Simultaneous Determination of Seven Active Flavonols in the Flowers of *Abelmoschus manihot* by HPLC

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Abstract

A high-performance liquid chromatography method is developed for the simultaneous quantification of seven flavonols, namely quercetin-3-O-robinobioside, hyperin, isoquercetin, hibifolin, myricetin, quercetin-3'-O-glucoside, and quercetin, in the flower of *Abelmoschus manihot*. These seven flavonols are selected as chemical markers because they are the major pharmacologically active constituents in the flower. The method involves the use of a Thermo ODS-2HYEPRSIL reversed-phase column (5 µm, 250 × 4.6 mm) at 25°C with a mixture of acetonitrile and aqueous H_3PO_4 as the mobile phase and detection at 370 nm. The recovery of the method is 94.31–107.08% with an RSD ≤ 3.14% and the linearity (r^2 > 0.9996) is obtained for all the flavonoids. The current assay method can be readily utilized for the determination of the flavonols present in the flower and is considered to be suitable for the quality control of *A. manihot* samples. The comparison of flowers collected from nine locations shows that flavonoid glucoside is more stable than aglycon in the flower. This is the first study that analyzes the stability of flavonoids in the flower of *A. manihot*. This research also provides important evidence that the flower is a potentially abundant resource for obtaining hibifolin.

Introduction

Abelmoschus manihot (Linn.) Medicus is a multipurpose plant, and its large yellow flower is a Traditional Chinese Medicine (1,2). The flower of *A. manihot* was found to have anti-inflammatory, antibacterial, and anticoagulant effects (3–5). It has been used for treatment of chronic renal disease, mouth ulcers, and burns (6–8). Previous phytochemical studies indicated that flavonoids were the major constituents separated from the flower of *A. manihot* (9–12). The flavonoids isolated from the flower of *A. manihot* by the author include quercetin-3-O-robinobioside (compound **1**), hyperin (comp. **2**), isoquercetin (comp. **3**), hibifolin (comp. **4**), myricetin (comp. **5**), quercetin-3'-O-glucoside (comp. **6**), and quercetin (comp. **7**). Their structures are shown in Figure 1. Five of them were discovered and determined in the plasma and urine of rat (13). Recent studies showed that flavonoids in *A. manihot* possess various biological activities, such as anti-inflammatory, antibacterial, antioxidant, and protective effects on the renal cellular membrane cells (14–17).

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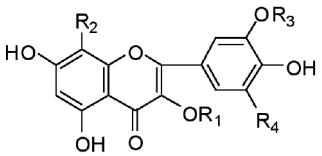
The previously reported UV–vis, TLC, and HPLC (18–20) quality control methods for the flower of *A. manihot* were only able to determine the total flavonoids or no more than three flavonoids, which could not comprehensively reflect the active compounds of the flower of *A. manihot* and were inadequate for quality control of the flower.

In our current study, a simple, reliable, and reproducible high-performance liquid chromatography (HPLC) method for the simultaneous determination of the seven major pharmacologic flavonols (comps. **1**–**7**) in the flower of *A. manihot* is developed for the quality control of the flower. The flowers collected from nine locations were analyzed and compared. This is the first study performed to analyze the stability of flavonoids in the flower of *A. manihot*. This research also provides important evidence that the flower is a potentially abundant resource for obtaining hibifolin.

Experimental

Chemicals, materials, and standards

HPLC-grade acetonitrile (CH_3CN) was purchased from Honeywell International Inc. (Burdick & Jackson, Muskegon, MI); analytical-grade methanol and phosphoric acid were purchased from Beijing Reagent Co. Ltd. (Beijing, P.R. China). Distilled and deionized water was used for the preparation of all solutions.



	R_1	R_2	R_3	R_4
1	rha(1-->6)-gal	H	H	H
2	gal	H	H	H
3	glc	H	H	H
4	H	O-glc	H	H
5	H	H	H	OH
6	H	H	glc	H
7	H	H	H	H

Figure 1. The structures of compounds 1–7.

The flowers of *A. manihot* from nine locations in the six provinces of China were collected, and then identified by Professor Hu-biao Chen (Peking University Health Science Center, China). The samples were stored at 20°C in the Natural Medicine Herbarium in the Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University Health Science Center, China.

The standards of flavonoids **1–7** were isolated from the flowers of *A. manihot* by the author. The isolation and purification of the seven standards were performed with chromatographic methods, such as polyamide column chromatography, Sephadex LH-20, RP-18, and HP-20 macroporous adsorption resin. The detailed separation method of hibifolin (comp. **4**) and myricetin (comp. **5**) were published by the author (21,9). Their structures were identified on the basis of spectral data (^{1}H-NMR, ^{13}C-NMR and UV) and comparing our data with published literatures (9,11,21–25). Purity analysis suggested that the purity of the standards were all > 98%.

Preparation of standard solutions and calibration

Standard stock solutions of compounds **1–7** were prepared separately with methanol. These seven stock solutions were mixed in proportion, vacuum-dried, and reconstituted with ethanol–water (50:50, v/v) to obtain the working standard solutions. All solutions were stored at 4°C.

The calibration ranged at the following concentrations: 5.03–125.87, 4.36–545.45, 2.91–363.64, 9.34–1167.27, 0.90–111.89, 1.91–239.16, and 0.84–20.98 µg/mL for compounds **1–7**, respectively. Calibration graphs were recorded by plotting the respective peak areas vs. the concentrations.

Preparation of samples

Five hundred milligrams dried flowers of *A. manihot* were accurately weighed and put into a 50-mL centrifuge tube. Twenty milliliters ethanol–water (50:50, v/v) solution was added. The sample was extracted in an ultrasonic water bath for 45 min, and then centrifuged at 3500 rpm for 15 min. This extraction procedure was repeated one more time. The extracted solution was mixed and the total volume of extract was adjusted to 50 mL with ethanol–water (50:50, v/v). The obtained solution was filtered through a membrane filter (0.45 µm pore size) prior to injection. Each sample was prepared with the described method for HPLC analysis.

Apparatus and operating conditions

HPLC was performed on a JASCO HPLC system and consisted of a solvent gradient delivery pump (Tokyo, Japan) and a diode array spectrophotometric detector (DAD). A Thermo ODS-2Hypersil reversed-phase column (5 µm, 250 × 4.6 mm) connected with a Phenomenex C_{18} (ODS, Octadecyl) guard column (5 µm, 4.0 × 3.0 mm) at a temperature of 25°C was applied for all analyses. An ultrasonic cleaner (KQ-500DB, Kunshan, China) was used for extraction.

The gradient solvent system consisted of acetonitrile (A) and 0.1% (v/v) phosphoric acid solution (B). The initial elution condition was A–B (15:85, v/v), linearly changed to A–B (16:84, v/v) at 18 min, and then linearly changed to A–B (20:80, v/v) at 26 min. The percentage of mobile phase A increased linearly to 40% at 35 min, and then held A–B (40:60, v/v) until 40 min. The quantification wavelength of these chromatograms was set at 370 nm. The flow rate was 1.0 mL/min, and the injection volume was 10 µL.

Results and Discussion

Optimization of HPLC conditions

According to the λmax values of the seven flavonols on the online UV spectra with three-dimensional chromatograms of HPLC and comparing the chromatograms detected at 255 and 370 nm, 370 nm was selected as detection wavelength as it resulted in better baseline. Four columns were screened with a gradient solvent system comprised of acetonitrile and 0.1% phosphoric acid solution. The Thermo ODS-2Hypersil reversed-phase column was selected because the best selectivity and resolution was observed with this column. Methanol–water, methanol–0.1% phosphoric acid solution, acetonitrile–water, and acetonitrile–0.1% phosphoric acid solution were used as mobile phase. The results (Figure 2) showed that hibifolin (comp. **4**), which contains a carboxyl group, was more susceptible to pH than other compounds, and acetonitrile was more suitable than methanol to separate the flavonol compounds. A number of gradient elution parameters were examined to optimize the resolution, and the optimal linear gradient condition was presented in detail earlier. Thus, it was decided to use the chromatographic system

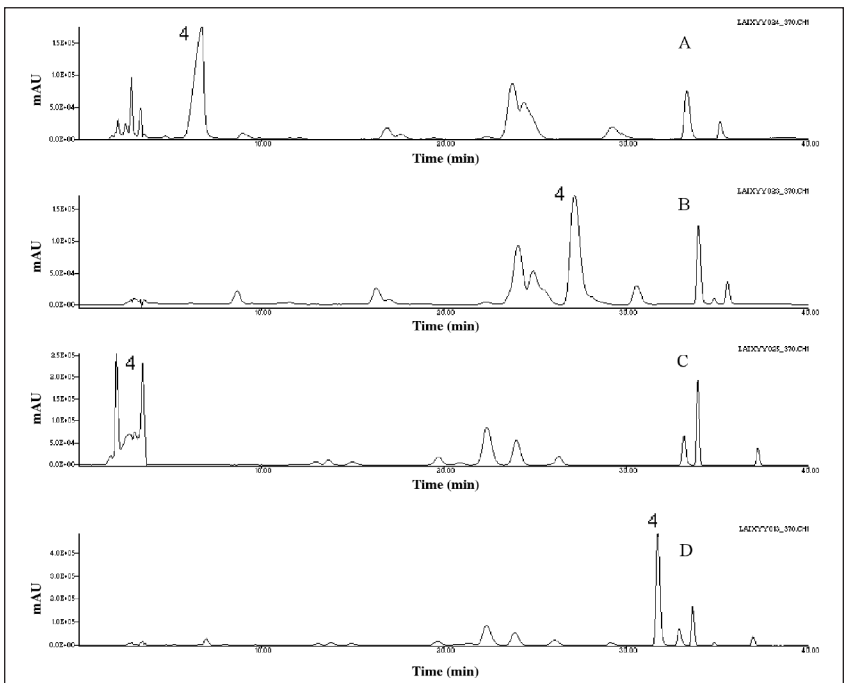


Figure 2. Chromatograms of the same sample run on a Thermo ODS-2HYEPRSIL C18 reversed-phase column under four different mobile phase systems at flow rate as 1.0 mL/min and the detection wavelength set at 370 nm. Methanol–water (A), methanol–0.1% phosphoric acid solution (B), acetonitrile–water (C), and acetonitrile–0.1% phosphoric acid solution (D). Peak 4 = hibifolin.

consisting of 370 nm detection wavelength, the Thermo ODS-2Hypersil reversed-phase column, and the acetonitrile–0.1% phosphoric acid solution linear gradient mobile phase for the simultaneous determination of the seven flavonols in the flowers of *A. manihot*.

Extraction method development

In order to evaluate the extraction efficiency of different solvents, methanol, 50% methanol, 95% ethanol, and 50% ethanol were employed as extraction solvents. The peak areas of the seven flavonols reached the highest values when the sample was extracted with 50% ethanol. The data is shown in Table I. Therefore, 50% ethanol was selected as the extraction solvent. For extraction method optimization, reflux extraction, ultrasonic extraction, and maceration extraction were compared. It was found that there were no significant differences between the three methods (Table I), but the operation of ultrasonic extraction was simpler and required less time. The ultrasonic extraction was chosen as a preferred method. To study the influence of the extraction time on the efficiency of extraction, samples were extracted with 50% ethanol in ultrasonic bath for 30, 45, and 60 min, respectively. The peak areas of the seven flavonols had no significant differences between the three kinds of extraction time (Table I). Forty-five minutes was selected as extraction time. Next, different extraction times were investigated. Samples were extracted once, twice, or three times to test the efficiency of extraction. The peak areas of the seven flavonols had significant differences between single and double extraction time but had no significant differences between three times and double extraction time (Table I). Twice was selected as the optimal extraction times. According to the experiments, the optimal condition was presented in detail in "Preparation of samples" section.

Table I. Area Numbers of the Seven Flavonols in the Flowers of *A. manihot* under Different Extraction Conditions (E + 06)

Extraction		1	2	3	4	5	6	7
Extraction solvent	methanol	0.514	2.863	1.645	6.112	1.222	1.912	0.122
	50% methanol	0.547	3.146	1.718	7.351	0.767	1.753	0.133
	95% ethanol	0.530	2.551	1.393	5.933	1.239	1.931	0.121
	50% ethanol	0.568	3.281	1.871	8.113	1.239	2.080	0.168
Extraction method	reflux	0.607	3.434	2.030	8.524	1.230	2.160	0.134
	ultrasonic	0.584	3.341	1.866	8.158	1.142	2.072	0.156
	maceration	0.587	3.344	1.893	8.207	1.174	2.079	0.199
Extraction time	30 min	0.556	3.356	1.877	7.889	1.156	2.055	0.147
	45 min	0.592	3.350	1.881	8.158	1.200	2.081	0.165
	60 min	0.601	3.336	1.983	8.565	1.254	2.130	0.205
Extraction times	once	0.455	2.902	1.501	6.261	0.903	1.706	0.108
	twice	0.587	3.342	1.950	8.334	1.294	2.126	0.166
	three times	0.573	3.337	1.894	8.293	1.203	2.103	0.171

Methodology validation

The analytical method described here was employed to simultaneously quantify the seven compounds in the flowers of *A. manihot*. Typical chromatograms of the standards and the flowers sample run under the optimized HPLC conditions are shown in Figure 3.

Linear regression analysis for each of the seven compounds was performed by plotting the peak area (y) versus concentration (x, μg/mL). The calculated results of regression equations, correlation coefficients, and linear ranges for the analysis of the seven compounds were presented in Table II. All seven compounds showed good linearity (r^2 > 0.9996) in the concentration range.

The limit of quantification (LOQ) and the limit of detection (LOD) were calculated based on the signal/noise (S/N) at levels approaching the lower limits using the following equations: LOQ = 10 (S/N) and LOD = 3.0 (S/N). The LOQ and LOD were in the range of 0.84–5.03 and 0.17–1.17 μg/mL for the seven flavonols, respectively. LOQ and LOD are reported in Table II for each compound.

The intra-day precision of the developed analytical method for each flavonol was evaluated by replicate analysis of the same samples for six times in a day. The inter-day precision was assessed by replicate analysis of the same samples for five continual days. The intra- and inter-day relative standard deviations (RSD) for the seven flavonols were ≤ 2.21%.

The sample stability was tested with one sample solution that was stored at room temperature and analyzed at 0, 2, 4, 8, 12, 24, 36, 48, and 72 h. The RSD of analytes were not more than 2.92%, and the results indicated that the analytes were rather stable within 72 h of preparation.

Measurement of the repeatability of the proposed method was

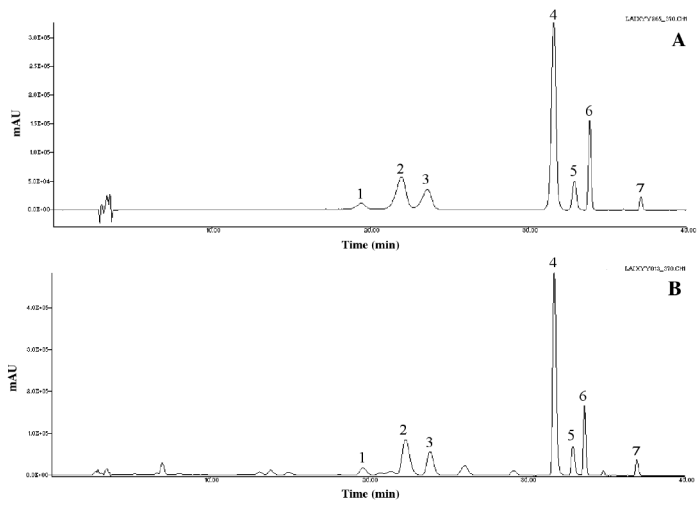


Figure 3. Typical chromatograms of the standards (A) and the flowers of A. manihot (B) run on a Thermo ODS-2HYEPRSIL C18 reversed-phase column under acetonitrile–0.1% phosphoric acid solution linear gradient mobile phase system at flow rate as 1.0 ml/min and the detection wavelength set at 370 nm. 1 (quercetin-3-O-robinobioside), 2 (hyperin), 3 (isoquercetin), 4 (hibifolin), 5 (myricetin), 6 (quercetin-3'-O-glucoside) and 7 (quercetin)

examined on seven independently prepared samples within one day. The RSD was calculated as the repeatability test precision. The results indicated that the RSD values of the seven flavonols were not more than 2.07%, which showed very good repeatability of the developed method.

Recovery experiments were carried out by adding different amounts of the mixed standard solution to known amounts of the flower samples with three different concentration levels (high, middle, and low) and triplicate experiments at each level. Next, the samples were extracted and analyzed with the proposed HPLC method. The ratio of detected versus added amount of standard was used to calculate the recovery. The average recoveries of the method was between 94.31% and 107.08%, with RSD ≤ 3.14%. This indicated that the proposed method has an adequate degree of accuracy for the determination of the seven flavonols in the samples.

Sample analysis

Using the optimized analytical method, seven flavonols in nine samples collected from various locations were successfully determined simultaneously (Table III). The contents of quercetin-3-O-robinobioside (comp. **1**), hyperin (comp. **2**), isoquercetin (comp. **3**), hibifolin (comp. **4**), myricetin (comp. **5**), quercetin-3'-O-glucoside (comp. **6**), and quercetin (comp. **7**) were 0.266–0.464, 0.761–1.626, 0.691–1.205, 0.996–3.411, 0.000–0.442, 0.229–0.837, and 0.023–0.431%, respectively.

Previous determination study of flavonoids in *A. manihot* only reported compounds **2**, **3**, and **6** in samples from three locations (20). The quantity of **2**, **3**, and **6** are in the range of our result, especially their contents in samples from Jiangyan, Jiangsu, which are very close to our determination of samples from the same location. But the biggest peak in the chromatography was missed in that study, which should be hibifolin (comp. **4**) based on our research and analysis. Our study reflects the comprehensive active compounds of the flower of *A. manihot* and is very useful for quality control of the flower.

Stability of flavonoids in the flower

The flower was recorded as unfolding in the morning and withering in the evening (26). The recent report shows that the flower's color and total flavonoid content in the flowering time were very different from its withering time (27). These reports indicate that changes occur in the flowering and withering time that was only one day, and some flavonoids were unstable in the flower. During the quantification study for *A. manihot*, we found that all flavonoid (comps. **1**–**7**) were stable both in the aqueous ethanol extracts and in dried flower. We conclude that some flavonoids were unstable only in the course when the flower was unfolding, withering, picked up, and dried. Flavonoid glucosides (comps. **1**–**4**, **6**) and aglycons (comps. **5**,**7**) are the two types of constituents in the flower. Another interesting fact was found when we compared the flavonoid contents of flowers from different locations. The variation between the highest and lowest flavonoid contents in nine locations samples were 1.7, 2.1, 1.7, 3.4, and 3.7-fold for flavonoid glucosides (comps. **1**–**4**, **6**), respectively. Flavonoid aglycon (comp. **5**) was not detected in two samples, and in the other seven samples the highest content was 7.8-fold more than the lowest contents. The content of the highest flavonoid aglycon (comp. **7**) was 18.8-fold more than the lowest. The result showed flavonoid aglycons (comps. **5**,**7**) were the unstable constituents in the flower and susceptible to locations and picking time variability.

Table II. Regression Equations, Correlation Coefficients, Linear Ranges, LOQ, and LOD for the Seven Flavonols (Comps. 1–7) (μg/mL)*

Compound	Regression equation	r^2	Linear range	LOQ	LOD
1	$y = 14859.65x + 6581.84$	0.99985	5.03–125.87	5.03	1.01
2	$y = 21784.59x - 6596.08$	0.99995	4.36–545.45	4.36	1.09
3	$y = 19511.89x + 1816.23$	0.99996	2.91–363.64	2.91	0.73
4	$y = 24888.25x + 37818.13$	0.99996	9.34–1167.27	2.33	1.17
5	$y = 37517.89x - 6080.12$	0.99994	0.90–111.89	0.90	0.45
6	$y = 32347.98x - 32047.09$	0.99967	1.91–239.16	0.96	0.48
7	$y = 58110.63x + 2\,955.86$	0.99994	0.84–20.98	0.84	0.17

* y = peak area; x = concentration of compound (μg/mL); r^2 = correlation coefficients; LOQ = limit of quantification; LOD = limit of detection.

Table III. Contents of the Seven Flavonols in the Flowers of *A. manihot* (%)

Locations	1	2	3	4	5	6	7
Baoying, Jiangsu	0.396	1.580	1.006	3.411	0.352	0.682	0.029
Haikou, Hainan	0.288	1.471	1.130	2.197	0.442	0.837	0.023
Qixian, Henan	0.437	1.626	0.993	3.255	0.109	0.691	0.049
Minquan, Henan	0.305	1.048	0.691	1.024	n.d.*	0.247	0.074
Xiayi, Henan	0.464	1.589	1.205	2.873	0.158	0.598	0.431
Daxing,Beijing	0.383	1.297	0.886	2.695	0.057	0.548	0.078
Jiangyan, Jiangsu	0.315	1.253	0.972	2.523	0.304	0.809	0.059
Neiqiu, Hebei	0.266	0.761	0.775	0.996	n.d.*	0.229	0.153
Shenyang, Liaoning	0.389	1.571	0.919	2.416	0.106	0.577	0.148

* n.d. = not detected.

Hibifolin resource

Hibifolin (comp. **4**) is not a compound that commonly occurs in plant species. To the best of our knowledge, hibfolin has only been identified before from six species of four families (28–33). Hibifolin was reported to possess anti-inflammatory activity through lipoxygenase inhibition (34) and a strong effect in neuroprotection (35). Our research indicates hibifolin is the most abundant constituent in the flower and its content is as high as 3.411%. This result provides important evidence that the flower is a potentially abundant resource for obtaining hibifolin.

Conclusions

In the present work, a simple method has been developed for the simultaneous quantification of seven major flavonols in the

flower of *A. manihot* by HPLC. This is the first report for the simultaneous quantification of seven flavonols in the flowers. The validation procedure confirmed that this method was reliable for the analysis of these flavonols and could be utilized as a quality control method for the determination of the flavonols in the flowers. The quantitative analysis of flavonoids in the flowers collected from nine locations showed that flavonoid glucoside is more stable than aglycon in the flower. This is the first study on the stability of flavonoids in the flower of *A. manihot*. Our research suggests that the *A. manihot* flower could represent a potentially abundant resource to obtain purified hibifolin.

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