Determination of Flavones in *Crataegus pinnatifida* by Capillary Zone Electrophoresis

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Abstract

A capillary electrophoretic method for the separation of four flavones in *Crataegus pinnatifida* is developed. The four flavones in *Crataegus pinnatifida* are separated on baseline within 15 min using 50mM borax buffer containing 15% acetonitrile and adjusted to pH 8.15 with phosphoric acid. The detection limits of vitexin-2"-rhamnoside, hyperside, rutin, and vitexin are 0.35, 0.30, 0.40 and, 0.29 µg/mL, respectively. The recovery of these flavones is as follows: vitexn-2"-rhamnoside 96.8%, hyperside 99.9%, rutin 97.1%, and vitexin 97.8%. The results are in accordance with those obtained in the high-performance liquid chromatography system. The content of flavones is higher in *Crataegus pinnatifida* leaves than in its fruits, and hyperside is not detected in either *Crataegus pinnatifida* fruits or flowers.

Introduction

Crataegus is widely used as a medical plant in both official and traditional medicine. The preparations of Crataegus can improve coronary and cerebral blood flow, protect against arrhythmias, increase cardiac activity, inhibit the aggregation of platelets, and delay thrombus formation and aging. Flavones are active compounds and the major constituents of this genus. It was reported that vitexin-2"-rhamnoside (VR), hyperside (H), rutin (R), and vitexin (V) are the four major flavones in Crataegus species (1,2) whose structures are shown in Figure 1 (1). It would further benefit pharmaceutical and pharmacological studies to be able to separate and identify the individual flavones in the preparations. Methods such as spectrophotometric (3–8), thin layer chromatography (TLC) (3), and high-performance liquid chromatography (HPLC) (3–8) have been reported for the determination of total and individual flavones. Because of the low sensitivity, spectrophotometric and TLC methods are not adequate. Currently, reversed phase HPLC (RP-HPLC) is the analytical separation technique commonly used for polyphenolic compounds such as flavones. Because of the variability of column filling materials and solvent systems, RP-HPLC exhibits a great potential in separating complex mixtures of flavonoids and other phenolic compounds (9). Compared with capillary electrophoresis (CE), the efficiency of HPLC is lower. It also consumes a large amount of solvents and is a high-priced column technique. As a modern separation method, CE has won increasing acclaim for its extremely high efficiency, flexibility, small volume, high speed, and good resolution, and its applications in the analysis of Chinese herbs are wide. As one mode of CE, capillary zone electrophoresis (CZE) is the most simple and mature technique. To date, no method on the determination of these four flavones in Crataegus by CE has been reported. In this paper, a CZE method for the separation of these four flavones was established, and the results were compared with those of HPLC.

Experimental

Chemicals

The borax, phosphoric acid, disodium hydrogen phosphate, methanol, and acetonitrile used in the CE system were guaranteed grade reagents, and the acetonitrile used in the HPLC system was analytical grade reagent. All were obtained from Beijing Chemical Factory (Beijing, China). Water used for buffer solu-

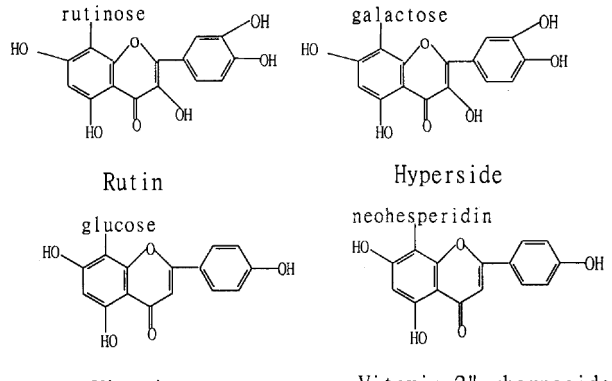

Figure 1. Structures of four flavones.

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tions in CE was deionized to the resistive of 15MΩ · cm, and the water used for dilution in HPLC was double distilled.

The four flavones [V, H, R, VR, and tris[hydroxymethyl]-aminomethane (tris)] were purchased from Sigma (St. Louis, MO).

Plant materials

The sample of Crataegus flowers and leaves were plucked from Lumei (Baoding, China). The fruits were purchased from Baoding Market (Baoding, China).

Preparation of plant samples

A 2.0-g sample was ground down with 0.5-mL 90% H_3PO_4 and extracted with 95% ethanol for 15 min then centrifuged for 5 min (4000 rpm). Extraction was repeated three times. The extracts were combined and diluted to 25 mL with 95% ethanol. The solution was then filtered through a 0.45-µm filter, and the resulting filtrate was used as the samples for analysis.

A blank test solution used to obtain detection limits for analytes was also prepared using the same procedures as the sample treating.

Apparatus and conditions

CE system

The electrophoretic experiments were performed on a BioFocus 3000 CE system (Bio-Rad, Hercules, CA) equipped with a high-speed scanning detector in the UV–vis region. A 50-µm-i.d. fused-silica capillary (Yongnian Optical Fiber Factory, Hebei province, China) with a 50-cm total length and 45.5-cm effective length was used. The CE conditions were as follow: cartridge temperature, 25ºC; carousel temperature, 25ºC; applied voltage, 15 kV; and pressure injection mode and injecting pressure 20 psi · s. The running buffer consisted of 50mM $Na_2B_4O_7$ and 15% acetonitrile, and the pH value was adjusted to 8.15 with H_3PO_4 .

HPLC system

All HPLC analyses were performed using an Elite instrument (Dalian Elite Limited, Dalian, China) equipped with an UV200 UV detector (350 nm), P200 II high-pressure pump and Echrom98 workstation. The separation was obtained with a reversed-phase column (4.6 × 200 mm) filled with 5-µm Hypersil basic deactivation silica (BDS) (Thermo Hypersil-Keystone, Cheshire, U.K.) at a flow rate of 0.6 mL/min. The mobile phase was CH3CN:H2O (20:80, v/v).

Results and Discussion

Effect of pH and electrolyte species of buffer

It is well known that the pH of the buffer not only controls the number of negatively-charged silianol on the inner wall of the capillary, but also determines the degree of dissociation of analytes. Flavone compounds are weak acids with ionization constants (pK_a) from 7.3 to 12.5 because of the presence of phenyl hydroxyl groups. Their apparent charge depends on their pK_a values and the pH of the running buffer (10).

In order to study the effect of the pH of the buffer on the separation, 50mM borax buffers with pH values ranging from 7.5 to 8.5 (adjusted by H_3PO_4) were initially used to separate the four

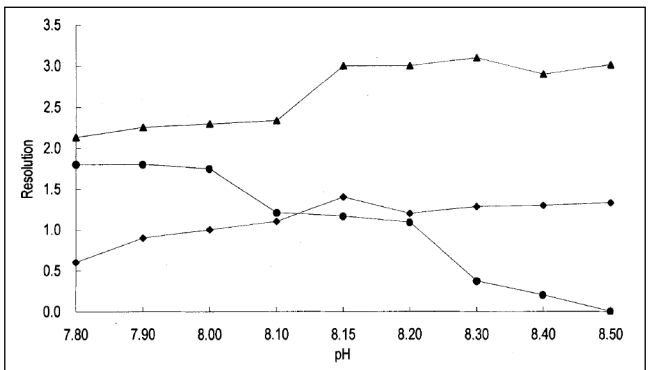

Figure 2. Effect of pH on resolution. Resolution of VR and H (♦), H and R (●), and R and V (▲).

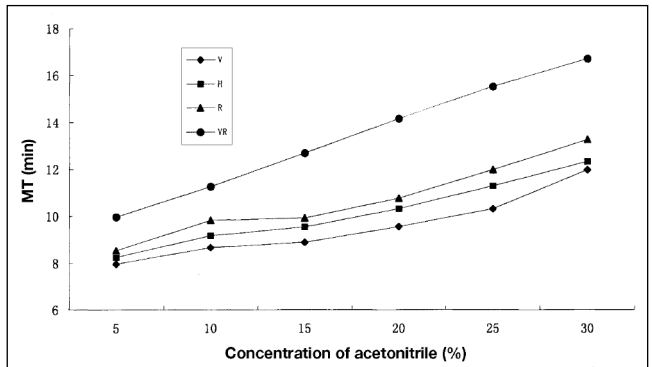

Figure 3. Effect of acetonitrile on migration time.

Table I. Data for Method Validation

	VR	H	R	V
Regression equation	$Y = 14027X - 13151$	$Y = 34141X - 85175$	$Y = 20615X - 15048$	$Y = 21753X - 6303$
R	0.999	0.9957	0.9956	0.9999
CE				
Detection limit (µg/mL)	0.35	0.30	0.41	0.29
Regression equation	$Y = 168.12X + 8.00$	$Y = 29.41X + 212.06$	$Y = 17.86X + 715.71$	$Y = 105.26X + 2105.26$
R	0.9999	0.9999	0.9998	0.9994
HPLC				
Detection limit (µg/mL)	0.40	0.36	0.52	0.30

analytes. It was found that the resolutions of these flavones were very poor when the pH value was lower than 7.5, and their migration time (MT) became too long when the pH was above 8.5. The resolutions of these flavones varied with the pH of the buffer. As seen in Figure 2, the best resolution of VR and H was 1.40 when the pH reached 8.15, which was where the resolutions of H and R and R and V were 1.2 and 3.0, respectively. Therefore, pH 8.15 was selected.

Tris. and phosphate (disodium hydrogen phosphate) buffer adjusted to pH 8.15 with phosphoric acid were tested. The separability was not enhanced and was worse than the borax buffer.

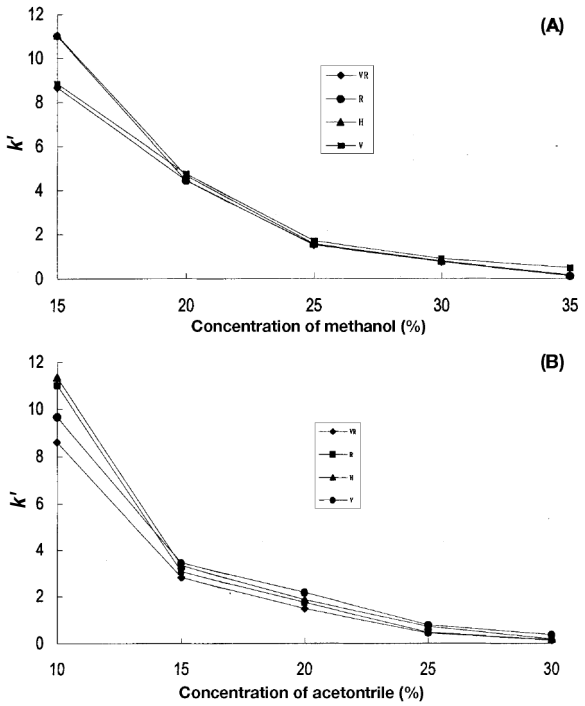

Figure 4. Effect of methanol (A) and acetonitrile (B) concentration on *k'*.

Table II. Content of Marker Substances in Creatagus Materials

Constituent		CE leaves	CE flowers	CE fruits	HPLC leaves	HPLC flowers	HPLC fruits
VR	C (mg/g)	1.910	0.010	0.024	1.890	0.012	0.023
	RSD* (%)	3.9	4.2	3.5	3.5	3.8	3.5*
	Added value (mg)	4.0050	0.0182	0.0500	4.0300	0.0200	0.0500
	Recovery value (mg)	3.8768	0.0177	0.0493	3.9293	0.0196	0.0493
	Recovery (%)	96.8	97.2	98.5	97.5	98.2	98.5
H	C (mg/g)	–†	–	–	–	–	–
	RSD (%)	0.3	0.8	0.5	0.4	0.5	0.5
	Added value (mg)	0.0100	0.0105	0.0124	0.0105	0.0095	0.0010
	Recovered value (mg)	0.0099	0.01048	0.0122	0.0105	0.0093	0.0098
	Recovery (%)	99.0	99.9	98.5	99.6	98.0	97.8
R	C (mg/g)	0.090	–	0.007	0.089	–	0.007
	RSD (%)	3.6	3.4	3.2	3.5	4.1	4.3
	Added value (mg)	0.2005	0.0100	0.0150	0.2000	0.0105	0.0150
	Recovered value (mg)	0.1947	0.0098	0.0148	0.2048	0.0102	0.0141
	Recovery (%)	97.1	98.3	98.5	102.4	96.8	94.3
V	C (mg/g)	0.220	–	0.034	0.220	–	0.036
	RSD (%)	2.5	3.2	2.9	2.9	4.2	4.5
	Added Value (mg)	0.4000	0.0100	0.0505	0.5025	0.0115	0.1005
	Recovered value (mg)	0.3912	0.0963	0.0482	0.4884	0.0117	0.0962
	Recover (%)	97.8	96.3	95.4	97.2	101.8	95.7

* RSD, relative standard deviation.
† Not detected.

Effect of organic modifier species and their concentrations

Organic modifier can alter the permittivity and viscosity of the medium and can directly affect the mobility of electroosmotic flow (EOF). It was found that the mobility of EOF decreases with the increase of the fraction of organic modifier. Therefore, the addition of organic modifier in the running buffer has been considered a convenient and useful procedure to improve the separation in both CZE and micellar electrokinetic chromatography (11,12). In order to study the effect of organic modifiers on the separation, methanol and acetonitrile were added to the 50mM borax buffer at pH 8.15, and their concentrations were varied from 5–30% in increments of 5%. The baseline separation of VR and H was obtained when methanol reached 10% and acetonitrile reached 5%, which was where the resolutions of H and R and R and V exceeded 1.5. For column efficiency, acetonitrile was better than methanol. The best resolution of VR and H was 1.8 when the concentration of acetonitrile reached 15%. The resolution decreased when acetonitrile was increased above 15%. The MT of these flavones increased with the increase of acetonitrile concentration. Their variation can be seen in Figure 3. Therefore, 15% acetonitrile was the best organic modifier condition for resolution and MT.

Effect of borax concentration

In order to decrease MT, different concentrations of borax buffers with 15% acetonitrile at pH 8.15 were used to separate the four flavones. There was an increase in the MT of the four markers with the increase of borax concentrations. However, when the concentration of borax buffers was below 40mM, the resolution of VR and H was significantly affected. To avoid partial overlap of VR and H, 50mM borax buffer was selected.

Analytical conditions for HPLC method

The effect of methanol and acetonitrile as the organic reagent added into the elution on the separability of four makers was studied. Figure 4 shows *k'* at different methonal and acetonitrile concentrations. It can be seen from the results that methanol was not a good organic reagent for the separation of the four markers; acetonitrile was a much better choice.

As shown in Figure 4, satisfactory separation was obtained when the acetonitrile concentration was below 20%. However, the retention time (RT) was affected significantly, and the peaks of the markers were broadened with the decrease of acetonitrile in mobile phase. Thus, a good resolution

with a satisfactory RT could be obtained by using of the mobile phase of 20% acetonitrile. The condition is simpler than others in which factors affecting separability are complex, strict, and difficult to control (3–8).

Method validation

The four flavones in the samples were identified by comparing their MT and UV spectra obtained from the high-speed scan detector of CE with those of authentic standards.

The linearity of the calibration curve of peak area over MT (*Y*) against concentration (*X*, µg/mL) in CE and peak area against content (*X*, ng) in HPLC for each of the flavones was investigated. In the test, the lowest concentration of standard solution was 0.5 mg/mL and the highest of VR, H, R, and V were 200, 150, 60, 150 µg/mL, respectively. The equations of the calibration curves of these flavones and their correlation coefficients are shown in Table I. Good correlations were confirmed by the values (R) obtained from the linear regressions, and the concentration ranges of standard flavones were suitable for the sample extraction analysis.

According to the suggestion of the International Union of Pure and Applied Chemistry, the detection limits of the four flavones in each method were obtained by the determination of the blank test solution. The results of these two methods are shown in Table I.

The recoveries of spiked samples for each method were determined five times. The recoveries were calculated by using the formula:

$$\text{Recovery (\%)} = \frac{\text{found value} - \text{sample value}}{\text{added value}} \times 100\% \qquad \text{Eq. 1}$$

The results are shown in Table II. Figure 5 shows the electropherograms and chromatograms of four flavone standards and the leaf extract.

As shown in Figure 5, the baseline separation of the four flavones in the CE system was finished in 15 min under the optimal conditions. It can be seen in Table II that the results obtained in the CE system were as good as those obtained in the HPLC system, and satisfactory recoveries of all constituents were obtained. Also, H was not detected in the *Crataegus pinnatifida* materials, which was not in accordance with those reports (1,2) in which H was detected and was a major flavone. Finally, the content of flavones was higher in *Crataegus pinnatifida* leaves than in its fruits, and only a small amount of VR was detected in its flowers.

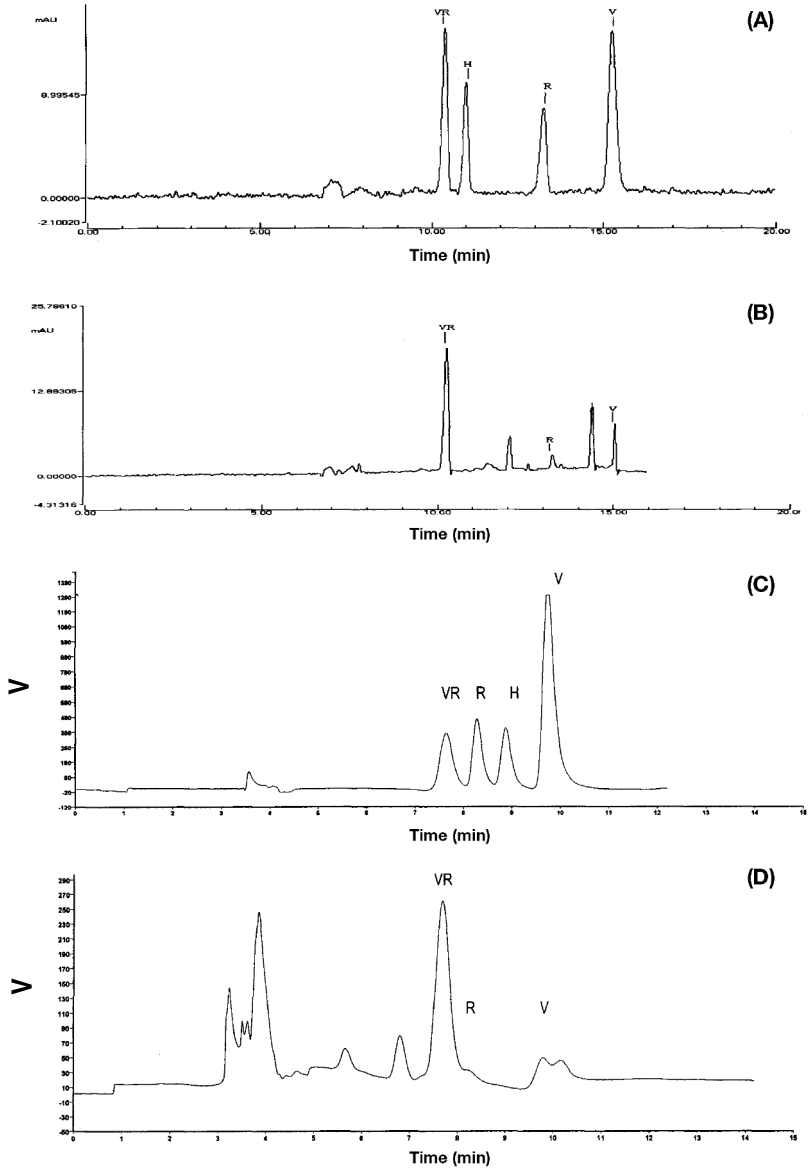

Figure 5. Electrophorgram and chromatogram of standard and mixture solution analytes. Electrophorgrams of (A) four markers and (B) leaf extract. Chromatograms of (C) four markers and (D) leaf extract.

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