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Optimization and Comparison of Extraction Methods of Lappaconitine from *Aconitum Sinomontanum nakai*

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The optimum extraction conditions for the maximum recovery of lappaconitine from *Aconitum sinomontanum nakai* with ultrasonic, microwave extraction were determined using response surface methodology. Box-Behnken design with three variables and three levels was employed and response surface plots were constructed according to a second order polynomial model. ANOVA showed that the quadratic model was well fitted. The optimum conditions were obtained through the overlapped contour plot. On the basis of which, a microwave-assisted ultrasonic extraction was built. The highest yields of lappaconitine extracted by the three methods were 0.887, 1.208 and 1.227 %, separately. Compared with the traditional extraction method, the extraction yield increase 10.9, 50.1 and 53.3 %; the extraction time save 87.50, 99.44 and 93.88 %. Finally a comparison of the lappaconitine content was conducted, indicating that *Aconitum Sinomontanum nakai* root cultured in Qinghai Province China has the highest lappaconitine content.

Keywords: Response surface methodology, Lappaconitine, Extraction, Optimization.

INTRODUCTION

Lappaconitine (LA), a kind of diterpenoid alkaloid extracted from the root of *Aconitum sinomontanum nakai*, possesses a wide range of biological effects including antifebric effect, apocatastasis, strong central analgesic and local anesthetic activities as well as antiarrhythmic and anti-inflammatory properties [1-6]. Being equivalent to pethidine, the analgesic effect of lappaconitine is 7 times as much as that of aminopyrine, furthermore, minor side effects and no addition are its characteristics. Lappaconitine has been applied clinically for the relieving of cancer and post-operative pain in China [7,8].

So far, few studies have been conducted on the extraction of lappaconitine from plant raw materials and most of them use traditional extraction methods involving organic solvents or heat-reflux method. Due to the low lappaconitine content and the potential hazards to humans from organic solvents used in the extraction processing, this paper, based on other studies, also the structure and unique properties of lappaconitine, identified a simple, safe and green extraction method offering further advantages including less solvent consumption and high efficiency.

In this study, regressive models were obtained through optimization of ultrasonic and microwave extraction of lappaconitine from the roots of *Aconitum sinomontanum nakai* using the response surface methodology, based on single factor experiments. On the basic of which, a microwave-assisted ultrasonic extraction was built. At the same time, a comparison between three extraction methods used in the paper and traditional extraction methods was operated to study the developmental advantages in industrial extraction of lappaconitine.

EXPERIMENTAL

Aconitum Sinomontanum nakai roots, originally cultivated in the Tibet autonomous region, Qinghai Province, Sichuan Province and Lanzhou Region in China. Standards of lappaconitine was purchased from Shida Institute of biological products (Lanzhou, China), Ethanol, Ethyl acetate and Petroleum ether were purchased from Factory of Damao Chemical (Tianjin, China). Methanol of HPLC grade was purchased from Tianjin Institute of fine chemicals retrocession, China.

Extraction: The mechanically pretreated *Aconitum Sinomontanum nakai* powder (10 g) was added to 95 % ethanol solution and extracted *via* ultrasonic, microwave or microwave-assisted ultrasonic extraction methods [9,10] for the required time periods. Then filtered, concentrated and acidified with hydrochloric acid at 0.1 mol/L. This solution was extracted with petroleum ether to remove impurities and the pH was

adjusted to desired value using 5 % NaOH. The mixture was extracted using ethyl acetate, followed by concentration and drying. Finally the lappaconitine was obtained by crysta-llized from ethanol. The final lappaconitine was diluted up to 100 mL with methanol and the diluent above was diluted 10 times. 1 mL extracts was injected through a 0.45 μm nanofilter for analysis.

Chromatographic conditions: HPLC analysis [11-13] was carried out using a C18 (4.6 × 150 mm, 5 μm) column. The mobile phase consisted of methanol (solvent A) and 0.2 mol/L of sodium dihydrogen phosphate (pH 4.0, solvent B) at a mix ratio of 60:40. Flow rate was 0.3 mL/min and injection volume was 10 μL. The monitoring wavelength of interest was 252 nm for lappaconitine.

Determination of lappaconitine content: The lappaconitine content was determined by HPLC analysis under the condition mentioned previously. Four quantification standard samples were used and the calibration curve was plotted for each standard compound on the basis of peak area.

Experimental design: The optimization procedure of ultrasonic extraction was designed based on a three-factor Box-Behnken design (BBD) consisting of temperature, X_1, extraction time, X_2 and value of pH alkalized, X_3, using three levels of each variable. Also, the effect of the independent variables extraction time (min), X_1, microwave power, X_2 and the liquid-solid ratio, X_3, at three variation levels were evaluated in the microwave extraction process (Table-1). The selection and range of these three factors was based on our preliminary experimental data (data not shown). Seventeen randomized experiments were assigned, based on Box-Behnken design [14-18]. Values of independent process variables are to be considered for response, as well as measured values.

The statistical analysis was operated with the Design Expert Version 8.0. A second-order polynomial equation presented as follow was used to fit the experimental data of the studied variables.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{2}\sum_{j=2}^{3}\beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, X_i and X_j are the independent variables affecting the responses; β_0, β_i (i = 1, 2, 3), β_{ii} (i = 1, 2, 3) and β_{ij} (i = 1, 2, 3; j = 2, 3) are the regression coefficients for the intercept, linear, quadratic and cross-product terms, respectively. The statistical significance of the terms in the regression equations was examined by ANOVA. The relationship between the independent variables (X_1, X_2 and X_3) and the response variables (Y_1 and Y_2) was demonstrated by the response surface plots. The optimized conditions of the independent variables were applied to verify the model, using the same experimental procedure as made previously. To evaluate the prediction power of the models, a comparison of theoretical predicted data and the experimental data was carried out. Triplicate samples of the optimized proportion were prepared and analyzed.

Microwave-assist ultrasonic extraction

Selection of the order of microwave and ultrasonic: Six parts of the same *Aconitum Sinomontanum nakai* powder (10 g) were randomly assigned to two groups (A and B), followed by extraction with different order of microwave and ultrasonic. The group of A was extracted by microwave extraction first and ultrasonic extraction followed, while the group of B was just opposite. Ultrasonic extraction was performed in a sonication cleaning bath (KQ5200V, Kun Shan Ultrasonic Instruments Co. Ltd) with temperature of 60 °C and extraction time of 20 min. Microwave extraction was carried out by using LWMC-205 (Nanjing Lingjiang Development CO. Ltd) operated at the power of 600 W, time of 2 min and the material-liquid ratio of 1:8.

TABLE-1
BOX-BEHNKEN DESIGN ARRANGEMENT OF ULTRASONIC AND MICROWAVE EXTRACTION

Test runs	Factors of ultrasonic extraction			Factors of microwave extraction		
	Time (min)	Temp. (°C)	pH	Time (min)	Microwave power (W)	Liquid/ solid ratio
1	1	0	1	0	0	0
2	0	0	0	0	1	1
3	0	0	0	-1	-1	0
4	-1	0	-1	0	-1	1
5	0	-1	-1	0	0	0
6	-1	-1	0	1	1	0
7	1	-1	0	-1	0	-1
8	0	1	1	1	-1	0
9	1	1	0	0	-1	-1
10	0	0	-1	0	0	0
11	1	0	-1	1	0	1
12	0	0	0	1	0	-1
13	0	0	0	0	1	-1
14	-1	0	1	-1	1	0
15	0	-1	1	0	0	0
16	0	0	0	0	0	0
17	-1	1	0	-1	0	1
True values[a]						
-1	30	55	12.0	1	500	1:8
0	45	60	12.5	2	600	1:10
1	60	65	13.0	3	700	1:12

[a]Values adopted for each factor in the Lappaconitine extraction experiment.

Selection of the ultrasonic extraction time: 10 g powder of lappaconitine was mixed with 100 mL of 95 % aqueous ethanol solution and the mixture was extracted with microwave-assisted ultrasonic extraction and the ultrasonic extraction followed by microwave extraction. The ultrasonic extraction temperature (60 °C), microwave extraction conditions (600 W, 2 min) were selected from the previous tests. Five ultrasonic extraction times (10, 20, 30, 40, 50 min) were used.

Comparison of lappaconitine content from *Aconitum Sinomontanum nakai* root in different areas: *Aconitum Sinomontanum nakai* root, originally cultivated in the Tibet autonomous region, Qinghai Province, Sichuan Province and Lanzhou Region in China, were extracted with the three method mentioned in the paper and the yields were compared.

RESULTS AND DISCUSSION

Identification of lappaconitine: Fig. 1 shows the analysis by HPLC at 252 nm of standard samples and extract. The two chromatograms indicate a common peak at about the 7th min.

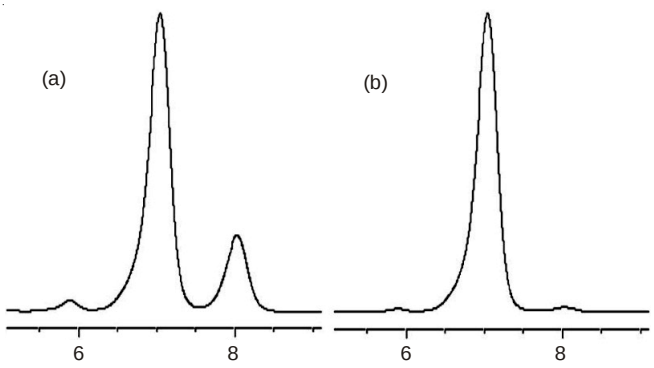

Fig. 1. HPLC chromatograms of reference substance (a) and samples (b)

Hence, extract was identified as lappaconitine. As we can saw in the figure, HPLC could provide better separation of lappaconitine and impurities.

Optimization of ultrasonic extraction: The mean values of the lappaconitine content extracted with ultrasonic extraction are shown in Table-2. The influence of three independent variables and their interactions towards lappaconitine content were reported through the significant ($p < 0.05$) coefficient of the second-order polynomial regression equation Table-3. The multiple regression analysis of total lappaconitine values showed that the model was significant ($p < 0.0001$) and did not present lack of fit ($p = 0.0737$), which indicated the suitability of models to accurately predict the variation and it could explain 96.31 % of all variance in data ($R^2_{adj} = 0.9631$) [19]. The fitted quadratic model is given bellow.

$$Y_1 = -432.76500 + 6 \times X_1 - 0.44458 \times X_2 + 43.75250 \times X_3 + 3.00000E\text{-}003 \times X_1 \times X_2 + 0.016000 \times X_1 \times X_3 + 0.053667 \times X_2 \times X_3 - 0.053350 \times X_1^2 - 4.38333E\text{-}003 \times X_2^2 - 1.9050 \times X_3^2 \quad (2)$$

Three dimensional surface and contour plots were generated based on eqn. 2 and are shown in Fig. 2, which present the relationship between every two process variables in the response. According to the Fig. 2, the mutual effects of the process variables are significant. When one of the factors is fixed, along with the increase of the other two factors, the yield of lappaconitine increased rapidly up to a certain extent and then decreased, which indicated that the control of the reaction conditions led to a higher yield of lappaconitine from the extraction of aconite. As the fitted model for the response was reliable within the region of the experiment based on the results of ANOVA [20,21] and the response surface plots showed that the maximal points of the responses were inside the experimental region, the optimal conditions were ultrasonic extraction time of 45 min, temperature of 60 °C and value of pH alkalized of 12.5, which gave estimated maximal values for lappaconitine (0.887 %).

TABLE-2
YIELDS OF LAPPACONITINE WITH ULTRASONIC AND MICROWAVE EXTRACTION

Standard order	Ultrasonic extraction (Y_1)	Microwave extraction (Y_2)
	Yields (%)	Yields (%)
1	0.773	1.209
2	0.883	1.015
3	0.885	0.908
4	0.769	0.935
5	0.721	1.218
6	0.681	0.883
7	0.648	0.969
8	0.679	0.934
9	0.654	0.941
10	0.716	1.220
11	0.747	1.052
12	0.861	0.894
13	0.887	0.913
14	0.634	1.015
15	0.668	1.211
16	0.869	1.194
17	0.597	0.961

Triplicate samples of the optimized proportion were prepared and analyzed to validate the prediction power of the models. The mean value of three parallel tests is 0.885 % and the relative deviation compared to the maximum yield of lappaconitine (0.887 %) from regression equation is 2.29 %, which indicates that the model can predict well of the actual lappaconitine extraction. Hence, the optimum extraction process is determined.

Optimization of microwave extraction: The mean values of the lappaconitine content extracted with ultrasonic extrac-

TABLE-3
RESULTS OF THE ANALYSIS OF VARIANCE TO THE RESPONSE SURFACE QUADRATIC MODEL FOR TOTAL LAPPACONITINE

Source	Sum of squares	df	Mean square	F value	P-value Prob > F	
Model	0.15	9	0.017	47.43	< 0.0001	Significant
X_1	6.48×10^{-4}	1	6.48×10^{-4}	1.79	0.2223	
X_2	2.49×10^{-3}	1	2.49×10^{-3}	6.88	0.0343	
X_3	4.95×10^{-3}	1	4.95×10^{-3}	13.71	0.0076	
X_1X_2	2.03×10^{-3}	1	2.03×10^{-3}	5.61	0.0498	
X_1X_3	6.40×10^{-5}	1	6.40×10^{-5}	0.18	0.6864	
X_2X_3	6.48×10^{-3}	1	6.48×10^{-3}	17.94	0.0039	
X^2_1	0.075	1	0.075	207.38	< 0.0001	
X^2_2	0.041	1	0.041	113.39	< 0.0001	
X^2_3	0.96	1	9.55×10^{-3}	26.44	0.0013	
Residual	9.55×10^{-3}	7	3.61×10^{-4}			
Lack of fit	2.53×10^{-3}	3	6.69×10^{-4}	5.15	0.0737	Not significant
Pure error	5.20×10^{-4}	4	1.30×10^{-4}			
Cor total	0.16	16				
R-squared	0.9839					
Adj R-square	0.9631					

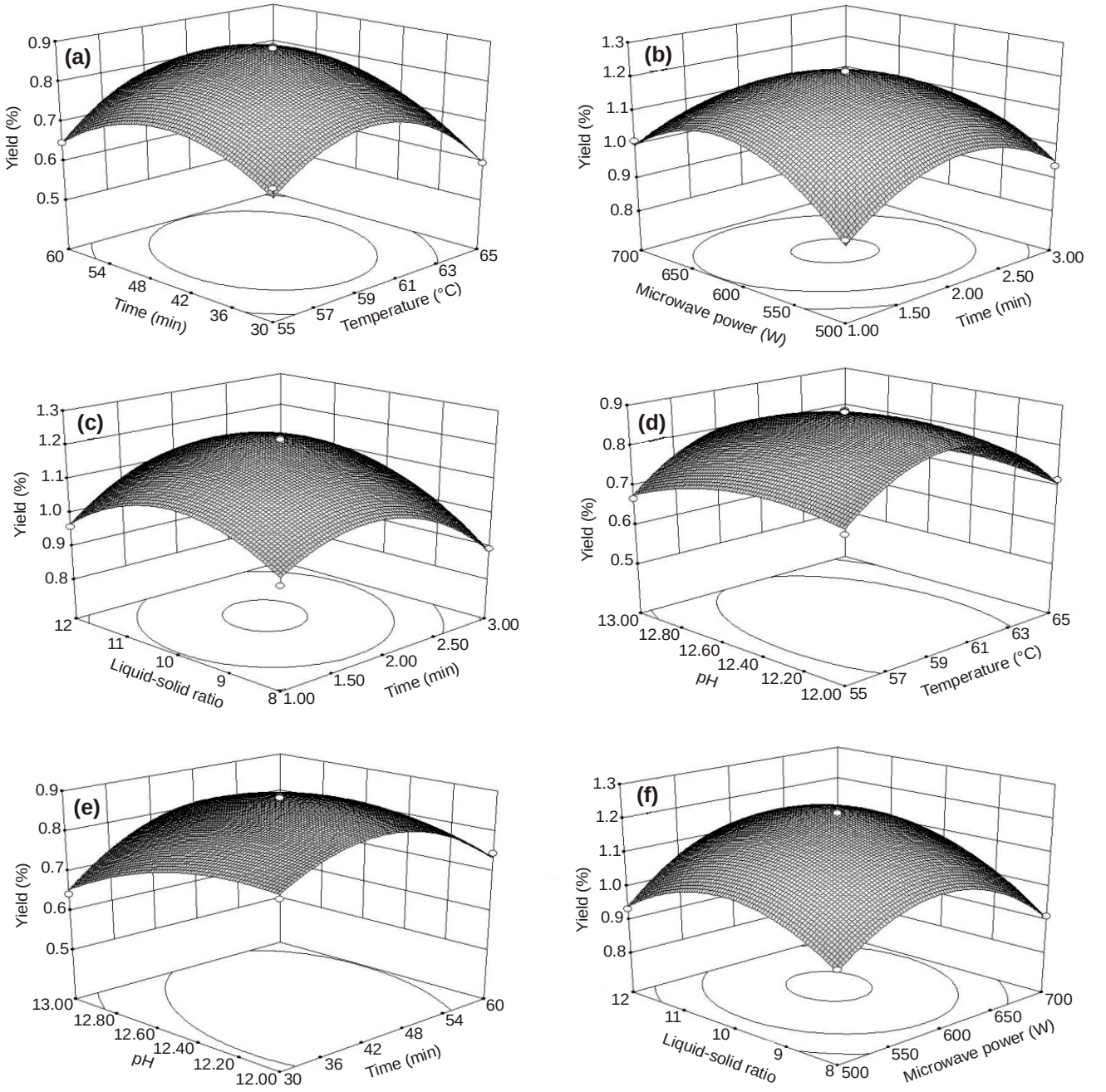

Fig. 2. Response surface plots showing the combined effect of extraction time and temperature (a), temperature and value of pH (b), value of pH and extraction time (c) extraction time and power of microwave (d), time and liquid-solid ratio (e) liquid-to-solid ratio and power of microwave (f) on the total Lappaconitine content of *Aconitum Sinomontanum nakai* extract

tion are shown in Table-2. Second order polynomial model is the empirical model most commonly used for optimizing methodology. The quality of the generated model was evaluated by analysis of variance (ANOVA), R square and the lack of fit of the model. The ANOVA results in Table-4 suggest that the model had very high F values and very low p values (< 0.0001) for the response. In addition, high R square values and insignificance of lack of fit (0.0537) were observed in Table-4, indicating that the quadratic model was highly significant and capable of describing the relationship between the extraction conditions and responses. It could explain 97.82 % of all variance in data (R^2_{adj} = 0.9782). The fitted quadratic model is given bellow.

$$Y_2 = -6.86090 + 0.53751 \times X_1 + 0.017127 \times X_2 + 0.45817 \times X_3 - 4.04250E\text{-}004 \times X_1X_2 + 0.020700 \times X_1 \times X_3 + 1.35500E\text{-}004 \times X_2 \times X_3 - 0.12814 \times X_1^2 - 1.46193E\text{-}005 \times X_2^2 - 0.028279 \times X_3^2 \quad (3)$$

Three dimensional surface and contour plots were generated based on eqn. 3, shown in Fig. 2, which present the relationship between every two process variables and its result is similar to ultrasonic extraction. The optimal conditions were microwave extraction time of 2 min, power of microwave of 600 W and liquid-to-solid ratio of 1:8, which gave estimated maximal values for lappaconitine (1.216 %).

TABLE-4
RESULTS OF ANALYSIS OF VARIANCE TO THE RESPONSE SURFACE QUADRATIC MODEL FOR TOTAL LAPPACONITINE

Source	Sum of squares	df	Mean square	F value	P-value Prob > F	
Model	0.26	9	0.029	80.70	< 0.0001	Significant
X_1	9.010×10^{-4}	1	9.010×10^{-3}	2.48	0.1592	
X_2	1.355×10^{-3}	1	1.355×10^{-3}	3.73	0.0947	
X_3	7.479×10^{-3}	1	7.479×10^{-3}	20.60	0.0027	
X_1X_2	6.537×10^{-3}	1	6.537×10^{-3}	18.00	0.0038	
X_1X_3	6.856×10^{-3}	1	6.856×10^{-3}	18.88	0.0034	
X_2X_3	2.938×10^{-3}	1	2.938×10^{-3}	8.09	0.0249	
X^2_1	0.069	1	0.069	190.40	< 0.0001	
X^2_2	0.090	1	0.090	247.82	< 0.0001	
X^2_3	0.054	1	0.054	148.37	< 0.0001	
Residual	2.542×10^{-3}	7	3.631×10^{-4}			
Lack of Fit	2.098×10^{-3}	3	6.994×10^{-4}	6.31	0.0537	Not significant
Pure Error	4.437×10^{-4}	4	1.109×10^{-4}			
Cor Total	0.27	16				
R-Squared	0.9905					
Adj R-Square	0.9782					

To determine the accuracy and reliability of the predicted model, as well as the deviation between actual and estimated values under the proposed optimal conditions, a verification experiment was conducted. No significant differences were observed between the observed (1.208 %) and estimated (1.216 %) values, verifying that the fitted model for the response was valid and reliable.

Optimization of microwave-assist ultrasonic extraction: The yields of lappaconitine obtained from group A and B were 1.203 and 1.202 % separately. It indicated that the order of microwave and ultrasonic processing in microwave-assist ultrasonic extraction has non-significance effect on the yield of lappaconitine.

Selection of the ultrasonic extraction time in microwave-assist ultrasonic extraction: With regard to the ultrasonic extraction time, the lappaconitine content increased with the increase in time, reaching up to a certain extent (30 min) and then decreased. This trend is same as the extraction using ultrasonic only. With the extension of extraction time, more lappaconitine dissolved from plant cells, while, after 30 min the yield of lappaconitine reduced, which may due to the damage of lappaconitine from the power of ultrasonic. Taking the energy consumed into consideration, a time of 20 min is used and hence, the optimal conditions were ultrasonic extraction time of 20 min, ultrasonic extraction temperature of 60 °C, microwave extraction time of 2 min and the power of microwave of 600 W.

Comparison of different extractions: The influence of extraction methods towards total lappaconitine content was reported in Table-5. The ultrasonic extraction, microwave extraction and microwave-assisted ultrasonic extraction can significantly improve the yield of lappaconitine and shorten extraction time compared to traditional extraction methods [22]. In addition, compared to microwave extraction, although the yield of lappaconitine extracted using microwave-assisted ultrasonic extraction is improved, the effect is not significant, at the same time, the microwave-assisted extraction method is more complicated. So, if a higher yield of lappaconitine is required, this method can be considered.

Comparison of lappaconitine content from *Aconitum Sinomontanum nakai* root in different areas: The yields of lappaconitine from *Aconitum Sinomontanum nakai* root cultured in Qinghai Province, Tibet, Sichuan and Lanzhou areas were 0.887, 0.872, 0.837 and 0.814 % with ultrasonic extraction and the ranking of the lappaconitine content from different areas reached agreement with other methods.

Conclusion

Response surface methodology was successfully implemented for optimization of total lappaconitine content. The ultrasonic extraction, microwave extraction and microwave-assisted ultrasonic extraction can significantly improve the yield of lappaconitine and shorten extraction time compared to traditional extractions. Considering the technical, economic and yield aspects, we can use microwave extraction method in the actual production. The lappaconitine content from *Aconitum Sinomontanum nakai* root originally cultivated in Qinghai Province is the highest, followed by the Tibet, Sichuan and Lanzhou, giving a reference to the development, introduction and cultivation of *Aconitum Sinomontanum nakai.*

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TABLE-5
COMPARISON OF DIFFERENT METHODS FOR EXTRACTING LAPPACONITINE

Method	Time (min)	Yield (%)	Relative improved yield (%)	Relative saved time (%)
Traditional extraction	360	0.800	–	–
Ultrasonic extraction	45	0.887	10.9	87.50
Microwave extraction	2	1.208	51.0	99.44
Microwave-assist	2+20	1.227	53.3	93.88

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