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Original Article

Optimization of ultrasonic extraction by response surface methodology combined with ultrafast liquid chromatography–ultraviolet method for determination of four iridoids in *Gentiana rigescens*

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ABSTRACT

Gentiana rigescens is a rich source of iridoids and is commonly used as a folk medicine for treatment of hepatitis and cholecystitis for over 1000 years. A rapid ultrafast liquid chromatography–ultraviolet method was developed for simultaneous determination of four major iridoid glycosides in *G. rigescens*. Response surface methodology based on the Box–Behnken design was applied to optimize the extraction conditions of iridoid glycosides. Using the Shim-Pack XR-ODS III, four iridoid glycosides were efficiently separated with an acetonitrile:0.1% formic acid aqueous solution gradient at a flow rate of 0.25 mL/min for 8 minutes. All the regression equations revealed a good linear relationship ($R^2 > 0.9995$). The intraday and interday variations were <1.95%. The recoveries ranged from 99.7% to 103.2%. The optimal extraction conditions were as follows: methanol concentration, 82%; the ratio of liquid to solid material, 68:1 (mL/g); and extraction time, 32 minutes. The yield of the four iridoid glycosides under the optimal process was found to be 63.08 mg/g, which was consistent with the predicted yield. In addition, the total content of 50 cultivated samples from Lincang, Yunnan, China, was within the range of 33.6–113.26 mg/g, which provides a more reasonable foundation for utilization of *G. rigescens*.

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1. Introduction

Herbal medicines have been widely used as folk medicines since ancient times to treat various diseases and improve health conditions. The quality of these medicines has captured worldwide attention. However, many factors such as sample preparation, target compounds, and analytical methods have a significant impact on the quality evaluation of herbal medicines [1,2].

Gentiana rigescens (Family: Gentianaceae) is a perennial species, which is mainly distributed in the Yunnan, Sichuan, Guizhou, Hunan, and Guangxi provinces in China. It is usually found in grassland slopes growing at elevations of 1100–3000 m [3]. The roots and rhizomes of *G. rigescens*, *Gentiana scabra*, and *Gentiana triflora* are recorded as the source materials of the important traditional Chinese medicine "Long Dan," which is commonly used as a hepatoprotective agent and as an anti-inflammatory agent in Chinese pharmacopoeia [4]. Modern phytochemical and pharmacological studies reported that *G. rigescens* is a rich source of iridoids. Among them, gentiopicroside, loganic acid, swertiamarin, and sweroside are the major chemical constituents and are commonly considered as the main indexes for quality evaluation of "Long Dan" [5–8]. According to a previous study, the total content of the four iridoid glycosides in *G. rigescens* was >4.5% [6].

Because of habitat loss and overharvesting, the numbers of wild grown *G. rigescens* are significantly reduced [9–11]. As *G. rigescens* is a major raw material of "Long Dan," the species is widely cultivated in Yunnan, China. The cultivated *G. rigescens* species is used as a supplement to the wild grown species in the preparation of "Long Dan" [5]. To accurately evaluate the constituents of *G. rigescens*, it is necessary to optimize the extraction of the four iridoid glycosides.

Response surface methodology (RSM) is a well-established tool for the optimization of analytical methods owing to its advantages over classical one-variable-at-a-time optimization [12]. At present, sample preparation by RSM is widely applied for analysis of foods and herbal medicines [13–19]. Wang et al [13] used RSM to optimize extraction conditions of polysaccharides from *G. scabra* to evaluate their antioxidant and antitumor activities *in vivo*. Liang et al [14] developed a high-speed counter-current chromatography method combined with RSM, which could effectively separate and extract six bioactive compounds from *Gentiana crassicaulis*.

In the present study, a rapid and reliable ultrafast liquid chromatography–ultraviolet (UFLC–UV) method for quantification of the four iridoid glycosides (i.e., loganic acid, swertiamarin, gentiopicroside, and sweroside) was developed and validated. Then, three main independent variables including methanol concentration, ratio of liquid to material, and extraction time were studied. Three-factor and three-level RSM based on the results of single-factor experiments were used to optimize the process variables of the four iridoid glycosides extracted from *G. rigescens*. Moreover, 50 samples were tested using the developed method for quality evaluation of cultivated *G. rigescens* collected from a major production area (Lincang, Yunnan, China).

2. Materials and methods

2.1. Materials and reagents

The roots and rhizomes of *G. rigescens* (50 samples) were collected from Lincang, Yunnan province of China in November 2012 and authenticated by Professor Hang Jin (Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming, China).

The high-performance liquid chromatography (HPLC)-grade solvents (acetonitrile and formic acid) were purchased from Tedia (Fairfield, USA) and Dikmapure (Lake Forest, USA), respectively. The methanol (Tianjin Feng Chuan Fine Chemical Research Institute, Tianjin, China) for extraction is of analytical grade. The pure water used in experiments was purified using a Milli-Q system (Millipore, Billerica, USA). The standards (1, loganic acid; 2, swertiamarin; 3, gentiopicroside; and 4, sweroside shown in Fig. 1) were provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All markers were determined to be of >98% purity by UFLC–tandem mass spectrometry (UFLC–MS/MS). The stock solutions of each marker were prepared in methanol by weighing them accurately and separately, and were finally stored at 4°C.

2.2. Apparatus

The HPLC system (Shimadzu Technologies, Kyoto, Japan) was equipped with an SPD-M10A VP photodiode array detector. Data acquisition was performed in the range of 200–400 nm.

The UFLC–MS/MS (LCMS-8030; Shimadzu, Kyoto, Japan) was equipped with an autosampler, binary gradient pumps, UV detector, and triple quadrupole mass analyzer with an

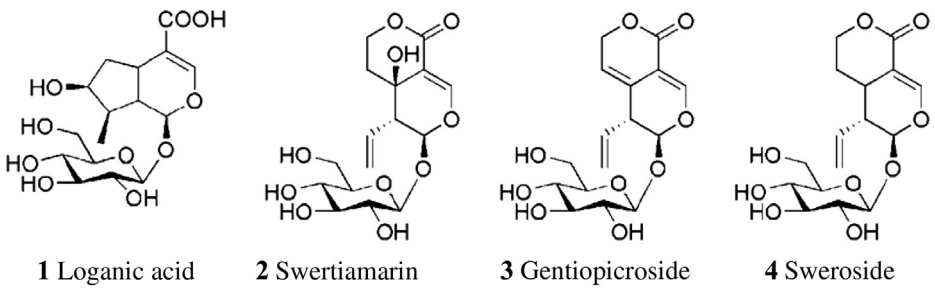

Fig. 1 – Structures of the four iridoid glycosides: 1, loganic acid; 2, swertiamarin; 3, gentiopicroside; 4, sweroside.

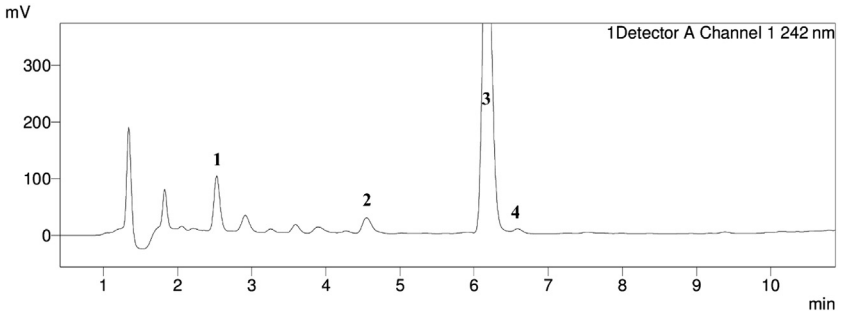

Fig. 2 – Chromatogram for the pooled sample at 242 nm.

electrospray ionization interface source. Chromatographic separation was performed on Shim-Pack XR-ODS III (150 × 2.0 mm, 2.2 μm, Shimadu, Kyoto, Japan). The mobile phase consisted of an acetonitrile (A):0.1% formic acid (B) aqueous solution gradient at a flow rate of 0.25 mL/min: 12% A at 0–2.5 minutes and 12–15% A at 2.5–7.3 minutes. The column temperature was maintained at 40°C and the injection volume was 3 μL. The detection wavelength was set at 242 nm (Fig. 2). The mass spectrometer parameters were set as follows: nitrogen was used as both the nebulizing gas and drying gas at a flow rate of 3.0 L/min and 15.0 L/min, respectively; the interface voltage was set at 4.5 kV. The desolvation line temperature was 250°C and the heat block temperature was 400°C.

2.3. *Sample preparation*

All samples were ground into powder form and then sieved through a 60-mesh stainless steel sieve before extraction. Samples of 0.25 g were extracted by ultrasonication under different conditions (extraction time, ratio of liquid to raw material, and methanol concentration). The pooled sample ($n = 50$) was selected for optimization of the extraction project. All extracts were stored at 4°C and filtered through a 0.22-μm membrane filter before being injected into the UFLC system.

2.4. *Experimental design and statistical analysis*

According to a previous study on *G. rigescens*, the four iridoid glycosides (gentiopicroside, swertiamarin, loganic acid, and sweroside) are well-established indexes for quality evaluation of *G. rigescens* [5]. The experimental iridoid glycosides yield was calculated based on the following equation:

$$\text{Yield (mg/g)} = \frac{m_i}{m_s} \quad (1)$$

where m_i and m_s are the weights of the four iridoid glycosides (mg) and dry weight of the *G. rigescens* sample (g), respectively.

Moreover, it was reported that methanol concentration, ratio of liquid to material, and extraction time had a significant impact on the yield of iridoid glycosides [20]. The single-factor experiments were performed to screen the three parameters. Methanol aqueous solutions with different concentrations (0%, 20%, 40%, 60%, 80%, and 100% v/v) were tested for optimization of the extraction solvent. The ratio of liquid to material was set at 40:1 (40 mL solvent to 1 g sample powder) and the extraction time was fixed at 30 minutes. Then, different ratios of liquid to material were optimized from 10:1 to 200:1 at the extraction time of 30 minutes using 80% methanol aqueous solutions as the extraction solvent. The extraction time was also tested. The sample powder (0.25 g) was extracted using 80% methanol aqueous solutions at a liquid-to-material ratio of 40:1 for 10, 20, 30, 40, 50, and 60 minutes, respectively.

According to the results of single-factor experiments, the appropriate range for each factor was tentatively determined, and then RSM was performed to optimize the experimental project. A three-factor modified Box–Behnken design was applied. Experimental factors and levels are presented in Table 1.

A nonlinear computer-generated quadratic model is given as follows:

$$Y = \beta_0 + \sum_{i=0}^{k} \beta_i x_i + \sum_{j=0}^{k} \beta_{ii} x_i^2 + \sum_{i=0}^{k} \sum_{j=0}^{k} \beta_{ij} x_i x_j \quad (2)$$

where Y is the response function (yield of iridoid glycosides); k is the number of independent variables (factors); β_0 is a constant; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively; The terms $x_i x_j$ and x_i^2 represent the interaction and quadratic terms, respectively [12].

Statistical analysis of the single-factor experimental data was performed using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). Stat-Ease Design-Expert 8.0.5 (Trial version; Stat-Ease Inc., Minneapolis, MN, USA) was used for the Box–Behnken design and regression analysis of the experimental data.

3. Results and discussion

3.1. *Optimization of UFLC–UV analysis conditions*

To achieve the best chromatographic separation, mobile phases, flow rate, and detection wavelength were optimized.

Table 1 – Variables and experimental design levels for response surface.

Code symbols	Variables	Levels		
		−1	0	1
x_1	Methanol concentration (%)	60	80	100
x_2	Ratio of material to liquid	1:10	1:15	1:20
x_3	Extraction time (min)	20	30	40

Table 2 – Linear regression data of standards.

Analyte	Regression equation	Linearity range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)
Loganic acid	$y = 7234.26x - 5616.32$	10–500	0.9998	0.21	0.74
Swertiamarin	$y = 5197.53x - 8421.72$	10–500	0.9995	0.12	0.46
Gentiopicroside	$y = 10{,}457.6x + 100{,}653$	300–3050	0.9999	0.17	0.73
Sweroside	$y = 7412.11x + 3339.87$	1–100	0.9997	0.24	0.88

LOD = limit of detection; LOQ = limit of quantification.

Chromatographic separation was performed on Shim-Pack XR-ODS III (150 × 2.0 mm, 2.2 μm, Shimadu Corporation). Several types of solvent systems including methanol–water and acetonitrile–water in various proportions and gradient durations were tested. The acetonitrile–water system for the four indexes was better than the methanol–water system with regard to the separation ability. Furthermore, 0.1% formic acid was added to the mobile phase, which was able to enhance resolution and eliminate peak tailing of the metabolites. In addition, gradient elution programs at a flow rate of 0.25 min/mL and column (Shim-Pack XR-ODS III (150 × 2.0 mm, 2.2 μm) at a column temperature of 40°C could also improve the separation performance by yielding narrow peaks and maintaining reasonable analytical time (8 minutes). The UV wavelength was set at 242 nm, where all the markers had adequate absorption.

3.2. *Method validation*

To develop a method for determination of the four iridoid glycosides, the linearity, precision, and accuracy were studied. The calibration curves were plotted with six different contents ranging from 0.5 μg/mL to 2500 μg/mL, and the correlation coefficient (R^2) was >0.9995. The limit of detection (LOD) and limit of quantification (LOQ) based on a signal-to-noise ratio of 3 and 10 were determined using serial dilution of the standard solution under the aforementioned conditions. Linearity data, LOD, and LOQ are presented in Table 2. The accuracy was confirmed by performing a recovery experiment. Three different amounts (low, medium, and high spike) of each standard were spiked to the pooled sample and the recovery was found to be in the range of 99.7–103.2%. The interday and intraday variation were selected to determine the precision of this method by analyzing known concentrations of standard solutions on 3 consecutive days in triplicate at seven different intervals (0, 4, 8, 12, 16, 20, and 24 hours) during a single day, respectively. The results showed that relative standard deviation of retention time and peak areas were <2%. Precision and accuracy results are presented in Table 3.

For selectivity, the four iridoid glycosides were identified by comparing their retention times with the standards at 242 nm and matching the precursor/product–ion pairs obtained by mass spectrum. In addition, the recovery test could also confirm the selectivity of this method.

3.3. *Results of single-factor experiments*

Single-factor experiments including methanol concentration, ratio of liquid to material, and extraction time were performed and the results are shown in Fig. 3. Fig. 3A indicates that the yield of the four iridoid glycosides increased with the increase in methanol concentration, and then reached the peak at 80%. It finally dropped from 90% to 100%. Therefore, 80% methanol was selected as the center point for further experiments. From Fig. 3B, it can be seen that the yield of the four iridoid glycosides continued to increase evidently with the increasing extraction time. However, the yield started to increase slowly but with slight decreases once the extraction time exceeded 30 minutes. In Fig. 3C, the yield increases with extraction time for a liquid-to-material ratio from 10:1 to 60:1, where a

Table 3 – Precision and accuracy of the developed method.

Analyte	Intraday RSD (%)		Intraday RSD (%)		Amount added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
	Rt	Pa	Rt	Pa				
Loganic acid	0.88	0.91	0.89	1.35	20	60.82	101.7	1.36
					40	80.61	100.3	0.97
					60	100.35	99.8	1.32
Swertiamarin	0.73	0.92	0.97	1.17	21	63.49	101.0	1.44
					42	84.97	101.6	1.56
					63	106.45	101.9	1.13
Gentiopicroside	0.79	0.84	0.96	1.47	370	1110.52	100.6	1.65
					740	1485.69	99.7	1.12
					1110	1846.31	99.8	1.03
Sweroside	0.93	1.12	0.82	1.95	2.5	7.78	102	1.73
					5	10.39	103.2	1.68
					7.5	12.88	102	1.81

Pa = peak areas; RSD = relative standard deviation; Rt = retention time.

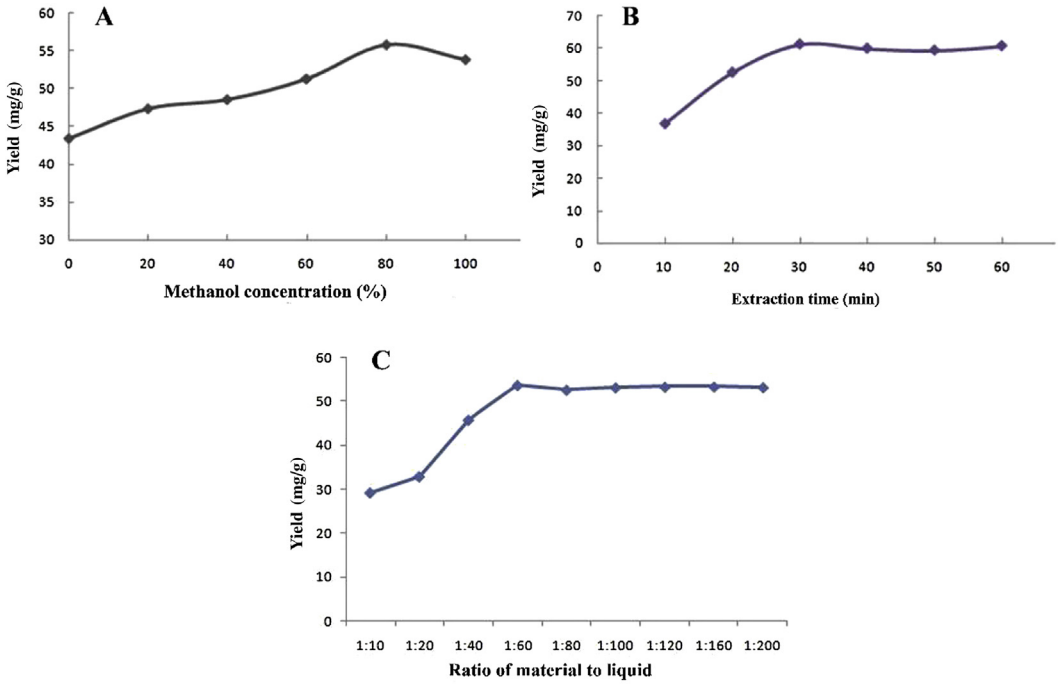

Fig. 3 – Effect of extraction parameters on the yield of the four iridoid glycosides: (A) methanol concentration; (B) ratio of liquid to material; (C) extraction time.

maximum yield was achieved. The yield did not change with further increases in the ratio of liquid to material, which indicated that 60:1 was the most appropriate liquid-to-solid ratio and more solvent was not necessary.

3.4. *Optimization of the extraction process*

According to the results of single-factor experiments, the Box–Behnken design with three independent variables (x_1, methanol concentration; x_2, ratio of liquid to material; and x_3, extraction time) at three levels was applied. The extraction of the four main iridoid glycosides from *G. rigescens* was optimized through the RSM approach. All 17 of the designed experiments were conducted to optimize the three individual variables in the current Box–Behnken design. The design matrix and corresponding results are displayed in Table 4. Multiple regression analysis carried out on the experimental data demonstrated that the relationship between the

Table 4 – ANOVA for response surface quadratic model analysis of variance table.

No.	x_1 (%)	x_2 (mL)	x_3 (min)	Y_1 (mg/g)	Y_2 (mg/g)	Y_3 (mg/g)	Y_4 (mg/g)	Y_0 (mg/g)
1	80	10	20	2.281	1.912	51.11	0.264	55.566
2	80	10	40	2.397	1.915	51.671	0.301	55.884
3	80	20	20	2.490	1.864	53.182	0.289	57.819
4	80	15	30	2.812	2.381	57.658	0.326	63.175
5	60	15	20	2.374	1.731	47.091	0.274	51.464
6	80	15	30	2.891	2.301	57.743	0.321	63.253
7	60	20	30	2.325	1.769	50.441	0.296	54.830
8	80	20	40	2.573	2.370	55.679	0.303	60.822
9	80	15	30	2.825	2.364	57.218	0.318	62.721
10	100	15	20	2.284	1.772	53.035	0.251	57.436
11	100	15	40	2.416	1.733	53.647	0.266	58.159
12	100	10	30	2.312	1.891	51.184	0.244	55.624
13	60	15	40	2.081	1.438	50.271	0.299	54.087
14	60	10	30	1.736	1.572	46.488	0.264	50.058
15	80	15	30	2.697	2.353	57.537	0.332	62.919
16	80	15	30	2.888	2.309	57.623	0.317	63.134
17	100	20	30	2.480	2.216	54.378	0.294	59.368

ANOVA = analysis of variance.

Table 5 – ANOVA for response surface quadratic model analysis of variance table.

Source	Sum of squares	*df*	Mean square	*F*	*p* Prob > *F*
Model	284.222	9	31.580	540.558	<0.0001
x_1	50.745	1	50.745	868.596	<0.0001
x_2	30.840	1	30.840	527.892	<0.0001
x_3	5.5561	1	5.556	95.104	<0.0001
x_1x_2	0.264	1	0.264	4.526	0.0709
x_1x_3	0.903	1	0.903	15.448	0.0057
x_2x_3	1.802	1	1.802	30.850	0.0009
x_1^2	111.820	1	111.820	1914.021	<0.0001
x_2^2	35.830	1	35.830	613.300	<0.0001
x_3^2	28.475	1	28.475	487.399	<0.0001
Residual	0.409	7	0.0584		
Lack of fit	0.220	3	0.073	1.554	0.3316
Pure error	0.189	4	0.047		
Cor total	284.631	16			

ANOVA = analysis of variance.
df = degree of freedom; Cor total (sum of squares and *df*) = model value + residual value.
F value = mean square of source / mean square of residual.
p (prob > *F*)(<0.05): the statistical significance of coefficient.

response and the test variables can be described by the following second-order polynomial equations:

$$Y_0 = -90.281 + 2.297x_1 + 3.696x_2 + 1.632x_3 - 2.571 \times 10^{-3}x_1x_2 - 2.375 \times 10^{-3}x_1x_3 + 0.013x_2x_3 - 0.013x_1^2 - 0.117x_2^2 - 0.026x_3^2 \quad (3)$$

$$Y_1 = -7.721 + 0.157x_1 + 0.395x_2 + 0.055x_3 - 1.048 \times 10^{-3}x_1x_2 + 5.325 \times 10^{-4}x_1x_3 - 1.850 \times 10^{-4}x_2x_3 - 9.465 \times 10^{-4}x_1^2 - 9.234 \times 10^{-3}x_2^2 - 1.571 \times 10^{-3}x_3^2 \quad (4)$$

$$Y_2 = -5.9 + 0.158x_1 + 1.13 \times 10^{-3}x_2 + 0.095x_3 + 3.175 \times 10^{-4}x_1x_2 + 3.15 \times 10^{-4}x_1x_3 + 2.535 \times 10^{-3}x_2x_3 - 1.034 \times 10^{-3}x_1^2 - 2.646 \times 10^{-3}x_2^2 - 2.604 \times 10^{-3}x_3^2 \quad (5)$$

$$Y_3 = -77.021 + 2.003x_1 + 3.211x_2 + 1.474x_3 - 1.886 \times 10^{-3}x_1x_2 - 3.21 \times 10^{-3}x_1x_3 + 9.69 \times 10^{-3}x_2x_3 - 0.011x_1^2 - 0.101x_2^2 - 0.021x_3^2 \quad (6)$$

$$Y_4 = -0.563 + 0.012x_1 + 0.022x_2 + 0.015x_3 + 4.5 \times 10^{-5}x_1x_2 - 1.25 \times 10^{-5}x_1x_3 + 1.15 \times 10^{-4}x_2x_3 - 8.131 \times 10^{-5}x_1^2 - 6.31 \times 10^{-4}x_2^2 - 1.778 \times 10^{-4}x_3^2 \quad (7)$$

where Y_0–Y_4 are the yield of the four iridoid glycosides, namely, loganic acid, swertiamarin, gentiopicroside, and sweroside, respectively.

The adequacy of response surface quadratic model on yield of four iridoid glycosides (Y_0) was evaluated by *F* value obtained from analysis of variance (ANOVA). The results are listed in Table 5. ANOVA of the quadratic regression model showed that the values of the determination coefficient (R^2) and the adjusted determination coefficient (Adj. R^2) were 0.9986 and 0.9967, respectively, which implied that the model could explain 99.86% variability of the response variable. Furthermore, the lack of fit commonly used to measure the model was insignificant because its *p* value was 0.332 (>0.05), which indicated the model was fitted well with this experimental project. The model *F* value of 540.56 suggested that this model was significant. Values of Prob > *F* of <0.05 indicated that the model terms were significant. In this case, x_1, x_2, x_3, x_1^2, x_2^2, x_3^2, x_1x_3, and x_2x_3 were significant model terms. These results show that the three variables had a significant influence on the yield of the four iridoid glycosides and their order is as follows: methanol concentration > ratio of liquid to material > extraction time. In addition, these corresponding parameters for Y_0–Y_4 were also within the required limits.

To directly reflect the interaction between various factors on the response values, three-dimensional surface and contour plot are presented in Fig. 4A–4C. Fig. 4A displays the effects of methanol concentration and the ratio of liquid to material on the yield of the four main iridoid glycosides. The yield is increased when the extractions are performed with a methanol concentration of around 80% and the ratio of liquid to material increased in the range from 10:1 to 80:1. In Fig. 4B and 4C, the effects of extraction time and the other two variables (i.e., methanol concentration and ratio of liquid to material) are presented, respectively. The results indicated that the yield insignificantly increased with 80% methanol concentration and ratio of liquid to material (60:1) when extraction time was changed. According to Fig. 4A–4C, extraction time represented a weaker effect whereas methanol concentration and ratio of liquid to material had a significant impact on the yield of the four iridoid glycosides.

The maximum response value for the yield of four iridoid glycosides was estimated using Design-Expert 8.0.5 software (Trial version; Stat-Ease Inc., Minneapolis, MN, USA) as 80.86% methanol, liquid-to-solid ratio of 67.56:1, and extraction time of 32.32 minutes. However, considering the operability in actual production, the optimal process would be modified as follows: methanol concentration, 81%; the ratio of liquid to material, 68:1; and extraction time, 32 minutes, respectively. Under the modified conditions, the yield of the four iridoid glycosides was 63.08 ± 0.68 mg/g (n = 3), and close to the predicted value (62.366 mg/g), which indicated that the model was also effective for the modified extraction process.

3.5. *Quantification*

To obtain the content information on cultivated *G. rigescens*, we collected a population of *G. rigescens* including 50 individuals from Lincang, the major producing area in the Yunnan province of China. Based on the result of the extraction project, the 50 individuals were subjected to the modified extraction process and the yield of the four iridoid glycosides was obtained from the developed UFLC–UV method. The four iridoid glycosides were confirmed by comparison of their

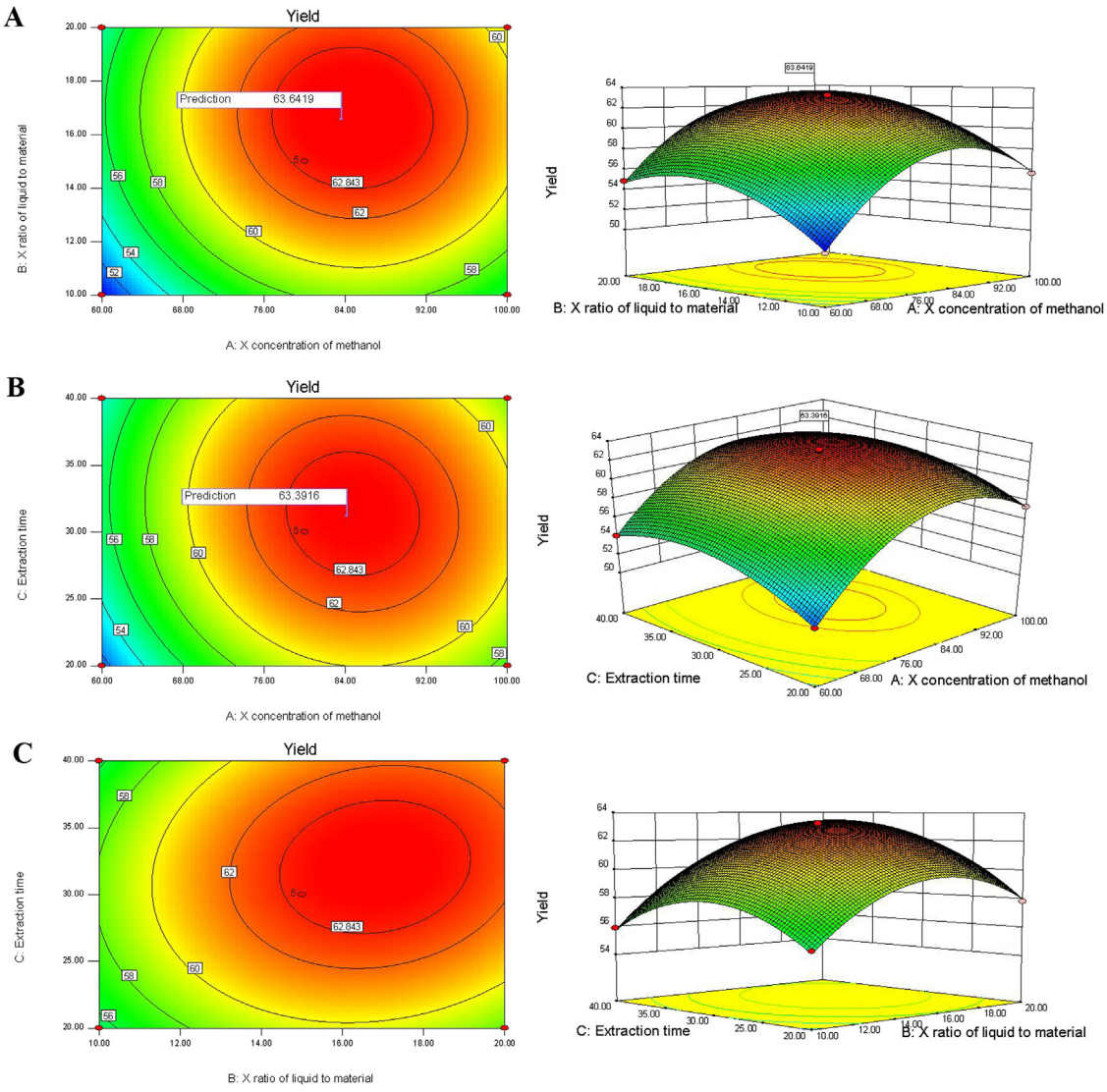

Fig. 4 – Response surface and contour plots showing the effects of parameters on the yield of the four iridoid glycosides: (A) concentration of methanol and ratio of liquid to material; (B) concentration of methanol and extraction time; (C) ratio of liquid to material and extraction time.

retention times and precursor/product–ion pairs obtained using the multiple- reaction monitoring (MRM) acquisition mode.

The distribution of the content of the four iridoid glycosides was compared using box plots (Fig. 5). The average content of the four major iridoid glycosides was 61.15 mg/g (6.12%), whereby the content of Samples 3 and 6 was >100 mg/g (10%), which might be responsible for the individual difference. Among the four iridoid glycosides, gentiopicroside and sweroside showed the highest and lowest contents in *G. rigescens*, respectively. In Chinese pharmacopoeia, the content of gentiopicroside is an important index for quality evaluation of "Long Dan." The quantification results demonstrated that all samples were qualified because their content of gentiopicroside was >1.5% [4].

In the present study, the developed UFLC–UV method was validated for the simultaneous determination of the four iridoid glycosides in *G. rigescens*. The RSM coupled with the Box–Behnken design was used to optimize the extraction conditions and to fairly accurately predict the yield of the four iridoid glycosides. The effect of the three independent variables (extraction time, extraction temperature, and ratio of liquid to material) was effectively determined. The highest content of the four iridoid glycosides compounds was obtained with 81% methanol concentration for 32 minutes at the liquid-to-material ratio of 68:1 (mL/g). This process could be considered a sustainable alternative for quality evaluation and provide a more reasonable foundation for utilization of *G. rigescens*.

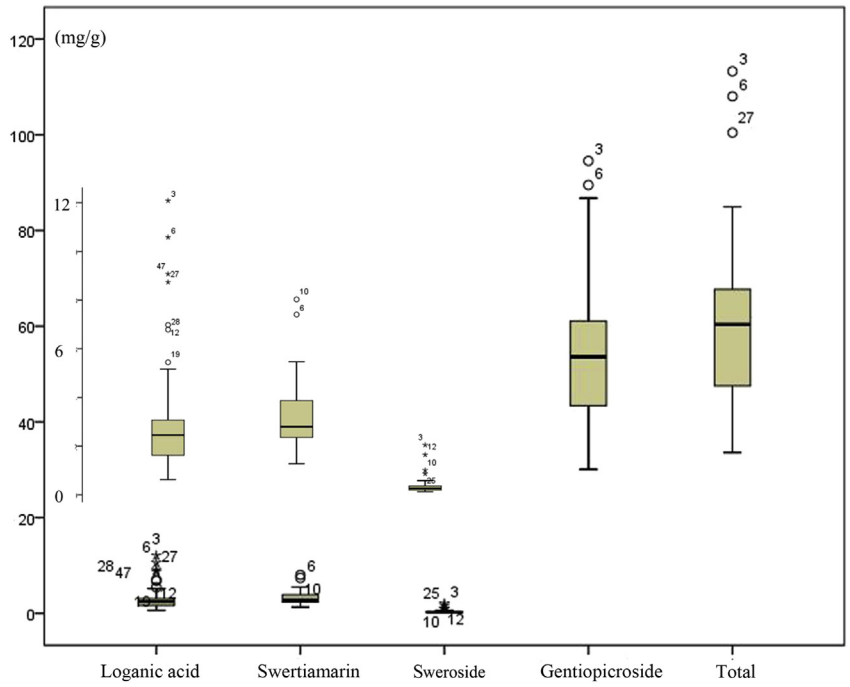

Fig. 5 – Box plots of the four iridoid glycosides in 50 samples.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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