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Shao Liu[ab]; Yi-Zeng Liang[b]; Jun Luo[a]; Dong-Sheng Cao[b]; Xin-Zhong Li[a]; Peng Lei[a]; Da-Lin Yuan[b]
[a] Xiangya Hospital, Central South University, Changsha, P.R. China [b] College of Chemistry and Chemical Engineering, Research Center of Modernization of Chinese Medicines, Central South University, Changsha, P.R. China

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Optimization of Liensinine, Isoliensinine and Neferine Extraction from the Embryo of the Seed of *Nelumbo nucifera* GAERTN

Shao Liu,[1,2] Yi-Zeng Liang,[2] Jun Luo,[1] Dong-Sheng Cao,[2] Xin-Zhong Li,[1] Peng Lei,[1] and Da-Lin Yuan[2]
[1]Xiangya Hospital, Central South University, Changsha, P.R. China
[2]College of Chemistry and Chemical Engineering, Research Center of Modernization of Chinese Medicines, Central South University, Changsha, P.R. China

Abstract: The influences of extraction solvents and techniques on the yield of alkaloids from the embryo of the seed of *Nelumbo nucifera* GAERTN, were comprehensively investigated in this work. After the preliminary tests (extraction solvents and extraction methods), several parameters, such as ethanol concentration, extraction time, the ratio of liquid to solid were optimized using an experimental design, response surface methodology, and accelerated random search algorithm (ARSA). The results showed the best experimental conditions for total alkaloids (T.A). Using ultrasound-assisted extraction were ethanol concentration: 75%, extraction time: 20 min, and the ratio of liquid to solid: 30:1.

Keywords: Accelerated random search algorithm, alkaloids, optimization, *Nelumbo nucifera*, response surface methodology

INTRODUCTION

The embryo of the seed of *N. nucifera* GAERTN., named "Lian Zi Xin" in Chinese, has been extensively used as a traditional medicinal herb in China for a long time. It was primarily used for nervous disorders, insomnia, high fevers with restlessness, and cardiovascular diseases such as

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Address correspondence to Yi-Zeng Liang, College of Chemistry and Chemical Engineering, Research Center of Modernization of Chinese Medicines, Central South University, Changsha 410083, P.R. China. Tel.: +86-731-8830831; Fax: +86-731-8825637; E-mail: Yizeng_liang@263.net

hypertension and arrhythmia (1–5). Liensinine(LIE) and its analogues, such as isoliensinine(ISO) and neferine(NEF), are three main isbenzylisoquinoline alkaloids components in the embryo of the seed of *N. nucifera* GAERTN. LIE has been shown to slow action potentials in myocardium and slow inward current in canine cardiac Purkinje fibers (6). ISO has exhibited a significant inhibitory effect on bleomycin-induced pulmonary fibrosis, probably due to its antioxidant and/or anti-inflammatory activities (7). NEF has been reported to possess a reversal effect of multidrug resistance (8) and these isbenzylisoquinoline alkaloids were reported to show anti-HIV activity recently (9). Consequently, it is important to develop effective methods for extracting these alkaloids from the embryo of the seed of *N. nucifera* GAERTN. Response surface methodology (RSM) is a collection of statistical and mathematical technique for developing, improving, and optimizing process (10–14). It can identify and quantify the various interactions among different parameters. Box-Behnken design is response surface methods used to examine the relationship between one or more response variables and a set of quantitative experimental parameters (15). 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 (16,17). Also, a method of numerical optimization, namely accelerated random search algorithm (ARSA) (18), is introduced to find the global maximum. The basic of this algorithm is simple: it first generates a previous record value randomly in definition domain D and search neighborhoods of this record, if a new record is found, the search neighborhood re-initialized to the entire space to start a new search with the new record being the center, and if not, shrink the neighborhood to start a new search. After some iteratives, ARSA can find the best record in the domain D. For this procedure, local maxima are avoided by automatic restart and reinitialize the search area when some shrink steps have been operated. For more details, the readers can see the appendix. The purpose of the present study is to apply a three-level Box-Behnken design combining with RSM and ARSA to optimize the extraction conditions for the embryo of the seed of *N. nucifera* GAERTN in order to maximize simultaneously the yield of LIE, ISO, and NEF.

MATERIALS AND METHODS

Materials and Chemicals

The embryo of the seed of *N. nucifera* GAERTN was purchased from Medicinal Materials Inc. of Hunan province, Changsha, China. The standards for LIE, ISO and NEF were prepared in our lab. The purity of the compounds was >98% (base HPLC, NMR and MS). Acetonitrile (purchased from Shanghai Chemicals and Reagents Co., Shanghai, China) of HPLC grade was used for HPLC analysis. Water used was Milli-Q grade (Millipore, Bedford, MA, USA). Other chemicals were of analytical grade. All samples and solvents used for HPLC measurements were filtered (0.45 μm) and degassed before their use.

Instrumentation and HPLC Method Conditions

An Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used for all experiments. This system is equipped with an auto injector with a sample tray cooler, a multi-solvent delivery system, and a temperature controlled column compartment. The wavelength of the detector was set to 282 nm. At this wavelength, the response of the detector was linear in the concentration range for all components investigated. The injection volume was 10 μL. The column temperature was 30°C. The flow rate used for all experiments was 1.0 mL/min. The analytical column (4.6 mm × 150 mm) packed with 5 μm C18 silica. The binary elution mobile consisted of acetonitrile (A) and 0.1% triethylamine (B). The gradient elution was programmed as follows: 0 min, 40% A; 10 min, 66.7% A calibration curves (correction coefficient) for LIE, ISO, and NEF were $Y = 6246X - 7.86$ ($r^2 = 0.9991$), $Y = 2502X - 7.66$ ($r^2 = 0.9990$) and $Y = 5876.5X - 34.97$ ($r^2 = 0.9996$), respectively.

Selection of Relevant Variables and Experimental Ranges

Before using RSM, a first set of tests was performed to select the relevant factors in LIE, ISO, and NEF extraction. First, the influence of extraction solvent was investigated; secondly, the impact of extraction methods on the content of LIE, ISO, and NEF was studied.

Comparison of Extraction Solvents

A attempt for comparing extraction efficiencies of different solvent was carried out. The extraction method was fixed in refluxing. 1.0 g of ground Lian Zi Xin was placed in an 100 ml beaker. Extractions were carried out with the same solid-to-solvent ratio and the same time but using seven different solvents, i.e., 95% aqueous ethanol (v/v); 70% aqueous ethanol (v/v); 50% aqueous ethanol (v/v); 90% aqueous methanol (v/v); 0.25% hydrochloric acid aqueous solution; 0.25% sulphuric acid aqueous solution and chloroform. Each extract was transferred to a 100 ml volumetric flask and the total volume was adjusted to 100 ml with the appropriate extraction solvent mixture. These aliquots of Lian Zi Xin extracts were filtered through a 0.45 μm PVDF syringe filter prior to analysis of alkaloids by HPLC assay. Four replicate extractions and duplicate HPLC analyses of each extract were carried out for each sample.

Comparison of Extractions Methods

Comparison with four extraction techniques (Maceration, Refluxing, Microwave-assisted extraction, Ultrasound-assisted extraction) was carried out under the optimized extraction solvent mixture (70% aqueous ethanol, v/v) with the same solid-to-solvent ratio and the same extraction time. Each extract was transferred to a 100 ml volumetric flask and the total volume was adjusted to 100 ml with the extraction solvent mixture. These aliquots of Lian Zi Xin extracts were filtered through a 0.45 μm PVDF syringe filter prior to analysis of alkaloids by HPLC assay. Four replicate extractions and duplicate HPLC analyses of each extract were carried out for each sample.

Experimental Design for the Response Surface Procedure

The ethanol concentration, the liquid-to-solid ratio and the extraction time, coded as x_1, x_2 and x_3, were selected as design variables, respectively (Table 1). The RSM used a three-factor and Box-Behnken design (BBD) consisting of 15 experimental runs including three replicates of the center point (see Table 2 for details). The response values were the yields of LIE, ISO, NEF, and T.A. (total alkaloids, the sum of the yields of LIE, ISO, NEF), respectively. For multiple linear regression and statistical analysis, SPSS version 11.5 software was used. The polynomial equation, response surface curve, contour plots, and the computer programs of ARSA were achieved with the help of MATLAB 6.5. The experimental data allowed the development of empirical models describing the interrelationship between operational and experimental variables by equations including linear, interaction, and quadratic terms:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

Where y is the dependent variable, and x_1, x_2 and x_3 denote the independent variables. The coefficients of the polynomial were represented by b_0 (intercept), b_1, b_2 and b_3 (linear effects), b_{11}, b_{22}, and b_{33} (quadratic effects), and b_{12}, b_{13} and b_{23} (interaction effects).

Table 1. Indepentent variables and their coded and actual values used for optimization

	Code levels		
Independent variable	−1	0	+1
Ethanol concentration (x_1)%	60	70	80
Extraction time (x_2)/min	10	15	20
Ratio of liquid to solid (x_3)/X:1	10	20	30

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at a probability (P) of 0.05, 0.01 and 0.001, respectively. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models. On the basis of the quadratic response surface regression model, ARSA was used to search the best experimental condition.

Table 2. The Box-Behnken design, Observed and predicted data for alkaloids extraction from Lian Zi Xin

	Independent variables			Observed dependent variables				Predicted dependent variables			
Run	x_1	x_2	x_3	LIE	ISO	NEF	T.A.	LIE	ISO	NEF	T.A.
1	−1	−1	0	0.288	0.208	0.718	1.213	0.290	0.213	0.718	1.216
2	−1	1	0	0.290	0.216	0.724	1.230	0.284	0.225	0.720	1.214
3	1	−1	0	0.297	0.223	0.735	1.254	0.302	0.231	0.740	1.304
4	1	1	0	0.326	0.249	0.786	1.361	0.324	0.251	0.786	1.348
5	0	−1	−1	0.305	0.243	0.753	1.301	0.297	0.238	0.749	1.278
6	0	−1	1	0.312	0.238	0.781	1.331	0.311	0.238	0.779	1.324
7	0	1	−1	0.300	0.238	0.760	1.297	0.299	0.238	0.761	1.294
8	0	1	1	0.317	0.257	0.810	1.384	0.325	0.262	0.791	1.396
9	−1	0	−1	0.262	0.214	0.690	1.166	0.267	0.214	0.694	1.169
10	1	0	−1	0.269	0.219	0.712	1.201	0.271	0.226	0.712	1.257
11	−1	0	1	0.267	0.225	0.709	1.201	0.265	0.220	0.710	1.243
12	1	0	1	0.319	0.243	0.783	1.345	0.313	0.244	0.780	1.385
13	0	0	0	0.309	0.250	0.793	1.352	0.305	0.248	0.797	1.345
14	0	0	0	0.306	0.248	0.787	1.341	0.305	0.248	0.797	1.345
15	0	0	0	0.300	0.246	0.810	1.356	0.305	0.248	0.797	1.345

RESULTS AND DISCUSSION

Influence of Different Solvents on the LIE, ISO, and NEF Extraction

The yields of LIE, ISO, and NEF during the extraction with different solvents were investigated, and the results are displayed in Table 3. In general, the extracting efficiency of acid aqueous solution for alkaloids is high. But from Table 3, it can be learnt that LIE was disrupted when extracted with acid aqueous solution, probably due to the extraction temperature, which caused increased disruption of the LIE. It was observed that extraction with 50% aqueous ethanol did not differ much from that of 90% aqueous methanol and chloroform. However, the concentration of ethanol is an important factor to affect the extraction efficiency. 70% aqueous ethanol is the most effective for extracting the alkaloids. Whichever the concentration of ethanol chosen above 70%, the yield of alkaloids extracted will remain the same. This allows choosing any value above this limit, but one should avoid the use of even higher concentration of solvent in the design of a process. So, 70% aqueous ethanol was selected as extracting solvent in following experiments.

Optimization of Different Extraction Methods

The comparison results for four extraction methods, that is, Maceration, Microwave-assisted extraction, Refluxing and Ultrasound-assisted extraction, are shown in Table 4. From the table, it could be easily seen that the best one for T.A. is Refluxing. Only marginal decrease (1.7%) in yields of alkaloids was obtained when ultrasound-assisted extractions were compared to Refluxing. The extraction efficiency of the other two procedures (Maceration and Microwave-assisted extraction) was between 74.9% and 86.4% as compared to Refluxing. However, the procedure of

Table 3. The yield of Lie, Iso, Nef, and T.A. with different solvents

Solvent	Lie yield (g/100 g)	Iso yield (g/100 g)	Nef yield (g/100 g)	T.A. yield (g/100 g)
95% aqueous ethanol (v/v)	0.311	0.263	0.839	1.413
70% aqueous ethanol (v/v)	0.318	0.279	0.882	1.479
50% aqueous ethanol (v/v)	0.282	0.213	0.667	1.162
Hydrochloric acid (0.25%)	—	0.282	0.881	1.163
Sulphuric acid (0.25%)	—	0.288	0.883	1.171
90% aqueous methanol (v/v)	0.369	0.231	0.780	1.380
Chloroform	0.251	0.232	0.798	1.281

Table 4. The yield of Lie, Iso, Nef, and T.A. with different extraction methods

Extraction methods	Lie yield (g/100 g)	Iso yield (g/100 g)	Nef yield (g/100 g)	T.A. yield (g/100 g)
Maceration	0.214	0.195	0.676	1.085
Refluxing	0.312	0.264	0.873	1.449
Microwave-assisted extraction	0.231	0.230	0.791	1.252
Ultrasound-assisted extraction	0.294	0.265	0.866	1.425

ultrasound-assisted extraction is simpler than that of refluxing. So, ultrasound-assisted extraction was selected as extraction methods in following experiments.

Optimization of Extraction by RSM

Statistical Analysis

The extraction of LIE, ISO, NEF, and T.A. from the embryo of the seed of *Nelumbo nucifera* GAERTN was further optimized through the RSM approach. The observed results and the predicted values obtained using model equations of LIE, ISO, NEF, and T.A. for all runs with different experiment conditions are listed in Table 2 and Fig. 1. As can be seen, the predicted values match the observed values reasonably well. Table 5

Table 5. Regression coefficients, R^2, and P or probability values for four dependent variables for the extraction of Lian Zi Xin

Regression coefficient	LIE yield	ISO yield	NEF yield	T.A. yield
b_0 (intercept)	0.305	0.248	0.797	1.345
b_1	0.013	0.009***	0.022***	0.044***
b_2	0.004	0.006**	0.012***	0.022***
b_3	0.010	0.006**	0.021***	0.037***
b_{12}	0.007	0.004	0.011**	0.023*
b_{13}	0.011***	0.003	0.013**	0.027**
b_{23}	0.003	0.006	0.006	0.014
b_{11}	– 0.017	– 0.021***	– 0.054***	– 0.093***
b_{22}	0.012	– 0.003	– 0.002	0.007
b_{33}	– 0.009	– 0.001	– 0.019***	– 0.029*
R^2	0.942	0.943	0.982	0.982
P or probability	0.03*	0.03*	0.002**	0.002**

*Significant at 0.05 level; **Significant at 0.01 level; ***Significant at 0.001 level; R^2, correlation coefficient.

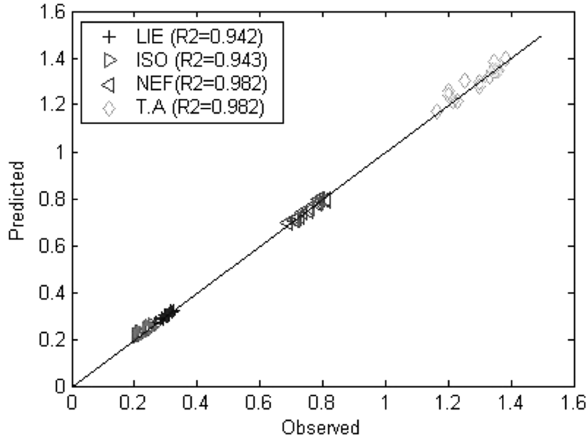

Figure 1. Relationship between observed and predicted values.

summarizes the results of each dependent variable with their coefficients of determination (R^2). The statistical analysis indicates that the proposed model was adequate, possessing no significant lack of fit and with very satisfactory values of the R^2 for all the responses. The R^2 values for LIE, ISO, NEF and T.A. yield were 0.942, 0.943, 0.982, and 0.982, respectively. The closer the value of R^2 to unity, the better the empirical model fits the actual data. On the other hand, the smaller the value of R^2, the less relevance the dependent variables in the model have in explaining the behavior of variations. The probability (p) values of all regression models were less than 0.05, without lack-of fit.

Effects of Ethanol Concentration, Extraction time, and Ratio of Liquid to Solid

The effect of different extraction conditions on the yields of LIE, ISO, NEF, and T.A. are reported (Table 5) by the coefficients of the second order polynomials. The response surface was used to illustrate the effect of ethanol concentration, extraction time, and the ratio of liquid to solid on the responses. Response surfaces and contour plots for T.A. yield are shown in Fig. 2a–c and Fig. 3a–c. Figure 2a shows the response surface plot for the effect of ethanol concentration and extraction time on T.A. yield. As shown in Table 5, the yield of T.A. mainly depends on the ethanol concentration as its linear ($P < 0.01$) and quadratic effects ($P < 0.01$) were significant giving an overall curvilinear effect. A negative quadratic effect indicates that there is a maximum in the T.A. yield at a certain

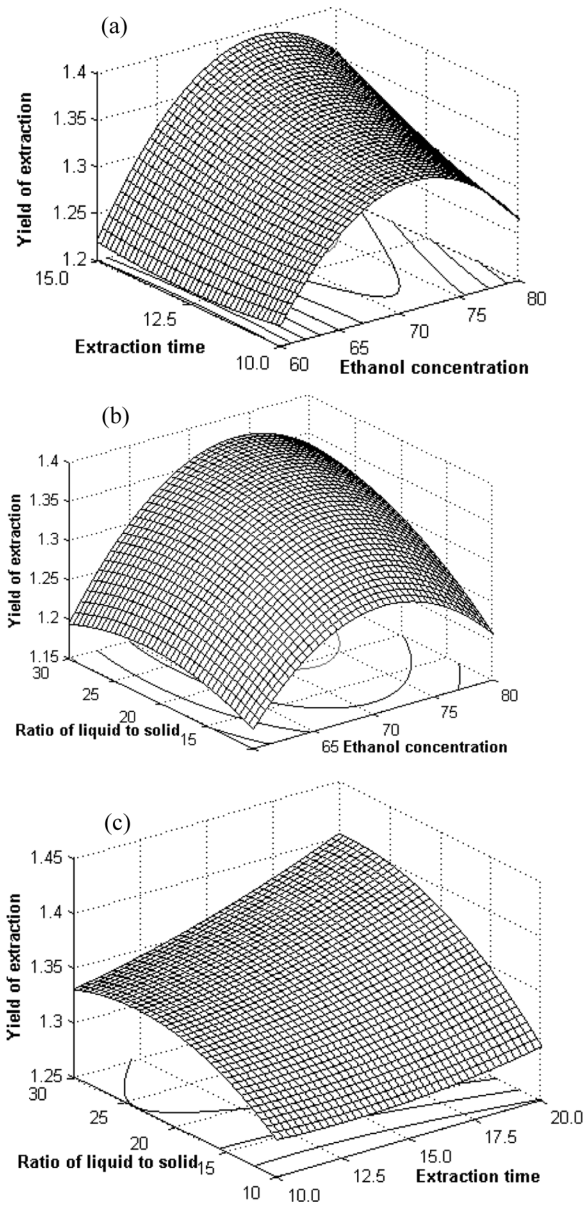

Figure 2. Response surface plots showing the effect of two variables on the yield of total alkaloids. Other two variables are held at center level. (a). Ethanol concentration and Extraction time; (b). Ethanol concentration and Ratio of liquid to solid; (c). Extraction time and Ratio of liquid to solid.

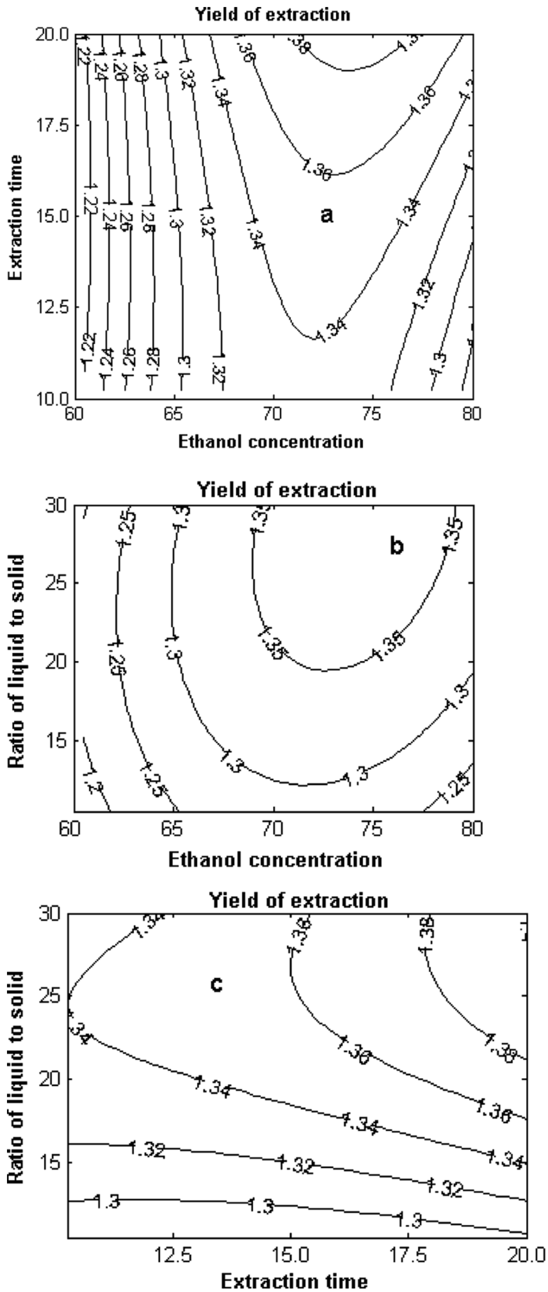

Figure 3. Contour plots showing the effect of two variables on the yield of total alkaloids. Other two variables are held at center level. (a). Ethanol concentration and Extraction time; (b). Ethanol concentration and Ratio of liquid to solid; (c). Extraction time and Ratio of liquid to solid.

ethanol concentration. The T.A. yield starts to decrease above this concentration. The quadratic terms in the model are not significant for the total yield of alkaloids, which results in a linear increase in yield with extraction time for all ethanol concentration. The interaction effect between the ethanol concentration and extraction time was significant ($P < 0.05$). The zone of optimization, as shown in Fig. 3a, depicts ethanol concentration should be between 72–77%, and extraction time should be over 18 min (Table 5) Fig. 2b shows the effect of the ethanol concentration and the ratio of liquid to solid on T.A. yield. It can be seen in Table 5 that the T.A. yield is found to be a function of the linear and quadratic effects of ethanol concentration and ratio of liquid to solid. The linear effect ($p < 0.001$) is positive, whereas the quadratic effect ($p < 0.05$) was negative, which results in a curvilinear increase in T.A. yield for all the ratio of liquid to solid employed (Fig. 2b). However, the effect of the ratio of liquid to solid on total alkaloids yield is the same as the effect of ethanol concentration. Figure 3b shows that the ethanol concentration should be be between 70–78% and ratio of liquid to solid should be over 20:1. Figure 2c shows the effect of extraction time and ratio of liquid to solid on T.A. yield. The T.A. yield is mainly a function of the ratio of liquid to solid with a positive linear effect ($p < 0.001$) and a negative quadratic effect ($p < 0.05$). The T.A. yield increases curvilinearly with an increase in ratio of liquid to solid for all extraction time but increases linearly with an elevation of extraction time at all ratio of liquid to solid (Fig. 2c). It can also be seen in Table 5 that T.A. yield is linearly related ($p < 0.001$) to the extraction time. However, the quadratic and interaction effects with the ratio of liquid to solid are insignificant. From Fig. 3c, the best combination of the response function can be determined. At very short extraction time and a low ratio of liquid to solid, the score value of the product was low. When the extraction time is above 18 min, high score values are recorded for the product processed at a ratio of liquid to solid that is over 22:1.

Further Optimization

From the foregoing account, it can be concluded the contour plots show the optimum conditions of the extraction process to T.A. yield were slightly different. There are a number of combinations of variables that could give maximum levels of T.A. yield and the optimum response for each dependent variable does not fall exactly in the same region. Thus, on the basis of the quadratic regression model, ARSA was used to further search the best experimental condition. With the help of the computer program of ARSA, between the ranges of independent variables (−1, 1), the optimum response is at the following conditions, that is, $x_1 = 0.5047$; $x_2 = 1$; $x_3 = 1$. For experimental conditions, these process variables for best combination are the ethanol concentration: 75% (v/v), extraction time: 20 min, and the ratio of liquid to solid: 30:1. The response function for the total alkaloids yield calculated from the polynomial model discussed above, was 1.423%. In order to verify the predictive capacity of the model, this optimum condition was used for an extra extraction test. The yield of T.A. was 1.409% (n = 4). The result confirms the predictability of the model used in this work.

CONCLUSIONS

Seven extraction solvents and four extraction techniques for alkaloids in "Lian Zi Xin" were systematically investigated and compared. Then, the different conditions (ethanol concentration, extraction time, and ratio of liquid to solid) for ultrasound-assisted extraction of "Lian Zi Xin" were further scrutinized by using the RSM. The results showed that all these variables markedly affect the yield of T.A., especially ethanol concentration. Using the RSM, the optimum set of the operating variables was obtained graphically. However, the optimum response for each dependent variable did not fall exactly in the same region. With the help of a newly developed global optimization method, say ARSA, the optima conditions predicted by the model are finally determined, that is, ethanol concentration: 75% (v/v), extraction time: 20 min, and the ratio of liquid to solid: 30:1.

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APPENDIX

Accelerated random search algorithm (ARSA)

The purpose of the accelerated random search algorithm is to find the maximum value in a region D, that is,

$$\max_{x \varepsilon D} f(x) \qquad D \subset \mathrm{R}^{\mathrm{d}} \tag{1}$$

The search space of ARSA is confined to a series of sphere surface with the radius smaller than or equal to 1. In such a way, the ARSA has the capability to converge fast to the global optimum of a given continuous objective function. Suppose that D is the unit hypercube and S(x, r) denotes the sphere surface with radius r and with the center x, the ARSA can be given as the following:

Step 0: Initialize $\mathrm{n} = 1$ and $\mathrm{r}_1 = 1$. Contraction factor $\mathrm{c} > 1$ and precision threshold value $\rho > 0$ are also predefined. Randomly produce x_1 uniformly distributed in D.
Step 1: In the nth step, for the given $x_n \in D$, randomly produce y_n in the domain of $S(x_n, r_n)$ where $r_n \in (0, 1)$.
Step 2: if $f(y_n) < f(x_n)$
$x_{n+1} = y_n$ *and* $r_{n+1} = 1$
Else if $f(y_n) \geq f(x_n)$
$x_{n+1} = x_n$ *and* $\mathrm{r}_{n+1} = \mathrm{r}_n/\mathrm{c}$
End
Step 3: if $\mathrm{r}_{\mathrm{n+1}} < \rho$ if $\mathrm{r}_{\mathrm{n+1}} < \rho$ let $\mathrm{r}_{\mathrm{n+1}} = 1$ end
Step 4: Let $\mathrm{n} = \mathrm{n} + 1$, then go back to Step 1.

It has been proved that the feasible solution sequence produced by the above algorithm can approximate the global optimum of formula (1) with the probability equal to 1. For this procedure, local maxima are avoided by automatic restart and reinitialize the search area when some shrink steps have been operated. Compared with the genetic algorithm (GA) (19–22), ARSA is more powerful in the ability of local search. More accurate optimization results are obtained from ARSA. Thus, ARSA will be a very useful tool for variable selection in nonlinear modeling.