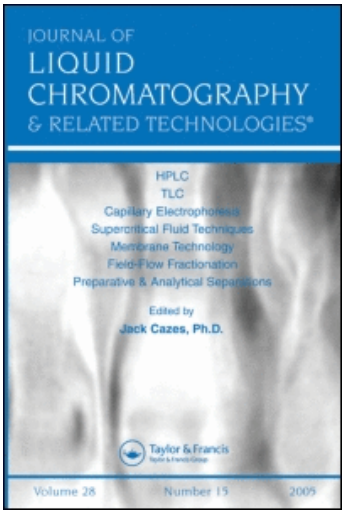

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Abstract: A facile method, based on high performance liquid chromatography (HPLC) with diode array detection (DAD), was developed for the simultaneous quantitative determination of nine xanthones in the root of *Polygala caudata* Rehd. et Wils., a Chinese folk medicinal herb. The HPLC fingerprint of xanthones was developed for the first time and applied to assess the quality of related *Polygala* medicinal plants. Ultrasonic extraction with chloroform-methanol (98 : 2) gave good yields of nine xanthone standards, the average recovery rates ranged from 94.1 to 113.4% (n = 5). The relative standard deviation (RSD) values for reproducibility were between 2.21 and 9.21% (n = 5). The fingerprint chromatograms of *P. tenuifolia* Wild. from six different planting regions were analyzed to set up a simulative mean chromatogram with 11 common peaks. The software of Similarity Evaluation System for Chromatographic Fingerprint of TCM (2004A version), developed by Chinese Pharmacopoeia Commission, was applied for data processing. The

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correlation coefficients of each chromatogram to the simulative mean chromatogram of *P. tenuifolia* were within the range of 0.807 ~ 0.927, and the correlation coefficients between each chromatogram were 0.689 ~ 0.907. The method can be used for the quality evaluation and control of Radix Polygalae, and provide a possibility for identifying the *Polygala* species rapidly.

Keywords: *Polygala caudata*, *Polygala L.*, Xanthones, HPLC, Quantitative determination, Fingerprinting

INTRODUCTION

Radix Polygalae, the root or bark of *Polygala tenuifolia* Wild. or *P. sibirica L.* (Polygalaceae), is a well-known traditional Chinese medicine (TCM), which has been traditionally used as a sedative, expectorant, and resuscitating agent. It is widely used in southern China as an expectorant, an anti-inflammatory for pharyngitis, and as an antibacterial agent.[1] *Polygala caudata* Rehd. et Wils., another plant of genus *Polygala L.*, has also been used as Radix Polygalae instead of *P. tenuifolia* and *P. sibirica* in the population of southwestern China.[2]

Previous chemical research demonstrated that plants of genus *Polygala* mainly contained xanthones,[3] sapoins,[4] and oligosaccharides.[5,6] In modern pharmacological researches, xanthones had shown various bioactivities, such as anti-tumor,[7,8] anti-inflammatory,[9] anti-thrombotic,[10] and antimicrobial activities.[11,12] Recently, xanthones with neuro-pharmacological properties have also been reported.[13,14] Xanthone derivatives isolated from *P. cyparissias* have been shown to inhibit neurogenic nociception in rats.[15] Euxanthone (1,7-dihydroxyxanthone) separated from *P. caudata* also showed effects on neuronal differentiation.[16] Only a little work on the analysis for xanthones in *Polygala L.* has been reported,[17] and it failed to assess the whole quality of xanthone components.

High performance liquid chromatography (HPLC) methods have been the most successful for constituent analysis in herbal medicine. It also has the advantage of sensitivity, high separation degrees, precision, and rapidness in the course of quantitative determination of xanthone components in *Polygala* plants. Furthermore, chromatographic fingerprinting was a recently developed technology for evaluating and controlling the quality of TCM.[18] Contrary to the conventional quality control approaches, chromatographic fingerprint analysis focuses on the whole characteristic chromatogram. It can comprehensively and clearly reveal the distribution and contents of each chemical ingredient in plant samples, and therefore assess the similarities among different samples. Samples were separated in a chromatographic column and detected at an appointed wavelength. With the retention time (RT) and peak area (PA), the quality and quantity of samples were assessed effectively. Furthermore, the professional software, Similarity

Evaluation System for Chromatographic Fingerprint of TCM developed by the Chinese Pharmacopoeia Commission, was applied for analysis of the chromatographic fingerprints, and the similarities between different samples were expressed as correlative coefficients visually.

In the present study, a HPLC method was developed for simultaneous quantitative determination of nine xanthones in *P. caudata*, and the HPLC fingerprint of xanthone was employed to evaluate the quality of related *Polygala* medicinal plants for the first time. The method can be used for determination of xanthone constituents in *Polygala L.*, as well as provide a possibility for identifying the *Polygala* species rapidly.

EXPERIMENTAL

Apparatus

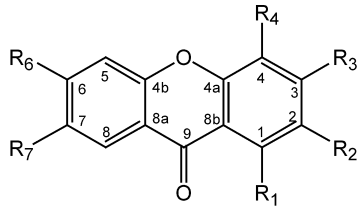
HPLC analysis was performed in the Agilent 1100 automatic HPLC system (HP Agilent Technologies, US), with the G1311A quatpump solvent delivery system, UV-Vis photodiode array detector (DAD) G1315B, and degasser G1379A. A reverse phase HPLC column, Synergi 4 μ Fusion-RP 80A C_{18} Column, 4 μm, 4.6 × 250 mm (Phenomenex, US) was employed.

Chemicals

Acetonitrile (HPLC grade) was from Fisher chemicals (Loughborough, UK). TFA (Trifluordcetic Acid, analytical grade) was from Acros Organic (US). Methanol (analytical grade) was from Tianjin Shield Company (Tianjin, China). Double distilled water was used throughout the experiment.

Preparation of Standards and Sample Solutions

Nine xanthone standards were all isolated from the roots of *P. caudata.* Their structures were established on the basis of spectral evidence as 2,7-dihydroxy-1-methoxyxanthone (**1**), 7-hydroxy-1-methoxyxanthone (**2**), 2-hydroxy-1,6,7-trimeth-oxyxanthone (**3**), 1,3,7-trihydroxylxanthone (**4**), 3-hydroxy-1,2-dimethoxyxanthone (**5**), 2-hydroxy-1,7-dimethoxyxanthone (**6**), 1,4-dimethoxy-2,3−methylenedioxy-xanthone (**7**), 1,7-dihydroxy-xanthone (**8**), and 1,3-dihydroxy-2-methoxyxanthone (**9**) (Fig. 1). The purities of the standards were tested by HPLC, and the contents were all over 98% after determination with the area normalization method. The nine xanthone standards were prepared in methanol to create a standard mixture solution with concentrations of 21.1, 24.4, 20.0, 22.2, 23.3, 20.0, 23.3, 24.4, and 23.3 $\mu g \cdot mL^{-1}$ for compounds **1** ∼ **9**, respectively.



1	R_1=OMe	R_2=R_7=OH	R_3=R_4=R_6=H
2	R_1=OMe	R_7=OH	R_2=R_3=R_4=R_6=H
3	R_1=R_6=R_7=OMe	R_2=OH	R_3=R_4=H
4	R_1=R_3=R_7=OH	R_2=R_4=R_6=H	
5	R_1=R_2=OMe	R_3=OH	R_4=R_6=R_7=H
6	R_1=R_7=OMe	R_2=OH	R_3=R_4=R_6=H
7	R_1=R_4=OMe	$R_{2,3}$=OCH_2O	R_6=R_7=H
8	R_1=R_7=OH	R_2=R_3=R_4=R_6=H	
9	R_1=R_3=OH	R_2=OMe	R_4=R_6=R_7=H

Figure 1. Structures of nine xanthone standards (**1** ∼ **9**).

The roots of *P. caudata* (C-1) used for quantitative determination were collected in September 2002 from the Guizhou Province of China. Fifteen samples were collected for fingerprint analysis, including three shrubs, *P. arillata, P. caudata*, and *P. fallax*, and five herbs, *P. glomerata, P. glomerata* Lour. var. *vallosa, P. japonica, P. sibirica*, and *P. tenuifolia.* All of the shrub samples were tested with the roots, while the herb samples were tested with the whole herbs, except for *P. tenuifolia*, which was tested with the roots as it is its traditional usage (Table 1). All samples were authenticated by Prof. Pei G. Xiao in the Institute of Medicinal Plant Development, Peking Union Medical College & Chinese Academy of Medical Sciences, where the voucher specimens were deposited.

An accurately weighed sample powder of 2.0 g was extracted with 60 mL of chloroform-methanol (98 : 2, v/v) in ultrasonic conditions for 30 min. The extract was filtered and concentrated in vacuo to a residue, then dissolved in methanol, and diluted to 2 mL in a volumetric flask. The solution was then filtered through a 0.45 μm filter to obtain a clear sample solution.

Conditions for HPLC

Chromatography was carried out with a linear gradient program. The solvents were (A) 0.05% TFA-H_2O and (B) acetonitrile. The gradient of A : B was 0–3 min, 77 : 23; 3–54 min, linear gradient up to 54 : 46; 54–74 min, up to 30 : 70; 70–80 min, B achieved 100%. The flow rate was kept constant at 0.8 mL · min^{-1} and injection volume was typically 20 μL. The DAD detector was set at 265 nm for acquiring a chromatogram in 80 min. UV

Table 1. A summary of the tested samples

No.	Sample code[a]	Species	Botanical characteristic	Sampling Parts	Sources	Date of collection
1	A-1	*P. arillata*	Shrub	Root	Yunnan	1989, 01
2	C-1	P. caudata	Shrub	Root	Xinyi, Guizhou	2002, 10
3	F-1	*P. fallax*	Shrub	Root	Jingxi, Guangxi	2001, 10
4	F-2	*P. fallax*	Shrub	Root	Yining, Guangxi	2001, 11
5	F-3	*P. fallax*	Shrub	Root	Shangrao, Jiangxi	2002, 10
6	G-1	*P. glomerata*	Herb	Whole	Naning, Guangxi	2004, 08
7	G-2	*P. glomerata* Lour. var. *vallosa*	Herb	Whole	Guangxi	2004, 10
8	J-1	*P. japonica*	Herb	Whole	Lipu, Guangxi	2004, 08
9	S-1	*P. sibirica*	Herb	Whole	Beijing	2004, 08
10	T-1	*P. tenuifolia*	Herb	Root	Zhengzhou, Henan	2004, 05
11	T-2	*P. tenuifolia*	Herb	Root	Shanxi	2003, 12
12	T-3	*P. tenuifolia*	Herb	Root	Bozhou, Anhui	2004, 03
13	T-4	*P. tenuifolia*	Herb	Root	Anguo, Hebei	2001, 11
14	T-5	*P. tenuifolia*	Herb	Root	Shanxi	2001, 10
15	T-6	*P. tenuifolia*	Herb	Root	Zhejiang	2004, 05

[a]Nine xanthone standards were all isolated from C-1, and their contents in C-1 were determined. Samples T-1 $\sim$ T-6 were analyzed by the software of Similarity Evaluation System for Chromatographic Fingerprint of TCM.

spectra was acquired from 200 to 400 nm. The column temperature was maintained at 24 $\pm$ 1°C.

Software

The Similarity Evaluation System for Chromatographic Fingerprint of TCM (2004A version) software, developed by the Chinese Pharmacopoeia Commission, was applied to generate a simulated mean chromatogram as a representative chromatogram for further fingerprint analysis. The correlation coefficient of each chromatogram to the simulative mean

chromatogram and those between every two chromatograms were calculated by this software.

RESULTS AND DISCUSSION

Development of Extraction Solvent

Three solvent systems of methanol, petrol-acetic ether, and chloroform-methanol were compared to develop the best extraction solvent for xanthones. The nine xanthone standards were selected as marker components. The methanol extract contained a great deal of high polarity components but fewer xanthone components corresponding to the markers. Xanthone ingredients failed to be extracted completely under the solvent system of petrol-acetic ether. The solvent systems of chloroform-methanol provided a good yield of marker components with little disturbance of high polarity components. The solvent system was finally optimized to be chloroform-methanol (98 : 2).

Quantitative Analysis of Nine Xanthones in *P. caudata*

The standard mixture solutions of 2, 4, 6, 8, and 10 μL were injected in a series to determine the linearity for all analytes. The calibration curves were evaluated by using the linear regression equation derived from peak area (Y) to amounts (X). The detection limits corresponding to amounts gave signal-to-noise ratio of 3 (S/N = 3). Data for the calibration curves and the detection limits were collected in Table 2. The precision of the method was evaluated by repetitive analyses of a single sample, and the RSD of retention time (RT) and peak area (PA) of compounds **1** ∼ **9**

Table 2. Data for the calibration curves and the detection limits

Compound	Regression equation (X: ng, Y: mAU)	Correlation coefficient	Linear range (ng)	Detection limit (ng)
1	$Y = 9.581X + 13.258$	0.9999	42.2 ∼ 211.1	0.127
2	$Y = 4.536X + 6.762$	0.9999	48.9 ∼ 244.4	0.367
3	$Y = 6.305X + 7.398$	0.9999	40.0 ∼ 200.0	0.300
4	$Y = 6.969X + 9.675$	0.9999	44.4 ∼ 222.2	0.222
5	$Y = 2.175X + 2.997$	0.9999	46.7 ∼ 233.3	1.167
6	$Y = 9.956X + 11.317$	0.9999	40.0 ∼ 200.0	0.200
7	$Y = 4.619X + 6.596$	0.9999	46.7 ∼ 233.3	0.700
8	$Y = 9.659X + 21.276$	0.9994	48.9 ∼ 244.4	0.061
9	$Y = 3.290X + 5.813$	0.9999	46.7 ∼ 233.3	0.933

showed the values of 0.19 ~ 0.33% and 1.99 ~ 4.50% (n = 6), respectively. Five replicates of the identical sample were prepared to test the reproducibility, and the RSDs of PAs lay in the range of 2.21 ~ 9.21% (n = 5). The recovery experiment was carried out to evaluate the accuracy of the method. Known amounts of analytes were added to the sample in which the original amounts of the nine analytes had been determined at first, then extracted, and analyzed accordingly. The amounts of these nine analytes were calibrated and calculated. The average recoveries and the RSDs were in the range of 94.1 ~ 113.4% and 2.24 ~ 7.36% (n = 5). The results of precision, reproducibility, and recovery experiments were shown in Table 3.

Contents of the nine xanthones in *P. caudata* samples (Table 1) were quantitatively determined. Results were presented in Table 3. The HPLC chromatograms of the mixture of standards and *P. caudata* sample were displayed in Fig. 2.

Analysis of HPLC Fingerprint

All the collected samples were prepared and analyzed under the conditions mentioned above. Their fingerprints were obtained as Fig. 3. A great diversity was shown in the fingerprints of different species or resources. The number and intensity of the peaks in the fingerprint chromatogram of

Table 3. Results of precision, reproducibility, recovery experiments and determination of the content of the nine xanthones in *P. caudata*

	Precision (RSD %, $n = 6$)		Reproducibility (RSD %, $n = 5$)	Recovery ($n = 5$)		Content in *P. caudata* ($n = 3$)[a]	
Compound	RT	PA	PA	Average %	RSD %	μg/g	RSD %
1	0.33	1.99	3.16	99.9	4.39	81.2	3.52
2	0.26	2.63	2.89	103.6	2.77	30.9	2.69
3	0.19	3.04	5.12	94.1	2.24	22.9	2.04
4	0.22	4.50	5.77	96.3	7.36	24.7	2.44
5	0.22	3.88	9.21	108.9	5.73	53.0	0.85
6	0.22	3.19	6.07	101.2	2.71	26.4	2.19
7	0.21	2.27	4.57	111.0	2.51	97.2	1.45
8	0.29	2.69	2.21	100.7	3.70	424.2	0.06
9	0.24	2.68	4.17	113.4	3.33	69.1	1.62

[a]Three individual samples of *P. caudata* were analyzed. The amounts of xanthones were expressed in μg/g of dried crude roots. Values shown were the average of three individual samples.

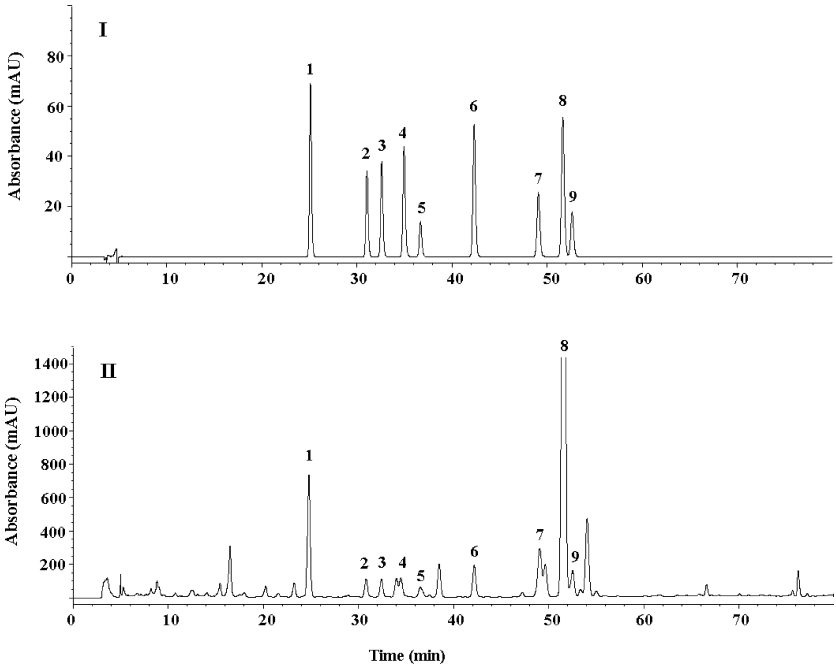


Figure 2. HPLC chromatograms of the mixture of standards (**I**) and *P. caudata* sample (**II**).

P. caudata were much higher than other species. Since there were little peaks with low abundance in the fingerprints of *P. arillata, P. glomerata*, and *P. glomerata* var. *vallosa*, it is hard to determine the common peaks in the fingerprint chromatograms from different species. The varieties in the chemical components may be related to their traditional medical usage. The relationships need to be studied further. On the other hand, the varieties in HPLC fingerprints provide the possibility for identifying the *Polygala* species rapidly.

The HPLC fingerprints of *P. tenuifolia* were analyzed to confirm 11 common peaks (A $\sim$ K) in spite of the difference in peak abundance (see Fig. 3). In addition, the correlation coefficient of each chromatogram to the simulative mean chromatogram was evaluated in the range of 0.807 $\sim$ 0.927. The correlation coefficients between every two chromatograms were within the range of 0.689 $\sim$ 0.907, as presented in Table 4. The contents of xanthones exhibit variation in the samples of different resources. This could probably be because of the influence of the growing soil, climate, or the growth stage. Results indicate that it is important to control the growing conditions for maintaining the quality of TCM.

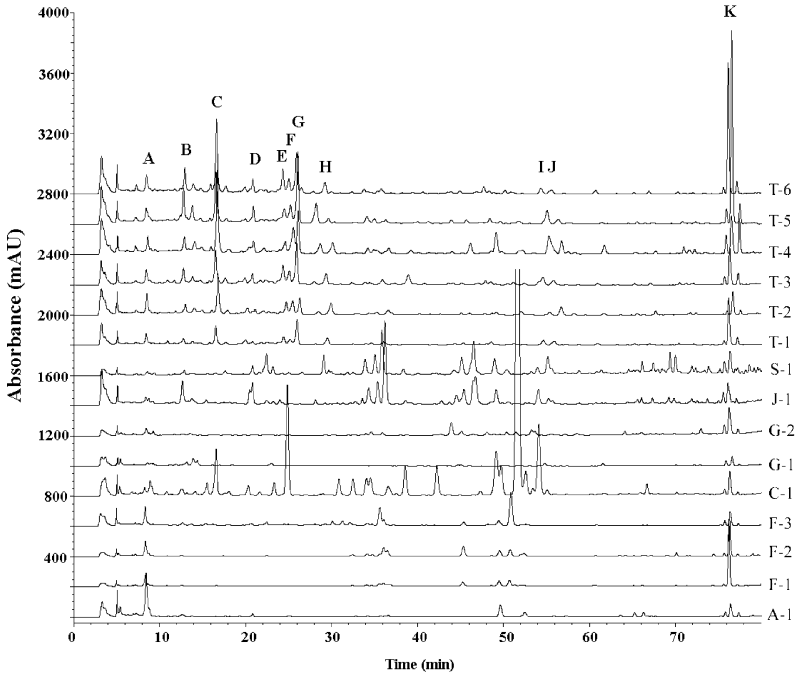


Figure 3. Fingerprints of all tested samples described in Table 1.

CONCLUSION

As the main constituents in Radix Polygalae, xanthone was a principal marker for the quality assessment and control of raw herbs. In the present study, a simple analysis method based on HPLC was successfully

Table 4. The correlation coefficients of each chromatogram to the simulative mean chromatogram and the correlation coefficients between each chromatogram of *P. tenuifolia* samples described in Table 1

	T-1	T-2	T-3	T-4	T-5	T-6	Simulative mean chromatogram
T-1	1.000	0.805	0.851	0.689	0.907	0.899	0.877
T-2	0.805	1.000	0.767	0.753	0.775	0.825	0.927
T-3	0.851	0.767	1.000	0.696	0.792	0.844	0.875
T-4	0.689	0.753	0.696	1.000	0.681	0.752	0.807
T-5	0.907	0.775	0.792	0.681	1.000	0.920	0.860
T-6	0.899	0.825	0.844	0.752	0.920	1.000	0.916
Simulative mean chromatogram	0.877	0.927	0.875	0.807	0.86	0.916	1.000

developed to simultaneously quantify nine xanthone compounds in the root of *P. caudate*. The current HPLC method was found to be rapid, accurate, and reproducible.

With HPLC fingerprint chromatography coupling with the aid of professional analysis software, the distribution profiling of xanthone and the similarities of each sample are determined. Consequently, this fingerprinting method could be applied for the quality control and specie identification of Radix Polygalae samples from different varieties or growing regions.

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