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OPTIMIZATION OF ULTRASONIC-ASSISTED EXTRACTION TOTAL FLAVONOIDS FROM *CORN COB* USING RESPONSE SURFACE METHOD

Xin Xie[1], Guoxin Zhao[2], Changbin Li[3*]

[1]Department of cooking and food, Henan polytechnic college, No.210, Pingan Road, Zhengzhou, Henan province, 450046, China;
[2] Institute of chemical industry and food, Zhongzhou University, No.18, Yingcai street, Zhengzhou, Henan province, 450044, China;
[3] College of Food Engineering, Henan University of Animal Husbandry and Economy, Zhengzhou, Henan, 450046,China
**656271158@qq.com*

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ABSTRACT
The aim of the study was to extract total flavonoids from Corn Cob through optimizing process assisted by ultrasonic. [Method] Based on single-factor experiments, ethanol concentration, extraction temperature, extraction time and ultrasonic power were set as independent variables and the yield of flavonoids was response value. Effects of each variable and their interactions on flavonoids were determined using Box-Behnken method. The quadratic polynomial regression equations between flavonoids and each variable were built using Design-Expert software. [Results] The results showed that the optimal conditions of total flavonoids from Corn Cob were set as follows: ethanol concentration of 70%, extraction temperature of 59℃, extraction time of 44 min and ultrasonic power of 55Hz, respectively. The yield of flavonoids was 3.719 ± 0.030mg/g (N=6) under the modified conditions. The relative error was 0.216% compared to predictive value, indicating the model fitted well with experimental data. In addition, the extraction yield was increased by 28.7% as compared to the traditional extraction method. [Conclusion] The results could provide a theoretical foundation and scientific basis for the development of natural antioxidant products of Corn Cob.

1. Introduction

Corn is one of the important traditional crops. The *corn* planting area is large and the output is high. After threshing *corn cob* accounted for 20%-30% of corn quality. Currently, *corn cob* is mainly used for the animal feed, making pulp and sugar. In addition to cellulose and starch, *Corn cob* is abundant in flavonoids (Jiaming et al., 2012). Nowadays more and more attention is cast on flavonoids by biochemical and nutritional researchers due to their various biological activities used in health-care food or medicine, especially antibacterial, antiviral and antioxidant effects (Zunlai et al., 2013). *Corn cob* is a type of underutilized natural resource**.** For the sake of making better use of the resource, more research is immediately required for efficient procedures of *Corn cob* extracts.

Response surface methodology (RSM), has been widely used to determine the optimal operating conditions. In this methodology, mathematical and statistical techniques are collected for designing experiments, building models, evaluating the effects of factors and searching optimum condition of factors for desirable responses (Box and Wilson, 1951). Box-Behnken (BBD) and central composite design (CCD) of the principal RSM have been widely used in various experiments. BBD, a spherical and revolving design, has been applied in optimization of chemical and physical processes because of its reasoning design and excellent outcomes (Ferreira et al., 2007; John and Borkowski, 1995; Yaqiang 2015). Ultrasonic-assisted extraction is widely applied in the extraction of food functional components because of its high efficient, time saving and simple operation (Lifen et al., 2011). In the present study, the total flavonoids content was considered as response value while ethanol concentration, extraction temperature, extraction time and ultrasonic power were considered for optimization parameters. Box-Behnken design, followed by canonical and ridge analyses, was employed to optimize the process parameters of total flavonoids extraction from the *corn cob*.

2. Materials and methods

2.1. Materials and instruments

Core cob was collected from Henan province (China). *Rutin* was purchased from the Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). Ethanol, Sodium nitrite**,** Aluminum nitrate and Sodium hydroxide were of analytical grade and purchased from Tianjin chemical reagent manufacturing co., LTD (Tianjin, China). Pure water was purchased from Hangzhou wahaha group co., LTD (Hangzhou, China). TU-1810 spectrophotometer (Persee Corporation, China) was used for total flavonoids analysis of samples. KX-1740QT Ultrasonic cleaner (Kexi Corporation, China) was used for ultrasonic-assisted extraction of total flavonoids from *Corn Cob*. TGL-158 centrifuge (Shanghai Anting Scientific Instrument Factory, China) was used for centrifugal separation.

2.2. Extraction of flavonoids

Dry corn cob was ground in a blender to obtain a fine powder (particle diameter: 0.2-0.3mm). Assisted with ultrasonic, Samples of 1 g were extracted by ethanol solvent in a designed ethanol concentration, temperature, extraction time, ultrasonic power and ratio of liquid to material. The extraction solution was separated from insoluble residue by centrifugation (2000rpm for 5 min), and then the supernatant fluid of 5 ml was sucked out for determination of flavonoids. The formula to calculate the yield of total flavonoids was given as:

$$\omega = \frac{m}{m_0} \qquad (1)$$

where ω-the yield of total flavonoids, mg/g; *m*-the quality of the total flavonoids in the sample, mg; m_0 - the quality of sample, g.

2.3. Determination of total flavonoids content

The content of total flavonoids was determined by the colorimetric method with some modification (Jianfu et al., 2014). The supernatant fluid of 5 ml was moved into the volumetric flask of 10 ml, mixed with 0.3mL of 5% (w/w) $NaNO_2$ for 5 min, and then 0.3mL of 10%的 $Al(NO_3)_3$ (w/w) was added and mixed. 6 min later, 2mL of 1mol/mL NaOH was added and diluted to 10mL. With 15 min standing, the absorbance of the solution was measured at 510 nm with TU-1810 spectrophotometer against the same mixture, without the sample as a blank. The calibration curve ($y = -0.0117+10.884x$, where y is absorbance value of sample, x is sample concentration) ranged 0.005-0.06 mg/mL (R^2=0.9998).

2.4. Experimental design and statistical analysis

The yield of total flavonoids was affected by numerous parameters. Because it was impossible to identify the effects of all parameters, it was necessary to select the parameters that had major effects. The total flavonoids content in *corn cob* was influenced main by ethanol concentration, ratio of liquid to material, extraction temperature, extraction time and ultrasonic power, so the five parameters were screened by single-factor experiment. Based on the preliminary results, the proper range for each factor was preliminarily determined, and a response surface methodology was conducted to design experimental project. As shown in Table 1, the four factors chosen for this study were designated as X_1, X_2, X_3, and X_4, and prescribed into three levels, coded +1, 0, –1 for high, intermediate and low value, respectively (Suresh et al., 2013).

Statistical analysis of the single-factor experimental data was performed with Microsoft Excel software. Design-Expert 8.0.6 was used for the experimental design and regression analysis of the experimental data. Student's t-test permitted the checking of the statistical significance of the regression coefficient, and Fischer’s F-test determined the second-order model equation at a probability (P) of 0.001, 0.01 or 0.05. The adequacy of the model was determination by evaluating the lack of fit, the coefficient of determination (R^2) and the F-test value obtained from the analysis of variance (ANOVA) that was generated.

Table 1. Factors and levels of response surface methodology

Factors	Coded symbols	Levels		
		-1	0	1
Ethanol concentration(%,v/v)	X_1	50	60	70
Extraction temperature(℃)	X_2	50	55	60
Extraction time(min)	X_3	40	45	50
Ultrasonic power(Hz)	X_4	50	60	70

3. Results and discussions

3.1. The effect of ethanol concentration on the total flavonoids yield

Ethanol concentration was an important parameter of the total flavonoids extraction (Guowen et al., 2010). Different concentrations of ethanol (40, 50, 60, 70, 80 %, V/V) were prepared when other experiments were set as follows: particle size 100 mesh, ratio of liquid to raw material 20:1(mL/g), extraction temperature 60℃, extraction time 1 h, and ultrasonic power 70 Hz. It could be seen from Figure 1a that different ethanol concentrations had important effects on yield of flavonoids. The extraction yield was the highest when 60% ethanol was used as extraction solvent, which was (3.63±0.16) mg/g. when the concentration of ethanol was less than 60%, the yields increased with the increased concentration of methanol. Then, the yields declined from 60 to 80% of ethanol. So 60% ethanol was selected as the center point for further response surface methodology (RSM) experiment.

3.2. The effect of the ratio of liquid to material on the total flavonoids yield

In order to study the effect of the ratio of liquid to material on the extraction performance, different ratio of liquid to material (10:1, 15:1, 20:1, 25:1, 30:1) were prepared when other experiments were set as follows: Particle size 100 mesh, ethanol concentration 60%, extraction temperature 60℃, extraction time 1 h, and ultrasonic power 70 Hz. The results were displayed in Figure1b. The yield increased greatly when the ratio increased from 10:1 to 20:1, and then it maintained a mild slope when the ratio of liquid to material increasing. If the ratio of liquid to material is too small, total flavonoids in raw material could not be completely extracted up. If the ratio of liquid to material is too big, this would cause high process cost. Therefore, suitable ratio of liquid to raw material should be selected for extraction of targeted flavonoids (Lei et al., 2013). Taking into account of cost, the ratio of liquid to material 20:1 was adopted in this work.

3.3. The effect of extraction temperature on the total flavonoids yield

The choice of the extraction temperature was another important step (Yu, 2014). Extraction temperature was not constant during the extraction stages. Here, it was, respectively, set at 30, 35, 40, 45, 50, 55, 60 and 65℃ to examine the influence of different temperature on the yield of the flavonoids extraction when other reaction conditions were as follows: particle size 100 mesh, ethanol concentration 60%, ratio of liquid to raw material 20:1(mL/g), extraction time 1 h, and ultrasonic power 70 Hz. Figure 1c indicated that the yield of total flavonoids rose gradually with the increase of temperature, and then reached the peak at 55℃, and finally dropped from 55 to 65℃. This phenomenon could be explained that solvent viscidity declined and movement of molecular accelerated with the increase of temperature, it was benefit for bioactive compounds to release from plant cells. However, much higher temperature promoted the degradation of some thermo-sensitive compounds. Therefore, the center point of extraction temperature was considered to be 55℃ in this experiment.

3.4. The effect of extraction time on the total flavonoids yield

Under the above optimal conditions of particle size 100 mesh, ethanol concentration 60%, ratio of liquid to raw material 20:1(mL/g), and ultrasonic power 70 Hz, effects of extraction time (20, 25, 30, 35, 40, 45, 50, 55, 60min) on the extraction yield of the flavonoids were tested. The results were displayed in Figure 1d. The yield of flavonoids was increased markedly with the increase of extraction time from 25min to 45min. Over 45min the yield lightly decreased. This might be due to the decomposition of active compounds during the prolonged extraction time. Therefore, the center point of extraction time chosen for RSM was 45min.

3.5. The effect of ultrasonic power on the total flavonoids yield

Ultrasonic power was also an important factor for extraction of active components from plant materials. It was associated with the final extraction efficiency, the energy cost and yield of total flavonoids (Qian et al., 2013). In this study, effect of different ultrasonic power (60, 70, 80, 90, 100, 120Hz) on the extracting yield was investigated. Six groups of samples were extracted with the optimal parameters obtained above. The results were displayed in Figure 1e. When the ultrasonic power was less than 60Hz, the yield of total flavonoids increased with the increased ultrasonic power. Then, the yields declined from 60Hz to 80Hz of ultrasonic power. This might be due to the much higher ultrasonic power promoted the decomposition of some structure- unstable compounds. Therefore, the 60Hz of ultrasonic power was adopted in this work.

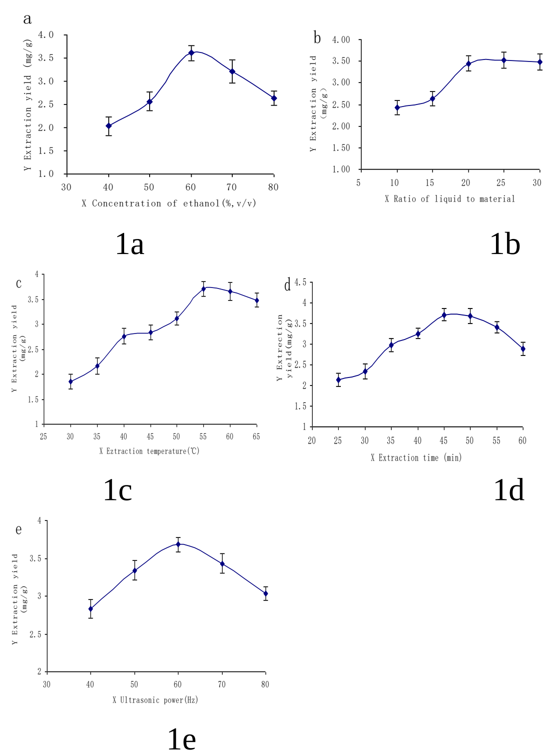

Figure 1. Effect of different extraction parameters (ethanol concentration, %, v/v; ratio of liquid to material; extraction temperature, ℃; extraction time, min; ultrasonic power, Hz) on yield of total flavonoids.

3.6. Optimization of the procedure

3.6.1. The model fitting and statistical analysis

The extraction of total flavonoids from *Corn Cob* was optimized through RSM approach. All 29 of the designed experiments were conducted for optimizing the four individual parameters in the current Box-Behnken design. Table 2 showed the experimental conditions and the results of total flavonoids yield according to the factorial design. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation: $Y = 3.64 + 0.13X_1 - 0.059X_2 - 0.007833X_3 - 0.11X_4 + 0.24 X_1 X_2 + 0.052X_1 X_3 - 0.008250X_1 X_4 - 0.13X_2 X_3 - 0.12X_2 X_4 + 0.016X_3 X_4 - 0.15X_1^2 - 0.17X_2^2 - 0.15X_3^2 - 0.22X_4^2$**,** where X_1, X_2, X_3 and X_4 were the coded values of ethanol concentration, extraction temperature, extraction time and ultrasonic power, respectively.

To determine whether or not the quadratic model was significant, the statistical significance of regression equation was checked by F-test, and ANOVE for response surface quadratic polynomial model were summarized in Table 3. The P-value was used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the P-values were, the bigger the significance of the corresponding coefficient. Here, the P-value of the model was smaller than 0.0001, which indicated the model was suitable for use in this experiment. The determination coefficient (R^2=0.9302) was close to 1, which indicated the satisfactory correlation between actual values and predicated ones. The Adj.R^2 value was 0.8605, which meat most variation (> 86.05%) of the total flavonoids content could be predicted by the models, which only 14% variation could not be explained by the model.

The lack-of-fit measured the failure of the model to represent the data in the experimental domain at points which were not included in the regression. The *F*-value of 1.37 and *P*-value of 0.4096 represented that the lack of fit was insignificant relative to the pure error. Insignificant lack of fit made the mode fit. Adequate precision compares the range of the predicted values at the design points to the average prediction error. A ratio greater than 4 indicated adequate model discrimination. In the present study, the value of 12.461 indicated an adequate signal. This model could be used to navigate the design space.

Table 2. Experiment design and result of response surface method analysis

NO.	X_1	X_2	X_3	X_4	**Extraction yield(mg/g)**
1	60	55	50	50	3.414
2	50	55	45	50	3.297
3	70	50	45	70	3.327
4	60	60	40	60	3.504
5	70	55	45	50	3.534
6	60	55	40	50	3.306
7	70	55	40	60	3.432
8	60	55	45	60	3.523
9	60	50	45	70	3.336
10	60	55	40	70	3.072
11	50	50	45	60	3.549
12	70	55	45	70	3.219
13	70	60	45	60	3.555
14	60	50	45	50	3.267
15	60	60	45	70	2.985
16	60	50	50	60	3.393
17	50	60	45	60	2.834
18	60	50	40	60	3.258
19	60	55	45	60	3.705
20	60	55	45	60	3.611
21	70	55	50	60	3.562
22	60	55	45	60	3.674
23	60	55	45	60	3.685
24	60	60	50	60	3.132
25	60	60	45	50	3.408
26	60	55	50	70	3.243
27	50	55	45	70	3.015
28	50	55	40	60	3.219
29	50	55	50	60	3.141

The regression coefficients and the corresponding *P*-values were also presented in Table 3. From the *P*-values of each model term, it could be concluded that the independent variables studied (X_1, X_2, X_4) and four quadratic term (X_1^2, X_2^2, X_3^2, X_4^2) significantly affected the total flavonoids yield. However, the analysis showed the interactions between two parameters (X_1X_2, X_2X_3, X_2X_4,) were in significant. The results of the study also represented that the ethanol concentration and the ultrasonic power were the most significant parameters which influenced total flavonoids yield followed by extraction temperature and time.

Table 3. Regression analysis results of extraction parameter of total flavonoids

Source	Sum of Squares	Degree freedom	Mean Square	F-Value	p-value (Prob>F)
Model	1.29	14	0.092	13.34	<0.0001
X_1	0.21	1	0.21	29.85	<0.0001
X_2	0.042	1	0.042	6.11	0.0269
X_3	0.0007363	1	0.0007363	0.11	0.7490
X_4	0.15	1	0.15	22.15	0.0003
$X_1 X_2$	0.22	1	0.22	32.14	<0.0001
$X_1 X_3$	0.011	1	0.011	1.56	0.2316
$X_1 X_4$	0.0002722	1	0.0002722	0.039	0.8456
$X_2 X_3$	0.064	1	0.064	9.29	0.0087
$X_2 X_4$	0.061	1	0.061	8.75	0.0104
$X_3 X_4$	0.0009922	1	0.0009922	0.14	0.7106
X_1^2	0.15	1	0.15	21.40	0.0004
X_2^2	0.18	1	0.18	26.48	0.0001
X_3^2	0.15	1	0.15	21.68	0.0004
X_4^2	0.33	1	0.33	47.29	<0.0001
Residual	0.097	14	0.006917		
Lack of fit	0.075	10	0.007490	1.37	0.4096
Pure error	0.022	4	0.005484		
Cor total	1.39	28			
R^2	0.9302				
Adj.R^2	0.8605				
Pred. R^2	0.6645				
Adequate precision	12.461				

Note: $P < 0.05$, difference significant;
$P < 0.01$ difference was extremely significant.

3.6.2. Analysis of response surface

Three-dimensional response surface plots and two-dimensional contour plots, as presented in Figure 2 and Figure 3, were very useful to see interaction effects of the factors on the responses. These types of plots showed effects of two factors on the response at a time. In all the presented figures, the other two factors were kept at level zero.

It could be seen from Figure 2a and Figure 3a that the effects of ethanol concentration and extraction temperature on the yield of flavonoids. The interaction effects between ethanol concentration and extraction temperature were very significant. The yield increased with the increased of ethanol concentration and extraction temperature when the two factors were kept at high level.

Figure 2b and Figure 3b represented the effects of ethanol concentration and extraction time on the yield of flavonoids. With an increase of extraction time, the yield increased when the extraction time was less than 44 min, but it decreased when the extraction time was more than 44 min. In addition, it could be seen that the yield changed earlier and greater in a higher level of ethanol concentration. As ethanol concentration was increased in the range from 50% to 55%, extraction time had little effect on the yield of flavonoids. However, when the ethanol concentration was increased in the range from 55% to 70%, the yield increased greatly with the increased extraction time.

According to Figure 2c and Figure 3c, as the ethanol concentration increased in the range from 50% to 65%, flavonoids yield increased. At low ethanol concentration levels, the curve did not level off, indicating that this concentration was well below optimum for flavonoids yield. There was a linear increase in the yield with increase in ultrasonic power from 50 Hz to 59 Hz, but beyond 59 Hz, the yield decreased with increasing ultrasonic power. In addition, the result was consistent with the preliminary experimental result and could determine the accurate value of the parameter.

The effects of extraction time and extraction temperature on the yield of flavonoids were shown in Figure 2d and Figure 3d. The yield mainly depended upon extraction temperature and resulted in a curvilinear increase until zero level 58℃, and then decreased in flavonoids yield. The effect of extraction time was less significant than the extraction temperature.

The effects of ultrasonic power and extraction temperature on the yield of flavonoids could be seen in Figure 2e and Figure 3e. It was obvious that the high yield of flavonoids could be achieved at a wide range of extraction temperature. However, the ultrasonic power should be strictly controlled. Increased ultrasonic power up to a threshold level led to increasing the yield. Beyond this level, the yield slightly decreased. The result was similar to other research findings (Chenxi et al., 2014; Javed et al., 2014).

Figure 2f and Figure 3f showed that extraction time exhibited a weaker effect whereas ultrasonic power represented a significant effect on the flavonoids yield. An increase in the yield of flavonoids could be significantly achieved with the increases of ultrasonic power, only at low levels of ultrasonic power. The yield of flavonoids decreased significantly at high levels of ultrasonic power.

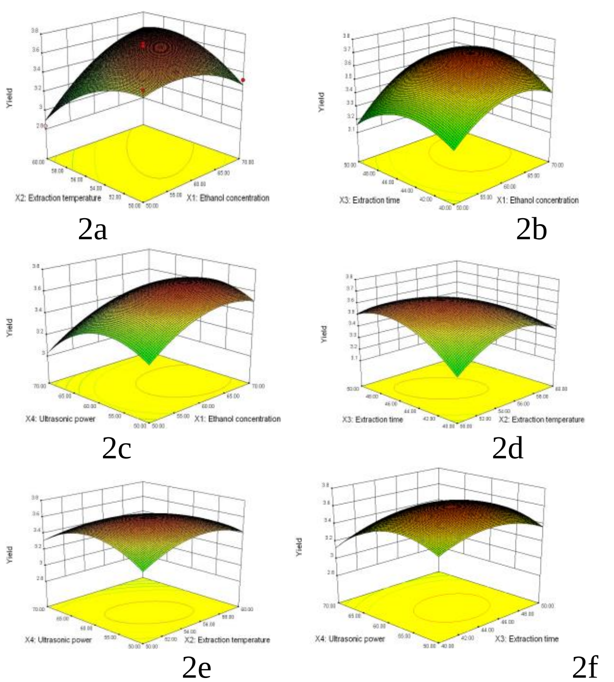

Figure 2. Response surface (3D) for the effect of different extraction parameters (X_1: ethanol concentration, %, V/V; X_2: extraction temperature,℃; X_3: extraction time, min; and X_4 ultrasonic power, Hz) added on the response *Y*.

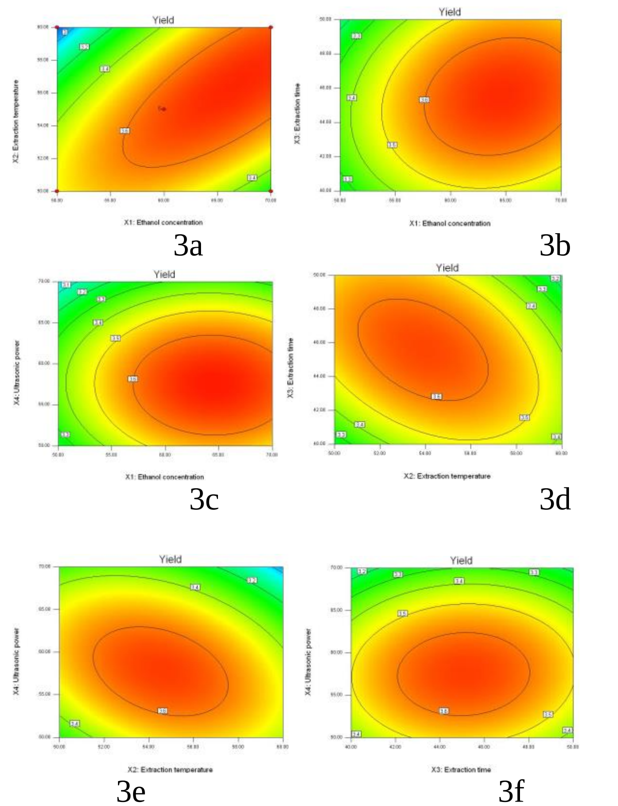

Figure 3. Contours (2D) for the effect of different extraction parameters (X_1: ethanol concentration, %, V/V; X_2: extraction temperature, ℃; X_3: extraction time, min; and X_4ultrasonic power, Hz) added on the response *Y*.

From what has been discussed above, the degree of influence on the extraction yield from big to small order is: ethanol concentration, ultrasonic power, extraction temperature and time.

3.6.3. Optimization of extracting parameters and validation of the model

In this study, the aim of optimization was to find the conditions which gave the maximum yield of flavonoids. The optimal values of the selected variables were obtained by solving the regression equation using Design-Expert 8.0.6 software. The optimum conditions for independent variables and the predicted values of the responses were also presented as follows: ethanol concentration of 70%, extraction temperature of 58.78℃, extraction time of 44.29 min and ultrasonic power of 55.19Hz, respectively. The predicted extraction yield was give by the Design-Expert 8.0.6 software under the above conditions was 3.711 mg/g.

Considering the operability in actual production, the optimal conditions could be modified as follows: ethanol concentration of 70%, extraction temperature of 59℃, extraction time of 45 min and ultrasonic power of 55Hz, respectively. Under the modified conditions, the experimental yield of flavonoids was 3.719 ± 0.030mg/g (N=6), the relative error was 0.216% compared to predictive value, indicating that the model was adequate for the extraction process (Table 4). In addition, the extraction yield was increased by 28.7% as compared to the traditional extraction method (Lixin et al., 2014).

4. Conclusions

The use of multivariate optimization was of paramount importance in order to select the optimal operating conditions of interrelated variables, avoid or minimize degradation and achieve the best yields in the extraction process. RSM proved to be fairly accurate in predictive modeling and optimization of conditions for the yield of flavonoids, and that the yield of flavonoids to be reasonably approximated by quadratic non-linearity. In addition, the yield of flavonoids under the optimized extraction conditions was great higher than that of the non-optimized condition. This process could be considered as a sustainable alternative for the industry since it allowed simplified handing and the quantity of targeted extracts to be improved.

Table 4. Predicted and experimental values of the responses at optimum conditions

Optimum conditions	Ethanol concentration(%)	70
	Extraction temperature(℃)	58.78
	Extraction time(min)	45
	Ultrasonic power(Hz)	55.19
Extraction yield (mg/g)	Experimental	3.719± 0.030
	Predicted	3.711

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