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Micelle to solvent stacking of two alkaloids in nonaqueous capillary electrophoresis

Hua-dong Zhu[a,b], Wen-juan Lü[a,b], Hong-hong Li[a,b], Yan-hua Ma[a,b], Shao-qiang Hu[a,b], Hong-li Chen[a,b], Xing-guo Chen[a,b,c,*]

[a] State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, China
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, China
[c] Key Laboratory of Nonferrous Metal Chemistry and Resources Utilization of Gansu Province, Lanzhou 730000, China

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ABSTRACT

This paper for the first time describes the development of micelle to solvent stacking (MSS) to nonaqueous capillary electrophoresis (NACE). In this proposed MSS-NACE, sodium dodecyl sulfate (SDS) micelles transport, release, and focus analytes from the sample solution to the running buffer using methanol as their solvent. After the focusing step, the focused analytes were separated *via* NACE. The focusing mechanism and influencing factors were discussed using berberine (BBR) and jatrorrhizine (JTZ) as model compounds. And the optimum condition was obtained as following: 50 mM ammonium acetate, 6% (v/v) acetic acid and 10 mM SDS in redistilled water as sample matrix, 50 mM ammonium acetate and 6% (v/v) acetic acid in pure methanol as the running buffer, −20 kV focusing voltage with 30 min focusing time. Under these conditions, this method afforded limits of detection (S/N = 3) of 0.002 μg/mL and 0.003 μg/mL for BBR and JTZ, respectively. In contrast to conventional NACE, the concentration sensitivity was improved 128–153-fold.

1. Introduction

Capillary electrophoresis (CE) is a powerful and versatile analytical separation technique due to the advantages of high efficiency, short analysis time and small sample requirements. Nonaqueous capillary electrophoresis (NACE) follows the same theoretical rules of aqueous CE, in which an organic solvent replaces the water. Therefore, besides the merits presented by aqueous CE, NACE affords several additional advantages including the superior separation performance of hydrophobic compounds [1–3], the improvement on selectivity owing to the decrease in wall adsorption effects, and the reduction in the migration time as a result of the allowable high electric field strength [4]. However, because of the short optical path length across the capillary, the low detection concentration sensitivity in conventional aqueous CE is still a problem in NACE.

To solve the problem of low concentration sensitivity, on-line sample preconcentration is often used because it requires no modification of current commercial instrumentation [5,6]. However, until now, there are few papers reporting on-line sample concentration for NACE [7–21], and the available techniques are only pseudo transient isotachophoresis (Pseudo t-ITP), large-volume sample stacking using the EOF pump (LVSEP), field amplified sample stacking (FASS), or different combinations, such as electrokinetic supercharging (EKS) and large-volume sample stacking using the EOF pump-anionic selective exhaustive injection (LVSEP-ASEI). The basic strategies of all these techniques except Pseudo t-ITP rely on creating distinct conductivity mismatch between the sample zone and the buffer zone, thereby causing the analytes' electrophoretic velocities change and the subsequent stacking at the sample–buffer boundary. Unfortunately, because the nonaqueous solutions have very low conductivity, it is difficult to meet the requirement of conductivity mismatch for sample stacking. Therefore, new developments of on-line preconcentration techniques for NACE are urgently demanded.

Recently, an interesting on-line preconcentration approach termed as analyte focusing by micelle collapse (AFMC) was established by Quirino [22,23], where the sample is prepared in a micellar matrix that contains a high mobility electrolyte salt. The micelles in the sample zone transport, release, and accumulate the bound analytes in the boundary zone between the sample and separation solution. Thereafter, different variations of AFMC were developed, such as micelle to solvent stacking (MSS) [24,25], and the combination of sweeping and MSS [26,27]. In MSS, the sample is prepared in

* Corresponding author at: Department of Chemistry, Lanzhou University, South Tianshui Road #222, Lanzhou 730000, Gansu, China. Tel.: +86 931 8912763; fax: +86 931 8912582.
E-mail address: chenxg@lzu.edu.cn (X.-g. Chen).

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a micellar solution without organic solvent, and the running buffer solution is modified by an organic solvent. Because the organic solvent in the running buffer affects the micelle interaction to the analytes, the effective electrophoretic directions of the analytes will reverse at the micelle to solvent stacking boundary (MSSB), therefore causing analyte focusing. Later, based on the same mechanism, micelle to trapping solution stacking [28,29] was introduced by inserting a section of trapping solution (containing high percents of organic solvent) to induce micelle collapse. The analytes change their electrophoretic mobilities in the trapping solution after being released by micelle collapse. However, these methods have not been applied in NACE. It is worth noting that the organic solvents used to affect the micelle interaction to the analytes or to induce micelle collapse in MSS are methanol, ethanol or acetonitrile, all of them are the commonly used separation media for NACE. Therefore, we reasoned that the technique of MSS using methanol to induce micelle collapse will be promising to improve detection sensitivity for NACE.

In this paper, based on methanol assistant micelle collapse coupled with sample matrix removing by polarity switching, MSS was applied to NACE (MSS-NACE) for on-line preconcentration for the first time. The mechanism of focusing and the strategy for increasing the enrichment factor were discussed. In order to demonstrate the feasibility of this stacking method, berberine (BBR) and jatrorrhizine (JTZ) were used as model analytes and their contents in spiked urine were determined.

2. Experimental

2.1. Instrumentation

All capillary electropherograms were recorded on a Beckman P/ACE MDQ electrophoresis 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 20 °C. New capillary was conditioned prior to its first use by flushing at 20.0 psi 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. Between runs, the capillary was rinsed at 20 psi sequentially with redistilled water (2 min), 0.1 M NaOH solution (2 min), redistilled water (2 min) and running buffer (3 min).

2.2. Regents and sample preparation

All solvents and reagents were of analytical grade and used without further purification. Berberine (BBR) and jatrorrhizine (JTZ) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ammonium acetate (NH_4Ac), glacial acetic acid (HAc), methanol and sodium dodecyl sulfate (SDS) were products of Tianjin Chemical Reagent Factory (Tianjin, China). Redistilled water was used throughout. Stock solutions of 0.2 M SDS and 1.0 M NH_4Ac were prepared in redistilled water. Another stock solution of 1.0 M NH_4Ac was prepared in pure methanol. Running buffer was 50 mM NH_4Ac and 6% (v/v) HAc (pH*, 8.23) in pure methanol. Sample matrix was 50 mM NH_4Ac, 6% (v/v) HAc (pH, 3.29) and 10 mM SDS in redistilled water. pH was adjusted with glacial acetic acid using a PHS-3B pH meter (Shanghai Precision & Scientific Instrument Co., Ltd.). Standard stock solutions of 0.50 mg/mL BBR and 0.50 mg/mL JTZ were prepared in methanol/water (10:90, v/v) and stored in refrigerator at 4 °C.

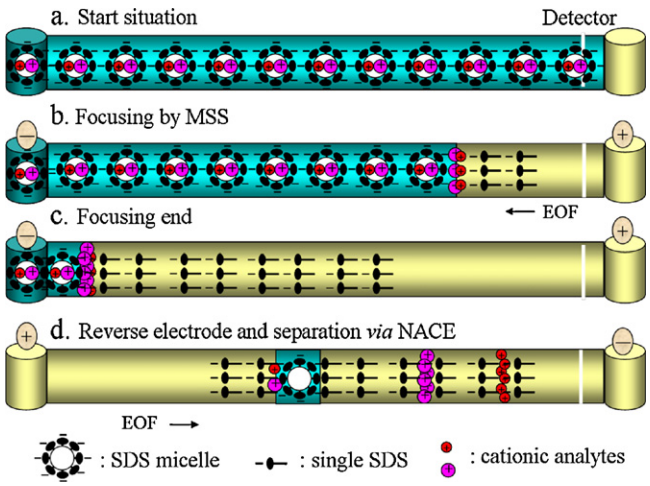

Fig. 1. Schematic evolution of analyte focusing in MSS-NACE: (a) filling the whole capillary with the micellar sample from the inlet vial and the outlet vial was filled with the running buffer, (b) removal of the sample matrix with the running buffer during sample focusing under a reverse potential, (c) the focusing process finished after a certain focusing time, (d) separation of the focused analytes by NACE.

Urine sample was prepared by the following procedures: fresh urine was collected from a healthy volunteer, after frozen in a refrigerator overnight, the urine was unfrozen at room temperature and centrifuged at 12,000 rpm for 3 min. Then, 1.0 mL of the supernatant was collected and diluted to 10.0 mL with the sample matrix, which was the urine sample for analysis. Spiked urine sample at different concentration levels was prepared by the standard addition of appropriate BBR and JTZ to the treated urine sample. All solutions were filtered through 0.45 μm filters prior to CE experiments.

3. Results and discussion

3.1. MSS-NACE model

Fig. 1 depicts the simple model of MSS-NACE. Initially (Fig. 1a), the whole capillary column is filled with the micellar sample solution (dark green blank parts) by hydrodynamic injection (20.0 psi, 1 min) from the inlet vial to a waste vial, and the outlet vial is filled with the running buffer. Then, under a reverse potential, the running buffer is brought into the capillary from the outlet vial by the electroosmotic flow (EOF) and a part of sample matrix is removed (Fig. 1b). Simultaneously, because the SDS micelles have negative charges, the micelles will continuously migrate and collapse into the running buffer where the concentration of the SDS drops below its critical micelle concentration (CMC), thereby releasing and focusing the transported organic cations at the MSS boundary (MSSB). After a certain focusing time, a majority of the sample matrix is replaced by the running buffer (Fig. 1c). Finally, the inlet vial of micellar sample solution is changed to a vial of running buffer, and then a positive voltage is applied (Fig. 1d), consequently, the focused analytes are separated *via* NACE.

3.2. Optimization of the stacking conditions

The experimental results indicate that the CZE (20 kV and 60.2 cm capillary) currents of the sample matrix and the running buffer are 23.2 and 13.5 μA, respectively, which means that the conductivity difference between the running buffer and the sample plug does not exceed 42%. Electric stacking is not considered to have a significant effect since the conductivity difference is less

than 10-fold [30]. According to the MSS-NACE model, though the entire capillary is filled with the sample solution for experimental convenience, only a portion of whole injected sample entered to the NACE after focusing process. In order to simplify the study, all the transported analytes are assumed focused at MSSB in the focusing process. So, under this premise, the focusing efficiency is proportional to the total amount of the analytes transported by SDS micelles, which is associated with the focusing time (t) and the apparent electrophoretic velocity of the analyte in the SDS micellar sample zone ($v_{s,app}$).

Generally speaking, the background electrolyte (BGE) concentration, pH, SDS micelle concentration, analysis time and applied voltage were the usually investigated aspects in CE study. Therefore, taking consideration of the routine CE study and the MSS mechanism, the factors including background electrolyte (BGE) concentration, the buffer pH*, focusing time, focusing voltage and SDS micelle concentration were all investigated. To simplify the investigation, the concentrations of BGE (the total concentration of ammonium acetate and acetic acid in BGE) were kept equivalent in the running buffer and sample matrix. The separation voltage was set as 20 kV. The test analytes are 1.0 μg/mL BBR and 1.0 μg/mL JTZ.

Under a constant concentration ratio of acetic acid to ammonium acetate (pH*, 8.32), the effect of the BGE concentrations was investigated in the range 30–70 mM, using 10 mM SDS, 30 min focusing time, and −20 kV focusing voltage. The results show that the peak heights and peak areas are almost at the same level at all the BGE concentrations, it is as expected that the focusing efficiency depends on the apparent electrophoretic velocities of the analytes, which associate to the electroosmotic flow and the analytes' effective electrophoretic migration velocities. As both magnitudes decrease with the increasing of BGE concentration, the BGE concentrations weakly affect the focusing efficiency. The suppression of electroosmosis is due to the increase in ionic strength, that causes a reduction of the thickness of the electric double layer, thereby decreasing the zeta potential. And the decrease of the analytes' effective electrophoretic velocities was caused by the increase in the mass transfer resistance and buffer viscosity, because of the increase of ionic strength [12]. As the variation of BGE concentration affects the focusing efficiency weakly, the median BGE concentration of 50 mM was used.

Under the condition of 50 mM ammonium acetate, 30 min focusing time, 10 mM SDS, and −20 kV focusing voltage, the effect of acetic acid percentage (buffer pH*) on the sensitivity was varied at 3%, 4%, 5%, 6%, 7% (v/v, corresponding to the buffer pH*, 8.42, 8.32, 8.23, 8.15). As shown in Fig. 2, both the peak heights and peak areas increase with the decreasing of the pH*. A low pH* condition leads to a low EOF, and $v_{s,app}$ increase, so the focusing efficiency will benefit from a low EOF. However, the resolution decreases with the decreasing of pH*. This attributes to that, in the focusing step, under the condition of a lower pH* (low EOF), the focusing MSSB migrated a relatively short distance within the focusing time from outlet to inlet. Then, in the subsequent separation step, this short MSSB migration distance in the capillary was used for separation, thus leading to the worse resolution. Compromising the focusing efficiency and resolution, the optimum pH* was chosen as 8.23.

The focusing time was investigated from 10 to 60 min. As shown in Fig. 3, when the focusing time was 10 min, the focusing efficiency was not good and the peaks for the two model analytes were overlapped. It seems that 10 min is too short to focus enough analytes, and the focusing boundary is still near the detection window. This could be indicated by the poor resolution as well as the short interval of the retention time between the analyte's peak and negative peak (aqueous sample zone). When the focusing time was prolonged to 30 min, the peak heights and peak areas were both increased. With further increasing the focusing time, the peak heights slightly altered, and the peak shape became broader and

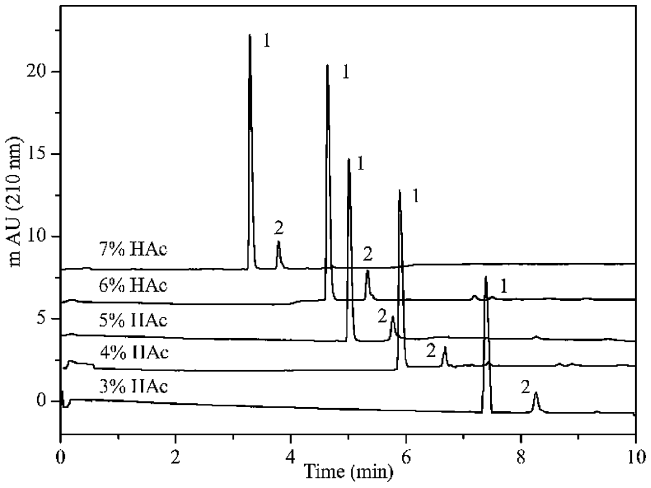

Fig. 2. Effect of acetic acid concentrations from 3% to 7% (v/v) on the sensitivity. CE conditions: 50 mM ammonium acetate; 30 min focusing time; 10 mM SDS (sample matrix); focusing voltage, −20 kV; separation voltage, 20 kV; detection wavelength, 210 nm; capillary, 60.2 cm total length (50.0 cm to detector). 1, 1.0 μg/mL BBR; 2, 1.0 μg/mL JTZ.

a little tailed, probably due to the diffusion effect. Thus, 30 min is the optimum focusing time and analytes focused with 30 min may be the maximum amount that can be concentrated by the present method. In contrast to the CE modes without on-line preconcentration and previous MSS method [24–29], 30 min seems a little longer for injection and focusing, however, it is worthy in some case of special trace analysis.

The effect of focusing voltage was investigated ranging from −10 to −30 kV under the condition of keeping constant the focusing voltage × focusing time. The results are shown in Table 1, in the case of equivalent focusing voltage × focusing time, focusing efficiencies (the peak heights and peak areas) are almost at the same level as the actual injection lengths are nearly equal. Considering the time consumption, higher voltage was in favor. But the optimum focusing voltage was −20 kV, further increasing focusing voltage causes the current breakdown because of the higher running current and the subsequent Joule heat. So −20 kV was selected as the focusing voltage.

The effect of SDS concentration was also investigated. First, SDS micelles were collapsed in the running buffer according to the

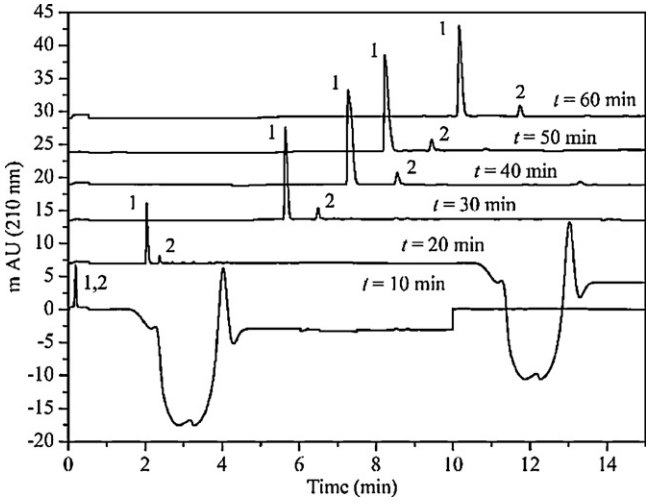

Fig. 3. Effect of focusing time from 10 to 60 min on the sensitivity. BGE, 50 mM ammonium acetate, 6% (v/v) acetic acid, and other conditions are the same as in Fig. 2.

Table 1
Effect of focusing voltage and time on the efficiency[a] (n = 5).

V (kV) × t (min)	Actual injection length (cm)	Peak heights (m AU)		Peak areas		Retention time (min)		R_S
		BBR	JTZ	BBR	JTZ	BBR	JTZ	
−10 × 60	42.2[b]	12.6	1.61	64104	6079	6.75	7.62	3.47
−15 × 40	43.9[b]	13.1	1.65	66607	6227	5.78	6.61	3.68
−20 × 30	45.6[b]	13.7	1.71	68612	6313	5.69	6.48	3.83

R_S, resolution.
[a] Other conditions are the same as those in Fig. 2.
[b] The actual injection length in each case was calculated as follows: measuring the weight of the sample matrix in the entire capillary, then measuring the weight of the sample matrix pumped out in each focusing conditions, finally, calculating its weight percentage, which is equal to its actual injection length percentage.

method described by Cifuentes et al. [31], using various concentrations of SDS from 1 to 20 mM, 50 mM NH_4Ac and 6% (v/v) HAc in pure methanol as the running buffer. By plotting the CZE electric current values versus the SDS concentrations, a good linearity was obtained with a correlation coefficient of 0.9992. Therefore, it was proved that SDS micelles were convincingly collapsed in the running buffer within the range of investigation. Then, the effect of SDS concentration in the sample matrix was investigated in the range 5.0–20.0 mM. The results were the same as described by Liu and Deng [28]: with the increase of SDS concentration, the peak heights and peak areas increased firstly and then decreased. The concentration corresponding to the optimum focusing efficiency is 10 mM, which is close to the CMC of SDS in water (around 8.0 mM). The results indicate that using concentrations of surfactant closer to its CMC in MSS will be more effective. So, 10 mM SDS was adopted.

In sum, the optimum condition was selected as follows: sample matrix, 50 mM ammonium acetate, 6% (v/v) acetic acid (pH, 3.29) and 10 mM SDS in redistilled water; running buffer, 50 mM ammonium acetate and 6% (v/v) acetic acid in pure methanol (pH*, 8.23); focusing time, 30 min; focusing voltage, −20 kV; separation voltage, 20 kV.

3.3. Performance of MSS-NACE

Under the optimum condition, the limits of detection (LODs), linearity, and repeatability were calculated and listed in Table 2. The linearity was obtained by plotting the peak heights of the analytes against the corresponding concentrations. The calibration curves exhibit good linear behavior over the concentration range of 0.01–2.4 μg/mL with correlation coefficients higher than 0.9990. As the peak areas revealed similar performance, peak heights were employed for quantification. The repeatability of the method was determined by five replicate injections of the standard mixture solutions at the concentration levels of 0.05, 0.1, 0.5, 1.0 and 2.0 μg/mL for both BBR and JTZ. The average relative standard deviations (RSDs) were 4.4, 3.7% with peak height evaluation and 3.6, 4.9% with migration time evaluation for BBR and JTZ, respectively.

3.4. Applications

To further examine the applicability of MSS-NACE, the proposed method was applied to determine trace BBR and JTZ in urine sample. As a comparison, first, the mixture solution of 100.0 μg/mL BBR and 100.0 μg/mL JTZ prepared in the running buffer was conventionally injected in normal NACE, as shown in Fig. 4a. Then, a blank control experiment for urine sample by MSS-NACE was done, as shown in Fig. 4b. Finally, 1.0 μg/mL BBR and 1.0 μg/mL JTZ in urine sample were analyzed by MSS-NACE, the result is shown in Fig. 4c. The peaks were identified by the standard addition methods and the magnitude of focusing efficiency was calculated as follows: the peak height obtained in Fig. 4c divided by that obtained in Fig. 4a, after correction for the dilution factor of 100. 128 and 153-fold enhancements on peak heights for BBR and JTZ were obtained, respectively.

The accuracy of the method was evaluated by analyzing BBR and JTZ spiked urine samples at different levels (0.05, 0.1, 0.5, 1.0 and 2.0 μg/mL for each one). The results are presented in Table 3.

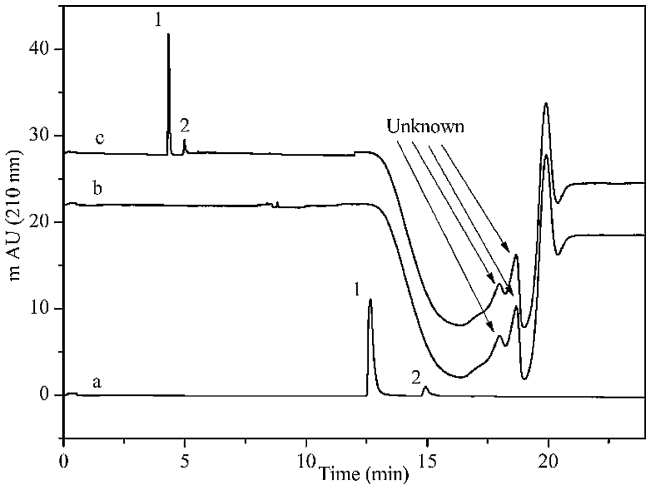

Fig. 4. The evaluation on MSS-NACE by applying it to analysis spiked urine sample and comparing with normal NACE. (a) Normal NACE, 5 s hydrodynamic injection of 100 μg/mL BBR and 100 μg/mL JTZ standards mixture; (b) blank control experiment of MSS-NACE; (c) MSS-NACE, 1, 1.0 μg/mL BBR; 2, 1.0 μg/mL JTZ; BGE, 50 mM ammonium acetate, 6% (v/v) acetic acid; other conditions are the same as in Fig. 2.

Table 2
Performance of MSS-NACE.[a]

	BBR	JTZ
Linear range (μg/mL)	0.01–2.4	0.01–2.4
Regression equation	$y = 14.1x + 0.17$[b]	$y = 1.7x + 0.61$[b]
Correlation coefficient	0.9991	0.9992
LOD (S/N = 3) (μg/mL)	0.002	0.003
RSD of migration time (n = 5)	3.6%	4.9%
RSD of peak height (n = 5)	4.4%	3.7%

[a] CE conditions were the same as those in Fig. 4c.
[b] y, peak height (m AU); x, concentration (μg/mL).

Table 3
Results for the determination of the two components in spiked urine samples[a] (n = 9).

		BBR	JTZ
0.05 (μg/mL)	Recovery (%)	97	105
	RSD (%)	4.2	3.3
0.10 (μg/mL)	Recovery (%)	95	103
	RSD (%)	4.4	3.5
0.50 (μg/mL)	Recovery (%)	99	98
	RSD (%)	3.7	3.1
1.00 (μg/mL)	Recovery (%)	102	97
	RSD (%)	3.9	3.3
2.00 (μg/mL)	Recovery (%)	95	95
	RSD (%)	3.8	3.3

[a] Conditions: the same as those in Fig. 4c.

Obviously, trace amount of rudimental metabolized medicine in human urine could be determined by the present focusing technique. In addition, it is worth noting that, in this proposed method, the urine sample is prepared by dilution with the sample matrix. Compared with other stacking techniques in NACE, in which the sample is prepared in organic solvent, the current sample pretreatments are straightforward and convenient. Furthermore, different from the previous stacking techniques in NACE, the analyte focusing in MSS-NACE occurs at the MSSB between the aqueous micellar sample and the nonaqueous running buffer. Therefore, this method could be applied to focus the aqueous samples in NACE.

4. Conclusions

In the present paper, MSS was applied to NACE to focus cationic analytes. This work represented the application of MSS in NACE for the first time. Experimental investigations of this new technique were carried out. The results indicated that the focusing efficiency was mainly associated with the focusing time, focusing voltage and the EOF. The proposed MSS-NACE was successfully applied to urine sample for focusing BBR and JTZ approximate 128 and 153-fold, respectively. These results demonstrated the feasibility of MSS in NACE.

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