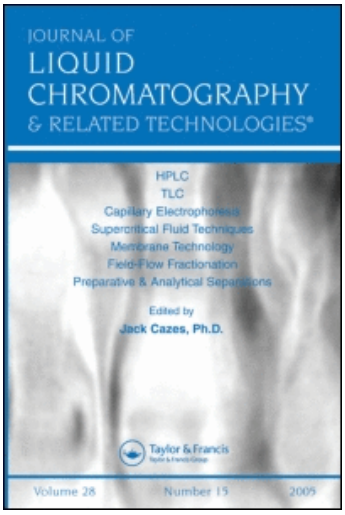

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Analysis of Five Pharmacologically Active Compounds from the Tibetan Medicine *Elsholtzia* with Micellar Electrokinetic Capillary Chromatography

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Abstract: A high performance capillary electrophoresis method with diode array detector detection for the determination of five bioactive ingredients in Tibetan medicine *Elsholtzia*, namely quercetin, rutin, saussurenoside, kaempferol, and oleanolic acid, has been developed. The effects of several factors, such as the acidity, concentration of running buffer, separation voltage, temperature, and SDS concentration were investigated. The optimal conditions were 44 mmol/L boric acid running buffer (pH 8.5), 45 mmol/L SDS, 16 KV voltage, 20°C, and 10.0% (V/V) of acetonitrile. Under the optimum conditions, five components could be separated with a good baseline resolution within 17 min. The calibration curves showed good linear relationship over the concentration range of 5×10^{-4}∼0.1 mg/mL for quercetin, rutin, saussurenoside, kaempferol, and 1×10^{-3}∼0.1 mg/mL for oleanolic acid. The average recoveries of the method and RSD were (99.2%, 3.2%) for quercetin, (102.1%, 2.1%) for rutin, (99.4%, 1.5%) for saussurenoside, (98.9%, 1.8%) for kaempferol, and (99.0%, 2.9%) for oleanolic acid, respectively. The detection limits (S/N = 3) were 1.1×10^{-4} mg/mL for quercetin, 2.6×10^{-4} mg/mL for rutin, 1.8×10^{-4} mg/mL for saussurenoside, 2.9×10^{-4} mg/mL for kaempferol, and 6.3×10^{-4} mg/mL

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for oleanolic acid, respectively. The method was simple, rapid, and reproducible and could be applied for the determination of quercetin, rutin, saussurenoside, kaempferol, and oleanolic acid in Tibetan medicine *Elsholtzia*, and the assay results were satisfactory.

Keywords: Micellar electrokinetic capillary chromatography, *Elsholtzia*, Oleanolic acid, Kaempferol, Saussurenoside, Quercetin, Rutin

INTRODUCTION

Within the pharmaceutical industry, the identification of novel active compounds through the use of powerful tools can be considered to be one of the most important discoveries for natural drug. Samples isolated for these compounds often are initially generated as complex mixtures consisting of multiple components, and powerful analysis tools are required for both the separation and characterization of the compounds in these mixtures in a rapid manner. Traditional chromatography has served as an effective technique for the separation of many of the components in complex mixtures. However, a great quantity of organic solvent must be employed, with drastic toxicity such as methanol, *n*-hexane, and benzene. In addition, the use of liquid chromatography (LC) sufferes from the problem that its column becomes easily contaminated for silica or alumina stationary phases; the column is hard to regenerate. Compared with LC, capillary electrophoresis (CE) is a conceptually simple technique that offers highly efficient separations with the requirement of only small amounts of samples. CE is also an effective tool for the separation of polar natural compounds with high efficiency, short analysis time, low consumption, and multiple modes to be chosen such as CZE, CEC, MEKC, CGE, CITP, and capillary isoeletric focus.

Although numerous publications were reported for the analysis of effective components from different traditional Chinese medicines (TCM),[1–5] the further composition analysis from TCM plants can be considered to be one of the most important discoveries in natural drugs. *Elsholtzia* is a traditional Tibetan medicine belonging to the family of Lamiaceae that is mainly distributed in East Asia, with about 40 species recorded in the world and 34 species and 16 varied species found in China.[6] The extracts of *Elsholtzia* exhibits a different efficacy as a result of clinical examination, such as inhibiting the central nervous system, anti-virus, anti-inflammatory, analgesic, anti-asthma, anti-cough, and anti-tumor.[7–11] The separation of several pure compounds from *Elsholtzia* plants by column chromatography was described.[12–21] However, the employment of CE as a tool for the simultaneous separation five natural polar compounds from *Elsholtzia* for drug discovery has not yet been investigated or exploited. In this study, the influence of parameters upon the retention and resolution of polar natural

product compounds was evaluated with the goal of optimizaing the utility of CE. The method has been successfully used for the determination of these analytes in *Elsholtzia* plants; the assay results were satisfactory.

EXPERIMENTAL

Instrumentation

Experiments were performed on an HP-3D CE system with a diode array detector (Agilent Technologies, USA). Data were collected on a PC computer using HP 3D ChemStation. In all experiments, 48.5 cm × 50 μm (40 cm to the detector) fused silica capillaries (Yongnian Optical Fiber Factory, Hebei, P. R. China) were used. The samples were detected at 221 nm. Before use, new capillaries were flushed with 1.0 M NaOH for 1 h, then flushed with redistilled water and background buffer for 20 min. Between runs the capillary was rinsed under pressure with 0.1 M NaOH, redistilled water, and running buffer for 3 min each. The temperature was maintained at 22°C. The other conditions were as follows: applied, voltage 16 KV; samples were injected by applying a pressure of 50 mbar for 4 s.

Chemicals

Potassium hydroxide was purchased from Jining Chemical Reagent Co (Shandong, China). HPLC grade of methanol, acetonitrile, were obtained from Shanghai Chemical Reagent Co (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used in this study were also of analytical grade unless otherwise stated. Quercetin and rutin (purity ≥ 98.0%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Saussurenoside, kaempferol, oleanolic acid, were isolated from *Elsholtzia ianthina*. Their structures were confirmed by comparing their melting points, ^{1}H-NMR, IR, UV, and MS data with those given in the literatures.[22–26] The chemical structures of all standards are shown in Fig. 1.

Preparation of Standard Solutions

The standard solutions of five components (1.0×10^{-3} mol/L) were prepared by dissolving corresponding amounts of pure compounds in 10 mL of 50% acetonitrile. A corresponding low concentration of solution

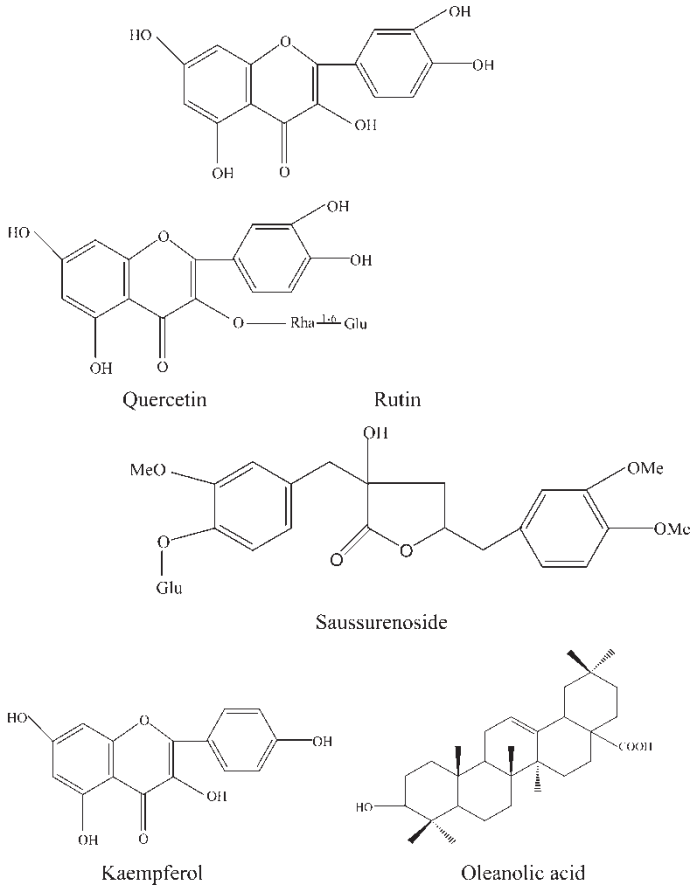

Figure 1. Structure of five natural product standards.

(1.0×10^{-5} mol/L) was obtained by diluting the stock solution with acetonitrile. When not in use, all reagent solutions were stored at 4°C or at −20°C in a refrigerator until CE analysis.

The four *Elsholtzia* samples were collected from Qinghai-Tibet Plateau (*Elsholtzia densa* was collected from mountainous Lijia Xining Qinghai, *Elsholtzia ianthina* was collected from mountainous Laji Qinghai, *Elsholtzia feddei* was collected from Duoba Xining Qinghai, *Elsholtzia calycocarpa* was collected from Menyuan Haibei Qinghai), and were washed successively with 20 mL each of water and deionized water. The washed *Elsholtzia* samples were dried under a stream of nitrogen and crushed into powdered samples. To a 10 mL round-bottom flask, 1.0 g powdered *Elsholtzia* and 10 mL 80% methanol was added. The contents of the flask were allowed to incubate at room temperature for 24 h, then immersed in

a sonicator water bath, and the samples were sonicated in 5 min intervals for 30 min. The contents were then centrifuged at a speed of 4000 rpm for 15 min. The supernatant was collected and stored at 4°C in a refrigerator until CE analysis.

RESULTS AND DISCUSSION

The main challenge of the present work was to test the feasibility for the separation of five natural pharmacologically active compounds from extracted *Elsholtzia* with CE in a variety of conditions including migration time, temperature, voltage, pH of buffers, and solvents.

Effect of Buffer Concentrations

Several types of buffers were tested in this study for separation of five polar compounds, including carbonate buffers, phosphate buffers, and borate buffers. The results indicated that a reasonably good separation of all five polar compounds was obtained with borate buffers at concentrations of 38–50 mmol/L with pH 8.5. The result is shown in Fig. 2. As can be seen from Figure 2, with borate buffer concentrations <38 and >50, quercetin and rutin were coeluted. To achieve optimal separation, operation at buffer

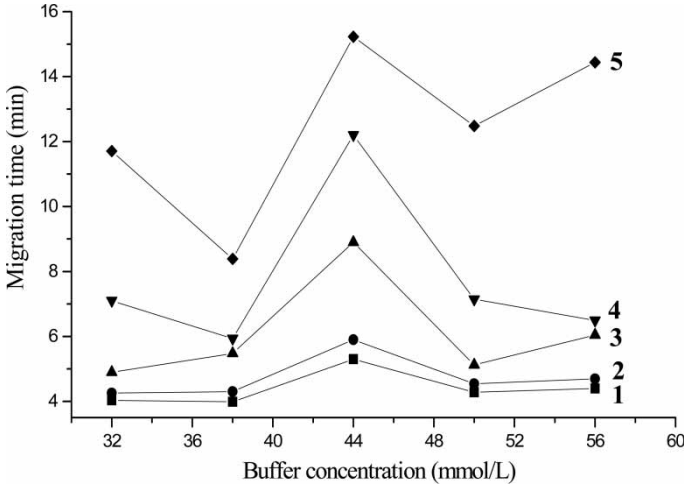

Figure 2. Effect of buffer concentrations on the separation of five compounds. Conditions: borate buffer at pH 8.5 containing 45 mmol/L SDS and 10% acetonitrile; applied voltage, 16 KV; temperature, 20°C; detection at 221 nm.

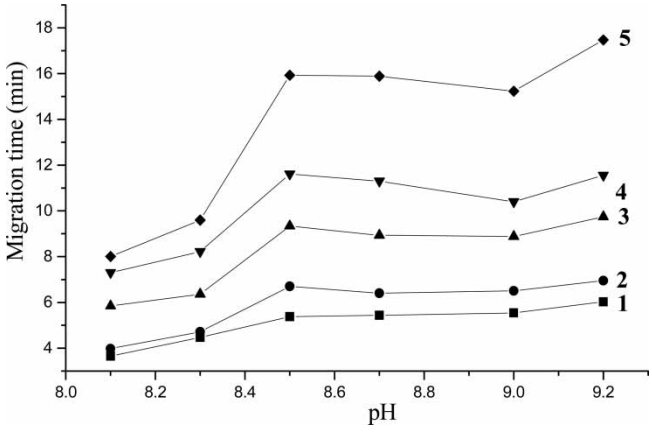

Figure 3. Effect of pH on the separation of five compounds. Conditions: borate buffer was 44 mmol/L borate, other conditions as in Figure 2.

concentrations at 38–50 mmol/L resulted in obvious baseline resolution. The final borate concentration was selected at 44 mmol/L.

Effect of pH

The effect of pH on the resolution was investigated with borate buffer in the pH range of 8.1–9.2. As shown in Fig. 3, the resolution of five polar

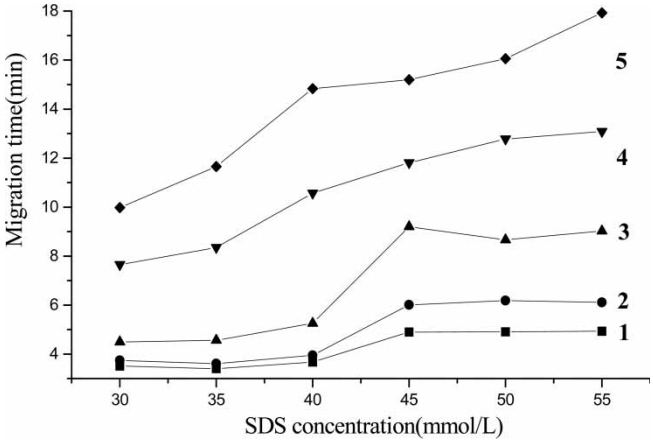

Figure 4. Effect of SDS concentration on the separation of five compounds. Conditions: borate buffer was 44 mmol/L, borate at pH 8.5, other conditions as in Figure 2.

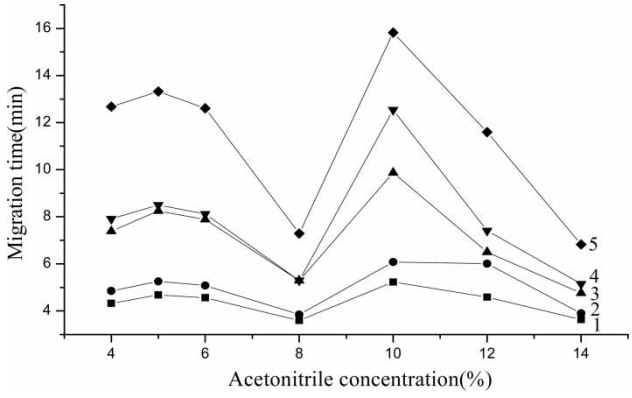

Figure 5. Effect of acetonitrile concentration on the separation of five compounds. Conditions: 45 mmol/L SDS, other conditions as in Figure 4.

compounds in CE running procedure increased progressively with increasing pH of buffers. As can be seen from Fig. 3, with pH < 8.3, quercetin and rutin were coeluted. To achieve optimal derivatization, the pH should be as high as possible. With pH 8.5, a complete baseline resolution of five polar compounds was observed. At higher pH values (>8.5), better resolution was obtained. However, the running time was prolonged at higher pH buffers. Therefore, 44 mmol/L borate buffer solution at pH 8.5 was used in the final optimization.

Effect of SDS Concentration

The effect of SDS concentrations on the resolution was investigated for five polar compounds. In this experiment, six CE running solutions, including 30–55 mmol/L SDS were, respectively, prepared with the optimized pH and buffers as described above. The effects of SDS on resolution of the compounds are shown in Fig. 4. As can be seen, the migration time and corresponding resolution of solutes increased with increasing SDS concentrations. Therefore, 45 mmol/L SDS was selected as the optimal SDS concentration, since it provided the shortest analysis time, and still gave good separation of all five polar compounds.

Effect of Organic Modifier

In this experiment, 4.0–14.0% of acetonitrile solutions were investigated for optimal separation of all five polar compounds. Figure 5 shows the effect of acetonitrile concentration on the resolution. When the acetonitrile

concentration was increased to 10.0%, the separation was improved and complete baseline separation was achieved. For the final method, 10.0% of acetonitrile in the eluent was chosen.

Effect of Temperature and Voltage

The effect of temperature on the resolution was investigated in the temperature range of 16 to 24°C. As shown in Fig. 6(A), when the temperature was lower than 18°C, quercetin and rutin were coeluted. When the temperature was above 18°C, the resolution of five polar compounds in the CE procedure increased progressively with the increasing temperature; a complete

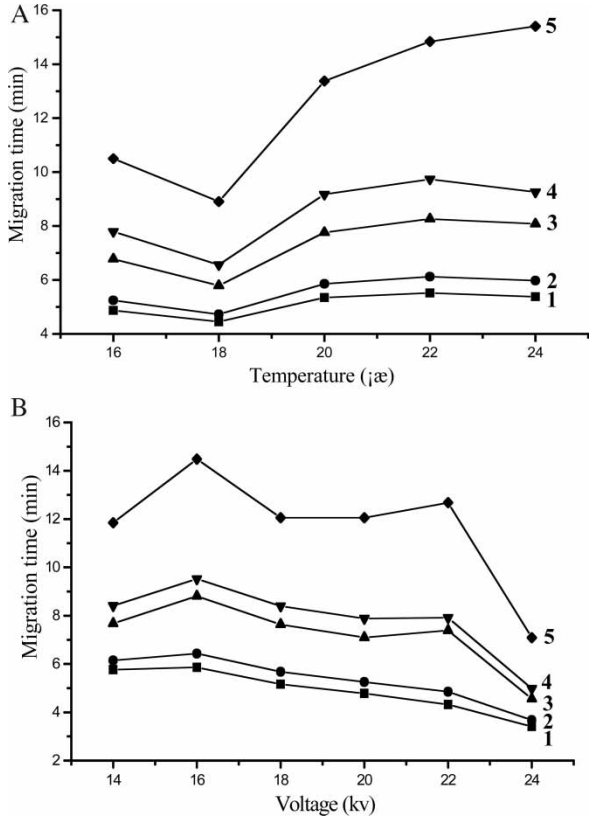

Figure 6. Effects of capillary temperature and applied voltage on the migration times of analytes. Conditions: 10% acetonitrile, other conditions as in Figure 5. In the top panel represents the effect of capillary temperature, in the bottom represents the effect of voltage.

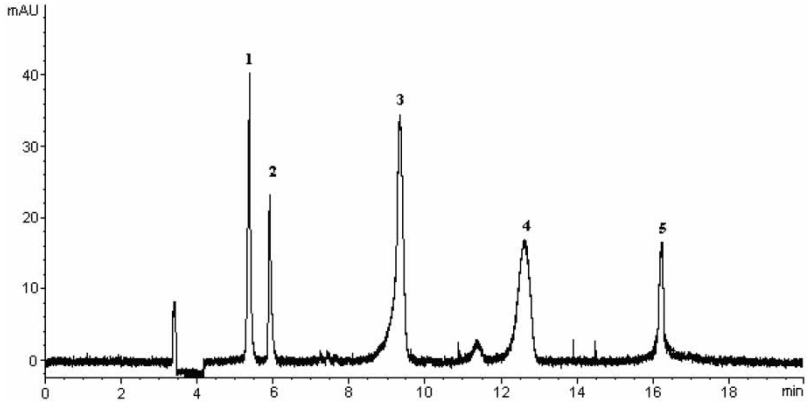

Figure 7. Electropherogram of standard solutions of five natural products. Running buffer: 44 mmol/L borate buffer containing 45 mmol/L SDS and 10% acetonitrile, other conditions and peak identification as in Figure 2.

Table 1. Relative standard deviation (RSD) of migration time and peak area

Analytes	Migration time RSD (%)	Peak area RSD (%)
Quercetin	0.35	3.26
Rutin	0.42	4.54
Saussurenoside	0.46	6.03
Kaempferol	0.82	7.13
Oleanolic acid	1.31	9.27

baseline resolution was observed at 20°C. Therefore, 20°C was used for the separation of solutes.

The effect of voltage on the resolution of the compounds is shown in Fig. 6(B). As can be seen, when the voltage increased from 14 to 16 KV, the migration time and corresponding resolution of solutes increased with

Table 2. Linear regression equations, linear range, correlation coefficents and detection limits of five active components

Analytes	Linear regression equations	Linear range (mg/mL)	Correlation coefficents	Detection limits (mg/mL)
Quercetin	$y = 53.89x + 2.46$	$5 \times 10^{-4} \sim 0.1$	0.9974	1.1×10^{-4}
Rutin	$y = 50.74x + 0.74$	$5 \times 10^{-4} \sim 0.1$	0.9986	2.6×10^{-4}
Saussurenoside	$y = 44.25x + 0.26$	$5 \times 10^{-4} \sim 0.1$	0.9980	1.8×10^{-4}
Kaempferol	$y = 46.44x + 4.35$	$5 \times 10^{-4} \sim 0.1$	0.9954	2.9×10^{-4}
Oleanolic acid	$y = 79.58x + 0.77$	$1 \times 10^{-3} \sim 0.1$	0.9938	6.3×10^{-4}

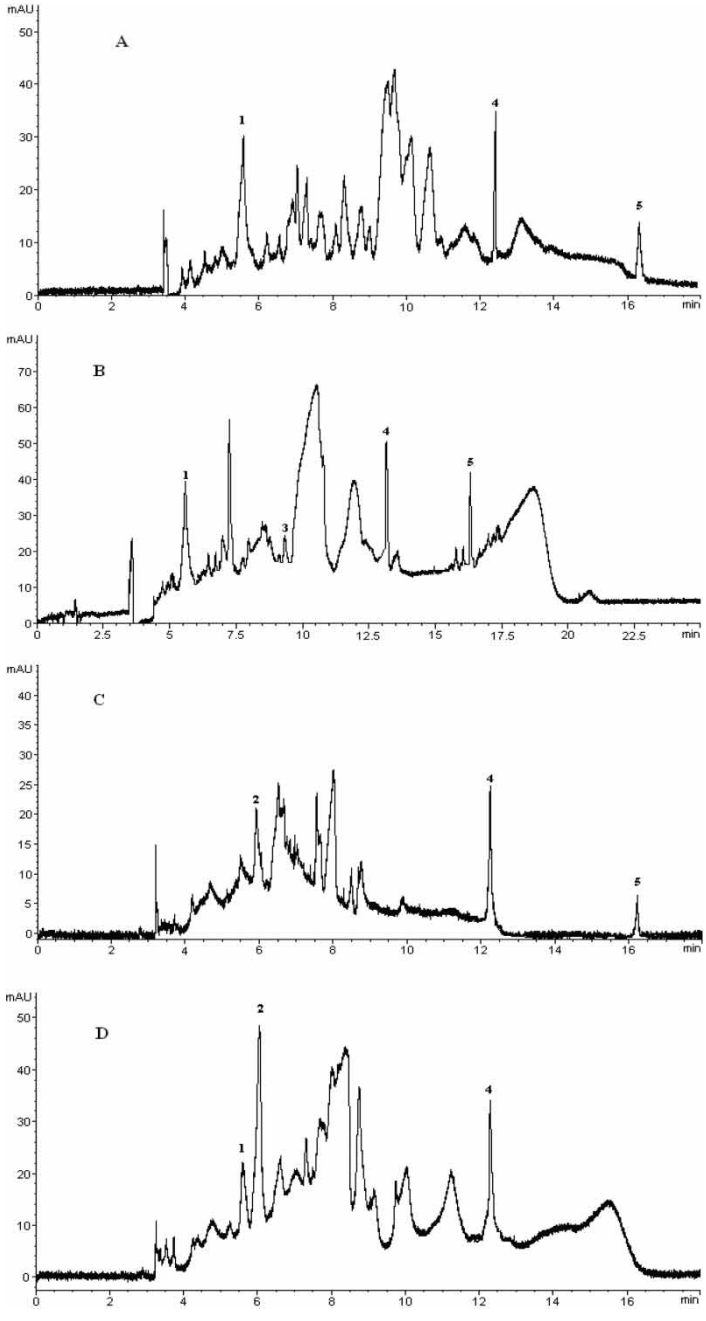

Figure 8. Electropherogram of extracts of *Elsholtzia densa* (A), *Elsholtzia ianthina* (B), *Elsholtzia feddei* (C), *Elsholtzia calycocarpa* (D) plants using methanol as extraction solvent. Separation conditions as in Figure 7.

the enhancement of the voltage. From 16 KV to 24 KV, the migration time and corresponding resolution of solutes decreased with the enhancement of the voltage. Taking both the short migration time and the good resolution into consideration, the applied voltage was selected at 16 KV. As a result of these experiments, the optimum separation conditions were set at 45 mmol/L SDS, 10.0% (V/V), acetonitrile containing 44 mmol/L borate buffer at 20°C, with 16 KV voltage. Figure 7 shows the electropherogram of the five standard polar compounds under the proposed conditions.

Reproducibility, Linearity, and Detection Limits

Repeatability was determined by carrying out six successive injections of standard solutions within one day. The relative standard deviation (RSD)

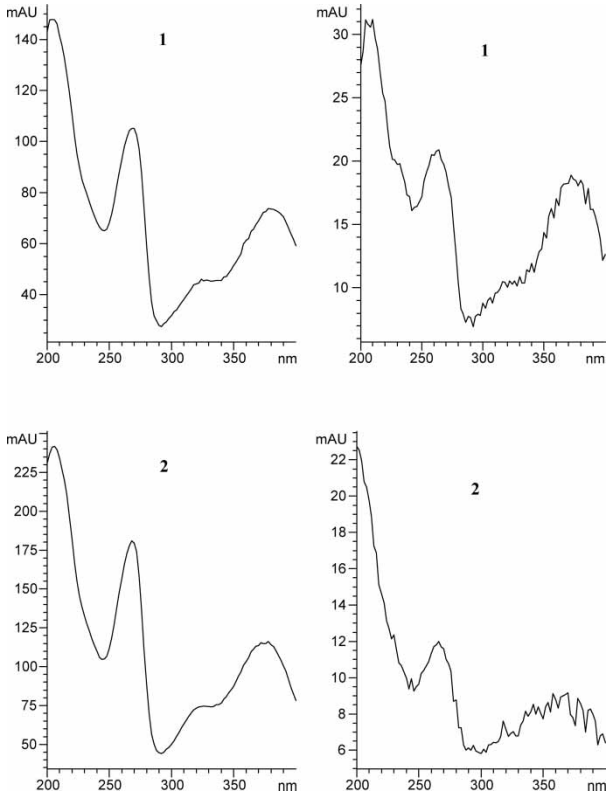

Figure 9. UV spectra of five standard natural products and on-line spectra of five standard natural products (left: standards, right: real samples). 1. quercetin; 2. rutin; 3. saussurenoside; 4. kaempferol; 5. oleanolic acid.

(*continued*)

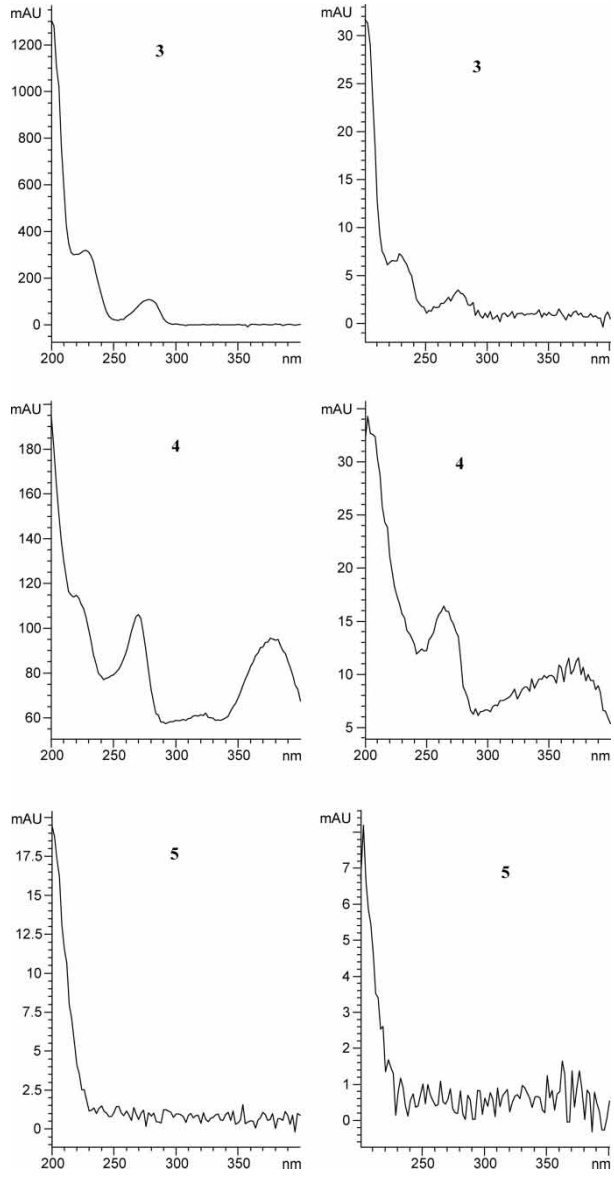

Figure 9. Continued

of migration times and peak areas are listed in Table 1. The linearities were established for the five active components with DAD detection (linearity range from 5×10^{-4}~0.1 mg/mL for quercetin, rutin, saussurenoside, kaempferol, and 1×10^{-3}~0.1 mg/mL for oleanolic acid). All of them

Table 3. Assay Results of the analytes in four kinds of real samples

Analytes	*Elsholtzia. densa* (mg/g)	*Elsholtzia. ianthina* (mg/g)	*Elsholtzia. feddei* (mg/g)	*Elsholtzia. calycocarpa* (mg/g)
Quercetin	0.75	0.63	[a]	0.45
Rutin	[a]	[a]	0.48	0.98
Saussurenoside	[a]	0.24	[a]	[a]
Kaempferol	0.64	0.59	0.46	0.53
Oleanolic acid	0.41	0.28	0.89	[a]

[a]Not detectable or below detection limits.

were found to give good linear responses over this range, with correlation coefficients >0.9938. The detection limits (at a signal-to-noise ratio $= 3:1$) were 1.1×10^{-4} mg/mL for quercetin, 2.6×10^{-4} mg/mL for rutin, 1.8×10^{-4} mg/mL for saussurenoside, 2.9×10^{-4} mg/mL for kaempferol, and 6.3×10^{-4} mg/mL for oleanolic acid, respectively. The linear regression equations, correlation coefficients, and detection limits are shown in Table 2.

Determination of Extracted Samples and Recovery

Extracted methanol solutions from several Elsholtzia plants were directly injected into the capillary. Separation of the five compounds was achieved within 17 min (Fig. 8). The peaks were identified with spiking standards. The on-line UV spectra of compounds in *Elsholtzia* (right) agreed with the five standard samples (left) (Fig. 9). The contents of quercetin, rutin, saussurenoside, kaempferol, and oleanolic acid from extracted *Elsholtzia* are listed in Table 3.

The recovery was determined by addition of a known amount of standards into the methanol extracts. The results were 99.2% for quercetin, 102.1% for rutin, 99.4% for saussurenoside, 98.9% for kaempferol, and 99.0% for oleanolic acid, respectively.

CONCLUSION

The work presented demonstrates that the five pharmacologically active compounds could be separated with a good baseline under the optimal conditions within 17 min using capillary electrophoresis. The developed method shows good repeatability for the analysis of active compounds from extracted *Elsholtzia* plants.

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