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Determination of Xanthii Constituents by High-performance Liquid Chromatography and Capillary Electrophoresis

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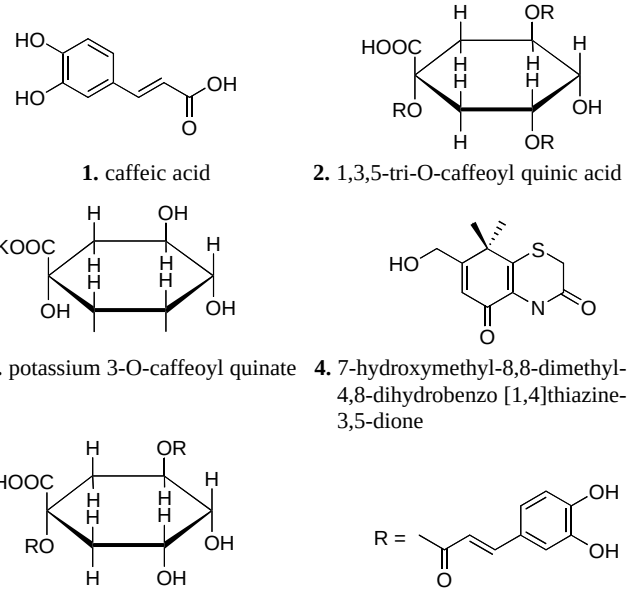
ABSTRACT

A high-performance liquid chromatographic method and a capillary electrophoretic method for the determination of potassium 3-O-caffeoyl quinate and 7-hydroxymethyl-8, 8-dimethyl-4,8-dihydro-benzo[1,4]thiazine-3,5-dione of *Xanthium Strumarium* L. were developed. In capillary zone electrophoresis, a buffer solution containing sodium borate and sodium acetate was found to be the most suitable approach to determine the contents of these marker substances within 50 minutes. In high-performance liquid chromatography, a buffer solution containing phosphate and acetonitrile was applied to analyze these marker substances within 60 minutes. The effect of buffers on the determination and validation of these two methods was discussed.

Key words: Xanthii Fructus, Xanthium strumarium, high-performance liquid chromatography, capillary electrophoresis

INTRODUCTION

Xanthii Fructus is the dried fruit of *Xanthium strumarium* L. of the Compositae family. In traditional Chinese medicine, it is used to treat sinusitis, headache and skin pruritus[(1)]. It contains caffeic acid, 1,3,5-tri-O-caffeoyl quinic acid, potassium 3-O-caffeoyl quinate, 7-hydroxymethyl-8, 8-dimethyl-4,8-dihydro-benzo[1,4]thiazine-3,5-dione and 1,5-di-O-caffeoyl quinic acid[(2-4)] as its main constituents (Figure 1). Most studies were concentrated on the separation of constituents and pharmacology[(5)]. However, with the lack of analysis by HPLC, CE or other methods, HPLC is now still the most popular tool for herbal analysis. Capillary electrophoresis is a widely applied technique in separation science due to its high efficiency, rapid rate of separation and small size sample requirement. Both methods could offer satisfactory results in the analysis of some Chinese herbs[(6-8)]. Here, we have developed HPLC and CE methods for determining the two Xanthii constituents simultaneously. The suitability of these two methods were compared and discussed.



1. caffeic acid

2. 1,3,5-tri-O-caffeoyl quinic acid

3. potassium 3-O-caffeoyl quinate

4. 7-hydroxymethyl-8,8-dimethyl-4,8-dihydrobenzo [1,4]thiazine-3,5-dione

5. 1,5-di-O-caffeoyl quinic acid

Figure 1. Structures of the five marker substances

MATERIALS AND METHODS

I. *Reagents and Materials*

Caffeic acid was purchased from Yoneyama (Osaka, Japan) and isolated from *Xanthium strumarium* L.. Potassium 3-O-caffeoyl quinate, 1,3,5-Tri-O-caffeoyl quinic acid, 7-hydroxymethyl-8, 8-dimethyl-4,8-dihydro-benzo[1,4]thiazine-3,5-dione, and 1,5-di-O-caffeoyl quinic acid were isolated from *Xanthium strumarium* L. The purity of these standards was judged by a photodiode array detector (Shimadzu SPD-M10A). Phosphoric acid, potassium dihydrogenphosphate, sodium acetate and sodium borate were purchased from Merck (Dermstadt, Germany). Acetonitrile and methanol were of the LC grade (Fisons, Loughborough, England). Deionized water from a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used to pre-

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pare all buffer and sample solutions. Xanthii Fructus samples were obtained from the Chinese herbal market in Taipei (Taiwan).

II. *Preparation of Xanthii Fructus Extracts*

A 1.0 g sample of pulverized Xanthii Fructus was extracted with 70% methanol (20 mL) by stirring at room temperature for 20 mins and then centrifuged at 1500 g for 5 mins. The extraction was repeated three times. The extracts were then combined and filtered through a No. 1 filter-paper. The filtrate was diluted to 100 mL with 70% methanol. This solution was passed through a 0.45 μm PVDF-filter and the filtrate was injected into the HPLC (20 μL) or CE system (8.5 nL).

III. *Calibration of the Marker Substances*

For each of the five components, stock solutions were prepared by dissolving 0.50-1.50 mg of marker substances (1.00 mg for **1,2** and **5**; 0.50 mg for **4**; 1.50 mg for **3**) in 10 mL of 70% methanol. Then diluting aliquots of the stock solution with the same solvent to give concentrations ranging from 1 to 150 μg/mL (dilution factor: 1, 2, 5, 10, 20, 30, 50 and 100). The linearity of the plot of peak-area (y) vs. concentration (x, μg/mL) for each of the marker substances in CE and HPLC was investigated.

IV. *Apparatus and Conditions*

(I) *CE system*

All analyses were carried out on a Waters Quanta 4000 CE system equipped with a UV detector set at 254 nm and 80 cm × 75 μm I.D. uncoated capillary (Polymicro Technologies, Phoenix, AZ, USA) with the detection window placed at 72.5 cm. The conditions were as follows: sampling time, 5 s hydrostatic (injection volume, 8.5 nL); running time, 50 mins; applied voltage, 20 kV (constant voltage, positive to negative polarity); and temperature, 25.0-25.5˚C. The electrolyte was a pH 9.2 buffer solution that contained 10 mM sodium borate and 40 mM sodium acetate.

(II) *HPLC system*

The HPLC system was consisted of two Waters Model 510 pumps, a Waters Model 680 automated gradient controller (Waters, USA) and an SPD-M10AVP photodiode array detector setting at 254 nm (Shimadzu, Japan). The separations were achieved with a 10 μL loop, a reversed-phase column (Cosmosil $5C_{18}$-MS, 5 μm, 25 cm × 4.6 mm I.D; Nacalai Tesque, Kyoto, Japan) and by linear gradient elution, using eluents A and B [A = 20 mM KH_2PO_4 buffer solution (adjusted to pH2.5 with H_3PO_4); B = CH_3CN-H_2O (4:1, *v/v*)] according to the following A-B profile: 0-10 mins, 100-95% A; 10-20 mins, 95-85% A; 20-35 mins, 85-65% A; 35-40 mins, 65-50% A; 40-45 mins, 50-0% A; 45-55 mins, 0% A. The flow-rate was kept at 1 mL/min. A precolumn of μ-Bondapak$^{TM}C_{18}$ (Millipore, Milford, MA, USA) was attached to protect the analytical column.

RESULTS AND DISCUSSION

I. *Analytical Conditions for CE Method*

Two xanthii constituents were determined by CZE under proper condition. The separation was achieved by optimizing the pH of buffer and the concentrations of CH_3COONa and $Na_2B_4O_7$. When acetate or borate was used alone, the peaks of marker substances were seriously overlapped with neighboring peaks. A system consisting of acetate and borate was used. The concentration of acetate was fixed at 0,10, 20, 30, 40 and 50 mM and the borate concentration was varied from 50, 40, 30, 20 10 to 0 mM, respectively. With 40 mM acetate and 10 mM borate solution, a fairly good separation effect was obtained. We found that the number of theoretical plates(N) was the highest at 10 mM borate and 40 mM acetate ($N = 2.6 \times 10^5$ for **3**). As for the pH value, it was optimized by adding 5% NH_4OH or CH_3COOH to the buffer solution. Varying the pH values from 9.0 to 10.0, 9.2 was found to be the best due to a higher theoretical plate number ($N = 3.1 \times 10^5$ for **3**) than other pH values. The electropherograms obtained by using the selected condition are shown in Figure 2A and 2B for the five marker standards and the methanol-water extract of Xanthii Fructus, respectively.

II. *Analytical Conditions for HPLC Method*

Since three out of the five marker substances were analyzed to be acids, based on our previous experience in separating Chinese herbs[7,8], a mobile phase consisting of acidic phosphate buffer, acetonitrile and methanol was used. After a series of modifications of the phosphate concentration, pH value and organic solvent, a good separation condition was obtained. By referring to the UV absorption of the compounds, 254 nm was selected as the detection wavelength.

When methanol was used as organic modifier, the numbers of theoretical plates of most peaks were low. As B pump solvent was fixed at the composition of H_2O/CH_3CN = 2/8 and the composition of A pump solvent phase was changed, it was found that without phosphate and phosphoric acid, the three carboxylic acids **1, 2** and **5** were eluted early and poorly separated. After addition of phosphate, the sharpness of various peaks increased markedly and **3** and an unknown peak **a** were separable (peak **a**, unidentified peak), but their numbers of theoretical plates were still low. As phosphoric acid was also added, it not only improved the peak shapes of **1** and **3** but also helped to sharpen the other peaks. Finally, it was found that a buffer solution consisting of 20 mM KH_2PO_4 and 0.1% H_3PO_4 offered an optimal separation.

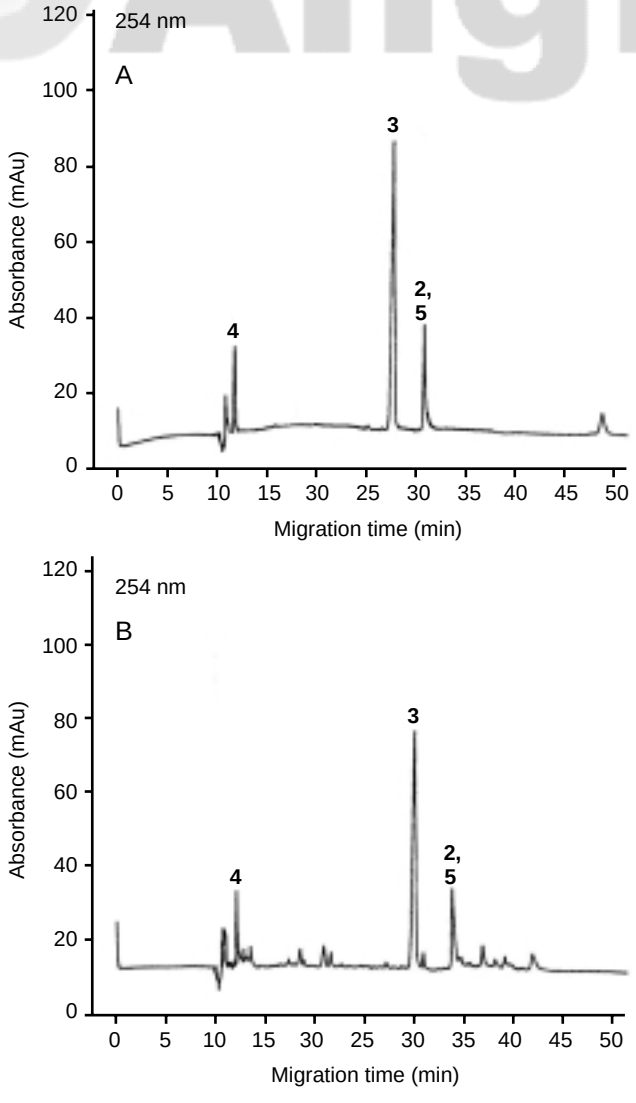

Figure 2. Electropherograms of (A) five authentic marker substances and (B) methanol-water extract of Xanthii Fructus.

Different concentration ratios of KH_2PO_4 buffer solution (for pump A) and H_2O/CH_3CN (for pump B) ranging from 0 to 50 mM were used for studying the effect of KH_2PO_4 on separability. As phosphate was added to the mobile phase, the numbers of theoretical plates of several compounds increased. This was due to the fact that the ion of phosphate salt covered the residual Si-OH groups. Finally, 20 mM phosphate was chosen owing to its higher resolution (Rs) (2.1 for **3**/**a**; below 2.0 for the others). To investigate the effect of pH on retention behavior, experiments were performed with 20 mM phosphate at different pH values ranging from 2.5 to 6.5 by mixing the buffer with 0.1% H_3PO_4. Results showed that a solution of pH 2.5 could separate the constituents well except **2** and **5**. At lower pH values, the peaks of **3** and **a** were partially overlapped. The peak of **1, 2** and **5** became broader at higher pH conditions (for **1:** N values were 18258, 15021 and 3564 at pH 3.5, 4.5 and 5.5, respectively). Hence, we chose 2.5 for pH value in the analysis.

The composition of mobile phase B was optimized to be 80/20 (acetontrile/water, *v*/*v*). The running time for the separation of all five compounds was about 60 mins (Figure 3A). As methanol-water extract of the Xanthii Fructus sample was injected directly and analyzed, the results were as good as those obtained with the standard solution (Figure 3B).

III. *Comparison Between HPLC and CE*

Due to the differences in separation mechanism, the migration time and retention time of compound **1** was 49 and 20 mins, for CE and HPLC respectively. From Figure 2B and 3B, peak **1** was present in HPLC chromatogram but

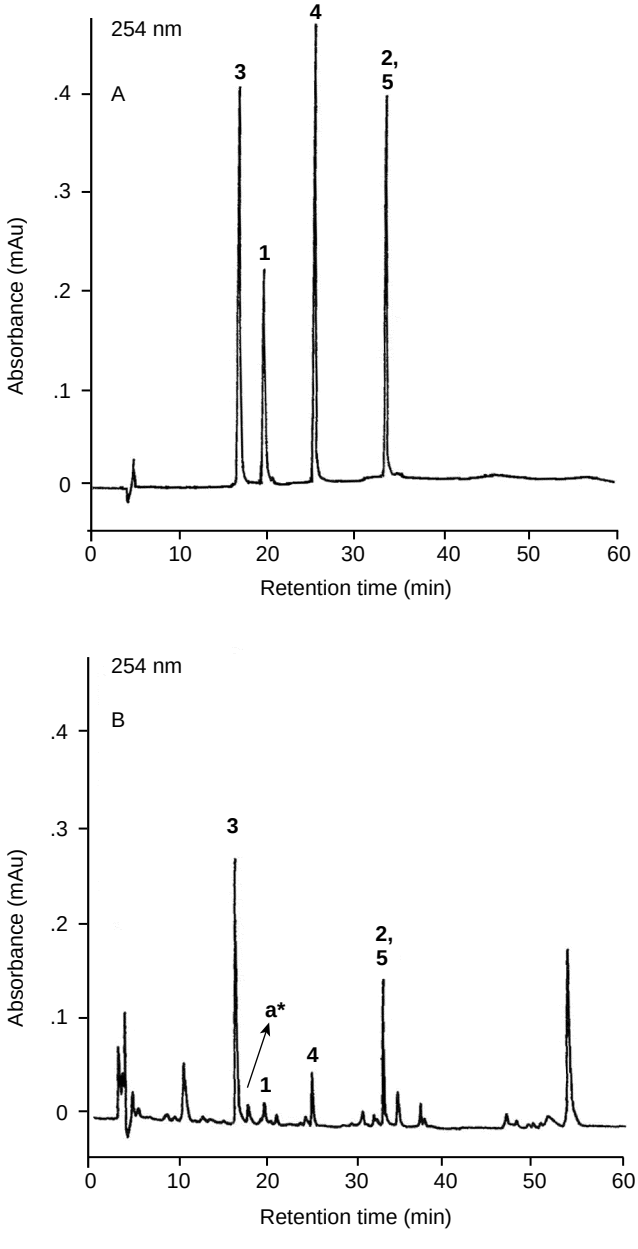

Figure 3. Chromatograms of (A) five authentic marker substances and (B) methanol-water extract of Xanthii Fructus.

Table 1. Data for linear ranges, correlation coefficients (r) and detection limits

Constituent	CE			HPLC		
	Linear range (μg/mL)	r	Detection Limit (μg/mL)	Linear range (μg/mL)	r	Detection Limit(μg/mL)
1	5.0-100.0	0.9965	4.35	1.0-100.0	0.9989	0.60
2	5.0-100.0	0.9983	3.34	5.0-100.0	0.9998	0.52
3	5.0-150.0	0.9975	4.83	5.0-150.0	0.9996	0.65
4	10.0-50.0	0.9954	8.51	1.0-50.0	0.9996	0.98
5	5.0-100.0	0.9964	3.86	5.0-100.0	0.9995	0.54

Table 2. Reproducibility of the marker substances

Constituent	CE (R.S.D, %, n = 6)				HPLC (R.S.D, %, n = 6)			
	Migration time		Peak-area		Retention time		Peak-area	
	Intraday	Interday	Intraday	Interday	Intraday	Interday	Intraday	Interday
1	3.72	4.65	3.25	3.67	0.37	0.42	0.98	1.12
2	3.76	5.26	3.38	3.98	0.43	0.46	0.88	0.86
3	3.00	3.98	2.96	3.00	0.65	0.60	1.70	2.15
4	2.82	2.89	2.24	3.14	0.42	0.51	0.69	1.02
5	3.65	5.02	2.56	4.07	0.45	0.50	0.87	0.98

Table 3. Recoveries of compound **3** and **4** (n = 3)

Constituent	CE		HPLC
	Added (μg)	Recovery	Recovery
3	20.0	102.43	99.99
	100.0	102.37	100.32
4	20.0	101.43	98.57
	100.0	99.79	97.88

not found in CE electropherogram. To investigate this phenomenon, when **2** and **5** were dissolved in 0.1 % H_3PO_4 aquous solution, Compound **1** was obtained. We could conjecture that **1** maybe not the ingredient of Xanthii Fructus but resulted from the acidic analysis condition. Compound **2** and **5**, derivatives of caffeic acid, were overlapped in CE or LC. Their separation should further be investigated.

IV. *Method Validation*

The linear ranges and the correlation coefficients for these compounds are given in Table 1. The regression equations of the constituents were as follows: **1**, $y = 1.356 \times 10^2x - 69.46$; **2**, $y = 4.684 \times 10^2x - 83.56$; **3**, $y = 2.156 \times 10^2x - 234.8$; **4**, $y = 2.377 \times 10^1x - 13.15$; **5**, $y = 4.146 \times 10^2x - 169.1$ (in CE system); and **1**, $y = 5.432 \times 10^2x - 134.6$; **2**, $y = 2.356 \times 10^3x + 11.13$; **3**, $y = 6.758 \times 10^2x - 169.22$; **4**, $y = 6.266 \times 10^2x - 35.86$; **5**, $y = 1.356 \times 10^3x + 52.96$ (in HPLC system).

Reproducibility (for initial stock solution) of the proposed methods, on the basis of the peak-area in six replicate injections), was 2.24 – 4.07% (for CE system) and 0.69 – 2.15% (for HPLC system). The relative standard deviation of the retention time of each peak for six replicate injections was less than 5.26% (CE system) or 0.65% (HPLC system). The detailed data for individual constituents are given in Table 2.

Suitable amounts (10.0 ~ 100.0 μg) of the marker substances were added to a pretreated sample of Xanthii Fructus. The recoveries of compound **3** and **4** determined by either method were around 97.88~102.43% (n = 3) as shown in Table 3. The tailing factors of all peaks were very close to unity. The detection limit (S/N = 3) of the individual marker substances in CE system varied from 3.34 to 8.51 μg/mL and in HPLC system from 0.52 to 0.98 μg/mL as shown in Table 1.

V. *Determination of the Marker Substances in Xanthii Fructus*

When the test solutions were analysed by HPLC and CE under the selected conditions, Figure 2B and 3B were obtained and the contents of two constituents in a Xanthii Fructus were calculated (mean ± S.D.; n = 3): **3**, 16.7 ± 0.42 mg/g; **4**, 5.38 ± 0.17 mg/g (by CE); **3**, 17.6 ± 0.38 mg/g; **4**, 5.53 ± 0.10 mg/g (by HPLC).

CONCLUSION

In conclusion, by optimizing the pH, buffer composition and buffer concentration of the mobile phase, potassium 3-O-caffeoyl quinate and 7-hydroxymethyl-8, 8-dimethyl-4,8-dihydro-benzo[1,4]thiazine-3,5-dione in Xanthii Fructus extract could be determined by HPLC within 60 mins and by CE within 50 mins. The two proposed methods showed good linear relationships between the peak-area and concentrations, acceptable reproducibility and high accuracy. Generally, the former method was somewhat superior to the latter method in either reproducibility or sensitivity, but longer analysis time.

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