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Optimization of Ultrasound Assisted Extraction of Isoliquiritigenin from Licorice by Response Surface Methodology

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Abstract: Isoliquiritigenin (ILG) of licorice is a potential cancer chemopreventive agent. In this paper, response surface methodology (RSM) integrating with Box-Behnken design (BBD) was firstly employed to optimize the ILG extraction, which based on the single-factor experiments. According to analysis, the effect of extraction time and ethanol concentration on yields was significantly. The optimum values were the ethanol concentration of 73.25%, extraction time of 27.82 min and liquid-solid ratio of 19.10: 1. Under these conditions, the highest yield of ILG from licorice was 0.26 mg/g.

Keywords: Response surface methodology, Isoliquiritigenin, Licorice, extraction.

1. INTRODUCTION

Licorice has been used as a flavoring agent in foods, beverages and tobacco. It was also used as a traditional natural medicine to treat individuals with duodenal ulcers [1], coughs, bronchitis, arthritis [2], and allergies [3]. Moreover, many studies also showed that flavonoides were the main bioactive components. Among them, ILG has reported to carry strong biological activity involving anti-oxidant [6-7], anti-inflammatory [4-5], anti-platelet [8], and estrogenic properties [9].

ILG, a compound with a chalcone structure, is one of the important compounds obtained from roots of licorice. It caused great interest because it have effects on inhibiting prolifera- tion of the human non-small cell lung cancer A549 cell line [10], inducing growth inhibition and apoptosis in oral cancer cells [11], suppressing pulmonary metastasis of mouse renal cell carcinoma [12], promoting mouse colon cancer cell apoptosis, inhibiting human breast cancer metastasis [13] and inhibiting the growth of prostate cancer [14-15]. The research showed that ILG is a special constituent in licorice, merited investigation as a potential cancer chemopreventive agent in humans.

The extraction method of ILG from licorice including Soxhlet extraction, water extraction, and supercritical CO_2 extraction was used to improve the extraction efficacy. However, it is usually involved with longer extraction time, higher investment costs, higher temperature, and energy requirements. Thus, ultrasound-assisted extraction has gained interest due to its simplicity, low cost, high extraction rate and time saving [16-17]. Up to now, there is no report that ultrasound-assistance extraction was used to extract ILG from licorice. Response RSM is an effective statistical tool for the optimization of multiple variables to get the best performance conditions, which has been widely used in food chemistry and natural products [18-19]. High-performance liquid chromatography (HPLC) has been used widely to analyze ILG in some Chinese traditional medicinal preparation and medicinal herbs [20]. It may be due to the very low contents of ILG in licorice, reports on its analysis are little. Moreover, little studies were devoted to the extract of ILG. Therefore, we reported the optimization of extract parameters for ILG from licorice.

In this study, the ultrasound-assisted extract of ILG from licorice was carried out using the BBD combined with RSM and the contents were measured by HPLC. Nowadays, the ultrasound assisted extract of ILG and its further optimization using RSM was reported for the first time.

2. MATERIAL AND METHODS

2. 1. Material

Licorice was collected from GAP cultivation base of Loufan County, Shanxi Province (China) in the year of 2013. The sample was prepared as powder by a milling machine. The powder was screened by a 60 size mesh and stored in the dry conditions with the air humidity of 10%. ILG was purchased from Kailai Biological Engineering Co., Ltd (Xi'an, China) and the purity was 98%. Ethanol (AR) came from Shentai Chemical Reagent Co., Ltd. (Tianjin, China). Methanol (HPLC) was from Honeywell B&J (USA) . Axwater water purification system (USA) was used to purify water.

HPLC analysis was a Dalian Elite 230+ system (Dalian, China), equipped with UV230+ ultraviolet-

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visible detector, P230 high-pressure constant flow pump, LU 230 low-pressure gradient mixer and 7725i manual filling value. The chromatographic was performed on a Hyporsil ODS_2 column (4.6 mm×250 mm, 5μm, Dalian Elite, China); Ultrasonic cleaner (Sheng Yuan instrument Co., Ltd, Zhengzhou, China) was used for ultrasonic extraction of ILG.

2. 2. Extraction of ILG

Licorice powder of 1.0000 g were placed into a 50 mL flat bottom flask and extracted by ethanol solvent in a designed ethanol concentration, extract time and liquid-solid ratio. Extraction was performed at a sonication cleaning bath with ultrasonic frequency of 80 KHz. The extraction solution was obtained by still and centrifugation (4000 r/min for 10 min). HPLC was used to determine the content of ILG.

2. 3. HPLC Analysis

The content of ILG was determined by HPLC. For HPLC analysis, methanol-water (45:55, v/v) was used as the mobile phase with 0.8 mL/min flow rate and 10 μL injection volumes at the temperature of 25 . The UV detection wave-lengths were 365 nm for ILG. All the samples were conducted in triplicate. Each sample was eluted 60 mins, using isocratic elution, and the retention times of ILG was 53.4 min. The high performance liquid chromatography graphs of standard substance and samples were shown in Fig. (**1**) and Fig. (**2**). For standard sample, various amounts of ILG were dissolved in methanol to prepare the stock solutions. Corresponding calibration curves was Y=0.0045X-1.1447 (R=0.9996).

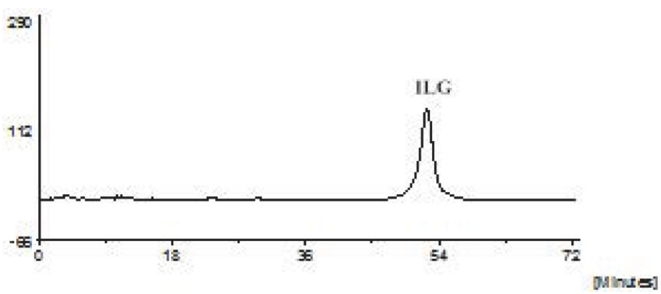

Fig. (1). The high performance liquid chromate- graphy graphs of ILG.

2. 4. Optimization of Ultrasonic- assisted Extract Method by RSM

Based on the single-factor experiments, the proper range of each factor was preliminarily chosen and used for experimental design of RSM. Independent variables of ethanol concentration, extraction time and liquid-solid ratio were respectively represented by X_1, X_2 and X_3. The coded and uncoded (actual) levels of the independent variables were shown in Table **1**. The yield of ILG was used as the response value. Design-Expert software 7.0.0 was used to generate the experimental design, statistical analysis and regression model.

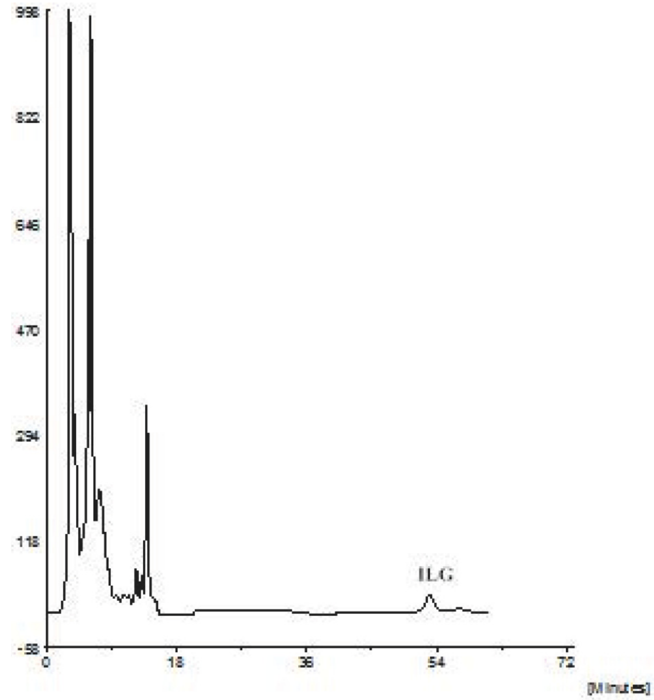

Fig. (2). The HPLC graphs of samples.

Table 1. Independent variables and their levels used in the BBD.

Factors	Levels		
	-1	0	1
X_1: ethanol concentration (%)	60	70	80
X_2: extraction time (min)	20	30	40
X_3: liquid-solid ratio (mL/g)	15:1	20:1	25:1

3. RESULTS AND ANALYSIS

3. 1. Single-Factor Experiment

3. 1. 1. The Effect of Ethanol Concentration on Extraction Yield of ILG

The ethanol used as extract solvent had several advantages including higher extract yields, environmental compatibility, lower cost and lower toxicity. However, the percentage of ethanol in water could affect the yields. So, different ethanol concentrations were tested in extraction process when other extraction conditions were as follows: extract time of 30 min and liquid-solid ratio of 20: 1 (mL/ g). From Fig. (**3**), the ILG yield increased with the increasing ethanol concentration. A noticeable increase was observed as the ethanol concentration was increased from 60% to 70%. The value was the highest at 70%, after which, the yield started to decrease. The reason for this might be that, with ethanol concentration increasing, fat-soluble matters such as ILG increased and water-soluble matters such as saccharides and proteins which were soluble in water decreased in extraction solvent. When ethanol concentration reached at 70%, higher ethanol concentration gave rise to larger osmotic pressure, thus extraction ratio of ILG would be affected and reduced to some extent. Therefore, the yield might be the maximum when ethanol concentration was 70%.

3. 1. 2. The Effect of Extraction Time on Extraction Yield of ILG

Time was also a factor that would influence the yield of ILG. The effect of different time on the ILG yield was shown in Fig. (**4**) The extracts were carried out at different time, and the other variable was set as follows: ethanol concentration of 70% and liquid-solid ratio of20: 1 (mL/ g).When the extract time was increased from 10 to 50 min, the ILG yield increased initially and then declined, and the yield reached highest when the extraction time at 30 min. All these results could be explained for following reasons: a short time (<30 min) might cause the insufficient solubilization of ILG. However, long extraction time (>30 min) might result in the degradation of the ILG, because of the thermal instability. In order to prevent the loss of the bioactivities, minimize the adverse effects of process and reduce the work costs, the optimal time was determined to be 30 min.

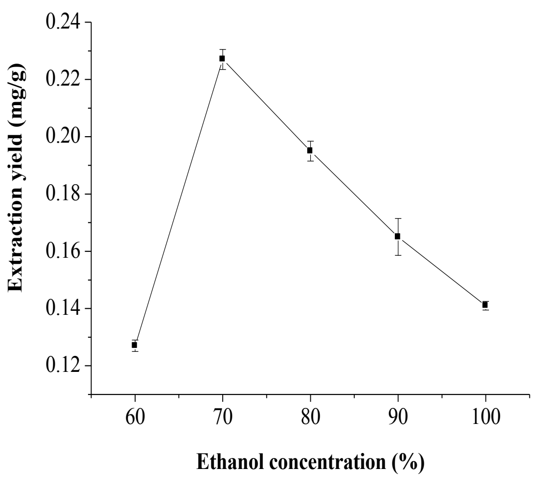

Fig. (3). The effect of ethanol concentration on extraction yield of ILG.

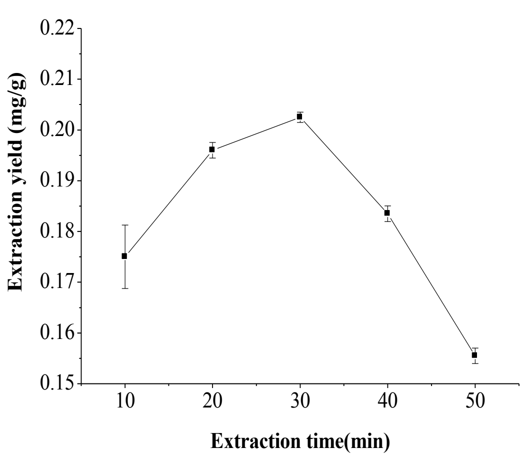

Fig. (4). The effect of extraction time on extraction yield of ILG.

3. 1. 3. The Effect of the Liquid-Solid Ratio on Extraction Yield of ILG

The liquid-solid ratio also was an important factor. If the liquid-solid ratio was too small, ILG in Licorice could be extracted incompletely. But once the liquid-solid ratio was superfluously high, it would cause complex procedure and high production cost. Therefore, suitable liquid-solid ratio should be selected for the extraction of ILG. In this study, effect of different liquid-solid ratio on the yield was investigated. The liquid-solid ratio was varied, and the other extraction variables as follows: ethanol concentration of 70% and extraction time of 30 min. The effect of different liquid-solid ratio on the yield of ILG was shown in Fig. (**5**). The yield increased significantly when the liquid-solid ratio was increased from 5: 1 to 20: 1, but decreased when the ratio was above 20: 1. The smaller liquid-solid ratio might cause the targets extraction incomplete and lower extraction yield. However, when the liquid-solid ratio was increased significantly, the yield was decreased. Generally, more solvent resulted in much dissolvable compounds. But superfluously high liquid-solid ratio was disadvantages because of the procedure complex, and could making solvent waste.

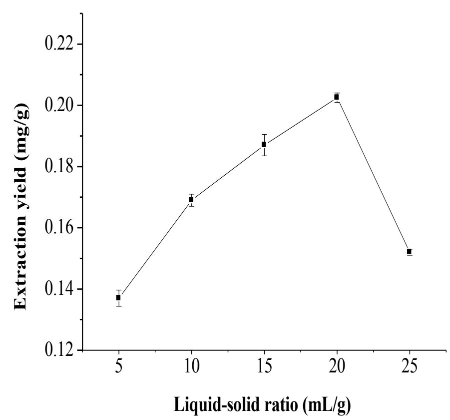

Fig. (5). The effect of the liquid-solid ratio on extraction yield of ILG.

3.2. Experimental Optimization

3. 2. 1. The Optimal Experiment of Response Surface

RSM was applied to optimize the three individual parameters for the yield based on the single-factor experiment. By using Design Expert 7.0.0, 17 experiments were designed and the corresponding yield were shown in Table **2**. The quadratic polynomial model between extracting conditions and the yield in terms of coded factors could be acquired as follow: $Y=0.26+0.026X_1-0.019X_2+0.0025X_3-0.015X_1X_2-0.023X_1X_3+0.018X_2X_3-0.051X_1^2-0.062X_2^2-0.024X_3^2$

The determination coefficient (R^2) and the adjusted determination coefficient (adjusted-R^2) obtained from the analysis of variance (ANOVA) were used to check whether the quadratic model was adequacy. The P-values were used to check the significance of each regression coefficient. And the smaller the p-value was, the more significant the corresponding coefficient was. As shown in Table **3**, the linear coefficients (X_1, X_2) and the quadratic coefficients (X_1^2, X_2^2) were extremely significant ($p<0.01$). The interaction coefficient (X_1X_3) and the quadratic coefficient (X_3^2) was found significant ($0.01<p<0.05$). The others (X_3, X_1X_2, X_2X_3) was not significant ($p>0.05$). A fairly high R^2 value of 0.9655 implied that the regression model was statistically significant and only 3.45% of the total variations were not explained by the model. The adjusted determination coefficiyent (adjusted-R^2 = 0.9213) also showed that the model had a high significance. The lack of fit was not significant (p = 0.7999). So, the predicted model could well represent the experimental values.

Table 2. Box-Behnken Design for independent variables and the response.

Run	X_1	X_2	X_3	Actual value(mg/g)
1	0	0	0	0.26
2	-1	-1	0	0.12
3	1	0	-1	0.22
4	0	-1	1	0.17
5	0	0	0	0.24
6	0	1	-1	0.14
7	-1	0	-1	0.13
8	1	-1	0	0.21
9	0	-1	-1	0.21
10	0	0	0	0.28
11	1	0	1	0.19
12	-1	1	0	0.11
13	0	0	0	0.27
14	0	0	0	0.24
15	-1	0	1	0.19
16	1	1	0	0.14
17	0	1	1	0.17

Table 3. Results of the ANOVA to the response surface quadratic model.

Source	Mean square	F	P
Model	4.998×10^{-3}	21.80	0.0003
X_1	5.512×10^{-3}	24.04	0.0017
X_2	2.812×10^{-3}	12.27	0.0100
X_3	5.000×10^{-5}	0.22	0.6547
X_1X_2	9.000×10^{-4}	3.93	0.0880
X_1X_3	2.025×10^{-3}	8.83	0.8754
X_2X_3	1.225×10^{-3}	5.34	0.0208
X_1^2	0.011	48.71	0.0541
X_2^2	0.016	69.46	0.0002
X_3^2	2.425×10^{-3}	10.58	<0.0001
Residual	2.293×10^{-4}		
Lack of fit	1.083×10^{-4}	0.34	0.7999

3.2.2. Response Surface Analysis

For studying the interaction among the different independent variables and their corresponding effects on the yield, three-dimensional response surfaces and two-dimensional contour plots were generated. The shapes of contour plots showed the effect of interactions between two tested variables. An elliptical nature of the contour plots indicated the interactions between the independent variables were significant. Otherwise, the interactive effect were slight. So, RSM established a more efficient relationship between the variable and response than the traditional design. Fig. (**6**) indicated the effects of ethanol concentration and extraction time on the yield of ILG. Ethanol concentration showed a significant effect, however, the effect of extraction time was weaker. The yield increased when ethanol concentration increased from 60% to 73%. Excessive increasing the ethanol concentration appeared to be disadvantageous for the yield. As shown in Fig. (**7**), the effects of ethanol concentration and liquid-solid ratio on the extraction yield of ILG. The yield of ILG presented the trend of increasing with liquid-solid ratio increasing up to 18: 1 after decreasing. According to Fig. (**8**), the extraction time exhibited a significant effect whereas liquid-solid ratio represented a weaker effect on the yield of ILG. With an increase in extraction time, the yield increased when the extraction time was under 28 min. But it decreased when

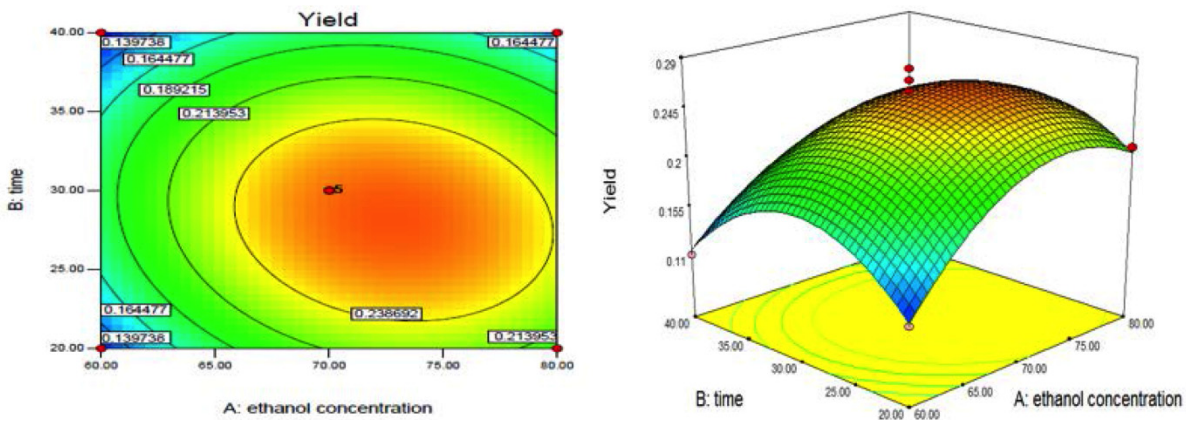

Fig. (6). The 2D contour plots and 3D surface of ethanol concentration and temperature

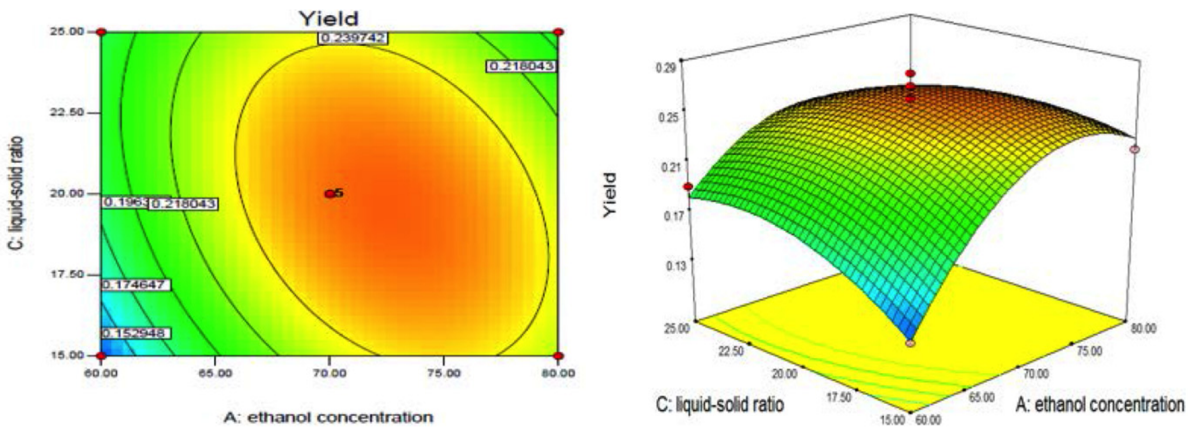

Fig. (7). The 2D contour plots and 3D surface of ethanol concentration and extraction time

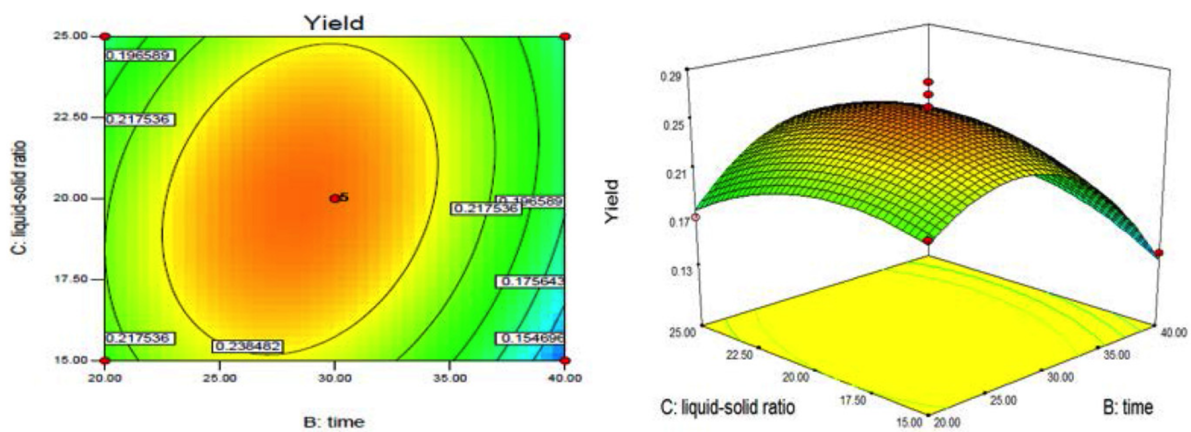

Fig. (8). The 2D contour plots and 3D surface of extraction time and liquid-solid ratio.

the extraction time was kept at levels higher than 28 min.

Making the regression equation equal to zero, the optimal level of three factors could be acquired. The optimal extraction condition was obtained as follows: the ethanol concentration of 73.25%, extraction time of 27.82 min and liquid-solid ratio of 19.10: 1. Under this condition the predicted yield of ILG would reached at 0.26 mg/g.

3. 2. 3. Model Test

The verification experiments were repeated three times under the optimized extraction conditions. The extraction yield of ILG were respectively 0.26 mg/g, 0.27 mg/g and 0.26 mg/g, and RSD was 0.41%. This indicated that the optimized extraction conditions was reasonable and reliable.

4. CONCLUSION

In this study, the ultrasound-assisted extraction was used to extract ILG from licorice. In addition, for finding the minimum number of experiments and the maximum yield, RSM with BBD as design method was chosen to establish the quadratic polynomial model and determine the optimal extraction condition to extract ILG. Under ethanol concentration of 73.25%, extraction time of 27.82 min and liquid-solid ratio of 19.10: 1, the maximum yield was 0.26 mg/g.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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