Fingerprinting Analysis of Four Variants of *Chrysanthemi Morifoli Flos* by RP-HPLC

SHEN Zhi[1], ZHANG Wen-ting[2], HUA Yun-fei[1], ZHAO Wei-liang[2*]

1. College of Pharmacy, Zhejiang University of Traditional Chinese Medicine, Hangzhou 310053, China
2. Zhejiang Institute for Food and Drug Control, Hangzhou 310004, China

Abstract: **Objective** To establish a RP-HPLC fingerprinting analysis method for quality evaluation and control of the four variants of *Chrysanthemi Morifoli Flos*. **Methods** RP-HPLC was used to establish the fingerprinting method. **Results** Despite of the similarity in terms of holistic HPLC chromatograms, the four variants of *Chrysanthemi Morifoli Flos* exhibit characteristic fingerprints and can be readily recognized by similarity clusters. **Conclusion** A simple and reliable HPLC fingerprinting method has been developed and validated to authenticate the four variants of *Chrysanthemi Morifoli Flos*, providing a scientific basis for quality control of *Chrysanthemi Morifoli Flos*.

Key Words: *Chrysanthemi Morifoli Flos*; fingerprint; quality control; RP-HPLC
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Introduction

Chrysanthemi Morifoli Flos, a traditional Chinese medicine (TCM), is the dried flowers of *Chrysanthemum morifolium* Ramat. *Boju*, *Hangju*, *Gongju*, and *Chuju* which are embodied in *Chrysanthemi Flos* of *Pharmacopoeia of the People's Republic of China* (Pharmacopia Committee of P.R.China, 2005), are similar to each other in their external appearance. In order to scientifically differentiate them, fingerprints of four variants of *Chrysanthemi Morifoli Flos* are studied by HPLC in this paper. Flavonoids and organic acids such as chlorogenic acid, luteolin, baicalin, apigenin, quercetin, and chrysin had been taken mainly as the reference substance in the study on fingerprint of *Chrysanthemi Morifoli Flos* by HPLC (Zhao *et al*, 2004; Cheng *et al*, 2000; Hu *et al*, 2004). In this paper, 3, 5-*O*- dicaffeoylquinc acid (Ma *et al*, 2005; Hu and Chen, 1997), and luteolin-7-*O*-β-*D*-glucoside (Gu and Qin, 2006) are taken as the reference substance in the study of fingerprint by HPLC, which has not been reported in related research. Forty batches of samples are deter- mined, and carried on the comparison to four variants of *Chrysanthemis Morifoli Flos*, of which the similarities have evident discrepancy. The study of so many samples has not been reported in related research, too. The acquired fingerprint of *Chrysanthemi Morifoli Flos* with the method provides the scientific evidence for the quality evaluation and control of *Chrysanthemi Morifoli Flos*.

Materials and methods

Apparatus

Shimadzu LC-20AT HPLC chromatograph (SPD-M20A with a diode array detector, a quaternary pump, DGU-20AS degasser, SIL-10AT auto sampler, and CTO-20AT column oven); KQ300VDB ultrosonic instrument (Kunshan, China); METTLER AE240 analytic balance (Mettler Toledo, Switzerland).

Materials

Forty batches of *Chrysanthemi Morifoli Flos* samples were purchased from different drugstores or factories in China, and were authenticated as flowers of *Chrysanthemum morifolium* Ramat by GUO Zeng-xi of Zhejiang Institute for Food and Drug Control. The various sources of *Chrysanthemi Morifoli Flos* samples were shown in Table 1. Chlorogenic acid (Batch no: 110753-200413), luteolin (Batch no: 111520-200504) and luteolin-7-*O*-β-*D*-glucoside (Batch no: 111720-200501)

* Corresponding author: Zhao WL Address: Zhejiang Institute for Food and Drug Control, 86 Jichang Road, Hangzhou 310004, China
Tel/Fax: +86-571-8645 2373 E-mail: zwl@zjyj.org.cn
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Table 1 The sources of samples

No.	Sample name	Collection location
1–13	*Hangju*	Tongxiang, Zhejiang
14–16	*Chuju*	Chuzhou, Anhui
17–25	*Boju*	Bozhou, Anhui
26	*Boju*	Baoding, Hebei
27–32	*Gongju*	Huangshan, Anhui
33–36	*Hangju*	Sheyang, Jiangsu
37–39	*Hangju*	Yuncheng, Shanxi
40	*Hangju*	Macheng, Hubei

were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. 3,5-*O*-dicaffeoylquinic acid is purchased from Delta company (purity > 98.0%, China). Acetonitrile was HPLC grade (Honeywell International Inc. USA). Water is double distilled and other reagents are of analytically grade.

HPLC conditions and systematic compatibility test

Column: Ultimate^{TM} XB-C_{18} (250 mm × 4.6 mm, 5 μm). Gradient elution was used with a mobile phase consisting of acetonitrile (A)-0.1% phosphoric acid (B). The initial condition was set at 10% of A, then gradient up to 18% of A in 11 min, 20% in next 19 min, 30% in next 15 min, and 70% in next 15 min and to keep consistent for 5 min. Detection wavelength: 348 nm. Flow rate: 1.0 mL/min. Column temperature: 25 ℃. Injection volume: 5 μL. Under the chromatographic conditions used, the number of theoretical plates calculated on the peak of 3,5-*O*-dicaffeoylquinic acid was not less than 10 000 and there was a complete baseline separation among other components with a good degree of resolution (*R*s > 1.5).

Preparation of samples

About 0.25 g of the dried powdered sample of Boju (Sample no: 20) was accurately weighed and extracted with 25 mL of 70% aqueous methanol in an ultrasonic water bath for 40 min, allowed to stand to cool. The extracted solution was filtrated through analytical filter paper. The filtered solution filtrated through a 0.45 μm membrane filter unit.

Preparation of standard solutions

The suitable amounts of chlorogenic acid, luteolin-7-*O*-β-*D*-glucoside, 3,5-*O*-dicaffeoylquinic acid, and luteolin were accurately weighed and dispersed together in 70% aqueous methanol, mixed. A stock mixing standard solution of chlorogenic acid (35 μg/mL), luteolin-7-*O*-β-*D*-glucoside (25 μg/mL), 3,5-*O*-dicaffeoyl-quinic acid (80 μg/mL) and luteolin (25 μg/mL) was obtained.

Instrumental precision

About 0.25 g of powdered sample was weighed accurately and extracted by the method of preparation of samples. The sample solution was analyzed repeatedly for six times under above chromatographic system. The chromatograms were recorded. Taking the first chromatogram as the reference atlas, they were analyzed with similarity evaluation system for chromatographic fingerprint of TCM (Version 2004A), which was published by Chinese Pharmacopoeia Commission. Their similarities were all above 0.997 (RSD = 0.1%). The results indicated that the instrumental precision was quite precise.

Test of reproducibility

Six times of 0.25 g of sample were weighed accurately and prepared respectively by the above method. Six chromatograms were recorded according to the above chromatographic system. Taking the first chromatogram as the reference atlas, their similarities were all above 0.998 (RSD = 0.1%) through analysis. The results indicated that the chromatographic method had a good reproducibility.

Test of stability

According to the above chromatographic system, 5 μL of the sample solution was measured in 0, 1, 2, 4, 8, 24, 48 h, respectively. Seven chromatograms were recorded according to the above chromatographic system. Taking the first chromatogram as the reference atlas, they were analyzed with Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A). Their similarities were all 1.000 (RSD = 0.0%). The results showed that the sample solution was stable within 48 h.

Results

Ten batches of *Boju* (Samples 17–26) samples were prepared respectively by the above method, and ten fingerprints were recorded under the above chromatographic system. According to Technical Requirement of the Fingerprint in Injection of Chinese Materia Medica, the fingerprints were matched by Similarity Evaluation System for Chromatographic fingerprint of TCM (Version 2004A). The reference fingerprint was built by

average number and shown in Fig. 1. The similarities all were above 0.930. All of the *Boju* samples had 16 common peaks.

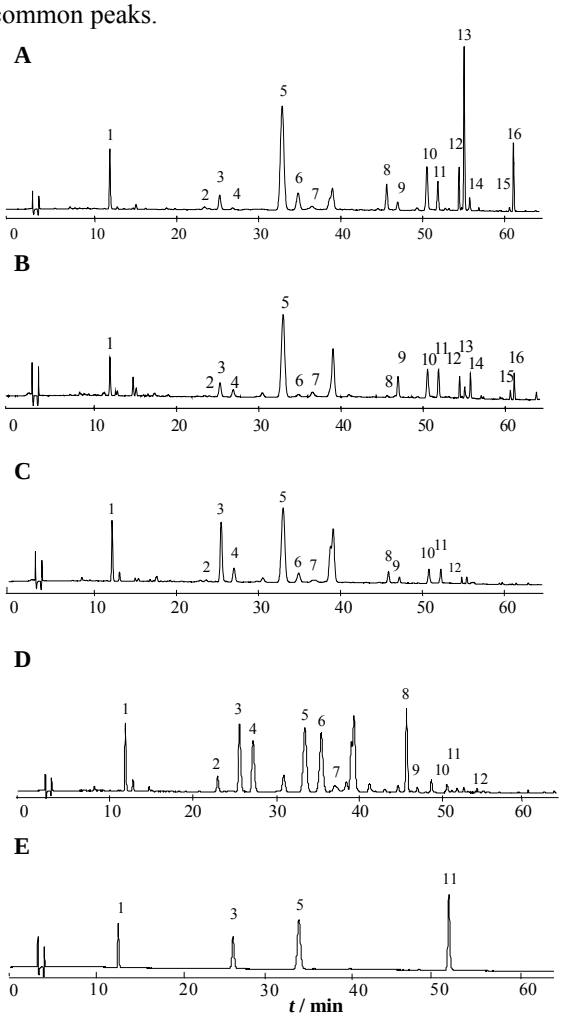

Fig. 1 Fingerprints of four variants of chrysanthemums

A: *Boju* reference fingerprint; B: *Chuju* sample; C: *Gongju* sample; D: *Hangju* sample; E: Reference substance

Peak 1: chlorogenic acid; Peak 3: luteolin-7-*O*-β-*D*-glucoside; Peak 5: 3,5-*O*-dicaffeoylquinic acid; Peak 11: luteolin

The fingerprints of other *Chrysanthemis Morifoli Flos* were recorded by the above method. Taking the *Boju* reference fingerprint as the reference atlas, the fingerprints of samples (40 batches) were matched by Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004B). Their similarities and fingerprints were shown in Table 2 and Fig. 2.

3,5-*O*-dicaffeoylquinic acid was used as the reference substance to select extractive method in this experiment. The content of 3,5-*O*-dicaffeoylquinic acid was determined under the above chromatographic system to compare ultrasonic method with reflux. The results showed that two extractive methods had no evident discrepancy. In addition, different extractive solvents such as methanol, 70% aqueous methanol, ethanol, and 70% aqueous ethanol, different extractive time such as 20, 40, and 60 min and different extractive solvent volumes such as 15, 25, and 50 mL were also tested. Finally, using 25 mL of 70% aqueous methanol as solvent and ultrasonic method to extract sample for 40 min was found to be a simple method with little disturbance.

Table 2 The similarity analysis results of samples

Sample no.	Similarity	Sample no.	Similarity
1	0.632	21	0.995
2	0.607	22	0.996
3	0.625	23	0.993
4	0.573	24	0.977
5	0.632	25	0.980
6	0.619	26	0.918
7	0.627	27	0.832
8	0.630	28	0.798
9	0.521	29	0.820
10	0.578	30	0.795
11	0.598	31	0.838
12	0.591	32	0.806
13	0.637	33	0.796
14	0.902	34	0.773
15	0.867	35	0.701
16	0.936	36	0.796
17	0.991	37	0.634
18	0.969	38	0.640
19	0.984	39	0.653
20	0.978	40	0.642

Different mobile phase systems with different solvent and proportions were compared to select mobile phase, such as acetonitrile-0.05% phosphoric acid, acetonitrile-0.1%phosphoric acid, acetonitrile-0.2% phosphoric acid, and methanol-0.1% phosphoric acid. The optimized effect for isolating primary components was obtained by acetonitrile-0.1% phosphoric acid. To select column temperature, different temperature was compared, such as 25, 30, and 40 ℃. The optimized effect for isolating primary components was achieved by 25 ℃. For the selection of column different types of columns were compared, such as Ultimate^{TM} XB-C_{18} (250 mm × 4.6 mm, 5 μm), Zorbax SB-C_{18} (250 mm × 4.6 mm, 5 μm), Diamonsil-C_{18} (250 mm × 4.6 mm, 5 μm), Venusil MP-C_{18} (250 mm × 4.6 mm, 5 μm), and Extend-C_{18} (250 mm × 4.6 mm, 5 μm). The optimized effect for isolating primary components was obtained by Ultimate^{TM} XB-C_{18}. Different kinds of apparatus were compared too, such as HP 1100, Agilent 1200, Waters 2695, and Shimadzu LC-20AT. The results

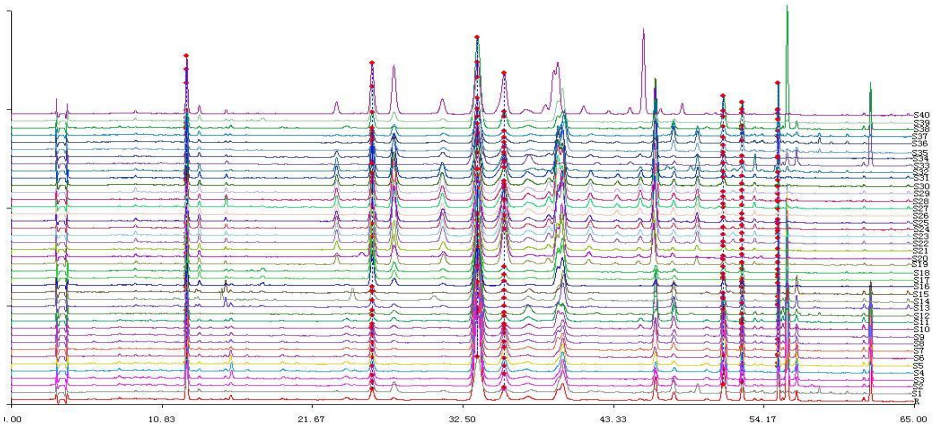

Fig. 2 Fingerprints of 40 batches of *Chrysanthemi Morifoli Flos*

S1–S40: Samples; R: common pattern of *Boju*

showed that they had a good degree of resolution.

The UV spectra of four components were measured under the chromatographic system (DAD detector, 200–400 nm). Maximum wavelength of chlorogenic acid, luteolin-7-*O*-β-*D*-glucoside, 3,5-*O*-dicaffeoylquinic acid and luteolin are 326, 348, 327, and 349 nm, respectively. The peak of luteolin-7-*O*-β-*D*-glucoside could not be separated from impurity at 326 nm. The base line is straight and the impurities have no interference at 348 nm. Finally, 348 nm is chosen as detection wavelength.

Discussion

To select reference substance: *Chrysanthemi Morifoli Flos* contained mainly flavonoids and organic acid. Biological activity and clinical effect of these components are evident. In this paper, taking 3,5-*O*-discaffeoylquinic acid and lutelin-7-*O*-β-*D*-glucoside as reference substance, and chlorogenic acid, luteolin are identified in the *Chrysanthemi Morifoli Flos*.

According to the similarity of samples, *Boju* sample atlas, *Chuju* sample atlas, and *Boju* reference atlas are similar to each other, above 0.910 and 0.860, respectively. The similarities of *Hangju*, *Gongju*, and *Boju* reference atlas are below 0.800 and 0.840, respectively. It is possibly caused by the different geographical environment. *Boju* and *Chuju* grow in north of the Yangtze River, and *Gongju* grows in south of the Yangtze River. Discrepancy of geographical environment results in discrepancy of some components in the *Chrysanthemis Morifoli Flos*. However, this is not unique reason. The similarities between *Hangju* reference atlas of Tongxiang and *Hangju* of Hubei, Jiangsu Province (grow in north of the Yangtze River) are among 0.520–0.796. The similarity between *Hangju* of Tongxiang and *Hangju* of Shanxi province (grow in north of the Yangtze River) is above 0.950.

Taking one of *Boju* (20) samples as the reference atlas, the similarity between *Hangju* and *Boju* is among 0.500–0.800; the similarity between *Chuju* and *Boju* was among 0.820–0.920; the similarity between *Gongju* and *Boju* is among 0.748–0.820. At the same time, the similarities among 13 batches of *Hangju* of Tongxiang samples are above 0.900. The similarities among 3 batches of *Chuju* samples are above 0.930. The similarities among 6 batches of *Gongju* samples are above 0.959. The results show that the similarity among the same type is good and the similarity among the different varieties is low. The method could be applied in authentication of four variants of *Chrysanthemis Morifoli Flos*.

References

Cheng HY, Feng QY, Cao YH, Gong LD, Hou JS, Wang Y, 2000. Determination of active ingredients in *Flos Chrysanthemi* and development of fingerprints by high performance liquid chromatography. *J Anal Sci* 23: 257-262.

Gu YH, Qin MJ, 2006. Chemical constituents of *Chrysanthemum morifolium* Ramat. Boju. *Chin Tradit Herb Drugs* 37(12): 1784-1786.

Hu BB, Jiang HD, Yang J, Zeng S, 2004. Determination of luteolin and luteolin-7-*O*-β-*D*-glucoside in *Chrysanthemum morifolium* Ramat. from different collection time by RP-HPLC. *J Zhejiang Univ (Med Sci)* 33: 29.

Hu LH, Chen ZL, 1997. Structure elucidation of a new *n*-pentyl fructofuranoside in *Dendranthema morifolium* (Ramat.) Tzvel. *Acta Bot Sin* 39: 181-184.

Ma SC, Pual BP, Vincent OE, Spencer HS, Lee SF. 2005. Determination of the antiviral caffeoyl quinic acids isolated from *Lonicera japonica* Thunb. *Chin J Pharm Anal* 25: 751-755.

Zhao YC, Liu GJ, Ren BZ, Qu LB, 2004. Fingerprint of *Flos Chrysanthemi* by HPLC. *Chin Tradit Herb Drugs* 36: 1552-1555.