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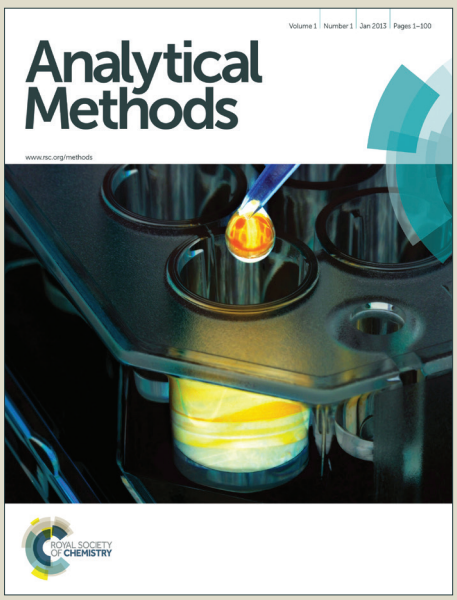

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On-line two-step stacking for the preconcentration and determination of quinolizidine alkaloids by capillary electrophoresis

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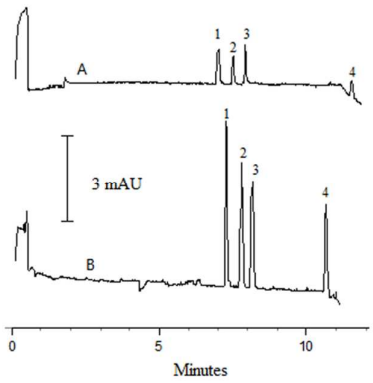

Comparison of the electropherograms obtained by typical CZE (A) and sweeping-MSS -CZE (B)

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Abstract

In this research, a novel on-line two-step stacking preconcentration method by sweeping and micelle to solvent stacking in capillary zone electrophoresis was developed and validated for the simultaneous determination of quinolizidine alkaloids (sophocarpine, matrine and oxymatrine) in traditional Chinese medicines. Strychnine was used as an internal standard. The main parameters that affect the separation and sensitivity were investigated and optimized. Under the optimum conditions, the sensitivity enhancement factors obtained by the developed method for the analytes were from 42- to 52-fold. The method showed a good linearity over the range of 0.1-10.0 μg mL^{-1} for sophocarpine, matrine and oxymatrine with the correlation coefficients (r) varying from 0.9992 to 0.9996. The limits of detection (S/N = 3) were 0.02-0.03 μg mL^{-1}. The intra-day (n = 8) and inter-day (n = 5) precisions of the method expressed as the relative standard deviation (RSD) were found to be less than 10%. The recoveries of the analytes by the method for the analysis of traditional Chinese medicines were in the range from 87.5% to 109.0% with RSDs (n = 3) less than 9.1%.

Keywords: Capillary electrophoresis; Micelle to solvent stacking; Quinolizidine alkaloids; Sweeping

Introduction

Capillary electrophoresis (CE) has been considered as an efficient and cost-effective analytical technique with many advantages, including high separation efficiency, fast separation speed and small sample and reagent consumption. Unfortunately, because of the small inner diameter of the capillary and the small sample injection amount, the CE detection sensitivity becomes a problem when the common UV detection was used. The most facile way to improve the concentration sensitivity in CE is on-line preconcentration since it can be performed easily by merely adjusting the composition of sample solution and background solution (BGS) without the need to modify the commercial CE instrument. In recent years, several on-line concentration techniques,[1-3] such as transient isotachophoresis,[4] dynamic pH junction (DypH),[5,6] sweeping,[7,8] field amplification (FA),[9,10] large-volume sample stacking (LVSS),[11] analyte focusing by micelle collapse[12] and micelle to solvent stacking (MSS),[13-15] have been developed. MSS, as one of the novel CE stacking techniques, was first introduced by Quirino in 2009.[14] The focusing was based on the reversal of the direction of the effective electrophoretic mobility of the analytes at micelle to solvent stacking boundary (MSSB) formed between the sample and BGS. MSS has been applied in different CE operation mode, such as capillary zone electrophoresis (CZE),[13,14,16-18] micellar electrokinetic chromatography (MEKC)[19,20] and non-aqueous capillary electrophoresis (NACE).[21]

In order to further improve the detection sensitivity of CE, the coupling of multiple modes of on-line stacking has received increasing attention in recent years. In this regard, two-step stacking featuring sweeping and MSS was first established for the analysis of organic cations[22,23] and anions.[24,25] Thereafter, the coupling of MSS with other on-line preconcentration techniques, such as large amount sample electrokinetic stacking injection,[26] simultaneous electrokinetic and

hydrodynamic injection[27] and field enhanced sample injection,[28] have been reported. Cheng et al combined three stacking techniques, i.e., LVSS, DypH and sweeping, for the concentration of methotrexate and its metabolites in whole blood and cerebrospinal fluid in MEKC mode.[29,30] The results indicated that the detection sensitivity of CE was much improved by using the multi-step stacking techniques.

An appropriate concentration of quinolizidine alkaloids exhibits potentially useful pharmacological activities such as sedative, depressant, analgesic, hypothermic, anti-tumor, antipyretic and cardiotonic activities.[31] However, they could become toxic to human and livestock at high concentrations.[32] Therefore, sensitive, rapid and effective analytical methods for the determination of quinolizidine alkaloids are of great interest. To date, several literature methods are available for the determination of quinolizidine alkaloids either by liquid chromatography (LC),[33] gas chromatography (GC)[34] or NACE.[10,35,36]

Recently, the combined use of both sweeping and MSS in capillary zone electrophoresis for the on-line preconcentration of strychnine and brucine from traditional Chinese herbal medicines has been developed by us.[23] As a continuation of our previous work, in this study, the application of the combination of sweeping with MSS was further explored for the simultaneous on-line preconcentration and determination of quinolizidine alkaloids in traditional Chinese medicines. The main factors that could affect the stacking efficiency were investigated and as a result, a simple on-line two-step stacking preconcentration method by CE was developed for the simultaneous determination of quinolizidine alkaloids in traditional Chinese medicines.

Experimental

Apparatus

All CE experiments were performed on a Beckman P/ACE MDQ Capillary Electrophoresis System (Fullerton, CA, USA) equipped with an auto sampler and a diode array detector. An uncoated fused-silica capillary (Yongnian Ruifeng Optical Fiber Factory, Hebei, China) of 50 cm (effective length, 41.5 cm) × 75 μm I.D. was used throughout the experiments. Data acquisition and instrument control were carried out using Beckman P/ACE MDQ 32 Karat software. The separation was performed at 25 ℃ with a voltage of 20 kV throughout this study. Unless otherwise stated, the detection wavelength was set at 200 nm. A PHS-3C pH meter (Hangzhou Dongxing Instrument Factory, Hangzhou, China) was used for pH measurements.

Reagents, chemicals and materials

Sophocarpine, matrine, oxymatrine and strychnine (I.S.) (all >99%) were purchased from Chinese National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). *Kushen tablets* were purchased from Liaoning Jindan Pharmaceutical Co., Ltd. (Liaoning, China) and *Kushen shiyang lotions* from Inner Mongolian Renhechuntian Bio-Technology Co., Ltd. (Inner Mongolia, China). Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate, sodium hydroxide, hydrochloric acid and MeOH (HPLC-grade) were the products of Kaitong Chemical Reagent Co., Ltd. (Tianjin, China). All reagents were of analytical grade or higher, and were used without further purification. All the solvents were filtered through a 0.45 μm Micro Science membrane filter (Tianjin Automatic Science Instrument Co., Ltd.

Tianjin, China). The water used throughout the work was double-distilled using SZ-93 automatic double-distiller (Shanghai Yarong Biochemistry Instrumental Factory, Shanghai, China).

A mixture stock solution containing sophocarpine, matrine and oxymatrine each at 1.0 mg mL^{-1}, and a stock solution of strychnine at 1.0 mg mL^{-1} were prepared in MeOH and stored in refrigerator at 4 ℃. Appropriate amounts of the stock solution were first dried under gentle stream of compressed air and then redissolved in 15 mM NH_4Ac at pH 2.0 to prepare a series of standard solutions. The BGS and micellar solution were prepared fresh every day and sonicated for 10 min prior to use.

Preparation of samples

Kushen tablets were ground and 0.4 g of the resultant powder was weighed. The powder was wetted with 500 μL of 25% (w/w) ammonium hydroxide for 5 min. After being soaked in 10.0 mL chloroform for half an hour, the mixture was refluxed for 0.5 h and then filtered. The chloroform was evaporated by reduced pressure distillation at 60 ℃ to dryness and the residue was dissolved with 10.0 mL MeOH, and the solution was filtered through a 0.45 μm syringe filters. To a 60 μL aliquot, 10.0 μL of strychnine (I.S.) stock solution was added. The mixture solution was evaporated under gentle stream of compressed air to dryness. Then the sample solutions were obtained by dissolving the residues with 10.0 mL 15 mM NH_4Ac at pH 2. All the sample solutions were filtered through a 0.45 μm syringe filter prior to CE experiments.

Kushenshiyang lotion sample (10.0 mL) was extracted by the addition of 500 μL of 25% (w/w) ammonium hydroxide and 10 mL chloroform. The mixtures were shaken for 5 min and the chloroform layer was isolated. The above extraction step was repeated three times and then all the

chloroform layers were collected together. Then, the chloroform was evaporated and the sample solution was prepared by the same procedures as described above for the preparation of *Kushen tablets*.

General electrophoresis procedure

Prior to first use, the new capillary was flushed in sequence with MeOH (20 min), water (10 min), 1.0 M sodium hydroxide (20 min), water (10 min) and finally BGS (10 min) at 20 psi. At the beginning of each working day, the capillary was conditioned with 1.0 M sodium hydroxide (5 min), water (5 min) and BGS (5 min). Between consecutive runs, the capillary was flushed with 1.0 M sodium hydroxide (3 min), then with water (3 min) and finally with the BGS (3 min).

For sweeping-MSS, the analytes were prepared in the electrolyte of 15 mM NH_4Ac at pH 2.0. The micellar solution was 15 mM SDS with 10 mM NH_4Ac (pH 2.0). The BGS was 50 mM NH_4Ac (pH 8.5) containing 40% MeOH (v/v). The ratio of the conductivity between the BGS, micellar solution and sample matrix was 1.20:1.15:1.00. After the injection of a short plug (0.5 psi, 30 s) of micellar solution, the sample was introduced into the capillary by hydrodynamic injection at 0.5 psi for 90 s.

Results and discussion

Focusing process

In the first step, the capillary was initially filled with the BGS (50 mM NH_4Ac at pH 8.5 containing 40% MeOH). Then, the micellar solution containing 10 mM NH_4Ac and 15 mM SDS

was introduced into the capillary by pressure injection as a short plug (0.5 psi, 30 s). Finally, the analytes dissolved in the electrolyte (15 mM NH_4Ac, pH 2.0) were injected into the capillary as a long plug (0.5 psi, 90 s). A similar conductivity for the BGS, micellar solution and sample solution was assumed to provide a homogenous electric field across the capillary.

After a positive voltage was applied, the electrokinetic velocities of the cationic analytes and anionic micelles were directed towards the cathode and anode, respectively. The micelles penetrated into sample zone and swept the cations. This continued until all the cations were swept by the micelles and the first step of sweeping stacking process was finished. Because the analytes were extracted into the anionic micellar phase, the effective electrophoretic mobility of the micelles-bound cations was directed towards the anode to micelle to solvent stacking boundary (MSSB).[13] At the MSSB, the analytes had less affinity towards the micelles due to the presence of high concentration of organic solvent. This caused the effective electrophoretic mobility of the analytes to be directed to the cathode. Then, the MSS progress began. With continuous electrophoresis, more and more of the anionic micelle-bound analytes migrated to anode and crossed the MSSB, and the analytes were accumulated leading to the enrichment of the analytes. After all the analytes were released from the micelle, the MSS process was accomplished. At last, the concentrated analytes were separated in CZE mode.

Effect of the concentration of MeOH in BGS

The presence of MeOH in the BGS is necessary for MSS to occur and the concentration of MeOH is important for achieving good peak shape and high separation efficiency. In this study, the effect of the percentage of MeOH in the BGS (50 mM NH_4Ac at pH 8.5) on the enrichment of the cationic alkaloids was studied in the concentration range between 20% and 50%. It can be seen

from Fig. 1A that the separation for sophocarpine, matrine and strychnine was poor when 20% MeOH was used; with the percentage of MeOH being increased, the resolutions and focusing efficiency of the alkaloids got better. The reason for this could be explained as follows. With high percentage of MeOH in BGS, the electroosmotic flow (EOF) would be reduced remarkably and a greater migration time difference between the analytes could be achieved. However, when the percentage of MeOH in the BGS was increased up to 50%, the peaks for the analytes became broader although the analytes were completely separated. Therefore, 40% MeOH was selected.

Effect of ammonium acetate concentration and pH value in BGS

The effect of the concentration of ammonium acetate in BGS was investigated by changing its concentration in the range from 25 to 100 mM (pH 8.5) while the MeOH concentration was kept constant at 40%. The results (Fig. 1B) showed that at 25 mM, the alkaloids could not be separated completely. As its concentration was changed to 50 mM or 75 mM, all of the analytes were baseline separated. However, when the concentration of ammonium acetate was increased to 100 mM, the peaks for the analytes became broadening with peak heights being decreased slightly. Based on the above results, 50 mM ammonium acetate in BGS was selected.

The pH of the BGS can affect the EOF, and therefore, will influence the migration time and resolution of the analytes. In this study, three different BGS pH values, i.e., 7.0, 8.5 and 10.0, were investigated. The results (see Fig. 1C) showed that the analytes could be baseline separated at pH 8.5. Hence, the pH of the BGS was chosen at 8.5.

Effect of the pH of sample solution

The pH of sample solution is one of the most important factors in the current study. Since the p*Ka* values of sophocarpine, matrine and oxymatrine are 7.22, 7.72 and 5.77,[37] the analytes will be

positively charged only when the pH value of sample solution is below 5.77, which is essential for MSS. Hence, the effect of sample pH on the separation and focusing of the analytes was examined in the range of 2.0-6.0. When the pH of sample solution was 6.0, a good separation and focusing for the analytes could not be achieved; when the pH was decreased, the resolution and focusing efficiency for the analytes were improved, and the best separation and sensitivity enhancement were obtained when the sample pH was at 2.0. Therefore, the sample pH was chosen at 2.0.

Effect of SDS concentration in micellar solution

The effect of surfactant concentration on sweeping is opposite to that on MSS. The increase of the surfactant concentration can improve the stacking ability of sweeping. However, if the surfactant concentration is too high, it will be difficult to release the analytes in the MSS process. Therefore, the best concentration of surfactant has to be selected so that the sweeping is sufficiently efficient but without much compromise for the MSS. In this study, the concentrations of SDS in micellar solution at 2, 5, 15 and 30 mM were investigated. As shown in Fig. 1D, when 2 mM SDS was used, the sweeping efficiency was poor. With the increase of the SDS concentration, the sweeping efficiency was increased. But when the concentration of SDS was increased to 30 mM, the separations between the analytes became deteriorated and the peaks of the analytes turned broad. Therefore, the concentration of SDS for further studies was selected at 15 mM.

Effect of injection time of sample solution and micellar solution

Effect of different injection times (30, 60, 90 and 120 s at 0.5 psi) of sample solution on the resolution of the analytes was studied. As a result, the peak heights were increased with the injection time being increased from 30 to 90 s. However, a substantial peak broadening and a significant loss of resolution were observed when the injection time was increased to 120 s.

Therefore, an injection of sample solution at 0.5 psi for 90 s was selected.

The effect of the injection time of the micellar solution on the stacking efficiency of the analytes was studied by changing the injection at 0.5 psi for 0, 15, 30 or 45 s, respectively, while the injection of the sample solution was kept constant at 0.5 psi for 90 s. The results indicated that the stacking efficiency of the analytes was increased with the injection time of micellar solution being increased from 0 s to 30 s and then decreased when the injection time was increased to 45 s. This may be because the too long flux of the micelles could broaden the focused zones at the MSSB. Therefore, the micellar solution injection at 0.5 psi for 30 s was chosen.

Method validation

A series of mixtur standard solutions containing sophocarpine, matrine and oxymatrine at seven concentration levels of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 μg mL^{-1} along with 1.0 μg mL^{-1} I.S. were prepared for the establishment of the calibration curves. For each concentration level, three replicate analyses were performed under the optimized conditions. . The ratio of the corrected peak area (peak area/migration time) of the analytes to that of the I.S. was used as the quantification signal. The results are summarized in Table 1. A good linearity was observed for the analytes in the range of 0.1-10.0 μg mL^{-1} with the correlation coefficients (r) ranging from 0.9992 to 0.9996. The limits of detection (LODs) at S/N =3 were between 0.02 and 0.03 μg mL^{-1}. The precision of the developed method was evaluated in terms of intra-day and inter-day variations through the analysis of the analyte standards at the concentration of 0.2 μg mL^{-1} (1.0 μg mL^{-1} I.S.) in the same day and on the five consecutive days, respectively. The intra-day and inter-day reproducibilities expressed as relative standard deviations (RSDs) of the method was from 5.2% to 6.5% and from 8.0% to 9.5%, respectively.

The focusing efficiency of the current method was assessed by the comparison of its performance with that of normal CZE. The mixture standard solution containing the three analytes each at 20.0 μg mL^{-1} and the I.S. at 10.0 μg mL^{-1} was prepared in the BGS. Then it was injected into the capillary at 0.5 psi for 3 s for normal CZE analysis and the electropherogram is shown in Fig. 2A. The LODs in normal CZE for sophocarpine, matrine and oxymatrine were estimated to be 0.8, 0.8 and 1.5 μg mL^{-1}, respectively. Fig. 2B presents the result by the current sweeping-MSS method with the sample solution containing the three analytes each at 2.0 μg mL^{-1} and the I.S. at 1.0 μg mL^{-1} being injected at 0.5 psi for 90 s. The sensitivity enhancement factor (SEF) in terms of peak area was calculated according to the following equation:

$$\text{SEF} = \frac{\text{quantification signal in the current method}}{\text{quantification signal in normal CZE}} \times \text{dilution ratio} \quad (1)$$

As a result, compared with the conventional CZE injection procedure, 44, 42 and 52-fold sensitivity enhancements for sophocarpine, matrine and oxymatrine were achieved by using the current on-line sweeping-MSS two-step stacking preconcentration method.

Applications

In order to test the applicability of the present method, two traditional Chinese medicines (*Kushen tablets* and *Kushenshiyang lotions*) were analyzed under the optimum conditions. The electropherograms for *Kushen tablets* and *Kushenshiyang lotions* are shown in Figs. 3 and 4, respectively. The recoveries of the method were evaluated by analyzing the spiked sample solutions with each analyte being spiked at 2.0 mg g^{-1} and 0.08 mg mL^{-1}, respectively. As shown in Table 2, the resultant recoveries of the alkaloids were in the range from 101.4% to 109.0% for *Kushen tablets* and from 87.5% to 98.4% for *Kushenshiyang lotions*, respectively, with RSDs (n = 3) less than 9.1%. These results show that the method is suitable for the determination of the quinolizidine

alkaloids in traditional Chinese medicines.

Comparison of the current method with other techniques

As listed in Table 3, in comparison with the previously reported methods used for the determination of quinolizidine alkaloids by CE,[38] NACE[35, 36] and GC-MS,[34] the present method provides a comparable or even better sensitivity except that the UPLC–MS/MS[33] method gave lower LODs. However, very expensive MS detector was used in that case.

Conclusions

A novel two-step stacking technique which combined sweeping and MSS in CE was developed for the simultaneous determination of three alkaloids in traditional Chinese medicines. Compared with typical CZE without any on-line preconcentration of the analytes, the sensitivity for the analytes with the current method was much improved. The method provides an alternative of choice for the determination of the quinolizidine alkaloids in traditional Chinese medicine samples

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Table Captions

Table 1. The linear ranges, limits of detection and sensitivity enhancement factors of the method.

Table 2. Results of the determination of the analytes in two Chinese medicines.

Table 3. Comparison of the current method with other techniques for the determination of the quinolizidine alkaloids

Figure Captions

Fig. 1. Optimization of the on-line concentration conditions. (A) Effect of concentration of MeOH in BGS. (B) Effect of NH_4Ac concentration in BGS. (C) Effect of pH value of BGS. (D) Effect of SDS concentration in micellar solution. Injection at 0.5 psi for 30 s of micellar solution followed by 90 s of sample solution (2.0 μg mL^{-1} each of quinolizidine alkaloids and 1.0 μg mL^{-1} I.S. in 15 mM NH_4Ac (pH 2.0)). Detection: 200 nm. Voltage: 20.0 kV. Peak identification: 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.

Fig. 2. Comparison of the electropherograms obtained by typical CZE (A) and sweeping-MSS -CZE (B). Sample solution, A: 20.0 μg mL^{-1} each of quinolizidine alkaloids and 10.0 μg mL^{-1} I.S.; B: 2.0 μg mL^{-1} each of quinolizidine alkaloids and 1.0 μg mL^{-1} I.S.

Fig. 3. Electropherograms of *Kushen tablets* (A) and the sample spiked at 2.0 mg g^{-1} of each of the analytes (B). Peak identification: U. unknown, 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.

Fig. 4. Electropherograms of *Kushenshiyang lotions* (A) and the sample spiked at 0.08 mg mL^{-1} each of the analytes (B). Peak identification: U. unknown, 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.

Table 1.

The linear ranges, limits of detection and sensitivity enhancement factors of the method.

	Sophocarpine	Matrine	Oxymatrine
Linear range (μg mL^{-1})	0.1-10.0	0.1-10.0	0.1-10.0
r	0.9996	0.9992	0.9996
LOD (μg mL^{-1})	0.02	0.02	0.03
Intra-day RSD (*n* = 8)(%)	5.2	6.1	6.5
Inter-day RSD (*n* = 5) (%)	8.0	9.1	9.5
SEF	42	44	52

Table 2.

Results of the determination of the analytes in two Chinese medicines.

Alkaloid	Kushen tablet (n = 5)					Kushenshiyang lotion (n = 5)				
	Content (mg g^{-1})	Spiked (mg g^{-1})	Found (mg g^{-1})	R[a] (%)	RSD (%)	Content (mg mL^{-1})	Spiked (mg mL^{-1})	Found (mg mL^{-1})	R[a] (%)	RSD (%)
Sophocarpine	ND[b]	2.0	2.18	109.0	8.6	0.18	0.08	0.25	96.2	8.9
Matrine	11.01	2.0	13.36	102.7	6.4	0.54	0.08	0.61	98.4	7.5
Oxymatrine	1.68	2.0	3.73	101.4	5.6	ND[b]	0.16	0.14	87.5	8.1

[a] R, recovery of the method. [b] ND, not detected.

Table 3. Comparison of the current method with other techniques for the determination of the quinolizidine alkaloids

Method	Sample	Linearity (μg mL^{-1})	LODs (μg mL^{-1})	RSDs (%) (n = 5)	Ref
NACE-UV	Lupinus species	17-1500	6.5-30.5	1.76-6.42	36
NACE-UV	Traditional Chinese herbal drugs	3-60	0.93-2.31	1.03- .68%	35
CE-DAD	Sophora medicinal plants	5.60–88.0		1.50 - 3.00	38
GC-MS	Genista sandrasica Hartvig & Strid				34
UPLC–MS/MS	Seeds of Sophora alopecuroides L.	10^{-2}-10^{2}	0.001-0.003	2.83-3.34	33
Sweeping-MSS-CE	Traditional Chinese drugs	0.1-10.0	0.02-0.03	5.2-9.5	This work

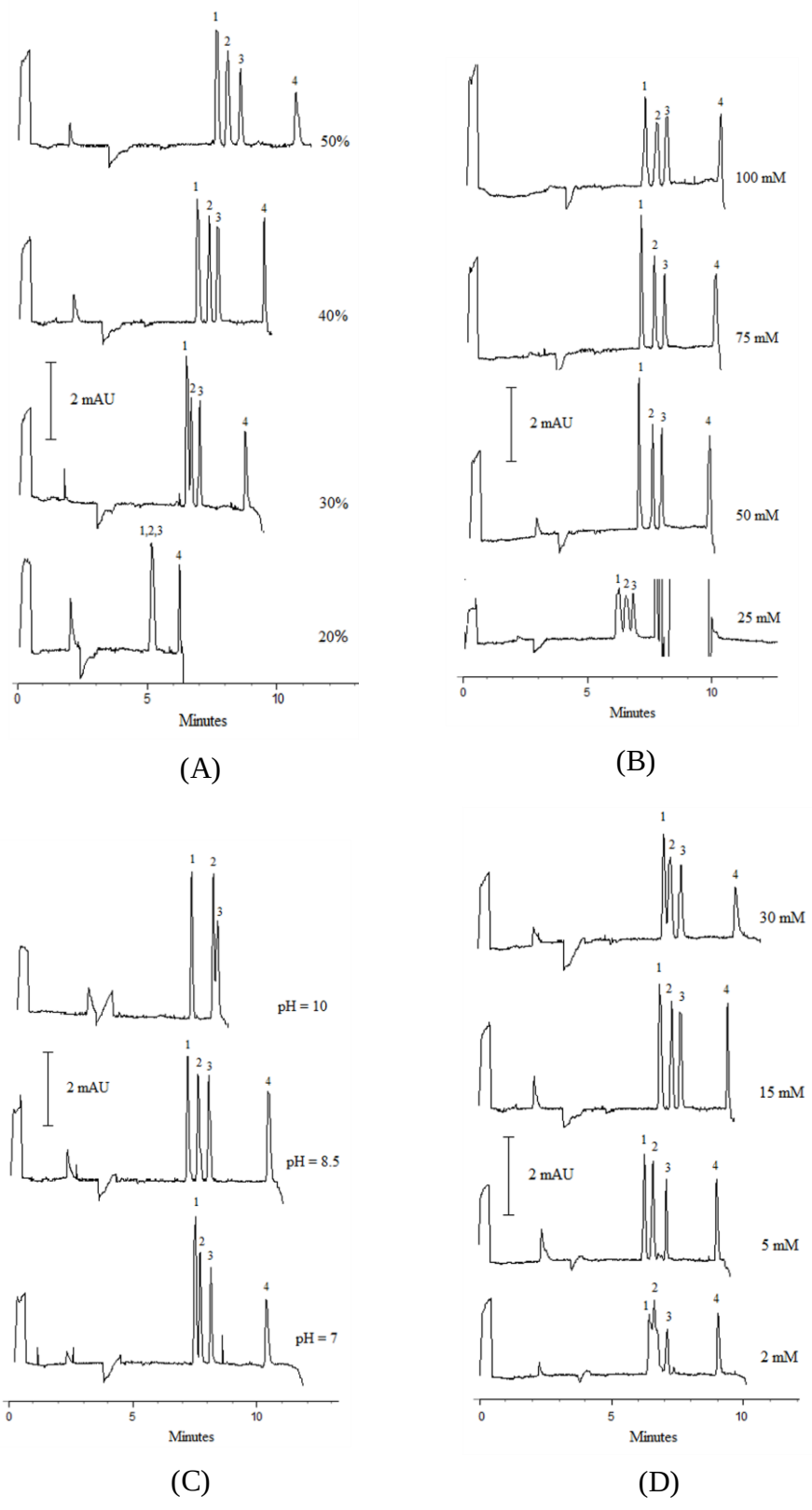

Fig. 1. Optimazation of the on-line concentration conditions. (A) Effect of concentration of MeOH in BGS. (B) Effect of NH_4Ac concentration in BGS. (C) Effect of pH value of BGS. (D) Effect of SDS concentration in micellar solution. Injection at 0.5 psi for 30 s of micellar solution followed by

90 s of sample solution (2.0 μg mL^{-1} each of quinolizidine alkaloids and 1.0 μg mL^{-1} I.S. in 15 mM NH_4Ac (pH 2.0)). Detection: 200 nm. Voltage: 20.0 kV. Peak identification: 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.

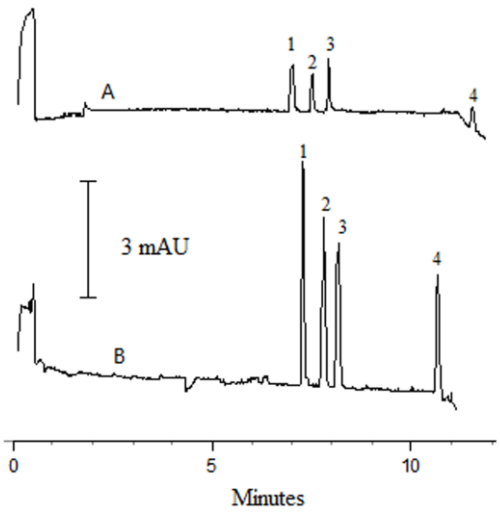

Fig. 2. Comparison of the electropherograms obtained by typical CZE (A) and sweeping-MSS -CZE (B). Sample solution, A: 20.0 μg mL^{-1} each of quinolizidine alkaloids and 10.0 μg mL^{-1} I.S.; B: 2.0 μg mL^{-1} each of quinolizidine alkaloids and 1.0 μg mL^{-1} I.S.

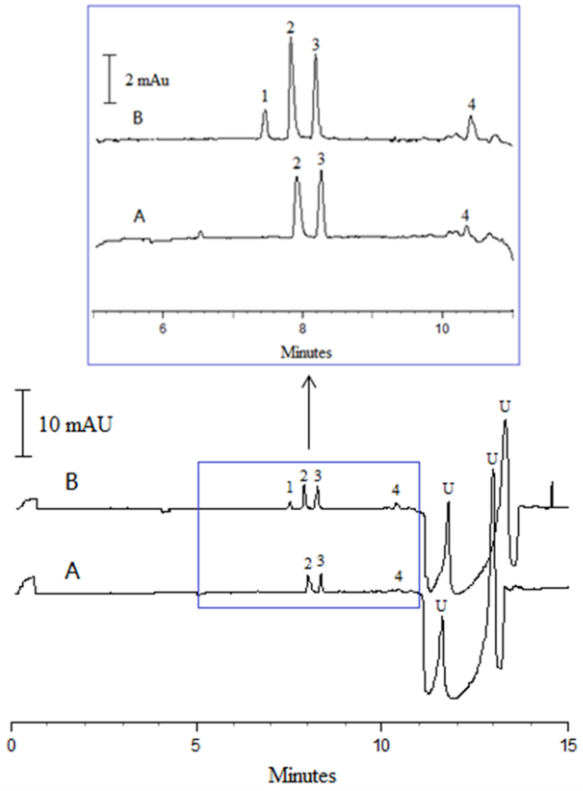

Fig. 3. Electropherograms of *Kushen tablets* (A) and the sample spiked at 2.0 mg g^{-1} of each of the analytes (B). Peak identifications: U. unknown, 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.

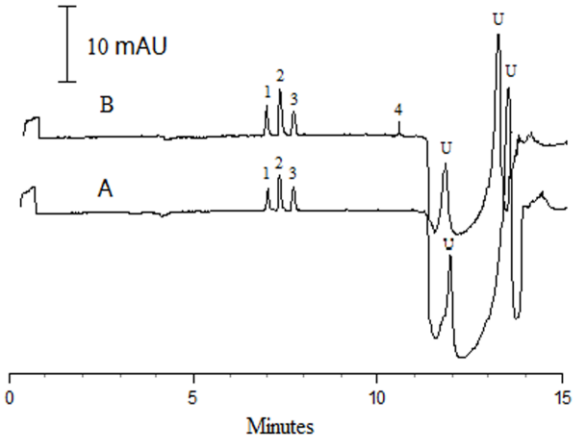

Fig. 4. Electropherograms of *Kushenshiyang lotions* (A) and the sample spiked at 0.08 mg mL^{-1} each of the analytes (B). Peak identifications: U. unknown, 1. sophocarpine, 2. matrine, 3. strychnine (I.S.), 4. oxymatrine.