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Full Length Research Paper

Simultaneous determination of three bioactive homoisoflavanones in rhizomes of *Polygonatum odoratum*

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An high-performance liquid chromatographic- photodiode array detector method (HPLC-PAD) method was established to simultaneously determine three bioactive homoisoflavanones in the rhizomes of *Polygonatum odoratum* (Mill) Druce. The optimal conditions of extraction were achieved using reflux extraction with methanol-water (80:20, v/v) for 2 h. The analysis was performed on a Diamonsil ODS column with a gradient elution of methanol and 1% acetic acid aqueous solution at a flow rate of 1.0 ml/min. The detection wavelength was 297 nm and the column temperature was 30°C. Linear relationships within the range of investigated concentrations were observed for three bioactive homoisoflavanones with their correlation coefficients greater than 0.9999. The method was validated for intra- and inter-day precision (RSD < 2.05%), stability (RSD < 2.40%), and repeatability (RSD < 2.38%) with recovery between 98.67 and 100.52% and the RSD less than 2.28%. The method was successfully applied to simultaneously determine three homoisoflavanones in the twelve samples of Rhizoma Polygonati Odorati collected from different regions. The results indicated that the developed assay could be considered as a suitable quality control method for the rhizomes of *P. odoratum*.

Key words: *Polygonatum odoratum*, homoisoflavanone, high-performance liquid chromatographic- photodiode array detector method (HPLC-PAD).

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INTRODUCTION

Rhizoma Polygonati Odorati, a commonly used herbal medicine called Yuzhu in Chinese, is derived from the dried rhizome of *Polygonatum odoratum* (Mill). Druce belonging to Liliaceae family. *P. odoratum* is a perennial plant that occurs in the wild and is also cultivated in the central and southwest areas of China. Traditionally it is used as removing dryness, promoting secretion of fluid and quenching thirst (The Pharmacopoeia Commission of PRC, 2010), and clinically applied for the treatment of diabetes and its complications (Li et al., 2004). Despite the popularity of usage, relevant quality control method for Rhizoma Polygonati Odorati is very limited. In the pharmacopoeia of China, the content of polysaccharide has been used for quantity assessment index using glucose as the marker compound (The Pharmacopoeia Commission of PRC, 2010). However, polysaccharide was commonly found in plants and not specified enough for the identification. Hence, it is very important to establish quantitative methods based on the bioactive substances for the quality control of Rhizoma Polygonati Odorati and its pharmaceutical preparations. Previous phytochemical investigations of the rhizomes of *P. odoratum* have resulted in the isolation of homo-isoflavanones (Rafi et al., 2007; Li et al., 2009; Wang et al., 2009a, b; Zhang et al., 2010). According to the report, the total flavonoids from the rhizomes of *P. odoratum* showed significant dose-dependent anti-diabetic activity (Shu et al., 2009). Recently we reported homoisoflavanones, (1) 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6-methoxy-8- methylchroman-4-one, (2) 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6,8-dimethylchroman-4-one and (3) 3-(4′-hydroxybenzyl)-5,6-dimethoxy-7-hydroxy-8-methylchroman-4-one from the rhizomes of *P. odoratum* exhibited much stronger inhibition of advanced glycation end products formation than aminoguanidine, a

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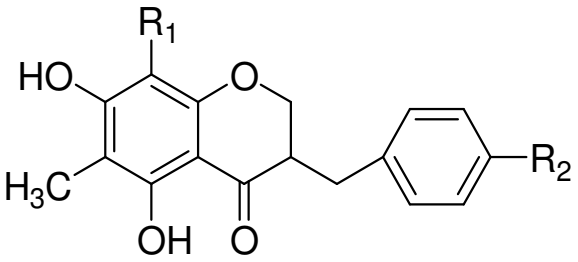

1 $R_1=OCH_3$ $R_2=OH$
2 $R_1=CH_3$ $R_2=OH$
3 $R_1=OCH_3$ $R_2=OCH_3$

Figure 1. Structures of characteristic homoisoflavanones in the rhizomes of *P. odoratum*. (1) 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6-methoxy-8-methylchroman-4-one, (2) 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6,8-dimethylchroman-4-one, (3) 3-(4′-hydroxybenzyl)-5,6-dimethoxy-7-hydroxy-8-methylchroman-4-one.

well-known glycation inhibitor (Dong et al., 2010). The structures of 1 to 3 are shown in Figure 1. In the present study, an high-performance liquid chromatographic-photodiode array detector method (HPLC-PVD) for the simultaneous determination of three bioactive homo-isoflavanones inthe rhizomes of *P. odoratum* was developed.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade methanol (Merck, Darmstadt, Germany) and deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) were used for preparation of mobile phase. Analytical grade methanol (Beijing Reagent, Beijing, China) was used for preparation of standard and sample extraction. Other chemicals were purchased from Beijing Chemical Engineering Factory (Beijing, China). 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6-methoxy-8-methylchroman-4-one (1), 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6,8-dimethylchroman-4-one (2), and 3-(4′-hydroxybenzyl)-5,6-dimethoxy-7-hydroxy-8-methylchroman-4-one (3) with more than 98 % of purity were isolated and purified from the rhizomes of *P. odoratum* in our laboratory. The structures were confirmed by comparison of their ESI-MS and NMR data with those in the literature (Wang et al., 2009a).

Plant materials

The rhizomes of *P. odoratum* were obtained from different regions and authenticated by Prof. Wenting Li, College of Pharmacy, Changchun University of Chinese Medicine, China. The corresponding voucher specimens are deposited at the Institute of Phytochemistry, Jilin Academy of Chinese Medicine Sciences, China.

Preparation of samples

The sample was pulverized to a homogeneous size, passed through a 40-mesh sieve, and dried to constant weight before use. Approximately 2.0 g sample power was accurately weighed and extracted with 100 ml methanol-water (80:20, v/v) under reflux condition for 2 h. And then the extract was made up to its volume for the losing volume with extraction solvent and was filtered by analytical filter paper.

The 50 ml filtrate was evaporated to dryness and the residue was dissolved in 4 ml water. The bioactive homoisoflavanones of crude extract were enriched on a solid phase extraction (SPE) C_{18} cartridge (Agela, Tianjin, China). The SPE C_{18} cartridge was sequentially conditioned with 5 ml methanol and 10 ml water without allowing the cartridge to dry. The concentrated extract was passed through the cartridge, washed with 5 ml water and eluted with 5 ml methanol. Theelute was transferred into 5 ml volumetric flack and was adjusted to the volume with methanol. The solution was then filtered through a 0.45 µm Nylon membrane filter and 20 µl were injected for HPLC analysis.

Preparation of standard solutions

The stock solutions were prepared separately by dissolving the reference substances in methanol to afford final concentrations of 0.712 mg/ml for 1, 0.376 mg/ml for 2, and 0.600 mg/ml for 3. The standard solutions were prepared by appropriate dilution of the mixed stock solution with methanol to give ten different concentrations with the ranges: 1, 10.68 to 106.8 µg/ml; 2, 5.64 to 56.4 µg/ml; 3, 1.08 to 10.8 µg/ml. All solutions were filtered through a 0.45 µm Nylon membrane filter and 20 µl were injected for HPLC analysis.

HPLC conditions

The HPLC analyses were performed using a Shimadzu 2010A HT series liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and SPD-M20A photodiode-array detector coupled with a CLASS-VP analytical workstation. The chromatographic separation was carried out on a Diamonsil ODS column (5 µm, 250 mm × 4.6 mm I.D., Dikma Technologies, Beijing, China).

The mobile phase consisted of methanol (A) and 1% acetic acid aqueous solution (B, v/v). A gradient program was used as follows: 0 to 5 min, isocratic elution with A to B (60:40, v/v); 6 to 25 min, linear change from A and B (60:40, v/v) to A and B (80:20, v/v); 26 to 35 min, isocratic elution with A to B (80:20, v/v). The total run time was 35 min and the column was reequilibrated for 10 min between runs. The flow rate was 1.0 ml/min and the column temperature was maintained at 30°C. The sample was detected by monitoring UV absorption at 297 nm.

RESULTS

Optimization of extraction conditions

Extraction method, extraction solvent, and extraction time

Table 1. The influences of extraction methods on extraction efficiency for compounds 1 – 3.

Extraction method	Compound[a]		
	1	2	3
Ultrasonication[b]	150.92 ± 2.10	96.47 ± 0.67	14.91 ± 0.10
Reflux[c]	166.82 ± 2.26	107.64 ± 1.81	16.35 ± 0.20
Soxhlet[d]	129.65 ± 0.22	71.05 ± 0.55	8.66 ± 0.08

[a] Mean ± S.D. (n = 3), μg/g[b.]. The extraction solvent was methanol, the extraction time was 1 h, ultrasound frequency was 40 kHz; [c] The extraction solvent was methanol, the extraction time was 2 h; [d] The extraction solvent was methanol, the extraction time was 4 h.

Table 2. The influences of extraction solvents on extraction efficiency for compounds 1 to 3.

Extraction solvent[b]	Compound[a]		
	1	2	3
Methanol-water (70:30, v/v)	169.03 ± 2.24	104.57 ± 1.94	15.82 ± 0.41
Methanol-water (80:20, v/v)	174.18 ± 0.85	117.52 ± 1.41	18.71 ± 0.25
Methanol-water (90:10, v/v)	173.04 ± 2.80	107.56 ± 0.51	16.71 ± 0.18
Methanol	166.82 ± 2.26	107.64 ± 1.81	16.35 ± 0.20

[a] Mean ± S.D. (n = 3), μg/g; [b] The extraction time was 2 h.

Table 3. The influences of extraction times on extraction efficiency for compounds 1 to 3.

Extraction time[b] (min)	Compound[a]		
	1	2	3
30	145.17 ± 0.58	93.16 ± 0.22	15.52 ± 0.10
60	162.26 ± 0.57	102.00 ± 0.69	15.72 ± 0.25
90	171.77 ± 0.53	103.74 ± 0.58	18.82 ± 0.12
120	174.18 ± 0.85	117.52 ± 1.41	18.71 ± 0.25
150	173.54 ± 1.35	112.80 ± 0.70	18.24 ± 0.46
180	174.30 ± 2.42	105.26 ± 0.51	16.36 ± 0.37

[a]Mean ± S.D. (n = 3), μg/g; [b] The extraction solvent was methanol-water (80:20, v/v).

were investigated so as to obtain the best extraction efficiency. Compared to three different extraction methods, the results suggested that reflux extraction was more effective than Soxhlet extraction and ultrasonic extraction (Table 1). Hence reflux extraction was chosen as the preferred method. Different concentrations of methanol, including methanol-water (70:30, v/v), methanol-water (80:20, v/v), methanol-water (90:10, v/v), and methanol were performed as extraction solvents to analyze the effect of the solvent on extraction efficiency. The results showed that methanol-water (80:20, v/v) was the most suitable extraction solvent (Table 2). Consequently, the samples were extracted with methanol-water (80:20, v/v) by reflux extraction for 30, 60, 90, 120, 150, and 180 min. The extraction yields were increased by increasing the extraction time, but when the extraction time increased above 2 h, there were no significant differences (Table 3). Based on these findings, reflux extraction with methanol-water (80:20, v/v) for 2 h was therefore selected as the preferred method of extraction.

Optimization of the separation conditions

On the basis of the absorption maxima of the three compounds in UV spectra acquired by use of the photodiode array detector, the monitoring wavelength was set at 297 nm. In order to select an appropriate elution system for HPLC separation of sample, different kind of mobile phases, including methanol and water, acetonitrile and water, methanol and 1% acetic acid aqueous solution (v/v), and acetonitrile and 1% acetic acid aqueous solution (v/v), were evaluated to find the best separation conditions. The optimum mobile phase was found to be methanol (A) and 1% acetic acid

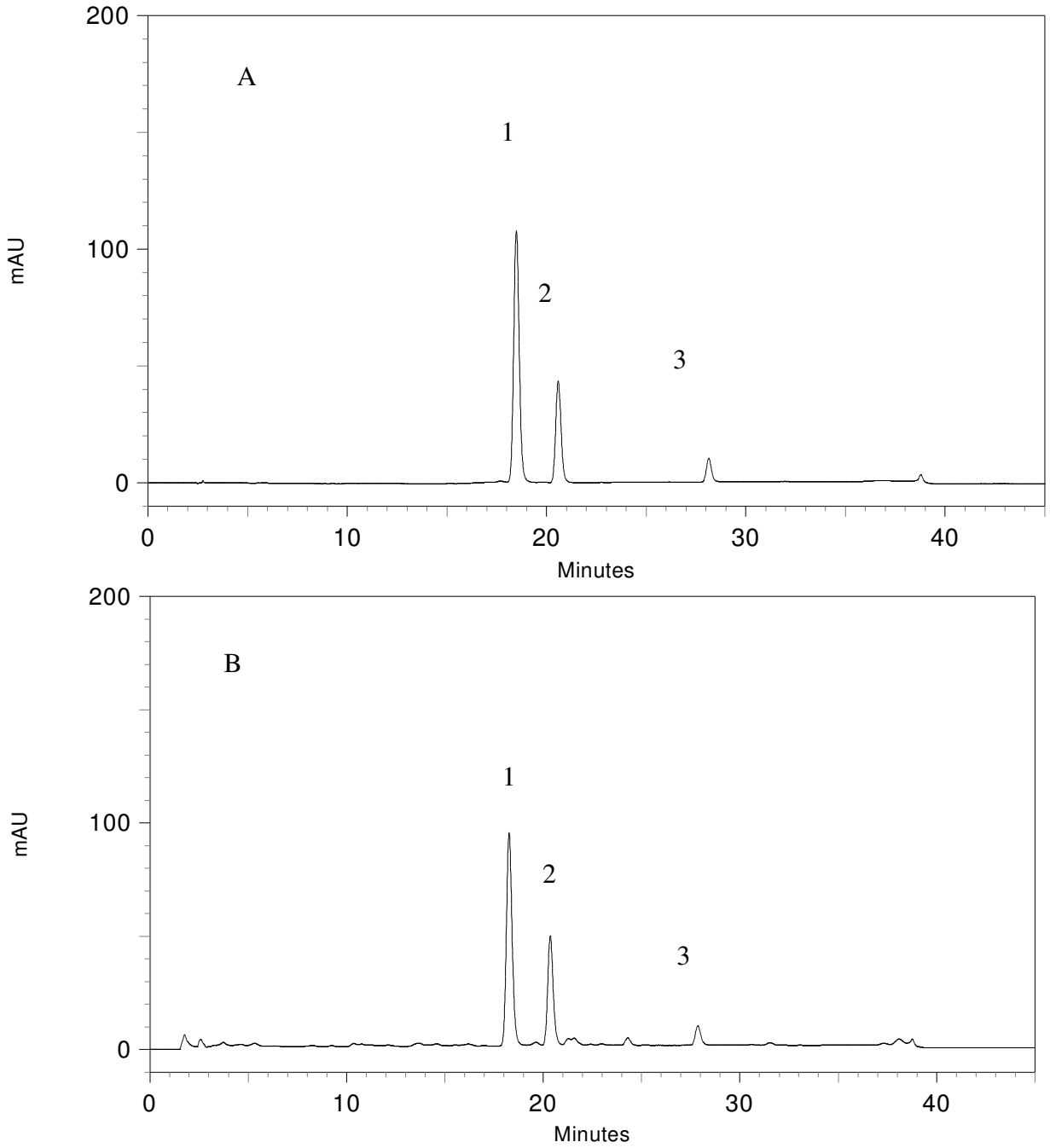

Figure 2. Typical HPLC chromatograms of (A) standard solution: 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6-methoxy-8-methylchroman-4-one (1) (35.6 µg/ml), 3-(4′-hydroxybenzyl)-5,7-dihydroxy-6,8-dimethylchroman-4-one (2) (18.8 µg/ml), 3-(4′-hydroxybenzyl)-5,6-dimethoxy-7-hydroxy-8-methylchroman-4-one (3) (3.6 µg/ml); (B) sample solution, respectively. HPLC conditions: column, Diamonsil ODS 250 mm × 4.6 mm I.D.; column temperature, 30°C; mobile phase, methanol (A) and 1% acetic acid aqueous solution (B, v/v); gradient program, 0 to 5 min, isocratic elution with A and B (60:40, v/v), 6 to 25 min, linear change from A and B (60:40, v/v) to A-B (80:20, v/v), 26 to 35 min, isocratic elution with A and B (80:20, v/v); flow rate, 1.0 ml/min; detection wavelength, 297 nm.

aqueous solution (B), and the ratios as well as gradient elution system were altered until all three compounds were satisfactorily resolved at the baseline in 35 min. The application of gradient elution may reduce the running time and provide a narrower peak for the three compounds. The typical chromatographic profiles of the standard solution and the sample solution were shown in Figure 2.

Table 4. Linear relationships between peak area and concentration of compounds 1 to 3.

Compound	Regression equation (y = ax + b)[a]	Correlation coefficient *R*	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
1	$y = 55918x + 20833$	0.9999	10.68-106.80	0.192	0.058
2	$y = 40762x + 1219.5$	0.9999	5.64-56.40	0.145	0.038
3	$y = 50608x + 3827.5$	0.9999	1.08-10.80	0.120	0.032

[a] In the regression equation y = ax + b, x denotes the concentration of the compound (μg/ml), y is the peak area, a is the slope and b is the intercept of the regression line.

Method validations

The developed method was validated in terms of linearity, intra- and inter-day precision, repeatability, stability, and recovery.

Linearity, limit of detection, and quantification

The linearity of the response for each compound was assessed by using ten standard solutions (each injected in triplicate) in an appropriate concentration range. The linear regression data were estimated and were reported in Table 4.

All the calibration curves were linear within the test range and followed the equation of the type of y = ax + b with high correlation coefficient (R > 0.9999). The limits of detection (LOD) and quantification (LOQ) were defined as the amounts for which the signal-to-noise ratios were 3 and 10, respectively. The LOD and LOQ values can be seen in Table 4.

Precision

Inter- and intra-day variations were examined to determine the precision of the developed chromatographic method. For the intra-day variability test, the same sample solution was analyzed in six replicateswithin one day, while for the inter-day variability test, the same sample solution was determined for three consecutive days. Variations were expressed by the relative standard deviation (R.S.D). The results were shown in Table 5, where it can be seen that the inter- and intra-day R.S.D values of the contents for compounds 1 to 3 were all less than 2.05%, confirming the good precision of the developed method.

Reproducibility and stability

Method reproducibility was evaluated by six replicated analyses of herbal samples. The values of R.S.D.s of the contents for compounds 1 to 3 were 0.91, 2.15, and 2.38%, respectively. Stability testing was performed on a sample solution after standing at time intervals of 0, 2, 4, 8, 12, 24 and 48 h in a volumetric flack. The results showed that the values of R.S.D.s of the contents for compounds 1 to 3 were 0.59, 0.46 and 2.40%, respectively.

RECOVERY

Recovery of the reference substance from sample is generally used to evaluate the accuracy of a newly developed analytical method. Three different concentrations (low, medium and high) of the individual standard solutions were added to known sample. Triplicate experiments were performed at each level. The mixture was extracted and analyzed using the method described above. The average percent recoveries were evaluated by calculating the ratio of the detected amount to the added amount. The recovery of the method was in the range of 98.67 to 100.52%, with R.S.D. less than 2.28% as shown in Table 6.

DISCUSSION

The established HPLC-PAD method was successfully applied to simultaneously determine three homo-isoflavanones in samples of Rhizoma Polygonati Odorati from different regions of China. The contents of compounds 1 to 3 in the samples are listed in Table 7. It can be seen that all compounds could be detected in the 12 samples. Among the analyzed compounds, the content of compound 1 was the highest in all of the samples. The results also showed that the total amounts of three homoisoflavanones in samples varied from 39.70 to 961.39 μg/g, with nearly 24 fold variation. Meanwhile, the single constituent was concerned. For example, the content of compound 1 in samples varied from 20.33 to 523.02 μg/g, with nearly 26 fold variation. A similar situation was also found in samples cultured in same region with different grades, which may consequently exist in different therapeutic effects.

Conclusion

The developed analytical methodology is the first method

Table 5. Intra- and Inter day precisions of the development method to determine compounds 1 -3.

Compound	Intra-day[a]						Inter-day[b]	
	Day 1		Day 2		Day 3		Conc.	R.S.D (%)
	Conc.	R.S.D (%)	Conc.	R.S.D (%)	Conc.	R.S.D (%)		
1	167.76 ± 0.29	0.17	168.84 ± 0.39	0.23	169.52 ± 0.24	0.14	168.71 ± 0.80	0.50
2	106.30 ± 0.06	0.06	106.23 ± 0.78	0.73	107.24 ± 0.11	0.10	106.59 ± 0.64	0.60
3	13.42 ± 0.13	0.97	13.18 ± 0.27	2.05	13.43 ± 0.17	1.27	13.34 ± 0.22	1.65

[a] Mean ± S.D (n = 6), μg/g, [b] Mean ± S.D. (n = 18), μg/g.

Table 6. Recoveries of compounds **1** to**3** from the rhizomes of *P. odoratum.*

Compound	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)[a]	R.S.D (%)
1	167.94, 169.63, 167.61	80.21	249.53, 248.81, 245.96	99.37	2.11
	166.09, 167.44, 168.95	160.42	327.76, 324.21, 325.37	98.67	1.85
	168.45, 166.43, 168.78	240.63	406.34, 412.15, 407.29	100.03	1.81
2	109.96, 111.39, 110.84	58.17	167.06, 168.86, 169.83	99.46	1.73
	111.06, 110.62, 113.58	116.34	227.62, 228.31, 226.95	99.60	1.93
	112.28, 112.16, 113.69	174.51	290.75, 286.45, 284.97	100.10	2.07
3	18.98, 18.26, 18.93	8.00	26.92, 26.40, 26.76	99.63	1.97
	17.75, 17.68, 18.17	16.00	33.52, 33.83, 34.50	100.52	1.78
	17.56, 17.91, 17.64	24.00	41.97, 41.24, 41.39	99.29	2.28

[a] Recory (%) = (found amount – original amount)/ spiked amount × 100, the data presented as average of three determinations.

Table 7. Contents of three homoisoflavanones in the samples of Rhizoma Polygonati Odorati.

No.	Region	Compound[a]			Total
		1	2	3	
1[b]	Hunan Province	32.92 ± 0.83	26.04 ± 0.69	3.09 ± 0.07	62.05
2[b]	Hunan Province	75.40 ± 1.81	59.15 ± 0.56	5.34 ± 0.14	139.89
3[b]	Hunan Province	190.75 ± 1.51	138.02 ± 1.64	17.11 ± 0.39	345.88
4[b]	Hunan Province	105.28 ± 1.16	100.88 ± 0.35	12.39 ± 0.37	218.55
5[b]	Hunan Province	103.23 ± 1.35	94.90 ± 0.90	12.45 ± 0.15	210.58
6	Hunan Province	251.08 ± 1.00	176.37 ± 1.91	17.78 ± 0.31	445.23

Table 7. Contd.

7	Inner Mongolia Region	95.92 ± 0.61	59.58 ± 1.05	2.56 ± 0.07	158.06
8	Zhejiang Province	136.79 ± 2.43	49.49 ± 1.02	7.04 ± 0.16	193.32
9	Liaoning Province	523.02 ± 1.63	405.38 ± 6.33	32.99 ± 0.96	961.39
10	Liaoning Province	301.81 ± 1.73	251.46 ± 4.18	37.18 ± 0.54	590.45
11	Liaoning Province	20.33 ± 0.28	17.65 ± 0.15	1.72 ± 0.02	39.70
12	Jilin Province	168.02 ± 0.84	111.71 ± 0.58	17.49 ± 0.29	297.22

[a] Mean ± S.D. (n = 3), μg/g, [b] The herbal samples were cultured in same region with different grades.

concerning the simultaneous determination of three bioactive homoisoflavanones in Rhizoma Polygonati Odorati, a traditional Chinese medicine with long history of usage due to its therapeutic properties on diabetes. The HPLC-PAD method is suitable for routine use to inspect quality of commercially available samples. Moreover, because variations in the bioactive component may influence the therapeutic potency of the medicine herb, the factors influencing the quality, such as growth environment, harvesting time, processing and storage conditions, should be standardized to obtain steady quality so as to improve the therapeutic effects.

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