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Sweeping under controlled electroosmotic flow and micellar electrokinetic chromatography for on-line concentration and determination of trace phlorizin and quercitrin in urine samples

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ABSTRACT

A novel sweeping under controlled electroosmotic flow scheme was developed for preconcentration and determination of neutral compounds by micellar electrokinetic chromatography (MEKC). An anionic surfactant, sodium dodecyl sulfate (SDS), was added into the buffer for sweeping and separation. By controlled electroosmotic flow (EOF) equal to the counter electrophoretic flow, the surfactants were at an immobile state in capillary. The neutral analytes with sample solution was injected electroosmotically into capillary and swept by SDS micelle for essentially an unlimited volume. The injected sample plug lengths for phlorizin and quercitrin under 18 kV for 70 min were experimentally estimated as 1532 cm, corresponding to 51-fold the effective capillary length. The sweeping under controlled EOF scheme resulted in increased detection factors for phlorizin and quercitrin of 2.3×10^4 and 2.1×10^4 using 70 min injection relative to a traditional pressure injection. The proposed method has been adopted to analyze trace phlorizin and quercitrin in urine samples successfully.

1. Introduction

Capillary electrophoresis is a powerful separation technique that has been applied in many areas. While it provides high-efficiency separations, the detection limits are higher due to the small sampling volume. A number of on-line concentration strategies have been developed, for example, field amplified sample stacking [1–3], isotachophoresis (ITP) [4,5], dynamic pH junction [6,7], pH-mediated stacking [8–10], and sweeping [11–13]. Sweeping is an effective and convenient way for doing on-line sample concentration in MEKC. The usual procedure for sweeping includes a hydrodynamic injection step followed by the subsequent sweeping and separation processes. The limitation is that the maximum volume of sample that can be injected can only be as high as the total capillary volume and improvements in sensitivity are therefore very limited. To overcome this sweeping barrier, electrokinetic injection has been used. Terabe's group and Zhu et al. developed cation- and anion-selective exhaustive injection and sweeping to improve the detection sensitivity by nearly 1 million fold [14–17]. However, this approach requires several initial liquid plugs in the capillary before sample injection and is only applicable to charged ions. Palmer et al. developed an electrokinetic stacking injection process for sweeping with SDS for neutral analytes, which has the capability of injecting 7-fold the effective capillary length of sample solution [18]. Their scheme was based on decreasing the velocity of the analyte/micelle complex without substantially reducing EOF, which essentially causes longer migration times for the analytes.

The improvement in sensitivity is mainly restricted by the amount of sample that can be injected and concentrated. Controlling the stacking boundary held stationary is a potential way to inject an unlimited sample volume without shortening the capillary length for subsequent separation. Gong et al. used EOF to balance electrophoretic migration of cationic micelles with an improvement in the sensitivity of neutral analytes of 4500-fold with a 60 min continuous electrokinetic injection [19]. Horáková et al. presented an "electrokinetic accumulation" system, in which weak acids were continuously electrokinetically injected into a low-pH electrolyte, thus accumulating on the sample/electrolyte interface. Mobilization of the neutral analytes after injection was achieved via sweeping, and an improvement in sensitivity of 4600 was obtained with a 120 min injection [20]. Breadmore used stationary ITP boundaries for concentration of anions with an improvement in the detection limits of 100,000 times better than normal hydrodynamic injection [21,22]. The stationary boundary was formed by balancing the anodic migration of an ITP boundary with a cathodic EOF. Zhang et al. modified the EOF with strong acids in sample

Abbreviations: MEKC, micellar electrokinetic chromatography; SDS, sodium dodecyl sulfate; EOF, electroosmotic flow.

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solutions to provide a stationary sweeping boundary for stacking of cationic molecules [23]. The technique was based on the modulation of movements of bulk flow inside capillary and maintaining the stacking frontier of the micelle zone staying constantly so that large samples volume can be injected and more than 1000-fold increase in sensitivity was obtained.

In this paper, we developed a new sweeping under controlled EOF preconcentration scheme with anionic surfactants. The EOF was used to balance the electrophoretic migration of SDS micelle to generate an immobile sweeping boundary. Sample solution was continually injected into capillary and sweeping by SDS micelles for essentially an unlimited volume. Phlorizin and quercitrin were chosen as model compounds to evaluate the effectiveness and reliability of the proposed system. To the best of our knowledge, this work represented the first demonstration of sweeping under controlled EOF coupled with MEKC for the preconcentration and determination of trace phlorizin and quercitrin in urine samples.

2. Experimental

2.1. Chemicals and reagents

The phlorizin and quercitrin were obtained from Zelang medicine Ltd. (Nanjing, China). Sodium tetraborate, sodium acetate, phosphate, etc. were obtained from Shanghai Chemical Plant (Shanghai, China). Sodium dodecyl sulfate (SDS) was from Sigma. Standard stock solutions of phlorizin and quercitrin at a concentration of 1.0 g/L were prepared in ethanol and diluted to the desired concentration with running buffer (without SDS) just prior to use. The phosphate buffer solution was prepared by adjusting the acidity of sodium dihydrogen phosphate (concentrations ranging from 5 to 150 mmol/L) solution to a desired pH with concentrated sodium hydroxide, and the pH value of the buffer solution was measured by a PHS-3C pH meter (Shanghai Dapu Instrument Co., Shanghai, China). All aqueous solutions were prepared with double-distilled water and filtered through 0.22 μm cellulose acetate membrane filters (Shanghai Yadong Resin Co., Shanghai, China) before use.

2.2. Apparatus

Capillary electrophoresis (CE) and electrochemical (EC) detection was carried out with a model MPI-A electrophoresis system (Xi'an Remax Electronics Inc., Xi'an, China), equipped with a high-voltage power supplier (0–30 kV) for electronic sampling and separation and an electrochemical (EC) potentiostat (0–2.5 V) for EC detection. A 30 cm fused-silica capillary with dimensions of 50 μm I.D. and 375 μm O.D. (Yongnian Optical Conductive Fiber Plant, Yongnian, China) was used as preconcentration and separation capillary. The EC detection was carried out with a three-electrode system consisting of a Pt working electrode, an Ag/AgCl reference electrode and a Pt auxiliary electrode. The arrangement of the end-column EC detection cell and the electrodes has been described in previous work [24]. The working electrode was cleaned by ultrasonication in ethanol and double-distilled water for 5 min before use.

2.3. Preparation of urine samples

Urine samples were acquired from a patient taking 10 mg doses/day in oral therapy. 5 mL urine sample was collected and then was mixed with 1 mL ethanol. After vortex mix 2 min and centrifuge 10 min at 4000 rpm, the supernatant was collected and diluted 10 times with 50 mmol/L sodium phosphate buffer solution (pH 5.0), and then was stored at the temperature of 4 °C. Aliquots

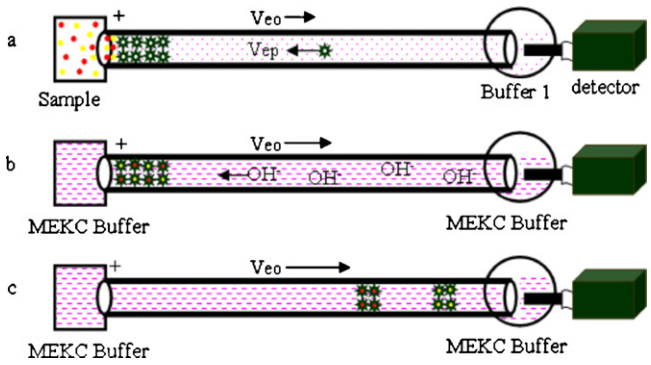

Fig. 1. Evolution of the sweeping under controlled EOF preconcentration and MEKC separation. V_{eo} is velocity of EOF. V_{ep} is electrophoretic velocity of SDS micelle. Buffer1 is 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 5.0); MEKC buffer is 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 8.0). (●) and (●) represent the analytes; ( ) represents SDS micelle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of supernatants were injected for sweeping under controlled EOF preconcentration and MEKC separation analysis.

Recoveries of phlorizin and quercitrin were calculated on the basis of calibration curves. Accurate amounts of the phlorizin and quercitrin were added to the post-dosed urine samples from patients, and the recovery values were obtained using their peak currents from the calibration curves under the same conditions.

2.4. Procedure

The capillary was flushed daily in the order of H_2O (1 min), 1.0 mol/L NaOH (15 min), H_2O (1 min) and conditioned with running buffer for 10 min by pressure. Between two runs, the capillary was conditioned with running buffer for 10 min. After the EC signal reached a constant value, the sample solution was injected with an injection voltage of 18 kV and an injection time of 70 min. Then a separation voltage of 18 kV was applied across the capillary. The detection potential of 0.85 V was applied at the working electrode and the EC responses were recorded.

3. Results and discussion

3.1. Sweeping under controlled EOF preconcentration and MEKC separation model

The main idea of sweeping under controlled EOF preconcentration and MEKC separation is illustrated in Fig. 1. The sweeping under controlled EOF preconcentration was carried out by inserting one end of the capillary, which was already filled with buffer 1 (50 mmol/L SDS in 50 mmol/L sodium phosphate buffer, pH 5.0), into a sample solution free of surfactant. An 18 kV voltage was then applied across the capillary through the sample and the buffer 1 cell (Fig. 1a). Analytes along with the sample buffer were electrokinetically pumped into capillary with EOF and captured by the negatively charged SDS micelle migrating toward the anode from buffer 1. The electrophoretic velocity (V_{ep}) of SDS micelle was the reverse of electroosmotic velocity (V_{eo}), and the apparent migration velocity of the micelle is zero at pH 5.0 [25]. Consequently, the SDS micelle at the capillary inlet stayed in an immobile state with an apparent velocity of zero. This immobile state was maintained for an extended period of time so that essentially unlimited volume of sample solution could be injected into the capillary. After 70 min duration for sweeping, the sample and buffer 1 were all exchanged with MEKC separation buffer (50 mmol/L SDS in 50 mmol/L sodium phosphate buffer, pH 8.0). The same voltage was applied between

the two separation buffer cells and hydroxyl ions in MKEC buffer solution entered the capillary from the cathode side and migrated toward the anode (Fig. 1b), resulting in an increase of the EOF. The immobile state of SDS micelle was destroyed and the micelle was pumped toward the detection end since the V_{eo} was larger than the V_{ep} of SDS micelle. Eventually, the pH in capillary reached the same magnitude as that of MEKC buffer in detection cell, and the analytes were separated by MEKC and detected (Fig. 1c).

3.2. Separation conditions

In order to optimize the MEKC separation conditions, buffer solutions at different concentrations and pH values, including sodium phosphate, sodium borate, sodium acetate, sodium citrate, sodium tartrate, etc. were examined for separation of phlorizin and quercitrin. The results showed that sodium phosphate buffer solution was more suitable because the resolution and separation efficiencies excel than those of other buffer solutions. The relationships of separation efficiencies to the concentration of sodium phosphate (varied from 10 to 150 mmol/L) and the buffer pH (ranging from 5.0 to 10.0) were tested. From the results obtained, when the concentration of phosphate was 50 mmol/L and the pH was 8.0, these compounds could obtain the best resolution and separation efficiencies.

SDS micelle (0–150 mmol/L) was added to 50 mmol/L phosphate (pH 8.0) buffer solution to carry out analyte separation. When no SDS micelle was added to pH 8.0 phosphate buffer solution, the phlorizin and quercitrin could not be separated on baseline. This showed the analytes were not deprotonated at this condition and could not be separated in the manner of CZE. As higher concentrations of SDS were added to buffer solution, gradual improvement in the resolution was observed. Optimum separation of phlorizin and quercitrin was realized when using 30–50 mmol/L SDS in running buffer. Concentrations of SDS > 50 mmol/L generated excessive current as well as longer analyte migration times in spite of improvement in the resolution. Considering the separation and the subsequent sweeping under controlled EOF preconcentration, 50 mmol/L SDS was used in subsequent studies.

3.3. Effect of pH on sample buffer

To obtain higher detection sensitivity while maintaining separation efficiencies and resolution during the separation, a sweeping under controlled EOF scheme was developed for sample preconcentration. If the swept zone in capillary reaches as far as the detection cell during injection, there would be no ability to separate the sample components. Moreover, to separate the concentrated analytes from one another after sweeping, a sufficient length of the capillary needs to be left free of analytes for subsequent separation in the separation buffer. In sweeping CE using SDS under normal polarity conditions, the micelles are negatively charged and have anodic V_{ep} against the EOF. By taking into account the sign of the migrating direction, the relationship between the V_{eo}, V_{ep} and the migration velocity of the SDS micelle (V_{mc}) is given as:

$$V_{mc} = V_{eo} + V_{ep} \quad (1)$$

The sign of each velocity is defined as positive when the migration is toward the negative electrode. When $V_{mc} = 0$, the micelle is at an immobile state although the bulk flow is still going on, which theoretically can permit unlimited volume injection of sample solution. According to Otsuka and Terabe's report [25], the apparent migration velocity of the micelle (V_{mc}) is zero at pH 5.0. As can be seen in Fig. 2a, analytes were not detected during the 70 min sweeping under controlled EOF and only appeared after the 70 min injection and sweeping once the separation voltages were applied because the background electrolyte was changed and

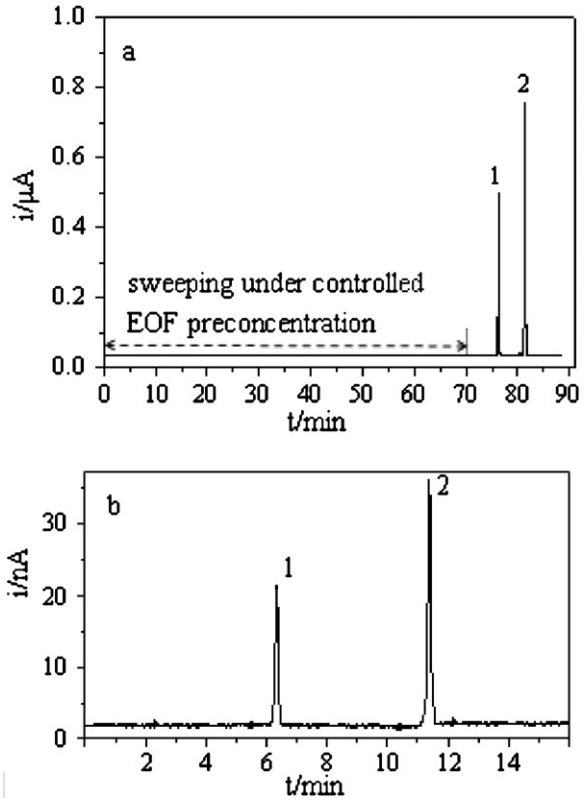

Fig. 2. Electropherograms showing a sweeping injection versus a typical injection. Experimental conditions: fused-silica capillary, 50 μm I.D. × 375 μm O.D., 30 cm length; separation voltage, 18 kV; detection potential, 0.85 V. Peaks 1 and 2 are phlorizin and quercitrin, respectively. (a) Electropherogram monitoring the sweeping under controlled EOF preconcentration and MEKC separation. Analyte injection was performed under 18 kV for 70 min for sweeping followed by the subsequent MEKC separation. The sample buffer was 50 mmol/L sodium phosphate buffer (pH 5.0); the sweeping under controlled EOF preconcentration buffer (buffer1 in Fig. 1) was 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 5.0); and the MEKC separation buffer was 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 8.0); analyte concentration was all 10 ng/mL. (b) Electropherogram with a typical hydrodynamic injection for 5 s at 0.2 psi. The sample buffer was 50 mmol/L sodium phosphate buffer (pH 8.0); analyte concentration was all 10 mg/L.

larger EOF was introduced to allow mobilisation of the micelles to the detector and facilitate a MEKC separation. Therefore, the analytes were prepared in 50 mmol/L phosphate buffer solution (pH 5.0), and the running buffer (Buffer 1 in Fig. 1) during the sweeping under controlled EOF preconcentration was 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 5.0). In order to comparison the sweeping injection and typical injection, the sample hydrodynamic injection was performed at 0.2 psi for 5 s with analytes prepared in 50 mmol/L sodium phosphate buffer with pH 8.0 and pH 5.0, respectively, and the MEKC separation buffer was 50 mmol/L SDS in 50 mmol/L sodium phosphate buffer (pH 8.0). Fig. 2b shows the electropherogram with analytes prepared in pH 8.0 phosphate buffer. In spite of the peaks efficiencies and migration times similar to that of Fig. 2a, the detection sensitivity with a typical hydrodynamic injection for 5 s at 0.2 psi was far lower than that of sweeping under controlled EOF injection at 18 kV for 70 min. The similar result was obtained when the analytes were prepared in 50 mmol/L sodium phosphate buffer with pH 5.0. However, if using a pH 5.0 buffer solution for MEKC separation, the SDS micelle was always keeping at immobile state and the analytes could not be separated and migrated to the detection end whether the analytes prepared in pH 5.0 or pH 8.0 solution because the analytes were not detected for a long period. By comparing of Fig. 2a with Fig. 2b we can see, the micelle was kept at an immobile state at pH 5.0 to

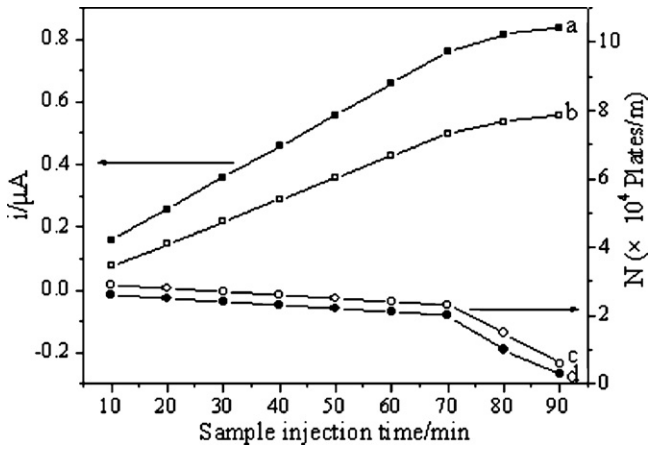

Fig. 3. Effect of sample injection time on peak current (a and b) and separation efficiency (c and d) of quercitrin (a and c) and phlorizin (b and d). The concentrations of quercitrin and phlorizin are 10 ng/mL in 50 mmol/L sodium phosphate buffer (pH 5.0). Other conditions are the same as in Fig. 2.

permit the sample injection for as long as 70 min and significant enhancement of sensitivity without loss of separation efficiencies.

3.4. Sample injection time

Although the immobile sweeping boundary permits an unlimited sample volume injection, there is still a potentially limiting factor for the large volume sweeping. The potential issue is the limited retention factor (k) of analytes in the micellar phase since the swept zone is controlled by the following equation [26],

$$l_{\text{sweep}} = \left(\frac{1}{1+k}\right) l_{\text{inj}} \quad (2)$$

where l_{sweep} and l_{inj} are the lengths of the swept zone and the injected sample solution, respectively. When the interaction between the analytes and micelles is too weak, the analytes may "leak" from the concentrated zone and flow to the detection end during the sweeping under controlled EOF preconcentration process. Therefore, the injection and sweeping duration needs to be optimized through experiments to determine the optimal values according to the analyte retention factor. The variation of peak current and separation efficiency with different sample injection times is shown in Fig. 3. In the figure, the increased peak current could be observed up to the injection time of 90 min, but the separation efficiency had decreased obviously when the injection time was longer than 70 min since the injected analytes were no longer held as a sharp zone at the front of the stationary sweeping boundary. Considering the sensitivity and separation efficiency, 70 min sample injection time was selected to do the remaining experiments.

3.5. Effect of high voltage on sample solution

The high voltage and the long voltage application time during the sweeping under controlled EOF preconcentration could lead to change of sample solution. With 18 kV for 70 min applied for the sweeping under controlled EOF preconcentration, successive injections from a 0.4 mL sample vial were conducted to study the change in the sample solutions. The typical electropherograms are shown in Fig. 4. The peaks were distorted after the third injection as well as shorter analyte migration times. Similarly, when the sample volumes of 0.8, 1.2 and 1.6 mL were used to conduct successive injections under 18 kV for 70 min each, the sixth, eighth and the twelfth injections, respectively, led to distorted peaks. When 2.0 mL sample volume was used, the peak shape still looked good after the fifteenth injection. The peak distortion and shorter migration time

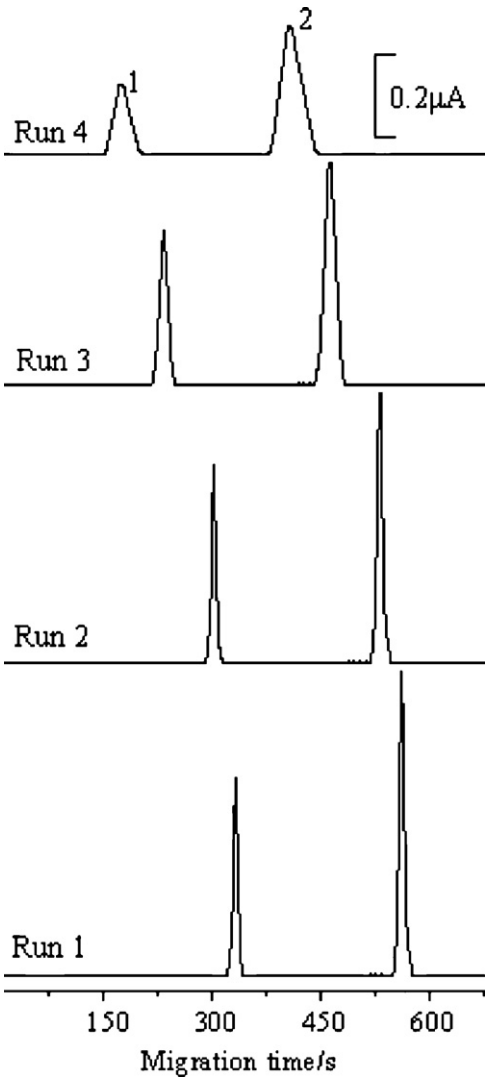

Fig. 4. High voltage effect on the sample solution during sweeping under controlled EOF preconcentration. Injection was performed under 18 kV for 70 min for several successive runs. Peak identification and other conditions are the same as in Fig. 2.

were mainly attributed to sample buffer change due to the electrolysis of water, which produced OH^- and increased the pH of the sample solutions. Furthermore, there was also the possibility of an increase in hydrodynamic flow as the volume of liquid in the sample was decreased during injection since 30 μL of sample solution was injected into the capillary for each run. Therefore, a large volume of sample solution needs to be prepared when 18 kV high voltage for 70 min was applied on the sample solution for sweeping under controlled EOF preconcentration.

3.6. Analytical characteristics

The effective sample volume injected into the capillary by the sweeping under controlled EOF for preconcentration is directly related to the apparent velocity of the analyte and the injection duration. Since the diameter of the capillary is fixed, the injected amount of an analyte can be quantified by the capillary length (l_{inj}) that would be occupied by the analyte at its original concentration. Phlorizin and quercitrin are phenolic substances and their pK_as are larger than 7.0 [27]. Therefore, the analytes are neutral compounds when the pH of sample buffer is 5.0 and their apparent velocity is

equal to the V_{eo}. So the injected length can be determined by the following equation,

$$l_{inj} = V_{eo}t = \mu_{eo}Et \quad (3)$$

where μ_{eo} is the EOF mobility. Under the experimental conditions, the μ_{eo} was 6.08×10^{-4} cm^2/(V s) determined with the current monitoring method [28]. For 70 min sample injection, the sample solution entering the capillary is about 1532 cm, equivalent to 51-fold the capillary length (30 cm), and the corresponding volume of sample solution is 3.0×10^4 nL. The retention factors of phlorizin and quercitrin in 50 mmol/L SDS buffer solution (pH 5.0) are 588 and 850, respectively. According to Eqs. (2) and (3), the swept zones of phlorizin and quercitrin are about 2.6 and 1.8 cm, respectively. The relatively high retention factor ensure the zone of phlorizin and quercitrin at the inlet end of the capillary during 70 min injection and sufficient capillary length remain after injection for subsequent separation. The detection enhancement was tested by 70 min ECBS preconcentration for 5.0 μg/L phlorizin and 5.0 μg/L quercitrin relative to the normal pressure injection for 5.0 mg/L phlorizin and 5.0 mg/L quercitrin at 0.2 psi for 5 s. The sample volume hydrodynamically injected with 0.2 psi for 5 s was 3.5 nL estimated by the following equation,

$$V_{inj} = \frac{\Delta P \pi d^4 t}{128 \eta L}$$

where ΔP is the pressure difference across capillary, d is capillary inside diameter, t is injection time, η is buffer viscosity and L is total capillary length. The enhancement factor was calculated by simply using the peak height ratio multiplied by the dilution factor. Signal enhancements of 2.3×10^4 and 2.1×10^4 fold were achieved for phlorizin and quercitrin, respectively. The limit of detection (LOD) for phlorizin and quercitrin under sweeping under controlled EOF preconcentration of 70 min at 18 kV was found to be 6.7 and 10.4 ng/L, respectively, which experimentally produced a signal-to-noise (S/N) ratio of 3. The limit of quantitation (LOQ) was 22.8 ng/L for phlorizin and 35.1 ng/L for quercitrin, which could be accurately quantified with an S/N of 10. The linear range for phlorizin and quercitrin were 0.01–20 ($n = 8$, $r = 0.9969$) and 0.006–15 μg/L ($n = 8$, $r = 0.9981$), with the equations of linear regression being $I = 49.6C + 0.38$ and $I = 73.8C + 0.62$ (I was peak current (nA) and C was analyte concentration, μg/L), respectively. The calibration curves for phlorizin and quercitrin were acquired by plotting the peak currents against individual analyte concentrations. Intraday relative standard deviations (RSD, $n = 5$) for migration time and peak area for the test mixture was less than 6 and 4%, respectively. Interday RSD ($n = 5$) was slightly worse, with 9 and 8% for migration time and peak area, respectively.

3.7. Analysis of phlorizin and quercitrin in urine sample

In the application of the proposed sweeping under controlled EOF preconcentration and MEKC separation system, phlorizin and quercitrin were analyzed in urine samples. Under the optimum preconcentration and separation conditions, the two analytes were determinated and the electropherogram is shown in Fig. 5. From Fig. 5a (blank urine sample) and b (postdosing urine sample), the electrochemical signals from the blank urine sample did not interfere with the model analyte peaks under experimental conditions. To check the validity and reliability of the proposed method, the concentration results with the proposed method were compared with those of the routine high performance liquid chromatography (HPLC) method from Pharmacopoeia of PR China for the determination of phlorizin and quercitrin as listed in Table 1. The concentrations of phlorizin and quercitrin in post-dosed urine samples determined with the proposed method were found to range

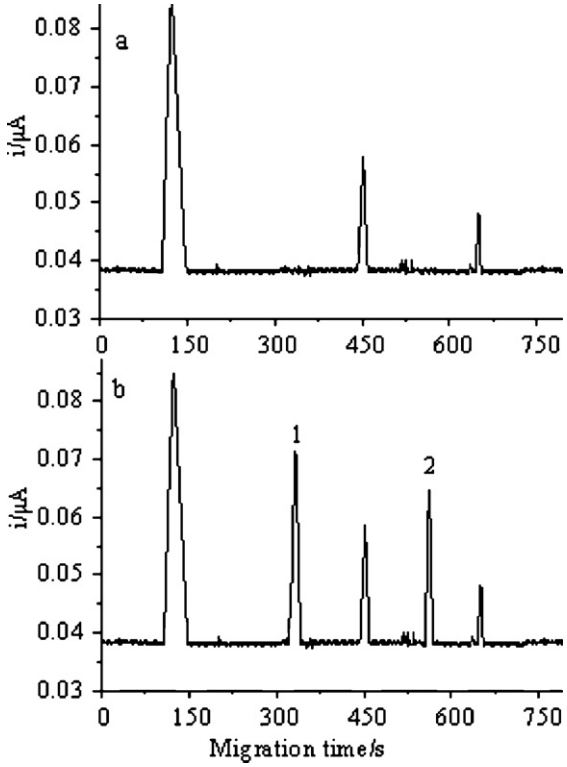

Fig. 5. Electropherogram of blank (a) and postdosing (b) urine samples. Peak identification and other conditions are the same as in Fig. 2.

Table 1
Comparison of concentration results of the proposed method with the routine HPLC method for the determination of phlorizin and quercitrin in different post-dosed urine samples.

Sample	Phlorizin (μg/L)		Quercitrin (μg/L)	
	The proposed method	HPLC method	The proposed method	HPLC method
1	14.3	16.1	8.7	10.1
2	7.9	9.4	12.3	13.6
3	18.5	17.3	0.05	0.07
4	0.6	0.9	3.5	4.9
5	4.7	3.2	0.4	0.7

from 0.6 to 18.5 μg/L and from 0.05 to 12.3 μg/L, respectively, which were in good agreement with the results of the routine HPLC method, indicating the high feasibility and validity of proposed method. The recoveries of phlorizin and quercitrin tested by spiking standard solutions into the post-dosed urine sample solutions from patients with the proposed method were 94.8 ± 0.7 and 97.1 ± 0.5%. The RSD ($n = 5$) of the reproducibility was 3.2 and 4.5%, respectively. The results demonstrate that the proposed method is very suitable for the determination of phlorizin and quercitrin in urine sample. This work also shows that the sweeping under controlled EOF preconcentration and MEKC separation is a powerful technique to study trace phlorizin and quercitrin in biomedical samples.

4. Conclusions

An easy, simple, and highly efficient on-line sweeping under controlled EOF preconcentration method for neutral compounds in CE was investigated. The EOF was controlled to balance the electrophoretic migration of SDS micelle to generate an immobile sweeping boundary. Samples solution was continually injected into

capillary and swept by SDS micelles for essentially a large volume. The detection sensitivity was improved more than 20,000-fold relative to a traditional pressure injection. The sweeping under controlled EOF scheme described in this paper may provide prospects for trace neutral or cationic analyte preconcentration.

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