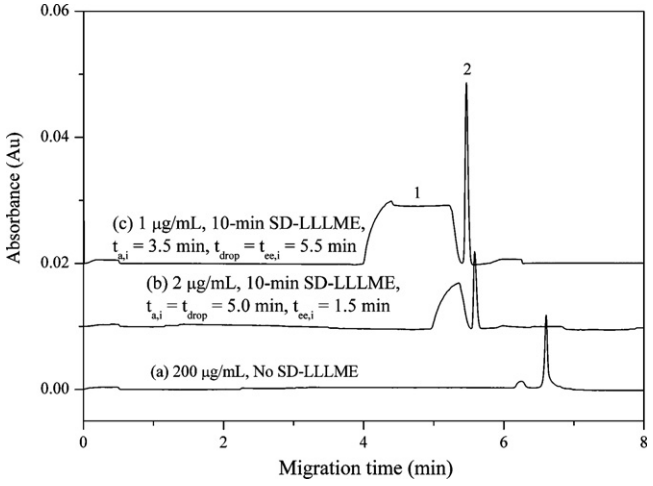

Fig. 4. Electropherograms of (a) 200 μg/mL adenine without enrichment (injection: hydrodynamic, 90 s at 0.5 psi), (b) 2 μg/mL adenine enriched by 10-min SD-LLLME in which $t_{a,i} = t_{drop} = 5.0$ min, $t_{ee,i} = 1.5$ min and (c) 1 μg/mL adenine enriched by 10-min SD-LLLME in which $t_{a,i} = 3.5$ min, $t_{drop} = t_{ee,i} = 5.5$ min. Other conditions as in Fig. 3.

when $t_{a,i}$ was 3.5 min. If $t_{a,i}$ was larger than 3.5 min, peak shape was unacceptable. Then, we kept $t_{a,i}$ at 3.5 min and studied the effect of t_{drop} in the range of 3.5–6.0 min while keeping $t_{drop} = t_{ee,i}$. The highest enrichment of 550-fold was achieved when t_{drop} was 5.5 min. To explain the great enhancement of the enrichment efficiency, a boundary, which may be not distinct, was assumed to exist between the acceptor phase and the running buffer (seen in Fig. 5). A pH gradient formed in the droplet and the H^+ concentration increased from the inside to the outside of the droplet (indicated in Fig. 5). Under this assumption, the concentration of H^+ in the outside of the droplet did not decrease much compared with the fresh acceptor phase while the surface area of the droplet increased greatly. This facilitated the back-extraction of adenine from the organic phase into the acceptor phase.

3.5. The extraction time

Mass-transfer is a time-dependent process. To investigate the effect of extraction time on the enrichment efficiency, peak area was studied as a function of the extraction time. As seen from Fig. 6, the amount of adenine extracted by SD-LLLME increased with increasing the extraction time form 5 to 60 min. However, further increase of the extraction time above 10 min resulted in unsatisfactory reproducibility. In addition, an extended extraction time went against a practical implementation of the technique. Therefore, as a compromise, a time of 10 min was selected.

3.6. Ionic strength of the donor phase

Generally, addition of salt decreases the solubility of analytes in the donor phase (salting-out effect) and, as a result, can improve extraction efficiencies of target analytes in SD-LLLME. For the purpose of improving sensitivity of this method, the effect of NaCl content was investigated. The obtained results indicated that the addition of NaCl offered no benefits to the extraction. As previous works indicated [31,32], next to the salting-out effect, a second effect, adverse for the extraction, may also take place. By this effect, the addition of salt can change the physicochemical properties of the organic plug, thus reducing the diffusions rates of the analyte into the drop. So no salt was added into the donor phase.

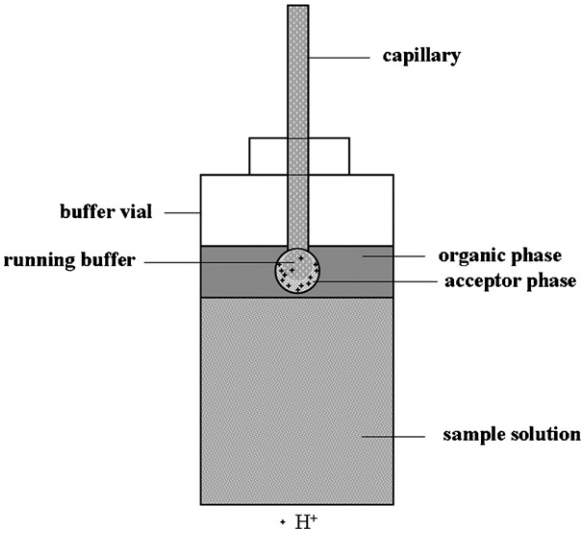

Fig. 5. Schematic of the drop formation. The organic phase was ethyl acetate saturated with water, and 0.05 M HCl was utilized as the acceptor phase. In the droplet, a boundary was assumed to exist between the acceptor phase and the running buffer.

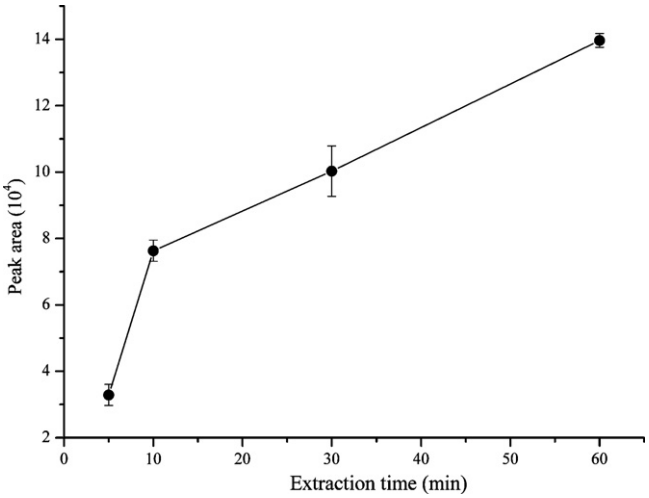

Fig. 6. Effect of extraction time on the enrichment efficiency of adenine. The concentration of adenine was 1 μg/mL; $t_{a,i}$ = 3.5 min and $t_{drop} = t_{ee,i}$ = 5.5 min; other conditions as in Fig. 3.

3.7. LVSS-sweeping-CE

In this work, LVSS-sweeping was utilized in CE to further concentrate the target analyte. This technique, designed by Zhang et al. [27], was carried out with pressure injection of large-volume sample followed by the electroosmotic flow (EOF) as a pump pushing the bulk of low-conductivity sample matrix out of the outlet of the capillary while analytes were swept by micelles and separated via micellar electrokinetic chromatography (MEKC) without the electrode polarity switching. Initially, the capillary was filled with the micellar running buffer. By applying a pressure, the plug of sample, which was usually prepared in low-conductivity solution, was introduced into the capillary. With the application of positive voltage, the sample plug was pumped toward the outlet end by EOF. Meanwhile, analytes contained in this low-conductivity plug were subjected to strong local electric field strength and consequently moved with a very high velocity. Once the analytes reached the boundary between the sample zone and the buffer zone, they would slow down and focus at this boundary, creating a sample zone which had much higher concentration than the original sample. At last, all the analytes were completely swept by the micelles and were separated via MEKC without the polarity-switching step. In this work, the running buffer, 10 mM tetraborate buffer containing 30% (v/v) ethylene glycol and 50 mM SDS, and other conditions were selected based on our previous works and not optimized in this work.

4. Method validation and applications

4.1. Validation

Performance characteristics of the developed method, including reproducibility, enrichment efficiency and linearity, were investigated under optimized conditions. The reproducibility was studied for five replicate experiments and the relative standard deviations (RSDs) of adenine were 5.24% and 2.29% for peak area and migration time, respectively. This was much better compared to the existing methods that combined sample preparation with CE [17,18,33,34]. The enrichment factor was used to evaluate the enrichment efficiency and it should be noticed that as high as 550-fold enrichment was achieved in only 10 min without agitation of the donor phase. Linearity was obtained over the range of 0.01–4.0 μg/mL and the

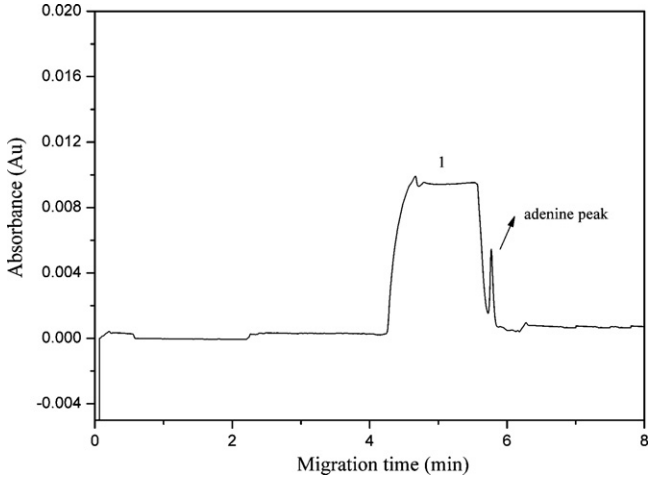

Fig. 7. Electropherogram of Green Tea sample solution. Enriched by 10-min SD-LLLME in which $t_{a,i}$ = 3.5 min, t_{drop} = $t_{ee,i}$ = 5.5 min. Other conditions as in Fig. 3.

coefficient of correlation (r^2) was 0.9993. Calculated for an S/N of 3, the limit of detection (LOD) was 0.002 μg/mL.

4.2. Application

To evaluate the practical applicability of the SD-LLLME-CE method, the developed system was applied to the analysis of adenine in Green Tea sample. As can be seen in Fig. 7, only adenine was detectable under the tested condition. Based on this fact, the proposed method was assumed to be selective. Further experiments indicated that while adenine was enriched 550-fold under the optimized condition, other compounds such as caffeine and theophylline, which usually existed in Green Tea and may affect the determination of adenine, cannot be enriched efficiently. This advantage allowed the proposed method to be able to determine adenine in Green Tea sample. Concerning to recovery, the standard addition methods with three concentration levels were employed and the average recovery was 71.7%. This meant that the matrix had some effect on SD-LLLME, which may be caused by the alteration of the ionic strength of the matrix. To address this issue, linear calibration was established utilizing Green Tea sample solutions spiked with adenine standard solutions. The coefficient of correlation (r^2) was 0.9990 and the recovery was 99.4%, which were satisfactory and supported the suitability of the proposed method for its application to real samples.

5. Conclusion

In this work, a novel approach to combining SD-LLLME with CE was proposed. This was the first report that SD-LLLME was employed as an on-line sample-preparation method for CE analysis. In combination with CE on-line, SD-LLLME not only allowed commercial CE instruments to directly handle complex matrices but also facilitated the automation and miniaturization of CE methodologies. Selectivity and strong enrichment capacity this technique displayed endowed it with more potential in analyzing trace target compounds in complex real samples. To evaluate the applicability of this method to the determination of various analyte classes, further work is currently underway in our laboratory.

Acknowledgement

This work was kindly supported by the National Natural Science Foundation of China (Grant No. 20875040).

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