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On-line concentration by field-enhanced sample injection with reverse migrating micelles in micellar electrokinetic capillary chromatography for the analysis of flavonoids in *Epimedium brevicornum* Maxim

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Abstract

A simple and sensitive micellar electrokinetic capillary chromatography (MEKC) method was developed for the separation and determination of six flavonoids in *Epimedium brevicornum* Maxim. Field-enhanced sample injection with reverse migrating micelles (FESI-RMM) was used for on-line concentration of the flavonoids. An electrolyte containing 20 mM H_3PO_4, 100 mM SDS, 20% acetonitrile and 2% 2-propanol (pH 2.0) was chosen as the electrophoretic buffer. By optimizing the stacking conditions, about 40–360-fold improvement in the detection sensitivity was obtained for the flavonoids.

Keywords: Field-enhanced sample injection; Reverse migrating micelles; Micellar electrokinetic chromatography; *Epimedium brevicornum* Maxim; Injection methods; Flavonoids

1. Introduction

Epimedium brevicornum Maxim from many species of the genus *Epimedium* (Berberidaceae), a commonly used Chinese medicine, has proven to have efficacy against cardiovascular diseases and much chronic illness, such as infertility, impotence and senile functional diseases, for over 2000 years [1]. Flavonoids are the major and active constituents of the genus. They are effective on dilation of the coronary, decreasing blood pressure and blood fat, inhibition of the platelet aggregation, delaying formation of thrombi, improving humoral and cellular immunity, increasing synthesis of DNA and anti-aging [1]. They can also be used to treat rheumatism, chronic tracheitis, infantile paralysis and neurasthenic in some clinical practices [2].

Several methods such as pulse polarography [3], thin layer chromatography (TLC) [4], high-performance liquid chromatography (HPLC) [5] and micellar electrokinetic capillary chromatography (MEKC) [1] have been used to study the flavonoids from *Epimedium* species. The former three methods had

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been only used to analyze the total flavonoids or icariin. MEKC has many advantages over other techniques, including high efficiency, technical simplicity and applicability to most analytes, small sample and reagent requirements. However, detection sensitivity is rather poor, being one to two orders of magnitude lower than that in HPLC. Therefore, it is necessary to develop a simple and sensitive MEKC for the analysis of flavonoids in *E. brevicornum* Maxim.

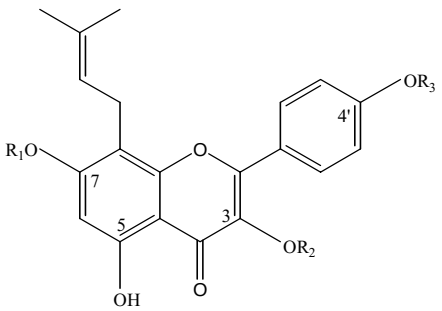
In 1998, Quirino and Terabe developed an on-line concentration technique in MEKC, field-enhanced sample injection with reverse migrating micelles (FESI-RMM) [6]. Low-pH buffer was used to reduce the electro-osmotic flow. The sample is dissolved with the aid of micelles in solution and injected into the capillary using voltage. This concentration technique has been shown to provide more than 100-fold increase in UV detector response with very high plate numbers. Sample stacking with a dynamic pH junction depends on the change in electrophoretic mobility as the charged analytes encounter the pH junction between the sample zone and the background eletrolyte (BGE) zone upon application of voltage. The pH junction is transient (dynamic) in nature, since the migrating buffer ions will gradually dissipate the pH difference during the separation. Thus, an analyte must possess an appropriate chemical functional group (e.g. amino, phenolic hydroxyl, or vicinal diol groups) so that it may exist in at least two distinct states, with different velocities, in the capillary. Concentration factors obtained for some zwitterionic catecholamines, weakly acidic compounds, and nucleotides are more than 50-fold [7,8].

In this paper, FESI-RMM was used for on-line concentration of the six flavonoids (the structures were shown in Fig. 1) in *E. brevicornum* Maxim. By optimizing the separation and stacking conditions, the analytes were successfully separated and about 40–360-fold improvement in the detection sensitivity was obtained for the flavonoids.

2. Experimental

2.1. Chemicals and materials

All the six flavonoids (icariin II (**1**), 2″-O-rhamnosylicariside I (**2**), ikarisoside A (**3**), icariin (**4**), epimedin B (**5**) and kaempferol-3-O-Rha (**6**)) isolated from *E. brevicornum* Maxim previously and the extract of *E. brevicornum* Maxim were obtained from National Laboratory of Applied Organic Chemistry, Lanzhou University. Phosphoric acid (H_3PO_4) and sodium dodecyl sulfate (SDS) were purchased from Beijing Chemical Plant and all chemicals used were of analytical grade.



	R_1	R_2	R_3
1	H	Rha	CH_3
2	H	Rha-Rha	CH_3
3	H	Rha	H
4	Glu	Rha	CH_3
5	Glu	Rha-Xyl	CH_3

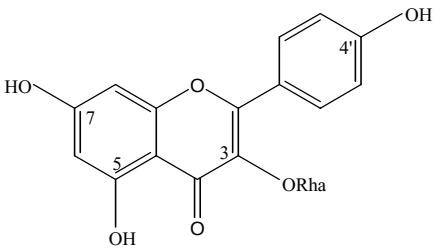


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Fig. 1. The structures of the analytes (Glu = glucose, Rha = rhamnose, and Xyl = xylose).

2.2. Solutions preparation

Stock solution of the standards containing 100 μg/ml of **1**, **3** and **6**, 200 μg/ml of **2**, 400 μg/ml of **4** and **5** was prepared in 60% methanol (methanol:water = 6:4). Solutions of lower concentration were prepared by diluting the stock solution with distilled water. The run buffer solution was prepared by mixing appropriate volumes of 0.2 M H_3PO_4, 0.2 M SDS, acetonitrile and 2-propanol in distilled water. All the solutions for CE were filtered through a 0.45 μm cellulose acetate filter (Shanghai Xinya Purification Apparatus Factory, Shanghai).

2.3. Apparatus

A Waters Quanta 4000 capillary electrophoresis system (Milford, MA, USA) controlled by a personal computer was used. Capillary electrophoresis was performed using a 70.0 cm (62.4 cm to the detector) × 75 μm i.d. fused silica capillary (Yongnian Photoconductive Fibre Factory, Hebei Province, China). Direct UV detection was employed at a wavelength of 254 nm. Data acquisition was carried out with a Maxima 820 chromatography workstation. The capillary was conditioned prior to its first use by consecutively flushing with 0.1 M NaOH for 5 min and distilled water for 5 min followed by the electrophoresis buffer for 5 min. To ensure good reproducibility, the buffer was renewed after every two runs and the capillary was rinsed between consecutive analyses with distilled water (for 1 min), followed by 1 M NaOH (1 min), methanol (1 min), 0.1 M NaOH (2 min), distilled water (2 min) and then the electrophoresis buffer (3 min). The temperature was controlled at 27.5 ± 0.5 °C. The separation voltage was 15 kV. A pHs-10A acidity meter (Xiaoshan Science Instrumentation Factory, Zhejiang, China) was used for the pH measurements. The pH of the buffer was adjusted with 0.2 M NaOH.

2.4. Operation procedure

A water plug was introduced into the capillary at the cathodic end by hydrodynamic injection where the water vial was raised by 10.0 cm. Voltage was applied at negative polarity with the sample solution in the cathodic vial and the buffer in the anodic vial. During sample injection, the current reached about −46 μA within 7 s, and then remained almost unchanged. After the sample was injected, the voltage was shut off and a buffer vial was placed at the cathodic end, and voltage was applied again at negative polarity until all peaks were detected.

2.5. Sample preparation

One hundred grams of powdered aerial part of *E. brevicornum* Maxim was refluxed with 1500 ml of distilled water for 1 h. Then, the refluxing was repeated two times with 1000 ml of distilled water. The extracts were combined and the water was evaporated under reduced pressure. The residue was dried below 100 °C and 11 g extract was obtained [9].

One hundred milligrams of powdered extract of *E. brevicornum* Maxim was dissolved with 10 ml of 60% methanol as the stock solution. The sample stock solution was diluted 20-fold for the determination of **3** and **6** in the sample and 200-fold for the determination of other four flavonoids in the sample. The solution was passed through a 0.45 μm filter and injected directly into the capillary electrophoresis system.

2.6. The stacking enhancement factor calculation

The stacking enhancement factors are calculated using the equations [6] $SE_{height} = H_{stack}/H$ and $SE_{area} = A_{stack}/A$, where the numerator is the peak height or peak area obtained with stacking and the denominator is the peak height or peak area obtained from 8 s hydrodynamic injection.

The effect of hydrodynamic injection time on the peak height of the analytes was investigated. When the injection time was increased from 2 to 8 s, the peak height increased. But on further increasing the injection time, the peak height of some of the analytes ceased to increase. So, we selected 8 s as the optimum hydrodynamic injection time. A time of 8 s for hydrodynamic injection was used as the evaluation base when SE_{height} and SE_{area} were calculated.

3. Results and discussion

3.1. Optimization of the separation conditions

The separation was optimized by studying the effects of the electrolyte pH and the concentrations of SDS and organic modifiers on the resolution between adjacent peaks. The optimum separation of the analytes was obtained with 20 mM H_3PO_4 containing 100 mM SDS, 20% acetonitrile and 2% 2-propanol at pH 2.0.

3.2. Optimization of the stacking conditions

3.2.1. Effect of SDS concentration on SE_{height}

As expected, SE_{height} increased with the SDS concentration increasing from 40 to 140 mM. This may be due to the increases of the retention factors of the analytes, and the difference between the electric field strength in sample zone and that in buffer zone with the increase of SDS concentration, which will increase the stacking efficiency. Though the SE_{height} was the highest at 140 mM SDS, some of the analytes were not baseline separated. Therefore, 100 mM SDS was selected.

3.2.2. Effect of the injection time of water plug on the peak height and reproducibility

With the injection time of water plug increasing from 0 to 3 s, the peak heights increased. The reason is that the water plug provides a higher electric field at the tip of the capillary, which will eventually improve the sample stacking procedure [10]. When the injection time was longer than 3 s, the peak heights began to decrease. This may be because when the water plug was too long, a strong laminar flow generated as a result of the mismatch of EOF velocity in the sample and buffer zones [11,12]. The introduction of water plug could also improve greatly the reproducibility of the peak heights [13]. Finally, the injection time of water plug was selected as 3 s.

3.2.3. Effect of the injection time of the sample on the peak height and peak shape

The peak heights of the analytes increased with the injection time increasing from 10 to 30 s. With further increase in the injection time, the peak heights of **4** and **5** began to decrease. At 30 s, the peaks shape of **1**, **2** and **3** became gradually broad and biforked, which may be due to sample overloading. Therefore, the injection time was chosen as 20 s to get compromise between the peak height and peak shape.

3.2.4. Effect of the injection voltage on the peak height

The peak heights of the analytes increased with the injection voltage increasing from −6 to −10 kV. However, further increase in the injection voltage led to the decrease of the peak heights. This may be because that higher voltage make the analytes get through the boundary between the water plug and the buffer and disperse into the buffer during injection procedure, which results in the decrease of the stacking efficiency [13]. Therefore, −10 kV was chosen.

3.2.5. Effect of the sample matrix on the peak height and peak area

Fig. 2 showed the effect of the composition of sample matrix on the peak heights and peak areas of the analytes. The sample matrix corresponding to the first group of data points was water. Others were 8 mM H_3PO_4 containing 8–32 mM SDS. As shown in Fig. 2, the peak heights and peak areas of **1**, **2**, **4** and **5** were higher, using 8 mM H_3PO_4 and 8 mM SDS as the sample matrix, than those obtained using water as the sample matrix. On the contrary, the peak heights and peak areas of **3** and **6**, two minor components in real sample, were higher when water was used as the sample matrix. This was because the concentration mechanisms were different when the two sample matrices were used. The molecules of investigated flavonoids have at least one ionization phenolic hydroxyl group. The pK_a values of phenolic hydroxyl groups at different positions for some monohydroxyflavonoids are 7.39 for 7-OH and 8.28 for 4′-OH [14]. According to Liang et al. [1], the pK_a values of **4** and **5** are 10.70 and 10.67, respectively. So, when water was used as the sample matrix, the six flavonoids could partly dissociate. But they were neutral in the background electrolyte (pH = 2). Hence, when the electric field was applied, they would migrate rapidly to the front edge of the sample plug (water plug) until they came into contact with the BGE where they would stop migrating. The flavonoids were focused because of the difference in their electrophoretic

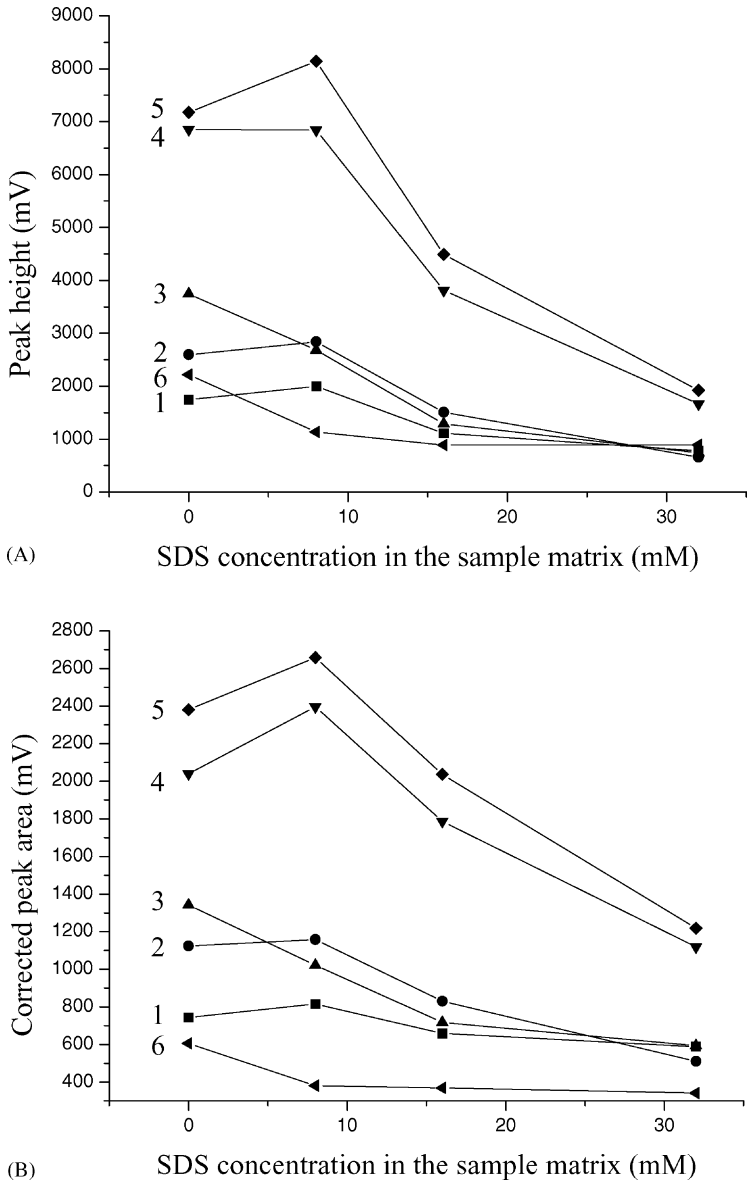

Fig. 2. Effect of the composition of sample matrix on the peak heights (A) and peak areas (B) of the analytes. The sample matrix corresponding to the first group of data points is water. Others are 8 mM H_3PO_4 containing 8–32 mM SDS. The sample was injected using −10 kV for 20 s after introduction of 3 s water plug. The concentrations of analytes were 2–8 μg/ml. Buffer: 20 mM H_3PO_4, 40–140 mM SDS and 20% (v/v) acetonitrile. Separation voltage was −15 kV.

mobilities in the high pH sample plug and the low pH BGE. Therefore, when water was used as the sample matrix besides FESI-RMM, a dynamic pH junction also had an effect on the concentration of flavonoids [7,8]. But when an electrolyte containing 8 mM H_3PO_4 and 8 mM SDS was used as the sample matrix, only FESI-RMM had effect on the concentration of flavonoids. The lower the conductivity of sample matrix, the better is enhancement of the field strength at inlet tip of the capillary during injection using

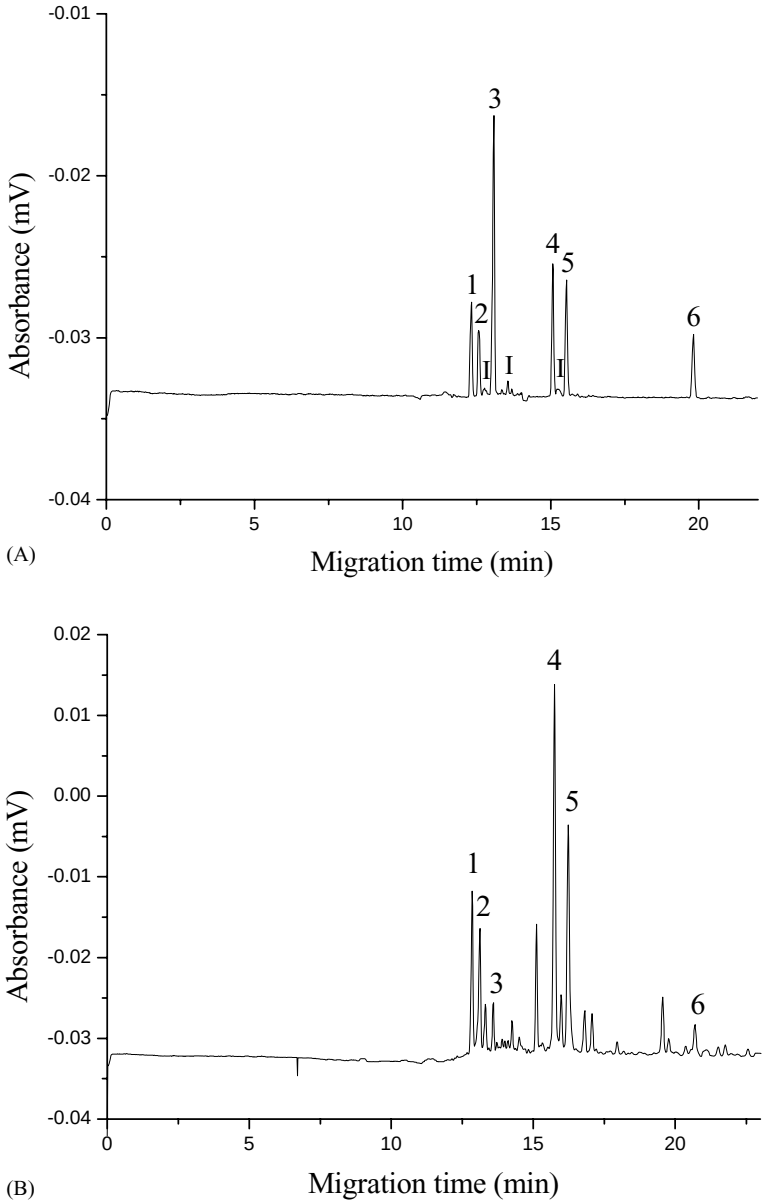

Fig. 3. The electropherogram of (A) the standards mixture and (B) the extract of *Epimedium brevicornum* Maxim. Other conditions were the same as in Fig. 2. *I* = impurity in the standards.

voltage. So, with the SDS concentration increasing from 8 to 32 mM, the peak heights and peak areas of the analytes decreased. Finally, water was used as the sample matrix.

Under the optimum separation and stacking conditions, the electropherogram of the standard mixture was shown in Fig. 3A. The impurity in Fig. 3A was associated with analytes **2**, **3** and **5**. All the six flavonoids were well separated within 21 min. The migration sequence coincided with that of the retention factor. The higher the retention factor, the shorter is the migration time. The stacking enhancement factors of the

Table 1
Stacking enhancement factors (SE_{height} and SE_{area}) of the analytes[a]

	1	2	3	4	5	6
Se_{height}	35	34	27	8	6	50
Se_{area}	30	31	41	8	5	52

[a] Concentration of the analytes: 10–40 μg/ml.

analytes, SE_{height} and SE_{area}, were given in Table 1. For low charge–mass ratio compounds (analytes **1**, **2**, **4** and **5**), the stacking factor mainly depended on the retention factors of the analytes. But for high charge–mass ratio compounds (analytes **3** and **6**), it mainly depended on the magnitude of charge–mass ratio of the analytes.

3.3. Linearity, reproducibility and limits of detection

Under the optimum conditions, the linear relationships between the peak heights of the analytes and the corresponding concentrations were shown in Table 2. The reproducibility of the method was determined with a standard mixture solution containing 2 μg/ml of **1**, **3** and **6**, 4 μg/ml of **2**, and 8 μg/ml of **4** and **5**. The R.S.D. values ($n = 5$) of the migration times and peak heights are 0.8–1.6 and 3.7–5.7%, respectively. The limits of detection for the analytes ($S/N = 3$) were 0.016–0.079 μg/ml. As a comparison, the linearity and limits of detection using the 8 s hydrodynamic injection were also given. The results demonstrated that about 40–360-fold increase in the detection sensitivity was obtained under the optimum conditions.

Table 3
Contents of the analytes in *Epimedium brevicornum* Maxim and R.S.D. ($n = 5$)

	1	2	3	4	5	6
Content (mg/g)	3.1	7.7	0.4	26.4	29.1	0.6
R.S.D. (%)	6.4	5.6	4.1	5.5	2.0	5.7

3.4. Application

The optimum conditions were applied to the separation and determination of **1**–**6** in the extract of *E. brevicornum* Maxim. The electropherogram is shown in Fig. 3B. The peaks were identified by comparing the migration times and spiking the standards to the real sample solution. The contents of the analytes in *E. brevicornum* Maxim together with R.S.D. ($n = 5$) are given in Table 3. The recovery of the method was determined with the addition of the standards in the real sample solution, with results from 97.5 to 104.5%.

Table 2
The results of regression analysis on calibration curves and the detection limits

Analyte number	Calibration curves $y = a + bx$[a]	Correlation coefficient	Linear range (μg/ml)	Detection limit[b] (μg/ml)
Stacking condition				
1	$y = -339.19 + 3374.35x$	0.9993	0.1–4	1.8×10^{-2}
2	$y = -258.72 + 1562.99x$	0.9995	0.2–12	3.9×10^{-2}
3	$y = -277.99 + 3691.69x$	0.9955	0.1–6	1.6×10^{-2}
4	$y = -559.54 + 1077.06x$	0.9988	0.4–16	5.6×10^{-2}
5	$y = 179.93 + 758.47x$	0.9977	0.4–16	7.9×10^{-2}
6	$y = -159.64 + 2175.93x$	0.9996	0.1–6	2.8×10^{-2}
8 s hydrodynamic injection				
1	$y = 155.50 + 54.35x$	0.9972	7.5–250	2.2
2	$y = -68.56 + 47.07x$	0.9997	15–500	2.6
3	$y = -67.57 + 90.62x$	0.9997	7.5–250	1.3
4	$y = -109.78 + 31.99x$	0.9995	20–400	3.8
5	$y = -65.91 + 38.37x$	0.9997	20–400	3.2
6	$y = 166.18 + 11.87x$	0.9989	15–500	10.2

[a] y and x are the peak heights and the concentrations of the analytes, respectively.
[b] The detection limit was defined as the concentration where the signal-to-noise ratio is 3.

4. Conclusion

Field-enhanced sample injection with reverse migrating micelles (FESI-RMM) was used for on-line concentration of the flavonoids in *E. brevicornum* Maxim. The detection sensitivity was improved about 40–360-fold compared with those obtained from 8 s hydrodynamic injection. For low charge–mass ratio compounds, low concentration micellar solution was more valuable and the stacking factor mainly depended on the retention factors of the analytes; while for high charge–mass ratio compounds, using water as the sample matrix was better and the stacking factor mainly depended on the magnitude of charge–mass ratio of the analytes. The results also demonstrated that the proposed method was applicable in the analysis of complex sample and was easy to use for the analysis of the minor component in the plant.

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