



Original Article

Optimization of microwave-assisted extraction of bioactive alkaloids from lotus plumule using response surface methodology[☆]Wei Xiong¹, Xianqiang Chen¹, Guangping Lv, Dejun Hu, Jing Zhao*, Shaoping Li

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ABSTRACT

In this work, a fast and efficient microwave-assisted extraction (MAE) method was developed to extract main bioactive alkaloids from lotus plumule. To optimize MAE conditions, three main factors were selected using univariate approach experiments, and then central composite design (CCD). The optimal extraction conditions were as follows: methanol concentration of 65%, microwave power of 200 W, and extraction time of 260 s. A high performance liquid chromatography–diode array detector (HPLC–DAD) method was established to quantitatively analyze these phytochemicals in different lotus plumule samples and in different part of lotus. Chromatographic separation was carried out on an Agilent Zorbax Extend-C₁₈ column (4.6 mm×150 mm, 3.5 μm). Gradient elution was applied with the mobile phase constituted with 0.1% triethylamine in water (A) and acetonitrile (B): 40%–70% B at 0–8 min, 70%–100% B at 8–9 min, 100% B for 2 min, and then equilibrated with 40% B for 2 min.

1. Introduction

Lotus (*Nelumbo nucifera* Gaertn) is a perennial aquatic plant and widely cultivated in Asia, especially in China [1]. The earliest origin of lotus is India and then spread to Persia and Egypt. In Hinduism and Buddhism, the lotus is regarded as a symbol of purity and beauty [2]. Almost all parts of lotus can be eaten as food or vegetable. As early as in ancient China, people already used different parts of lotus for various medicinal purposes. The first monograph which recorded lotus as a kind of food with therapeutic effects in China is Shi Liao Ben Cao by Shen Meng (AD 621–713) [3].

Lotus plumule, the green embryo of lotus seeds, is a traditional medicine while people usually drink it as a kind of tea popularly in China for overcoming restlessness and hypertension [2]. There are already many commercial products sold in stores. The seeds of lotus are also made as a folk dish in Southeast Asia, Japan and India [4]. As a plant which can be used as both food and medicine, lotus plumule has high nutritional values [5] and pharmacological effects, such as treating cardiovascular diseases, nervous disorders, insomnia and high fevers, etc [2,6–8]. Various chemical constituents including alkaloids, flavonoids and volatile oil have been isolated from lotus plumule [9–12]. Among these compounds, alkaloids are the main composition in lotus plumule [13]. Liensinine, dauricine isoliensinine, neferine and nuci-

ferine show good bioactivity and healthcare function [14–16]. The alkaloids in lotus plumule have many benefits to cardiovascular system like antihypertensive effect, antiarrhythmic activity and antifibrosis activity [17,18]. Besides, nuciferine also shows good antiobesity activity [19]. Thus, these alkaloids are chosen as markers for quality control of lotus plumule. Presently, alkaloids from *N. nucifera* Gaertn are mainly extracted using ultrasonic method [20,21] and heating reflux extraction [22,23], which are time-consuming and laborious. The ultrahigh pressure-assisted extraction [24], supercritical fluid extraction [25] and microwave-assisted extraction [26,27] have also been reported for the extraction of alkaloids from *N. nucifera*.

In order to establish a fast and efficient method to extract these bioactive alkaloids, microwave-assisted extraction (MAE) was selected because of its promising extraction efficiency [28,29]. MAE is most commonly used for alkaloids extraction at present, including sinoacutine from *Stephania sinica* [30], camptothecin from *Nothapodytes foetida* [31], senkirkine from *Tussilago farfara* [32] and sanguinarine from *Macleaya cordata* [33]. The principle of microwave extraction is to apply microwave that will penetrate the samples and convert into heat, and as a result extract bioactive compounds from the samples [34]. Compared with traditional reflux extraction, soxhlet extraction and ultrasonic extraction, microwave-assisted extraction has the advantages of short extraction time, high extraction rate and good

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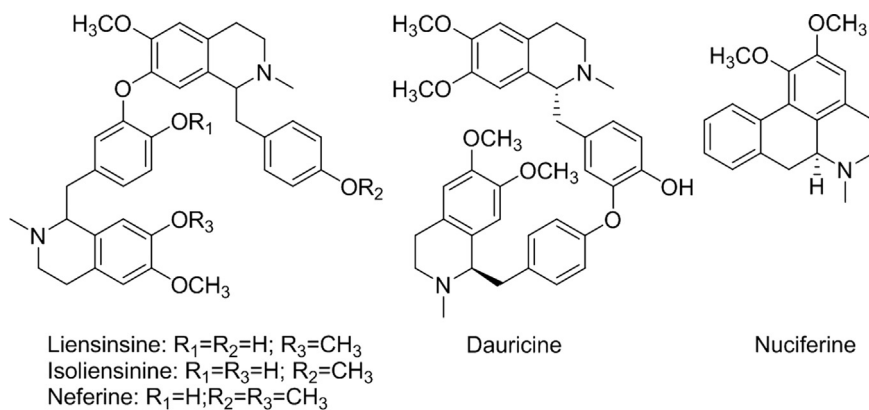


Fig. 1. Chemical structures of the five alkaloids from lotus plumule.

Table 1
Factors and levels of single experiment.

Factor	Level						
Methanol concentration (%)	0	20	40	60	80	100	
Microwave power (W)	100	200	300	400	500	600	
Extraction time (min)	2	4	6	8	10	12	
Liquid-to-solid ratio (mL/g)	5	10	15	20	25	30	
Powder size (mesh)	< 20	20	20–40	40	60–80	120	

product quality [35]. Besides, MAE is a green extraction and green chemistry in terms of energy, solvents and sample preparation [36–38].

The objective of this study was to establish an MAE method to extract five bioactive alkaloids from lotus plumule, i.e., liensinsine, dauricine, isoliensinsine, neferine and nuciferine, and use response surface methodology (RSM) with central composite design (CCD) to optimize the parameters for the best extraction method. At the same time, a high performance liquid chromatography (HPLC) method was developed to quantitatively evaluate the selected alkaloids.

2. Experimental

2.1. Materials and chemicals

Alkaloids standards including liensinsine, dauricine, isoliensinsine, neferine and nuciferine (Fig. 1) were purchased from Preferred (Chengdu, Sichuan, China). Acetonitrile, methanol and triethylamine were purchased from Merck (Darmstadt, Germany). The water used for HPLC was from the Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). All the reagents and chemicals were of analytical grade.

The lotus plumules were collected and purchased from Macao (MA), Zhuhai (ZH), Hubei (HB), Fujian (FJ), Hunan (HN), Jiangxi (JX) and Zhejiang (ZJ). Among these samples, lotus plumules in Macao and Zhuhai were picked from fresh lotus, while the others were purchased from drugstores. All the samples were dried under 60 °C and crushed into powder before extraction.

2.2. MAE extraction

The powder of lotus plumule (0.1 g, 60–80 mesh) was extracted by Multiwave 3000 Reaction System (Anton Paar GmbH, Graz, Austria). This microwave system has 64-position rotor which can provide high performance and reliable quality. This extraction was carried out at a power of 200 W with 2 mL of 60% (v/v) methanol aqueous solution for 5 min. The solution was centrifuged after cooled to room temperature, and 1 mL of the supernatant was transferred into a 2 mL volumetric

flask and methanol was used to make up to the volume. At last, the solution was filtered through a 0.45 µm filter for HPLC analysis.

2.3. HPLC analysis

All of the analyses were performed on a Dionex (Germering, Germany) Ultimate 3000 UHPLC system equipped with Ultimate 3000 degasser, pump, RS autosampler and column compartment, coupled with a diode array detector (DAD). Data processing was carried out with Chromeleon 6.8 software (Dionex). Chromatographic separation was carried out on an Agilent Zorbax Extend-C₁₈ column (4.6 mm×150 mm, 3.5 µm). The column temperature was set at 25 °C. Gradient elution was applied with the mobile phase constituted with 0.1% triethylamine in water (A) and acetonitrile (B): 40%–70% B at 0–8 min, 70%–100% B at 8–9 min, 100% B for 2 min, and then equilibrated with 40% B for 2 min. The flow rate was 1.0 mL/min and injection volume was 5 µL. The chromatograms were monitored at 280 nm and the ultraviolet and visible (UV) spectra was recorded from 180 nm to 400 nm.

2.4. RSM

2.4.1. Univariate approach experimental design

To select a suitable range for variables, the classic univariate approach was used. The powder of lotus plumule was treated under different conditions of MAE as shown in Table 1.

2.4.2. CCD

In this study, the CCD was employed to optimize the conditions for alkaloids extraction of lotus plumule. The experimental results were analyzed by Design-Expert 8.0.6. Methanol concentration, microwave power and extraction time were selected as three independent variables in the CCD. Five levels, coded –1.682, –1, 0, 1, and 1.682, respectively were applied to each variable and the CCD matrixes covered 20 experiments for setting quadratic models. The data were fitted to an equation as shown below:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^n b_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n b_{ij} X_i X_j + e_i$$

Where Y represents the response, which is the peak area of the alkaloids; n is the number of factors; X_i and X_j are the coded variables; b₀ is the offset term; b_i, b_{ii}, and b_{ij} are the first-order, quadratic, and interaction effects, respectively; i and j are the index numbers for factor; and e_i is the residual error. According to the polynomial model, the response surface and counter plots were obtained.

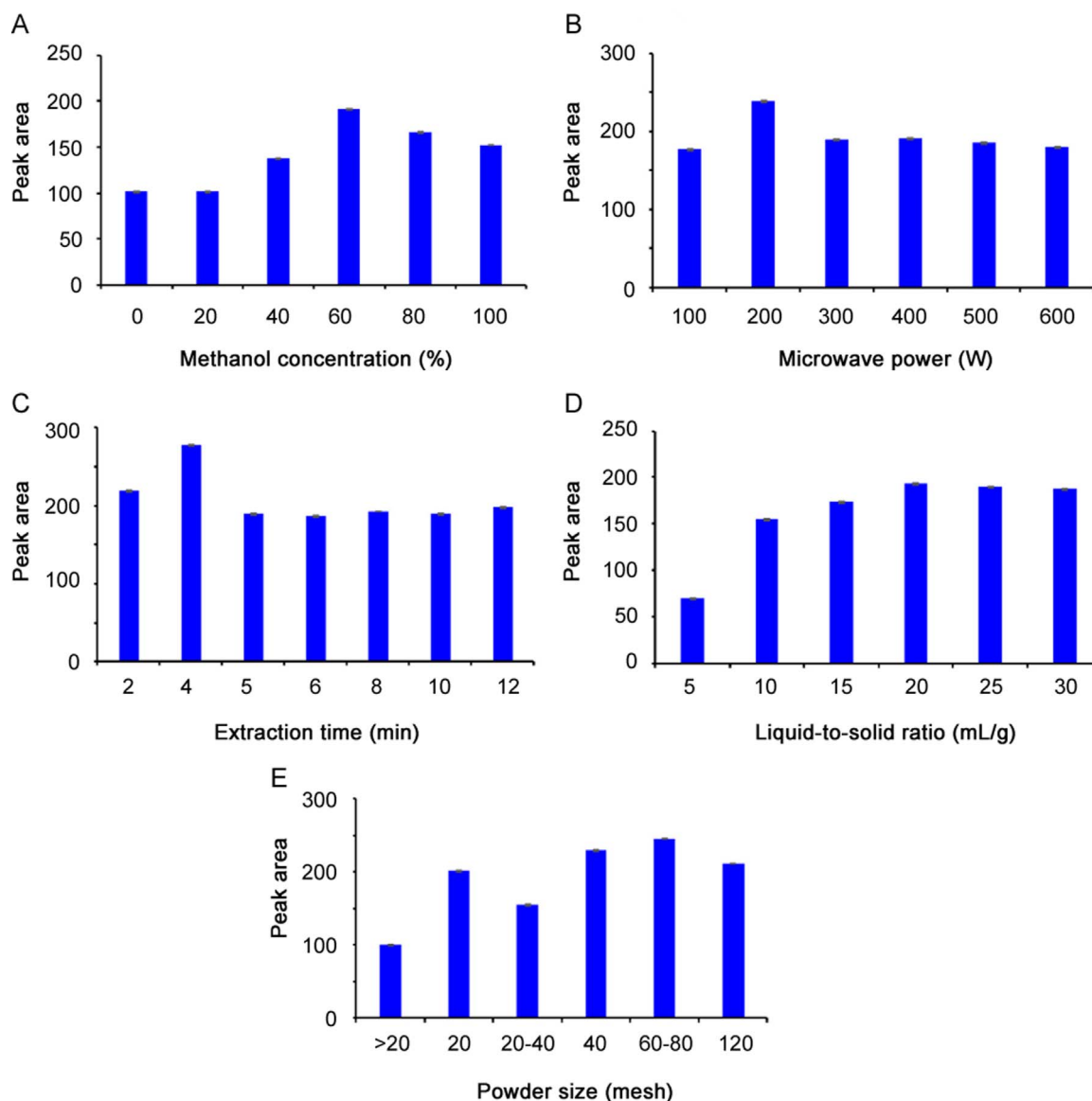


Fig. 2. Effects of (A) methanol concentration, (B) microwave power, (C) extraction time, (D) liquid-to-solid ratio, and (E) powder size on the sum of selected five alkaloids peak area in sample JX-1.

3. Results and discussion

3.1. Optimization of MAE conditions by CCD

3.1.1. Univariate approach experiment

To quantitatively extract alkaloids from lotus plumule, MAE is an attractive technique. Compared with the traditional methods, MAE has more significant advantages and it has already been applied to extract alkaloids from different plants. The factors which influence MAE performance were studied, including methanol concentration, microwave power, extraction time, liquid-to-solid ratio and powder size. The effects of different MAE factors are shown in Fig. 2.

The results show that solvent is one of the main factors in the extraction process. When the concentration of methanol increased from 0% to 100%, the yield of alkaloids growth reached a maximum by using 60% methanol and decreased afterwards. According to the similarity-intermiscibility theory, the solubility of alkaloids enhances as the concentration of methanol increases. However, once the concentration of methanol is over 80%, the yield of alkaloids declines. This may result from the lower microwave energy absorption and

poorer endothermic capacity. Considering the two factors, 60% methanol was selected.

The effect of microwave power on the yield of alkaloids is also important. The yield was the highest around 200 W, and when the microwave power increased to more than 300 W, there was no significant change during different power and therefore 200 W was chosen. In the same way, when the extraction time extended to more than 4 min, the yield maintained a stable state. Besides, the higher power and longer extraction time may damage the structure of the target components; thus 4 min was selected as the extraction time.

In order to select an appropriate liquid-to-solid ratio, the ranges between 5:1 and 30:1 were studied. The yield of alkaloids rose when the liquid-to-solid ratio increased; however, the yield no longer rose as the ratio continued to increase to more than 20:1. By absorbing microwave energy, the solvent penetrated the cell wall so that the effective components released. But once reaching the maximum value, the yield of alkaloids may decline as the percentage of solvent increased.

The particle size of powder can also influence the yield of target compounds. Increased size of the powder may lead to smaller surface

Table 2
CCD experiment and the results of RSM.

Run	Experiment design			Response (Y)
	X ₁	X ₂	X ₃	
1	36	141	2.9	6.99
2	84	141	2.9	9.89
3	36	260	2.9	6.85
4	84	260	2.9	9.61
5	36	141	5.3	7.19
6	84	141	5.3	10.30
7	36	260	5.3	7.59
8	84	260	5.3	10.70
9	20	200	4	6.26
10	100	200	4	8.36
11	60	100	4	9.10
12	60	300	4	9.97
13	60	200	2	10.40
14	60	200	6	12.00
15	60	200	4	13.20
16	60	200	4	13.00
17	60	200	4	12.80
18	60	200	4	13.00
19	60	200	4	12.90
20	60	200	4	13.00

X₁: methanol concentration (%); X₂: microwave power (W); X₃: extraction time (min); Y: the sum of peak area(×10²).

The sample of JX-1 was used for CCD test.

area and result in low yield of target compounds. However, if the particle size is too small, it is easy to agglomerate, thereby resulting in yield reduction.

3.1.2. CCD

According to the results of univariate approach, three main factors including methanol concentration, microwave power and extraction time, were selected to be studied using RSM by CCD. RSM is a method for optimization of different variable conditions. The most commonly experiment designs for RSM are CCD and Box–Behnken design (BBD) [26,27]. The CCD consisted of a full factorial 2³ design, 6 axial points and 6 central points in the cube. 20 runs in the design matrix and the response are shown in Table 2. The second order regression model was carried on the coded values of three independent variables. The response (Y) for the sum of peak area of five alkaloids is presented by the equation below:

$$Y = 13.00 + 1.13X_1 + 0.13X_2 + 0.38X_3 - 0.017X_1X_2 + 0.07X_1X_3 + 0.15X_2X_3 - 2.11X_1^2 - 1.32X_2^2 - 0.73X_3^2$$

The statistical significance of the model was carried out by Fisher's test and *p*-value. The model *F*-value of 32.72 implies that the model is significant. The *p*-value was used to evaluate the coefficient and the *p*-value was less than 0.05 showing that the model is significant. On the basis of the ANOVA results, the linear coefficient for X₁, X₂ and X₃, the interactive coefficient for X₂X₃, and quadratic coefficient for X₁², X₂² and X₃² were significant, thus indicating that the yield of alkaloids was significantly influenced by the methanol concentration, microwave power and extraction time. The “Lack of Fit *F*-value” of 39.19 indicates that the Lack of Fit is significant.

The R² of 0.9672 indicates that the model fits well. The “Pred R-Squared” of 0.7559 is in reasonable agreement with the “Adj R-Squared” of 0.9376. “Adeq Precision” measures the signal to noise of the model and the ratio of 18.663 indicates an adequate signal. This means the model can be used to navigate the design space. The coefficient of variation (CV) evaluates reproducibility of the model, which is the ratio of the standard error to the mean value. The CV of 5.87% indicates the model has good reproducibility.

The model graphs were used to show the influence of each variable

on response, including 3D response surface and contour plot. Fig. 3 shows the interactive effects of methanol concentration, microwave power and extraction time on the yield of alkaloids. These 3D plots depict the influence of any two independent variables on the response and the maximum value for each variable can be obtained. The contour plots (Fig. 3) can clearly present the correlation between any two variables and conveniently find the optimum value range.

The optimal condition for extraction of the five alkaloids was obtained by the Design Expert software as follows: methanol concentration 66.63%, microwave power 204.32 W and extraction time 4.43 min. Considering the practical situation and instruments limit, the optimal condition was adjusted to methanol concentration of 65%, microwave power of 200 W and extraction time of 260 s. Three parallel experiments were carried out under the condition; the sum of the peak area of the five alkaloids was 1294.92, 1313.2 and 1310.65. Moreover, the mean was close to the predicted value 1320, so the model is reliable.

3.2. HPLC method validation

The linearity, regression and linear ranges of the five alkaloids were validated by the developed HPLC-DAD method. As shown in Table 3, the calibration curves of the five alkaloids had good linearity (R² > 0.999), the LODs of the alkaloids ranged from 0.2 to 0.8 µg/mL, while the LOQs ranged from 0.4 to 1.6 µg/mL. The intra-day variation and inter-day variation were used to evaluate the precision of the method. The mixed standard solutions were analyzed for six times within one day and three consecutive days. The RSDs of intra-day and inter-day precision ranged from 0.7% to 3.8%. In order to evaluate the repeatability of the method, three different quantities (low, medium, and high) of the standard solutions were tested, and each concentration was tested for three times. The RSDs ranged from 0.5% to 4.0%. Accuracy of the method was validated by recovery test. Accurate amounts of the standards were spiked into the samples (0.05 g) in the form of solution. The spiked samples were extracted, processed, and quantified. The recoveries ranged from 97.6% to 103.9%.

3.3. Quantitative determination of alkaloids in lotus plumule

The alkaloids in different lotus plumule samples and in different parts of lotus were analyzed by the developed HPLC-DAD method. The data are shown in Table 4. Fig. 4 depicts the HPLC-DAD chromatograms of the mixed standard solution and the content of alkaloids in different samples. According to these data, the content of alkaloids in lotus plumule from different regions is quite different while the alkaloids in fresh lotus plumule are higher than those in the medicine sold in the market. The contents of alkaloids from lotus plumule in Hubei were higher than those in other regions. This may be related to different growing environments and storage conditions. Of course, this is not a typical property because there were not enough samples. Moreover, variances between all the parts of lotus were obvious. As for lotus plumule, neferine was the highest content, followed by isoliensinine and liensinine and the content of nuciferine was the lowest. However, the dauricine was not detected in all samples. This may be because the collected samples were partial. Compared with different parts of lotus, the alkaloids were mainly contained in lotus plumule and lotus leaves, while there were almost no alkaloids in the lotus seeds without the embryos and lotus stamens.

4. Conclusion

In this study, it is the first time to optimize the MAE method for extracting alkaloids from lotus plumule using response surface methodology. The central composite design was used for the optimization of MAE parameters. An HPLC-DAD method was developed to analyze the five alkaloids in lotus plumule, and the method is rapid, accurate and specific. It can be used to analyze alkaloids in different parts of lotus.

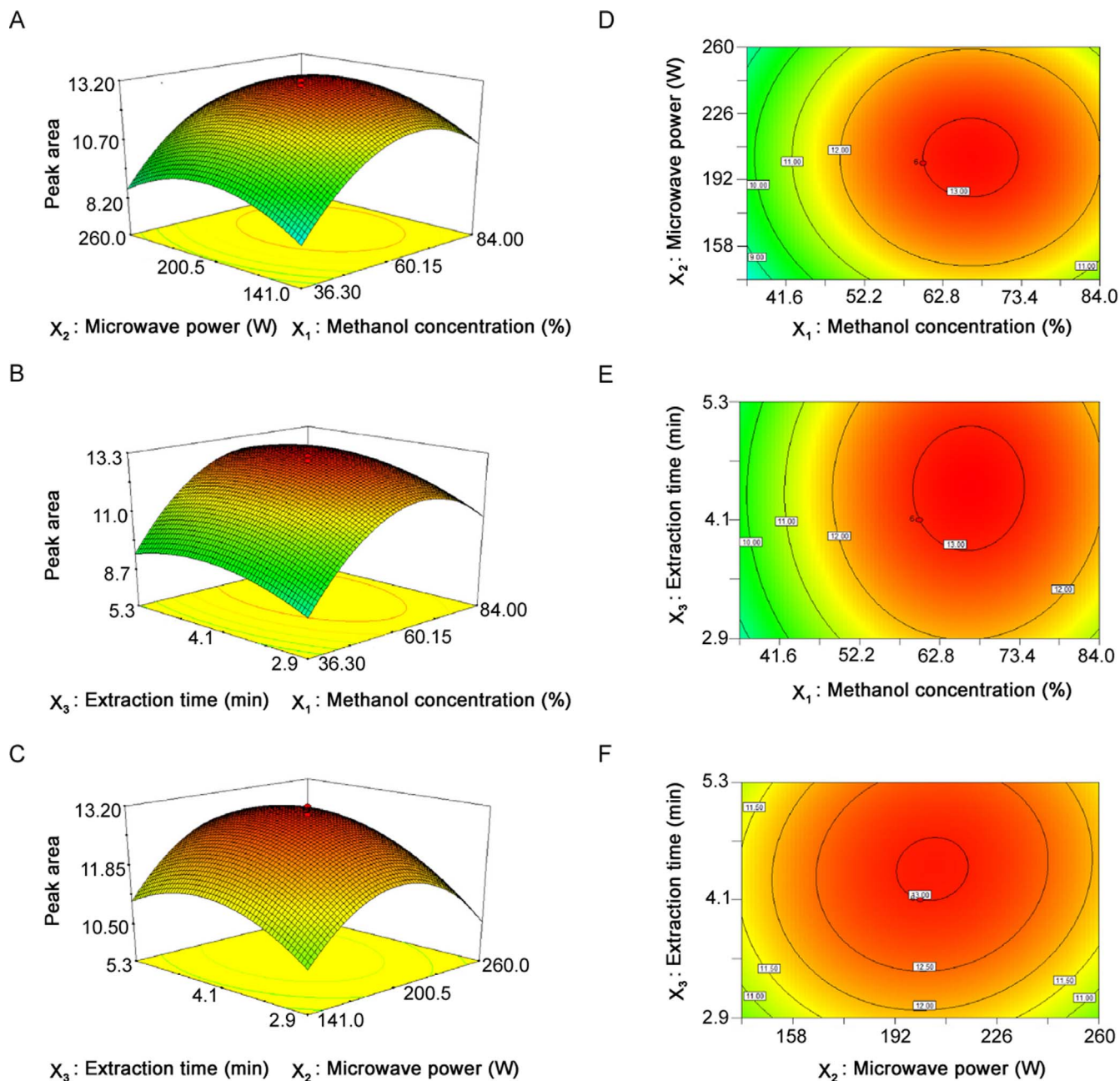


Fig. 3. The 3D response plots (A, B, C) showing the interactive effects of methanol concentration (X_1), microwave power (X_2) and extraction time (X_3) on the yield of alkaloids; Contour plots (D, E, F) showing the interactive effects of methanol concentration (X_1), microwave power (X_2) and extraction time (X_3) on the yield of alkaloids.

Table 3
 Linear regression, LOD, LOQ and recovery of the investigated compounds.

Analytes	Regression equation	R ²	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Recovery (%)
Liensinine	Y=0.0195X-0.0003	0.9997	3.2–103.0	0.8	1.6	98.4
Dauricine	Y=0.0351X+0.0067	0.9995	0.8–25.3	0.4	0.8	102.8
Isoliensinine	Y=0.0331X-0.0705	0.9998	6.7–215.0	0.8	1.7	103.9
Neferine	Y=0.0378X-0.0044	0.9997	3.3–106.5	0.4	0.8	97.6
Nuciferine	Y=0.1474X+0.0005	0.9999	0.4–13.4	0.2	0.4	99.3

LOD: limit of determination; LOQ: limit of quantitation.

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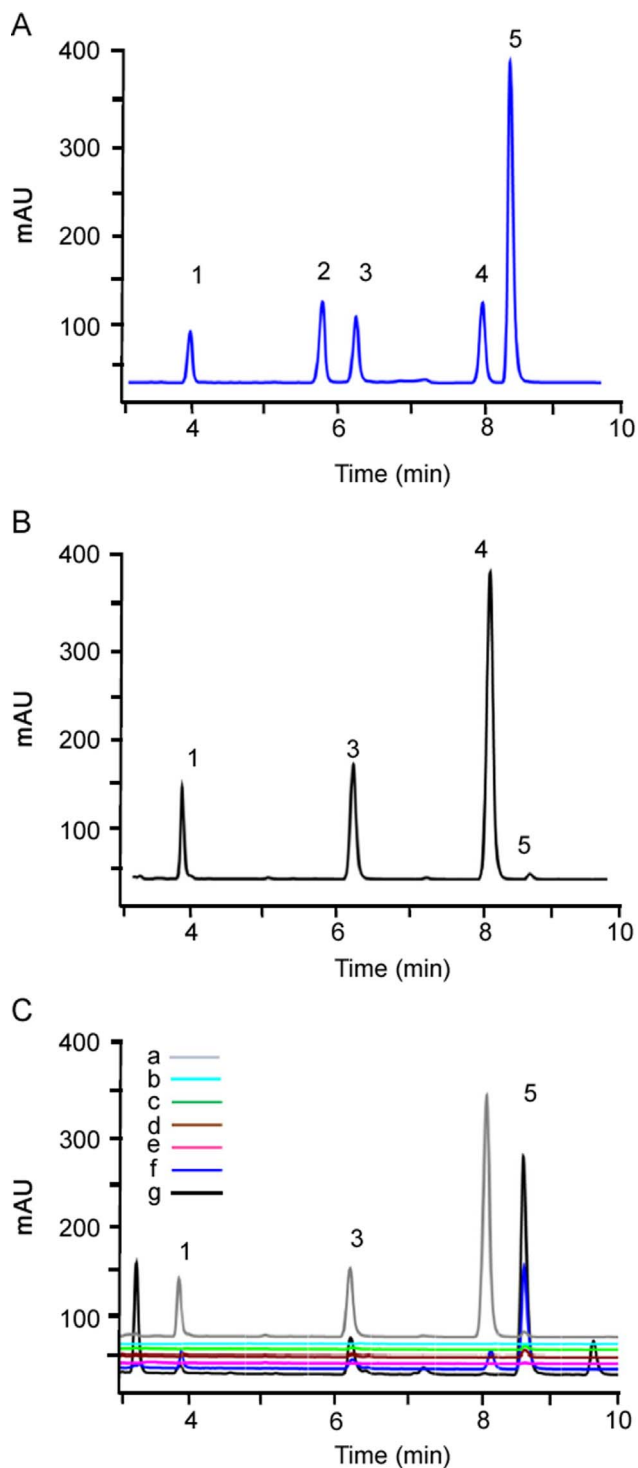


Fig. 4. The HPLC-DAD chromatograms of (A) the mixed standard solution, (B) the content of alkaloids in LZX and (C) different parts of lotus. (1) liensinine, (2) dauricine, (3) isoliensinine, (4) neferine, (5) nuciferine; a: lotus plumule; b: lotus seed; c: the hull of lotus seed; d: receptaculum nelumbinis; e: lotus stame; f: the flower of lotus; g: lotus leaves.

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Table 4

The alkaloids in lotus plumule and in different parts of lotus.

Samples	Analytes ^a (mg/g)				
	Liensinine	Dauricine	Isoliensinine	Neferine	Nuciferine
HB-1	1.86	nd	2.11	5.71	0.01
HB-2	2.12	nd	2.95	6.20	0.01
HB-3	1.70	nd	1.96	5.23	0.01
FJ-1	1.77	nd	1.47	5.11	0.02
FJ-2	0.72	nd	2.21	5.27	0.01
FJ-3	0.92	nd	2.01	5.20	0.01
HN-1	1.13	nd	1.64	6.00	0.03
HN-2	0.94	nd	2.14	5.23	0.02
HN-3	1.27	nd	1.81	5.74	0.03
JX-1	1.62	nd	2.25	5.23	0.02
JX-2	1.67	nd	2.05	4.88	0.02
JX-3	1.75	nd	2.11	5.18	0.02
ZJ-1	1.51	nd	1.81	4.68	0.02
ZJ-2	1.72	nd	1.86	4.93	0.02
ZJ-3	1.38	nd	2.02	4.84	0.02
MA-LZX	2.13	nd	2.14	6.56	0.03
MA-HY	1.65	nd	1.24	nd	1.11
MA-HH	0.41	nd	0.32	0.38	0.61
MA-LX	nd	nd	nd	nd	nd
MA-LF	0.05	nd	0.11	nd	0.04
MA-LZK	nd	nd	0.10	nd	0.02
MA-LZ	nd	nd	nd	nd	nd
ZH-LZX	0.36	nd	4.36	11.70	0.02
ZH-HY	2.36	nd	2.39	nd	3.02
ZH-HH	0.19	nd	0.14	0.39	0.41
ZH-LX	nd	nd	nd	nd	nd
ZH-LF	nd	nd	0.44	nd	0.21
ZH-LZK	nd	nd	0.08	nd	0.02
ZH-LZ	nd	nd	nd	nd	nd

LZX: lotus plumule; HY: the leaves of lotus; HH: the flower of lotus; LX: the stamens of lotus; LF: receptaculum Nelumbinis; LZK: the hull of lotus seed; LZ: lotus seed; nd: not detect.

^a The data was average of two determination with the variation of less than 5%.

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