

# Optimization of the Glycyrrhizic Acid Extraction from Licorice by Response Surface Methodology

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**ABSTRACT:** *This study aims to extract Glycyrrhizic Acid (GA) from Glycyrrhiza glabra L. (licorice) using a novel, high efficiency, and low-cost extraction method. Water was chosen as the proper solvent for the extraction process to eliminate the requirement of harmful and toxic solvents, prepare an environmentally friendly process, and reduce the costs. The effect of different process variables (extraction time, feed to solvent ratio, pH of extraction medium, and temperature) on the extraction, yield was investigated experimentally and statistical analysis using the RSM method was performed to analyze the effect of variables and obtain a proper model correlating the yield of extraction to variables. The obtained model was found successful in fitting the experimental results. It was found that all the four variables affect the yield of extraction significantly, and only the interaction between pH and extraction time was found important in increasing the GA% in the precipitate. The highest yield was achieved experimentally 54.9%, for the case with the extraction time of 17 h, feed to solvent ratio of 10 g/ml, pH of 10, and temperature of 60°C. On the other hand, the optimum condition obtained by the model was found at extraction time of 10.12 h, feed to solvent ratio of 10.71 g/ml, pH of 9.8, and temperature of 119.7°C.*

**KEYWORDS:** *Glycyrrhiza glabra L.; Glycyrrhizic Acid; Licorice; Response Surface Method (RSM).*

## INTRODUCTION

*Glycyrrhiza glabra* L. (Licorice) belongs to the Fabaceae family and the plant is considered to be a good source of a large number of bioactive substances [1]. It has been extensively used as a traditional herb and has been frequently utilized to treat diverse diseases such as arthritis, peptic ulcers, adrenal insufficiency, asthma, pharyngitis, bronchitis malaria, abdominal pain, insomnia, infections, and allergies [2-6]. The important

bioactive constituents of licorice are glycyrrhizin, flavonoids, amino acids, coumarins, triterpenoids and essential oils [3, 7, 8]. The major active and the most abundant component in licorice is Glycyrrhizic Acid (GA) [3]. Some therapeutic activities such as antiviral, anti-inflammatory, and anti-cancer have been reported for GA [7, 9]. There are different technologies for separating and recovery of bioactive compounds like GA from licorice

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extract such as solvent extraction, adsorption separation with different adsorbents, chromatography, crystallization, etc. [9]. These methods suffer from various drawbacks such as large solvent supplies, low recovery, high cost and low yield [4, 8]. Furthermore, due to these disadvantages, controlling the process conditions and optimizing the parameters could be difficult. In order to overcome such drawbacks of traditional extraction methods, an alternative extraction technique has been introduced with unique advantages such as simplicity, rapid process, high efficiency, environmentally friendly and requirement of low cost. Different solvents such as water, methanol, ethanol, and ammonia have been used for extraction of GA. In order to reduce or eliminate the use of organic solvent and improve the yield of extraction processes water was employed as a universal and environmentally friendly solvent in this study. It has a better solubility of GA. Hence it was used as a solvent in this work and other parameters were optimized (including licorice powder to water ratio, time, pH and temperature of extraction). Effective analyzing the experimental data is a very significant step in every experimental study. The obtained data were statistically analyzed using Response Surface Methodology (RSM). Actually, RSM combines statistical and mathematical techniques for fitting the experimental data to the model in optimization processes [10]. RSM methodology has been extensively applied to develop quadratic response surface models [11, 12]. The optimum conditions for substantial variables were obtained using RSM.

## EXPERIMENTAL SECTION

### Chemicals

Licorice powder was purchased from FT-Sanat Co. [Kermanshah, Iran]. A voucher specimen of the plant was deposited in the FT-Sanat Co. and authenticated by quality control of them. Mono-ammonium glycyrrhizic acid (as the standard substance for HPLC analysis) was procured from Sigma [Sigma Co. St. Louis, MO, USA]. All the other performed materials were in analytical grade (HPLC grade).

### The glycyrrhizic acid extraction procedure

The extract of licorice roots was used as feedstock in this. The different ratios of licorice powder to water (Table 1) were mixed [13]. The extraction was carried out at different temperatures and different time durations (Table 1). Then sulfuric acid was added to the solution,

until pH of the solution became 2-3, and stirred regularly for 20 min. Acidification of the solution causes GA precipitation for isolation of GA from other water-soluble components and further purification [8]. The obtained solution was centrifuged (Heidolph, 4000 rpm, 20 min) to separate the precipitate. The precipitate was dissolved in water and was neutralized with ammonia at different pH (Table 1) and then ethanol was added to obtain 40% (v/v) solution. The liquid extract and the precipitate were collected separately. The liquid extract was evaporated under vacuum at 40°C to form a dried powder. The diagram of the different steps of the experimental procedure is shown in Fig. 1.

### HPLC analysis of glycyrrhizic acid

The obtained GA was analyzed using a High-Performance Liquid Chromatography [HPLC, Knauer V7603, Berlin, Germany]. A reverse type Agilent RPC-18 column (250×6.4 mm ID, pore size 5µm) was used. Detection was carried out at 254 nm with a UV detector. The mobile phase comprised of a mixture of acetic acid, water, and acetonitrile (1:61:38 volume ratio) and was delivered at a flow rate of 2 ml/min.

### Experimental design method

In the present study, Box-Behnken experimental design was performed to carry out an optimizing study. The Design Expert software [Version 8.0.7.1, Stat-Ease, Inc., USA] was used for regression and graphical analysis of the data. The Response Surface Model (RSM) using Box-Behnken was chosen as a proper method for optimizing the percentage of GA in the precipitate because it is a desired experimental design tool for analyzing a lot of variables at different levels with a limited number of experiments [14]. In order to minimize the number of experiments and variables, before experimental design, selection of the most affecting variables was done based on different experiments and the effect of different variables (pressure, temperature, the extraction time, pH of solvent, the feed to solvent ratio, type of solvent, the size of plant, and number of experimental stages) on response was examined separately. Consequently, the four variables possessing the most significant role in increasing the percentage of GA was chosen. Four selected independent process variables (extraction time, the feed to solvent ratio, pH of extraction medium, and temperature) and one response variable (percentage of

Table 1: The range of designed independent variables.

Independent variable	Range and level of variables (coded)		
	-1	0	+1
X <sub>1</sub> = Extraction time (h)	4	17	30
X <sub>2</sub> =Feed to solvent (licorice powder to water) ratio (g/mL)	10	25	40
X <sub>3</sub> =pH	8	10	12
X <sub>4</sub> =Temperature (°c)	60	90	120

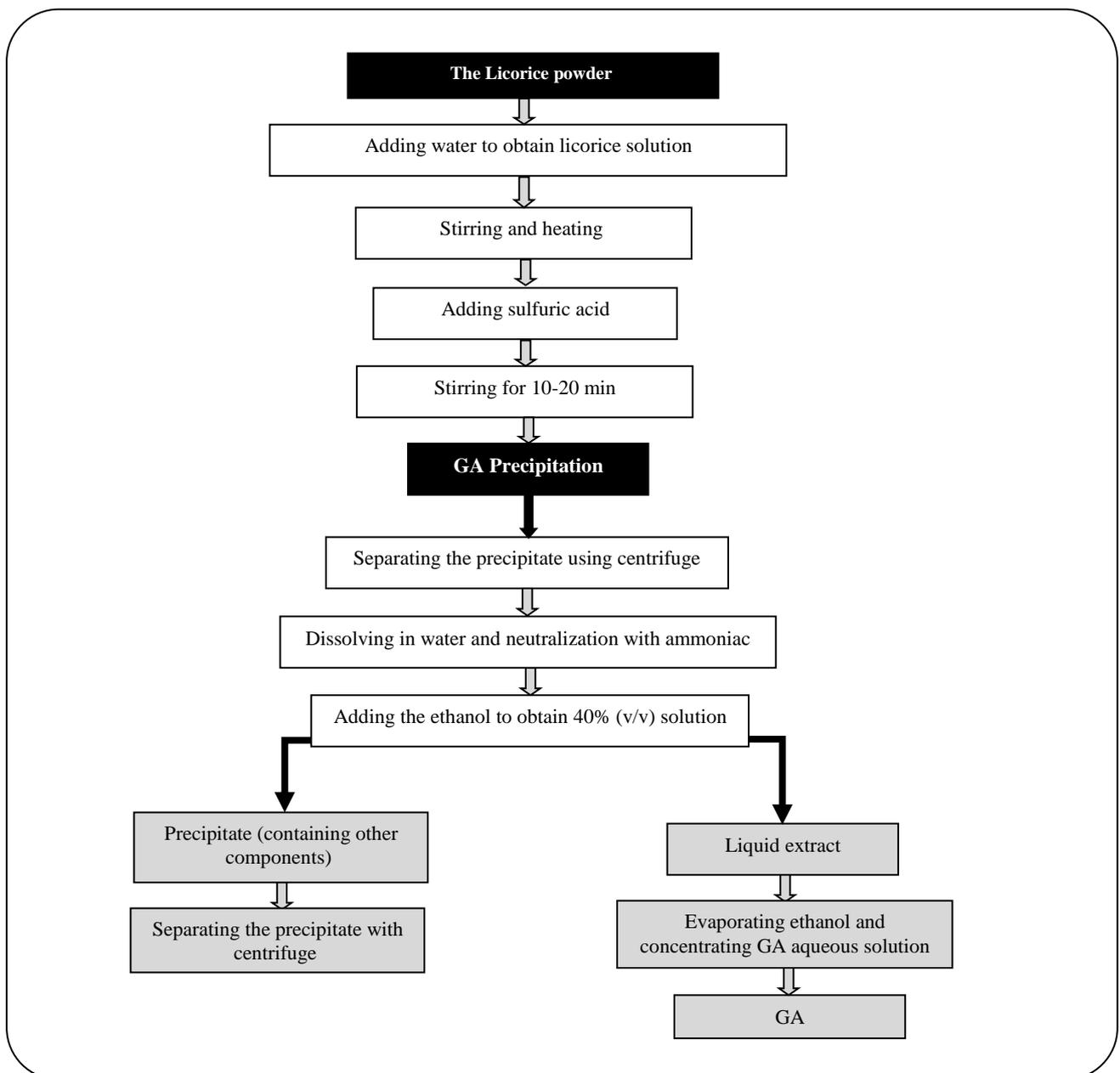


Fig. 1: The diagram of Glycyrrhizic acid extraction procedure.

GA in precipitate) were defined in the model, and other variables were kept constant. A total of 27 experiments were conducted separately to get the experimental response of GA%. The range of designed variables is tabulated in Table 1.

According to the performed model, the Design Expert software could result in a quadratic polynomial equation to correlate the response to operating variables (Eq.(1)).

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

Where Y is the response,  $\beta_0$  is constant coefficient,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are coefficients for the linear, quadratic and interaction effects respectively,  $X_i$  and  $X_j$  are independent variables,  $\varepsilon$  is the error, and k is the number of variables.

Furthermore, the software could prepare a graphical analysis of the experimental data, which is important to determine the optimum combinations of variables [15]. The accuracy of the model on fitting the polynomial equation could be evaluated by multiple correlation coefficients,  $R^2$  and adjusted  $R^2$ .

## RESULTS AND DISCUSSION

### *Fitting the Response Surface models*

In this study, Box-Behnken was performed as a favorable experimental design method to analyze the effect of processing parameters on the percentage of GA in the precipitate (GA%). RSM experimental design has been used in other studies to achieve a set of optimized conditions in extraction and isolation of GA from licorice and could successfully predict the effect of experimental parameters on extraction yield using quadratic equations. Hedayati and Ghoreishi [16] performed RSM for optimizing of GA extraction in a procedure using supercritical carbon dioxide. A quadratic equation was obtained for correlating GA extraction yield to four independent parameters: temperature, pressure, dynamic extraction time, and carbon-dioxide flow rate. The RSM experimental design could successfully predict the optimum conditions as well as the effect of parameters on the extraction yield. In another study carried out by Li et al., [17] RSM was found a proper method for optimization of GA and flavonoids extraction from licorice and yielded a quadratic equation with no significant prediction error. Table 2 demonstrates the set of experiments and the actual amounts of response (GA%).

According to the experimental results and using the Box-Behnken method, the response function was fitted by a quadratic polynomial equation. This equation is given as follows in terms of coded and actual factors:

$$R = +24.46 - 8.29A - 7.96B + 2.44C - 0.11D - 11.88AC + 13.28B^2 + 11.75D^2 \text{ (Coded)} \quad (2-1)$$

$$R = +101.62 - 3.93A - 3.48B + 8.99C - 2.35D - 0.46AC + 0.06B^2 + 0.013D^2 \text{ (Actual)} \quad (2-2)$$

Where, A is the extraction time, B is the ratio of licorice powder to water, C is pH, D is the temperature, and R is the percentage of GA in precipitate.

The analysis of variance (ANOVA) was used to analyze the statistical significance of the model and the regression coefficients. This analysis introduces competence measures for the model, such as rejection probability (Model p-value),  $R^2$ , adjusted  $R^2$ , predicted  $R^2$ , and adequate precision. The ANOVA results of the fitted model are shown in Table 3. The low p-value of the model (p-values < 0.05) indicates the significance of the model terms. It is important to note that the smaller magnitude of p-value shows the more significant of the corresponding variable. According to the obtained p-values, all the four independent variables significantly affect the response, but the extraction time has the most significant effect on GA %. Considering the interaction effects, it was found that only the interaction between the extraction time and pH is significant and should be considered in optimizing the response. According to the F-test, a confidence limit of 95% was achieved and the F-value of 12.89 for model confirmed the good agreement between experimental and model values. The lack of fit F-value indicates that the lack of fit was not significant in the response, which confirms the adequate accuracy of the model and low error in regression (Table 3).

### *Validation of the models*

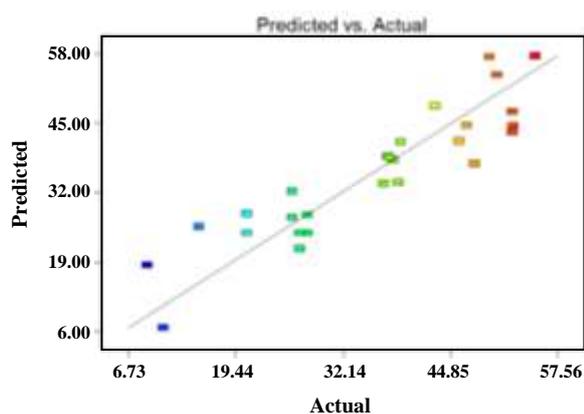
The coefficient of determination ( $R^2$ ), adjusted  $R^2$  and predicted  $R^2$  of the model was found at 0.8260, 0.7219, and 0.5382 respectively. These values show the good agreement between the model and actual results. Another criterion for ensuring the validity of the model was the prediction error of the model. For this purpose, the prediction error of the model for the experimental result was calculated for the case with maximum yield (GL%=54.9%). The maximum GA% was obtained

**Table 2: The set of experiments and experimental results of extraction yield.**

Test number	Extraction time (h)	Licorice powder to water ratio (g/mL)	pH	T (°C)	GA% in precipitate
1	4	25	10	60	46.8
2	30	25	12	90	10.8
3	17	25	8	60	38.7
4	17	40	10	60	45.9
5	30	25	10	60	20.7
6	17	10	10	120	49.5
7	30	40	10	90	27
8	4	25	12	90	52.2
9	4	25	10	120	52.29
10	30	10	10	90	47.7
11	17	40	8	90	26.1
12	17	25	10	90	20.7
13	17	25	8	120	36.9
14	17	25	12	120	37.62
15	4	10	10	90	50.4
16	17	25	12	60	37.44
17	17	10	8	90	52.2
18	17	25	10	90	27
19	17	40	12	90	26.1
20	17	40	10	120	38.97
21	30	25	8	90	15.03
22	17	25	10	90	27.9
23	17	10	10	60	54.9
24	4	40	10	90	38.07
25	30	25	10	120	27.9
26	4	25	8	90	8.91
27	17	10	12	90	43.02

**Table 3: The ANOVA results of the designed model.**

Source	Sum of Squares	D <sub>f</sub>	Mean Square	F-Value	p-value	
Model	3903.08	7.00	557.58	12.89	<0.0001	Significant
A-A	825.68	1.00	825.68	19.08	0.0003	
B-B	761.29	1.00	761.29	17.60	0.0005	
C-C	71.74	1.00	71.74	1.66	0.2133	
D-D	0.13	1.00	0.13	3.058E-003	0.9565	
AC	564.54	1.00	564.54	13.05	0.0019	
B <sup>2</sup>	1128.48	1.00	1128.48	26.08	<0.0001	
D <sup>2</sup>	883.41	1.00	883.41	20.42	0.0002	
Residual	822.09	19.00	43.27			
Lack of Fit	791.31	17.00	46.55	3.02	0.2770	Not significant
Pure Error	30.78	2.00	15.39			
Cor Total	4725.17	26.00				

**Fig. 2: Comparison between model predicted and actual values of GA extraction yield.**

at the conditions of extraction time of 17h, feed to solvent ratio of 10 g/mL, pH of 10, and temperature of 60°C. For this case, the experimental and predicted GA% was 54.9% and 57.56%. The prediction error was 0.48, which is less than 5%, and confirmed the validity of the model in predicting and fitting the experimental data.

The graph of the predicted and actual values of GA% is shown in Fig. 2. The predicted values were found quite close to the experimental values, which confirmed the acceptable accuracy and validation of the model developed for establishing a correlation between the process variables and the percentage of GA in the precipitate.

#### ***Effect of the extraction parameters on the percentage of GA in the precipitate***

The optimal values of the independent variables could be obtained using the regression equation together with analyzing the response surfaces and contour plots. The effect of each variable on the response could be examined using the regression equation. Regarding the sign of coefficients in Eqs. (2-1) and (2-2), it is clear that the GA% increases with decreasing the extraction time, the ratio of feed to solvent, and temperature; but increasing the pH causes increasing the GA% in the precipitate. The relationships between the percentage of GA in the precipitate and the variables could be visualized by two-dimensional contours or three-dimensional (3D) response surface plots. Fig. 3 illustrates the response surface 3D plots exhibiting the effect of interaction between the extraction time and pH. The interaction between the pH of solvent and extraction time plays a significant role in increasing the percentage of GA in the precipitate. A glance at figure obviously indicates that the GA% could be increased by shortening the time of extraction and simultaneously increasing the pH of the medium. At pH equal to 12 (highest pH) and extraction time of 4 h (shortest time) the GA% was found in the maximum level, according to the figure. The increasing trend of GA% with elevating pH could be due to the specific structure of GA at different pH values. At acidic pH values (pH<3), GA is presented in a molecular form, but it is dissociated at higher pH values and

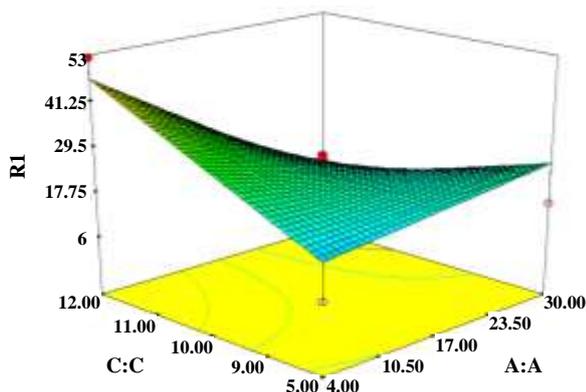


Fig. 3: The 3-D plot of the effect of pH- extraction time interaction on GA% in the precipitate (A is the extraction time (h) and C is the pH value).

a dissociated form of GA is more hydrophilic. So, at higher pH values, the extraction of GA could be facilitated and GA could be efficiently extracted into a solvent (water) [9, 18]. The effect of pH on the extraction of GA from licorice was also examined by Hashemi *et al.*, who extracted GA from licorice using lighter than water organic solvents (n-hexane and acetone). At acidic pH values (in which GA is present in molecular form) the highest extraction yields were achieved because they used organic solvents and the better extraction could be achieved when GA is present in the protonated molecular form [19]. The extraction yield was also decreased by elevating the temperature. This also thought to be caused by the structure of GA, which is formed by two parts: glucuronic acid (glycon) and glycyrrhetic acid (aglycon). This study was conducted at high temperatures and at higher temperatures increasing the temperature caused hydrolysis and decomposition of GA into one molecule of glycyrrhetic acid and two molecules of glucuronic acid [20]. The hydrolysis of GA could be facilitated by increasing the temperature [21].

#### Optimization of the extraction process

The maximum percentage of GA in the precipitate was achieved experimentally 54.9%, for the case with an extraction time of 17 h, feed to solvent ratio of 10 g/mL, pH of 10, and temperature of 60°C. The optimum conditions could be obtained using Design Expert software. To determine the optimum conditions, some solutions would be suggested by the software. The final

result of three proper suggestions for optimization of GA% is given in Table 4. The optimum conditions were selected among these suggestions, extraction time of 10.12 h, feed to solvent ratio of 10.71 g/ml, pH of 9.8, and temperature of 119.7°C. These remarkably high values for predicted and actual maximum yield approved the successful performance of the obtained method. In a similar study carried out by Mukhopadhyay and Panja [8], the maximum yield of extraction of GA from licorice was 13.6%. They used high-pressure hot water for extraction of GA at the temperature range of 30-120°C, extraction time of 60-120 min, and solvent to feed ratio of 2.0-80 mL/g. the optimum conditions for a time, temperature, and solvent ratio were found respectively 90 min, 110 °C, and 60 mL/g. Furthermore, the extraction could not be continued after 100 min and the solvent got saturated by the extract. In another similar study, Shabkhiz *et al.* [21] used superheated water as the solvent for isolation of GA and the maximum yield of extraction was achieved 54.76 mg/g under the temperature of 100°C, extraction time of 120 min and water flow rate of 15 mL/min.

#### CONCLUSIONS

The extraction of GA from licorice using water, as a solvent, was the main objective of the present study. In order to prepare statistical analysis, RSM using Box-Behnken was performed to model the experimental results and obtain a proper design for a limited set of experiments. Among different affecting parameters, four parameters were chosen as the most significant variables in increasing the yield of extraction (extraction time, the feed to solvent ratio, pH of extraction medium, and temperature). The effect of each variable on the yield was examined and the ANOVA results revealed that all the four variables significantly affect the yield of extraction. Additionally, the interaction between pH and extraction time played a significant role in increasing the extraction yield. According to the model results, the shorter extraction time and the higher pH values result in the higher GA extraction yield. The model achieved a quadratic correlation between significant experimental variables and the response, which was acceptably fitted by experimental results. The model results demonstrated that the yield of extraction of GA (GA%) increases with decreasing the extraction time, the ratio of the feed to

Table 4: The list of optimum conditions obtained from the model.

Solutions Number	A	B	C	D	R	Desirability	
1	10.12	10.71	9.80	119.70	59.0398	1.000	Selected
2	10.80	10.85	10.74	70.01	56.0143	1.000	
3	5.84	10.39	11.54	89.09	57.6827	1.000	

solvent, and temperature; but increasing the pH resulted in increasing the GA% in the precipitate. The results confirmed that the performed extraction method was successful in achieving GA with high extraction yields. The maximum GA% was obtained at conditions correspond to the extraction time of 17 h, feed to solvent ratio of 10 g/ml, pH of 10, and temperature of 60°C, in which the GA% was 54.9%. According to the model, the optimum conditions for extraction time, feed to solvent ratio, pH, and the temperature was 10.12 h, 10.71 g/mL, 9.8, and 119.7°C, respectively.

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