Separation and Determination of Flavonoids in *Ixeridium gracile* by Capillary Electrophoresis

Yu Zhang[1,2], Liang Zhao[1], and **Yan-Ping Shi[1,*]**
[1]Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, P.R. China and [2]Graduate University of Chinese Academy of Sciences, Beijing 100039, P. R. China

Abstract

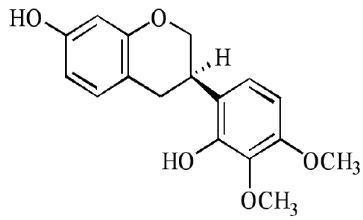
A simple and rapid capillary electrophoresis method has been developed for the quantitative analysis of three active compounds: (3R)-7,2'-dihydroxy-3',4'-dimethoxy-isoflavan, kaempferol, and quercetin in *Ixeridium gracile*. The buffer solution used in this method is 20 mmol/L borate at pH 9.5. The effects of pH value, borate concentration, and applied voltage on migration behavior are systematically investigated. Regression equations reveal good linear relationships (correlation coefficients: 0.9986, 0.9997, and 0.9999) between the peak area of each compound and its concentration. The relative standard deviations of the migration time and peak area are less 1.12% and 3.11% (intraday), and 1.43% and 3.52% (interday), respectively, under the optimized separation conditions. The contents of the three flavonoids in *I. gracile* are successfully determined within 7.8 min, with satisfactory repeatability and recovery.

Introduction

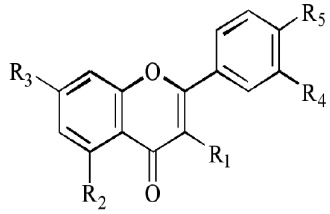
Natural medicine preparations are widely used in Eastern Asia. The research and development of bioactive ingredients in these traditional medicines have attracted serious international scientific attention in recent decades (1–3). However, because a great number of these medicines may show a complicated profile of constituents, efforts are quite necessary to develop a simpler and more facile analytical method that can assay as many bioactive components as possible, especially for quality control.

Ixeridium gracile belongs to Ixeridium genus and occurs only in the Sitsang region in China. It is used as a traditional Tibetan herb for the treatment of hyperplasia of the mammary gland, appendicitis, celiac abscess, hepatitis, and phthisis (4). Up to now, few articles have been reported about its components. Through phytochemical investigation, three active flavonoids: (3R)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan (DDI), kaempferol (KA), and quercetin (QU) have been first isolated by our laborary from the extract of *Ixeridium gracile*, and their molecular structures have been elucidated by the 1H, 13C NMR, and MS data (molecular structures are shown in Figure 1). QU and KA have been shown to inhibit osteoclastic bone resorption and osteoclast apoptosis (5-7), and isoflavan was reported to exhibit multiple pharmacological activities (8). Thus, identification and determination of QU, KA, and DDI will play an important role in the efficacy, safety, and therapeutic reproducibility of *I. gracile* and its medicinal preparation.

Due to their strong pharmacological effects, analyses of flavonoids have attracted the attention of many analysts. So far, the analysis for flavonoids has been accomplished using different chromatographic techniques, including thin-layer chromatography (9,10), supercritical fluid chromatography (11), gas chromatography (12), and liquid chromatography (13–16). Among these methods, high-performance liquid chromatography (HPLC) shows good reproducibility and can provide structural information of the analytes if combined with MS. However, it has



(3R)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan (DDI)



Quercetin $R_1 = R_2 = R_3 = R_4 = R_5 = OH$ (QU)
Kaempferol $R_1 = R_2 = R_3 = R_4 = R_5 = OH$, $R_4 = H$ (KA)

Figure 1. Molecular structures of DDI, QU, and KA.

* Author to whom correspondence should be addressed: email hiyp@lzb.ac.cn.

some shortcomings, such as long analysis times and large amounts required of organic reagent, and because of the complex chemical nature of natural medicines, the applicability for these analytical techniques is greatly restricted by the relatively large consumption of material and time.

Since CE, in its modern form, was first described by Jorgenson and Lukacs (17), its application for the separation and determination of a variety of samples has been increasingly widespread because of its minimal sample volume requirement, short analysis time, and high separation efficiency. During the past years, CE has been focused on the determination of flavonoids in the extracts of medicinal plants due to their strong pharmacological activities (18–23). Deng et al. reported an MEKC method for the determination of QU, KA, and Isorhamnetin in *Ginkgo biolba* within 13 min (24). Yue et al. (22) developed a CE method for the determination of QU, KA, and Isorhamnetin in the extract of *H. rhamnoides* using DM-β-CD as modifier. Suntornsuk (25) reported a CE method for analysis of QU, KA, etc. in selected Thai plants. To the best of our knowledge, there are no reports on the separation and determination of DDI, KA, and QU in *I. gracile*. In this paper, a simple, sensitive, and accurate CE method coupled with a diode-array detector (DAD) was described for the simultaneous determination of DDI, KA, and QU in the extract of *I. gracile* within 7.8 min. The optimum conditions for the analytical method were investigated for the best resolution and highest sensitivity of detection.

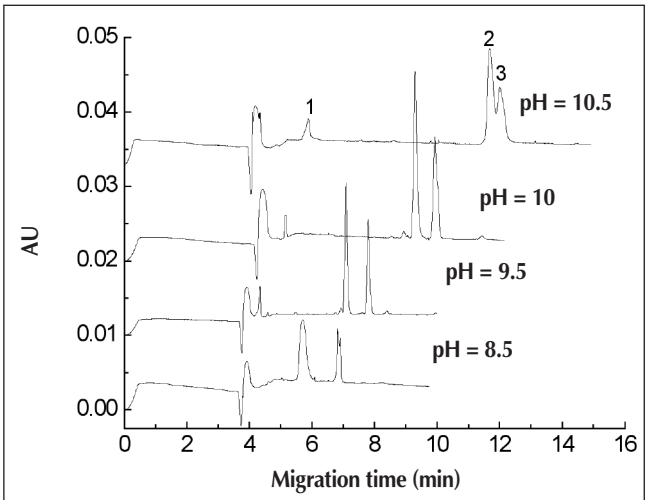


Figure 2. Effect of buffer pH on the migration time. DDI, 1; KA, 2; QU, 3. Electrophoretic conditions: 20 mmol/L borate; applied voltage, 25 kV; temperature, 25°C; UV detection wavelength, 270 nm.

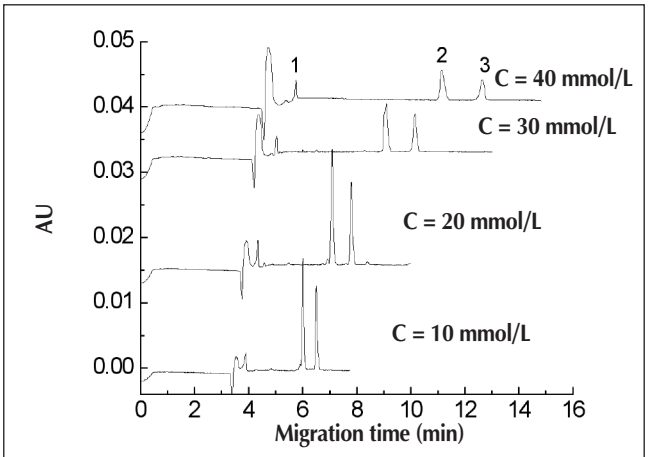


Figure 3. Effect of borate concentration on the migration time. DDI, 1; KA, 2; QU, 3. Electrophoretic conditions: pH = 9.5; other conditions as in Figure 2.

Experimental

Reagents and materials

Standards, (3R)-7,2'-dihydroxy-3',4'-dimethoxyisoflavan (DDI), kaempferol (KA), and quercetin (QU) were obtained by our own laboratory (26).

The fresh air-dried material of *I. gracile* was bought from the Tibetan Hospital of Qinghai Province, China.

All chemicals were analytical-grade and purchased from Beijing Chemical Reagents Plant (Beijing, China). Deionized water was used throughout.

Sample preparation

The stock solution of each component was prepared by dissolving small amounts of standard in methanol into a 5-mL volumetric flask. The concentrations of DDI, QU, and KA were 0.94, 0.56, and 0.64 mg/mL, respectively, and various concentrations of the sample solutions were prepared by appropriate dilution from the stock solution when needed.

One gram of *I. gracile* dried powder was accurately weighed and added into a 50-mL flask together with 20.00 mL 85% (v/v) methanol. Then the flask was weighed with a precision of 10^{-4} g and extracted with an ultrasonic bath for 20 min, then allowed to settle for 10 minutes, and the previously described procedure was repeated 4 times. After that, 85% (v/v) methanol was added to the primary weight. The extracts were filtered through a filter paper and a 0.45-µm syringe filter and injected directly after degassed by ultrasonication.

Apparatus and conditions

All the experiments were carried out on a Beckman PACE/MDQ capillary electrophoresis instrument equipped with an on-column DAD (Beckman Coulter, Fullerton, CA). 32 Karat System Software version 5.0 (Beckman Coulter) was used for data acquisition and evaluation. Samples were injected by applying a pressure of 0.5 psi for 8 s (psi = 6894.76 Pa). A voltage of 25 kV was applied during all separations, with a constant temperature of 25°C. The detection wavelength was set at 270 nm. An ultrasonic bath, Model SB5200 (Branson, Shanghai, China) was used to degas the buffer. Uncoated fused-silica capillaries (Yong-Nian Optical Fiber Factory, Hebei Province, China) with an inner diameter of 75 µm and a total length of 70.5 cm (effective length of 60 cm) were used.

Before use, the capillary was rinsed with 0.1 mol/L NaOH for 15 min, then with deionized water for 10 min; it was then conditioned with running electrolyte for 4 min. Between runs, the capillary was rinsed as follows: 2 min with 0.1 mol/L sodium hydroxide, 2 min with deionized water, and 2 min with running electrolyte.

Results and Discussion

Effect of buffer pH

Buffer pH is a very important parameter for its effect on zeta potential (ζ), the electroosmotic flow (EOF), and the overall charge of all the analytes, which affects the migration time and separation of the analytes. So, a series of buffers with pH ranging from 8.5 to 10.5 were investigated. At pH 9.0, no signals were detected within 15 min. Figure 2 shows the influence of buffer pH (8.5, 9.5, 10.0, and 10.5) on the migration behavior. At pH 8.5, DDI had no signal, and the peak shapes of KA and QU were poor. When the pH increased from 9.5 to 10.5, the migration time increased whereas the resolution decreased with peak broadening simultaneously. At pH 10.5, KA and QU could not be baseline separated. Therefore, pH 9.5 was chosen as the running buffer pH, considering the peak areas, resolution, and the analytical time.

Effect of borate concentration

As the buffer concentration influences the viscosity coefficient of the solution, so do the diffusion coefficient of analytes and the ζ-potential of the inner surface of capillary tube, which affect both the resolution and migration time of the analytes. Figure 3 summarizes the effect of borate concentration in the range of 10–40 mmol/L on the three flavonoids' migration behavior.

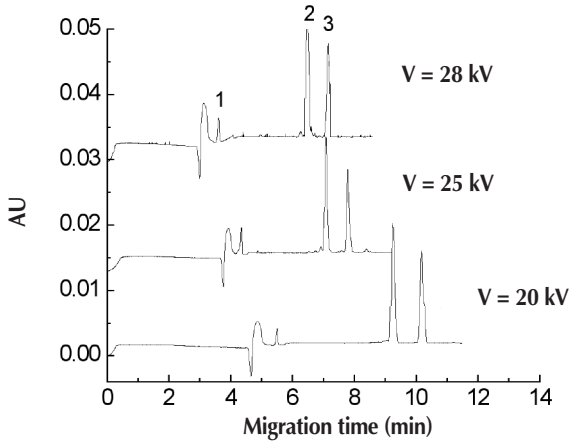


Figure 4. Effect of separation voltage on the migration time. DDI, 1; KA, 2; QU, 3. Electrophoretic conditions: pH = 9.5; other conditions as in Figure 2.

According to this figure, the migration time and the resolution increased with an increased buffer concentration. However, higher buffer concentrations (> 20 mmol/L) lead to poor peak shapes and also had a negative effect on the detection limits, because the peak areas of all analytes decreased, and the effect of Joule heat became more pronounced, so 20 mmol/L borate buffer (pH 9.5) was selected for further experiments.

Influence of separation voltage

The separation voltage affects the electric field strength, which in turn affects the EOF and the migration velocity of charged particles (27); these two factors determine the migration time of analytes accordingly. So higher voltage is necessary for a rapid CE analysis; however, higher separation voltage may result in higher Joule heating. The effect of different separation voltage (20 kV, 25 kV, and 28 kV) on the migration time of the analytes is shown in Figure 4. Increasing the voltage gives shorter migration time, but it also increases the background noise, resulting in a higher detection limit. Although the resolution of analytes can be improved to some extent, too low a separation voltage will increase the analytical time considerably, which in turn causes peak broadening. Based on experiments, 25 kV was chosen as the optimum voltage to accomplish a good compromise.

Linearity, detection limit, and reproducibility

The detection limits (signal = 3), linear ranges, and linear relationships between the concentration of the three analytes and the corrected peak area are shown in Table I. The method was validated for reproducibility of the migration time and the peak area of the analytes. The relative standard deviations of the migration time and the peak area of each peak for six replicate injection were 0.82–1.12% and 2.61–3.11% (intraday), and 1.01–1.43% and 3.16–3.52% (interday), respectively.

Application and recovery

The extracted solution of *I. gracile* was injected directly after dilution and separated under the optimum conditions described. The desired compounds in the plant were identified by comparing both the migration times and UV spectra with that of the standards. The analytes were further confirmed by spiking the standards in actual sample. Figure 5 illustrates the typical electropherogram of the extract. The three flavonoids and other unknown constituents were baseline separated within 7.8 min. The contents of DDI, KA, and QU in *I. gracile* were 0.70, 0.81, and 0.11 mg/g, respectively. The recovery and reproducibility experiments were also conducted under the optimum conditions to evaluate the precision and accuracy of the method. Accurate amounts of DDI, KA, and QU were added to the diluted extract of *I. gracile*, and the recovery values were obtained using their peak areas from the calibration curve under the same conditions. The results, listed in Table II, indicate that this method is accurate, sensitive, and reproducible, providing a useful quantitative method for the analysis of active ingredients in *I. gracile*.

Table I. Results of Regression Analysis on Calibration and Detection Limits

Compounds	Regression equation $y = a + bx$*	Correlation coefficient	Linear range (μg/mL)	Detection limit† (μg/mL)
DDI	$y = 81.22x - 16.18$	0.9988	1.7–478	1.2
KA	$y = 1339.9x - 345.67$	0.9996	1.0–365	0.3
QU	$y = 967.55x - 757.36$	0.9999	1.2–508	0.4

* y and x stand for the corrected peak area and the concentration (μg/mL) of the analytes, respectively, and five different concentrations were used to define each linear range.
† The detection limit was defined as the concentration where the signal-to-noise ratio is 3.

Table II. Recoveries of Three Flavonoids (RSD, *n* = 3)

Compound	Original amount (mg)	Added amount (mg)	Found amount (mg)	Recovery (%)	RSD (%)
DDI	0.63	1.19	1.16	97.5	2.24
			1.18	99.2	
			1.21	101.7	
KA	0.73	1.24	1.22	98.2	1.89
			1.25	100.8	
			1.22	98.2	
QU	0.10	0.78	0.77	98.7	3.38
			0.76	97.4	
			0.81	103.8	

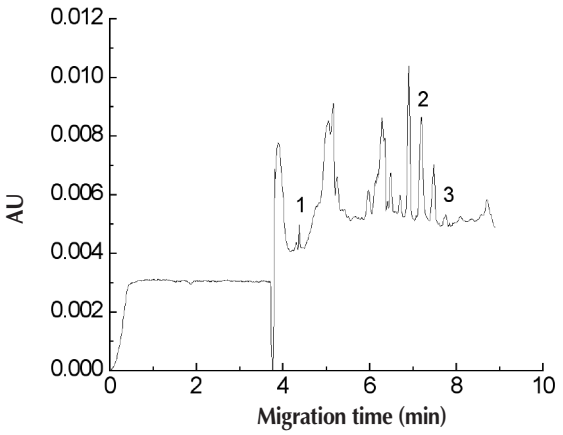


Figure 5. Chromatogram of the extract of *I. gracile*. DDI, 1; KA, 2; QU, 3. Electrophoretic conditions: pH = 9.5, other conditions as in Figure 2.

Conclusion

In this paper, a CE method was successfully developed for the simultaneous analysis of DDI, KA, and QU in the Tibetan medicine plant *I. gracile*. Compared with other existing method, the proposed methodology has major advantages, such as simple running buffer, low detection limits, and rapid analysis time. The results demonstrate that this method has higher resolution, excellent reproducibility, and low cost, and is suitable for use as a safety assurance tool for herbal medicines and finished products that are suspected to contain these flavonoids.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (No. 20475057) and the Natural Science Foundation of Gansu Province (No. 3ZS051-A25-077).

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Manuscript received March 20, 2007;
Revision received July 23, 2007.