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Simultaneous analysis of flavonoids from *Hypericum japonicum* Thunb.ex Murray (Hypericaceae) by HPLC-DAD–ESI/MS

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Abstract

A novel and sensitive HPLC-UV method has been developed for the simultaneous determination of five major flavonoids in *Hypericum japonicum* hydroalcoholic extract. The chemical profile of five flavonoids, including taxfolin-7-*O*-α-L-rhamnoside (**1**), isoquercitrin (**2**), quercitrin (**3**), quercetin-7-*O*-α-L-rhamnoside (**4**) and quercetin (**5**) was acquired by using high-performance liquid chromatography-diode array detector coupled to an electrospray tandem mass spectrometer (HPLC-DAD–ESI/MS). The analysis was performed on a ZORBAX SB-C18 analytical column (5 μm, 250 mm × 4.6 mm, i.d.) with a gradient solvent system of acetonitrile-0.5% aqueous formic acid. The validation was carried out and the linearities ($r^2 > 0.9997$) and recoveries (ranged from 98.4% to 99.8%) were acceptable. The limits of detection (LOD) of these flavonoids ranged from 0.5 to 7.5 ng. The results indicated that the contents of investigated flavonoids in *H. japonicum* varied significantly from habitat to habitat with contents ranging from 2.00 to 34.18 mg/g. The proposed method is simple, effective and suitable for the quality control of this traditional Chinese medicine (TCM).
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Keywords: Flavonoids; *Hypericum japonicum*; Hypericaceae; HPLC-DAD–ESI/MS; Simultaneous analysis

1. Introduction

Hypericum japonicum, locally called 'tianjihuang', is prepared from the entire herbs of *H. japonicum* Thunb.ex Murray (Hypericaceae). It is one of traditional Chinese medicine (TCM), widely distributed in south drainage area of the Changjiang River, China [1]. *H. japonicum* has been used for the treatment of bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal hemorrhage and tumors [2–6]. As reported previously, *H. japonicum* mainly contains xanthones [6,7], chromenes [8], flavonoids [9,10], dipeptide derivatives [11] and phloroglucinol derivatives [12]. Of these flavonoids are commonly considered as the major bioactive constituents.

In our previous study [13], eight flavonoids had been isolated from *H. japonicum* by column chromatography (CC). Their chemical structures were identified by spectral analysis (^{1}H NMR, ^{13}C NMR, 2D NMR, MS, UV and IR). Most of the flavonoids were found to have antihepatitis activity [14]. Isoquercitrin, quercitrin, quercetin-7-*O*-α-L-rhamnoside were found to have hepatoprotective and jaundice-relieving effects [15]. Therefore, the simultaneous determination of the flavonoids is significant to ensure the quality of *H. japonicum*. The presently employed quality control methods for the flavonoids in *H. japonicum* and its preparations are mainly based on thin layer chromatography (TLC) [16,17], and high-performance liquid chromatography (HPLC) [17–20]. Until now, to the best of our knowledge, no data have been reported on the simultaneous determination of these flavonoids in *H. japonicum*.

In this paper, a simple and efficient HPLC–ESI/MS method is proposed for the quantification of the major flavonoids in twenty batches of *H. japonicum* from different habitats.

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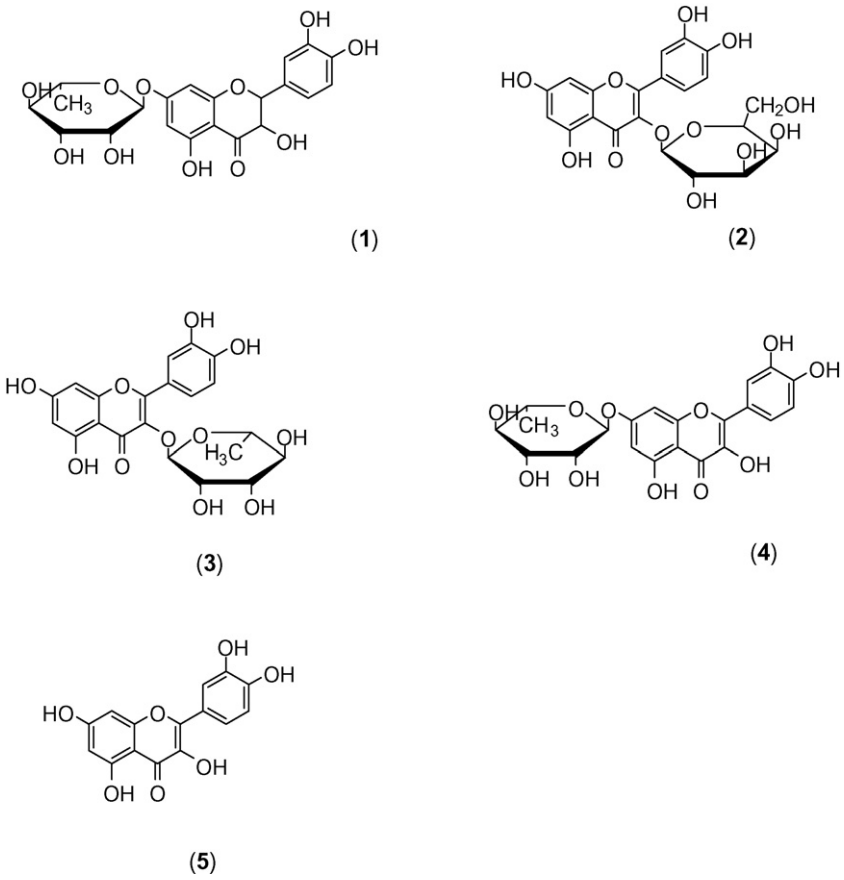

Fig. 1. Chemical structures of the investigated flavonoids in *H. japonicum*. (**1**) taxfolin-7-O-α-L-rhamnoside, (**2**) isoquercitrin, (**3**) quercitrin, (**4**) quercetin-7-O-α-L-rhamnoside and (**5**) quercetin.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile, methanol and formic acid were purchased from Merck Company (Merck, Darmstadt, Germany). Ultrapure water was prepared by Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA). All other solvents used in this study were of analytical grade from Shanghai Chemical Reagent Corporation (Shanghai, China).

The reference standards of the five flavonoids (taxfolin-7-*O*-α-L-rhamnoside, isoquercitrin, quercitrin, quercetin-7-*O*-α-L-rhamnoside, quercetin) were isolated in our laboratory (over 99.5% purity) and their chemical structures (Fig. 1) were identified by spectral analysis [21–24]. The ethanol extract of the whole plant of *H. japonicum* was evaporated *in vacuo*. The residue was suspended in water, and then partitioned with petroleum ether, $CHCl_3$ and EtOAc. The EtOAc extract was repeatedly submitted to column chromatography over silica gel, reverse phase gel (ODS), and Sephadex LH-20 to yield the five flavonoids.

Twenty batches of *H. japonicum* were collected from various habitats as follows: Wuhan City, Hubei Province (lot no. 060705), Tiandeng City, Guangxi Province (lot no. 060802), Zigong City, Sichuan Province (lot no. 060715), Chongqian City (lot no. 060814), Dabie Mountain, Anhui Province (lot no. 060901), Yingtan Ctiy, Jiangxi Province

Table 1
HPLC-ESI/MS data of the MeOH extract of *H. japonicum*

Peak	R_t (min)	MS (*m*/*z*)	MS^2 fragment ion (*m*/*z*)	Identification
1	21.5	451[*M*+H]$^+$	305[*M*+H-Rhamnose]$^+$	Taxfolin-7-O-α-L-rhamnoside
2	30.5	465[*M*+H]$^+$ 487[*M*+Na]$^+$	303[*M*+H-Glucose]$^+$	isoquercitrin
3	37.9	449[*M*+H]$^+$	303[*M*+H-Rhamnose]$^+$	Quercitrin
4	48.6	449[*M*+H]$^+$	303[*M*+H-Rhamnose]$^+$	Quercetin-7-O-α-L-rhamnoside
5	61.0	303[*M*+H]$^+$	285[*M*+H–H_2O]$^+$, 257[*M*+H–H_2O–CO]$^+$, 137[*M*+H–$C_6H_4O_2$–$C_2H_2O_2$]$^+$	Quercetin

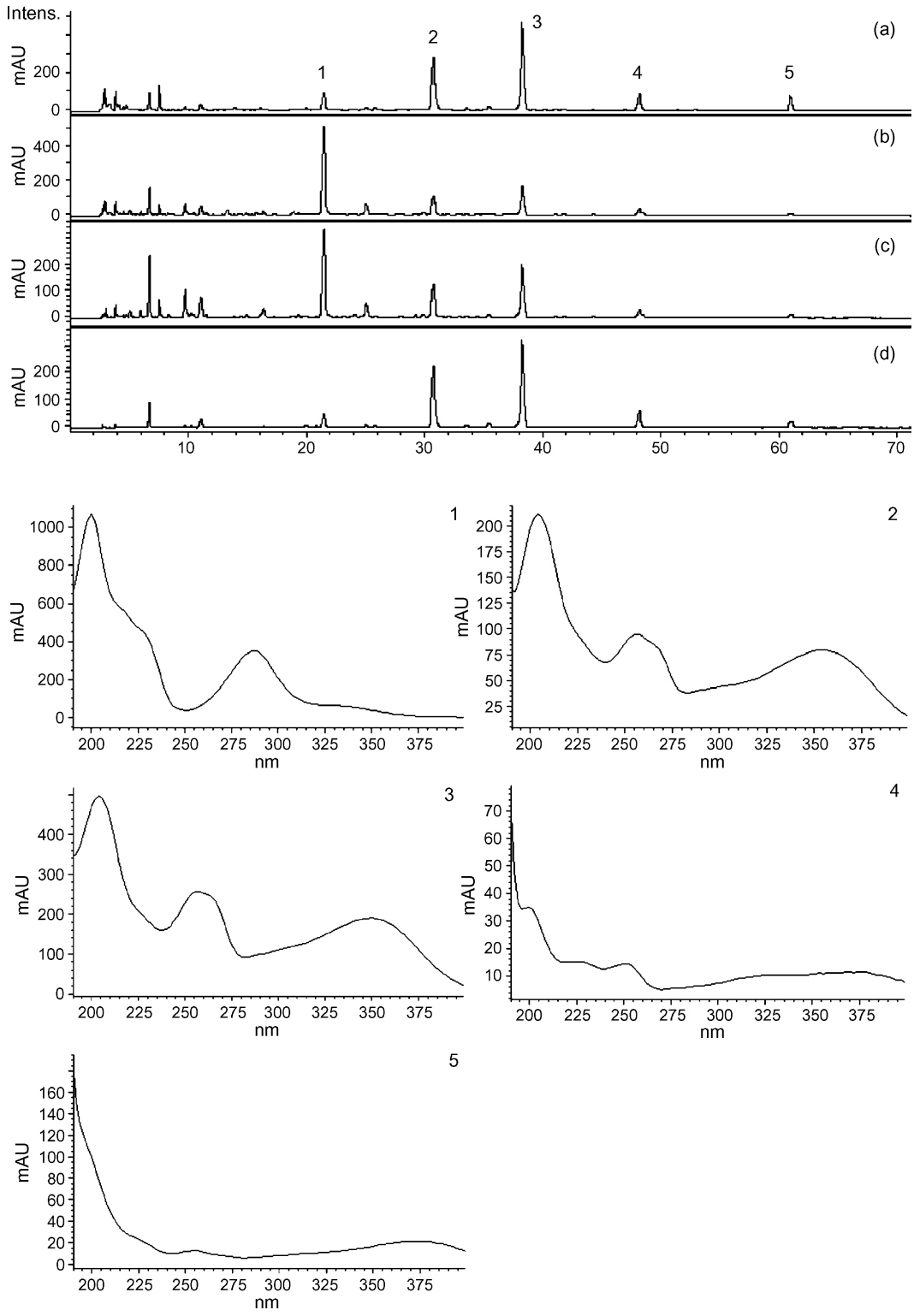

Fig. 2. HPLC-DAD chromatograms of *H. japonicum* (lot no. 060812) using the DAD detector at the wavelength of (a) 256 nm, (b) 280 nm, (c) 300 nm and (d) 354 nm. Identified flavonoids and their UV spectra: (1) taxfolin-7-O-α-L-rhamnoside, (2) isoquercitrin, (3) quercitrin, (4) quercetin-7-O-α-L-rhamnoside and (5) quercetin.

(lot no. 060713), Nanchuan City, Jiangxi Province (lot no. 060801), Jiujiang City, Jiangxi Province (lot nos. 040901, 050411, 050715, 060721, 060726, 060809, 060812, 060817, 060820, 060901, 060907 and 060912) and Tongrentang, Beijing City (lot no. 061015). A voucher specimen of these collections has been identified and deposited at Herbarium of School of Pharmacy, Second Military University, Shanghai, China.

Table 2
Statistical results of linear regression equation analysis in the determination of the five flavonoids

Compound	Regression equation				
	Linear range (μg/ml)	Slope (*a*)	Intercept (*b*)	r^2 ($n=6$)	LOD (ng)
Taxfolin-7-O-α-L-rhamnoside	20.8–416	2.612	−7.527	0.9998	5.1
Isoquercitrin	6.15–123	30.136	20.047	0.9999	0.8
Quercitrin	8.05–161	34.754	30.430	0.9999	0.5
Quercetin-7-O-α-L-rhamnoside	22.76–455.2	3.273	−3.447	0.9998	7.5
Quercetin	1.73–69.2	42.830	8.217	0.9999	0.7

In the regression equation $y = ax + b$, y refers to the peak area (A), x concentration of each reference flavonoid (μg/ml), r^2 the correlation coefficient of the equation and LOD is the limit of detection (S/N = 3).

Table 3
Statistical results of precision of the five flavonoids ($n=5$)

Compound	Intra-day precision		Inter-day precision	
	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)
Taxfolin-7-O-α-L-rhamnoside	10.91 ± 0.06	0.55	10.87 ± 0.11	1.01
Isoquercitrin	1.55 ± 0.01	0.65	1.53 ± 0.02	1.31
Quercitrin	4.22 ± 0.03	0.71	4.19 ± 0.06	1.43
Quercetin-7-O-α-L-rhamnoside	2.33 ± 0.02	0.86	2.31 ± 0.04	1.73
Quercetin	0.64 ± 0.01	1.56	0.65 ± 0.01	1.54

2.2. Preparation of standard solutions

Each flavonoid was accurately weighted, dissolved in methanol and diluted to appropriate concentration. A mixed methanolic stock solution of standards, containing taxfolin-7-*O*-α-L-rhamnoside (208 μg/ml), isoquercitrin (61.5 μg/ml), quercitrin (80.5 μg/ml), quercetin-7-*O*-α-L-rhamnoside (227.6 μg/ml), quercetin (34.6 μg/ml), was prepared. A set of standard solutions were prepared by appropriate dilution of the stock solution with methanol, containing 20.8–416 μg/ml of taxfolin-7-*O*-α-L-rhamnoside, 6.15–123 μg/ml of isoquercitrin, 8.05–161 μg/ml of quercitrin, 22.76–455.2 μg/ml of quercetin-7-*O*-α-L-rhamnoside, 1.73–69.2 μg/ml of quercetin. All solutions were stored in the refrigerator at 4 °C before analysis.

2.3. Preparation of samples

H. japonicum was dried at 60 °C until constant weight. The dried material was pulverized to 100 mesh. Approximately 1.0 g powder was accurately weighted and then ultrasonically extracted twice with 70% methanol (2 × 20 ml) for 20 min. The supernatant solution was combined, filtrated, then cooled down to ambient temperature. The solution was transferred to a 50 ml volumetric flask, and 70% methanol was added to make up to volume. The obtained solution was filtered through a syringe filter (0.45 μm) and aliquots (10 μl) were subjected to HPLC analysis.

2.4. HPLC-DAD–ESI/MS analysis

An Agilent-1100 HPLC system with diode array detector was coupled with a LC/MSD Trap XCT electrospray ion mass spectrometer (Agilent Corporation, MA, USA), equipped with quaternary pump, vacuum degasser, autosampler, column heater-cooler (Agilent Corporation, MA, USA). The chromatographic separation was performed on a ZORBAX SB-C_{18} analytical column (5 μm, 250 mm × 4.6 mm i.d., Agilent Corporation, MA, USA) with the column temperature set at 25 °C. A linear gradient elution of water, containing 0.5% formic acid (A) and MeCN (B) was used (12–30% of B in 70 min, v/v). The flow rate was 1.0 ml/min, and the injection volume was 10 μl. By solvent splitting, 0.2 ml/min portion of the column effluent was delivered into the ion source of the mass spectrometer.

LC–MS detection was performed directly after UV-DAD measurements. Analyses were performed using a LC/MSD Trap XCT mass spectrometer (Agilent Corporation, MA, USA) equipped with an ESI source. The MS conditions were as

Table 4
Statistical results of recovery of the five flavonoids ($n=5$)

Compoud	Added amount (mg)	Recorded amount (mg)	Recovery (%)	R.S.D. (%)
Taxfolin-7-O-α-L-rhamnoside	5.31	5.23 ± 0.05	98.4 ± 0.9	0.9
isoquercitrin	0.738	0.728 ± 0.009	98.7 ± 1.3	1.3
Quercitrin	2.10	2.10 ± 0.04	99.8 ± 2.0	2.0
Quercetin-7-*O*-α-L-rhamnoside	1.16	1.15 ± 0.03	99.5 ± 2.65	2.6
Quercetin	0.346	0.341 ± 0.005	98.5 ± 1.5	1.6

follows: positive ion mode; collision energy (Ampl): 1.0 V; collision gas: He; drying gas N_2: 8 l/min; temperature: 350 °C; pressure of nebulizer: 30 psi; HV voltage: 3.5 kV; scan range: 100–800 u; target mass: 350 u; smart parameter setting: active. Data acquisition was performed using a Chemstation software (Agilent Corporation, MA, USA).

2.5. HPLC-UV analysis

An Agilent-1100 HPLC system coupled with UV detector was used for quantitative determination. The chromatographic conditions were as described above. The chromatograms were monitored at 256 nm while UV spectra (DAD) were recorded between 200 and 400 nm. Data acquisition was performed using a Chemstation software (Agilent Corporation, MA, USA).

3. Results and discussion

3.1. Optimization of chromatographic conditions

Optimized chromatographic conditions for good resolution of adjacent peaks were achieved after several trials with elution systems of acetonitrile–water, methanol–water, acetonitrile–acidic aqueous solution, methanol–acidic aqueous solution in various proportions. It was found that the presence of 0.5% aqueous formic acid in mobile phase lead to a significant improvement on the retention behavior of the different components; otherwise, the peaks were rather broad with poor separation. The optimal mobile phase, consisting of acetonitrile-0.5% aqueous formic acid, was finally employed, which produces high resolution and symmetrical peak shape. The column temperature was set at 25 °C.

DAD detection was employed at wavelength range of 200–400 nm to investigate the UV spectra of the five flavonoids. It was found that 256 nm is the best wavelength for the detection because almost all the investigated constituents have the maximum absorption there. Fig. 2 shows the chromatogram of the 70% methanol extract from *H. japonicum*.

3.2. Identification of five flavonoids from H. japonicum

The MS spectra of major flavonoids from *H. japonicum* was acquired in positive ion mode. Table 1 contains their retention times (R_t), MS and MS^2 fragmentation ions. The five flavonoids exhibited their quasi-molecular ions $[M+H]^+$ or $[M+Na]^+$. Fragment ions, obtained e.g. by the loss of glycose $[M-146]^+$ or $[M-162]^+$, H_2O $[M-18]^+$, CO, etc., could also be observed in the MS^2 spectra. Among the five flavonoids, quercetin showed typical fragmentation patterns as previously reported [25].

On the basis of the MS and UV spectra and comparison of the chromatographic retention times with those of authentic standards, the five flavonoids were identified in twenty batches of *H. japonicum*. The results further revealed that the five flavonoids investigated compounds were the main constituents in *H. japonicum*.

3.3. Method validation

3.3.1. Linearity

Linear calibration curves were constructed by six assays of each reference compounds. The regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and concentration of each reference compound, respectively. The high correlation coefficients ($r^2 > 0.9997$) and the regression parameters listed in Table 2 indicated good linearity in relatively wide concentration ranges. The limits of detection (LOD; S/N > 3) ranging from 0.5 to 7.5 ng show high sensitivity.

3.3.2. Precision

As shown in Table 3, both intra-day and inter-day reproducibility of the determination of the five investigated components was good: R.S.D. less than 1.73% ($n = 5$).

3.3.3. Accuracy

The recoveries of the flavonoids were determined by the method of standards addition. Suitable amounts (about 50% of the content) of the five flavonoids were spiked into a sample of *H. japonicum* (lot no. 060812), which were determined previously. The mixture was extracted and analyzed by using the proposed procedure. For comparison, an unspiked sample was prepared and analyzed simultaneously. As shown in Table 4, the mean recoveries of the flavonoids were 98.4–99.8%, with R.S.D. values ranging from 0.9% to 2.6% ($n = 5$).

3.4. Quantification of five flavonoids in H. japonicum

The five predominant flavonoids in *H. japonicum* were simultaneously determined by the proposed HPLC-UV method (shown in Fig. 3). The quantitative analyses were performed by means of the external standard method. In some cases, where the flavonoids were present in very low concentration (e.g. taxfolin-7-O-α-L-rhamnoside and isoquercitrin in lot no. 060705), the samples were concentrated to proper volume in order to match with the linear range. Data of the quantitative analyses were expressed as mean ± deviation (listed in Table 5).

The total flavonoid content in each sample was calculated and the results showed that it was higher in three samples (lot nos. 060802, 050715 and 060726) than in the other samples. Since flavonoids were considered to be the bioactive ingredients in *H. japonicum*, the results suggested that these three batches might have stronger pharmacological effects than others.

The results in Table 5 show that the contents of the five flavonoids are quite different in the twenty batches of *H. japonicum*. The content of taxfolin-7-O-α-L-rhamnoside was higher than that of the other four flavonoids with average values of 10.95 mg/g.

3.5. Chromatographic fingerprint analysis

Samples with similar chromatographic fingerprint will likely have similar properties. Therefore, we can compare the chromatographic fingerprints of samples with the reference

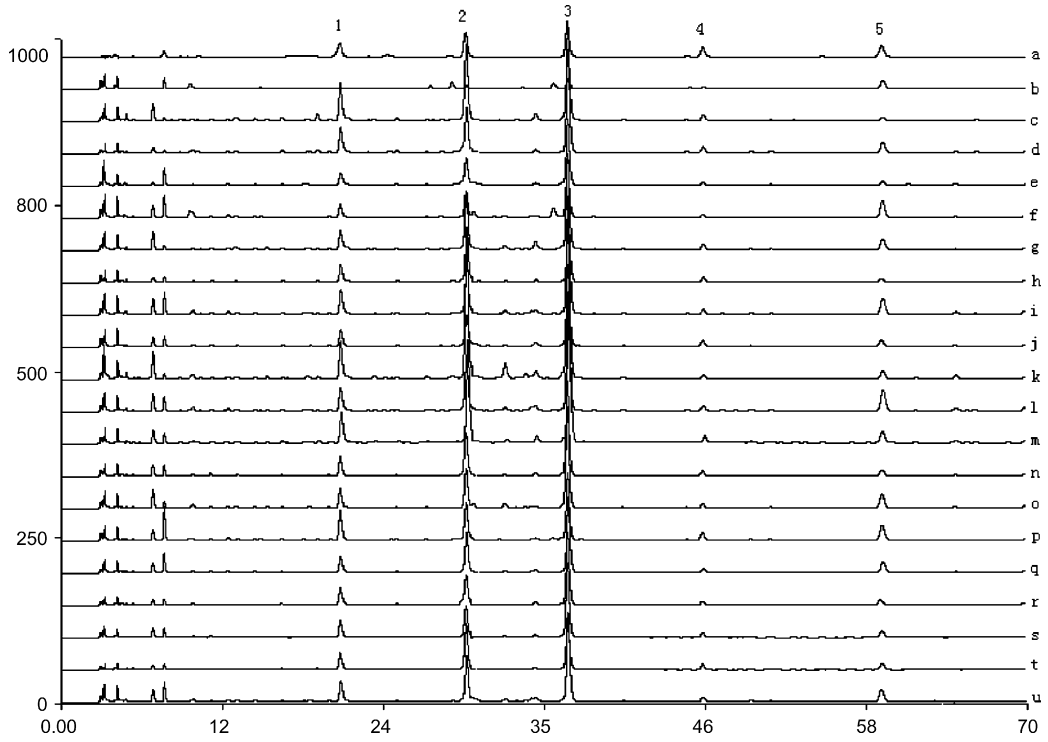

Fig. 3. HPLC chromatograms of (a) standard mixture and batches of *H. japonicum* (b–u). The five standard substances are (1) taxfolin-7-O-α-L-rhamnoside, (2) isoquercitrin, (3) quercitrin, (4) quercetin-7-O-α-L-rhamnoside and (5) quercetin. The batch numbers (lot nos.) were (b) 060705, (c) 060802, (d) 060715, (e) 060814, (f) 060901, (g) 060713, (h) 060801, (i) 040901, (j) 050411, (k) 050715, (l) 060721, (m) 060726, (n) 060809, (o) 060812, (p) 060817, (q) 060820, (r) 060901, (s) 060907, (t) 060912 and (u) 061015, respectively. See Section 2.4 for the chromatographic conditions.

fingerprints for their quality evaluation. To standardize the fingerprint of *H. japonicum*, twelve samples of *H. japonicum* from Jiujiang City, which were considered as standard were analyzed. Five peaks of flavonoids in the chromatograms of these reference samples were considered as the fingerprint peaks, and a reference chromatographic fingerprint was generated by utilizing their mean values. The content values of these five flavonoids in each chromatogram were used to calculate the similarity value.

Table 5
Contents (mg/g) of the investigated flavonoids in the twenty batches of *H. japonicum* (mean ± S.D., $n = 5$)

Lot no.	Content of flavonoids (mg/g)					Total flavonoids	Similarity
	Taxfolin-7-*O*-α-L-rhamnoside	Isoquercitrin	Quercitrin	Quercetin-7-*O*-α-L-rhamnoside	Quercetin		
060705	0.55 ± 0.04	0.12 ± 0.01	0.37 ± 0.02	0.61 ± 0.04	0.35 ± 0.03	2.00 ± 0.07	0.2585
060802	17.99 ± 0.52	2.83 ± 0.16	3.44 ± 0.22	2.69 ± 0.11	0.12 ± 0.01	27.07 ± 0.49	0.9144
060715	12.70 ± 0.40	1.72 ± 0.06	4.03 ± 0.19	2.90 ± 0.13	0.45 ± 0.02	21.8 ± 0.36	0.9899
060814	8.23 ± 0.24	0.91 ± 0.03	2.89 ± 0.14	1.32 ± 0.03	0.17 ± 0.01	13.52 ± 0.27	0.8297
060901	7.36 ± 0.17	1.05 ± 0.03	2.53 ± 0.08	1.49 ± 0.05	0.75 ± 0.04	13.18 ± 0.30	0.8659
060713	12.36 ± 0.37	2.15 ± 0.12	3.87 ± 0.15	2.82 ± 0.13	0.46 ± 0.02	21.66 ± 0.35	0.9939
060801	8.92 ± 0.26	2.11 ± 0.11	2.66 ± 0.09	2.41 ± 0.10	0.13 ± 0.01	16.23 ± 0.24	0.9191
040901	12.16 ± 0.32	1.92 ± 0.06	4.89 ± 0.23	2.22 ± 0.08	0.66 ± 0.02	21.85 ± 0.41	0.9781
050411	9.53 ± 0.27	1.19 ± 0.04	3.40 ± 0.14	2.95 ± 0.14	0.29 ± 0.02	17.36 ± 0.29	0.9597
050715	19.02 ± 0.55	5.89 ± 0.33	7.03 ± 0.34	1.87 ± 0.06	0.37 ± 0.03	34.18 ± 0.64	0.8244
060721	13.38 ± 0.45	2.24 ± 0.15	5.08 ± 0.27	2.44 ± 0.12	0.99 ± 0.05	24.13 ± 0.39	0.9118
060726	14.89 ± 0.41	3.58 ± 0.18	6.11 ± 0.24	2.81 ± 0.11	0.45 ± 0.02	27.84 ± 0.48	0.9450
060809	10.11 ± 0.38	1.55 ± 0.04	3.69 ± 0.13	2.34 ± 0.07	0.25 ± 0.02	17.94 ± 0.27	0.9729
060812	10.99 ± 0.29	1.57 ± 0.03	4.20 ± 0.14	2.35 ± 0.06	0.62 ± 0.03	19.73 ± 0.35	0.9862
060817	14.40 ± 0.31	1.31 ± 0.05	2.55 ± 0.07	3.75 ± 0.17	0.60 ± 0.02	22.61 ± 0.47	0.9188
060820	8.56 ± 0.19	1.56 ± 0.05	3.18 ± 0.11	2.02 ± 0.07	0.49 ± 0.02	15.81 ± 0.35	0.9703
060901	8.90 ± 0.21	1.23 ± 0.06	2.92 ± 0.13	1.58 ± 0.08	0.22 ± 0.02	14.85 ± 0.32	0.8973
060907	8.85 ± 0.17	1.15 ± 0.06	2.97 ± 0.15	2.22 ± 0.05	0.27 ± 0.02	15.46 ± 0.31	0.9465
060912	8.47 ± 0.20	1.72 ± 0.08	2.26 ± 0.08	2.47 ± 0.13	0.26 ± 0.02	15.18 ± 0.29	0.9335
061015	11.72 ± 0.42	1.86 ± 0.08	3.88 ± 0.11	2.41 ± 0.14	0.58 ± 0.03	20.45 ± 0.42	0.9934
Average	10.95 ± 4.05	1.88 ± 1.19	3.60 ± 1.44	2.28 ± 0.68	0.42 ± 0.23	19.14 ± 6.72	–

Assume that vector $X = [x_1–x_5]$ and $Y = [y_1–y_5]$ respresent fingerprint X and the reference fingerpint Y, respectively, where x_i denotes the content of ith peaks of fingerprint X, and y_i denotes the content of ith peaks of the reference fingerprint, then the similarity value of this sample can be calculated as below [26]:

$$\sigma(X, Y) = \frac{2\sum_{i=1}^{5} x_i y_i}{\sum_{i=1}^{5} x_i^2 + \sum_{i=1}^{5} y_i^2} = \frac{2XY^{\mathrm{T}}}{XX^{\mathrm{T}} + YY^{\mathrm{T}}}$$

where σ is the value of similarity; X and Y are two vectors consist the contents of five flavonoids in their fingerprints; the superscript T indicates the transpose of vector.

Results of chromatographic fingerprint analysis are shown in Table 5. It was considered that the holistic quality of samples was quite similar when the similarity was higher than 0.9. The result showed that the quality of samples from Wuhan City, Chongqian City and Dabie Mountain was extremely different from those from Jiujiang City. Also, the similarities of two batches from Jiujiang City (lot nos. 050715 and 060901) were less than 0.9, which indicated that the quality of samples from the same habitat can be different. The quality of this TCM can also be influenced by the method of preparation and storage.

4. Conclusion

An HPLC-UV method has been proposed to determine the five major flavonoids in *H. japonicum*. This method can be applied as a convenient, effective technique to control the bioactive chromatographic profile and thus the quality of *H. japonicum*.

Acknowledgments

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