Research on flavonoids contents in *Fructus sophorae* with capillary zone electrophoresis

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Abstract: Genistin, genistein, kaempferol, quercetin and rutin, five kinds of flavonoids in *Fructus sophorae*, have been analyzed by capillary zone electrophoresis with internal standard calibration. Buffer pH and concentration, applied voltage, β-cyclodextrin and ethanol concentration were optimized and the optimum conditions are: 20 mmol/L borax (pH 9.5) with 8 mmol/L β-cyclodextrin and 5% (*v/v*) ethanol and at a voltage of 25 kV. The contents of five flavonoids in *Fructus Sophorae* grown in different area of Dezhou, Shandong Province of China were determined by the developed method and with satisfactory results. The distributions of the studied flavonoids were also investigated.

Keywords*:* β-cyclodextrin (β-CD); Capillary Zone Electrophoresis (CZE); flavonoids; genistin; genistein; kaempferol; quercetin; rutin.

INTRODUCTION

The flavonoids, which have a large family of over 4000 compounds and possessed the capacity of radical absorbance, have many medical effects and are active constituents of many Chinese herbal medicines (Merken and Beecher, 2000). So, identification and determination of flavonoids occupied a very important place in the efficacy, the safety and therapeutic reproducibility for Chinese herbal medicine and their medicinal preparation.

Many methods (Maleš and Medić-Šarić, 2001; Sutthanut *et al*., 2007; Bian *et al*., 2005; Chen *et al*., 2001; Zeng *et al*., 1990; Fiamegos *et al*., 2004; Qu *et al*., 2001; Bo *et al*., 2002; Chen *et al*., 2002; Cao *et al*., 2004; Chen *et al*., 2000; Jiang *et al*., 2004; Lu *et al*., 2004; Lu *et al*., 2005; Lu *et al*., 2008; Tian, 2002; Pan *et al*., 2004; He *et al*., 2012; Liu *et al*., 2011) have been used in flavonoids analysis. Of all the reported methods, high performance capillary electrophoresis (HPCE) has the advantages of rapidity, high resolution, high efficiency, low cost and has won the acceptance of many analysts from all over the world in recent years.

Fructus sophorae, the fruit of Leguminosea plant *Spohera japonica L*, is a traditional Chinese herbal medicine, which has the function of anti-inflammatory, anti-cancer, cooling the blood and staunching (Tian, 2002) and has long been used for osteoporosis, cancer and other illness since ancient time (Ma and Lou, 2006). Genistin, genistein, kaempferol, quercetin and rutin are five flavonoids in *Fructus sophorae* (Jiang and Xiao, 1986). Accurate determination of the five flavonoids is very important for its quality control and its medicinal preparation development.

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So far, flavonoids in *Fructus sophorae* have been analyzed by high-performance liquid chromatography (HPLC) (Bian *et al*., 2005; Pan *et al*., 2004; He *et al*., 2012; Liu *et al*., 2011) and high-performance capillary electrophoresis (Chen *et al*., 2002). Nevertheless, the analysis time is relatively long (Bian *et al*., 2005; Pan *et al*., 2004; He *et al*., 2012; Liu *et al*., 2011) and only a few of the above analytes have been analyzed (Chen *et al*., 2002). In this paper, a capillary electrophoretic method with internal standard calibration has been developed for determination of genistin, genistein, kaempferol, quercetin and rutin in *Fructus sophorae*. The method was applied to analyze the five flavonoids in *Fructus sophorae* of different growth area in Dezhou, Shandong Province, China. The method has the advantages of accuracy, rapidity and low cost and was very important for the quality control of *Fructus sophorae* and its medicinal preparations. As the producing area are very important for the chemical constituents variation and curative effect of Chinese herbal medicine, the results was helpful for the medicinal resource development of *Fructus sophorae* in Dezhou, Shandong Province, China.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, unless otherwise stated, were of analytical reagent grade. All flavonoids and 4-methylumbelliferone (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Genistin stock solution of 1.0 mg/mL was prepared in 80% (*v/v*) ethanol solutions. Other standard solutions of 1.0 mg/mL were prepared in ethanol. Buffer solutions were prepared from borax (concentration range: 5-25 mmol/L), β-cyclodextrin (concentration range: 0-10 mmol/L) and ethanol (concentration range: 0-10%, *v/v*) by dissolving them in

ultrapure water. The final pH values were adjusted with 0.1mol/L sodium hydroxide and 0.1mol/L sodium dihydrogen phosphate. All buffer solutions were filtered through a 0.45μm membrane filter and sonicated for 10 min before use.

Electrophoretic conditions

The analysis were carried out in a P/ACE MDQ HPCE system (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a diode array detector operated at 254 nm. A 60.2 cm×50 μm I. D. fuse-silica capillary (Yong Nian Optical Fiber Factory, Hebei Province, China) was utilized with an effective length of 50 cm, and its temperature was maintained at 25°C. Data were collected and analyzed using the Karat 7.0 software from Beckman Coulter running on a IBM Pentium IV-1GHz computer. The working buffer of capillary electrophoresis was 20 mmol/L borax (pH 9.5), with 8 mmol/L β-cyclodextrin and 5% (*v/v*) ethanol. Buffer pH was adjusted with a pHS-3C pH meter (Leici Instrumentation Factory, Shanghai, China). Electrophorectic procedures, including capillary flushing, quantification and quantitation are the same as reported (Lu *et al*., 2008).

Sample preparation

Fructus Sophorae samples were collected from Pingyuan (37.16N, 116.44E), Linyi (37.21N, 116.86E), Ningjin (37.64N, 116.80E), Lingxian (37.34N, 116.58E), Qingyun (37.37N, 117.37E), Yucheng (36.95N, 116.66E), Leling (37.74N, 117.22E), Xiajin (36.95N, 116.00E), Wucheng (37.21N, 116.08E), Qihe (37.79N, 116.76E) and Decheng district (37.45N, 116.29E), 11 different areas of Dezhou, Shandong Province in later October, 2007. Afterwards they were washed with ultrapure water and then air dried in the sun for several days after which they were ground in a mill. The samples were sieved to obtain particles of 0.25 mm in diameter. They were dried for 6 hours at 60°C before use.

Thereafter, 0.1000 g of the powder was extracted with 7 mL of 80% (v/v) ethanol by ultrasonication at room temperature for 20 min, then centrifuged at 3000 rpm for 10 min. The resulted supernatant solution was moved into a volumetric flask of 25 mL. The extraction process was repeated three times and the extracts were combined and diluted to 25 mL. Afterwards, 5 mL of the above sample extracts was transferred to a 10 mL volumetric flask and diluted to mark after 0.8 mL internal standard stock solution was added before analysis.

Method verification

The linearity of the five analytes in standard solutions was investigated.The calibration graphs were plotted by concentration (x,μg/mL) against peak area ratio (y, analyte/internal standard). The detection limits were acquired based on three times noise. The reproducibility is estimated by making eight replicate injection of a standard mixture solution under the selected optimum conditions.

Accurate amount of the five flavonoids were added to a *Fructus sophorae* sample (from Leling city) to do recovery experiments and the recovery value were achieved by the corresponding calibration curve on the same conditions.

RESULTS

Linearity, repeatability and detection limits

The linearity, detection limits and repeatability of the method were shown in table 1 and table 2. The calibration plots of the five analytes were linear in the concentration ranges of 1-220 μg/mL, 0.5-100 μg/mL, 0.5-100 μg/mL, 1-200 μg/mL and 1-220 μg/mL for genistin, genistein, kaem-pferol, quercetin and rutin, respectively. The variation in migration time and peak area were in the range of 0.39-0.85% and 1.91-3.23%, respectively. The detection limits of the five analytes were in the 0.11-0.32μg/mL range.

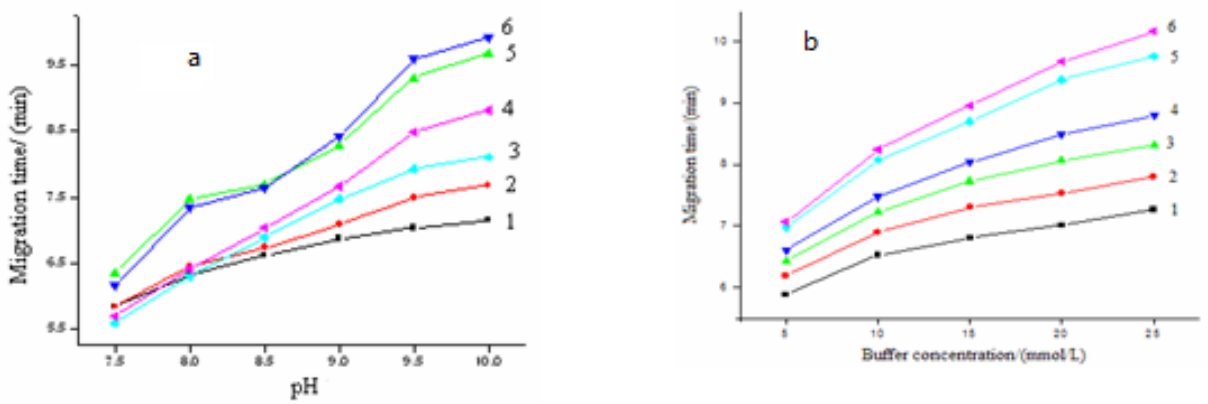

Fig. 1: Effects on migration time of the six compounds by buffer pH (a), and concentration (b): 1. genistin; 2. genistein; 3. 4-methylumbelliferone; 4. kaempferol; 5. quercetin; 6. rutin (The meaning of 1, 2, 3, 4, 5, 6 were the same in the figures of the whole paper).

Recovery

The recoveries of the five flavonoids were shown in table 3 and were in the 93.6-105.2% range, which were satisfactory.

DISCUSSION

Effect of buffer pH and buffer concentration

Structures of the six analytes suggest that their pKa should be in the pH 7.81-9.93 range (Morin *et al*., 1997; Liang *et al*., 1997; McGhie and Markham, 1994; Wolfbeis *et al*., 1984). Borax buffer was employed as the running buffer and the effects of buffer pH on the separation was investigated in the pH7.5-10.0 range. As shown in fig. 1 (a), the migration time of the six compounds increase with the increase of the buffer pH. The six compounds can be well separated when the buffer pH is higher than 9.0. However higher pH value results in longer analysis time and the analytes are more susceptible to oxidation. In consideration of the analysis time and good separation, pH 9.5 was chosen as a compromise.

Keep the buffer pH at 9.5 and other conditions the same as the pH optimization the influence of buffer concentration was investigated in the 5~25 mmol/L concentration range. As indicated in fig. 1 (b), the migration time and the resolution of the six compounds increase with increasing buffer concentration, the six compounds can be well separated when buffer concentration was higher than 15 mmol/L. The results also showed that the capillary current increase with the increase of the buffer concentration, which will increase the Joule heating effect and in the long run sacrifice the detection limits. In consideration of resolution, analysis time and detection limits, 20 mmol/L was selected as the optimum buffer concentration.

Effect of applied voltage

The effect of applied voltage on the separation was examined in the range of 19~27 kV. The results showed that with the increase of applied voltage, the migration time of the six compounds decreased, which results in shorter analysis time and an improvement of the efficiency. However, the baseline noise increased apparently when the applied voltage exceeded 25 kV, which can make the detection limits deteriorate due to the pronounced Joule heating caused by the applied voltage increase. So, 25 kV was selected as the optimum applied voltage.

Effect of β-CD concentration

Incorporation of β-CD into the running buffer can form some inclusion-complex between hydrophobic analytes and β-CD thus improving the separation (Hoffstetter-Kuhn *et al*., 1991). In an attempt to attain improvement in the separation of the six compounds, the effect of β-cyclodextrin (β-CD) concentration was examined in the 0~10 mmol/L concentration range. The results were shown in fig. 2, the migration time of rutin didn't change significantly, while those of other compounds decreased with the increase of the β-CD concentration. Simultaneously, optimum separation can be achieved when the β-CD concentration was at 8 mmol/L, so 8 mmol/L β-CD was adopted in the further experiments.

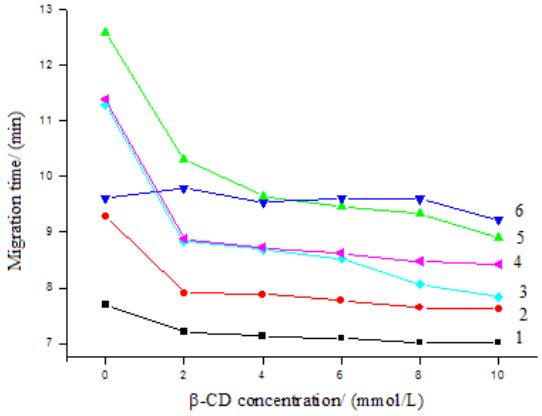

Fig. 2: The effects of β-CD concentration on migration time of the analytes.

Effect of ethanol

Organic solvent can be added to the buffer solution to reduce the electro-osmotic flow (EOF) thus increasing the time window and improving separation. Different organic modifiers including MeOH, EtOH, n-PrOH and n-BuOH were tested. Based on experiments, EtOH was chosen as the organic modifier. Setting the other conditions as above, 0-10% (V/V) EtOH was used as organic modifier to improve the separation. The results were shown in fig. 3, the migration time of the six compounds increased with the increase of the ethanol concentration. And optimum separation was achieved when ethanol concentration was 5% (v/v). Hereby, 5% (v/v) ethanol was adopted as organic modifier in the following experiments. Under the above optimized conditions, good separation of the six compounds was achieved in 10 min, fig. 4 shows a typical electropherogram of the six compounds.

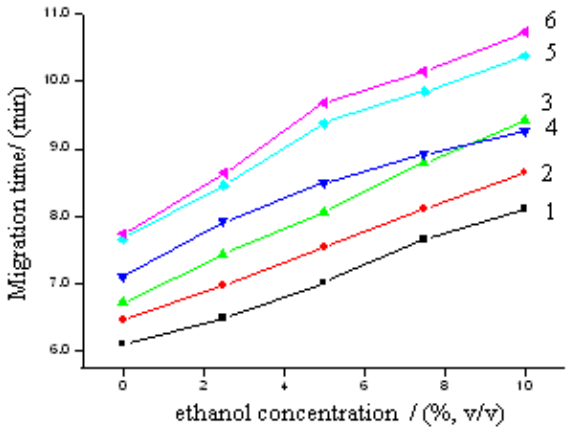

Fig. 3: Effects of ethanol concentration.

Table 1: The regression equations and detection limits[a]

Compound	Regression equation[b]	Correlation Coefficient	Linear range (μg/mL)	Detection limit[c] (μg/mL)
genistin	Y=0.0113X-0.00450	0.9998	1~220	0.27
genistein	Y=0.03037+0.00061	0.9999	0.5~100	0.11
kaempferol	Y=0.02249X-0.00557	0.9998	0.5~100	0.14
quercetin	Y=0.0123X-0.00468	0.9999	1~200	0.25
rutin	Y=0.00870X-0.00083	0.9995	1~220	0.32

a. CE conditions are the same as in fig. 4
b. In the regression equation, the X value is the concentration of analytes (μg/mL), the y value is the peak area ratio (analyte/internal standard)
c. The detection limit is evaluated on the basis of a signal-to-noise of 3.

Table 2: Reproducibility of the peak area and migration time of the compounds (n=8).

Compound	Concentration (μg/mL)	Migration time(min)		Peak area (μAU·sec)	
		Mean	R.S.D (%)	Mean	R.S.D (%)
genistin	50	7.015	0.59	6973	1.91
genistein	50	7.507	0.39	15748	2.47
4-methylum-belliferone	80	8.016	0.65	11357	2.32
kaempferol	50	8.503	0.54	12938	2.90
quercetin	50	9.329	0.85	7165	2.21
rutin	50	9.618	0.76	4879	3.23

Table 3: The recoveries in this method with *Furctus sophorae* grown in Leling City (n=5).

Compounds	Original amount (mg/g)	Added amount (mg/g)	Found (mg/g)	Recovery (%)	R.S.D. (%)
genistin	89.68	10.0	99.04	93.6	3.1
genistein	1.21	10.0	10.89	96.8	3.5
kaemferol	7.92	10.0	18.13	102.1	4.2
quercetin	N.F.	10.0	10.52	105.2	3.7
rutin	9.55	10.0	19.47	99.2	2.5

Table 4: The contents of the five analytes in *Fructus Sophorae* in different areas of Dezhou(mg/g, n=5)

Growth area	genistin	genistein	kaempferol	quercetin	rutin
Pingyuan County	70.93 (2.35%)	0.53 (3.40%)	2.15 (3.25%)	2.42 (3.72%)	9.23 (2.18%)
Linyi County	65.52 (3.17%)	0.52 (2.96%)	6.81 (2.36%)	1.06 (2.79%)	9.55 (3.12 %)
Ningjin County	78.94 (3.14%)	0.48 (4.43%)	5.33 (3.17%)	1.08 (3.05)	9.05 (3.40%)
Lingxian County	55.73 (1.45%)	0.37 (3.91%)	4.88 (2.93%)	1.08 (3.48%)	11.49 (1.89%)
Qingyun County	54.48 (2.82%)	0.54 (2.43%)	7.98 (2.04%)	0.74 (4.2%)	14.12 (2.77%)
Yucheng County	53.98 (3.46 %)	0.42 (3.38%)	2.60 (3.61%)	1.65 (2.60%)	16.32 (2.61%)
Leling County	89.68 (1.84%)	1.21 (2.79%)	7.92 (2.76%)	N. F[b]	9.55 (2.52%)
Xiajin County	80.38 (2.13%)	0.42 (4.32%)	9.32 (1.81%)	N. F.	5.23 (3.36%)
Wucheng County	80.49 (2.59%)	0.51 (3.25%)	2.85 (3.49%)	1.42 (2.80%)	9.67 (3.07%)
Qihe County	86.20 (1.98%)	1.35 (2.29%)	5.24 (3.23%)	1.13 (3.06%)	13.32 (2.22%)
Decheng District	42.04 (4.02%)	0.42 (3.75%)	8.79 (2.18%)	0.58 (3.83%)	16.89 (1.74%)

[1]The data in the parentleses denote RSD, [2]NF = not found

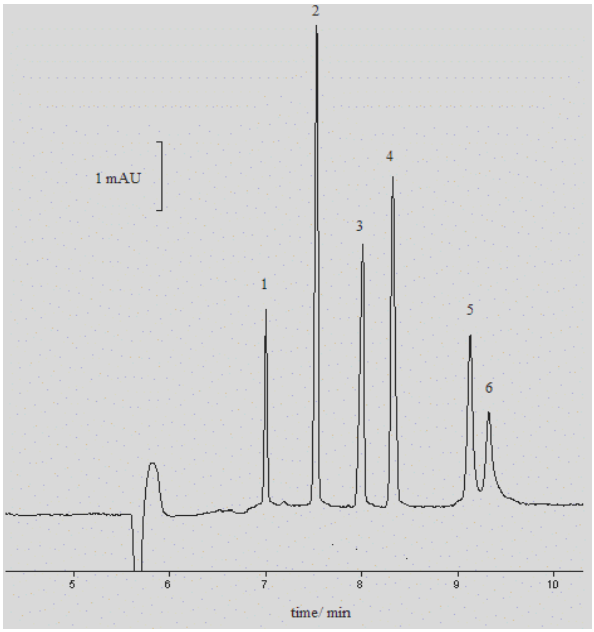

Fig. 4: Electropherogram of a mixture of five flavonoids and the internal standard Conditions: Borax, 20 mmol/L; pH 9.5; ethanol, 5% (v/v); β-cyclodextrin, 8 mmol/L; applied voltage, 25kV; temperature 25°C UV detection wavelenghth 254nm.

Sample analysis

The developed method was applied to determine genistin, genistein, kaempferol, quercetin and rutin in *Fructus sophorae* grown in different areas of Dezhou, Shandong Province, China. The analysis results were shown in table 4. As can see from table 4, the contents of genistin and kaempferol are higher than what Pan *et al*. (genistin, 17.1 mg/g) (Pan *et al*., 2004) and He *et al*. (genistin, 4.48-7.09 mg/g and kaempferol, 0.09-0.22 mg/g) (He *et al*., 2012) have reported. The contents of rutin and quercetin are comparable to what Liu *et al*. (rutin, 8.7 mg/g) (Liu *et al*., 2011), Chen *et al*. (rutin, 27.5-29.6 mg/g) (Chen *et al*., 2002) and Bian *et al*. (quercetin, 0-2.42 mg/g) (Bian *et al*., 2005) have reported. The contents of genistein are lower than the results of He *et al*., (2.36-3.05 mg/g) (He *et al*., 2012).

ACKNOWLEDGEMENTS

This work was jointly supported by the Key Technologies R&D Programme of Shandong Province (2010GSF10615).

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