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Simultaneous quantification of five major bioactive flavonoids in *Rhizoma Smilacis Glabrae* by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of five flavonoids, taxifolin, neoastilbin, astilbin, neoisoastilbin and isoastilbin, contained in *Rhizoma Smilacis Glabrae*. The optimal conditions of separation and detection were achieved on a Lichrospher C18 column (250 mm × 4.6 mm, 5 μm), with a gradient elution program, detected at 291 nm. The correlation coefficients of all the calibration curves showed good linearity ($r > 0.999$) within test ranges. The relative deviation of this method was less than 3% for intra- and inter-day assays, and the average recoveries ($n = 3$) were between 96.2 and 103.1%. The extraction process was also optimized as 2 h immersion and 30 min sonication in 60% ethanol. Eight samples of *Rhizoma Smilacis Glabrae* from different locations in China were analyzed. The results indicate that the assay is reproducible and precise and could be readily utilized for the quality control of *Rhizoma Smilacis Glabrae*.

Keywords: *Rhizoma Smilacis Glabrae*; Flavonoids; Reversed-phase high-performance liquid chromatography; Taxifolin; Neoastilbin; Astilbin; Neoisoastilbin; Isoastilbin

1. Introduction

Rhizoma Smilacis Glabrae is the rhizome of the *Smilax glabra* Roxb plant. It is a perennial plant that grows on dry slopes throughout China. In traditional Chinese medicine, *Rhizoma Smilacis Glabrae* is called tufuling, and has been used clinically for a long time to treat leptospirosis, dermatitis, syphilis, brucellosis, eczema, acute bacterial dysentery, and acute and chronic nephritis [1,2]. Recently, *Rhizoma Smilacis Glabrae* is also found being used in Thailand and some other Southeast Asian countries as traditional medications for cancer and AIDS patients [3–5]. Several papers have reported that the ethanolic extract from *Rhizoma Smilacis Glabrae* showed anti-tumor and anti-atherosclerosis properties as well as inhibition of HIV-1 protease, integrase, and reverse transcriptase [6]. Our previous study has demonstrated that the aqueous extract from the rhizome showed inhibition against the delayed-type hypersensitivity (DTH) reactions mainly through affecting the effector phase of DTH with an anti-inflammatory action, but without inhibiting humoral immune response [7].

From the *Rhizoma Smilacis Glabrae*, a flavanol (taxifolin) and four stereoisomeric flavanones (neoastilbin, isoastilbin, astilbin and neoisoastilbin) were isolated [8,9], whose structures were shown in Fig. 1. Among the components, astilbin was reported to inhibit coenzyme A reductase [10], aldose reductase [11] as well as to display anti-oxidant properties [12,13]. In our previous works, astilbin was shown to significantly inhibit both ear contact dermatitis and liver injury induced by DTH. The mechanism of inhibition involved a selective induction of apoptosis of activated T cells with no influence on naive T cells and other tissue cells [14–16]. It also significantly suppressed collagen-induced arthritis by causing the dysfunction of lymphocytes and inhibiting lymphocyte migration [17,18]. Such a selective activity of astilbin is quite different from the non-selective activity of current immunosuppressors [19]. For example, astilbin could stimulate endogenous interleukin-10 and up-regulate its downstream proteins, which are suppressors of cytokine signaling-1 (SOCS1) and -3 (SOCS3). Astilbin is distinct from the immunosuppressant cyclosporin A, which inhibited the pro-inflammatory cytokines, but neither influenced interleukin-10 nor SOCS1 and SOCS3 [20]. The four other components, taxifolin, neoastilbin, isoastilbin and neoisoastilbin displayed antibacterial and antitumor activities [21,22]. Thus, these five flavonoids could be considered as marker components

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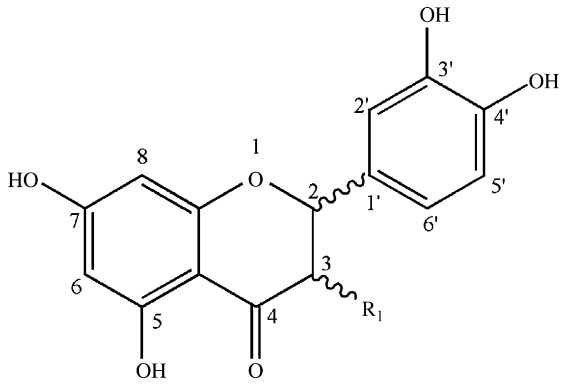

Fig. 1. Structures of five compounds isolated from *Rhizoma Smilacis Glabrae*.

	R_1	Absolute configuration	
		C-2	C-3
Taxifolin	OH	*R*	*R*
Neoastilbin	ORha	*S*	*S*
Astilbin	ORha	*R*	*R*
Neoisoastilbin	ORha	*S*	*R*
Isoastilbin	ORha	*R*	*S*

of *Rhizoma Smilacis Glabrae*. It is necessary to analyze the contents of these flavonoids in the consumer-available herbal drug in order to evaluate its quality. Because the four flavanones were all diastereomers, with their only difference being the absolute configurations of C-2 and C-3: astilbin is (2*R*, 3*R*), and neoisoastilbin (2*S*, 3*R*), isoastilbin (2*R*, 3*S*), neoastilbin (2*S*, 3*S*), a robust analytical separation is required.

Most plants require a balance of resources-energy, water, and mineral nutrients to maintain optimal growth. Plants in nature often encounter multiple stresses from environment, and as a response to the growth environment, the percentages of components in a plant vary with the growth environments [23]. In addition, as the different compounds have different chemical characteristics, the methods and solvents of extraction will influence the extraction yields and purities.

Although an analytical method has been developed for the determination of astilbin in *Rhizoma Smilacis Glabrae* by HPLC [24], there is still a lack of a robust assay for taxifolin, neoisoastilbin, isoastilbin or neoastilbin in the herb extract. Therefore, the aim of this work was to develop a relatively simple HPLC method for the simultaneous quantification of the above five flavonoids. A simple reversed-phase HPLC method was developed and evaluated for the assay for *Rhizoma Smilacis Glabrae* samples from different locations in China.

2. Experimental

2.1. Chemicals and materials

Analytical-grade acetic acid (Nanjing Chemical Regent No. 1 Factory, Jiangsu, China) and HPLC grade methanol (Hanbang Sci. & Tech. Co., Ltd., Jiangsu, China) were used for the HPLC analysis. Deionized water was obtained with a Milli-Q Water purification system (Millipore, MA, USA). All the other organic solvents used in this study were of analytical grade and came from Nanjing Chemical Regent Company.

Taxifolin, neoastilbin, astilbin, neoisoastilbin and isoastilbin were isolated from *Rhizoma Smilacis Glabrae* obtained from Bozhou market of medicinal materials in Anhui province (Serial No. 20050634). The dried *Rhizoma Smilacis Glabrae* (6 kg) was extracted with methanol (1 × 30 l, 2 × 18 l, 2 h each) under reflux. After being evaporated, and suspended in water (1 l), the solution was successively partitioned with EtOAc (3 × 1 l) to obtain the EtOAc fraction (80 g). A portion of EtOAc-soluble fraction (70 g) was subjected to silica gel column (1000 g) and eluted with a gradient of $CHCl_3$–MeOH (19:1, 4:1, 7:3, each 2 l) to give three fractions: Fr. 1 (2.5 l, 32.5 g), Fr. 2 (2 l, 28 g), and Fr. 3 (1.5 l, 6 g). Fr. 1 (32.5 g) and Fr. 2 (28 g) were re-chromatographed with Sephadex LH-20 (Pharmacia, USA) (500 g), respectively. By using MeOH–H_2O (2:8, 3 l, respectively) as eluent, taxifolin (0.2 l, 0.8 g), isoastilbin (0.5 l, 1.9 g) and astilbin (0.8 l, 8.2 g), were obtained from Fr. 1, while neoastilbin (0.3 l, 1.1 g) and neoisoastilbin (0.2 l, 1.0 g) were from Fr. 2. These fractions were also evaporated under reduced pressure and purified by preparative HPLC to achieve standards with higher purity. The purities of the standards were determined by injecting 5 μl solution of each substance (at the concentration about 0.5 mg/ml) into analytical HPLC-UV with the conditions described in Section 2.2. As a result, the purities were all above 98% by calculating the peak area percentages. The five substances were dissolved in methanol respectively and introduced into a Mariner Biospectrometry Workstation (Applied Biosystems) electrospray ionisation/time-of-flight (ESI-TOF) mass spectrometer at 10 μl/min for positive ion analysis. The electrospray source was set at a capillary temperature of 140 °C and a nozzle potential of 180 V using a spray voltage of 5.0 kV and curtain and nebuliser gas flow rates were set at 1.6 and 0.4 l/min, respectively. Mass spectra were acquired for 2 s/spectrum over the *m/z* range 40–3000 in profile mode. The chemical structures of all the five flavonoids as standards were also confirmed by ^{1}H NMR and ^{13}C NMR. The data of ESI-MS, ^{1}H NMR and ^{13}C NMR were consistent with those in the literature [25,26].

The eight samples of *Rhizoma Smilacis Glabrae* were collected from eight locations in China. All the rhizome samples were sundried and then sealed up for preservation. The samples were milled into powder and dried at 80 °C for 4 h before determination of the flavonoids. A sample with a serial number 20050634 was used to determine the precision and accuracy, limits of detection, type of extraction method and solvent composition.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters 600 pump, a 2487 UV–vis detector, an online degasser, a 5 μl injection loop, a LC workstation equipped with Empower™ software for data collection. A Lichrospher C18 column (250 mm × 4.6 mm, 5 μm) maintained at a temperature of 25 °C was used. Detection wavelength was set at 291 nm. Mobile phase (A) was methanol, while mobile phase (B) was 0.3% (v/v) acetic acid in water. The

gradient elution program was 0 min, 35:65; 25 min, 37.2:62.8; 30.6 min, 10.2:89.8; 32.0 min, 10.2:89.8; 37.0 min, 37.6:62.4; 41.0 min, 39:61; 45.0 min, 58:42; 50.0 min, 35:65, respectively. The flow rate was 0.8 ml/min.

2.3. Method validation

The proposed method was validated to determine the linearity, precision, accuracy and stability of each analyte.

2.3.1. Standard solutions and calibration curves

Taxifolin, neoastilbin, astilbin, neoisoastilbin and isoastilbin are stable in methanol and can be stored for at least 6 months at 4 °C. The standard solution mixture of the five flavonoids was prepared by dissolving the reference substances in methanol to final concentration of 31 μg/ml for taxifolin, 2.2 μg/ml for neoastilbin, 309 μg/ml for astilbin, 495 μg/ml for neoisoastilbin and 194 μg/ml for isoastilbin, respectively. Then, the standard solution mixture was diluted to 75%, 40%, 20%, 10% and 5% of the concentration of the original solution. The above standard solutions of six concentrations (each 5 μl) were injected and run for establishment of calibration curves. Linearity of each compound was determined with three injections for each concentration and plotted using linear regression of the mean peak area versus concentration.

2.3.2. Sample preparation

About 10 g of dried *Rhizoma Smilacis Glabrae* samples was milled into powder and filtered through 20–40 mesh. An accurately weighed mass of the powder (0.60 g) was transferred into a 25 ml volumetric flask adjusted with 60% (v/v) ethanol allowed to soak for 2 h and then sonicated for 30 min. The mixture was allowed to cool for 15 min and filtered through analytical filter paper and adjusted to 25 ml with 60% ethanol. The sample solution was filtered through a 0.45 μm membrane (Millipore, USA) and then 5 μl was injected into HPLC.

2.3.3. Precision and accuracy studies

The measurements of intra- and inter-day variability were utilized to determine the repeatability of the developed method. The precision was examined on standard solutions containing all five standards at two different concentrations. Quantities for the analytes were calculated from their corresponding calibration curves. The intra-day variability was determined by analyzing each sample at four times within the same day, and the inter-day reproducibility was performed on four different days. The relative standard deviation (R.S.D.) was considered as the measure of precision.

To evaluate the accuracy of the developed HPLC method, a sample was prepared according to Section 2.3.2, and analyzed with the method. The extracts from the tested plants (2 g), which were evaporated by lyophilization, were spiked by three different concentration levels of mixture standard solutions, and triplicate analyses were taken at each level. The accuracy was evaluated by calculating the mean recoveries of the five compounds from the spiked standard solutions.

2.3.4. Limits of detection (LOD) and quantification (LOQ)

The standard stock solutions were diluted with methanol to provide a series of solutions with the appropriate concentrations. The limits of detection and quantification for each analyte were determined by the signal-to-noise (S/N) ratio for each compound by analyzing a series of diluted solutions until the S/N ratio was about 3 for LOD and 10 for LOQ.

2.4. Optimization of the extraction process

Two regular extraction, hot solvent extraction and sonication, were compared. After being immersed in 25 ml methanol for 2 h, six samples of 0.6 g dried powder were separately extracted for 30 min, three by hot solvent (60–80 °C) and the other three by sonication. The extracted solutions were filtered through an analytical filter paper, cooled and adjusted to 25 ml with methanol. Then, these sample solutions were filtered through a 0.45 μm membrane and analyzed by HPLC.

To evaluate the extraction efficiency of different solvents, the following solvents methanol, 100% ethanol, 80% ethanol, 60%

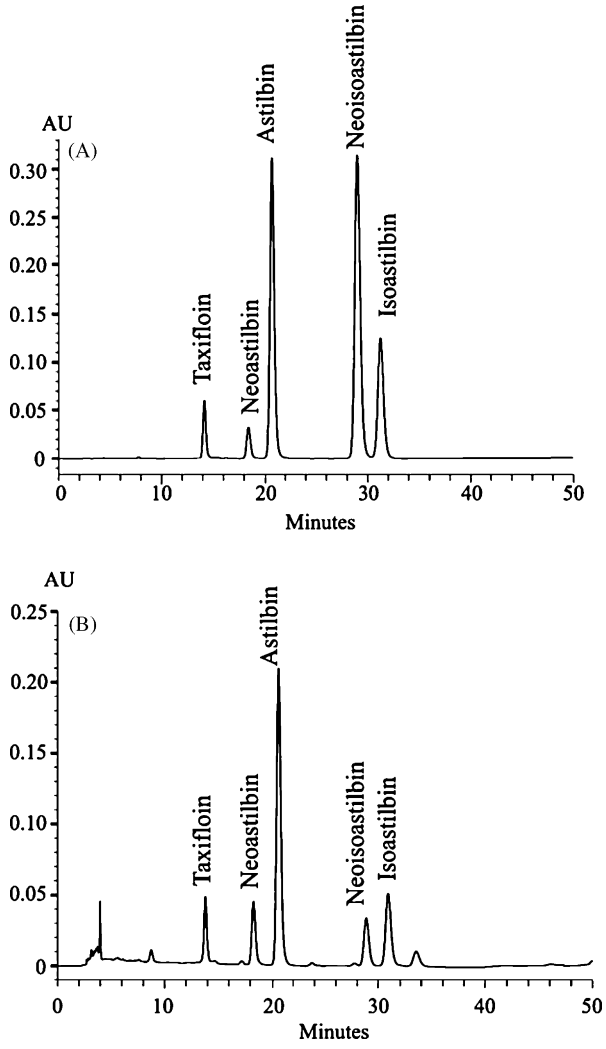

Fig. 2. HPLC chromatograms of standard mixture at a high concentration (A) and the extract from *Rhizoma Smilacis Glabrae* with 60% ethanol by sonication detected at 291 nm (B).

Table 1
Regression equations, correlation coefficients, linearity ranges, limit of detection (LOD) and limit of quantification (LOQ) for the five components in *Rhizoma Smilacis Glabrae*

Compound	Regression equation	Linear range (μg/ml)	r	LOD (μg/ml)	LOQ (μg/ml)
Taxifolin	$Y = 43279.5x + 45.4$	0.3–31	0.9999	0.188	0.632
Neoastilbin	$Y = 369981.2x - 42.1$	0.024–2.4	0.9995	0.021	0.068
Astilbin	$Y = 33915.3x + 44.6$	3.09–309	0.9996	0.242	0.792
Neoisoastilbin	$Y = 26712.1x + 7.7$	4.95–495	0.9993	0.304	0.996
Isoastilbin	$Y = 27379.5x - 46.5$	1.94–194	0.9997	0.296	0.972

Y: peak area; *x*: content of each compound injected (μg/ml); *r*: correlation coefficient; limit of detection: S/N = 3; limit of quantification: S/N = 10.

ethanol, and 40% ethanol were used as extraction solvents with the efficient extraction method selected in the above assay with fewer impurities.

The immersion time before extraction and the extraction time were also examined. By using the optimized extraction method and solvent, five replicate samples were prepared. The immersion time ranged from 30, 60 to 120 min, the extraction time was either 30 or 60 min. The extraction method yielding the highest concentration of the five components was selected.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The UV absorption maxima of taxifolin, neoastilbin, astilbin, neoisoastilbin and isoastilbin were between 290.6 and 291.3 nm; thus 291 nm was selected as the wavelength of the detector. The optimization of the gradient elution program was aimed at obtaining chromatograms with the best possible resolution within a reasonable run time. Several mobile phases, including methanol–water, acetonitrile–water, and methanol–water containing acetic acid, were tested. As the result, methanol–water (containing 0.3% acetic acid (v/v)) resulted in the best separation of the five flavonoids at 25 °C and at a flow rate of 0.8 ml/min. The final gradient elution program chosen is shown in Section 2.2. Chromatograms of the standard solution mixture are shown in Fig. 2(A), while the chromatograms of a testing sample of *Rhizoma Smilacis Glabrae* extract are shown in Fig. 2(B). To obtain reproducible retention time, the system was equilibrated with methanol–water for 10 min.

3.2. Linearity, precision, accuracy, and stability

The five calibration curves exhibited liner regressions of at least ($r > 0.999$). With the exception of neoastilbin, the LOD and LOQ were in the range of 0.188–0.304 and 0.632–0.996 μg/ml, respectively. The LOD and LOQ of neoastilbin are lower: 0.021 and 0.068 μg/ml, respectively. These results are presented in Table 1.

The results in Tables 2 and 3 show that the developed analytical method was reproducible with good recovery and stability for all analytes. The intra-and inter-day variations of all the analytes were less than 3%. The accuracy assays of these five flavonoids were carried out by adding the standards to the treated samples, and the result demonstrated that the recoveries for all these five compounds were in the range of 96.2–103.1%.

3.3. Optimization of the process of extraction

As reported in Fig. 3, sonication was more efficient than hot solvent extraction for obtaining taxifolin and neoisoastilbin. Furthermore, since hot solvent extraction has been reported to easily

Table 2
Recoveries of the five components in *Rhizoma Smilacis Glabrae*

Compounds	Spiked (μg/ml)	Detected (μg/ml)	Recovery (%)	R.S.D. (%, $n = 3$)
Taxifolin	23.25	23.30	100.2	0.97
	6.20	6.01	96.9	1.81
	1.55	1.49	96.3	2.45
Neoastilbin	1.8	1.81	100.6	1.92
	0.48	0.46	96.8	2.29
	0.12	0.11	96.2	2.89
Astilbin	231.75	238.93	103.1	0.81
	61.8	62.22	100.7	1.36
	15.45	15.07	97.5	2.13
Neoisoastilbin	371.25	379.12	102.1	2.77
	99	97.33	98.3	2.15
	24.75	24.05	97.2	2.91
Isoastilbin	145.5	146.85	100.9	1.84
	38.8	37.81	97.5	2.46
	9.7	9.39	96.8	2.73

Table 3
Intra- and inter-day variations for the determination of the five compounds

Compounds	Amount injected (μg/ml)	R.S.D. (%)	
		Intra-day ($n=3$)	Inter-day ($n=3$)
Taxifolin	12.40	0.82	1.43
	3.11	1.29	2.07
Neoastilbin	0.96	1.03	2.19
	0.24	1.48	1.84
Astilbin	123.63	0.95	2.46
	30.90	1.31	1.96
Neoisoastilbin	198.01	1.42	1.81
	49.54	2.33	2.77
Isoastilbin	77.62	2.16	2.34
	19.41	2.27	2.85

convert astilbin to isoastilbin [27], sonication was chosen for extraction.

Various solvents were examined for extraction by sonication. As shown in Fig. 4, 60% ethanol extracted the highest concentration of taxifolin, neoastilbin, astilbin and neoisoastilbin. Although 100% ethanol resulted in the smallest concentration of impurities, it was not suitable for neoisoastilbin and isoastilbin. Interestingly, 80% ethanol resulted in impurities, while 20% ethanol allowed for the lowest extraction efficiency. Thus, 60% ethanol was evaluated as the best extraction solvent tested, using sonication.

To find the optimal extraction time, five replicate samples were immersed in 60% ethanol for 30, 60, 120 min and then sonicated for 30 or 60 min, respectively. The peak areas of five

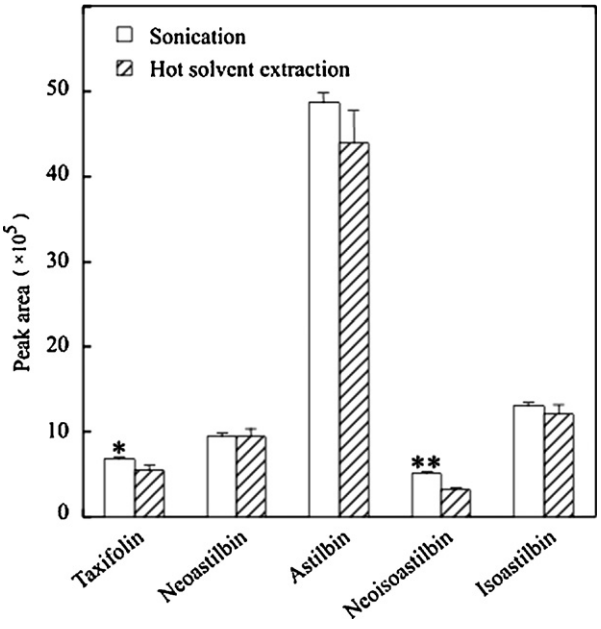

Fig. 3. Comparison of the extract efficiency of the five compounds from *Rhizoma Smilacis Glabrae* by sonication and hot solvent. Values are expressed as means ± S.D. of three individual experiments, performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$ (Student's two-independent-sample *t*-test), significantly different from those by the hot solvent extraction.

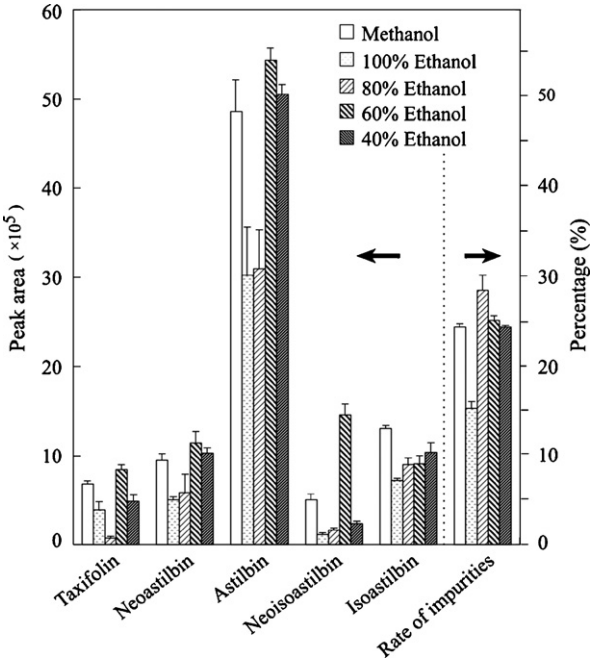

Fig. 4. Extraction efficiency of five compounds from *Rhizoma Smilacis Glabrae* by different solvents. Each column represents the mean ± S.D. of three independent experiments.

compounds obtained by different immersion times are shown in Fig. 5. The 2 h immersion and the 30 min sonication resulted in the highest concentration of all five compounds.

3.4. Sample analysis

The determination for the *Rhizoma Smilacis Glabrae* samples from eight locations in China indicates that the contents of the five flavonoids vary with the growth locations at the range of 29.0–1056.7, 12.8–54.3, 1535.3–11299.6, 449.2–13695.0 and 551.9–4836.9 μg/g crude drugs for taxifolin, neoastilbin, astilbin, neoisoastilbin and isoastilbin, respectively. All the analytical results obtained low R.S.D.s (<3%), which indicated that

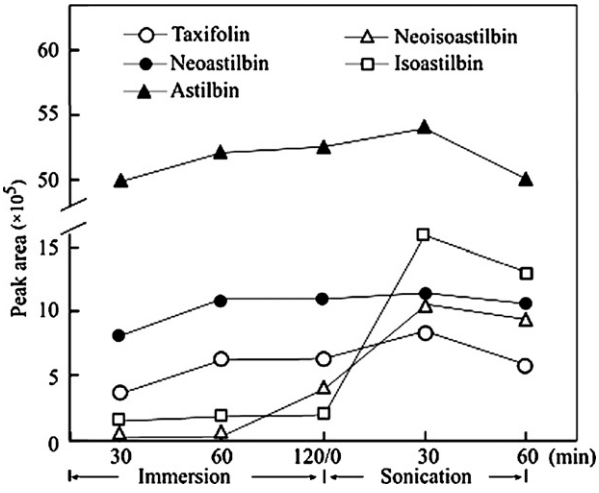

Fig. 5. Extraction kinetics of the five compounds from *Rhizoma Smilacis Glabrae* in different extraction phases.

this HPLC method was reproducible, precise, and useful for analyzing the samples of *Rhizoma Smilacis Glabrae*.

4. Conclusion

This is the first report on the simultaneous determination of five bioactive flavonoids in *Rhizoma Smilacis Glabrae* by a HPLC-UV method. An optimal gradient elution program was developed to separate the four diastereomers and taxifolin effectively. The assay is simple, reproducible, sensitive, rapid, and reliable and has been fully validated. An optimal method of extraction was also validated and applied to the preparation of samples. Furthermore, the HPLC method was successfully applied to analyze samples from eight locations in China. The results indicated that the herbs from different places showed an obvious difference of the contents of these five flavonoids. All the validation procedure confirmed that this developed method was suitable for not only the quantification of five flavonoids, but also the quality evaluation of *Rhizoma Smilacis Glabrae* to ensure the therapeutic benefits.

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