

Simultaneous Determination of Five Flavonoids in *Saussurea involucrata* by Capillary Electrophoresis

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Summary: A method of determination of five flavonoids in *Saussurea involucrata* by β -cyclodextrin modified capillary zone electrophoresis has been developed. The effects of buffer pH and buffer concentration, applied voltage and β -CD concentrations on the separation were systematically investigated. The optimum condition providing baseline separation of all compounds within 8 min was obtained in the 20 mmol·L⁻¹ borax buffer (pH 9.2), 20 kV applied voltage and 8 mmol·L⁻¹ β -CD. The linearity, detection limits, limits of quantification, reproducibility and recovery were satisfactory. The β -cyclodextrin modified capillary zone electrophoresis method proposed here has been satisfactorily employed to analyze *S. involucrata* samples.

Keywords: *Saussurea involucrata*; Flavonoids; β -cyclodextrin modified capillary zone electrophoresis (β -CD – CZE).

Introduction

Saussurea involucrata is a rare traditional Chinese medicinal herb which belongs to Asteraceae family and is on the verge of extinction. It distributes in the alpine zone of Tianshan, A'er Tai, and Kunlun areas and was listed as a second-grade national protected wild plant in China [1]. In folk medicine, it has long been used under the name "Snow Lotus" for the treatment of rheumatoid arthritis, cough with cold, stomachache and dysmenorrhea [2]. Now, pharmacological studies have demonstrated that *S. involucrata* has biological activity, such as anti-fatigue [3], anti-inflammation [4], anti-cancer [5], and cardiogenic [6]. However, a great number of medicinal plants show complicated profile of constituents, so the quality control and quantitative analysis of active components in traditional herbal medicines has great importance.

Flavonoids are important polyphenolic secondary metabolites in medicinal plants and have been reported to have anti-inflammatory [7, 8], antineoplastic [9], anti-oxidant [10-12], antiviral [13] and anticarcinogenic [14, 15] activity. Thus, the content of flavonoids demonstrated to be an important index to evaluate the quality of *S. involucrata*. Several methods such as thin-layer chromatography (TLC) [16], gas chromatography (GC) [17] and high-performance liquid chromatography (HPLC) [18] have been developed for flavonoid analysis. Nowadays, more and more

attention has been paid to capillary electrophoresis owing to its remarkable separation efficiency, rapid analysis, and minimum consumption of samples, chemicals and wider applications in the analysis of flavonoids [19-28]. However, there were few reports about analysis of flavonoids by capillary electrophoresis in *S. involucrata* [23], and nonetheless further study on improvement of separation efficiency is also required.

In this work, we have established a procedure for simultaneous determination of five flavonoids in *S. involucrata* by capillary zone electrophoresis with 4-methylumbelliferone as internal standard. The structures of the analytes and the internal standard were shown in Fig. 1. With the developed method, the five analytes and the internal standard can be separated in 8 min, which is much faster than Chu et al. have reported [23].

Results and Discussion

Effects of Buffer pH and Buffer Concentration

Borax can chelate with the analytes to form more soluble complex anions, so it was tried in this work [29]. According to the literature [29-31], the pKa of the analytes should be between pH 7.0-9.0, so the effects of buffer pH were investigated in the pH

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7.0-9.5 range. As shown in Fig. 2, the migration time and separation of all the compounds increased with the increase of the buffer pH and the compounds can be well separated in the pH 9.0-9.5 range. For good separation and the simplicity of preparing buffer solutions, pH 9.2, the pH of 20 mmol L⁻¹ borax at 25 °C, was selected as the optimum buffer pH. At this pH, the effect of the buffer concentration was studied over the range of 5–25 mmol L⁻¹. The results showed that the migration time of the analytes and the internal standard increased with the increase of buffer concentration, and the analytes could be well separated when buffer concentration was higher than 15 mmol L⁻¹. Based on experimental results, 20 mmol L⁻¹ borax buffer was selected as the optimum electrolyte concentration.

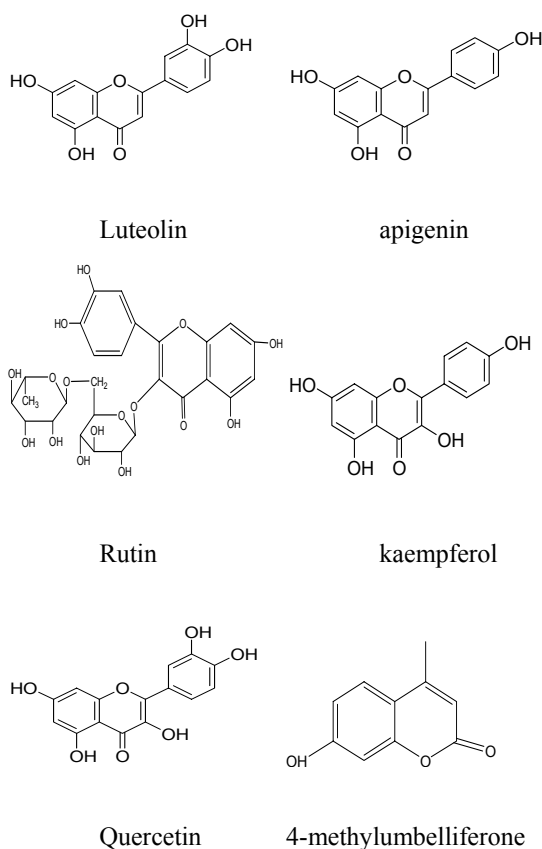


Fig. 1: Chemical structures of Flavonoids.

Effects of Applied Voltage

The effect of applied voltage on the separation was examined in the range of 14-22 kV. The results showed that with the increase of applied voltage, the migration time of the compounds

decreased, which results in shorter analysis time and an improvement of the efficiency. At the same time, the resolution didn't change significantly. However, the baseline noise increased apparently when the applied voltage exceeded 20 kV, which can make the detection limits deteriorate. This was due to the pronounced Joule heating caused by the applied voltage increase. So, 20 kV was selected as the optimum, which combined sufficient separation, moderate analysis time and adequate detection limits.

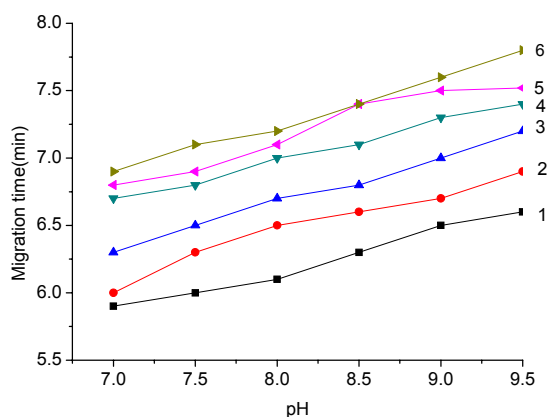


Fig. 2: Effects of pH on the migration time of the analytes : 1. 4-methylumbelliferone; 2. kaempferol; 3. apigenin; 4. luteolin; 5. quercetin; 6. rutin. (The meaning of 1, 2, 3, 4, 5, 6 has the same meaning in the whole paper).

Effects of β -CD Concentration

β -CD can form inclusion complexes with various compounds (guest) ranging from polar reagents such as acids, amines, small ions, to highly apolar aliphatic and aromatic hydrocarbons. These guest compounds are included in the cavity of the cyclodextrins (host). The nature of the binding force is mainly attributed to Vander Waals interactions between guest and host and/or hydrogen bonding between the guest and the hydroxyl groups of cyclodextrin. The mutual affinity between the analyte and β -CD can affect the migration of the analyte. As mutual affinity increases, the analytes' migration time would prolong, which can make obvious contribution to the separation [32]. The effect of β -CD concentration was examined in the range of 0-10 mmol L⁻¹. As shown in Fig. 3, the migration time of the investigated compounds decreased significantly except for rutin and the separation improved moderately with the increase of the β -CD

concentration. At the same time, the peaks of the compounds improved after β -CD was added into the buffer solution. In consideration of separation, peak shape and analysis time, $8 \text{ mmol}\cdot\text{l}^{-1}$ β -CD was adopted in the further experiments.

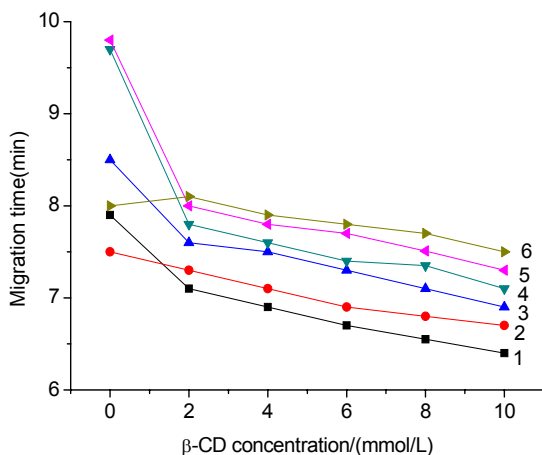


Fig. 3: Effects of β -CD concentration on migration time of the analytes.

Analytical Performance

Under the optimized conditions, a good separation of five flavonoids and the internal standard was achieved in 8 min. The electropherogram of the standard solution was shown in Fig. 4. The linearity of five flavonoids in standard solutions was investigated. The linearity (x , the concentration of the analytes; y , the ratio of peak area of the component to that of the internal standard), the detection limits as well limits of quantification (LOQ) for the analytes are given in Table-1. The reproducibility is estimated by making eight replicate injection of a standard mixture solution under the selected optimum conditions and the results were shown in Table-2.

Sample Analysis

Sample determination was carried out under the optimal conditions according to the procedures stated above. The determination of the five flavonoids in *S. involucre* extracts was performed and the contents of the five analytes were shown in Table-3. Accurate amounts of the five analytes, equivalent to $0.100 \text{ mg}\cdot\text{g}^{-1}$ in solid samples, were added into the real samples to do recovery experiments using the developed CZE method. As

shown in Table-3, the recoveries of kaempferol, apigenin, luteolin, quercetin and rutin were 97%, 98%, 92%, 96% and 102%, respectively, which indicated that this method was accurate and practical.

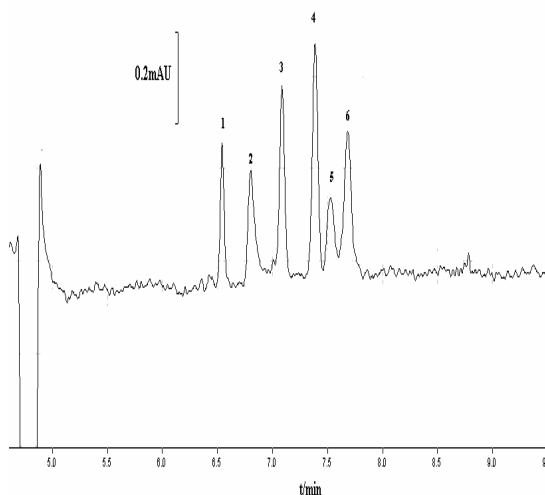


Fig. 4: Electropherogram of the analytes and the internal standard.

Conditions: 20 mmol/L borax (pH 9.2) containing $8 \text{ mmol}\cdot\text{l}^{-1}$ β -CD; applied voltage, 20 kV ; UV detection wavelength, 254 nm

Experimental

Instrumentation and Separation Conditions

A P/ACE MDQ Capillary Electrophoresis system with a photodiode array detector (Beckman Coulter, Fullerton, CA, USA) was used in this work.

Operating solution was $20 \text{ mmol}\cdot\text{l}^{-1}$ borax buffer (pH 9.2), adding $8 \text{ mmol}\cdot\text{l}^{-1}$ β -CD as organic modifier. The dimensions of the capillary were $60.2 \text{ cm} \times 50 \mu\text{m}$ i.d. The effective length of the capillary was 50 cm . The temperature of the capillary was kept at $25 \text{ }^\circ\text{C}$. The applied voltage was 20 kV . Samples were introduced under pressure: 3445 Pa , 5 s . The detection wavelength was 254 nm . Before each run, the capillary tube was washed with $0.1 \text{ mol}\cdot\text{l}^{-1}$ NaOH, water and the operating buffer for 10 min , separately. Between consecutive analysis, the capillary tube was washed with water for 3 min , with $0.1 \text{ mol}\cdot\text{l}^{-1}$ NaOH for 3 min , with water for 4 min , with operating solution for 4 min sequentially to maintain proper reproducibility of run-to-run injections.

Table-1: The linearity, detection limits^a and limits of quantification (LOQ)^b of the analytes.

Compound	Regression equation	Correlation coefficient	Linear Range (mg·l ⁻¹)	Detection limit (mg·l ⁻¹)	LOQ (mg·l ⁻¹)
kaempferol	Y=0.1482x-0.2749	0.9957	2~400	0.6	2.0
apigenin	Y=0.1380x+0.2345	0.9994	2~500	0.5	1.7
luteolin	Y=0.2068x+0.2374	0.9963	1~600	0.3	1.2
quercetin	Y=0.1452x+0.0958	0.9943	2~200	0.5	1.7
rutin	Y=0.1102x+0.0584	0.9971	3~500	0.6	2.0

^a the detection limits were based on three times noise^b LOQ were based on ten times noise

Table-2: Reproducibility of the compounds (n = 8).

Compound	Concentration (mg·l ⁻¹)	RSD (%)	
		Migration time	Peak area
4-methylumbelliferone	10	0.78	1.23
kaempferol	10	0.96	1.42
apigenin	10	0.94	1.28
luteolin	10	0.66	1.35
quercetin	10	0.73	1.68
rutin	10	0.82	1.56

Table-3: Results of sample analysis and the recovery (n=5).

Analytes	Original(mg·g ⁻¹)	Added(mg·g ⁻¹)	Found(mg·g ⁻¹)	Recovery(%)
kaempferol	0.128±0.004	0.100	0.225±0.007	97±7
apigenin	0.119±0.005	0.100	0.217±0.010	98±10
luteolin	0.103±0.003	0.100	0.195±0.007	92±7
quercetin	0.0699±0.002	0.100	0.166±0.008	96±8
rutin	0.140±0.000	0.100	0.242±0.009	102±9

Reagents and Materials

Kaempferol, apigenin, luteolin, quercetin, rutin, β -CD and 4-methylumbelliferone was obtained from Sigma-Aldrich (St. Louis, MO, USA). The ultrapure water, used throughout, was prepared with a milli-Q system (Millipore, Bedford, MA, USA). All chemicals were of analytical grade. Stock standard solutions of 1 mg·ml⁻¹ were prepared by dissolving the compounds in methanol and kept in a refrigerator at 8-10°C. Buffer solutions were prepared with borax (concentration range: 5-25 mmol·l⁻¹) and β -CD (concentration range: 0-10 mmol·l⁻¹) by dissolving them in 18 m Ω /cm ultrapure water. The final pH values were adjusted with 0.1 mol·l⁻¹ NaOH and 0.1 mol·l⁻¹ HCl.

Preparation of Samples

S. involucrate sample were purchased from the local drugstore in Dezhou. The sample was ground in mill and dried for 4 hours at 50°C before use. 1.0000g powders were weighed accurately and dispersed in 50 ml of methanol. The mixture was refluxed in 75°C water bath for 5 h. After cooling, it was filtered through a filter paper. The extract was transferred to a 50 ml flask, adding 0.4 ml 4-methylumbelliferone of 1mg·ml⁻¹ as an internal standard, and then diluted to mark with methanol.

Conclusions

A simple, rapid, and accurate β -cyclodextrin modified capillary zone electrophoresis (CZE) technique has been developed for the separation and

determination of the flavonoids in *S. involucrate*. The separation of the five flavonoids was achieved successfully within 8 min in the 20 mmol L⁻¹ borax buffer (pH 9.2), 20 kV applied voltage and 8 mmol L⁻¹ β -CD. The linearity, the detection limits, limits of quantification (LOQ), reproducibility, and recovery were satisfactory. The analytical result of sample provides basic data for the quality evaluation and control of *S. involucrate*.

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