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Simultaneous determination of active ingredients in *Erigeron breviscapus* (Vant.) Hand-Mazz. by capillary electrophoresis with electrochemical detection

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Abstract

A high-performance capillary electrophoresis (CE) with electrochemical detection (ED) method was developed for the determination of the pharmacologically active ingredients in *Erigeron breviscapus* (Vant.) Hand-Mazz. and its extract phytopharmaceuticals in this work. Under the optimum conditions, nine analytes, baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid were separated within 24 min in a borax buffer (pH 8.7). Notably, excellent linearity was obtained over two orders of magnitude with detection limits ($S/N = 3$) ranged from 1.0×10^{-7} g/mL to 5.6×10^{-7} g/mL for all nine analytes. This method was successfully used in the analysis of *E. breviscapus* (Vant.) Hand-Mazz. and its phytopharmaceuticals with a relatively simple extraction procedure, and the assay results were satisfactory.

Keywords: *Erigeron breviscapus* (Vant.) Hand-Mazz.; Capillary electrophoresis; Electrochemical detection

1. Introduction

Erigeron breviscapus (Vant.) Hand-Mazz. belongs to the family of Compositae [1]. It has been used as a medicinal herb in the folklore of China for promoting blood circulation, expelling the cold and relieving exterior syndrome, dispelling the wind and dampness, as well as removing stagnancy of indigested food and relieving pain. Intensive research has been undertaken on this plant, revealing a wide range of profound and important therapeutic effects, which can be grouped into hematic and circulatory system's effects [2]. Especially, as a therapeutic drug, it has no side effect for long use. Modern research has revealed that flavonoids show cancer chemopreventive effects in several animal models [3,4] and some phenolic acids, namely ferulic, chlorogenic and caffeic acid, have been found to be pharmacologically active as antioxidant, antimutagenic and anticarcinogenic agents [5,6]. Chemical constituent investigations show that it mainly contains flavonoids, phenolic acids and their glucuronides, etc. [1]. Baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid are important active constituents in this plant [7]. The molecular structures of these compounds are shown in Fig. 1. Hence, it is necessary to develop some simple, economical and efficient methods for the analysis and quantitative measurement in order to establish the quality standard.

The analysis of active ingredients of *E. breviscapus* (Vant.) Hand-Mazz. is often a challenging task because of complicated compositions, significant concentration difference of active constituents, as well as effects of many factors such as climates, regions of growth, seasons of harvest on the contents of active ingredients. For the analysis of ingredients in *E. breviscapus* (Vant.) Hand-Mazz., there have been several analytical methods including high performance liquid chromatography (HPLC) with UV [8,9], or tandem mass spectrometry [10,11], UV [12], spectrophotometry and TLC [13], as well as wave spectrum or NMR [14,15]. HPCE with

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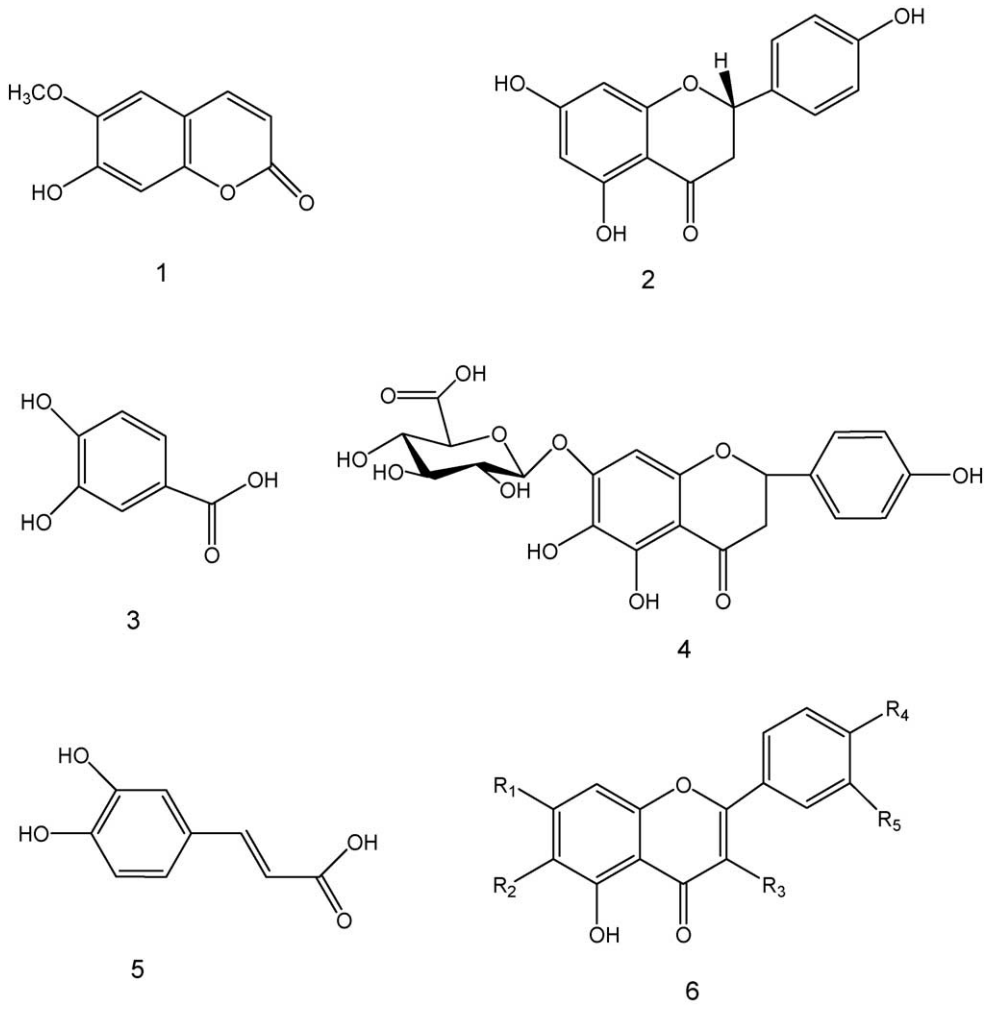

Fig. 1. Molecular structures of (1) scopoletin, (2) naringenin, (3) protocatechuic acid, (4) scutellarin, (5) caffeic acid, (6) baicalein (R_1, R_2 = OH, R_3, R_4, R_5 = H); kaempferol (R_1, R_3, R_4 = OH, R_2, R_5 = H); apigenin (R_1, R_4 = OH, R_2, R_3, R_5 = H); luteolin (R_1, R_4, R_5 = OH, R_2, R_3 = H).

UV [17] has also been employed for this purpose. Furthermore, CE–ED [18] has been applied for the analysis of six flavonoids in *Ginkgo biloba* L. including apigenin and luteolin.

However, HPLC, as a currently prevailing method for the analysis of traditional Chinese medicines, often has some shortcomings, including long analysis time, low resolution and short column lifetime owing to numerous co-existing interfering compounds, some of which can be adsorbed strongly onto the packing materials of HPLC column, resulting in fast column degradation or even irreversible damage [19,20]. Capillary electrophoresis (CE) is increasingly recognized as an important analytical separation technique because of its speed, efficiency, reproducibility, ultra-small sample volume, and little consumption of solvent and simple cleaning-up. In addition, with electrochemical detection (ED), CE–ED affords high sensitivity and good selectivity for electroactive species. Therefore, CE–ED has inherent potential for the analysis of traditional Chinese medicines. However, to our knowledge so far only a few traditional Chinese medicines have been successfully separated and detected by CE–ED method [21], and thus far this method has not been applied to the analysis of *E. breviscapus* (Vant.) Hand-Mazz. samples.

In this work we have successfully developed a sensitive, dependable, and simple method for the determination of nine active ingredients of *E. breviscapus* (Vant.) Hand-Mazz. based on CE–ED approach.

2. Experimental

2.1. Apparatus

The laboratory-built CE–ED system has been constructed and described previously [22]. A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) provided separation voltage between the ends of the capillary. The inlet of the capillary was held at a positive potential and outlet end of capillary was maintained at ground.

The separation was proceeded in a 75 cm length of 25 μm i.d. and 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). In order to prevent operator from the high voltage and assure the safety of the CE–ED system, the entire capillary, the buffer reservoir for CE, and all electrodes were enclosed in a Plexiglas box with a safety switch wired to turn off the power supply whenever the box was opened [23].

A carbon-disk electrode with 300 μm diameter was employed as the working electrode as described previously [24]. Before use, the surface of the carbon-disk electrode was polished with emery sand paper, sonicated in deionized water, and then positioned carefully opposite the capillary outlet with the aid of an Oriel Corp. (Stratford, CT, USA) Model 14901 micropositioner. A three-electrode cell system consisting of a carbon-disk working electrode, a platinum auxiliary electrode and a SCE reference electrode was used in combination with a BAS LC-4C amperometric detector (Biochemical System, West Lafayette, IN, USA). The electropherograms were recorded using a chart recorder (Shanghai Dahua Instrument factory, China).

2.2. Reagents

Baicalein, naringenin, scopoletin, kaempferol, apigenin, luteolin, and caffeic acid were purchased from Sigma (St. Louis, MO, USA). Scutellarin was provided by Second Military Medical University (Shanghai, China). Protocatechuic acid was obtained from Shanghai Reagent Factory (Shanghai, China), and used as received. *E. breviscapus* (Vant.) Hand-Mazz. and its phytopharmaceuticals were purchased from Yunnan (China). Stock solutions of nine analytes (1.00×10^{-3} g/mL, each) were prepared in anhydrous ethanol (A.R. grade), stored in the dark at 4 °C, and were diluted to the desired concentrations with the running buffer (H_3BO_3–$Na_2B_4O_7$ buffer ranging from 20 to 100 mmol/L with a pH value from 8.0 to 9.0). Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Sample preparation

About 3 g of dried *E. breviscapus* (Vant.) Hand-Mazz. and 3 g of *E. breviscapus* (Vant.) Hand-Mazz. extract tablets (10 tablets) were ground into powder and accurately weighed. Each weighed sample (1.5006 g *E. breviscapus* (Vant.) Hand-Mazz. powder, or 1.5000 g *E. breviscapus* (Vant.) Hand-Mazz. extract tablets) was extracted with 10 mL of the mixed solvent of anhydrous ethanol (A.R. grade) and water (4:1 in volume) for 30 min in an ultrasonic bath. Then each of the samples was filtered through a filter paper first, then through a 0.22 μm syringe filter, and made up to 5 mL in volume. *E. breviscapus* (Vant.) Hand-Mazz. extract injection was directly filtered through 0.22 μm nylon filters, 1.5 mL filtrate was obtained. Sample solution was stored in the dark. The above filtrate was diluted to the desired concentration with the running buffer of 50 mmol/L (pH 8.7).

2.4. Sample analysis

All the world-real samples were analyzed by the laboratory-built CE–ED system. To ensure reproducibility, the capillary was washed every day with 0.1 M sodium hydroxide, followed by the running buffer for 15 min, and then equilibrating with the buffer for 15 min, while applying the separation voltage. The samples could be well separated in a 75 cm length capillary at the separation voltage of 16 kV in a 50 mmol/L borax running buffer (pH 8.7) with the carbon-disk electrode at potential of +950 mV (versus SCE). Samples were all injected electrokinetically, applying 16 kV for 8 s.

3. Results and discussion

3.1. Method development

3.1.1. Effect of the potential applied to the working electrode

In amperometric detection the potential applied to the working electrode directly affects the sensitivity, detection limit and stability of this method. Therefore, hydrodynamic voltammetry was investigated to obtain optimum detection results. As shown in Fig. 2, when the applied potential exceeds +0.40 V (versus SCE), all nine analytes can generate oxidation current at the working electrode, and this oxidation current increases rapidly except baicalein, scutellarin, caffeic and protocatechuic acids. When an applied potential is between +0.95 and +1.00 V (versus SCE), the increase of peak current becomes slow. When the applied potential is

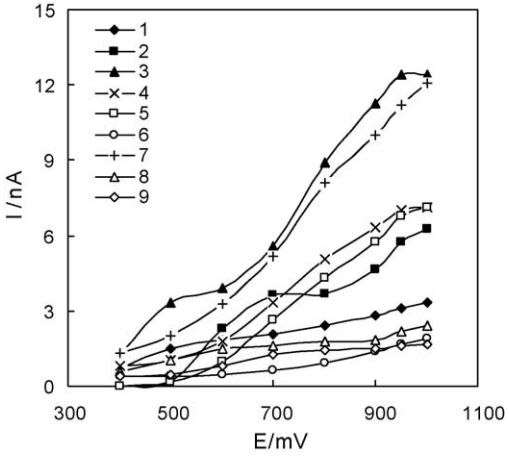

Fig. 2. Hydrodynamic voltammograms (HDVs) of baicalein (1), naringenin (2), scopoletin(3), kaempferol (4), apigenin (5), scutellarin (6), luteolin (7), caffeic acid (8), and protocatechuic acid (9) in CE. Fused-silica capillary: 25 μm i.d. × 75 cm; working electrode: 300 μm diameter carbon disk electrode; running buffer: 50 mmol/L (pH 8.7); separation voltage: 16 kV; injection time: 8 s (at 16 kV); concentrations of nine analytes: 2.0×10^{-5} g/mL, each.

greater than +1.00 V (versus SCE), both the baseline noise and the background current increase very strongly, resulting in an unstable baseline, which is a disadvantage for sensitive and stable detection. Therefore the applied potential to the working electrode was maintained at +0.95 V (versus SCE) where the background current is not too high and the *S*/*N* ratio is the highest. Moreover, the working electrode showed good stability and high reproducibility at this optimum potential.

3.1.2. Effect of the buffer pH value

Borate buffer was employed as the running buffer in this work because borate can chelate with the analytes to form more soluble complex anions [24]. The pH dependence of the migration time was investigated in the pH range of 8.0–9.0. As shown in Fig. 3, the migration time of all analytes increases with increasing pH value, separation of the analytes can be achieved from pH 8.7 to 9.0. When pH is lower than 8.7, baicalein and naringenin, kaempferol and apigenin (at pH 8.0), or scopoletin and kaempferol (at pH 8.4) cannot be separated from each other. When pH value is greater than 9.0, naringenin cannot be separated from kaempferol. Moreover, higher pH value results in long analysis time, and also makes analytes more susceptible to oxidation. Therefore, pH 8.7 was selected as the optimum pH value.

3.1.3. Effect of the buffer concentration

Besides the pH value, the running buffer concentration is also an important parameter. The effect of the running buffer concentration on migration time was also studied ranging from 20 to 100 mmol/L, and the optimum running buffer concentration lies between 40 and 60 mmol/L (at pH 8.7), at which good separation can be obtained for all analytes within relatively short migration time.

3.1.4. Effect of separation voltage

The influence of the separation voltage on the migration time and the separation of the analytes were also studied in this experiment. Higher separation voltages give shorter migration time for all analytes. However when the separation voltage exceeds 18 kV, separation of naringenin and scopoletin cannot be achieved, besides, baseline noise becomes larger. Therefore the optimum separation voltage is 16 kV, at which good separation can be obtained for all analytes within 24 min.

3.1.5. Injection time

The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current was studied by varying injection time from 2 to 12 s at 16 kV, as shown in Fig. 4. When the injection time is longer than 8 s, peak current levels off. In this experiment, 8 s (16 kV) is selected as the optimum injection time.

Through the experiments above, the optimum conditions for determining baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid have been decided. The typical electropherogram for a standard solution of the nine analytes is shown in Fig. 5, from which we can see that good separation can be achieved within 24 min.

3.2. Method validation

Appropriate method validation information concerning new analytical techniques for analyzing pharmaceuticals is required by regulatory authorities. Validation of such methods includes assessment of the stability of the solutions, linearity, reproducibility, detection and quantification limits, and robustness.

3.2.1. Stability of the solutions

The stability of standard and sample solutions was determined by monitoring the peak current of standard mixture solutions and sample solutions over a period of 1 week. The

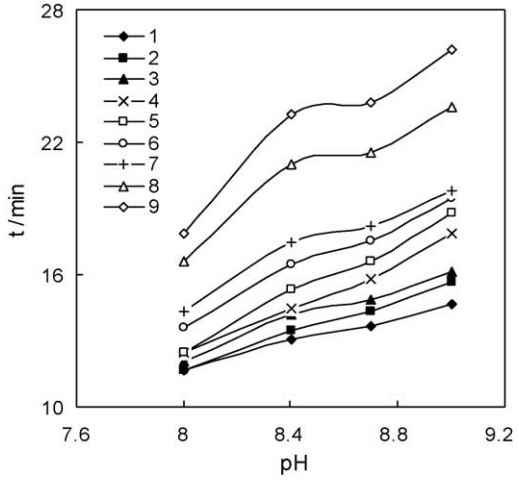

Fig. 3. Effect of pH on migration time. Working electrode potential is +0.95 V (vs. SCE); other conditions are the same as in Fig. 2.

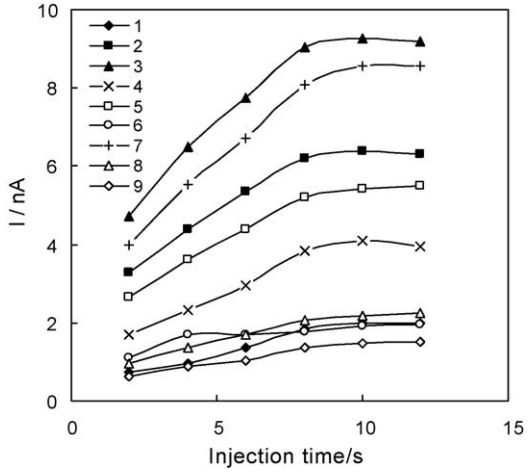

Fig. 4. Effect of injection time on peak current. Experimental conditions are the same as in Fig. 3.

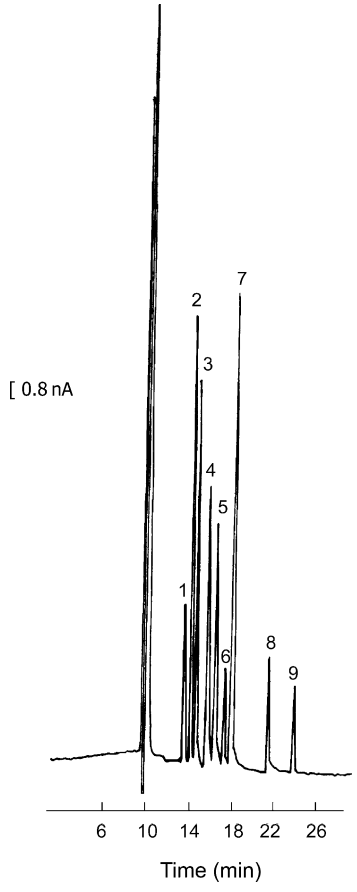

Fig. 5. The electropherogram of standard solution (2.0×10^{-5} g/mL each). Peak identification: (1) baicalein, (2) naringenin, (3) scopoletin, (4) kaempferol, (5) apigenin, (6) scutellarin, (7) luteolin, (8) caffeic acid, and (9) protocatechuic acid. Experimental conditions are the same as in Fig. 3.

results showed that the migration time and peak current of each analyte were almost unchanged (R.S.D.% <2.1) and that no significant degradation is observed within the given period, indicating the solutions are stable for at least 1 week without the results being affected.

3.2.2. Linearity

To determine the linearity of baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid, a series of standard solutions from 5.0×10^{-7} g/mL to 2.0×10^{-4} g/mL in analyte concentration were tested. The results of regression analysis on calibration curves are summarized in Table 1.

3.2.3. Reproducibility

The reproducibility of the peak currents is estimated by making repetitive injections of a standard mixture solution (2.0×10^{-5} g/mL for each analyte) under the selected optimum conditions. The relative standard deviations (R.S.D.s) of the peak current are 1.9, 0.7, 1.2, 1.9, 1.0, 3.1, 1.3, 3.6 and 2.4% for baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid, respectively ($n = 7$).

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

The detection limit is evaluated on the basis of a signal-to-noise ratio of 3. The LOD of nine analytes were ranged from 1.5×10^{-7} to 6.0×10^{-7} g/mL, which detailed data were shown in Table 1.

The LOQ is defined as the level at, or above, which the measurement precision is satisfactory for quantitative analysis. In our case, LOQ was estimated by taking 10 times the standard deviation of the peak height obtained from the nine analytes and subsequently calculating the corresponding concentration. The LOQ were 10.7×10^{-7} g/mL, 3.3×10^{-7} g/mL, 5.0×10^{-7} g/mL, 13.3×10^{-7} g/mL, 10.7×10^{-7} g/mL, 18.7×10^{-7} g/mL, 5.3×10^{-7} g/mL, 9.0×10^{-7} g/mL, and 15.0×10^{-7} g/mL for baicalein, naringenin, scopoletin, kaempferol, apigenin, scutellarin, luteolin, caffeic acid and protocatechuic acid, respectively.

3.2.5. Robustness

Robustness is defined as the capability of an analytical procedure to remain unaffected by small but deliberate changes in the method parameters. A good efficiency of the analyte peaks was always obtained within variations of ±10% of the optimum value of the electrophoretic parameters, buffer concentration, injection time and the potential applied to working electrode.

Table 1
The regression equations and detection limits[a]

Compound	Regression equation[b]	Correlation coefficient	Linear range (g/mL)	Detection limit (10^{-7} g/mL)
Baicalein	$y = 1.76 \times 10^5 x - 0.03$	0.9994	1×10^{-6} to 1×10^{-4}	3.2
Naringenin	$y = 3.95 \times 10^5 x - 0.26$	0.9999	1×10^{-6} to 1×10^{-4}	1.0
Scopoletin	$y = 4.42 \times 10^5 x - 0.91$	0.9992	2×10^{-6} to 1×10^{-4}	1.5
Kaempferol	$y = 2.15 \times 10^5 x - 0.56$	0.9990	1×10^{-6} to 1×10^{-4}	4.0
Apigenin	$y = 2.16 \times 10^5 x - 0.14$	0.9992	1×10^{-6} to 1×10^{-4}	3.2
Scutellarin	$y = 9.50 \times 10^4 x + 0.02$	0.9993	2×10^{-6} to 1×10^{-4}	5.6
Luteolin	$y = 3.77 \times 10^5 x - 0.66$	0.9996	1×10^{-6} to 1×10^{-4}	1.6
Caffeic acid	$y = 1.22 \times 10^5 x + 0.01$	0.9992	2×10^{-6} to 1×10^{-4}	2.7
Protocatechuic acid	$y = 8.48 \times 10^4 x + 0.09$	0.9991	1×10^{-6} to 1×10^{-4}	4.5

[a] CE–ED conditions are the same as in Fig. 3.
[b] In the regression equation, the x value is the concentration of analytes (g/mL), the y value is the peak current (nA).

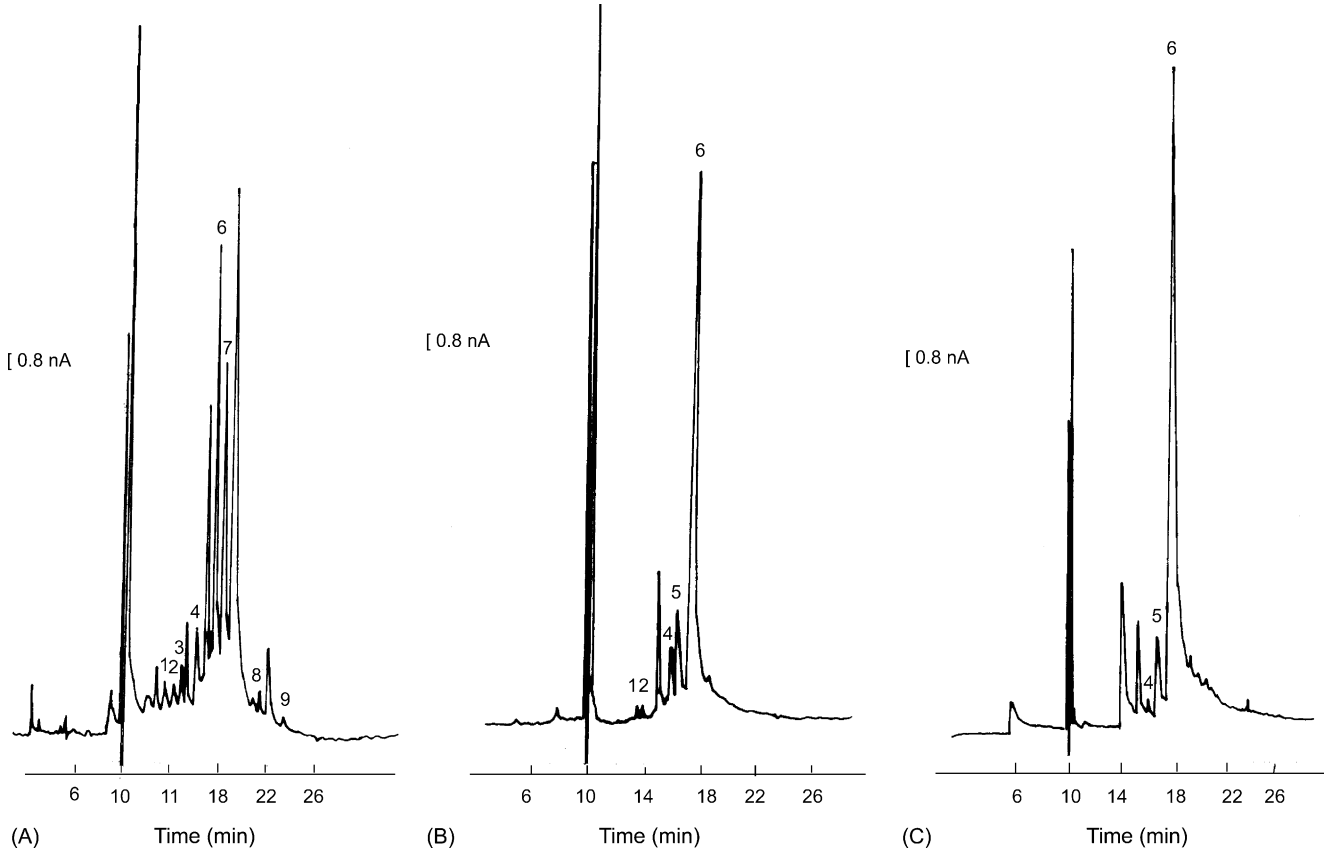

Fig. 6. The electropherograms of *E. breviscapus* (Vant.) Hand-Mazz. herbs (A), *E. breviscapus* (Vant.) Hand-Mazz. extract tablets (B) and *E. breviscapus* (Vant.) Hand-Mazz. extract injection (C). Peak identifications and determination conditions are the same as in Fig. 5.

Table 2
Assay results for *E. breviscapus* (Vant.) Hand-Mazz. herbs and its extract granules ($n = 3$)[a]

Sample	Ingredients	Found (μg/g)	R.S.D. (%)
E. breviscapus (Vant.) Hand-Mazz. herbs	Baicalein	222.1	2.1
	Naringenin	64.7	3.3
	Scopoletin	122.6	3.2
	Kaempferol	328.1	2.1
	Apigenin	N.F.	
	Scutellarin	5270.6	1.0
	Luteolin	764.2	1.7
	Caffeic acid	222.1	3.6
	Protocatechuic acid	133.3	4.3
E. breviscapus (Vant.) Hand-Mazz. extract tablets	Baicalein	74.1	4.3
	Naringenin	25.9	3.9
	Scopoletin	N.F.	
	Kaempferol	194.9	3.4
	Apigenin	363.6	2.5
	Scutellarin	5697.0	1.1
	Luteolin	N.F.	
	Caffeic acid	N.F.	
	Protocatechuic acid	N.F.	

[a] CE–ED conditions are the same as in Fig. 3.

3.3. Applications

Under the optimum conditions, nine active ingredients in *E. breviscapus* (Vant.) Hand-Mazz. samples were determined by CE–ED. Typical electropherograms of *E. breviscapus* (Vant.) Hand-Mazz. herbs (A), *E. breviscapus* (Vant.) Hand-Mazz. extract tablets (B) and *E. breviscapus* (Vant.) Hand-Mazz. extract injection (C) are shown in Fig. 6. By comparing with the electropherogram of the standard solution (Fig. 5), the active ingredients namely baicalein (peak 1),

Table 3
Assay results for *E. breviscapus* (Vant.) Hand-Mazz. extract injection ($n = 3$)[a]

Sample	Ingredients	Found (μg/mL)	R.S.D. (%)
E. breviscapus (Vant.) Hand-Mazz. extract injection	Baicalein	N.F.	
	Naringenin	N.F.	
	Scopoletin	N.F.	
	Kaempferol	8.6	3.0
	Apigenin	54.5	2.8
	Scutellarin	1086.4	1.2
	Luteolin	N.F.	
	Caffeic acid	N.F.	
	Protocatechuic acid	N.F.	

[a] CE–ED conditions are the same as in Fig. 3.

Table 4
Determination results of recovery in this method with *E. breviscapus* (Vant.) Hand-Mazz. herbs sample ($n = 3$)[a]

Ingredient	Original amount (g/mL)	Added amount (g/mL)	Found (g/mL)	Recovery (%)	R.S.D. (%)
Baicalein	0.33×10^{-5}	2.0×10^{-5}	2.30×10^{-5}	98.7	3.2
Naringenin	0.10×10^{-5}	2.0×10^{-5}	2.14×10^{-5}	101.9	4.6
Scopoletin	0.18×10^{-5}	2.0×10^{-5}	2.21×10^{-5}	101.4	4.1
Kaempferol	0.49×10^{-5}	2.0×10^{-5}	2.53×10^{-5}	101.6	3.5
Apigenin	N.F.	2.0×10^{-5}	2.05×10^{-5}	102.5	2.4
Scutellarin	7.91×10^{-5}	2.0×10^{-5}	9.97×10^{-5}	100.6	2.2
Luteolin	1.15×10^{-5}	2.0×10^{-5}	3.25×10^{-5}	103.2	2.7
Caffeic acid	0.33×10^{-5}	2.0×10^{-5}	2.24×10^{-5}	96.1	3.9
Protocatechuic acid	0.20×10^{-5}	2.0×10^{-5}	2.16×10^{-5}	98.2	4.3

[a] CE–ED conditions are the same as in Fig. 3.

naringenin (peak 2), scopoletin (peak 3), kaempferol (peak 4), apigenin (peak 5), scutellarin (peak 6), luteolin (peak 7), caffeic acid (peak 8) and protocatechuic acid (peak 9) in the actual samples can be determined. The assay results are listed in Tables 2 and 3.

Under the optimum conditions the recovery and reproducibility experiments were also conducted to evaluate the precision and accuracy of the method. Recovery was determined by standard addition method the *E. breviscapus* (Vant.) Hand-Mazz. samples, and the results are summarized in Table 4.

4. Conclusions

The above assay results indicate that this method is accurate, sensitive and reproducible, providing a useful quantitative method for the analyses of *E. breviscapus* (Vant.) Hand-Mazz. and its phytopharmaceuticals. Furthermore, it suggests that it is valuable to deep explore the phytopharmaceuticals of *E. breviscapus* (Vant.) Hand-Mazz.

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