Analysis of Flavonoids in *Flos Genkwa* by β-cyclodextrin and Ionic Liquid Modified Capillary Zone Electrophoresis

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Summary: A *β-cyclodextrin (β-CD)* and ionic liquid (1-ethyl-3-methylimidazolium tetrafluroborate, 1E-3MI-TFB) modified capillary zone electrophoretic (CZE) method has been developed to determine three flavonoids (genkwanin, apigenin and luteolin) in *Flos Genkwa* with 4-methylumbelliferone as internal standard. The effects of buffer pH and concentration, β-CD concentration and ionic liquid on separation were investigated. The optimum electrophoretic conditions are as follows: 20 mmol·L^{-1} tetraborate (pH 9.0)- 6 mmol·L^{-1} β-cyclodextrin-0.04 % 1E-3MI-TFB (*V/V*) as the running buffer, applied voltage of 25 kV. Under the optimized conditions, the calibration curves of the three flavonoids all showed excellent linearity in the 1~100 μg·mL^{-1} range. The relative standard deviation in migration time and peak area was 0.21 % and 2.92 % for genkwanin, 0.15 % and 2.33 % for apigenin, 0.28 % and 3.35 % for luteolin. The detection limits of the three flavonoids ranged from 0.26 to 0.32 μg·mL^{-1}. The recoveries of the three flavonoids ranged between 94.3 % and 103.5 %.

Introduction

Flavonoids have many medical effects and are active constituents of many Chinese herbal medicines [1], so identification and determination of flavonoids play a very important role in insuring the efficacy, the safety and therapeutic reproducibility for Chinese herbal medicine and their medicinal preparations.

Several methods such as gas chromatography (GC) [2], thin layer chromatography (TLC) [3, 4], high performance liquid chromatography(HPLC) [5, 6] and capillary electrophoresis (CE) [7-14] have been been used to flavonoids analysis in medicinal plants. GC has the shortcoming of long time and complexity of process. TLC can't be applied to determine several components in a single crude herb or in a medicinal preparation simultaneously. HPLC suffers from limitation such as low resolving power, high consumption of organic solution and time. Compared to the above methods, capillary electrophoresis (CE) has the advantages of rapidity, high resolution, high efficiency, low consumption of sample and the reagents, long longevity and easy elution of the capillary column, and has gained the acclaims of many analysts from all over the world since its emergence in the 1980s [7-19].

Ionic liquids (Ils) are organic salts that are liquids at room temperature. They present a variety of desirable properties. They are environmentally benign, nonvolatile and nonflammable, and in addition, they have high thermal stability. Besides, Ils are good solvents for many inorganic and organic materials. In recent years, a great attention has been paid to their utilization in capillary electrophoresis. In capillary electrophoresis, Ils have been reported to be used as electrolytes [14, 15], additives for electrolytes [16, 17] and covalent coating reagents of the capillary [18, 19].

Flos Genkwa, the flower bud of Thymelaeaceae family plant *Daphne genkwa* Sieb. et Zucc, is a kind of Chinese traditional folk medicine. It has the function of removing water retention, anthelminthic, relieving cough, eliminating sputum, easing pain, diminishing inflammation and detoxifcating, and it has been used for asthma, oedema, turgor, dropsy, acariasis, chilblain, crusted ringworm and many other illness since ancient time [3, 20]. Genkwanin, apigenin and luteolin are three kind of flavonoids in *Flos Genkwa* [21], the structures of which were shown in Fig. 1. Accurate determination of the three flavonoids is very

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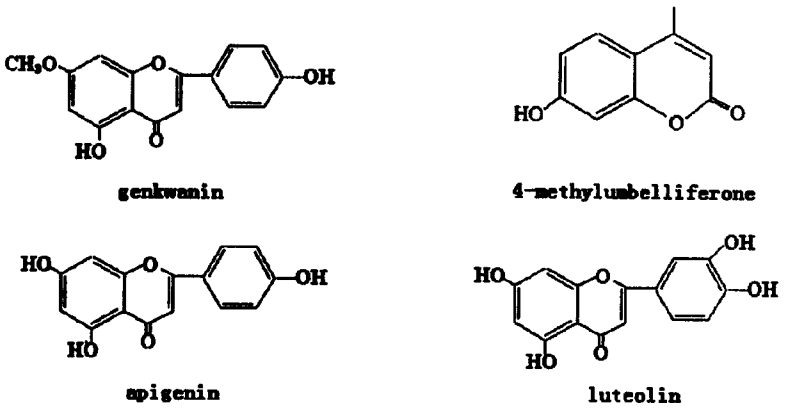

Fig. 1: Molecular structures of genkwanin, 4-methylumbelliferone, apigenin and luteolin.

important for the quality control and medicinal preparation development of *Flos Genkwa*.

Until now, TLC [3] and HPLC [6] have been applied to analysis of flavonoids in *Flos Genkwa*. Nevertheless, due to their inherent disadvantages, the analytical results are not satisfactory enough in consideration of efficiency and cost. In this paper, a *β-cyclodextrin (β-CD)* and ionic liquid (1E-3MI-TFB) modified capillary zone electrophoretic (CZE) method has been developed to determine genkwanin, apigenin and luteolin in *Flos Genkwa* with 4-methylumbelliferone as internal standard. The method has the advantages of accuracy, rapidity and low cost and was very important for the quality control of *Flos Genkwa* and its medicinal preparations.

Results and Discussion

Effect of Buffer pH and Buffer Concentration

Buffer pH affects both electroosmotic flow (EOF) of the capillary and the electrophoretic mobility of the analytes. So it can affect the migration time and separation of the analytes. The effect of buffer pH on the separation was investigated in the pH 8.0-10.0 range. As shown in Fig. 2a, the migration time and resolution of the four compounds increase with the increase of the buffer pH. The four compounds can be well separated when the buffer pH is in the pH 9.0~10.0 range. However, higher pH value results in longer analysis time and the analytes are more susceptible to oxidation when the buffer pH is high. In consideration of the analysis time and good separation, pH 9.0 was chosen as a compromise.

Buffer concentration influence the viscosity coefficient of the solution, the diffusion coefficient of the analytes, the zeta-potential of the inner surface of capillary tube, and ultimately affects the migration time and separation of the analytes. Keep the buffer pH at 9.0 and other conditions the same as the pH optimization, the influence of buffer concentration was investigated in the 5~25 mmol · L^{-1} concentration range. As indicated in Fig. 2b, the migration time and the resolution of the four compounds increase with increasing buffer concentration. The four compounds can be well separated when buffer concentration is higher than 15 mmol · L^{-1}. The results also showed that the capillary current increase with the increase of the buffer concentration, which will increase the Joule heating effect and sacrifice the detection limits in the long run. In consideration of resolution, analysis time and detection limits, 20 mmol · L^{-1} was selected as the optimum buffer concentration.

Effect of Applied Voltage

The driving force of the migration of the analytes in capillary zone electrophoresis is the electric field and ultimately the applied voltage all over the capillary length. The effect of applied voltage on the separation was examined in the range of 19~27 kV. As shown in Fig. 3, with the increase of

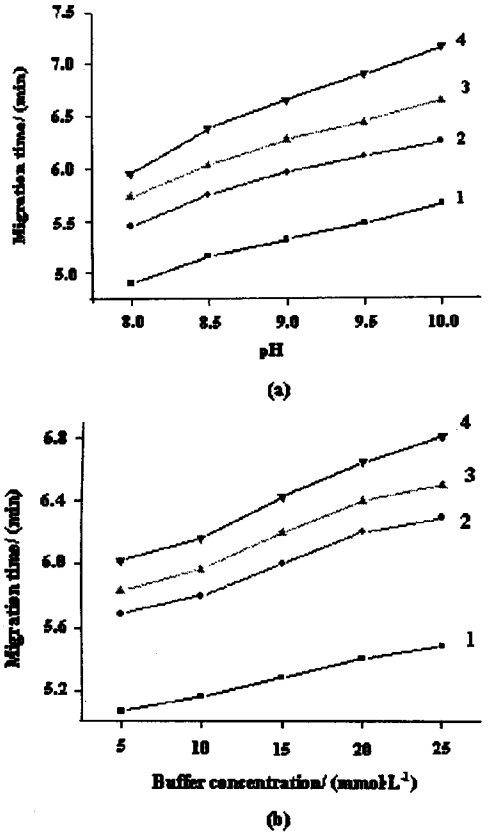

Fig. 2: Effects of buffer pH (a) and buffer concentration (b) on migration time of the four compounds: 1. genkwanin; 2. 4-methylumbelliferone; 3. apigenin; 4. luteolin (The meaning of 1, 2, 3, 4 were the same in the Figures of the whole paper).

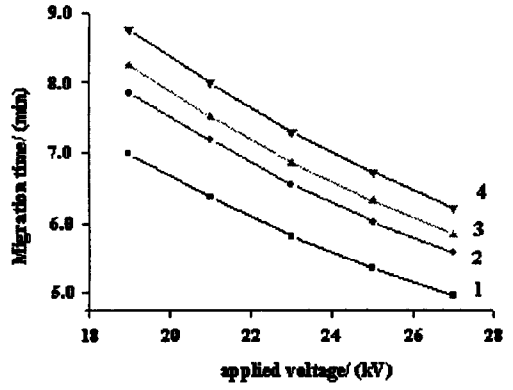

Fig. 3: Effect of applied voltage on migration time of the four compounds.

applied voltage, the migration time of the four compounds decreased, which results in shorter analysis time and an improvement of the column efficiency. However, the baseline noise increased apparently when the applied voltage exceeded 25 kV, which can make the detection limits deteriorate. This was due to the pronounced Joule heating caused by the applied voltage increase. 25 kV was selected as the applied voltage, which combined sufficient separation, moderate analysis time and adequate detection limits.

Effect of β-CD Concentration

In CE analysis, β-cyclodextrin (β-CD) was often used to improve separation. It has a hydrophilic surface and a hydrophobic cavity. The hydrophobic cavity can include proper guest molecules into it, so the inclusion-complexes are formed. Different inclusion-complexes have different stability, hence the separation was improved [22]. In an attempt to attain improvement in the separation of the four compounds, the effect of β-cyclodextrin (β-CD) concentration was examined in the 0~8 mmol·L^{-1} concentration range. The results was shown in Fig. 4 and Fig.5. As shown in Fig. 4, the migration time and resolution of the four compounds decreased with the increase of the β-CD concentration. However, the peak shapes of the four compounds (especially genkwanin, the sensitivity was also enhanced) improved with the increase of the β-CD concentration. Fig. 5 gives a apparent comparison of electropherograms between that obtained without β-CD (Fig. 5a) and with β-CD (Fig. 5b). In consideration of

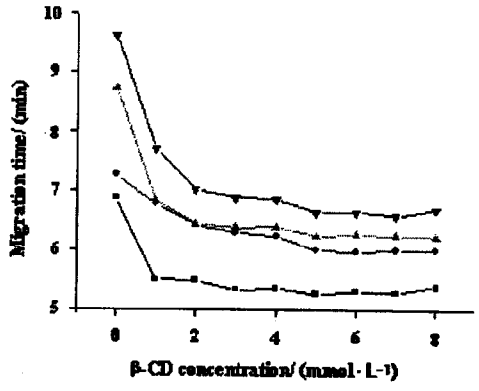

Fig. 4: Effects of β-CD concentration on migration time of the four compounds.

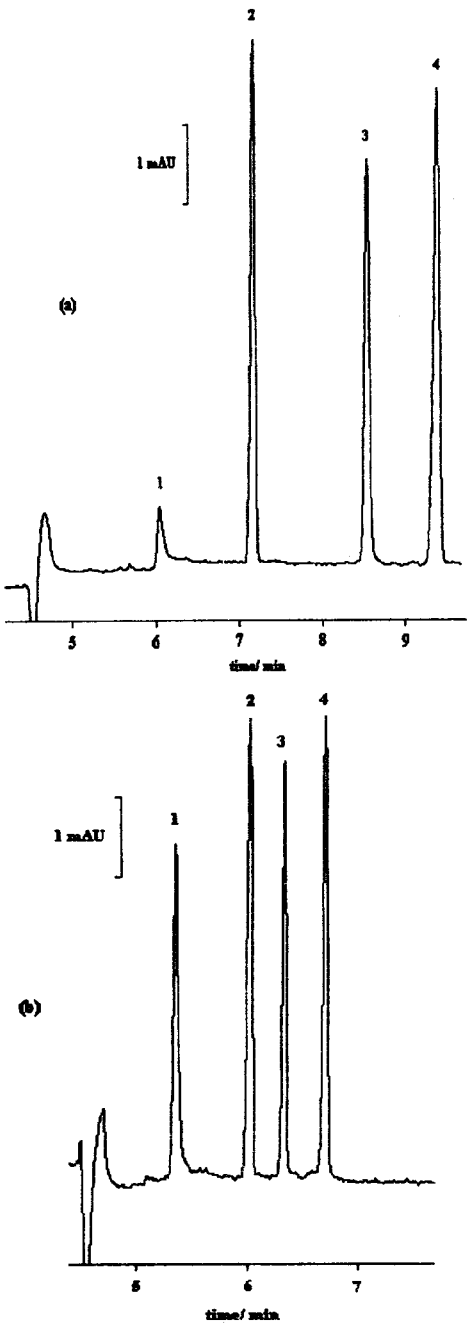

Fig. 5: Electropherogram of the four compounds obtained without β-CD (a) and with 6 mmol·L^{-1} β-CD in the buffer (b): Other conditions: 20 mmol·L^{-1} tetraborate (pH 9.0); 0.04 % 1E-3MI-TFB *(v/v)*; applied voltage, 25 kV; ; temperature, 25 °C; UV detection wavelenghth, 254 nm.

separation, peak shape and sensitivity, 6 mmol·L β-CD was adopted in the further experiments.

Effects of Ionic Liquid Concentration

1-Ethyl-3-methylimidazolium tetrafluoroborate (1E-3MI-TFB) was chosen as ionic liquid modifier in this experiments. As reported in the literature, the imidazolium cations of 1E-3MI-TFB can interact with the wall of the capillary or the analytes, which will affect the migration of the analytes and the separation as well [14]. The effects of 1E-3MI-TFB concentration was studied in the 0~0.10 % *(V/V)* range. As shown in Fig. 6, the migration time of the four compounds increase with the increase of the 1E-3MI-TFB concentration. The separation of the four compounds improved with the increase of 1E-3MI-TFB concentration in the 0~0.04 % range. However, the resolution between 4-methylumbellirone and apigenin decreased with the increase of 1E-3MI-TFB concentration in the 0.04~0.10 % range. So 0.04 % *(V/V)* 1E-3MI-TFB was adopted in the further experiments.

3.5 Validation of the Method

Under the optimized conditions, a good separation of the four compounds was achieved in 7 min. Fig. 5b shows a typical electropherogram of the four compounds. The linearity of the three analytes in standard solutions was investigated. The calibration graphs were plotted by peak area ratio (y, analyte/ internal standard) against concentration (x, μg·mL^{-1}). The detection limits were acquired based on three

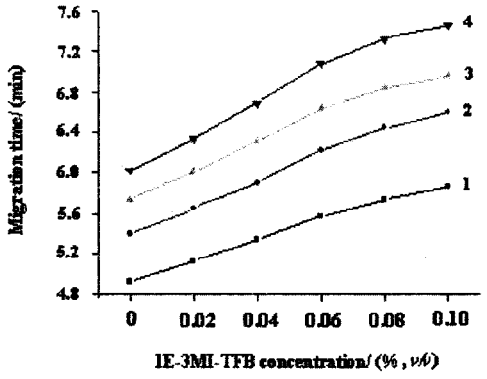

Fig. 6: Effect of 1E-3MI-TFB on the migration of the four compounds.

times noise. The calibration and detection limits results were all summarized in Table-1, which were satisfactory. The reproducibility is estimated by making eight replicate injections of a standard mixture solution under the selected optimum conditions. As shown in Table-2, the relative standard deviation of the four compounds based on migration time and peak area were in the 0.15~0.70 % and 2.33~3.35 % range, respectively.

Table-1: The regression equations and detection limits[a]

Compound	Regression equation[b]	Correlation Coefficient	Linear range ($\mu g \cdot mL^{-1}$)	Detection limit[c] ($\mu g \cdot mL^{-1}$)
genkwanin	=0.01544X+0.00499	0.9999	1~100	0.29
apigenin	=0.01431X+0.00335	0.9999	1~100	0.32
Luteolin	=0.01750X+0.00748	0.9998	1~100	0.26

a. CE conditions are the same as in Fig. 5(b)
b. In the regression equation, the X value is the concentration of analytes($\mu g \cdot mL^{-1}$), the y value is the peak area ratio(analyte/internal standard)
c. The detection limit is evaluated on the basis of a signal-to-noise ratio of 3.

Table-2: Reproducibility of the peak area and migration time of the four compounds (n=8).

Compound	Concentration ($\mu g \cdot mL^{-1}$)	Migration time (min)		Peak area ($\mu AU \cdot sec^{-1}$)	
		Mean	R.S.D. (%)	Mean	R.S.D. (%)
genkwanin	50	5.317	0.21	10870	2.92
4-methylumbelliferone	80	5.979	0.70	13777	2.35
apigenin	50	6.271	0.15	10043	2.33
luteolin	50	6.646	0.28	12750	3.35

Results of Sample Analysis and Recovery

The developed method was applied to determine genkwanin, apigenin and luteolin in *Flos Genkwa*. Electropherogram of *Flos Genkwa* extracts was shown in Fig. 7 and the analysis results were shown in Table-3.

Accurate amount of genkwanin, apigenin and luteolin were added to *Flos Genkwa* sample to do recovery experiments. As shown in Table-4, the recoveries of genkwanin, apigenin and luteolin were 94.3 %, 103.5 % and 99.1 %, respectively. The recovery results were satisfactory.

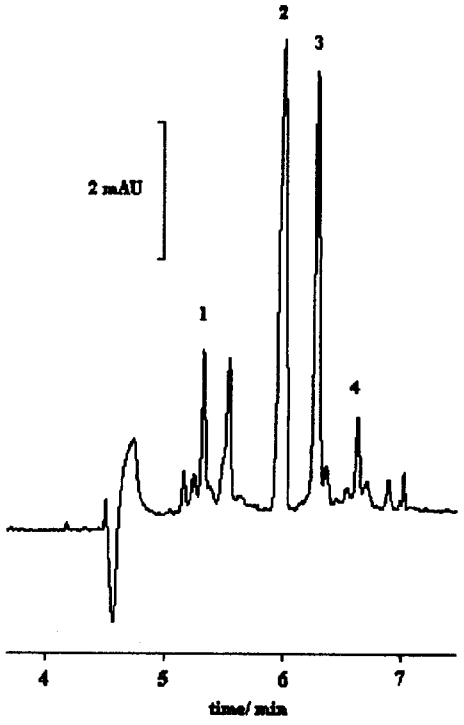

Fig. 7: Electropherogram of the *Flos Genkwa* extracts. Peak identification and determination conditions are the same as that in Fig. 5b.

Table-3: Results of *Flos Genkwa* sample analysis (n=5).

Component	Found ($mg \cdot g^{-1}$)	Average ($mg \cdot g^{-1}$)	RSD/ %
genkwanin	1.70、1.67、1.62、1.67、1.72	1.68	2.25
apigenin	5.79、5.73、5.66、5.44、5.32	5.59	3.58
luteolin	0.86、0.86、0.85、0.84、0.80	0.84	2.96

Table-4: The recoveries in this method (n=5).

Compounds	Original amount (mg/g)	Added amount (mg/g)	Found (mg/g)	Recovery (%)	R.S.D. (%)
Genkwanin	1.68	1.0	2.62	94.3	4.31
apigenin	5.59	1.0	6.62	103.5	2.94
luteolin	0.84	1.0	1.83	99.1	3.72

Experimental

Apparatus

CE analysis were carried out in a P/ACE MDQ capillary electrophoresis system with a photodiode array detector for absorbance measurements at 254 nm (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capillaries purchased from Yongnian Optical Fiber Plant (Yongnian, Hebei Province, China) was used. The dimensions of the capillary were 60.2 cm×50 μm i. d. The effective length of the capillary was 50 cm. The temperature of the capillary was kept at 25 °C. The applied voltage was 25 kV. Samples were introduced under pressure (5s, 0.5 psi). The CE system was interfaced with a

computer. 32 karat software (version 7.0) of Beckman was used for data acquisition. A pHS-3C pH meter (Leici Instrumentation Factory, Shanghai, China) was employed with a precision of ± 0.02 pH unit. The ultrapure water, used throughout, was prepared with a milli-Q system (Millipore, Bedford, MA, USA).

Chemicals

Genkwanin was purchased from Alfa Aesar (Heysham Lancashire, England). Apigenin, luteolin and 4-methylumbelliferone (internal standard) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade. Analytes Standard solutions of 0.5 mg mL^{-1} and 4-methylumbelliferone standard solutions of 1.0 mg mL^{-1}were prepared in methanol. All stock solutions were stored in refrigerator with temperature at 4 °C. Buffer solutions were prepared with tetraborate (concentration range: 5-25 $mmol \cdot L^{-1}$), β-cyclodextrin (concentration range: 0-8 $mmol \cdot L^{-1}$) and 1-ethyl-3-methylimidazolium tetrafluoroborate (concentration range: 0-10 %, *V/V*) by dissolving them in 18 MΩ/cm ultrapure water. The final pH values were adjusted with 0.1 $mol \cdot L^{-1}$ sodium hydroxide and 0.1 $mol \cdot L^{-1}$ HCl. All buffer solutions were filtered through a 0.45 μm membrane filter and degassed by ultrasonication for approximately 10 min before use.

Electrophoretic Procedure

The capillary was conditioned daily by washing with 0.1 $mol \cdot L^{-1}$ sodium hydroxide for 10 min, with water for 10 min and with the running buffer for 10 min sequentially. Between consecutive analysis , the capillary was rinsed with water for 3 min, with 0.1 $mol \cdot L^{-1}$ sodium hydroxide for 3 min, with water for 4 min, with running buffer for 4 min sequentially to maintain proper reproducibility of run-to-run injections. Duplicate injection of the solutions were performed and average peak areas were used for the quantification. Peak identification was conducted by spiking the sample with the analytes to be identified. Comparing the on-line ultraviolet spectrum of real sample with that of the standard solution also served as a complementary method for peak identification in this work.

Sample preparation

Flos Genkwa samples was purchased from a local drugstore in Dezhou (Shandong Province, China). At first, they were washed with ultrapure water and air dried, then they were pulverized. 0.5000 g of the powder was extracted with 7 mL of methanol by ultrasonication at room temperature for 20 min, then centrifuged at 3000 rpm for 10 min. The resulted supernatant solution was moved into a volumetric flask of 25 mL. The extraction process was repeated three times. Next, the total extracted solutions were diluted to 25 mL with methanol. Before analysis, 5 mL of the extracted solution was transferred to a 10 mL volumetric flask and diluted to mark with methanol after 0.8 mL internal standard stock solution was added.

Conclusion

A β-cyclodextrin and ionic liquid (1E-3MI-TFB) modified capillary zone electrophoretic method has been developed to determine genkwanin, apigenin and luteolin in *Flos Genkwa* with 4-methylumbelliferone as internal standard. The method was accurate, fast, and could be very effective for quality control of *Flos Genkwa* and its medicinal preparations. The analytical results were also meaningful for the medicinal resource development of *Flos Genkwa*. No such work has been reported in the literature.

Acknowledgement

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