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Analysis of Bioactive Flavonoid-O-glycosides in *Saussurea mongolica* Franch by Capillary Zone Electrophoresis Using Ionic Liquids as the Additive

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ABSTRACT

In this study, a capillary zone electrophoretic method, in which 1-alkyl-3-methylimidazolium-based ionic liquids (IL) was used as the additive, was established to separate and identify six bioactive flavonoid-O-glycosides in *Saussurea mongolica* Franch. In order to investigate the traits of IL in capillary electrophoresis (CE), the influences of the alkyl group, the anionic part and the concentration of IL were investigated and discussed. Optimum separation was achieved with 20 mM borate buffer at pH 9.00 containing 5 mg/mL 1-ethal-3-methylimidazolium tetrafluoroborate. The applied voltage was 15 kV and the capillary temperature was kept constant at 25°C. The relative standard deviations (RSD) of the migration time and the peak area of each peak for six replicate injections were 0.75% - 1.45% and 3.18% - 3.91%, respectively. Moreover, it was worth noticing that the mechanism of interaction between IL and analytes was investigated preliminarily.

Key words: ionic liquid, capillary electrophoresis, flavonoid-O-glycosides, *Saussurea mongolica*

INTRODUCTION

Flavonoid is one of the largest groups of naturally occurring phenols and are widespread components in all parts of plants[1]. During the past years, much attention has been devoted to the analysis of flavonoids-containing plants for identification and quantification purposes. The analysis has been performed by different techniques, including thin-layer chromatography (TLC)[2], pulse polarography[3] and liquid chromatography (LC)[4]. However, due to the inherent structural similarity of most flavonoids and the complex characteristics of the sample matrices, these methods suffer from materials and time consuming because of large amounts of organic reagent and many operation steps being often required. Recently, owing to its high resolving power, low solvent consumption and simple pretreatment, high-performance capillary electrophoresis (HPCE) has proved to be a valuable method for the separation and detection of flavonoids occurring in complicated plant extracts of traditional Chinese herbal medicine[5-9].

Room temperature ionic liquids (IL), recently recognized as possible environmentally benign solvents, are those compounds composed of organic cations and inorganic anions which are liquids at room temperature or have melting points slightly higher than ambient temperature[10]. Most of IL had good solubility in water and were stable in air[11]. The IL could be designed with various cations and anions, and more attractively, the alkyl groups of cations could be varied and thus the properties of the compound could be finely tuned for specific purposes[12]. Therefore, IL's have been used in different chemical processes such as synthesis[13], catalysis[14] and electrochemistry[15] *etc*. Recently, IL's have raised enormous interests in separation science. They were employed as stationary phase in gas chromatography (GC)[16,17] and as eluent or additive in high performance liquid chromatography[18,19]. The applications of IL's in capillary electrophoresis (CE) were focused in their roles as additive, background electrolyte or coating material in aqueous/nonaqueous CE[20-23]. Our research team has reported the separation of bioactive flavonoid-O-glycosides for *Saussurea mongolica* Franch by CE on a simple and low cost buffer system[24]. In order to explore the mechanism of interaction

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between IL and analytes, in this paper, IL's were used as additives in CE to separate the six bioactive flavonoid-O-glycosides in *S. mongolica* Franch. Our previous work[25] indicated that the hydrogen bonding interaction between the hydrogen on the C-2 carbon of the imidazolium cation and oxygen of hydroxy in flavonoids were important. However, the result in this work showed that the resolution of flavonoid-O-glycosides did not change when the hydrogen on the C-2 carbon of the imidazolium cation was substituted with methyl, This suggested that the separation mechanism was not the hydrogen bonding between IL and analytes while the electrostatic, hydrophobic, or ion-dipole/ion-induced-dipole interaction between the IL and flavonoid-O-glycosides played an important role in the separation. Moreover, the method described here is relatively rapid with the retention time of the method is about 28 min with the use of methanol.

MATERIALS AND METHODS

I. *Apparatus and Conditions*

All the experiments were carried out on an Agilent HP^{3D} capillary electrophoresis system (Agilent, USA). The applied voltage was held constant at 15 kV. Separation capillary was an untreated fused silica capillary with total length of 38.5 cm and an effective length of 30 cm (50 μm i.d., 365 μm O.D.) (Yongnian, Hebei province, China). The UV detector was operated at 280 nm. The temperature of the capillary cartridge was maintained at 25°C. Before used, the capillary was rinsed with 0.5 M NaOH for 10 min, and then with deionized water for 4 min; it was then conditioned with running electrolyte for 4 min. Between running, the capillary was rinsed with electrolyte only for 2 min. Samples were introduced under pressurized injection at 50 mbar for 5 s.

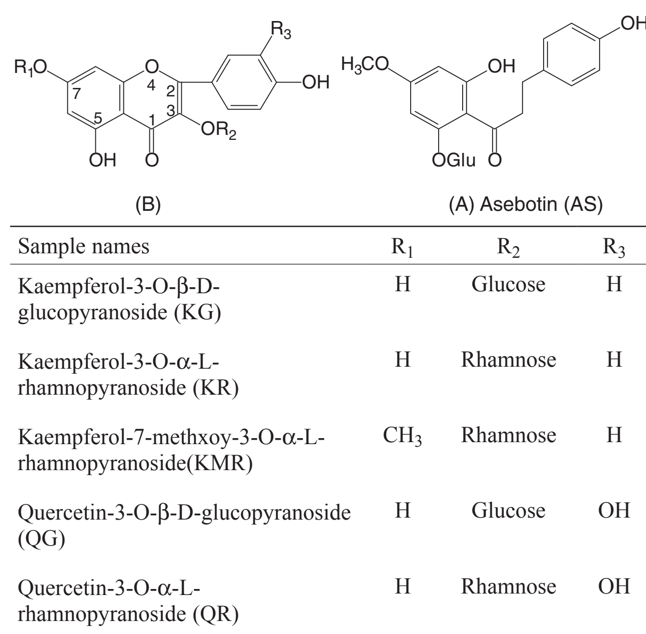
II. *Materials and Reagents*

The *S. mongolica* were collected from Zhang County of Gansu Province, China. Asebotin (AS), kaempferol-3-O-β-D-glucopyranoside (KG), kaempferol-3-O-α-L-rhamnopyranoside (KR), kaempferol-7-methxoy-3-O-α-L-rhamnopyranoside(KMR), quercetin-3-O-β-D-glucopyranoside (QG) and quercetin-3-O-α-L-rhamnopyranoside (QR) were isolated and elucidated from the extract of the whole plant of *S. mongolica* in our laboratory (molecular structures were shown in Figure 1). 1-ethyl-3-methyl-imidazolium tetrafluoroborate (1E-3MI-TFB), 1-propyl-3-methyl-imidazolium tetrafluoroborate (1P-3MI-TFB), 1-butyl-3-methyl-imidazolium tetrafluoroborate (1B-3MI-TFB), 1-amyl-3-methyl-imidazolium tetrafluoroborate (1A-3MI-TFB), *etc* were the kind gifts from Yan-Long Gu of Chinese Academy of Sciences, Lanzhou, China (molecula structures were shown in Figure 2). All chemicals were of analytical grade and purchased from Beijing Chemical Reagents Plant (Beijing, China). Deionized water was used throughout the experiment. All solutions and samples were filtered through a 0.45 μm syringe filter.

Standard stock solutions of six flavonoid-O-glycosides at concentration of 4000 μg/mL were prepared in methanol, and the sample solutions of various concentrations were prepare by appropriate dilution of the stock solution when needed. The pH values of borate buffer solutions were adjusted by mixing 0.1 M HCl or 0.1 M NaOH solution with sodium tetraborate solution. Methanol was used as the electroosmotic flow (EOF) mark.

III. *Sample Preparation*

The air-dried *S. mongolica* F. (7.2 g) was powdered and extracted with 10 mL methanol in ultrasonic bath for 2 h. After filtered through a filter paper and a 0.45 μm membrane filter, the extract was injected directly.



Sample names	R_1	R_2	R_3
Kaempferol-3-O-β-D-glucopyranoside (KG)	H	Glucose	H
Kaempferol-3-O-α-L-rhamnopyranoside (KR)	H	Rhamnose	H
Kaempferol-7-methxoy-3-O-α-L-rhamnopyranoside(KMR)	CH_3	Rhamnose	H
Quercetin-3-O-β-D-glucopyranoside (QG)	H	Glucose	OH
Quercetin-3-O-α-L-rhamnopyranoside (QR)	H	Rhamnose	OH

Figure 1. Molecular structures of (A) AS, (B) KG, KR, KMR, QG and QR.

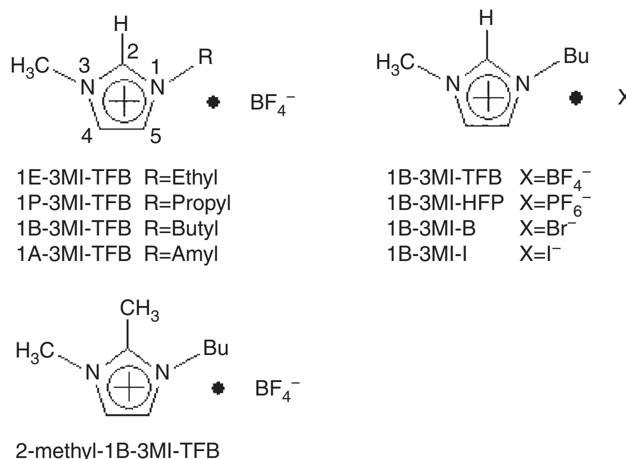


Figure 2. Structures of ionic liquid (IL).

RESULTS AND DISCUSSION

I. *Effect of Alkyl Group on the Imidazolium Cations and the Mechanism Research*

In order to study the effect of the alkyl group on the separation for six bioactive flavonoid-O-glycosides, 1E-3MI-TFB, 1P-3MI-TFB, 1B-3MI-TFB and 1A-3MI-TFB were used as additives. All separations were operated at 20 mM borate buffer with 5 mg/mL ionic liquids at pH 9.00. Figure 3 shows the representative electropherograms for the separation of flavonoid-O-glycosides with and without 1-alkyl-3-methylimidazolium-based IL's. It should be noted that the resolutions of flavonoid-O-glycosides improved with the addition of each ionic liquids was added. In order to investigate the mechanism of interaction of IL and analytes, the results were carefully examined. Our study[25] indicated that the hydrogen bonding interaction between the hydrogen on the C-2 carbon of the imidazolium cation and oxygen of hydroxy in flavonoids were important. So, instead of the above IL's, 2-methyl-1B-3MI-TFB was added as additive. As shown in Figure 3, the resolutions of flavonoid-O-glycosides did not change. Moreover, the resolutions of glycosides were destroyed completely when the $NaBF_4$ was added as additive. All of the results indicated that the separation mechanism be the interaction of electrostatic, hydrophobic, or ion-dipole/ion-induced-dipole between the IL and flavonoid-O-glycosides. As to the resolution, it was apparent that all the alkyl groups provided better resolution. However, taking account of the analytical time and baseline 1B-3MI-TFB we used for further experiments.

II. *Effect of Inorganic Counterions*

To investigate the effect of the counterion, IL's composed of the organic cation 1B-3MI with four different counterions (BF_4^-, PF_6^-, Br^- and I^-) were studied (the typical electropherograms were shown in Figure 4). The resolution improved with all the IL as addtive, but PF_6^- lengthened the analytical time. Taking account of the analytical time, baseline and peak shape, 1B-3MI with BF_4^- was used for further experiments. However, when the $NaBF_4$ was added as additive, the migration time increased and KMR and QG, did not separate, indicating that IL has played an important role in the separation.

III. *Effect of 1B-3MI-TFB Concentrations*

The effect of the concentration of the 1B-3MI-TFB in range of 2 to 10 mg/mL on the separation was examined. The result was displayed in Figure 5. The results indicated that the resolution of the analyte improved with the increase of 1B-3MI-TFB

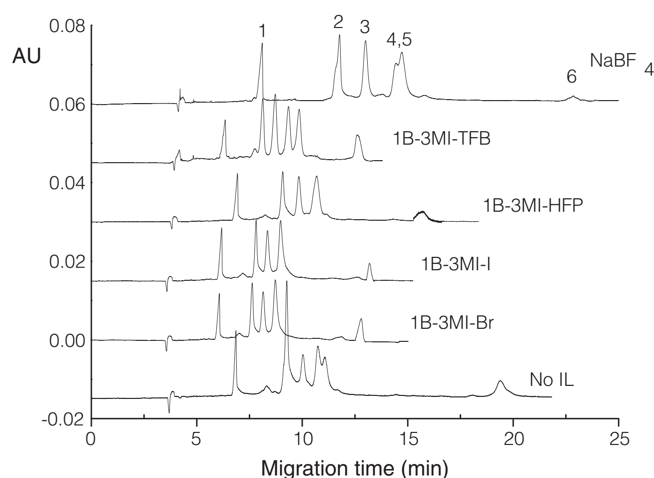


Figure 4. Effect of inorganic counterions. Analytical conditions: 20 mM borate buffer with 5 mg/mL IL at pH 9.00. Voltage, 15 kV; temperature, 25°C; UV detection at 280 nm. Other conditions were similar to Figure 3. 1: AS; 2: KG; 3: KR; 4: KMR; 5: QG; 6: QR.

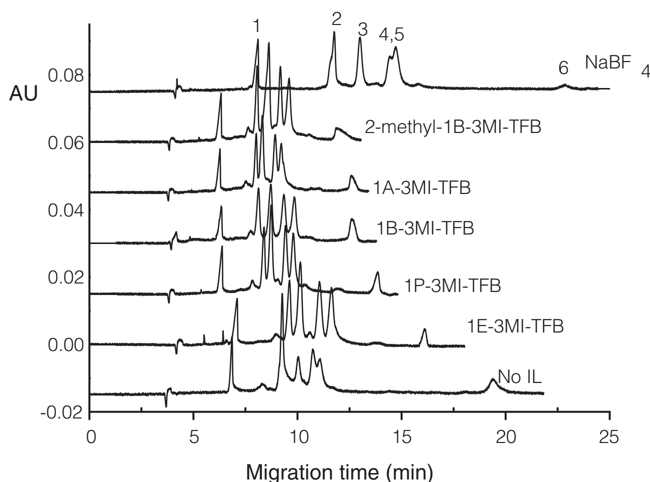


Figure 3. Effect of alkyl group on the imidazolium cations. Analytical conditions: 20 mM borate buffer with 5 mg/mL IL at pH 9.00. Voltage, 15 kV; temperature, 25°C; UV detection at 280 nm. 1: AS; 2: KG; 3: KR; 4: KMR; 5: QG; 6: QR.

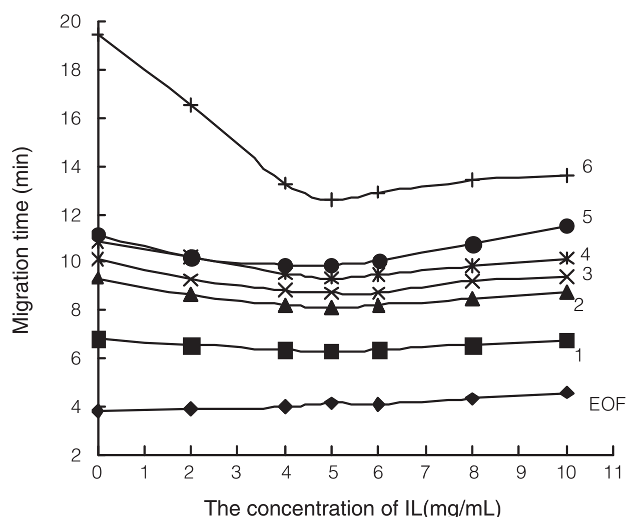


Figure 5 Effect of 1B-3MI-TFB concentrations. Analytical conditions: 20 mM borate buffer at pH 9.00. Voltage, 15 kV; temperature, 25°C; UV detection at 280 nm. Other conditions were similar to figure 3. 1: AS; 2: KG; 3: KR; 4: KMR; 5: QG; 6: QR.

concentration. When the concentration of the 1B-3MI-TFB was lower than the 5 mg/mL, KMR and QG overlapped. When the concentration of the 1B-3MI-TFB was higher than 5 mg/mL, the KMR and QG separated well. However, with the increase of the 1B-3MI-TFB concentration, the migration time of the analyte increased too. So, taking account of the resolution and analytical time, 5 mg/mL 1B-3MI-TFB was the ideal selection. In summary, the best condition was obtained, an electrolyte containing 20 mM borate with 5 mg/mL 1B-3MI-TFB at pH 9.0. Figure 6 shows the CE chromatogram of a mixture of containing six flavonoid-O-glycosides.

IV. *Linearity, Reproducibility, Detection Limit and Recovery*

Calibration curves [peak area (*y*) *vs.* concentration (*x*) (μg/mL)] were constructed over the concentration ranges: 10 - 350 μg/mL for AS, and KG, 2 - 200 μg/mL for KR, KMR and QG, 5 - 400 μg/mL for QR. The regression equation of these curves and their correlation coefficients (r) were calculated as following: AS: $y = 27.52x - 45.07$ ($r = 0.9990$); KG: $y = 20.25x + 9.86$ ($r = 0.9985$); KR: $y = 80.68x + 443.34$ ($r = 0.9995$); KMR: $y = 74.10x + 223.5$ ($r = 0.9997$); QG: $y = 21.65x - 199.20$ ($r = 0.9990$); QR: $y = 100.46x + 19.54$ ($r = 0.9987$).

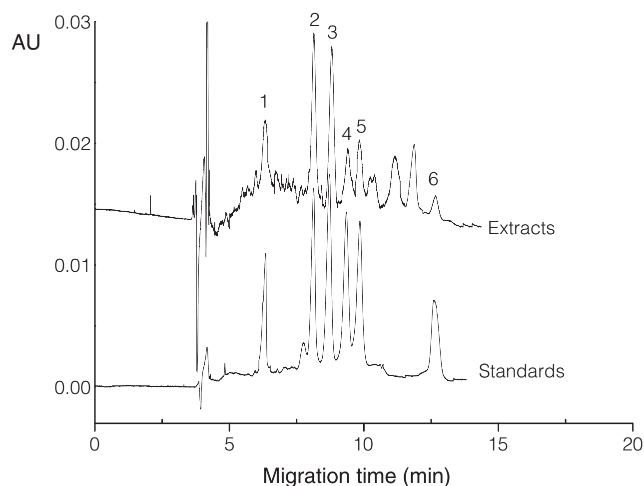


Figure 6. Electropherograms of the extract of *S. mongolica* Franch and the mixture of flavonoids glycosides. Analytical conditions: 20 mM borate buffer with 5 mg/mL 1B-3MI-TFB at pH 9.00. Voltage, 15 kV; temperature, 25°C; UV detected at 280 nm. 1: AS; 2: KG; 3: KR; 4: KMR; 5: QG; 6: QR.

Table 1. The Reproducibility and Recovery of the analytes

Analytes	Reproducibility (n = 6)		Recovery (%)
	Migration time (RSD%)	Peak area (RSD%)	
AS	0.75	3.28	90.4
KG	1.24	3.18	93.5
KR	1.08	3.46	98.6
KMR	1.45	3.87	99.7
QG	1.36	3.91	101.5
QR	1.42	3.69	100.8

The method was validated for the reproducibility of the migration time and the peak area of the analytes. The relative standard deviations (RSD) of the migration time and the peak area of each peak for six replicate injections were 0.75% - 1.45% and 3.18% - 3.91%, respectively. The results were summarized in Table 1. The detection limit (S/N = 3) of AS, KG, KR, KMR, QG and QR was 2.5, 5, 0.5, 1, 1 and 2 μg/mL, respectively.

Accuracy of the method was determined by spiking suitable amounts of the analytes of known concentration into plant extracts. The recoveries were between 90.4 - 101.5% for the six flavonoid-O-glycosides. The results of the recovery were summarized in Table 1.

V. *Separation and Determination of the Flavonoid-O-glycosides in Herb*

Methanol solution of extract was injected directly and separated under the optimum condition mentioned above. Figure 6 shows the electropherogram of extract of *S. mongolica* and peaks were identified by addition of standards. The calculated contents of the six flavonoid-O-glycosides were as following: AS: 0.059 mg/g, KG: 0.48 mg/g, KR: 0.085 mg/g, KMR: 0.062 mg/g, QG: 0.64 mg/g and QR: 0.0393 mg/g.

CONCLUSIONS

The results demonstrate that the determination of flavonoid-O-glycosides in the extract of the whole plant of *S. mongolica* has been achieved using 1B-3MI-TFB as additive. This work also shows that HPCE is a powerful technique to study flavonoid compounds in the complex extract of the plants.

The properties of IL's in the borate buffer were obviously complex and actually only qualitative interpretation of these propertied could be made. The interaction of electrostatic, hydrophobic, or ion-dipole/ion-Induced-dipole between the IL and flavonoid-O-glycosides was believed to be the main separation mechanism.

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