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Separation and determination of flavonoids in *Lamiophlomis rotata* by capillary electrophoresis using borate as electrolyte

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Abstract

A capillary electrophoresis method was developed for the simultaneous determination of five flavonoids such as luteolin-7-*O*-glucoside, isorhamnetin, apigenin, luteolin and quercetin in *Lamiophlomis rotata* (Benth.) Kudo. Optimal conditions were obtained at pH 9.0 with 30 mM borate as buffer containing 8% (v/v) acetonitrile, 20 kV as driving voltage and 210 nm as detection wavelength. The association constant K and the change in Gibbs free energy (ΔG) of the interaction of flavonoids with borate anion ion (a typical ion-dipole or ion-induced dipole interaction) were calculated for the quantitative evaluation and characterization of the interaction. The described method was successfully applied for the rapid and efficient quality control by quantifying flavonoids in *L. rotata*. Repeatability tests showed that the R.S.D.s of both intra- and inter-day migration times and peak areas were less than 5%. The LOD of the five flavonoids was less than 3.75 mg/l. Recovery results ranged from 94.2% to 105.1%.

Keywords: Flavonoid; Capillary electrophoresis; *Lamiophlomis rotata*; Association constant

1. Introduction

Flavonoids attract increasing interest in the recent years due to their antiphlogistic, antiviral [1], antioxidant [2,3], antitumor [4] properties. Many medicines and foods contain flavonoids, among them the Chinese folk medicine *Lamiophlomis rotata* (Benth.) Kudo which has been used to promote blood circulation, remove blood stasis, subdue swelling and alleviate pain [5].

Several analytical methods have been described for the separation and determination of flavonoids, such as high performance liquid chromatography (HPLC) [6–8], high performance liquid chromatography–mass spectrometry (HPLC–MS) [9–11], capillary electrophoresis (CE) [12–16], and gas chromatography–mass spectrometry (GC–MS) [17,18], etc. CE has many advantages such as small injection sample volume, high efficiency, and short analysis time, etc., which can be useful in the rapid and efficient determination of flavonoids in complex extracts.

Borate was used as electrolyte in the separation of flavonoids by CE, because the borate anion forms complexes with flavonoid aglycones and/or saccharides enhancing the selectivity of separation [19]. According to Schmitt-Kopplin [19], the borate ions form stable five-membered-ring complexes with 1,2-diols and six-membered-ring complexes with 1,3-diols. The flavonoids in this study have 5,7-diols on A-ring and/or 3′,4′-diols on B-ring, and hence they can easily form complexes with borate.

The aim of this study was to develop for the first time a CE method based on borate complex formation for the quantitative determination of five flavonoids (polyhydroxy flavonols and flavons) in *L. rotate* as an alternative to the HPLC method. The structures of the studied flavonoids are shown in Table 1.

The association constant K and the changes in Gibbs free energy (ΔG) of the 1:1 complexes between borate and flavonoids were also studied for the better understanding of the interaction of flavonoids with borate. The advantages of CE for the determination of association constants are obvious: it only requires small amounts of sample at low solute concentration, because the procedure only requires migration time; the calculations are straightforward.

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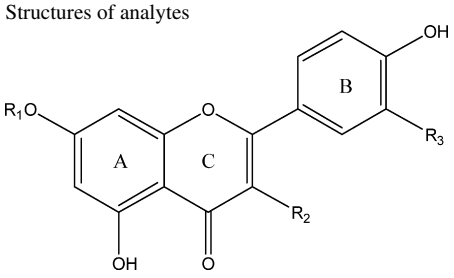
Table 1
Structures of analytes



Compound	Name	R_1	R_2	R_3
1	Luteolin-7-*O*-glucoside	Glc[a]	H	OH
2	Isorhamnetin	H	OH	OCH_3
3	Apigenin	H	H	H
4	Luteolin	H	H	OH
5	Quercetin	H	OH	OH

[a] Glc: glucose.

2. Experimental

2.1. Reagents and chemicals

The standards of apigenin, luteolin, quercetin were purchased from Sigma (USA), isorhamnetin was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China), and luteolin-7-*O*-glucoside was isolated from the plant in our laboratory (data not shown, identified by MS and NMR). Methanol and acetonitrile were HPLC grade (Merck, Germany). All other chemicals were of analytical grade supplied by reagents companies in China. *L. rotata* was gathered from Maqu County, Gansu Province, China.

2.2. Apparatus

CE experiments were performed using an Agilent CE system equipped with a diode-array detector (Agilent Technologies). The 68.5 cm (60 cm to the detector) length, 75 μm i.d. uncoated fused silica capillary was obtained from Ruifeng Inc. (Hebei, China). It was conditioned by flushing sequentially with methanol for 5 min, water for 10 min, 1 M NaOH for 10 min and water for 10 min. When not in use, the capillary was rinsed with water for 5 min and dried with an air flow for 10 min. Between analyses the capillary was flushed with the buffer for 2 min. The temperature of capillary was set at 20 °C. The system control and data acquisition were performed using the ChemStation software Rev. A. 09. 03.

2.3. Preparation of standard solutions

The standard solutions (5, 10, 25, 50 mg/l) of the five flavonoids were obtained by dilution with methanol of a stock solution (100 mg/l in methanol, containing some drops of DMSO to enhance dissolution).

2.4. Preparation of samples

Sample 1. *L. rotata* was pulverized and 25 ml of 70% (v/v) methanol aqueous solution was added to 2.00 g portion of the powder. The mixture was sonicated at room temperature for 30 min. After centrifugation, the supernatant was diluted to 50 ml with methanol, and then the solution was filtered through 0.45 μm filter before analysis.

Sample 2. Three milliliters of hydrochloric acid was added to a 21 ml portion of sample 1, the solution was heated in a boiling water bath for 1 h. The cooled mixture was diluted to 50 ml and filtered through 0.45 μm filter, and then analysed.

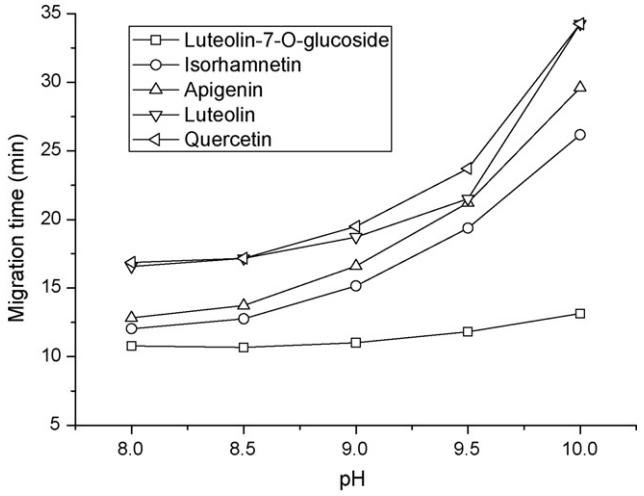


Fig. 1. Effect of pH on migration times with the buffer of 30 mM borate, at the voltage of 20 kV.

2.5. Preparation of buffer solutions

The pH of 0.5, 1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60 mM borate buffer solutions was adjusted to pH 9.0 with boric acid.

3. Results and discussion

3.1. Effect of pH on separation

The effect of pH was studied by varying it from 8.0 to 10.0 at fixed borate concentration (30 mM) at applied voltage of 20 kV. Fig. 1 shows the change of migration times of the flavonoids. It was found that the migration times were increasing with the increase of pH, so the apparent mobilities (μ_{app}) of flavonoids were decreasing which were calculated from the following equation:

$$\mu_{app} = \frac{LL_{tot}}{Vt} \quad (1)$$

where L_{tot} is the total length of capillary, L the length to detector, V the applied voltage and t is the migration time of the analyte.

As it is well known, the mobility of the electroosmotic flow (EOF) (μ_{eof}) is increasing with the increase of pH. In accordance with Eq. (2), negative effective mobility ($-\mu_{eff}$) was increasing with pH. It was mainly caused by the enhancement of the ionization of flavonoids with increasing pH. Finally we choose pH 9.0 as the optimum pH, because good resolution was thus obtained:

$$\mu_{eff} = \mu_{app} - \mu_{eof} \quad (2)$$

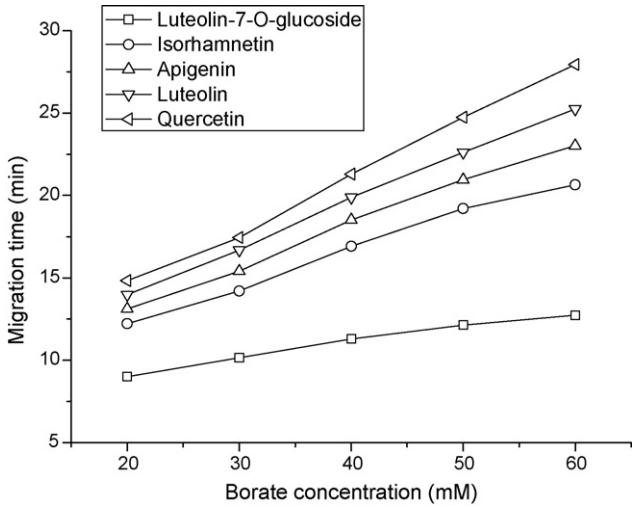


Fig. 2. Effect of borate concentration on migration times at pH 9.0 with voltage of 20 kV.

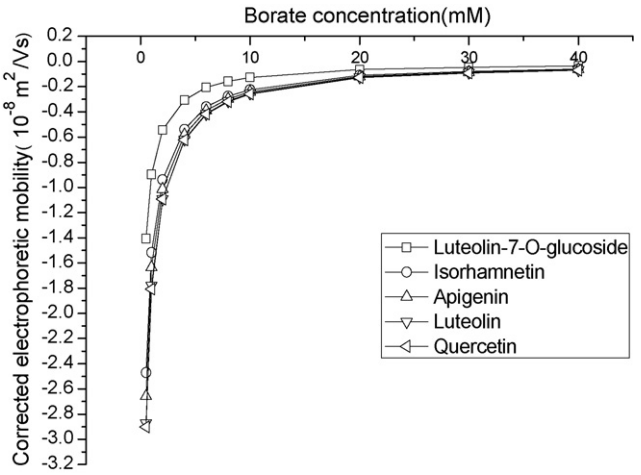


Fig. 3. The change of corrected electrophoretic mobility of five analytes with the borate concentration ranging from 0.5 to 40 mM at pH 9.0 with the voltage of 20 kV.

3.2. Effect of borate concentration on separation

3.2.1. The optimal borate concentration

Effect of borate concentration ranging from 20 to 60 mM (pH 9.0) on migration times was studied (Fig. 2). It was found that the migration times of the analytes increased with the increase of the borate concentration due to the strong interaction of borate and flavonoids at high borate concentration.

The peak area increased with the increase of the borate concentration since the interaction of borate ions and flavonoids significantly enhances UV absorbance of the analytes [20]. Taking into account the migration time and peak area, 30 mM borate was found as the optimum buffer solution.

3.2.2. The calculation of association constant (K)

In order to investigate the interaction of borate and flavonoids, the association constant K and the changes in Gibbs free energy (ΔG) were studied assuming the formation of 1:1 complexes between borate and flavonoid.

The association constant K can thus be defined as

$$K = \frac{[\text{FL–B}]}{[\text{FL}][\text{B}]} \tag{3}$$

where [FL] is the concentration of flavonoid, [B] the concentration of borate, and [FL–B] is the concentration of the 1:1 complex.

Besides, electrophoretic mobility of the flavonoid is the weighted sum of the mobility of the free flavonoid and the formed complex as shown in Eq. (4):

$$\mu_\text{i} = \frac{[\text{FL}]}{[\text{FL}] + [\text{FL–B}]}\mu_\text{f} + \frac{[\text{FL–B}]}{[\text{FL}] + [\text{FL–B}]}\mu_\text{c} \tag{4}$$

where μ_i is the corrected electrophoretic mobility, μ_f the electrophoretic mobility of the free flavonoids for [B] = 0, μ_c the electrophoretic mobility of the formed complex. With regard to the corrected electrophoretic mobility μ_i, it is corrected according to Eq. (5) [21] because not only the concentration of borate but also viscosity of buffer solution can affect the electrophoretic mobility of analytes:

$$\mu_\text{i} = \frac{\eta_i}{\eta_0}\mu_\text{eff} = \frac{I_0}{I_i}\mu_\text{eff} \tag{5}$$

where η_0, I_0 are respectively the viscosity of the buffer solution and the produced current when borate concentration is zero, η_i, I_i are respectively the viscosity of the buffer solution and the produced current, μ_eff the effective electrophoretic mobility. From the borate concentration values extrapolated to zero versus I_i, rough value of produced current I_0 was obtained.

The combination of Eq. (3) with Eq. (4) gives Eq. (6):

$$\frac{1}{\mu_\text{f} - \mu_\text{i}} = \frac{1}{\mu_\text{f} - \mu_\text{c}}\frac{1}{K}\frac{1}{[\text{B}]} + \frac{1}{\mu_\text{f} - \mu_\text{c}} \tag{6}$$

In this work, μ_i was investigated with the borate concentration ranging from 0.5 to 40 mM at pH 9.0 with the voltage of 20 kV (Fig. 3). From the borate concentration values extrapolated to zero versus μ_i, rough values of absolute mobility μ_f were obtained for the analytes. By plotting $(\mu_\text{f} - \mu_\text{i})^{-1}$ versus $[\text{B}]^{-1}$ the association constant K (intercept/slope) was estimated (Table 2). The apparent Gibbs free energy ΔG was calculated from Eq. (7):

$$\Delta G = -RT \ln K \tag{7}$$

where R is molar gas constant which is equal to 8.314 J/kmol and T is the absolute temperature which is equal to 293.15 K.

Table 2 shows that the values of association constant K of luteolin-7-*O*-glucoside, isorhamnetin and apigenin increase which indicates the strong interaction of borate and flavonoids leading to the increase of μ_eff and migration time. But the values of K of luteolin and quercetin were decreasing presumably because of the formation of 2:1 complex owing to the simultaneous presence of 5,7-diols and 3′4′-diols on A- and B-ring of flavonoid so that Eq. (3) did not fit for calculating the values of K of luteolin and quercetin. From Table 2, the association constants of borate with flavonoids is same as that of borate

Table 2
Results of μ_f, K, ΔG of five flavonoids

Analyte	Regression equation[a]	R^2	μ_f ($\times 10^{-8}$ m^2/V s)	K (l/mol)	$-\Delta G$ (kJ/mol)
Luteolin-7-*O*-glucoside	$Y = -3.59 \times 10^7 - 1.84 \times 10^4 X$	0.9989	−2.77	1952.20	18.47
Isorhamnetin	$Y = -1.78 \times 10^7 - 6.88 \times 10^3 X$	0.9990	−5.62	2588.60	19.15
Apigenin	$Y = -1.64 \times 10^7 - 6.27 \times 10^3 X$	0.9991	−6.10	2624.34	19.19
Luteolin	$Y = -1.58 \times 10^7 - 6.46 \times 10^3 X$	0.9992	−6.34	2443.38	19.01
Quercetin	$Y = -1.64 \times 10^7 - 7.48 \times 10^3 X$	0.9989	−6.07	2198.86	18.76

[a] $Y = 1/(\mu_f - \mu_i)$ and $X = 1/[B]$.

Table 3
Intra- and inter-day R.S.D. of migration times and peak areas and the limits of analytes

Analytes	Intra-day R.S.D.% ($n = 5$)		Inter-day R.S.D.% ($n = 5$)		LOD (mg/l)	Recovery (%) ($n = 3$)
	Migration time	Peak area	Migration time	Peak area		
Luteolin-7-*O*-glucoside	0.51	3.25	1.44	2.86	3.75	105.1
Isorhamnetin	2.76	2.69	3.46	4.95	2.81	100.7
Apigenin	1.89	2.70	3.89	3.14	3.75	98.5
Luteolin	1.43	3.69	3.50	3.37	1.71	94.2
Quercetin	2.12	3.19	3.99	1.67	1.88	96.8

with humic acids [19], and the changes in the apparent values of Gibbs free energy are approximately 19 kJ/mol, which seem reasonable for typical ion-dipole or ion-induced-dipole interactions.

3.3. Effect of voltage on separation

The effect of voltage ranging from 10 to 30 kV on migration times was investigated (Fig. 4). The results showed that the voltage had a negative correlation with migration times of the analytes. Increasing high voltage enhanced the EOF and electrophoretic velocity resulting in short analysis time. But high voltage induced high Joule heating and peak broadening and harms the capillary. Accordingly, we chose 20 kV as the voltage in this analysis.

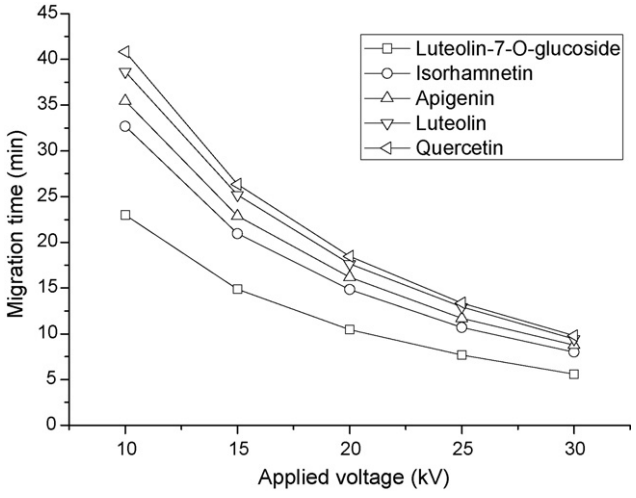


Fig. 4. Effect of voltage on migration times with the buffer of 30 mM borate at pH 9.0.

3.4. Effect of organic modifier on separation

The purpose of using organic solvent in CE was to enhance the solubility of analytes in the aqueous phase and to slow down the EOF by increasing the viscosity of the buffer system to improve resolution. The effect of 5% (v/v) methanol, ethanol, 1-propanol, 1-butanol and acetonitrile in buffer with 30 mM borate at pH 9.0 was studied. Resolution was enhanced as the chain length of alcohol increased. The best peak shapes were obtained by using acetonitrile as organic modifier. Investigating acetonitrile concentrations ranging from 2% to 10% optimum separation efficiency was obtained at 8% (Fig. 5).

3.5. Optimum separation conditions

The optimum conditions for the CE method are as follows: capillary size, 68.5 cm × 75 μm i.d.; running buffer, 30 mM borate, (containing 8% acetonitrile (v/v)), pH 9.0; hydrodynamic injection, 50 mbar for 5 s; running voltage, 20 kV from positive to negative; UV detector, 210 nm, 20 °C.

3.6. Intra-day and inter-day precision and limits of detection

The repeatability of the developed CE method was expressed as relative standard deviation (R.S.D.) of migration time and peak areas, respectively. The intra-day precision was determined by analyzing the same standard solution in five replicates, and the inter-day precision was examined over 5 days by the analysis of the same standard solution.

The determination of the limits of detection (LOD) was based on the signal-to-noise ratios of 3.

Standard solutions of concentration of 5, 25, 50 mg/l were added to the powder of *L. rotata* followed by handling them according to the procedure of preparation of sample 1 in Section

Table 4
Regression equations of five flavonoids, and their content in sample 1 and sample 2

Flavonoid	Linear range (mg/l)	Regression equation	*R*	Sample 1 without hydrolysis (mg/g)	Sample 2 after hydrolysis (mg/g)
Luteolin-7-*O*-glucoside	5–100	$Y = 5.67 + 3.06X$	0.9984	3.26	n.d.[a]
Isorhamnetin	5–100	$Y = 30.82 + 5.04X$	0.9974	n.d.	n.d.
Apigenin	5–100	$Y = -10.95 + 10.08X$	0.9990	n.d.	0.074
Luteolin	5–100	$Y = 15.12 + 8.72X$	0.9996	0.041	0.91
Quercetin	5–100	$Y = 24.54 + 9.22X$	0.9982	n.d.	n.d.

[a] Not detectable.

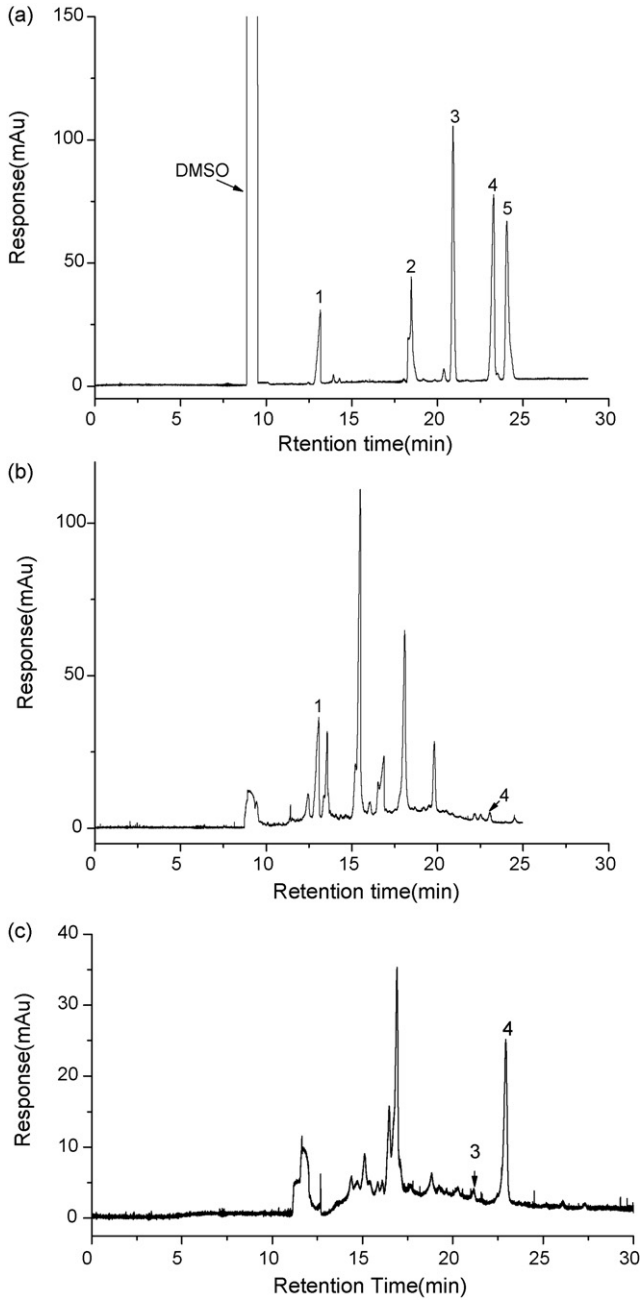


Fig. 5. Electropherograms of standards (a), sample 1 (b) and sample 2 (c). (peaks: 1, luteolin-7-*O*-glucoside; 2, isorhamnetin; 3, apigenin; 4, luteolin; 5, quercetin). For experimental conditions see Section 3.5.

2.4. Through CE analysis, the recovery rates were calculated ($n = 3$).

The R.S.D., LOD and recovery data of the method in Table 3 show that the method is suitable for the determination of the five flavonoids in *L. rotata*.

3.7. Linearity and quantitative result

The linear regression equations (peak area versus concentration), correlation coefficients and the quantitative results are shown in Table 4. The quantitative results show that before hydrolysis glucosylated flavonoids are the main components and the content of flavonoid aglycones was not detectable or is very low.

4. Conclusions

The contents of luteolin-7-*O*-glucoside, isorhamnetin, apigenin, luteolin and quercetin in *L. rotata* (Benth.) Kudo and its hydrolysates were determined by the proposed CE method with satisfactory repeatability and good accuracy. This method could be useful in the quality control of *L. rotata*.

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