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Determination of kava lactones and flavonoid glycoside in *Scorzonera austriaca* by capillary zone electrophoresis

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Abstract

A capillary zone electrophoretic method has been developed for the quantitative analysis of three active comppounds, 12-hydroxy-desmethoxyyangonin (HD), 12-β-D-glucopyranoside-desmethoxyyangonin (GD) and luteolin 3′-(6-E-*p*-coumaroyl-β-D-glucopyranoside) (LG) in *Scorzonera austriaca* with UV detection at 254 nm. The applied voltage was 25 kV and the capillary temperature was kept constant at 25 °C. The effect of buffer pH, the concentration of electrolyte and organic modifier on migration were studied systematically. Optimum separation was achieved with 20 mM borate buffer at pH 10.00 containing 10% (v/v) methanol. Daphnetin was used as internal standard for quantification. Regression equations revealed good linear relationship between the ratios of the peak area of each compound and its the ratios of concentration. All the correlation coefficients were higher than 0.9990. The relative standard deviations of migration time and the peak area were <1.46% and 5.13% (inter-day), and <1.65% and 5.16% (intra-day), respectively. The contents of the three compounds in *S. austriaca* were successfully determined with satisfactory repeatability and recovery.

Keywords: Kava lactone; Flavonoid glycoside; *Scorzonera austriaca*; Capillary electrophoresis

1. Introduction

Scorzonera austriaca occurs in the northwestern mountainous regions in China. Its root is used as Tibetan traditional medicine for the treatment of many diseases, such as curing fever, carbuncle and mastitis [1]. Li, et al. [2] first reported the structural elucidation of a new guaianolide isolated from the acetone extract of *S. austriaca*. Through phytochemical investigation, three active compounds, 12-hydroxy-desmethoxyyangonin (HD), 12-β-D-glucopyranoside-desmethoxyyango -nin (GD) and luteolin 3′-(6-E-*p*-coumaroyl-β-D-glucopyranoside) (LG) have been first isolated from the extract of *S. austriaca* in our laboratory and their molecular structures have been elucidated by the ^{1}H, ^{13}C NMR and MS data (molecular structures were shown in Fig. 1). All NMR data were identical to those of the corresponding known compounds previously reported in the literature [3,4]. In the three compounds, HD is kavalactone and GD is kavalactone glucoside. Kavalactones have also been employed in the western medicine for the sedative, muscle relaxant, analgesic, anticonvulsive, anaesthetic, anti-arrhythmic, anti-thrombosis, neuroprotective and anti-spasmodic effects [5]. It has been reported that flavonoids display a wide range of pharmacological activities, such as anti-inflammatory [6], anti-neoplastic [7] and anti-oxidant [8]. So the identification and determination of the three active compounds in *S. austriaca* will play an important role in the further development and use of this herb.

In general, due to their strong pharmacological effects, the analyses of kavalactones and flavonoids have attracted the attention of many analysts. So far, the analyses for kavalactones compounds have been accomplished using different chromatographic techniques, including thin layer chromatography (TLC) [9], supercritical fluid chromatography (SFC) [10], gas chromatography (GC) [11,12], and liquid chromatography (LC) [13]. The techniques of analyses for flavonoids compounds including thin layer chromatography (TLC) [14], gas chromatography (GC) [15], and liquid chromatography (LC) [16] have been reported. However, because of the complex chemical nature

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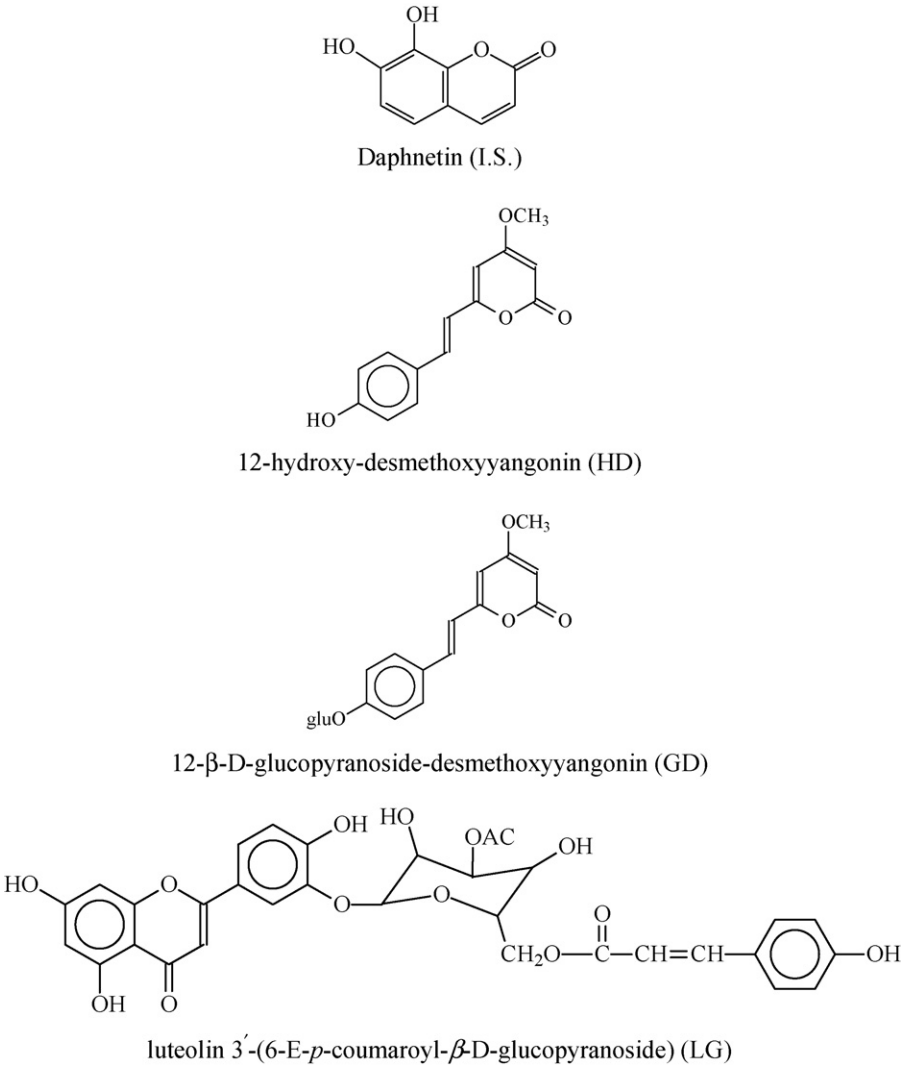

Fig. 1. Molecular structures of daphnetin (I. S.) and 12-hydroxy-desmethoxyyangonin (HD), 12-β-D-glucopyranoside-desmethoxyyangonin (GD) and luteolin 3′-(6-E-*p*-coumaroyl-β-D-glucopyranoside) (LG).

of traditional Chinese medicines, the applicability for these analytical techniques was greatly restricted by relatively large consumption of material and time. Recently, owing to its high resolving power, low solvent consumption and simple pretreatment, capillary electrophoresis (CE) has been used as an attractive method for separating and monitoring Chinese traditional medicines [17–21]. Although Lechtenberg [22] has described CE method for determination of kavalactones from dry extracts of *Piper methysticum* Forst. To the best of our knowledge, there are no reports on the separation and determination of the kava lactones and flavonoid glycoside in the *S. austriaca*. In this paper, we first developed a CZE method for the simultaneous determination of HD, GD, and LG in the *S. austriaca*.

2. Experimental

2.1. Apparatus and conditions

Experiments were carried out on an Agilent HP^{3D} capillary electrophoresis system (Agilent, USA). The applied voltage was held constant at 25 kV. The column was an uncoated fused-silica capillary with a total length of 50 cm and an effective length of 41.5 cm (Yongnian, Hebei Province, China). The temperature of the capillary cartridge during electrophoresis was maintained at 25 °C and UV detection was done at 254 nm. Before each use, the capillary was rinsed with 1 M NaOH for 10 min, then with water for 10 min; it was then conditioned with running electrolyte for 10 min. Between runs, the capillary was rinsed with water and electrolyte for 5 min each. Samples were loaded by pressure injection at 50 mbar for 5 s.

2.2. Materials and reagents

S. austriaca was purchased from Xinin city, Qinghai province, China. Daphnetin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (molecular structures were shown in Fig. 1). HD, GD and LG were isolated from extracts of *S. austriaca* as following: The air-dried whole plant (2.0 kg) was powdered and extracted with 95% EtOH at room temperature (20 L × 4, each extrac-

tion lasted 7 days). The combined extracts were evaporated to dryness under reduced pressure. The residue (250 g) was then suspended in H_2O (1.5 L), extracted with $CHCl_3$ (1.5 L) and *n*-BuOH (1.5 L), respectively. The *n*-BuOH extract (90 g) was subjected to column chromatography (CC) on resin (HPD100) using H_2O and 95% EtOH, respectively. The 95% EtOH fraction (80 g) was subjected to CC on silica gel (1.0 kg) using $CHCl_3$ with increasing volume of CH_3OH (V:V = 50:1, 40:1, 15:1, 8:1, 5:1, 3:1, 1:1, each about 3.0 L) as eluent. Fractions were examined by TLC and combined to afford five pooled fractions (Fr. A-Fr. E). Fr. A (3.2 g) was isolated by CC on silica gel firstly using $CHCl_3$–CH_3COOCH_3 (V:V = 3:1), then $CHCl_3$–CH_3OH (V:V = 30:1) and lastly by CC on RP-18 using CH_3OH–H_2O (V:V = 7:3) to yield pure HD (30 mg). Fr. C (4.0 g) was isolated by CC on silica gel with $CHCl_3$–CH_3OH (V:V = 5:1), $CHCl_3$–CH_3COCH_3–H_2O (V:V:V = 9:45:7) and $CH_3COOC_2H_5$–CH_3OH–H_2O (V:V:V = 20:2:1) to yield pure GD (50 mg). Fr. E (6.2 g) was isolated by CC on silica gel with $CHCl_3$–CH_3OH–H_2O (V:V:V = 12:5:1) and $CH_3COOC_2H_5$–CH_3OH–H_2O (V:V:V = 20:4:1), further isolated by CC Sephadex LH-20 with CH_3OH and by CC on polyamide with CH_3OH–H_2O (V:V = 4:5) to obtain pure LG (43 mg). The structures of HD, GD and LG were confirmed by the ^1H, ^{13}C NMR and MS data (molecular structures were shown in Fig. 1). All NMR data were identical to those of the corresponding known compounds previously reported in the literature [3,4]. All chemicals were analytical grade and purchased from Beijing Chemical Reagents Plant. Deionized water was used throughout. All solutions and samples were filtered through 0.45 μm syring filter.

Stock solutions of HD, GD, and LG at the concentration of 1500 μg mL^{-1} were prepared in methanol, and various concentrations of the sample solution were prepared by appropriate dilution from the stock solution when it was needed. The pH values of borate buffer solution were adjusted by mixing 0.1 M HCl or 0.1 M NaOH solution with borate solution. Methanol was used as the electroosmotic flow (EOF) marker.

2.3. Sample preparation

The air-dried *S. austriaca* (2.0 g) sample were powdered and extracted, with 50 mL methanol in ultrasonic bath for 0.5 h for three times. The three extracts were combined and dried under reduced pressure, and finally dissolved in 2.5 mL methanol. Then, the solution was filtered through a 0.45 μm membrane filter. The injected solutions were prepared from this stock solution by the addition of internal standard and dilution by methanol.

3. Results and discussion

At the beginning of this work, 20 mM borate buffer (pH 9.00), 20 mM H_3PO_4–NaOH (pH 9.00) and 40 mM Tris–H_3PO_4 (pH 9.00), respectively, were used running electrolytes. However, it is difficult to obtain baseline separation and stability when H_3PO_4–NaOH and Tris–H_3PO_4 were evaluated. Therefore, further study was focused on the evaluation of borate buffer.

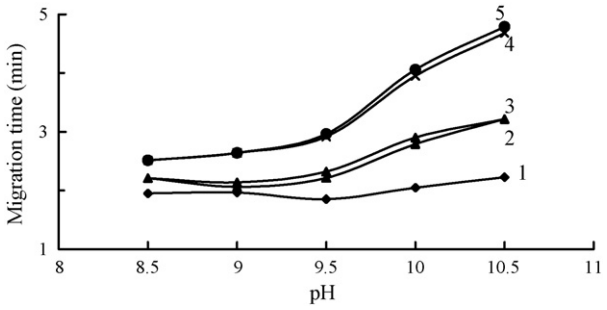

Fig. 2. Effect of pH on the migration time: 1, EOF; 2, I.S.; 3, HD; 4, GD; 5, LG. Analytical conditions: borate, 20 mM; voltage, 25 kV; temperature, 25 °C; UV detection wavelength, 254 nm.

3.1. Effect of buffer pH

The different borate buffers, with the same concentration (20 mM) but different pH values (8.50–10.50), were used to resolve the HD, GD, and LG. As can be seen from Fig. 2, the migration time increased at basic pH and resulted in retardation of migration. At pH < 8.50, I.S. and HD, GD and LG could not separated because of all analytes no charge. The analytes and I.S. are weak acids with pK_a value of 9.0–11.0, therefore these compounds will be constantly ionized in higher pH and migration times and resolution will increase. The additional reason of retardation could be that the complex formation reaction of borate and glycosides was a strongly pH-dependent equilibrium [23]. At pH lower than 9.50, the co-elution of GD and LG was observed. Also, HD and I.S. overlapped at pH 10.50. Thus, taking account of the resolution and analytical time, we used a pH of 10.00 for further experiments. Moreover, the effect of analytes' structures on the complex formation reaction of borate and glycosides was clear. From Fig. 2, as can be seen that the complex formation reaction of borate and glycosides was a strongly related to the number of hydroxyl of analytes. So, the two glycosides of GD and LG were eluted later than the kava lactone of HD.

3.2. Effect of buffer concentration

To verify the effect of buffer concentration on migration behavior, the running buffer consisting of sodium tetraborate at different concentration (10, 20, 30, 40 and 50 mM) at pH 10.00 were investigated. The result was shown in Fig. 3. The migration time and resolution of the three analytes increased with

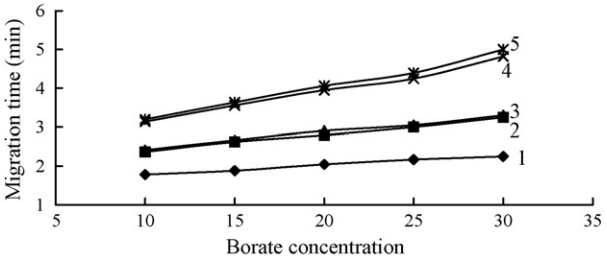

Fig. 3. Effect of buffer concentration on the resolution: 1, EOF; 2, I.S.; 3, HD; 4, GD; 5, LG. Analytical conditions: pH 10.00, other conditions as for Fig. 2.

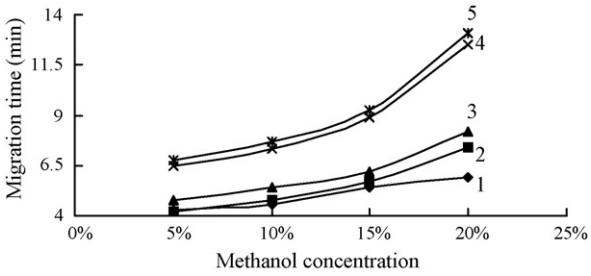

Fig. 4. Effect of methanol concentration on the resolution: 1, EOF; 2, I.S.; 3, HD; 4, GD; 5, LG. Analytical conditions: borate concentration 20 mM, other conditions as for Fig. 3.

an increase of the concentration of running buffer. This is as a result of the decreased EOF since this effect is directly related to the decrease of the zeta potential at the capillary wall–solution interface. Additionally, with the increase of buffer concentration, the complex formation reaction between borate and the natural occurings became more and more strong. Moreover, the increase of the migration time of each analyte was almost equal and the migration order was not changeable. The best resolution was achieved at 20 mM borate buffer. Although HD, GD, and LG were completely separated under the above optimal conditions, when the extracts of *S. austriaca* were introduced to capillary, LG was poor separated from other unknown compounds. Therefore, further attempts were made to improve the separation by adding the organic modifier to the electrolyte.

3.3. Effect of organic solvents

Methanol, acetone and isopropanol were used to enhance the separation. Addition of acetonitrile to the buffer solution could not resolve this problem. Although addition of isopropanol made LG to be separated from the unknown components, it gave poor reproducibility and even block the capillary. Methanol was selected as modifier because of short analysis time, good separation and reproducibility. Fig. 4 shows the effect of methanol proportion on the migration time. With the increase of methanol concentration, the resolution of the analytes was improved. When methanol was added to the buffer, the dissociation of silanol in the inner wall of the capillary were reduced and EOF was reduced, which improve the resolution. However, at higher concentration of methanol, the migration times of the analytes were increased too. At 10% (v/v) methanol percentages, the HD, GD, and LG were completely separation and LG was excellent separated from other unknown compounds.

From the above results, the best condition was obtained, an electrolyte containing 20 mM borate and 10% (v/v) methanol at pH 10.00. The applied voltage was 25 kV. Fig. 5 A shows the CE electropherogram of a mixture of I.S. and three analytes.

3.4. Construction of calibration curves of HD, GD, and LG

Calibration curves were constructed in the concentration range of 18–450 $\mu g\,mL^{-1}$ for HD, 10–150 $\mu g\,mL^{-1}$ for GD, 56–1200 $\mu g\,mL^{-1}$ for LG. The regression equations of these curves and their correlation coefficients (r) were calculated as follows: HD, $Y = 0.1243X + 0.4466$ ($r = 0.9996$); GD, $Y = 0.1148X + 2.314$ ($r = 0.9994$); LG, $Y = 0.3255X + 13.908$ ($r = 0.9997$), where Y and X are the peak area ratio of the analytes and internal standard and the concentration ratio of the analytes and internal standard, respectively. In every calibration curves, the six concentration levels were included.

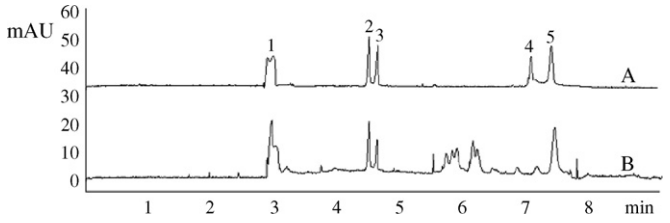

Fig. 5. Chromatogram of mixture of I.S., HD, GD and LG. (A) and methanol extract of *S. austriaca* (B): 1, EOF; 2, I.S.; 3, HD; 4, GD; 5, LG. Analytical conditions: 10% methanol, other conditions as for Fig. 4.

3.5. System suitability test, detection limit and recovery

The method was validated for reproducibility of the migration time and the peak area ratio of the analytes and internal standard. The relative standard deviations (R.S.D.) of the migration time ratio of the analytes and internal standard for six replicate injections were 1.33% (HD), 1.39% (GD) and 1.46% (LG) for inter-day, respectively. The peak area ratio of the analytes and internal standard for six replicate injections were 2.64% (HD), 4.38% (GD) and 5.13% (LG) for inter-day, respectively. The results indicated that the reproducibility of the proposed CE method was excellent. The detection limits ($S/N = 3$) of HD, GD, and LG were 5, 5 and 10 $\mu g\,mL^{-1}$, respectively. Recovery experiments were performed four times adding a 50 $\mu g\,mL^{-1}$ of aliquot HD, GD, and LG into the methanol extracts from *S. austriaca.* The recoveries of HD, GD, and LG were 99.6, 101.3, and 92.6%, respectively. The RSD of HD, GD, and LG were 3.46, 4.68, and 4.97%, respectively.

3.6. Applications

Methanol solutions of extracts by the addition of internal standard were for five replicate injections directly and separated under the optimum condition described above. A typical electrophogram for a sample is shown in Fig. 5B. Peaks were identified by the addition of standards of three plant hormones. The analytical results were summarized in Table 1.

Table 1
Contents of HD, GD and LG in *S. austriaca*

Samples	Average contents (mg/g) $n = 5$		
	HD	GD	LG
S. austriaca	0.034	0.047	0.462

4. Conclusion

The results demonstrate that CE is a useful, simple and rapid technique for identification and determination of kava lactones and flavonoid glycoside in *S. austriaca*. This work also shows that CE is a powerful technique to study active compounds in the complex extract of the medicinal plants.

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