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Simultaneous determination of flavonoids in *Ixeridium gracile* by micellar electrokinetic chromatography

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Abstract

A micellar electrokinetic chromatography (MEKC) method has been developed for the quantitative analysis of five flavonoids: luteolin 7-*O*-glucoside (LG), 2′,4′-dihydroxy-dihydrochalcone (DD), 2′,4′-dihydroxy-chalcone (DC), 7-hydroxy-flavanone (HF) and quercetin 3-*O*-galactoside (QG) in *Ixeridium gracile* with UV detection at 275 nm. The applied voltage was 25 kV and the capillary temperature was kept constant at 25 °C. The effects of buffer pH, the concentration of electrolyte and organic modifier on migration behavior were studied. Optimum separation condition was achieved with 15 mM borate, 30 mM sodium dodecyl sulfate (SDS) and 10% (v/v) ethanol at pH 10.5. Regression equations showed good linear relationships (correlation coefficients: 0.9984, 0.9991, 0.9994, 0.9995 and 0.9997) between the peak area of each compound and their concentrations. The relative standard deviations (R.S.D.) of the migration time and peak area were less 1.67 and 3.53% (intra-day), and 1.82 and 3.73% (inter-day), respectively, under the optimum separation conditions. The contents of the five active compounds in *I. gracile* were determined with satisfactory repeatability and recovery.

Keywords: Flavonoids; *Ixeridium gracile*; Micellar electrokinetic chromatography

1. Introduction

Natural medicines have been used to treat human diseases in Eastern Asia for centuries. However, a great number of medicinal plants show complicated profiles of constituents, so work on the quality control and quantitative analysis of active components in traditional and herbal medicines has great importance.

Ixeridium gracile (DC) Shih (Compositae family) is a traditional Tibetan herbal grown only in Sitsang region and it is believed to help patients affected by hyperplasia of mammary gland, appendicitis, celiac abscess and hepatitisand phthisis [1]. Up to now, few articles have been reported about its components. Our laboratory first carried out the systemic phytochemical investigation of *I. gracile,* and five active constituents: luteolin 7-*O*-glucoside (LG), 2′,4′-dihydroxy-dihydrochalcone (DD), 2′,4′-dihydroxy-chalcone (DC), 7-hydroxy-flavanone (HF) and quercetin 3-*O*-galactoside (QG) have been isolated from the extract of *I. gracile* and their molecular structures were elucidated by the ^{1}H, ^{13}C NMR and MS data (molecular structures are shown in Fig. 1) [2].

Flavonoids represent a highly diverse class of secondary plant metabolites with about 9000 structures which have been identified up to now. These compounds are abundant in fruits, vegetables and medicinal plants. They can be divided into five subclasses: flavonols, flavones, flavans, chalcones and flavonones [3,4].

Flavonoids have been reported to have anti-inflammatory [5,6], antineoplastic [7], anti-oxidant [8–10], antiviral [11] and anticarcinogenic [12] activity. Analytical methods of flavonoids described in the literatures are mainly based on thin-layer chromatography (TLC) [13,14], gas chromatography (GC) [15], and liquid chromatography (LC) [16–19]. None of these methods is entirely adequate because of their more or less shortcomings such as: long analysis time, large amounts of organic reagent, limited resolution or low sensitivity. CE has been applied widely owing to its minimal sample volume requirement, short analysis time and high separation efficiency. During the past years,

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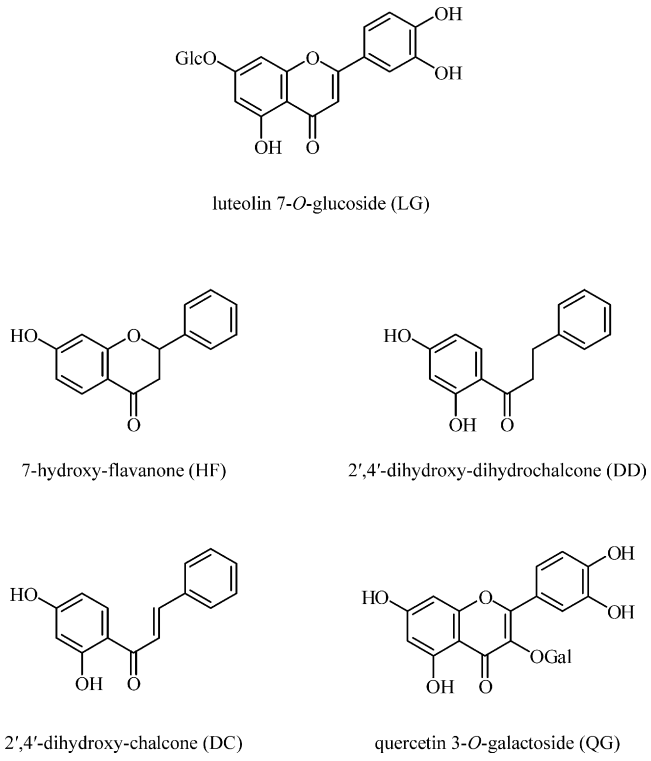

Fig. 1. Molecular structures of LG, HF, DD, DC and QG.

CE has been applied for the determination of flavonoids in the extracts of medicinal plants due to their extensive pharmacological activities [20–27]. To the best of our knowledge, there are no reports on the separation and determination of LG, HF, DD, DC and QG in *I. gracile*. In this paper, we first developed an MEKC method for the simultaneous determination of LG, HF, DD, DC and QG in the extract of *I. gracile*. The optimum conditions for the analytical method were investigated for the best resolution and highest detection sensitivity.

2. Experimental

2.1. Apparatus and conditions

A Beckman PACE/MDQ capillary electrophoresis instrument equipped with an on-column diode-array detection (DAD) (Beckman coulter, Fullerton, CA, USA) was used with a software of 32 Karat system and version 5.0 (Beckman coulter, Fullerton, CA, USA) for data acquisition and evaluation. Samples were injected by applying a pressure of 0.5 psi for 8 s. A voltage of 25 kV was applied during all separations, with a constant temperature of 25 °C. The detection wavelength was set at 275 nm. An ultrasonic bath Model SB5200 (Branson, Shanghai China) was used to degas the buffer. PB-10 pH meter (Beijing Sartorius Instrument & System Engineering Co., Ltd.) was used to adjust the BGE solution to the desired pH value with 0.1 M NaOH or 0.1 M HCl. Uncoated fused-silica capillaries (Yong-Nian Optical Fiber Factory, Hebei, 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 M NaOH for 15 min, then with deionized water for 10 min; it was then conditioned with running electrolyte for 4 min. Between the two runs, the capillary was rinsed as follows: 2 min with 0.1 M sodium hydroxide, 2 min with deionized water and 2 min with running buffer.

2.2. Reagents and materials

Standards, luteolin 7-*O*-glucoside, 2′,4′-dihydroxy-dihydrochalcone, 2′,4′-dihydroxy-chalcone, 7-hydroxy-flavanone and quercetin 3-*O*-galactoside were separated in our own laboratory [2].

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

The optimal buffer was composed of 15 mM sodium tetraborate, 30 mM SDS and 10% (v/v) ethanol at pH 10.5 and all chemicals were analytical grade and purchased from Beijing Chemical Reagents Plant (Beijing, China). Deionized water was used throughout.

2.3. Sample preparation

Standard stock solutions of five flavonoids at concentration of 0.5 mg/ml were prepared by dissolving about 2.5 mg of standard in methanol in a 5 ml volumetric flask, and various concentrations of the sample solutions were prepared by appropriate dilution from the stock solution when needed.

1.0 g dried powder of the whole plant of *I. gracile* was accurately weighed and added into a 50 ml flask together with 20.00 ml 85% (v/v) methanol. Then the flask was weighed and extracted with an ultrasonic bath for 20 min, then put still 10 min and the above procedure was repeated four 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.

3. Results and discussion

3.1. Effect of buffer pH

Buffer pH is a very important parameter for its effect on zeta potential (ζ), the electroosmotic flow (EOF), as well as the overall charge of all the analytes, which affect the migration time and the separation of the analytes. So a series of buffers with pH ranging from 8.5 to 10.5 was investigated. At pH 8.5 no peaks has been detected within 15 min. Fig. 2 shows the influence of buffer pH on separation from 9.0 to 10.5. It can be seen that DD, DC and QG could not be baseline separated, and the peak shape of LG was poor at pH 9.0. With the buffer pH increasing, the resolution and the migration time of all flavonoids were increased, and the peak shapes became better. Although all compounds were baseline separated at pH 9.5, when extract of *I. gracile* was introduced into the capillary, DD and QG were poorly separated from other unknown compounds. So pH 10.5 was selected for further experiments.

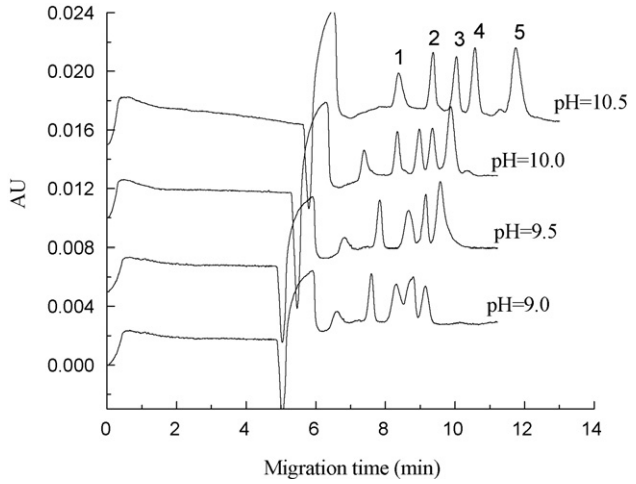

Fig. 2. Effect of buffer pH on the migration time. (1) LG; (2) HF; (3) DD; (4) DC; (5) QG. Electrophoretic conditions: 15 mM borate, 30 mM SDS, 10% ethanol (v/v); applied voltage, 25 kV; temperature, 25 °C; UV detection wavelength, 275 nm.

3.2. Effect of borate concentration

As the buffer concentration influences the viscosity of the solution, the diffusion coefficient of analytes and the ζ-potential of the inner surface of capillary tube as well, it affects both the resolution and migration time of the analytes [28]. The influence of the sodium tetraborate concentration at the range of 10–30 mM on the separation of the five flavonoids was examined with 30 mM SDS and 10% ethanol (v/v) at pH 10.5. As Fig. 3 shows, with increasing buffer concentration, the migration times of the five flavonoids and the resolutions of HF, DD, DC and HF increased, but the resolution between LG and HF changed little. When buffer concentration increased to 15 mM, five components could be separated completely from each other. Therefore, 15 mM borate was selected to keep separation efficiency and shorten analytical time.

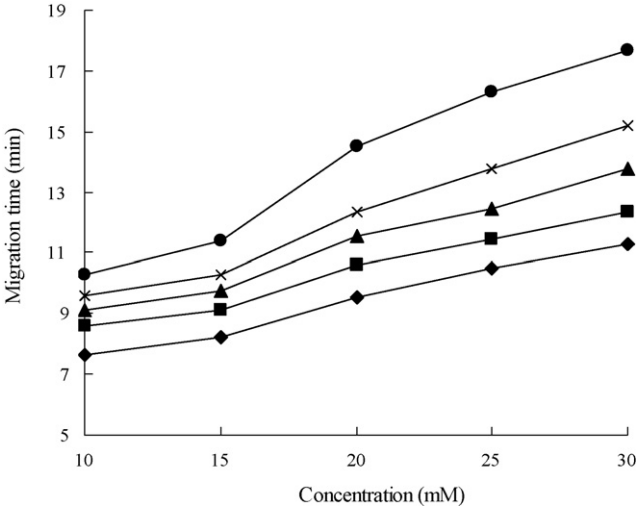

Fig. 3. Effect of borate concentration on the migration time. (♦) LG; (■) HF; (▲) DD; (×) DC; (●) QG. Electrophoretic conditions: 30 mM SDS, 10% ethanol (v/v); pH 10.5; other conditions as in Fig. 2.

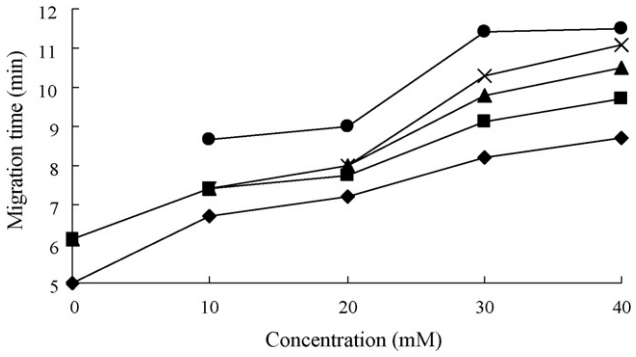

Fig. 4. Effect of SDS concentration on the migration time. (♦) LG; (■) HF; (▲) DD; (×) DC; (●) QG. Electrophoretic conditions: 15 mM borate, 10% ethanol (v/v); pH 10.5; other conditions as in Fig. 2.

3.3. Effect of SDS and organic modifier concentration

Effect of SDS concentration ranged from 0 to 40 mM was verified (as shown in Fig. 4). With increasing SDS concentration, the migration times and resolutions increased. Before adding SDS to the system, the peaks of HF and DD combined, and DC and QG had no signal. When the concentration of SDS increased to 10 mM, LG and QG were separated, but HF, DD and DC were overlapped. When SDS concentration exceeded 30 mM, the five compounds could be completely separated. Considering the analysis time and resolution, 30 mM SDS was chosen for further experiments.

Organic modifiers were also employed to improve resolution and separation selection. Methanol, ethanol, acetonitrile were investigated and ethanol was selected. With the increase of ethanol concentration (0–15%), the analysis time increased and the resolutions improved. Considering the migration time and resolution, 10% (v/v) ethanol was appropriate.

3.4. Linearity, reproducibility, LOD and LOQ

The limits of detection (LODs) (S/N = 3), the limits of quantitation (LOQs) (S/N = 10), linear ranges and the linear relationships between the concentration of the five analytes and the corrected peak area are shown in Table 1. The method was validated for reproducibility of the migration time and the peak area of the standards. The relative standard deviations of the migration time and the peak area of each of peak for six replicate injections were 1.15–1.67 and 2.42–3.53% (intra-day), and 1.41–1.82 and 2.96–3.73% (inter-day), respectively.

3.5. Extraction of the analytes in I. gracile

According to the literature, an ultrasonic bath at room temperature was a suitable extraction system for flavonoids [29,30]. So in our study, the ultrasonic bath extraction was chosen as preferred method. The influence of extraction solvents on the extraction of herbal plant samples was investigated. Pure and aqueous methanol (85%, 60%) was employed as extraction solutions in this study. Eighty five percent methanol was better for all the analytes exhibiting a significantly larger signal than those

Table 1
Results of regression analysis on calibration, LOD and LOQ

Compounds	Regression equation $y = a + bx$[a]	Correlation coefficient	Linear range (μg/ml)	LOD[b] (μg/ml)	LOQ[b] (μg/ml)
LG	$y = 603.79x - 5416.6$	0.9984	4.3–448.3	3.0	9.2
HF	$y = 447.53x + 2409.7$	0.9991	4.5–464.9	2.1	6.9
DD	$y = 307.24x + 1363.5$	0.9994	5.0–608.5	1.5	5.0
DC	$y = 888.35x + 2044.9$	0.9995	1.9–304.2	1.2	4.0
QG	$y = 1414.31x + 2826.2$	0.9997	1.1–368.4	0.5	1.6

[a] y and x stand for the corrected peak area and the concentration (μg/ml) of the analytes, respectively.
[b] The LOD was defined as the concentration where the signal-to-noise ratio is 3 and the LOQ was defined as the concentration where the signal-to-noise ratio is 10.

Table 2
Recoveries of five flavonoids (R.S.D., $n = 3$)

Compound	Original amount (mg)	Added amount (mg)	Found amount (mg)	Recovery (%)	R.S.D. (%)
LG	2.78	1.52	1.48	97.4	2.5
HF	0.52	0.49	0.50	102.0	3.9
DD	0.24	0.56	0.55	98.2	4.2
DC	0.65	0.77	0.74	96.1	2.8
QG	0.10	0.21	0.22	104.8	3.6

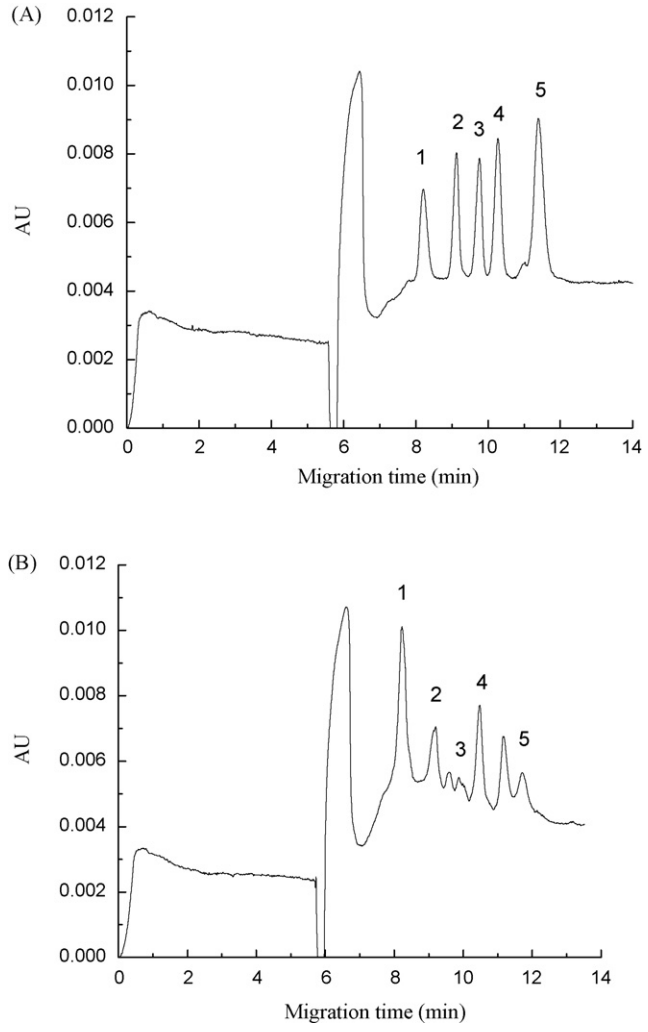

Fig. 5. Chromatograms of the five flavonoids mixture (A) and extract of the *Ixeridium gracile* (B). (1) LG; (2) HF; (3) DD; (4) DC; (5) QG. Electrophoretic conditions: pH 10.5, other conditions as in Fig. 2.

extracted by the other. The result of the recovery test also demonstrated that the extraction method was adequate and appropriate for the analysis.

3.6. Application and recovery

The extracted solution of *I. gracile* was injected directly after appropriate dilution and separated under the proposed method. Fig. 5 illustrates the typical electropherograms of the five standards and 85% methanol extract from *I. gracile*. The five flavonoids and other unknown constituents were separated within 12 min. The contents of LG, HF, DD, DC and QG in *I. gracile* were 2.32 ± 0.03, 0.43 ± 0.02, 0.20 ± 0.01, 0.54 ± 0.03 and 0.08 ± 0.02 mg/g (mean ± S.D., $n = 3$), respectively.

Recovery and reproducibility experiments were also conducted to evaluate the precision and accuracy of the method. Accurate amounts of LG, HF, DD, DC and QG were added to the diluted extract of *I. gracile*, and three duplicate injections were performed. The results are shown in Table 2. The results indicate that this method is accurate, sensitive and reproducible, providing a useful quantitative method for the analysis of active ingredients in *I. gracile*.

4. Conclusion

In this paper, a sensitive MEKC method has been firstly developed for simultaneous analysis of LG, HF, DD, DC and QG in *I. gracile*. The reproducibility of quantitative analysis is satisfactory. This work shows that MEKC is a suitable and powerful technique to study flavonoid compounds in the complex extract of the medicinal plants.

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