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Quantification and Stability Studies on the Flavonoids of *Radix hedysari*

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Radix hedysari is not only a restorative food but also a famous Traditional Chinese Medicine. In this study, a simple, reliable, and reproducible high-performance liquid chromatography−ultraviolet method was developed for the first time to determine the true contents of five major flavoniods, naringenin-7-*O*-α-L-rhamanopyranosyl-(1→2)-β-D-glucopyranoside, ononin, formononetin, medicarpin, and an unstable flavonoid malonate (formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate, FGM), and the stability of FGM was also investigated. Flavoniods were selected as chemical markers because they have appeared as one of the major bioactive compound groups in *Radix hedysari*. The stability results of FGM at different pH showed that it remained relatively stable in acidic aqueous methanol of pH 3. The HPLC assay with an improved sample preparation scheme can be readily utilized as a suitable "chemical" quality control method for *Radix hedysari*.

KEYWORDS: ***Radix hedysari*; formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate; flavonoids; HPLC-UV**

INTRODUCTION

Radix hedysari, the dry roots of *Hedysarum polybotrys*, known as hongqi in China, is esteemed as the uppermost class of *Radix astragali* (huangqi) which is one of the most important tonics in Traditional Chinese Medicine (TCM) and widely used as a restorative food and a famous TCM to reinforce "Qi" (vital energy) (*1*, *2*). It comprises a number of chemical constituents such as flavonoids, benzofuranoids (*3*), coumestans (*4*), organic acids, olysaccharides, and phenols, among which flavonoids are one of the major compound groups. Flavonoids, in general known to be biologically active compounds, may offer several benefits to human health, including immune system stimulative, antioxidative, antiallergic, anticarcinogenic, antiviral, and metal chelative activities (*5*, *6*). The flavonoids isolated from *Radix hedysari* mainly include isoflavonones, chalcones, and pterocarpans, such as ononin, formononetin, afromosin, liquiritigenin, isoliquiritigenin, and medicarpin, etc. (*3*). As the bioactive "marker compounds" of *Radix hedysari*, two isoflavonoids (formononetin and calycosin) have been quantified for the quality control of *Radix hedysari* (*7*).

In this paper, the stability of an unstable flavonoid malonate, formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (FGM, **3**), was described in detail, and a simple, reliable, and reproducible HPLC method with an improved sample preparation scheme for permitting veritably simultaneous determination of four major flavonoids, naringenin-7-*O*-α-L-rhamanopyranosyl-(1→2)-β-D-glucopyranoside (**1**), ononin (FG, **2**), formononetin (F, **4**) and medicarpin (**5**) (**Figure 1**), and indirectly valuating the true content of FGM (**3**), was developed for the quality control of *Radix hedysari* first. Medicarpin, the characteristic compound with antibacterial activity differentiating *Radix hedysari* from *Radix astragali* (*2*), was also used as a reference compound for the quantitative analysis of *Radix hedysari* for the first time.

MATERIALS AND METHODS

Chemicals and Materials. HPLC grade acetonitrile (CH_3CN) was purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was purifed by Milli-Q system (Bedford, MA). Methanol (MeOH), phosphoric acid (H_3PO_4), and formic acid (HCOOH) for analysis were of analytic grade from Beijing Reagent Company (Beijing, PR China).

Radix hedysari was purchased from Wudu Micangshan of Gansu Province, which is the major cultivated area, in September 2004 and was identified by Prof. Hu-Biao Chen, Department of Natural Medicines, Peking University. A voucher specimen was deposited at the Herbarium, School of Pharmaceutical Sciences, Peking University Health Science Center.

Reference Compounds. Naringenin-7-*O*-α-L-rhamanopyranosyl-(1→2)-β-D-glucopyranoside (**1**), FG (**2**), FGM (**3**), F (**4**), and medicarpin (**5**) were purified from *Radix hedysari* and characterized by chromatography methods. The purity of the five reference compounds was greater than 97% by the peak area normalization method using HPLC-UV with detection at 230 nm.

Apparatus. A Jasco HPLC system (Tokyo, Japan), equipped with a quaternary pump, a diode array spectrophotometric detector (DAD),

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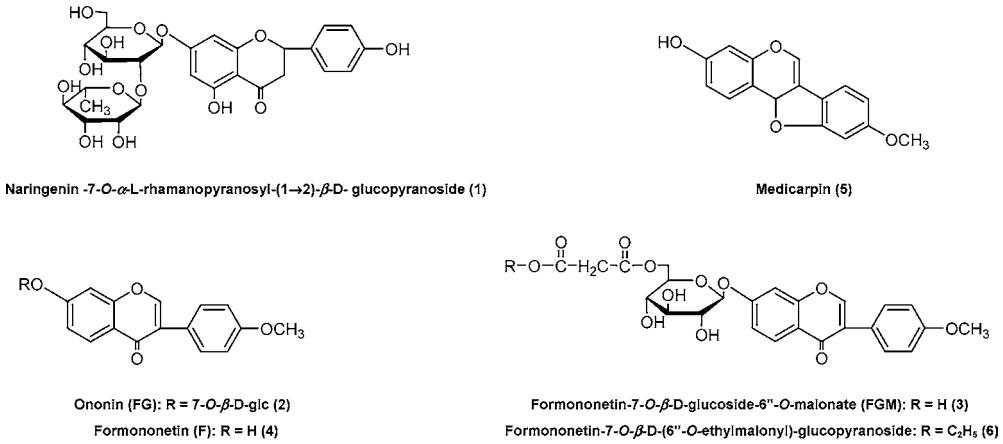

Figure 1. The structures of compounds **1**–**6**.

and a column oven, was used for analysis of the flavonoids. The column configuration consisted of a Phenomenex analytical column (Torrance, CA) with 5 μm C18-reversed phase material (250 × 4.6 mm i.d.) and a Phenomenex guard column with C18-reversed phase material (10 × 4.0 mm i.d.). An ultrasonic cleaner KQ-500DB (KunShan, PR China) was used for extraction. The pH meter PHS-2C (ShangHai, PR China) and pH combination electrode SHKY E-201-C (ShangHai, PR China) were used to value the pH accurately. The NMR spectra were recorded on a Bruker Avance DRX-500 NMR spectrometer equipped with 5 mm probes. ESI-TOF mass spectra were obtained on a Qstar mass spectrometer coupled to an Agilent 1100 HPLC system.

Preparation of Samples. The roots of *Radix hedysari* were powdered to a homogeneous size in a mill, sieved through a no. 40 mesh, and dried at 40 °C in the oven for 5 h. The powder sample (1.000 g) was extracted with 20 mL of methanol−water−formic acid (8:2:0.02, v/v/v) solution of pH 3 in an ultrasonic water bath for 30 min. This extraction was repeated two times. The mixed extracted solution was filtered, and the residue was washed with 20 mL of fresh extraction solvent. The combined filtrates were evaporated at 40 °C to dryness by vacuum. The dry extract was made up to exactly 10 mL of solution with fresh extraction solvent using a volumetric flask and filtered through a 0.45-μm-membrane filter before injecting a 20 μL sample. Each sample was prepared with the above protocol for HPLC analysis.

For valuating the true content of FGM, 80% aqueous methanol (without acid) was chosen as the extraction solvent, and the extraction liquid was refluxed for about 4 h to allow FGM to translate into FG completely. The remnant process was kept the same as the sample preparation above.

Quantification of Flavonoids. *Chromatographic Conditions.* The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% phosphoric acid, v/v) with a gradient elution (0−5 min, 21% A; 5−13 min, 21−23% A; 13−25 min, 23% A; 25−40 min, 23−40% A; and 40−60 min, 40−60% A). The detection wavelength and column temperature were set at 230 nm and 30 °C, respectively.

Calibration. Calibration was performed in the range of 1.11−208.00 mg/L using dilutions of the respective stock solutions. Calibration graphs were recorded by plotting the respective peak areas [230 nm] vs the concentrations (mg/L). Each calibration curve was performed with 5−7 different concentrations in triplicate. Consequently, calibration curves were constructed.

Validation of the Quantification Method. The measurements of intra- and interday variability were utilized to determine the precision of the developed assay method. The relative standard deviation (RSD) was taken as a measure of precision. For the same sample, the intra- and interday reproducibility were examined on five time-dots a day and three consecutive days, respectively.

A sample of *Radix hedysari* was extracted and analyzed in duplicate. The procedure was repeated five times to evaluate the reproducibility of extraction protocol.

The stability of extracts at room temperature was studied at different time intervals, i.e., 0, 2, 4, 8, 12, 24, and 48 h.

Extraction recovery was carried out by mixing a powdered root sample (0.500 g) and three control levels (high, mid, and low levels of the calibration range) of reference compounds **1**, **2**, **4**, and **5**. The experiments were repeated three times for each level. The extraction procedure was the same as described in "Preparation of Samples". The extracted samples were analyzed by HPLC, and the amounts of the reference compounds **1**, **2**, **4**, and **5** were calculated. Recoveries were calculated as follows

$$\%\ \text{recovery} = 100 \times (\text{amount from sample and added reference} - \text{amount from sample})/\text{amount from added reference}$$

RESULTS AND DISCUSSION

Optimization of HPLC Conditions. Compared to systems with methanol, systems with acetonitrile had a better resolution and a smoother baseline. Different ratios of water and acetonitrile were tried, but no satisfied separation was reached. Finally, a gradient program (0−5 min, 21% A; 5−13 min, 21−23% A; 13−25 min, 23% A; 25−40 min, 23−40% A; and 40−60 min, 40−60% A) of mobile phase consisting of solvent A (acetonitrile) and solvent B (0.1% phosphoric acid, v/v) was chosen for its well baseline resolution and suitable duration for analysis. In order to obtain sufficient sensibility of quantified peaks on the HPLC chromatogram, the UV spectra of correlative peaks were investigated, and 230 nm was selected as the detection wavelength. Representative chromatogram of the extract of *Radix hedysari* sample was shown in **Figure 2**.

Optimization of Extraction Conditions. An orthogonal experiment was employed in order to optimize the extraction conditions. It involved four factors: (A) solvent volume; (B) proportion between methanol and water; (C) extraction times; and (D) sonication time. The experimental factors and corresponding levels were shown in **Table 1**, and orthogonal designs L_9 (3^4) were presented in **Table 2**. The optimal conditions for the extraction of *Radix hedysari* could be obtained by intuitionistic analysis of the experimental results of the orthogonal design. In order to fully show the flavonoid profiles of *Radix hedysari*, the relative sum area of five principal peaks, standing for the five major flavonoids, was used as a criterion for the selection of optimal extraction conditions. Optimized factors' ordering was obtained according to range analysis (**Table 1**). The efficiency of extraction twice and trice varied slimly, so washing up of the residue substituted for the third ultrasonic extraction practicably. The optimal conditions were presented in detail in "Preparation of Samples".

Stability of FGM in *Radix hedysari* Extracts. In the study on *Radix astragali*, known as huangqi in China, unstable flavonoid malonates were found (*8*). Malonates BGM (biocha-

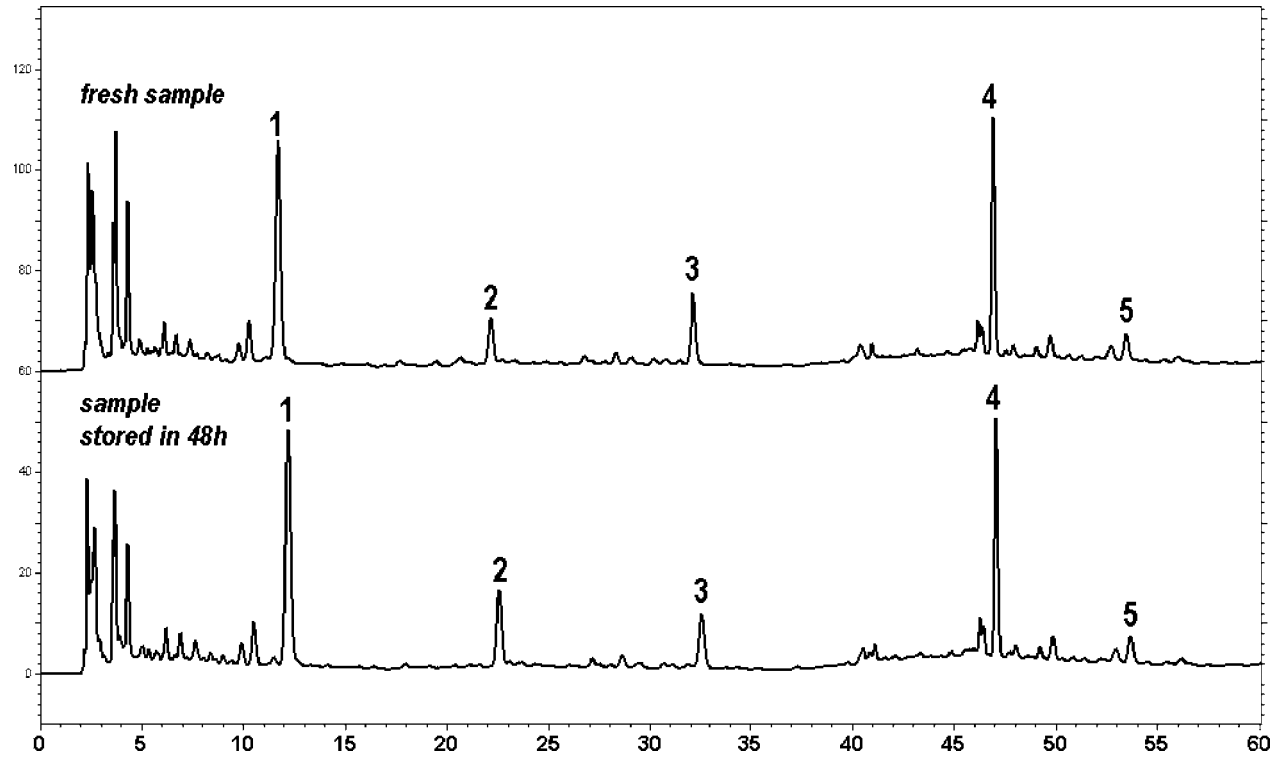

Figure 2. HPLC-UV chromatograms of samples before-and-after stored at room temperature for 48 h. Peak numbers correspond to the assignments given in **Figure 1**.

Table 1. Factors and Levels for the Optimization of Extraction Conditions

	levels		
factors	1	2	3
A: solvent volume (mL)	10	20	40
B: methanol concentration (%)[a]	50	80	100
C: extraction times	1	2	3
D: sonication time (min)	20	30	40

[a] Including 0.2% formic acid.

nin-7-*O*-*β*-D-glucoside-6″-*O*-malonate) and FGM were gradually converted to their related glucosides during the malonate-free heating process (*9*).

During the optimization of extraction conditions for *Radix hedysari*, an unstable chromatogram peak was also found (peak 3, **Figure 2**). It decreased gradually, while the peak of FG (peak 2, **Figure 2**) increased correspondingly, as the aqueous methanol extract was stored at room temperature. Furthermore, the unstable constituent could only be found in trace amounts in the extracts obtained by using aqueous methanol through refluxing. A detailed HPLC analysis showed that this constituent in aqueous methanol (80%) extract disappeared almost completely at reflux for 4 h or by storing it at room temperature for about 2 weeks. Obviously, heating accelerated the convertion course.

The above evidence suggested that the unstable constituent might be a flavonoid malonate which was very popular in leguminous plants (*10*). Then, it was isolated from the extracts of *Radix hedysari* and identified as formononetin-7-*O*-*β*-D-glucoside-6″-*O*-malonate (FGM) by comparison of the UV,

Table 2. Results and Analysis of Orthogonal Design

run no.	A: solvent volume (mL)	B: methanol concn (%)	C: extraction times	D: sonication time (min)	A_{5p}[a]
1	10	50	1	20	1277155
2	10	80	2	30	1992629
3	10	100	3	40	1792133
4	20	50	2	40	2063857
5	20	80	3	20	2051698
6	20	100	1	30	1376005
7	40	50	3	30	2098460
8	40	80	1	40	1432708
9	40	100	2	20	1599850
K_1	1687306	1813157	1361956	1642901	
K_2	1830520	1825678	1885445	1822365	
K_3	1710339	1589329	1980763	1762899	
range	143214	236349	618807	179464	
optimized scheme	A2	B2	C2	D2	
primary and secondary order	4	2	1	3	

[a] A_{5p} represents the area sum of 5 principal peaks.

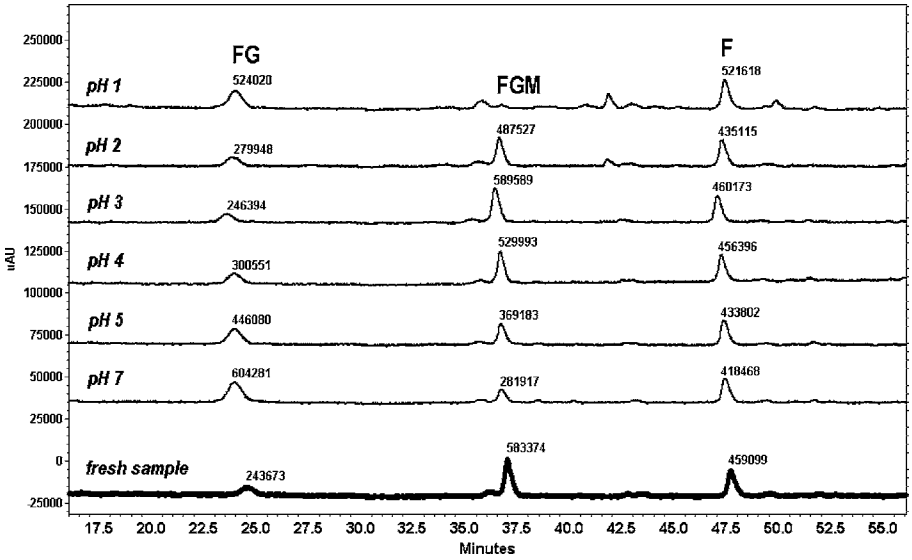

Figure 3. HPLC-UV chromatograms (including peak area) of samples refluxed at different pH.

mass, proton, and carbon NMR data with those published in the cited references (*11*, *12*).

It was reported that enzymolysis and thermolability were the reasons that corresponded to the instability of isoflavone malonylglucosides in red clover (*Trifolium pratense* L.) extracts (*13*). In our study, it was found that FGM was still not very stable, while its NMR spectra were recorded in DMSO-d_6, which indicated that enzymolysis and thermolability were not the only reasons that caused the instability of FGM in plant extracts. Additionally, it was interesting to find that both FG, the corresponding isoflavonoid glucoside of FGM without a malonate group, and formononetin-7-*O*-*β*-D-(6″-*O*-ethylmalonyl)-glucopyranoside (**6**, **Figure 1**), the ethyl ester of FGM, which was a novel isoflavone glucoside isolated from *Millettia nitida* var. *hirsutissim* in our laboratory (*14*), were very stable in the extracts. So, the free carboxyl group presented in FGM might be an important factor that caused FGM's instability, and then the effect of different pH environments on the stability of FGM was studied in detail.

The 80% methanol extract of *Radix hedysari* was divided into several portions and adjusted to pH 1, 2, 3, 4, 5, and 7, respectively, using a suitable microamount of acid liquor. Subsequently, each portion was refluxed for 2 h in a water bath of 90 °C. After having been cooled to room temperature, the samples were examined by HPLC-UV in turn. The results (**Figure 3**) showed that, between pH 2−4 value range, FGM remained relatively stable, while its corresponding glucoside FG did not show a simultaneous increase. At other pH values (pH 5−7), the conversion occurred in somewhat different degrees. Additionally, it was also found that during the heating process the content of F, the corresponding aglycone of FGM and FG, remained relatively steady over the range of pH 2−7, except for in the intensely acidic environment of pH 1.

Based on the above evidence, we concluded that in aqueous methanol extracts of *Radix hedysari*, FGM easily lost the malonate group and subsequently converted to its corresponding glucoside FG at room temperature or under the refluxing conditions. However, the conversion process could be inhibited at a suitable pH environment. Obviously, for quantification purposes an acidic extraction solvent of pH 3 is to be preferred, and this is the reason for the use of 0.2% formic acid in the extraction solvent.

Quantification of Flavonoids in *Radix hedysari*. Based on the above results of FGM's stability in *Radix hedysari* extracts, it is obvious that the use of regular extraction methods without acid may well result in the underestimation of malonylglucoside isoflavone FGM and consequently overestimation of corresponding isoflavone glucoside FG. So, in this study the sample pretreatment was mended, and 0.2% formic acid was adopted in the extraction solvent to inhibit the conversion of FGM to FG (detail in "Preparation of Samples").

Table 3. Calibration Curves of Four Flavonoids in *Radix hedysari*

compds	regression equation[a]	r^2	test range (mg/L)	limit of detection[b] (ng)
1	$y = 32671.67267x - 9569.38971$	0.99987	3.07–120.00	20
2	$y = 29357.73992x + 32453.64236$	0.99998	2.59–40.40	17
4	$y = 137385.68064x - 31414.45833$	0.99992	1.11–43.20	7
5	$y = 48355.21436x + 14589.04389$	0.99994	1.30–208.00	8

[a] *y*: peak area; *x*: concentration of analyte (mg/L). [b] Limit of detection: $S/N = 3$.

Table 4. Intra- and Interday Variability of Four Flavonoids in *Radix hedysari*

	intraday ($n = 5$)		interday ($n = 3$)	
compds	found (area)	RSD (%)	found (area)	RSD (%)
1	704877 ± 3001	0.43	705626 ± 524	0.07
2	183394 ± 2195	1.20	184051 ± 2470	1.34
4	638212 ± 1438	0.23	635591 ± 1360	0.21
5	114428 ± 1730	1.51	114356 ± 2067	1.81

Under the chromatographic conditions used in this study, all four calibration curves exhibited good linear regressions as shown in **Table 3**, and the limit of detection (LOD) was in the range of 8−20 ng for compounds **1**, **2**, **4**, and **5**. The results in **Table 4** demonstrated that the developed analytical method was reproducible with good accuracy and sensitivity for all analytes examined. The overall intra- and interday variations were less than 2% for all four analytes. The recovery assays of the flavonoids were carried out by adding standards to crude drug powders, and the results were shown in **Table 5**, from which it was clear that the recoveries for all four flavonoids determined were in the range of 90−110%.

Valuation of FGM Content in *Radix hedysari*. In the literature quantification of glucoside malonates has not received much attention; some relative studies (*15*−*17*) were simply

Table 5. Recoveries of Four Flavonoids in *Radix hedysari* at Three Levels of Calibration Ranges

compds	low level, mean ± SD[a] (RSD %)	mid level, mean ± SD[a] (RSD %)	high level, mean ± SD[a] (RSD %)	recovery, mean ± SD[b]	RSD %[b]
1	106.7 ± 5.0 (4.71)	107.3 ± 1.2 (1.10)	105.4 ± 3.1 (2.94)	106.5 ± 1.0	0.90
2	102.7 ± 2.3 (2.21)	99.7 ± 2.3 (2.27)	94.0 ± 3.0 (3.15)	98.8 ± 4.4	4.50
4	109.3 ± 6.3 (5.76)	105.2 ± 2.4 (2.28)	106.8 ± 3.6 (3.42)	107.1 ± 2.1	1.93
5	101.4 ± 5.9 (5.79)	94.6 ± 3.1 (3.28)	102.0 ± 5.5 (5.42)	99.4 ± 4.1	4.14

[a] $n = 3$. [b] $n = 9$.

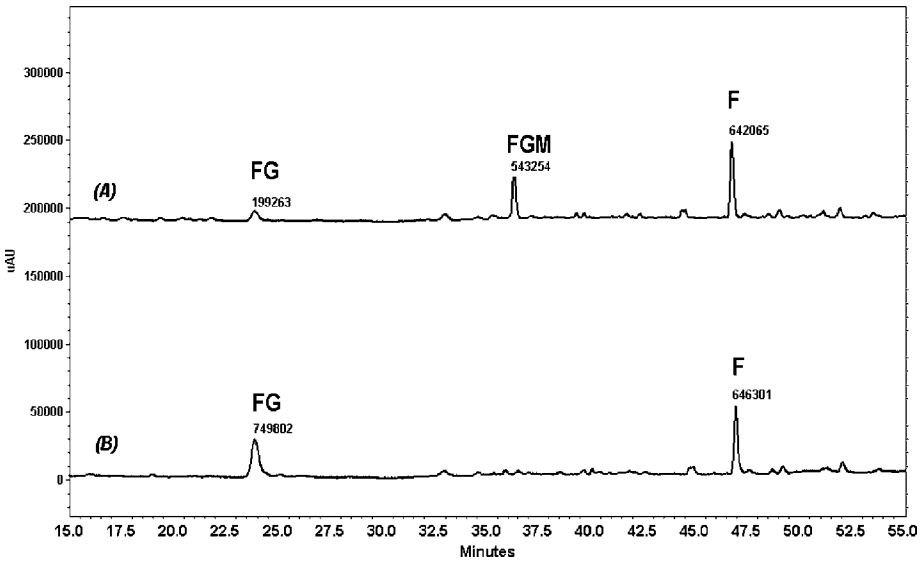

Figure 4. HPLC-UV chromatograms (including peak area) of (**A**) sample extracted with acid (pH 3) and (**B**) sample extracted without acid at reflux for 4 h.

based on the formation of aglycone after hydrolysis. In this paper, the true content of FGM in *Radix hedysari* was valuated indirectly.

During the course of the quantification study for *Radix hedysari*, we found that flavonoid glucoside FG in the aqueous methanol extracts without acid was not further degraded into the corresponding aglcone F, whether after long-term storage at room temperature (48 h) or refluxing (4 h), while FGM could convert to FG completely after refluxing for 4 h (**Figure 4**). So, the true content of FGM in *Radix hedysari* could be determined indirectly by subtracting the content of FG determined above from that found at reflux for 4 h, in spite of the instability of FGM itself. The mole concentration of FG and FGM was adopted for the convenience of calculating, since analyte conversion was considered. All analyses were carried out in triplicate.

This newly developed HPLC-UV assay method was subsequently applied to simultaneous determination of the four major flavonoids and valuation of FGM in 10 commercial medicinal materials (**Table 6**). The contents of naringenin-7-*O*-α-L-rhamanopyranosyl-(1→2)-β-D-glucopyranoside (**1**), FG (**2**), FGM (**3**), F (**4**), and medicarpin (**5**) were 125.2−221.7, 49.0−216.5, 167.4−550.4, 49.2−196.3, and 18.6−263.7 μg/g, respectively.

This is the first report on the stability of FGM in different pH environments and the determination of true contents of five major flavonoids, including an unstable isoflavone malonylglucoside, in *Radix hedysari*. The HPLC method with a modified sample pretreatment scheme developed in this study was proved to be simple, sensitive, accurate, and reproducible. This HPLC assay can be readily utilized as a suitable quality control method for the determination of the major bioactive flavonoids in *Radix hedysari*.

Table 6. Content of Compounds **1**–**5** in Commercial *Radix hedysari* (hongqi)

sample	content of compds **1**–**5** (μg/g)				
	1	**2**	**3**	**4**	**5**
1	125.2	89.8	174.1	196.3	263.7
2	nd[a]	82.6	173.5	101.5	44.2
3	nd[a]	167.5	530.4	140.5	106.8
4	nd[a]	114.8	249.2	78.3	122.4
5	nd[a]	68.4	199.1	71.1	55.7
6	nd[a]	55.9	179.1	92.9	48.6
7	nd[a]	216.5	550.4	75.4	27.3
8	nd[a]	62.0	225.7	85.2	21.9
9	nd[a]	49.0	167.4	163.2	45.8
10	221.7	56.9	210.6	49.2	18.6

[a] nd: not detected.

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