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Thousand fold concentration of an alkaloid in capillary zone electrophoresis by micelle to solvent stacking

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ABSTRACT

In this paper, the co-solvent of methanol–water was used to facilitate the sodium dodecyl sulfate (SDS) micelles collapse, thereby inducing the on-line sample focusing technique of micelle to solvent stacking (MSS). To demonstrate this stacking method, the mechanism of micelles collapse in co-solvent was discussed. The details of the required conditions were investigated and the optimized conditions were: running buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); micellar sample matrix, 20 mM SDS, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); co-solvent buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 in methanol/water (90:10, v/v). The validity of the developed method was tested using cationic alkaloid compounds (ephedrine and berberine) as model analytes. Under the optimized conditions, this proposed method afforded limits of detection (LODs) of 0.5 and 1.1 ng/mL with 300 and 1036-fold improvements in sensitivity for ephedrine and berberine, respectively, within 15 min.

1. Introduction

Capillary electrophoresis (CE) has been developed as a powerful analytical technique. Its advantages include high efficiency, short analysis time, and small sample requirements. It has been widely used in different areas of chemistry and biochemistry. However, because of the short optical path length across the capillary, one of the major limitations of CE is the low detection sensitivity with absorbance detection for trace analytes. Over past decades, a number of approaches have been developed to improve the detection sensitivity of CE, these investigations include extending the detection path length with a bubble cell [1] or z-shape capillary [2], using powerful detectors like laser-induced fluorescence, off-line and on-line sample preconcentration. The on-line sample preconcentration is based on focusing a large volume of injected sample to a minimum volume inside the capillary, requiring no modification of current commercial instrument. Therefore, on-line sample preconcentration is a useful technique to improve the concentration sensitivity.

Over the last decades, hundreds of articles were published on on-line sample stacking in CE [3,4], including field amplified sample stacking [5–10], transient isotachophoresis (tr-ITP) [11–13], dynamic pH junction [14,15] and transient moving reaction boundary (tMCRBM) [16] as well as sample sweeping [17–29], etc. Each method relies on creating difference between the background electrolyte (BGE) or buffer solution zone and the sample zone or a special inserted zone for enrichment. For example, the field amplified sample stacking relies on the changes in analytes' electrophoretic velocities caused by the mismatch in concentrations or electrical conductivities between the sample solution and the separation solution. tr-ITP uses an imposed electrophoretic mobility gradient to create concentrated analyte zones with nondispersing interfaces. The stacking effect of dynamic pH junction is based on pH discontinuity between the sample and the electrolyte, which causes significant changes in ionization states or electrophoretic velocities of the analytes. tMCRBM relies on non-steady-state isoelectric focusing. Sweeping is based on chromatographic partition, complexation or any other interaction between the analytes and additives, so the additives 'sweep' the long sample band into a narrow zone.

Recently, a new on-line focusing method termed as analyte focusing by micelle collapse (AFMC), has been developed by Quirino and Haddad [30]. Neutral analytes are associated to the sodium dodecyl sulfate (SDS) micelles in the sample matrix which contains

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anions of high electrophoretic mobility, and the running buffer contains high concentration of micelles but low concentration of the same anions in the sample matrix. When voltage is applied, the anionic micelles transport the associated sample molecules from the high conductivity zone to the low conductivity zone. Micelle dilution occurs when the micelles and electrolyte anions move into a dilution zone. Under the suitable conditions, the concentration of SDS falls below its critical micelle concentration (CMC), and the micelles collapse at the boundary of the two zones, thereby releasing and focusing the loaded molecules. This technique has been successfully applied to on-line concentration of some neutral analytes and the detection sensitivity was increased by one to two orders of magnitude [31–33]. Thereafter, micelle to solvent stacking (MSS) [34,35] has been introduced by the same group. In MSS, the sample was prepared in a micellar solution without organic solvent, the separation solution was modified by an organic solvent. The focusing was based on change in the effective electrophoretic mobilities of the analytes at the boundary between the micellar sample solution and the separation solution.

The change in electrophoretic mobility due to the presence of organic solvent in MSS [35] can also be caused by micelle collapse. This was demonstrated by Liu et al. [36], in their report, the sample was prepared in a 8.0 mM SDS micellar matrix, the running buffer was 75 mM H_3PO_4, 2% (v/v) Tween 20, 5% (v/v) methanol buffer, and a section of trapping solution composed of 50 mM H_3PO_4, 55% ethanol was inserted between the sample solution and the running buffer. The analytes change their electrophoretic mobilities in the trapping solution when released by SDS micelles collapse. After focused by MSS, the analytes were separated via micellar electrokinetic chromatography (MEKC). This technique afforded 113 and 123-fold improvements in the detection sensitivity for tetrandrine and fangchinoline, respectively. In this paper, methanol–water co-solvent was applied to induce micelles collapse, thereby leading to MSS. The micelles collapse in co-solvent and the required conditions were discussed in detail. Using the proposed MSS-CZE, good concentration sensitivity enhancements were obtained for the two test alkaloids (berberine and ephedrine).

2. Experimental

2.1. Instrumentation

All capillary electropherograms were recorded on a Beckman P/ACE MDQ system (Fullerton, CA), 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 375 μm o.d. obtained from Handan Xinnuo Fiber Chromatogram Co., Ltd. (Handan, China). All capillaries were 60.2 cm long with an effective length of 50.0 cm, and were thermostated at 18 °C.

2.2. Chemicals and reagents

All solvents and reagents were of analytical grade and were used without further purification. Berberine and ephedrine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sodium dihydrogen phosphate, boric acid, methanol, ethyl acetate and sodium dodecyl sulfate (SDS) were products of Tianjin Chemical Reagent Factory (Tianjin, China). Redistilled water was used throughout.

2.3. Preparation of solutions and samples

Stock solutions of 0.4 M SDS, 0.4 M NaH_2PO_4, 0.4 M H_3BO_3 and 0.1 M H_3PO_4 were prepared in redistilled water. Running buffer was the mixture solution of 20 mM H_3BO_3 and 20 mM NaH_2PO_4 (pH 4.0), prepared by diluting the stock solutions of NaH_2PO_4 and H_3BO_3 with redistilled water. The micellar sample matrix comprised of 20 mM SDS, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0), prepared by diluting the corresponding stock solutions with redistilled water. pH was adjusted with 0.1 M H_3PO_4 using a PHS-3B pH meter (Shanghai Precision & Scientific Instrument Co., Ltd.). Co-solvent buffer (20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution in methanol/water (90:10, v/v) was prepared by diluting 0.5 mL 0.4 M NaH_2PO_4 and 0.5 mL 0.4 M H_3BO_3 to 10 mL with pure methanol. The conductivities of the three solutions are determined by measuring the CZE electric current values using a CE instrument. The stock solutions of 0.50 mg/mL ephedrine and 0.50 mg/mL berberine were prepared in methanol/water (10:90, v/v) and stored in refrigerator at 4 °C. The standard test sample solutions at various concentrations were prepared by appropriate dilution of the stock solution with the micellar sample matrix.

Fresh urine was collected from a healthy volunteer, after frozen in a refrigerator overnight, the urine was unfrozen at room temperature and centrifuged at 1200 rpm for 3 min the supernatant was collected. Spiked urine samples at various concentrations were prepared by appropriate addition of stock solutions of 0.50 mg/mL ephedrine and 0.50 mg/mL berberine to 1.0 mL of the supernatant, which was extracted with 2.0 mL ethyl acetate three times, the ethyl acetate layer was collected and evaporated at 60 °C to dryness under nitrogen protection. Then the sample solution was obtained by dissolving the residues with 1.0 mL micellar sample matrix. All the sample solutions were filtered through a 0.45 μm syringe filter prior to CE experiments.

2.4. Procedures

Prior to use, new capillary was conditioned by flushing at 20.0 psi (1.0 psi = 56894.76 Pa) sequentially with methanol for 10 min, redistilled water for 3 min, 1.0 M NaOH solution for 20 min, redistilled water for 3 min, and running buffer for 20 min, and finally, equilibrated at 25 kV with running buffer for 60 min. At the beginning of each run, the capillary was rinsed at 20 psi sequentially with redistilled water (2 min), 0.5 M HCl (2 min), 0.1 M NaOH solution (2 min), redistilled water (2 min), and running buffer (3 min). Sample and co-solvent buffer introduction were facilitated by applying a certain pressure for a period of time. The approximate injecting length of sample plug and that of co-solvent buffer plug were calculated using BACKMAN EXPERT software.

3. Results and discussion

3.1. Design of SDS micelle collapse

As reported that the CMC is known to change in organic solvent [37], and as presented by Palepu et al. [38], the log(CMC) of surfactants in the solvent mixtures follows Eq. (1):

$$\log(\mathrm{CMC}) = \log(\mathrm{CMC})_{\mathrm{water}} + KC \quad (1)$$

where C is the solvent/water ratio in wt%, K is a constant and CMC is quoted in mol/L.

CMC at different solvent/water ratio was determined by CE method [39], the results were listed in Table 1. When log(CMC) was plotted against C, a straight line was obtained. The slope of the line was the K, which was equal to 0.02. From Table 1, it is obvious that the CMC increases with the increase in organic solvent content. The conclusion is in good agreement with previous reports that at higher organic solvent contents, the CMC is markedly increased, and the addition of significant amounts of organic solvent will likely cause the micelles to disintegrate [40]. Therefore,

Table 1
Determination K in the system of SDS in methanol–water co-solvent.[a]

Methanol/water (v/v)	Wt% (methanol/water)	CMC (mM)	Log(CMC)
0	0	8.7	0.9395
10	8.2	11.4	1.057
20	16.7	15.3	1.185
30	25.5	21.6	1.334
40	34.8	31.5	1.498
50	44.4	45.2	1.655
60	54.5	59.2	1.772
70	65.1	93.6	1.971

[a] K = 0.016, CMC was measured by CE method, the CE conditions: applied voltage, +25 kV; capillary, 60.2 cm total (50 cm to detector); running buffer, a series of different concentrations of SDS in various methanol/water (v/v) co-solvents.

micelles are not formed under the surfactant concentrations commonly used. In other words, the micelles will collapse when they enter the co-solvent buffer plug.

3.2. Demonstration of the focusing model

The focusing model of MSS for the efficiently on-line focusing of cationic analytes with the aid of micelle collapse in CZE is similar to the focusing theory described by Liu et al. [36]. In the starting situation, the capillary was conditioned with a low-pH running buffer, then a long plug of sample solution prepared with SDS micellar sample matrix, and a short plug of co-solvent buffer were sequentially injected. When positive voltage was applied, the cationic analytes carried in anionic micelles (SDS) migrated to the anode, the co-solvent plug moved toward the cathode with same velocity of electroosmotic flow (EOF). Once the micelles load analytes to the co-solvent buffer plug, the micelles collapsed and released the cationic analytes. Simultaneously, the released cationic analytes migrated back to the micellar sample zone. As the SDS micelles continuously collapsed into the co-solvent, the analytes were focused at the boundary of the sample solution zone and the co-solvent buffer zone. After the focusing step, the SDS from the sample matrix was depleted, and then the focused analytes were separated in this region via a simple CZE.

3.3. Factors affecting focusing efficiency

As ionic strength was related to the mass transfer resistance and buffer viscosity, which influenced the electrophoretic mobilities of SDS and the electroosmosis, thereby affecting focusing efficiency. The background electrolytes (BGE) were investigated from 10 mM to 50 mM (pH 4.0), using 1.28 cm (0.5 psi, 30 s) co-solvent buffer plug and a sample zone length of 35.7 cm (7.0 psi, 60 s) with 20 mM SDS in the sample matrix. As shown in Fig. 1, the 'block' after the analyte's peak is the co-solvent peak and the negative dip is the co-solvent buffer-running buffer interface peak. The analyte's peak heights firstly increase with the BGE concentrations increasing from 10 mM to 30 mM. With further increasing the BGE concentration, the peak heights decreased to almost the same level. This maybe due to the increase in ionic strength suppresses the electroosmosis and electrophoretic mobility of SDS in different extent. An increase in ionic strength causes a reduction of the thickness of the electric double layer, therefore, the zeta potential decreases, resulting in electroosmosis suppression. And the increase of ionic strength hinders the analytes transferring in the capillary [41] therefore leading to the decrease of electrophoretic mobility of SDS. As pH influences both the electroosmosis and the degree of protonation of analytes therefore influences the focusing efficiency. In this study, using the BGE of 20 mM H_3BO_3 and 20 mM NaH_2PO_4, the pH was varied at 3.50, 3.75, 4.00, 4.15, and 4.65. Experimental results showed that the focusing efficiency enhanced

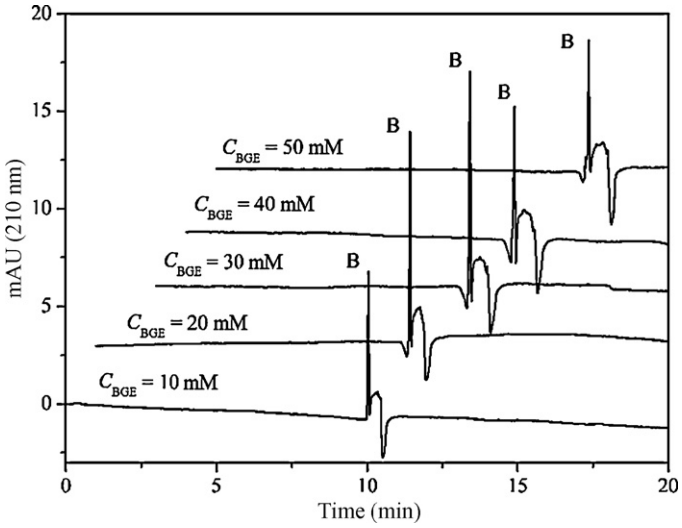

Fig. 1. Effect of the background electrolytes (BGE) concentration, BGE, H_3BO_3 and NaH_2PO_4 solution (pH 4.0); micellar sample matrix, 20 mM SDS in the BGE; sample zone length, 35.7 cm (7.0 psi, 60 s); co-solvent buffer plug, 1.28 cm (0.5 psi, 50 s) BGE in methanol/water (90:10, v/v); B, 1.0 μg/mL berberine in micellar sample matrix; applied voltage, +25 kV; detection wavelength, 210 nm; capillary, 60.2 cm total length (50.0 cm to detector).

slightly with the decrease of pH mainly because of the electroosmosis suppression, but a lower pH resulted in longer analysis time. So, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 (pH 4.0) was selected as running buffer.

The influence of co-solvent buffer plug length on the focusing efficiency was investigated from 0 to 2.56 cm (0.5 psi, 10–60 s). As illustrated in Fig. 2, there was a very weak focusing effect when the co-solvent buffer plug length was 0.43 cm; when it was changed in the range of 0.64–0.77 cm, the focusing effect was weak; when the co-solvent buffer plug length was increased to 0.85 cm, good focusing efficiency was obtained. And, the focusing efficiency did not increase obviously with further increase of co-solvent buffer plug length. This is because a necessary amount of co-solvent to support micelles collapse and large volume can associate with more analytes. Considering the partial diffusion, a longer than 0.85 cm co-

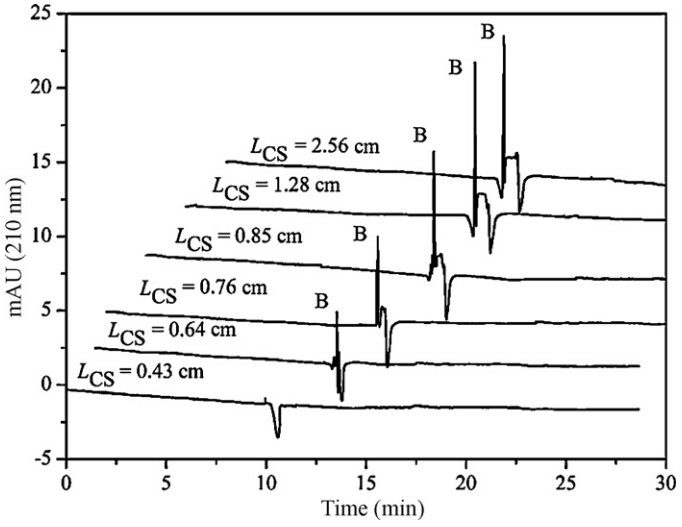

Fig. 2. Effect of co-solvent plug length, running buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); micellar sample matrix, 20 mM SDS, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); co-solvent buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 in methanol/water (90:10, v/v); other conditions are the same as in Fig. 1.

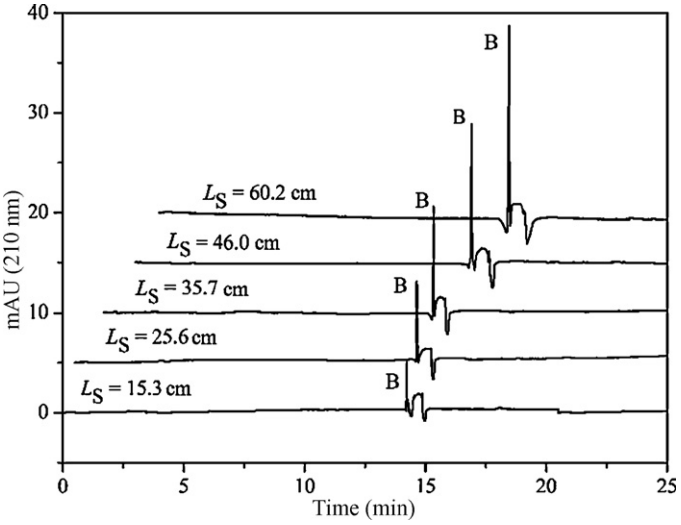

Fig. 3. Effect of injected sample zone length, co-solvent buffer plug length, 1.28 cm (0.5 psi, 50 s); other conditions are the same as in Fig. 1.

solvent buffer plug was favored, thus 1.28 cm was selected as the optimum co-solvent buffer plug length.

The injected sample zone length was studied from 5.1 cm to 60.2 cm (60 s, 1.0–12.0 psi), as shown in Fig. 3, and the focusing efficiency enhanced as the sample zone length increase. It can be deduced that the injected micellar sample has been completely focused and the focusing efficiency was proportional to the injected sample volume.

In this proposed preconcentration method, the concentration of SDS in the sample matrix was a significant factor. Quirino and Haddad studied the effect of SDS concentration in AFMC, and they noted that the CMC of SDS in their system was 3 mM and a practical SDS concentration of 5 mM was used. The concentration of SDS in AFMC needs to be at a relatively low level to ensure micelle collapse, because micelle collapse in AFMC is based on the micelle dilution. Here we also used micelle collapse technique, but the novelty is that the micelle collapse in the current technique is based on that SDS does not form micelles in methanol-water co-solvent. Therefore, SDS concentrations can be used in a higher range. Fig. 4 shows the effect of SDS concentrations in the range from 5 to 40 mM. It can be seen that the peak height did not increased with the increas-

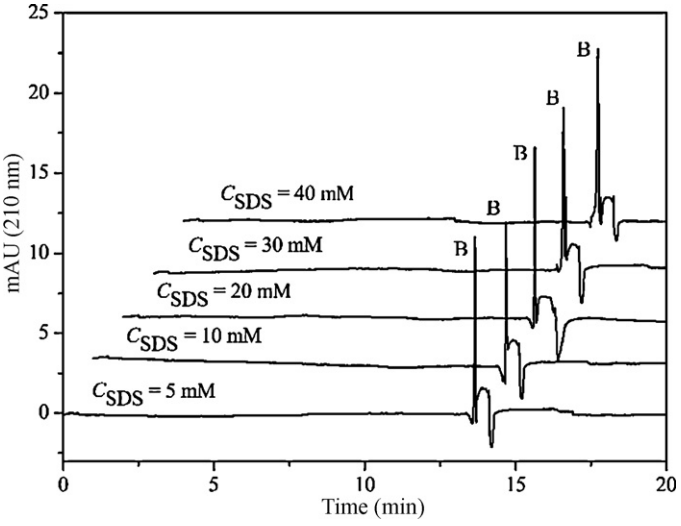

Fig. 4. Effect of SDS concentration, co-solvent buffer plug length, 1.28 cm (0.5 psi, 50 s); other conditions are the same as in Fig. 1.

Table 2
Performance of the current MSS-CZE method.[a]

	Berberine	Ephedrine
Linear range (ng/mL)	10–2400	100–12,000
Regression equation	$y = 10.9x + 0.19$[b]	$y = 3.6x - 0.040$[b]
Correlation coefficient	0.9955	0.9987
Limit of detection (S/N = 3) (ng/mL)	0.5	1.1
RSD of migration time (n = 5) (%)	5.1	5.5
RSD of peak height (n = 5) (%)	9.4	10.3

[a] *Conditions*: the same as those in Fig. 5.
[b] y: peak height (mAU), x: concentration (μg/mL).

ing of SDS concentration. But the peak became a little broaden. This maybe due to the sample destacking effect, which was caused by the mismatch in conductivities between the sample zone and the co-solvent buffer plug became more and more serious. As indicated by the obtained CE (25 kV and 60 cm capillary) currents for the sample matrix, with SDS concentration increasing from 5 to 40 mM, it changed from 10.5 μA to 11.9 μA. The currents obtained using the running buffer and co-solvent buffer was 10.3, and 7.2 μA, respectively. Since the difference in conductivity between the sample and co-solvent solution is less than 10-fold [8], the destacking affect weekly to the focusing efficiency. And the results show that the peak area did not change significantly. It suggested that a high concentration of SDS can be used.

Considering the focusing efficiency, analysis time, and peak shape together, the optimum conditions were selected as follows: Running buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); micellar sample matrix, 20 mM SDS, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); co-solvent buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 in methanol/water (90:10, v/v); co-solvent buffer plug length, 1.28 cm (0.5 psi, 30 s); sample zone length, 35.7 cm (7.0 psi, 60 s).

3.4. Method validity verification

Under the optimum conditions, the limits of detection (LODs), linearity, repeatability of the method were investigated using ephedrine and berberine as test analytes. The linearity was obtained by plotting the peak heights of the analytes against the corresponding concentrations, and the peak heights were employed for quantification. The repeatability of the method was determined by repeated injection (n = 6) of the standard mixture solutions at the concentration level of 1.0 μg/mL using the current method. The results were listed in Table 2. The LODs were 1.1 ng/mL for berberine and 0.5 ng/mL for ephedrine. In contrast to the results (1.2, 1.3, 5 ng/mL for berberine, and 0.15, 30, 670 ng/mL for ephedrine) found in literatures [42–47], the present method shows a little better in sensitivity. The focusing efficiency of the method was assessed by comparison its performance with that of normal CZE, as illustrated in Fig. 5. First, 0.25 cm (0.5 psi, 5 s) 300.0 μg/mL ephedrine and 300.0 μg/mL berberine mixed sample prepared in running buffer were conventionally injected in normal CZE, as shown in Fig. 5a. And then, a blank control experiment was done by the current MSS-CZE with 35.7 cm blank sample zone injected, as shown in Fig. 5b. Finally, 35.7 cm sample zone (1.0 μg/mL ephedrine and 1.0 μg/mL berberine) was injected by the current MSS-CZE, the result was shown in Fig. 5c. Comparing Fig. 5c with Fig. 5a, sensitivity was improved, the peak shapes of ephedrine and berberine became narrower about 2.3-folds and 2.1-folds, respectively, while the resolution significantly decreased. This was due to the fact that, in the present MSS-CZE, a large sample volume (150-folds of the normal CZE injection volumes) was injected, thus shorter effective capillary length was left for separation after the focusing process, thereby causing decrease in resolution. Therefore, the injection volume should be limited to

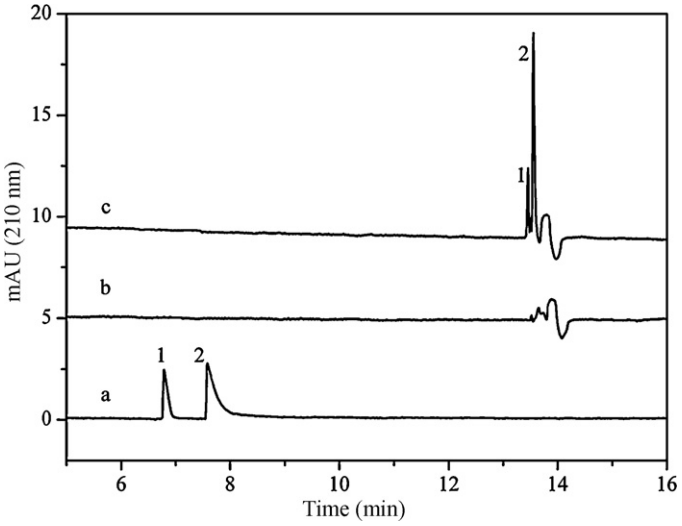

Fig. 5. Focusing efficiency calculation. (a) Normal CZE, injection was maintained for 5 s under the pressure of 0.5 psi, concentrations of ephedrine and berberine: 300 μg/mL, (b) the current MSS-CZE, blank sample, (c) the current MSS-CZE, concentrations of ephedrine and berberine: 1.0 μg/mL; sample zone length, 35.7 cm (7.0 psi, 60 s); 1.28 cm (0.5 psi, 50 s) co-solvent plug. Running buffer, 20 mM H_3BO_3 and 20 mM NaH_2PO_4 solution (pH 4.0); micellar sample matrix, 20 mM H_3BO_3, 20 mM NaH_2PO_4 and 20 mM SDS solution (pH 4.0); applied voltage, +25 kV; detection wavelength, 210 nm; capillary, 60.2 cm total length (50.0 cm to detector). 1, ephedrine; 2, berberine.

an appropriate amount. On the other hand, the shorter separation capillary leaded to less diffusion. This effect and the MSS focusing together improved the peak shapes. Though there were some limitations of the maximum injection volumes to meet the sufficient resolution, 300 and 1036-fold enhancements in peak heights for ephedrine and berberine were still obtained, respectively, as calculated through dividing the peak height in Fig. 5c by that in Fig. 5a, after correction for the dilution factor. It was worth noting that MSS showed higher efficient to berberine than ephedrine. This maybe attributes to that berberine is more hydrophobic and therefore has stronger affinity to SDS micelles than ephedrine, which suggested that MSS maybe more suitable for hydrophobic analytes. The high sensitivity enhancement factor and short analysis time (only 15 min) suggest the high efficiency of the method.

3.5. Application

In order to check the applicability of the proposed MSS-CZE, urine samples were analyzed. Under the optimum conditions discussed above, recovery experiments were carried out by determination of the two test alkaloids in spiked urine sample. The urine samples were spiked with a mixture of the two alkaloids (ephedrine and berberine) at different levels (0.5, 1.0, 1.5 and 2.0 μg/mL for each one) according to the procedure described in Section 2.3. Each level was prepared in triplicate and each sample was injected three times. The peaks were identified by the standard addition methods. The results were shown in Table 3, the recovery of the two alkaloids ranged from 92 to 103%. A typical electropherogram of 1.0 μg/mL ephedrine and berberine spiked urine sample was presented in Fig. 6. According to reports [48–50], the sensitivities for detecting berberine in human urine were 1 ng/mL in lower limit of quantification (LLOQ), 0.1 ng/mL, and 2.3 ng/mL in LOD, respectively. So, it indicated that this method was suitable for detecting berberine in urine with a LOD of 1.1 ng/mL. Moreover, as the LOD for ephedrine was 0.5 ng/mL, which was found to be adequate for the usual analytical requirements (10 μg/mL for ephedrine) in doping control [51]. The method proposed here demonstrated to be useful for the quantitation of the two test alkaloids as possible residues in urine sample, where the presence of drug residues should be avoided.

Table 3
Recovery study for the two test alkaloids at different spiked level in urine sample[a] (n = 9).

		Berberine	Ephedrine
0.5 μg/mL	Recovery (%)	95	97
	RSD (%)	8.5	5.2
1.0 μg/mL	Recovery (%)	92	103
	RSD (%)	7.2	7.7
1.5 μg/mL	Recovery (%)	101	95
	RSD (%)	7.3	8.4
2.0 μg/mL	Recovery (%)	94	96
	RSD (%)	5.8	6.8

[a] *Conditions*: the same as those in Fig. 6.

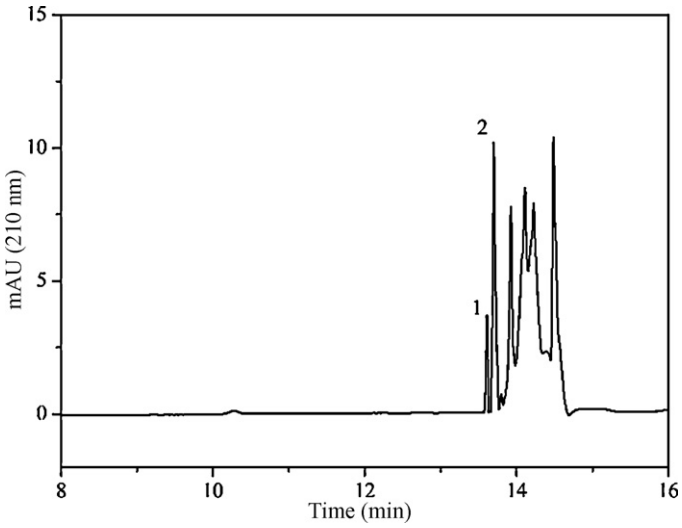

Fig. 6. Electropherogram of 1.0 μg/mL ephedrine and berberine spiked urine sample. Co-solvent plug length, 1.28 cm (0.5 psi, 50 s); other conditions are the same as in Fig. 1.

4. Conclusion

In the present paper, we described the use of micelle collapse in co-solvent (methanol–water) for MSS. The application of this proposed MSS in CZE for the analysis of two alkaloids (ephedrine and berberine) was demonstrated. Under the optimal conditions, 300 and 1036-fold sensitivity enhancements were obtained for ephedrine and berberine, respectively, within 15 min. These results demonstrated that this method is highly efficient. We expect this work to be helpful in environmental and biological analysis as well as further development of new on-line preconcentration methods.

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