Asian Journal of Chemistry; Vol. 24, No. 7 (2012), 2965-2968

ASIAN JOURNAL OF CHEMISTRY

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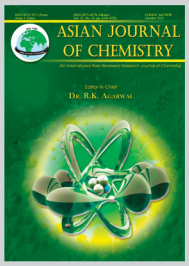

Analysis of Flavonoids by β-Cyclodextrin Modified Capillary Zone Electrophoresis in Traiditional Chinese Medicine

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(*Received*: 27 June 2011; *Accepted*: 6 February 2012) AJC-11043

A β-cyclodextrin modified capillary zone electrophoresis method was developed to determine flavonoids in traiditional Chinese medicines. After optimization, the optimum separation conditions were selected as: a background electrolyte of 20 mmol L^{-1} Borax (pH 7.5) containing 6 mmol L^{-1} β-cyclodextrin and 20 % (v/v) acetonitrile and injection time of 65 s. Under these conditions the sensitivity was enhanced 15 to 17 times when compared to a normal hydrodynamic injection. Detection limits for the five flavonoids acids were in the range of 15-30 ng mL^{-1}. The developed method was further verified by application to traditional Chinese medicine real sample analysis with good results.

Key Words: Field amplified sample stacking, β-Cyclodextrin, Flavonoids, Traiditional Chinese medicine.

INTRODUCTION

Flavonoids occur as a group of active constituents in many traiditional Chinese medicines[1], so to analyze them accurately is of much importance. The traiditional method for flavonoids analysis is high performance liquid chromatography(HPLC)[2-4]. Althout HPLC is very accurate, it is often inadequate in terms of speed, running cost and resolving power. Capillary electrophoresis has the advantages of low consumption of reagents and samples, rapidity and high resolution and has been widely appllied in flavonoids analysis[5-13].

However, the short optical path length associated with on-column UV detection imposes an inherent detection problem for capillary electrophoresis. To address this problem, on-line extaction[13], coupling of capillary isotachophoresis with capillary zone electrophoresis[8-10] and to extend the optical length by processing the capillary[14] were developed. But all these methods either involves complex operation or their sensitivity enhancements effects are often limited, which hinders their application. The on-line concentration methods are based on changes in analyte migration due to differences between the sample and electrolyte conductivity or pH or through association with moving additives such as surfactants and various combination of these effects. These methods enjoyed the advantages of simplicity and easiness for automation and have been developed to enhance the sensitivity for capillary electrophoresis[11-11,14-20].

When contents of flavonoids are low, some preconcentration methods have to be introduced in capillary electrophoresis. Pospíšilová *et al.*[8] have determined flavonoids and other antioxidants in Hypericum perforatum leaves and flowers, red wine[9] and Melissae herba[10] by isotachophoresis and capillary zone electrophoresis in the column-coupling configuration. Wang *et al.*[11] have applied field-enhanced sample injection with reverse migrating micelles (FESI-RMM)to determine six flavonoids and 40-360-fold improvement in detection sensitivity have been achieved. Zhu *et al.*[12] have compared two on-line preconcentration techniques named stacking with reverse migrating micelles (SRMM) and anion selective electrokinetic injection and a water plug-sweeping with reverse migrating micelles (ASIW-sweep-RMM) for concentration of flavonoids and detection sensitivity were enhanced 27-37-fold and 45-194-fold, respectively. Later, the same authors[13] used stacking with anion selective electrokinetic injection and a water plug in a reverse-migrating microemulsion (SASIW-RMME) and sweeping with a reverse migration microemulsion (sweeping-RMME) to focus flavonoids and detection sensitivity enhancements of 20-76-fold and 35-79-fold have been aquired, respectively.

In this work, field amplified sample stacking (FASS), the simplest and most straightforward concentration scheme was used in β-cyclodextrin modified capillary zone electrophoresis to analyze five kinds of flavonoids with sensitivity enhancements of 15-17-fold were achieved. The developed method

was applied to flavonoids analysis in three kinds of traditional Chinese medicines with good results.

EXPERIMENTAL

Capillary electrophoresis analysis were carried out in a P/ACE MDQ capillary electrophoresis system with a photodiode array detector for absorbance measurements at 199 nm (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capillaries purchased from Yongnian Optical Fiber Factory (Hebei, China) were used. The dimensions of the capillary were 60.2 cm × 50 μm i.d. The effective length of the capillary was 50 cm. The temperature of the capillary was kept at 25 ºC. The applied voltage was 25 kV. Samples were introduced under pressure (65 s, 0.5 psi). The CE system was interfaced with a computer. 32 karat software (version 7.0) of Beckman was used for data acquisition.

The back ground electrolyte (BGE) solution had a concentration of 20 mmol L^{-1} of sodium tetraborate (pH 7.5) containing 6 mmol L^{-1} β-cyclodextrin and 20 % acetonitrile (v/v). The buffer solutions were prepared freshly each day, sonicated for 5 min and filtered through a 0.45 μm membrane filter before use. The buffer solutions were prepared freshly each day, sonicated for 5 min and filtered through a 0.45 μm membrane filter before use.

4-Methylumbelliferone, kaempferol, apigenin, quercetin, rutin and β-cyclodextrin were from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC-Grade) was from Tianjin Yongda Chemical Reagent Development Centre (Tianjin China). Acetonitrile was of analytical reagent grade and was from Tianjin Huayue Chemical Reagent Co. (Tianjin, China). Water of 18.2 mΩ was treated with a CascadaTM lab water system (Pall Life Science, China).

A stock standard solution of 0.5 mg $mL^{-1}$of each analyte and internal standard were prepared in methanol. A mixed standard solution of the five analytes was prepared at a concentration of 0.1 mg mL^{-1} in methanol and was used as stock standard solution. The working standard solutions were prepared daily by diluting the stock standard solution with methanol and the concentration of internal standard was kept at 1 μg mL^{-1}. All solutions were stored in dark containers at 4 ºC.

Procedure: Before use each day, the capillary was rinsed with 1 mol L^{-1} sodium hydroxide, water and separation medium for 10 min sequentially. Between analysis, the capillary was washed with water for 3 min, with 0.1 mol L^{-1} sodium hydroxide for 3 min, with water for 4 min and with the back ground electrolyte for 4 min to maintain proper reproducibility of run-to-run injections.Duplicate injection of the solutions were performed and average peak heights were used for quantification.

Preparation of samples: *Lysimachia christinae hance*, *Tribute chrysanthemum* and *Lonicera japonica* Thunb samples were purchased from local drug store in Dezhou. Afterwards, they were washed with ultrapure water and then air dried and finally pulverized and homogenized. Thereafter, 0.5 g of the powder was extracted with 100 mL of methanol in a soxhlet extractor for 3 h, then centrifuged at 3000 rpm for 10 min. The extracts were diluted to 100 mL. Then, 1 mL of the above sample extracts was transferred to a 10 mL volumetric flask and diluted to mark after 20 μL internal standard solution was added.

RESULTS AND DISCUSSION

Effects of buffer pH: Buffer pH can affect electroosmotic flow (EOF) and the ionization of the analytes, which in the long run will influence the analytes migration and separation. The effects of buffer were investigated in the pH 6.5-8.5 range with a 20 mmol L^{-1} borax buffer. Results showed that with the increase of buffer pH, the six compounds all migrate faster, which is due to the increased ionization. At the same time, the separation of the compounds first improved with the pH increase, then the separation decreased when the buffer was higher than pH 7.5. So pH 7.5 was chosen in the further experiments.

Effects of β-cyclodextrin: β-Cyclodextrin has a hydrophobic cavity which can host some hydrophobic compounds to form inclusion-complexes. The stability of different inclusion-complexes is different, which can help the separation in capillary electrophoresis. The effects of β-cyclodextrin concentration were studied in the 0-8 mmol L^{-1} range. Results showed that the migration time of the six compounds decreased reapidly with the increase of β-cyclodextrin concentration in the 0-2 mmol L^{-1}. When β-cyclodextrin concentration is higher than 2 mmol L^{-1}, the changes in migration time of the compounds become not obvious. At the same time, when β-cyclodextrin concentration is lower than 6 mmol L^{-1}, the peak shape of the six compounds (especially kaempferol and apigenin) are very poor. In the whole β-cyclodextrin concentration range studied, there is no apparent variations in separation for the six compounds. Taking peak shape, analysis time and separation into consideration, 6 mmol L^{-1} β-cyclodextrin was adopted in the further work.

Effects of organic modifier: Adding organic solvent into the buffer solution can lower the zeta potential of the capillary inner surface and reduce the electroosmosis flow to improve the separation. The effects of methanol, ethanol and acetonitrile were investigated. Preliminary results showed that methanol and ethanol can decrease the separation, so their effects were not further studied. The effects of acetonitrile were in the 0-20 % (v/v) range. The results showed that the migration time and the separation of the six compounds increased with the increase of acetonitrile concentration. When the acetonitrile concentration was 20 % (v/v), the six compounds can be well separated. On further increase the acetonitrile concentration will make the analysis time even longer, 20 % (v/v) acetonitrile was chosen as a compromise.

Effects of injection time: The effects of injection time was explored in the 25-75 s range. Results showed that with the increase of injection time, the peak height increased in the 25-65 s range. When the injection time is more than 65 s, the increase of peak height is not apparent and the peak broadening is significant, which will effect the separation efficiency. To maintain the highest sensitivity and a good separation efficiency, 65 s was adopted as the optimum injection time.

Analytical characterization: Under the optimum conditions, the normal CZE and FASS-CZE separation of the six

compounds were shown in Fig. 1a-b. The sample-to-sample time was less than 10 min. As shown in Table-1, sensitivity enhancement were from 15-17. The relative standard deviation was achieved by five consective injection of a standard mixture (Table-1), were in the range of 0.7-1.8 and 3.9-5.9 % for migration time and peak height, respectively. The detection limits and calibration were given in Table-2. The detection limits of the five analytes were in the 15-30 ng mL^{-1} range, based on three times noise. The calibration graphs were plotted by peak height ratio (y, analyte/internal standard) against concentration (x, μg mL^{-1}) and were linear over the range of 0.05-2, 0.05-2, 0.1-4, 0.1-4 and 0.05-2 μg mL^{-1} for kaemperol, apigenin, rutin, quercetin and luteolin, respectively.

TABLE-1
ENHANCEMENT FACTORS AND
REPEATABILITY OF FASS-CZE

Compounds	Normal	FASS			
	Height	Height	E.F.	RSD (n = 5 %)	
				Time	Height
4-Methy-lumbelliferone	66	1047	16	0.7	4.7
Kaemperol	201	3306	16	0.8	3.9
Apigenin	198	3060	15	1.3	5.2
Rutin	104	1751	17	1.8	5.9
Quercetin	122	1997	16	1.0	4.5
Luteolin	184	2883	15	1.6	4.2

Note: In both normal and FASS-CE, a standard mixture containing 2 μg mL^{-1} analytes and 1 μg mL^{-1} internal standard was introduced.

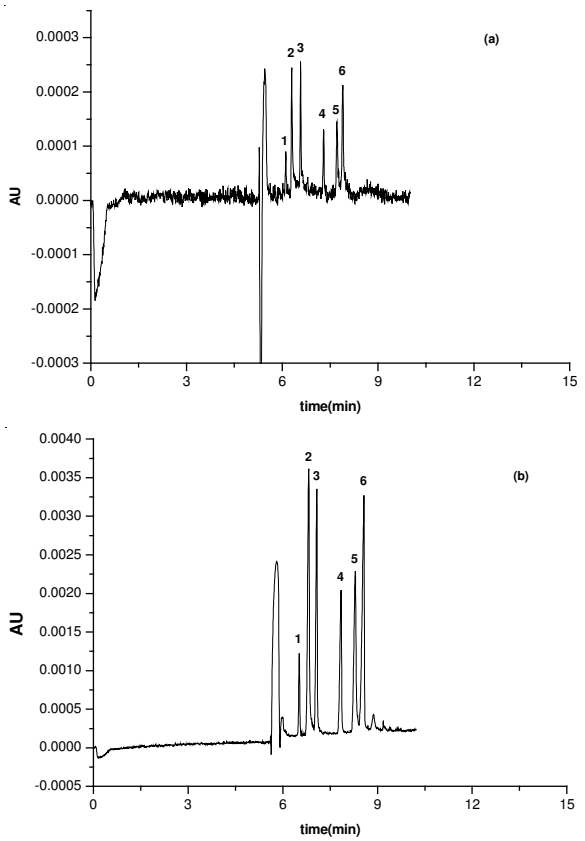

Fig. 1. Electropherogram of normal (a) and FASS-CE (b) separation of the six compounds. Conditions: (a) fused silica capillary 60.2 cm × 50 μm i.d.; BGE, 20 mmol L^{-1} sodium tetraborate buffer (pH 7.5); separation voltage: 25 kV; hydrodynamic injection of mixture of 2 μg mL^{-1} of standards and 1 μg mL^{-1} internal standard at 0.5 psi for 5 s; detection, UV at 254 nm. (b) hydrodynamic injection of mixture of 2 μg mL^{-1} of standards and 1 μg mL^{-1} internal standard at 0.5 psi for 65 s; other conditions are the same as (a) 1. 4-methylumbelliferone; 2. kaemperol; 3. apigenin; 4. rutin; 5. quercetin; 6. luteolin (the meaning of 1-6 were the same in the whole paper)

Real sample analysis: The developed method was applied to analyze the extracts of *Lysimachia christina hance*, *Lonicera japonica thunb* and *Tribut chrysanthemum*. The electropherograms were shown in Fig. 2. The results were shown in Table-3. Comparable to 0.2 mg g^{-1} of the analytes mixture was added into 1 mL of the extract of *Lysimachia christina hance* and diluted to 10 mL to carry out the recovery experiments. The recoveries were 91.2, 83.5, 87.6, 94.5 and 104.7 %, respectively.

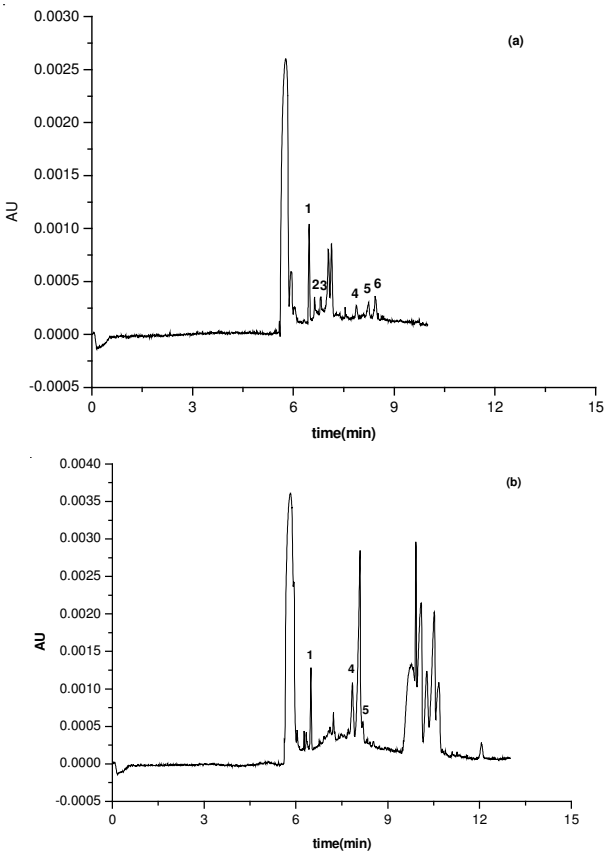

TABLE-2
REGRESSION EQUATIONS AND DETECTION LIMITS IN FASS-CZE

Compound	Regression equation*	Correlation coefficient	Linear range (μg mL^{-1})	Detection** limits (ng mL^{-1})
Kaemperol	Y = 1.487 X + 0.1288	0.9986	0.05-2	15
Apigenin	Y = 1.4198 X + 0.0073	0.9998	0.05-2	15
Rutin	Y = 0.7660X + 0.04347	0.9989	0.10-4	30
Quercetin	Y = 0.9237X + 0.00477	0.9997	0.10-4	24
Luteolin	Y = 1.3101X + 0.06248	0.9995	0.05-2	17

*In the regression equation, the X value is the concentration of analytes (ng/mL), the y value is the peak height ratio of analytes to the internal standard. **Based on three times noise.

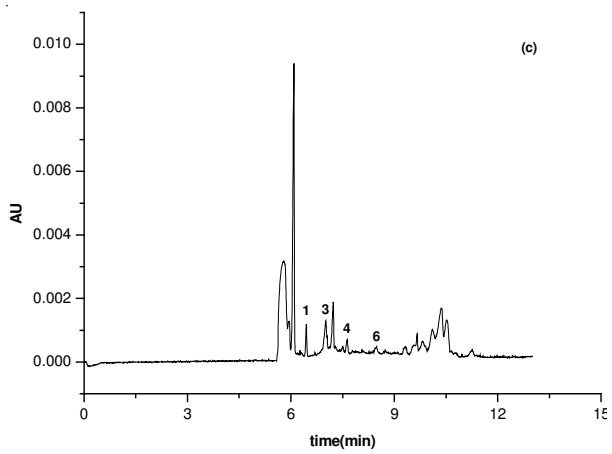

Fig. 2. Electropherogram of methanol extracts of *Lysimachia christina hance* (a), *Lonicera japonica thunb* (b) and *Tribut chrysanthemum* (c). CE conditions is the same as in Fig. 1b

TABLE-3
RESULTS OF SAMPLE ANALYSIS (n = 5, mg g^{-1})

	Lysimachia christina hance	*Lonicera japonica thunb*	*Tribute chrysanthemum*
Kaempferol	0.16	Not found [a]	Not found
Apigenin	0.84	Not found	1.29
Rutin	0.26	1.54	0.68
Quercetin	0.24	0.43	Not found
Luteolin	0.22	Not found	0.22

Conclusion

A FASS-CE method was developed to determine kaempferol, apigenin, rutin, quercetin and luteolin five kinds of flavonoids. Using this method, the detection sensitivity of the five analytes enhanced 15-17 times. This method was applied to analyze the five flavonoids in *Lysimachia christina hance*, *Lonicera japonica thunb* and *Tribut chrysanthemum* three kinds of traiditional Chinese medicine. The recovery experiments were also carried out and the recovery for the five flavonoids werr in the 83.5-104.7 % range, which is satisfactory.

ACKNOWLEDGEMENTS

This work was jointly supported by funding from the Scientific Project of the Education Branch of Shandong Government (No. J08LC52), the Natural Science Foundation of Shandong Province (No. Q2008B08) and the Key Technologies R & D Programme of Shandong Province (No. 2010GSF10615).

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