Talanta 84 (2011) 155–159

Contents lists available at ScienceDirect

Talanta

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

Separation and sweeping of flavonoids by microemulsion electrokinetic chromatography using mixed anionic and cationic surfactants

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ARTICLE INFO

Article history:
Received 25 August 2010
Received in revised form
15 December 2010
Accepted 21 December 2010
Available online 8 January 2011

Keywords:
Flavonoids
Microemulsion electrokinetic chromatography
Mixed anionic and cationic surfactants
Radix Astragali
Sweeping

ABSTRACT

In this report, a novel means for the separation and sweeping of flavonoids (quercetin, rutin, calycosin, ononin and calycosin-7-O-β-D-glucoside) by microemulsion electrokinetic chromatography using mixed anionic and cationic surfactants as modified pseudostationary phase was presented. The optimized background electrolyte consisted of 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 10 mM sodium borate or 25 mM phosphoric acid. We systematically investigated the separation and preconcentration conditions, including the concentrations of surfactant, types of sweeping, sample matrix, the effect of high salt or acetonitrile, and sample injection volume. It was found that the use of mixed surfactants significantly enhanced the separation efficiency through the change of the efficient electrophoretic mobility of analytes. Compared with normal sample injection, 185–508-fold sensitivity enhancement in terms of limit of detection was achieved through effective sweeping of large sample volume at 50 mbar pressure (up to 45% capillary length). At last, the proposed method was suitable for the determination of Radix Astragali sample.

1. Introduction

Microemulsion is thermodynamically stable, optically isotropic and clearly transparent submicron dispersions of oil and water, stabilized by the presence of a surfactant and co-surfactant. Because of the remarkable analytical feature of microemulsion buffer, microemulsion electrokinetic chromatography (MEEKC) has become an active field of research in CE and obtained a diverse range of successful applications during the last years [1–5]. When performing MEEKC separations, the choice of surfactants affected the charge on the microemulsion droplet, decreased the interfacial tension between the oil droplet and aqueous phase, and changed the partition of the solutes. So far, in many published MEEKC investigations, anionic, cationic and nonionic types of surfactants have been extensively used to separate a large variety of compounds with high efficiency [6–9]. Notably, anionic sodium dodecyl sulfate (SDS) was the preferred surfactant in most of the MEEKC applications due to its inexpensive and flexible property. Mixed surfactant systems, which primarily focused on two different types of anionic and nonionic surfactants to provide the novel separation selectivity, have been reported in MEEKC [10,11]. Currently, the MEEKC applications did not involve the mixed use of anionic and cationic surfactants. Also, some studies demonstrated that the use of oppositely charged surfactants system significantly influenced the retention behaviors and separation selectivity in micelles and vesicles [12–14]. Therefore, efforts should be made to explore the great potential to widen elution window and enhance resolution in microemulsion media using mixed surfactant systems.

Although CE provided many analytical benefits when applied to detecting small ions or large biological molecules [3,15], an obvious drawback using ultraviolet detection was poor concentration sensitivity due to a limited amount of sample injected. On-line sample concentration techniques based on stacking or sweeping have received wide attention in CE, and have been applied in recent years to enhance sensitivity by introducing a large injected sample volume into the capillary [16–22]. It should be mentioned that a single surfactant system for sample preconcentration in micelle or microemulsion was the most widely used, while the studies of mixed surfactants system were limited [23]. Furthermore, preconcentration techniques caused by oppositely charged surfactants system in CE were not exploited previously. The addition of cationic surfactants to the electrophoretic buffer can reverse the direction of the electroosmotic flow (EOF) and make the wall charge positive owing to the formation of a bilayer in the capillary. Obviously, the preconcentration process would become more complicated in the presence of the mixture of anionic and cationic surfactants. Hence, we attempted to investigate the possibility of sweeping with an anionic and cationic mixed microemulsion for reducing the limit of

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doi:10.1016/j.talanta.2010.12.037

detection (LOD) and to establish a suitable method for the determination of multipolar components in plant sample.

Flavonoids are a class of attractive planar molecules with a chromane-type skeleton, which exhibit a wide range of biological and physiological characteristics, such as antioxidant, anti-allergic, anti-inflammatory and anti-cancer activities [24]. Recently, special attention was devoted to developing on-line concentration techniques for the analysis of flavonoids, including field-enhanced sample injection [25], stacking with reverse migrating micelles [26], and anion selective electrokinetic injection [26]. In this paper we reported for the first time a highly effective separation and sweeping method for detection of flavonoids in MEEKC using the mixture of anionic and cationic surfactants. Some experimental parameters were optimized by varying the concentrations of surfactants, types of sweeping, sample matrix, the effect of high salt or acetonitrile, and sample injection volume. In addition, the sensitivity enhancement was improved about 185–508-fold compared with conventional hydrodynamic injection method without apparent decrease of separation efficiency.

2. Experimental

2.1. Apparatus

All electrophoresis experiments were performed with an Agilent 3D capillary electrophoresis system and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). Uncoated fused-silica capillaries with 50 μm i.d. and 375 μm o.d. were obtained from Yongnian optic fiber factory (Hebei, China). The total capillary length was 55.0 cm, and the effective length was 46.5 cm from the inlet end to the detection position. Capillary temperature was 25 °C and UV absorbance detection was performed at 200 nm. A PHS-3C meter (Shanghai Precision Scientific Instrument Co., Ltd., China) was used to measure the pH of microemulsion buffers.

The LC–MS experiments were performed using an Agilent 1100 series LC system coupled with single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation was carried out on an Agilent Zorbax Extend C_{18} column (5 μm, 150 mm × 4.6 mm) at a temperature of 30 °C. The mobile phase consisted of 0.1% formic acid water (A) and acetonitrile (ACN) (B) using a gradient elution of 20–27% (v/v) B at 0–12 min; 27–30% B at 12–20 min; 30–60% B at 20–22 min; 60–100% B at 22–24 min. The flow rate was 0.8 mL min^{-1}, and the injection volume was 10 μL. ESI-MS analysis was performed in the negative ionization mode. The nebulizer gas pressure, drying gas flow rate and drying gas temperature for the ESI source were set at 35 psi, 9 L min^{-1} and 300 °C, respectively. The fragmentor voltage was 120 V, and capillary voltage was set at 3500 V.

2.2. Chemicals and reagents

Quercetin and rutin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Calycosin, ononin and calycosin-7-O-β-D-glucoside were supplied by Ronghe Pharmaceutical Technology Development Co., Ltd. (Shanghai, China). SDS and dodecyltrimethylammonium chloride (DTAC) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Ethyl acetate, 1-butanol and sodium borate were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Phosphoric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium acetate, ACN and sodium hydroxide were purchased from Hangzhou Chemical Reagent Factory (Hangzhou, China). All reagents are the analytical grade available in our experiments.

2.3. Procedure

Before first use, a capillary was conditioned with 1.0 M sodium hydroxide (10 min), followed by 0.1 M sodium hydroxide (10 min), deionized water (10 min), and finally microemulsion buffer (5 min) by flushing at 935 mbar. Between runs, the capillary was flushed sequentially with 0.1 M sodium hydroxide (1 min), water (3 min), and separation buffer (5 min). The sample solutions were injected from the cathode end of capillary at 50 mbar, and then run under a constant voltage of 25 kV or −25 kV.

Running buffer for MEEKC was prepared by mixing 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 10 mM sodium borate (pH 9.0) or 25 mM phosphoric acid (pH 2.0) in distilled water. The mixture was then sonicated for 30 min in a bath sonicator to obtain stable and transparent microemulsion. Sample solutions of five analytes were prepared by dilution of methanol stock solutions with distilled water or phosphoric acid appropriately. All these solutions were filtered through 0.22 μm filters prior to analysis.

2.4. Sample preparation

Approximately 1.0 g of the powder samples of Radix Astragali (40 mesh), accurately weighed, were extracted with 20 mL methanol for 60 min in an ultrasonic bath, followed by centrifugation for 5 min at 12,000 rpm. The methanol solutions were then analyzed by conventional or sweeping MEEKC methods.

3. Results and discussion

3.1. Effect of mixed surfactant systems on separation efficiency

It is well-known that the inner walls of bare fused silica capillaries used in CE intrinsically generate a negative charge because of the presence of dissociated silanol groups [27,28]. When a BGE containing a cationic surfactant adsorbs on the capillary surface of the opposite charge, positive counterions can form an electrical double layer and reverse the direction of EOF depending on the sufficient surfactant concentrations [29–31]. In this work, we studied the possibility of using mixed anionic and cationic surfactants as modified pseudostationary phase (PSP) to improve separation in MEEKC. The concentration of anionic surfactant (SDS) was kept unchanged at 2.0% in the microemulsion solutions, and then different concentrations of cationic surfactant DTAC (0–15 mM) were added. Under the experimental conditions, the electrostatic properties of the bare silica surface could be dynamically modified by DTAC, but adsorbed amount of DTAC was very limited; therefore the buffer system was not necessary to reverse electrode polarity. Fig. 1 shows the effect of modified PSP on the separation of five flavonoid compounds (calycosin-7-O-β-D-glucoside, rutin, calycosin, ononin and quercetin) under conditions of constant ionic strength and pH. As can be seen, the separation efficiency of tested analytes was improved when the concentrations of DTAC were increased from 0 to 15 mM. This is particularly true in the case of calycosin-7-O-β-D-glucoside, rutin, ononin and quercetin because a significant improvement in the resolution was observed compared to the use of a single SDS microemulsion, definitely suggesting that these analytes had strong interactions with mixed surfactant molecules. It is important to note that the migration order of five analytes in the mixed surfactant system was the same as those obtained using only SDS microemulsion. Furthermore, the elution range could be effectively extended in the modified PSP with raising concentration of DTAC in the positive electrode mode, without loss of separation efficiency. It was obvious that the slight decrease of EOF (from 5.39×10^{-4} to 5.33×10^{-4} $cm^2 V^{-1} s^{-1}$) was observed with

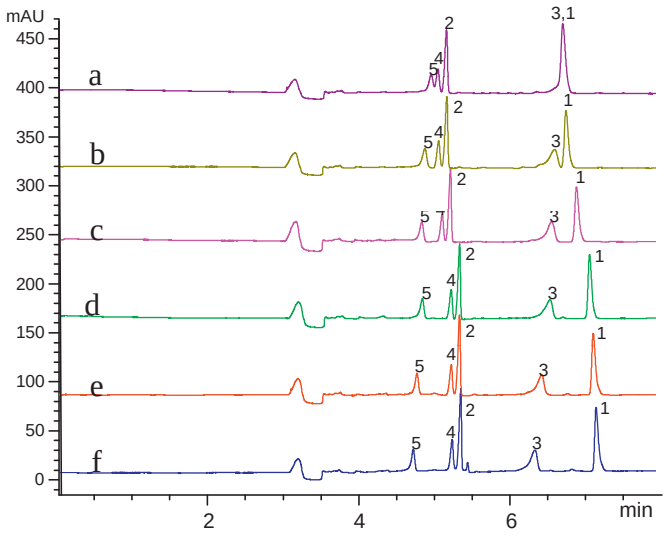

Fig. 1. Effect of cationic surfactants on the resolution and migration of five flavonoids. The microemulsion buffers: 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 0–15 mM DTAC, 4.0% (w/v) 1-butanol and 10 mM sodium borate buffer of pH 9.0. Capillary: 55.0 cm (46.5 cm effective length) × 50 μm of uncoated fused-silica capillary; temperature: 25 °C; separation voltage: 25 kV; sample injection: 50 mbar, 3 s; analytes concentrations: 0.8 mg mL^{-1} of each flavonoid; cationic surfactant: (a) 0 mM DTAC, (b) 3 mM DTAC, (c) 6 mM DTAC, (d) 9 mM DTAC, (e) 12 mM DTAC, (f) 15 mM DTAC; chromatographic peaks 1–5: (1) quercetin, (2) calycosin, (3) ononin, (4) rutin, (5) calycosin-7-O-β-D-glucoside.

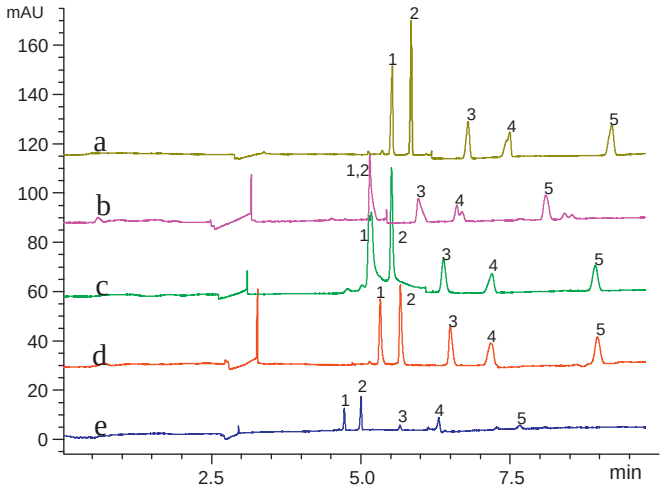

Fig. 2. The effect of sample matrix on flavonoids sweeping in reversed electrode polarity mode. BGE: 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 25 mM phosphoric acid solution of pH 2.0; separation voltage: −25 kV; sample matrix: (a) 25 mM phosphoric acid, (b) 25 mM disodium hydrogen phosphate, (c) 25 mM sodium dihydrogen phosphate, (d) 25 mM sodium acetate, (e) 25 mM sodium borate; sample concentration: 2.5 μg mL^{-1}; sample injection: 50 mbar, 100 s.

increasing DTAC concentration. In addition, the influence of buffer concentration and pH on MEEKC separation was evaluated. In this case, the two parameters also affected the mobility of analytes, the dissociation of the solutes and the interaction between compounds and PSP (data not shown), and this result was consistent with other CE studies [32–36]. As described above, the presence of DTAC in microemulsion had a marked influence on the electrophoretic behavior of these analytes in the study. It also reflected that the MEEKC separation was directly related to inherent electrostatic interaction between the charged headgroups of two types of surfactants. Hence, the modified PSP provided additional selectivity of microemulsion droplets which contributed to significant improvement in the resolution. Considering the resolution and analysis time, 9 mM DTAC was chosen to do the further experiments.

3.2. Sweeping with mixed anionic and cationic surfactants in MEEKC

To improve the detection sensitivity of analytes with mixed anionic and cationic surfactants, we investigated the effects of some experimental parameters of the sweeping-MEEKC method, including the polarity of the voltage applied, the composition of sample matrix, the high salt matrix, the organic modifier and the injection time.

3.2.1. Effect of the composition of the sample matrix

In initial sweeping experiments, the use of positive electrode polarity sweeping in MEEKC was ineffective for on-line concentration and separation of tested analytes (data not shown). In order to achieve a more efficient preconcentration in MEEKC, reversed electrode polarity sweeping mode was examined in detail. Previous studies demonstrated that the choice of the sample matrix had a strong influence on sweeping efficiency and solute mobility [6]. Thus, a series of electrolytes, such as phosphoric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium acetate and sodium borate, were used as sample matrices to mix with five analytes, and their influence on sweeping MEEKC was investigated. While a separation buffer was composed of 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 25 mM phosphoric acid of pH 2.0. The analytes at a concentration of 2.5 μg mL^{-1} were injected for 100 s. As shown in Fig. 2, flavonoid compounds prepared in a phosphoric acid solution of pH 2.0 was able to provide the best peak enhancement without loss of separation efficiency, while the lowest detection sensitivity was obtained when a borate buffer of high pH (9.0) was used as the sample matrix. Interestingly, the migration order of the analytes was different from what was observed in positive electrode polarity sweeping mode. These results indicated that the small difference of conductivity between sample matrix and running buffer affected the affinity of the analyte toward microemulsion droplets and electric field of the sample zone, and therefore influenced the sweeping efficiency. As a result, an acidic 25 mM phosphoric acid of pH 2.0 was selected as the optimal sample matrix in reversed electrode polarity sweeping.

3.2.2. Effect of high salt or ACN in the sample matrix

In recent years, high-salt stacking and sweeping in CE were developed as a high-efficiency method for the preconcentration of analytes [37,38]. To investigate the effect of salt matrix on the sweeping efficiency, experiments were performed at negative polarity with a microemulsion buffer consisting of 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 25 mM phosphoric acid of pH 2.0. The concentrations of five flavonoids in the salt matrices were the same as those in the salt-free samples of Fig. 2; the injection times were all 100 s under a constant pressure of 50 mbar. Herein, sample matrices containing sodium chloride from 25 to 150 mM were examined. As shown in Fig. 3, the preconcentration ability of tested analytes was obviously enhanced at 50 mM sodium chloride, while their detection sensitivity become poorer and poorer as salt concentrations increased from 50 to 150 mM. This means that the sweeping of analytes in the salt matrixes was carried out to some extent; beyond that range zone broadening with such large injection began to dominate under the given experimental conditions. In addition, an increase of salt concentrations in sample solutions resulted in a large increase in the operating current (from 65 to 93 μA), which was deleterious to sweeping experiments. Therefore, in this case, high-salt sweeping did not lead to improved sensitivities and well-shaped peaks.

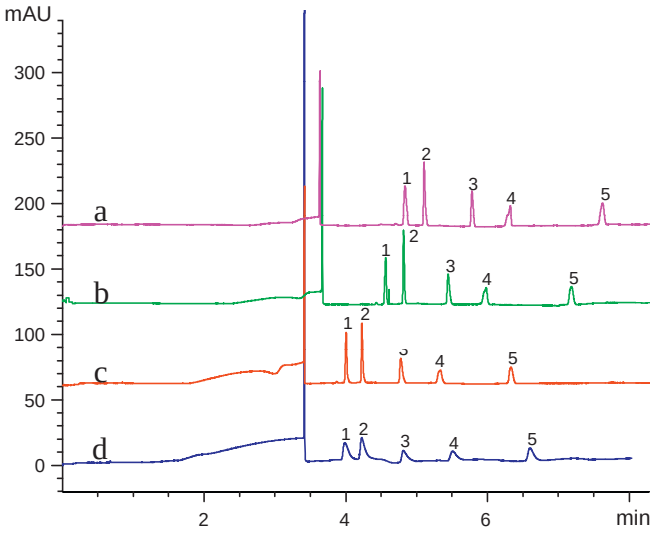

Fig. 3. Electropherograms of MEEKC with the sweeping of analytes in the salt matrixes with (a) 25 mM, (b) 50 mM, (c) 100 mM, and (d) 150 mM sodium chloride. All other conditions were the same as in Fig. 2.

Previous research has indicated that the nature and concentration of organic additives in buffers could effectively improve selectivity and resolution, influence electrophoretic parameters such as EOF and the solutes, and increase the solubility of analytes in the aqueous phase [39–41]. To obtain good separation and enhance the sensitivity with mixed SDS and DTAC as surfactants, we investigated the influence of ACN in sample matrix on sweeping MEEKC method in initial experiment. It was found that the sweeping efficiency was dramatically decreased with 5–15% ACN concentrations (data not shown). The obtained results were attributed to the addition of ACN, which might weaken affinity interaction between analytes and microemulsion.

3.2.3. Effect of the injection time

The increase of peak intensity is generally related to the effective sample plug injected into the capillary [42]. Next, the effect of injection time on sweeping ability based on mixed surfactants was investigated by different sampling time from 50 s to 400 s using a 50 mbar hydrodynamic injection. As can be seen in Fig. 4, the sweeping efficiency increased linearly with the raising of sample injection time for peak areas in MEEKC. In addition, peaks

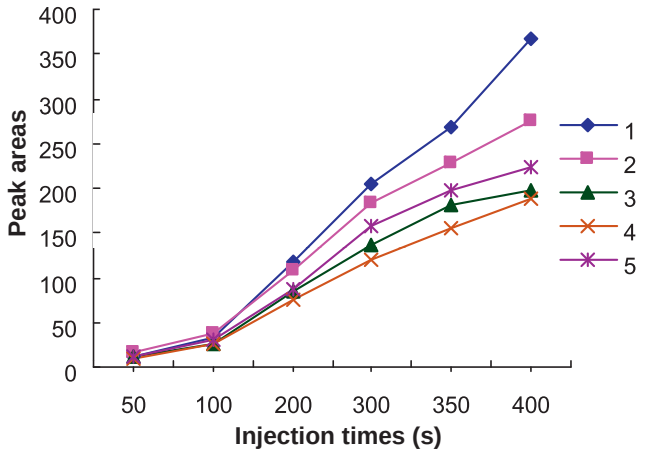

Fig. 4. The effect of injection time on sweeping sensitivity. Sample matrix: 25 mM phosphoric acid solution; sample concentration: 1.25 μg mL^{-1}; injection volume: 50 mbar, 50–400 s. All other conditions were the same as in Fig. 2.

Table 1
LODs with conventional MEEKC, sweeping MEEKC and LC–MS.

Analytes	LODs (μg mL^{-1})		
	Conventional MEEKC[a]	Sweeping MEEKC[b]	LC–MS
1	2.25	0.008	0.019
2	2.03	0.004	0.012
3	2.46	0.013	0.028
4	4.08	0.022	0.037
5	3.37	0.017	0.023

[a] Pressure injection was performed at 50 mbar for 3 s in conventional MEEKC.
[b] 50 mbar for 350 s in sweeping MEEKC.

became broaden and the baseline was disrupted when the injection time was longer than 350 s, which might be produced by the degradation of the steady state of the microemulsion frontier. As a matter of fact, the EOF decreased greatly from 6.43×10^{-4} to 4.44×10^{-4} cm^2 V^{-1} s^{-1}, resulting in longer migration times when the injection time was varied between 50 s and 400 s. Therefore, it was found that a sampling time of 350 s provided satisfactory resolution and maximum peak enhancement.

3.2.4. Method validation and detection enhancement

To evaluate the proposed sweeping MEEKC method, some parameters such as precision, repeatability, linearity, recovery and LOD were determined under the optimum conditions. A standard mixture solution of the five compounds was carried out to determine the precision of sweeping MEEKC. As was observed in the experiment, the relative standard deviations (RSDs) of migration time and peak area were in the range of 0.6–1.9% and 1.2–4.5% based on five consecutive runs. Besides, the repeatability of the developed method was examined with five sample solutions that were extracted and analyzed from the same crude drug as mentioned above. The results showed that the RSD of migration time was less than 2.1% and that of peak area was less 5.3%. Although the migration time and peak area changed from run to run, the repeatability of the method was acceptable. Calibration curves of five analytes were constructed via least-square regression with six appropriate concentrations in triplicate. The results indicated that good linearity was obtained in the concentration range of 0.1–8.0 μg mL^{-1} with correlation coefficients ranging from 0.9936 to 0.9994. Recoveries were carried out by spiking known amounts of five standards to the real samples prior to solvent extraction. As a result, the mean recoveries were in the range from 80% to 92%. The detection enhancement was evaluated by using injection times (50 mbar, 350 s) for sweeping MEEKC relative to the common pressure injection at 50 mbar for 3 s for conventional MEEKC. The mixture solutions of five analytes in two MEEKC methods are performed to determine LOD values under the same experimental conditions, and the results are summarized in Table 1. The LODs based on a signal-to-noise ratio of 3 ranged from 0.004 to 0.022 μg mL^{-1} for sweeping mode, and from 2.03 to 4.08 μg mL^{-1} for conventional MEEKC, respectively. Compared to the usual injection, a roughly 185–508-fold increase in signal enhancement was achieved for five analytes in proposed sweeping method. When compared with LC–MS (Table 1), the proposed sweeping MEEKC method also provided similar detection sensitivity for the analysis of these ionizable compounds in CE-UV system. The above results have demonstrated that sweeping-MEEKC using mixed surfactants indeed provided good sensitivity for the determination of charged analytes.

3.2.5. Sample analysis

Finally, to test the applicability of the proposed sweeping MEEKC, the simultaneous quantification of five compounds in Radix Astragali was performed using 0.5% (w/v) ethyl acetate, 2.0% (w/v)

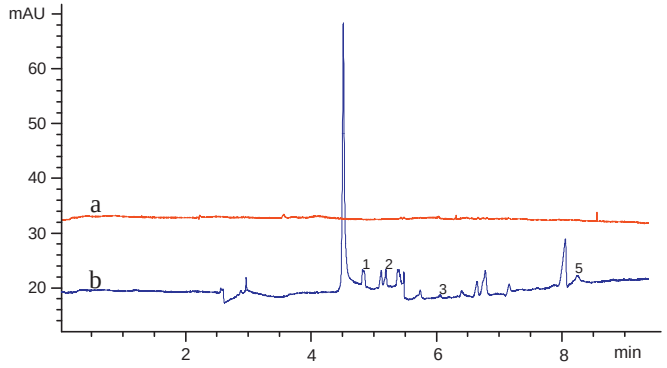

Fig. 5. Electropherograms of Radix Astragali sample determined by the conventional MEEKC or sweeping methods. Running buffer: 0.5% (w/v) ethyl acetate, 2.0% (w/v) SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 25 mM phosphoric acid solution of pH 2.0. Sample solution concentration: 1.25 mg mL^{-1}. (a) Injection was performed at 50 mbar for 3 s in the conventional MEEKC method. (b) Injection was performed at 50 mbar for 100 s in the sweeping MEEKC. All other conditions were the same as in Fig. 2.

SDS, 9 mM DTAC, 4.0% (w/v) 1-butanol and 25 mM phosphoric acid of pH 2.0. Radix Astragali samples (1.25 mg mL^{-1}) were hydrodynamically injected into the capillary for the conventional MEEKC (50 mbar, 3 s) and sweeping MEEKC (50 mbar, 100 s) methods. The typical electropherograms obtained from the plant extract are shown in Fig. 5. As shown in Fig. 5a, we could not detect any of five analytes by normal CE method. Furthermore, it was observed that a large number of peaks were strikingly determined without any interference in sweeping MEEKC mode (Fig. 5b). The peaks were identified in terms of the migration times, standard additions, and UV spectra. Quantitative analytical results of Radix Astragali sample were 0.12 mg g^{-1} quercetin, 0.23 calycosin mg g^{-1}, 0.04 mg g^{-1} ononin and 0.31 mg g^{-1} calycosin-7-O-β-D-glucoside, respectively. Next, in order to further confirm the accuracy of sweeping MEEKC measurement, a reverse phase LC–MS method was applied to determine flavonoids in Radix Astragali sample. Consequently, the contents of all analytes tested were basically similar to that of sweeping MEEKC (correlation, $r^2 = 0.9402$), indicating a good quantitative performance for the newly established method.

4. Conclusion

In this study, we have demonstrated that the separations achieved in MEEKC could be favorably improved by a new microemulsion system using the mixture of anionic/cationic surfactants. The results showed the unique advantage of the modified PSP over single surfactant system in the MEEKC separation, resulting in higher resolution and selectivity. Additionally, sweeping using oppositely charged surfactants was a highly effective way for the online concentration of charge solutes in MEEKC. This proposed method was further applied to the analysis of active compounds in Radix Astragali.

Acknowledgement

This study was supported by China Postdoctoral Science Foundation (20090461388).

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