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Response surface optimization of microwave-assisted extraction for HPLC-fluorescence determination of puerarin and daidzein in *Radix Puerariae thomsonii*

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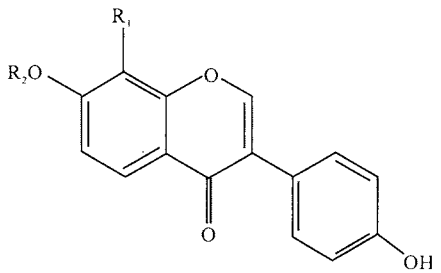
Abstract: Microwave-assisted extraction was optimized with response surface methodology for HPLC-fluorescence determination of puerarin and daidzein in *Radix Puerariae thomsonii*. The optimized extraction procedure was achieved by soaking the sample with 70% methanol (1 : 15, v/v) for 30 min, and then microwave irradiation for 11 min at a power of 600 W. Coupling the extraction process with HPLC-fluorescence presented good recovery, satisfactory precision, and good linear relation. Compared with a method from the Chinese Pharmacopoeia, the proposed method enables higher extraction efficiency and more accurate analytical results. It can be of potential value in quality assessment of *Radix Puerariae thomsonii* medicinal materials.

Keywords: microwave-assisted extraction (MAE); response surface methodology (RSM); puerarin; daidzein; HPLC-fluorescence detection; *Radix Puerariae thomsonii*

1 Introduction

Radix Puerariae thomsonii ("Fen-ge" in Chinese) is the dried root of *Pueraria thomsonii* Benth. (Fabaceae), a perennial leguminous plant mainly distributed in eastern Asia. It is an important Chinese crude herb used to therapy shoulder or wrist stiffness, common cold, influenza, vascular hypertension, etc. [1] Puerarin and daidzein (Figure 1) are two main active isoflavones of *Radix Puerariae thomsonii*. Pharmacological and clinical studies have shown that puerarin could be used to treat hypertension, angina pectoris, and acute myocardial infarction [2]; and that daidzein has an effect on anoxia, cerebral ischemia [3] and angiocardiopathy [4,5]. Increasing demand from the pharmaceutical industry has resulted in mass extraction of puerarin and daidzein from *Radix Puerariae thomsonii*. This has necessitated establishment of a highly efficient and low-cost extraction and determination.

Many attempts have been made to assay puerarin in *Radix Puerariae thomsonii* and its medicinal preparations, such as ultraviolet spectrophotometry (UV) [6], high-performance liquid chromatography with UV detection (HPLC-UV) [7-9] and liquid chromatography/tandem mass spectrometry (LC-MS) [10]. Separation and purification of pueraria isoflavones with high-speed counter-current chromatography have also been developed [11]. Determination of puerarin and other bioactive constituents from *Radix Puerariae* or its medicinal preparations has been attempted using capillary zone electrophoresis with UV detection [12,13], near-infrared spectroscopy (NIRS) [14] and HPLC-UV [15,16]. Fluorescence detection with HPLC was developed for the determination of puerarin to improve sensitivity [17,18].



	R_1	R_2
1. Puerain	glc	H
2. Daidzein	H	H

Figure 1 Structures of puerarin and daidzein procedure.

Pueraria isoflavones must be extracted from *Radix Puerariae thomsonii* before HPLC analysis. Conventional extraction techniques such as heat reflux, alcohol percolation and impregnation [19,20] have low extraction efficiency and high ratio of organic solvent. Some new techniques such as supercritical fluids, ultrasound and microwaves have been used for the extraction of pueraria isoflavones [21]. Microwave-assisted extraction (MAE) for the analysis of active compounds in plant herbs has generated widespread interest [22-26].

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Response surface methodology (RSM) is a collection of statistical and mathematical technique for developing, improving, and optimizing process [27]. It can identify and quantify the various interactions among different parameters. Box-Behnken design is one method of RSM used to examine the relationship between one or more response variables and a set of quantitative experimental parameters [28]. It has fewer design points and fewer experiments to be performed for the quadratic model. Furthermore, each factor requires only three levels instead of five required for central composite designs (unless alpha is equal to one), which is experimentally more convenient and less expensive to perform than central composite designs with the same number of factors [29].

The objective of this study was to optimize the microwave-assisted extraction of bioactive compounds from *Radix Puerariae thomsonii* with response surface methodology, and to simultaneously determine puerarin and daidzein in the extract by high-performance liquid chromatography with fluorescence detection (HPLC-FD). This study carried out such a procedure for the first time.

2 Experimental

2.1 Materials and chemicals

The commercial herb sample of *Radix Puerariae thomsonii*, produced in Guangxi, China, was purchased from the drug market of Xi'an (China). It was authenticated by Dr. Yi Ren (Professor of Key Laboratory of the Ministry of Education for Medicinal Plant Resources and Natural Pharmaceutical Chemistry, Shaanxi Normal University, Xi'an, China). Standard compounds of puerarin and daidzein were obtained from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were obtained from Honeywell International Incorporated (Muskegon, ML, USA). Ultra-pure water was prepared with the Millipore Milli-Q system (Bedford, MA, USA).

2.2 HPLC analysis

HPLC analysis was made on a Waters Breeze liquid chromatography system (Waters Corporation, Milford, MA, USA). This comprised a Waters 1525 binary high-pressure pump and a thermostated column compartment controlled by Empower Workgroup. A Waters 2996 diode-array detector recorded the absorption spectra of analytes. A Waters 2475 multi-wavelength fluorescence detector was used for quantitative detection. Separations were carried out on an Agilent HC-C18 column (250 mm × 4.6 mm i.d., 5.0 μm) with a guard column (4.6 mm × 20 mm; packed with HC-C18; Agilent Corporation, Wilmington, DW, USA).

Acetonitrile (A) and KH_2PO_4-triethylamine buffer solution (pH 7.5, 0.01 M) (B) were used as the mobile phase. Gradient elution was programmed 0 – 25 min, 15 : 85 (A-B, v/v) – 40 : 60 (A-B, v/v); and 25 – 30 min, 40 : 60 (A-B, v/v) – 15 : 85 (A-B, v/v). Flow rate of the mobile phase was 0.8 mL/min. Injection volume was 10 μL, and column temperature was maintained at 30 ℃. Absorption spectra were recorded from 220 nm to 400 nm. Fluorescence excitation and emission wavelengths were 350 nm and 472 nm (bandwidth, 18 nm), respectively.

2.3 Preparation of standard solutions

Five milligrams of puerarin and one milligram of daidzein were accurately weighed and placed in a 5-mL volumetric flask. Seventy percent of aqueous methanol was used to prepare the stock solution of 1000 mg/L puerarin and 200 mg/L daidzein, respectively. An appropriate quantity of stock solution was taken and diluted with 70% aqueous methanol to give a series of mixed standard solutions (5, 25, 125, 250, 500, and 1000 mg/L for puerarin; and 1, 5, 25, 50, 100, and 200 mg/L for daidzein) for plotting calibration curves.

2.4 Preparation of sample solutions with MAE

The MAE was carried out in an XH-100B microwave preparation system (Xianghu Science and Technology Co., Ltd., Beijing, China). The sample was powdered by an electrical grinder and passed through a 50-mesh sieve. Two grams of each powdered sample was soaked in 30 mL of 70% aqueous methanol (methanol/water = 70 : 30, v/v) for 30 min, and then irradiated at microwave power of 600 W for 11 min. A condenser with a continuous flow of water was used to avoid solvent loss during the MAE process. The resultant mixture was filtered under a reduced pressure of 0.09 MPa and the residue was washed with 4 mL of extractant. The merged filtrate was evaporated to near dryness on a rotary evaporator RE-52A (Ya Rong Biochemical Instrument Factory, Shanghai, China). Then the extract was dissolved in 25 mL of 70% aqueous methanol to get the final solution. The final solution was filtered through a 0.45 μm millipore filter membrane (Jinteng, Tianjin, China) and 10 μL was injected for HPLC analysis. Three extractions were done for each sample following the procedures described above. Each extract was determined in triplicate.

2.5 Design of MAE experiment with Box-Behnken design (BBD)

The independent variables chosen were extractant volume (V, mL), microwave power (P, W), and extraction time (t, min). A three-variable and three-level of BBD (and one method of RSM) were adopted to optimize the extraction procedure. Three levels of each variable were coded as −1, 0, and +1 (Table 1). The chromatographic peak area of puerarin was taken as response, Y. Regression analysis was made for the experimental data to fit into an empirical second-order polynomial model.

$$Y = \beta_0 + \beta_1 V + \beta_2 P + \beta_3 t + \beta_{11} V^2 + \beta_{22} P^2 + \beta_{33} t^2 + \beta_{12} VP + \beta_{13} Vt + \beta_{23} Pt$$

where β_0 is the intercept; β_1, β_2, β_3 are linear coefficients; β_{11}, β_{22}, β_{33} are squared coefficients; and β_{12}, β_{13}, β_{23} are interaction coefficients. A software Design-Expert 7.1.3 Trial (State-Ease Inc., Minneapolis, MN, USA) was used to design the experiment and analyze the results. Quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an *F*-test.

3 Results and discussion

3.1 Optimization of microwave-assisted extraction

3.1.1 Influence of extractant and soak time on extraction

Seventy percent of aqueous solution of methanol and 70% aqueous solution of ethanol were tested as the extractant for MAE with soak time of 30 min and extractant volume of 30 mL (2.0 g sample). The results showed that there were some strong impurity peaks when aqueous solution of ethanol was used as extractant. Therefore, aqueous solution of methanol was chosen as extractant in the following experiments.

Extractions were carried out under the same condition except for using five concentrations of an aqueous solution of methanol (40%, 55%, 70%, 85% and 100%, v/v). Chromatographic peak areas of puerarin and daidzein increased with the increase of methanol concentration up to 70%, and did not change for a methanol concentration of over 70%. An aqueous solution of methanol of 70% was therefore used as the extractant for MAE.

Soaking sample adequately prior to MAE was indispensable to make a solvent-absorbed sample and to have the capacity of absorbing sufficient microwave energy during MAE process. In the experiment, 2.0 g of the sample was soaked into 30 mL of 70% methanol for 10, 20, 30, 60, 90 and 120 min, followed by microwave irradiation for 10 min. The extract efficiency was increased with the increase of the soak time up to 30 min, and the prolonged soak time did not significantly increase the peak area of puerarin and daidzein. Therefore, 30 min was chosen as the optimal soak time.

3.1.2 Optimization of extraction parameters with BBD

The variables and levels for each variable in BBD were determined according to the results of preliminary experiments. Based on the experimental results of the BBD (Table 1), puerarin extraction followed a second-order polynomial model:

$$Y = -1231600 + 20152.5V + 16383.7P - 48759.8t - 34.8VP + 1242.4Vt + 253.9Pt - 237.4V^2 - 15.8P^2 - 5901.5t^2$$

where Y represents response, chromatographic peak area of puerarin ($\mu V \cdot s$); and V, P and t correspond to three independent variables, extractant volume (mL) (2.0 g sample), microwave power (W), and extraction time (min), respectively.

A summary of the analysis of variance for the model and experimental results is listed in Table 2. R^2 was 0.9513, indicating that the regression model had low dispersion. A coefficient of variation (CV) of 6.62% showed that the model was reproducible with high precision. The *P* value of the model was 0.0086, predicting significance of the model. P, t, Pt and P^2 were significant model terms ($P < 0.05$). This indicated that the linear terms of microwave power and extraction time, the quadratic terms of microwave power, as well as the interaction terms between microwave power and extraction time, had significant effects on extraction efficiency for puerarin.

Table 1 Box-Behnken design for optimization of MAE

Run	Factor			Response	
	Extractant volume (mL)	Microwave power (W)	Extraction time (min)	Peak area ($\mu V \cdot s$)	
				Observed	Predicted
1	30 (0)	800 (1)	3 (-1)	1.58186E+006	1.621E+006
2	50 (1)	800 (1)	9 (0)	1.89750E+006	2.036E+006
3	50 (1)	400 (-1)	9 (0)	2.95694E+006	2.964E+006
4	30 (0)	600 (0)	9 (0)	3.24037E+006	3.305E+006
5	10 (-1)	600 (0)	15 (1)	2.95408E+006	3.132E+006
6	30 (0)	600 (0)	9 (0)	3.32437E+006	3.305E+006
7	10 (-1)	800 (1)	9 (0)	2.47419E+006	2.467E+006
8	30 (0)	600 (0)	9 (0)	3.34926E+006	3.305E+006
9	10 (-1)	400 (-1)	9 (0)	2.97644E+006	2.838E+006
10	50 (1)	600 (0)	15 (1)	3.24605E+006	3.278E+006
11	30 (0)	800 (1)	15 (1)	2.81756E+006	2.647E+006
12	30 (0)	400 (-1)	15 (1)	2.72618E+006	2.687E+006
13	10 (-1)	600 (0)	3 (-1)	3.04664E+006	3.014E+006
14	50 (1)	600 (0)	3 (-1)	2.74227E+006	2.564E+006
15	30 (0)	400 (-1)	3 (-1)	2.70947E+006	2.880E+006

Table 2 Analysis of variance (ANOVA) for the model and experimental results[a]

Source	Sum of squares	df[b]	Mean square	*F*	*P*
Model	3.359E+012	9	3.733E+011	10.85	0.0086 [c]
V	4.630E+010	1	4.630E+010	1.35	0.2985
P	8.436E+011	1	8.436E+011	24.51	0.0045 [c]
t	3.460E+011	1	3.460E+011	10.05	0.0248 [c]
VP	7.761E+010	1	7.761E+010	2.26	0.1935
Vt	8.891E+010	1	8.891E+010	2.58	0.1689
Pt	3.715E+011	1	3.715E+011	10.79	0.0218 [c]
V^2	3.329E+010	1	3.329E+010	0.97	0.3705
P^2	1.482E+012	1	1.482E+012	43.05	0.0012 [c]
t^2	1.667E+011	1	1.667E+011	4.84	0.0790 [c]
Residual	1.721E+011	5	3.442E+010		
Lack of fit	1.656E+011	3	5.519E+010	16.95	0.0562
Pure error	6.511E+009	2	3.255E+009		
Cor total	3.532E+012	14			

[a] Determination coefficient (R^2), 0.9513; Variation coefficient (CV), 6.62%. [b] df, degree of freedom. [c] Significance, $P<0.05$.

Three-dimensional (3D) surface plots and contour plots were constructed (Figures 2 – 4) according to the model equation. The 3D surface plots showed visually the effects of extractant volume, microwave power, and extraction time on the extraction yield of puerarin and their interaction. The contour plots shown in Figure 2 revealed that the optimal combination of extraction parameters was microwave power of 570 – 590 W and extraction time of 10 – 12 min. The contour plots in Figure 3 showed the optimal combination of microwave power to be 570 – 590 W and the extractant volume (2.0 g sample) to be 30 – 31 mL. The optimal combination was an extraction time of 11 – 12 min and an extractant volume of 25 – 31 mL from the contour plots in Figure 4.

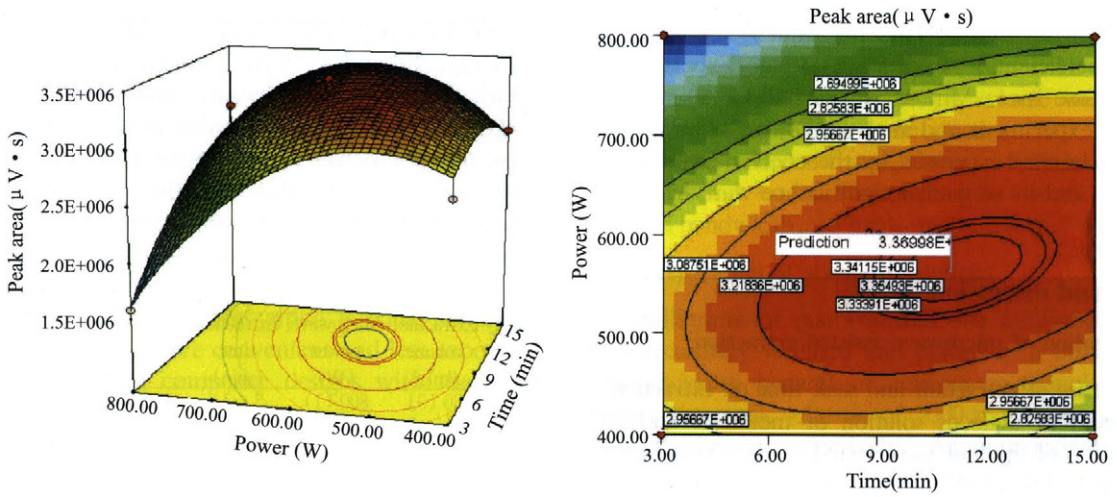

Figure 2 Response surface and contour plots for the effects of extraction time and microwave power on puerarin extraction

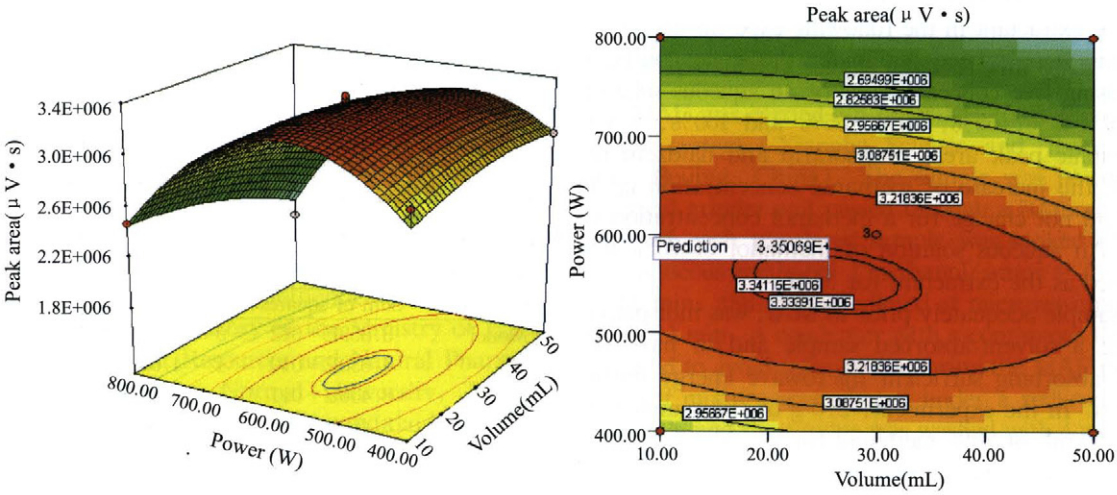

Figure 3 Response surface and contour plots for the effects of extractant volume and microwave power on puerarin extraction

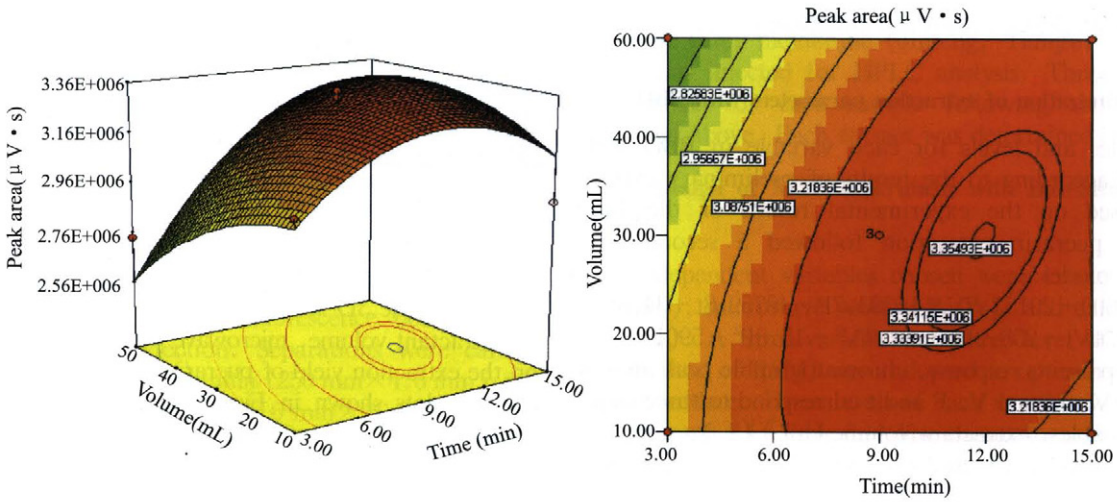

Figure 4 Response surface and contour plots for the effects of extraction time and extractant volume on puerarin extraction

The shape of the contour reflects the strength of the interaction effect. The ellipse of the contour represents the interaction effect of two factors. Figures 2 – 4 show that the interaction effect of microwave power and extraction time was remarkable, that of extraction time and extractant volume was relatively significant and that of microwave power and extractant volume was not noticeable. These results were consistent with ANOVA results.

Optimal calculated values of the variables affecting the extraction yield of puerarin from the regression equation were extractant volume of 30.4 mL, microwave power of 560.9 W, and extraction time of 11.3 min. The value of 560.9 W was not chosen as the operating microwave power due to the limitation of instrument. The actual extraction condition chosen was extractant volume of 30 mL (for 2.0 g sample), microwave power of 600 W, and extraction time of 11 min.

The necessity of multiple extractions was further investigated under the optimized extraction conditions. Sample components were nearly completely extracted after triplicate extractions. Therefore, a triplicate extraction for one sample was chosen.

3.2 Optimization of the chromatographic system

The chromatographic system was optimized in order to match with the fluorescence detector. Puerarin and daidzein are pueraria isoflavones; they have similar fluorescence spectra, excitation wavelengths at about 260 nm and 350 nm, respectively, and a maximum emission wavelength at 472 nm (17, 18). Compared with 260 nm, stronger fluorescence intensity was observed at an excitation wavelength of 350 nm. Therefore, 350 nm and 472 nm were selected as excitation and emission wavelengths, respectively, for HPLC detection.

The pH of the mobile phase affects the retention times of analytes and sample separation; it also has a great influence on the fluorescence intensity of puerarin and daidzein. It was reported that puerarin had the strongest fluorescence intensity at pH 8 – 9 [17]. After several attempts, a pH value of 7.5 for the mobile phase was chosen to obtain rational retention times and relatively strong fluorescence intensities for both puerarin and daidzein.

To effectively separate puerarin and daidzein from other compounds, various HPLC mobile phase systems were tested. Acetonitrile presented a better separative capability compared with methanol. KH_2PO_4-NaOH, Na_2HPO_4-KH_2PO_4, Tris-HCl and KH_2PO_4-triethyla mine buffer solutions were examined. KH_2PO_4-triethylamine buffer solution was found to give the best resolution, and the optimized KH_2PO_4 concentration was 0.01 M.

Gradient elution is widely applied to improve mixture separation in analytic liquid chromatography. In the present work, the optimal gradient was ascertained through many attempts. Using acetonitrile and KH_2PO_3-triethylamine buffer solution (pH 7.5, 0.01 M) as the mobile phase, gradient elution was programmed as 15% – 40% acetonitrile at 0 – 25 min and 40% – 15% acetonitrile at 25 – 30 min. Good separation was achieved within 30 min with the retention time of (6.558 ± 0.089) min for puerarin, and (22.075 ± 0.075) min for daidzein. The typical chromatograms for standard and sample solutions are shown in Figure 5. Virtually no interference was observed in chromatographic separation, and each target peak had good resolution. System suitability was conducted using standard solutions and evaluated using five replicate injections.

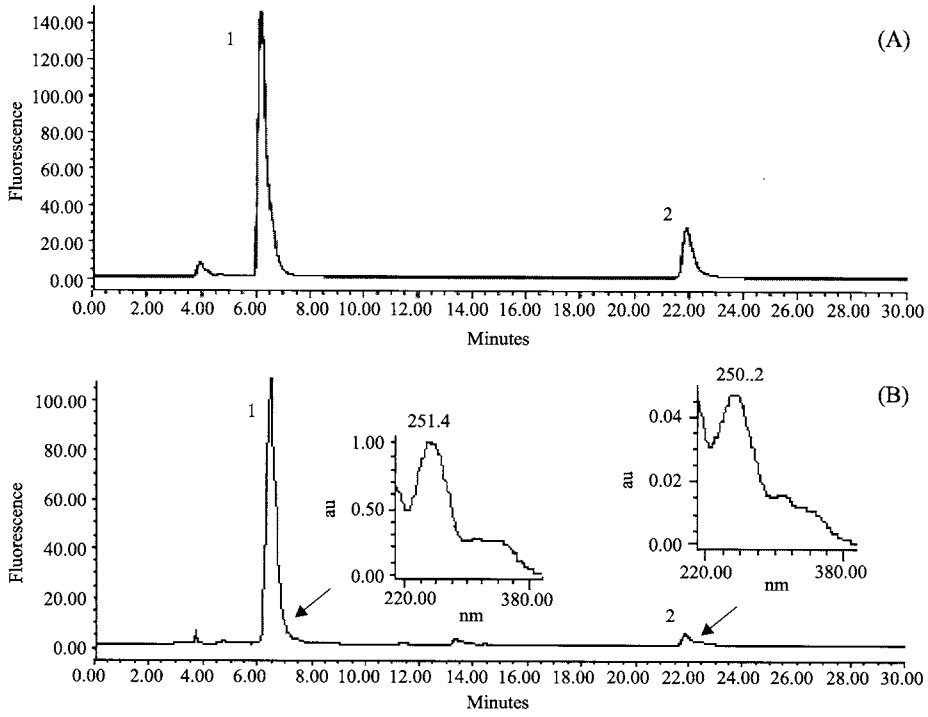

Figure 5 HPLC-FD chromatograms of the standard (A) and sample (B) solutions. 1, puerarin; 2, daidzein.

3.3 Method validation

This analytic method was validated for specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ).

The specificity of the method was determined by comparing retention times and characteristics of the UV spectra with those of the standard solution of the mixture.

Calibration curves were constructed for quantitative analysis of puerarin and daidzein. The regression equation, peak area (Y) against concentration (X, mg/L), was derived as $Y = 6345.5X - 25286$ ($r = 0.9998$) for puerarin, and $Y = 5205.6X - 8081.4$ ($r = 0.9996$) for daidzein. The linear ranges were 5 – 1000 mg/L for puerarin and 1 – 200 mg/L for daidzein, respectively. The LOD, defined as the lowest analyte concentration which could be detected (S/N >3), was 0.005 mg/L for puerarin, and 0.008 mg/L for daidzein. The LOQ, defined as the lowest analyte concentration which could be quantitatively determined (S/N> 10), was 0.02 mg/L for puerarin, and 0.03 mg/L for daidzein.

Precision (reproducibility) of the method was evaluated by calculating the relative standard deviation (RSD) of repeated injections of the standard mixture solutions at concentrations of 5, 125, and 1000 mg/L for puerarin, and 1, 25, and 200 mg/L for daidzein. Intra-day precision was determined by six replicate injections within one day, whereas inter-day precision was determined by six injections for six consecutive days, for retention times and peak areas. Intra-day precision of <1.9% and inter-day precision of < 3.2% were obtained (Table 3).

A recovery study was conducted to validate the accuracy of this method. Samples were spiked with standard compound of the analytes in triplicate at three different amounts. Puerarin and daidzein in spiked samples were determined. Recoveries were obtained by comparing the amount of analytes added to sample with the amount of analytes detected. Mean recovery was calculated at three concentration levels for each analyte. Recovery was 90% – 110% with an RSD lower than 7.5% (Table 4). This result demonstrated good accuracy and reproducibility of the method in the concentration range tested.

Table 3 Method precision ($n = 6$)

Analyte	Concentration (mg/L)	Retention time RSD (%)		Peak area RSD (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Puerarin	5	1.40	1.90	1.10	2.30
	125	1.80	2.40	0.27	0.64
	1000	1.50	1.80	0.35	0.42
Daidzein	1	0.79	1.20	0.73	3.20
	25	0.42	0.44	1.20	1.50
	200	0.31	0.53	1.90	2.40

3.4 Determination of puerarin and daidzein in *Radix Puerariae thomsonii*

MAE optimized with RSM coupled with HPLC-fluorescence was applied to determine puerarin and daidzein in *Radix Puerariae thomsonii* (Table 5). A series of control experiments were done using a method from the Chinese Pharmacopoeia [1] in which puerarin was extracted with heat reflux and determined with HPLC-UV to evaluate the accuracy of the proposed method. The results showed that MAE optimized with RSM had a higher extraction efficiency than heat reflux extraction, and that there were no significant differences between HPLC-fluorescence detection (FD) and HPLC-UV for puerarin determination.

Table 4 Recovery test of analytical method for puerarin and daidzein

Analyte	Spiked (mg/L)	Intra-day ($n = 6$)			Inter-day ($n = 3$)		
		Found		Recovery (%)	Found		Recovery (%)
		Mean (mg/L)	RSD (%)		Mean (mg/L)	RSD (%)	
Puerarin	125	130.8	3.7	104.6	132.4	4.3	105.9
	250	268.2	4.2	107.3	242.1	5.9	96.8
	500	498.3	4.6	99.7	510.8	5.1	102.2
Daidzein	12.5	13.1	5.8	104.8	13.7	7.4	109.6
	25.0	24.3	2.6	97.2	26.7	5.3	106.8
	50.0	52.1	3.4	104.2	46.8	4.5	93.8

Table 5 Determination of puerarin and daidzein in *Radix Puerariae thomsonii* ($n = 6$)

Method	Puerarin (%)	Daidzein (%)
MAE-HPLC-FD[a]	0.586 ± 0.028	0.0405 ± 0.0032
HR-HPLC-FD[b]	0.378 ± 0.021	0.0307 ± 0.0026
HR-HPLC-UV[c]	0.353 ± 0.031	
MAE-HPLC-UV	0.549 ± 0.033	

[a] Proposed method, MAE coupled with HPLC-fluorescence detection. [b] Extracted with heat reflux (HR) and determined with HPLC-fluorescence detection. [c] Chinese Pharmacopoeia method, extracted with HR and determined with HPLC-UV.

4 Conclusion

RSM optimization could consider the effects and interaction of MAE parameters and enhance the extraction efficiency. MAE coupled with HPLC-fluorescence detection is a simpler and more accurate method for simultaneous determination of puerarin and daidzein in *Radix Puerariae thomsonii*. MAE is more effective than heat reflux extraction as a sample treatment procedure, and the fluorescence detector provides higher selectivity and sensitivity. This method will be valuable for routine quality control and standardization of *Radix Puerariae thomsonii* and other crude medicinal mate-

rials and formulations containing puerarin and daidzein.

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