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Full Length Research Paper

Optimization of the extraction of flavonoids from clovers by response surface methodology (RSM)

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The extraction of flavonoids from clovers (*Trifolium*) was optimized to maximize flavonoid yield in this study. A central composite design of response surface methodology involving extracting time, liquid-solid ratio, extracting temperature, and ethanol concentration was used, and second-order model for flavonoid yield was employed to generate the response surfaces. The optimum condition for flavonoid yield was determined as follows: 24 min extracting time, 20 liquid-solid ratio, 80°C extracting temperature, and 72% ethanol concentration. Under the optimum condition, the flavonoid yield was 2.29%.

Key words: Clover, extraction, response surface methodology (RSM), flavonoid.

INTRODUCTION

Clover is a kind of wild plant, which is mainly distributed in China. Its leaves have been consumed as herbal medicine for hundreds years, which is reported to have many curative effects, such as reducing blood pressure, antibacterial, antitumor activities, etc (Ponce et al., 2004; Setchell and Cassidy, 1999; He et al., 1996). Flavonoids, abundant in fruits, teas, vegetables, and medicinal plants, have received the greatest attention and have been investigated extensively, since they are highly effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are suspected of being carcinogenic and causing liver damage (Pekkarinen et al., 1999; Engelman et al., 2002; Xie et al., 2001). In this study, a flavonoid-rich extract from leaves of clovers was prepared by different ethanol concentration extraction.

When many factors and interactions affect desired responses, response surface methodology (RSM) is an effective tool for optimizing the process. RSM uses an experimental design such as the central composite design (CCD) to fit a model by least squares technique. If the proposed model is adequate, as revealed by the diagnostic checking provided by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum (Rustom et al., 1991). The purpose of this work was to optimize the extraction of flavonoids from clovers by RSM.

MATERIALS AND METHODS

The clovers for this study were collected in Xinxiang. Rutin was from Sigma. Ethanol, $NaNO_2$, $Al(NO_3)_3$, and NaOH were of analytical grade and were used as received. One response was used: flavonoid yield, Y, defined as the ratio of flavonoids in the extract to total amount of raw material expressed as percentage. Each of the variables to be optimized was coded at 3 levels: -1, 0, and 1. Table 1 showed the variables, their symbols and levels. The selection of variable levels was based on our preliminary study.

A CCD, shown in Table 2, was arranged to allow for fitting of a second-order model. The CCD combined the vertices of a hypercube whose coordinates are given by the 2^n factorial design with the "star" points. The star points were added to the factorial design to provide the estimation of curvature of the model. Five replicates (run 7, 8, 11, 16, and 17) at the center of the design were used to allow the estimation of "pure error" sum of squares. Experiments were randomized in order to minimize the effects of unexplained variability in the observed response due to extraneous factors.

Extraction of flavonoids

According to the experiment design in Table 2, the clover (1 g)

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Table 1. Variables and their levels for central composite design.

Variable	Symbol	Coded variable level		
		-1	0	1
Extracting time (min)	X_1	10	20	30
Liquid-solid ratio	X_2	15	20	25
Extracting temperature (°C)	X_3	60	70	80
Ethanol concentration (%)	X_4	50	70	90

Table 2. Central composite design arrangement and results.

Experiment	Coded Levels				Y (%)
	X_1	X_2	X_3	X_4	
1	30	25	80	50	1.7
2	20	20	80	70	2.46
3	30	15	80	90	2.1
4	10	15	60	50	1.09
5	20	20	70	50	1.49
6	10	25	80	90	1.75
7	20	20	70	70	1.99
8	20	20	70	70	1.95
9	20	15	70	70	1.31
10	30	15	60	90	1.64
11	20	20	70	70	1.81
12	30	25	60	50	0.76
13	10	15	80	50	1.34
14	10	20	70	70	1.72
15	30	20	70	70	1.64
16	20	20	70	70	2.26
17	20	20	70	70	1.9
18	20	20	60	70	1.89
19	20	20	70	90	1.7
20	20	25	70	70	1.58
21	10	25	60	90	1.78

were extracted with different volumes of ethanol at different concentrations for different times and then filtered under vacuum. After being filtered, the supernatant was diluted to determine the content of flavonoids.

Determination of the content of flavonoids

The content of flavonoids was measured as rutin equivalents from a rutin standard curve (Liu and Zhu, 2007). One milliliter of the sample extract was transferred to a test tube, the solution was redissolved in 30% ethanol to 12.5 ml, and 0.7 ml of 5% $NaNO_2$ reagent was added. After an incubation period of 5 min, 0.7 ml of $Al(NO_3)_3$ was added, mixed well, and was kept for 6 min at room temperature, and then, 5 ml of 1 M NaOH reagent was added. The solution was redissolved in 30% ethanol to 25 ml .The aforementioned solution was incubated for 10 min, and then the absorbance was readed at 500 nm using a spectrophotometer.

Statistical analysis

A software package (Design Expert7.0) was used to fit the second-order models and generate response surface plots. The model proposed for the response (Y) was:

$$Y = b_0 + \sum_{n=1}^{4} b_n x_n + \sum_{n=1}^{4} b_{nn} x_n^2 + \sum_{n \neq m-1}^{4} b_{tm} x_n x_m$$

where b_0 is the value of the fitted response at the center point of the design, which is point (0, 0, 0), while b_n, b_{nn}, and b_{nm} are the linear, quadratic, and cross-product regression terms, respectively.

RESULTS AND DISCUSSION

Diagnostic checking of the fitted model

When many factors and interactions affect desired responses, RSM is an effective tool for optimizing the process. RSM uses an experimental design such as the CCD to fit a model by least squares technique. If the

Table 3. ANOVA for the fitted model.

Source	Sum of squares	d_f	Mean square	F value	Prob > F
Model	2.69	14	0.192	5.522	0.0226
X_1	0.003	1	0.003	0.092	0.7719
X_2	0.036	1	0.036	1.048	0.3454
X_3	0.479	1	0.479	13.79	0.0099
X_4	0.022	1	0.022	0.634	0.4562
X_1X_2	0.059	1	0.059	1.705	0.2395
X_1X_3	0.174	1	0.174	5.005	0.0666
X_1X_4	0.039	1	0.039	1.141	0.3264
X_2X_3	0.005	1	0.005	0.143	0.7176
X_2X_4	0.007	1	0.007	0.225	0.6517
X_3X_4	0.072	1	0.072	2.076	0.1997
X_1^2	0.051	1	0.051	1.479	0.2695
X_2^2	0.362	1	0.362	10.43	0.0179
X_3^2	0.318	1	0.318	9.149	0.0233
X_4^2	0.131	1	0.131	3.782	0.0998
Residual	0.208	6	0.034	-	-
Cor Total	2.897	20	-	-	-

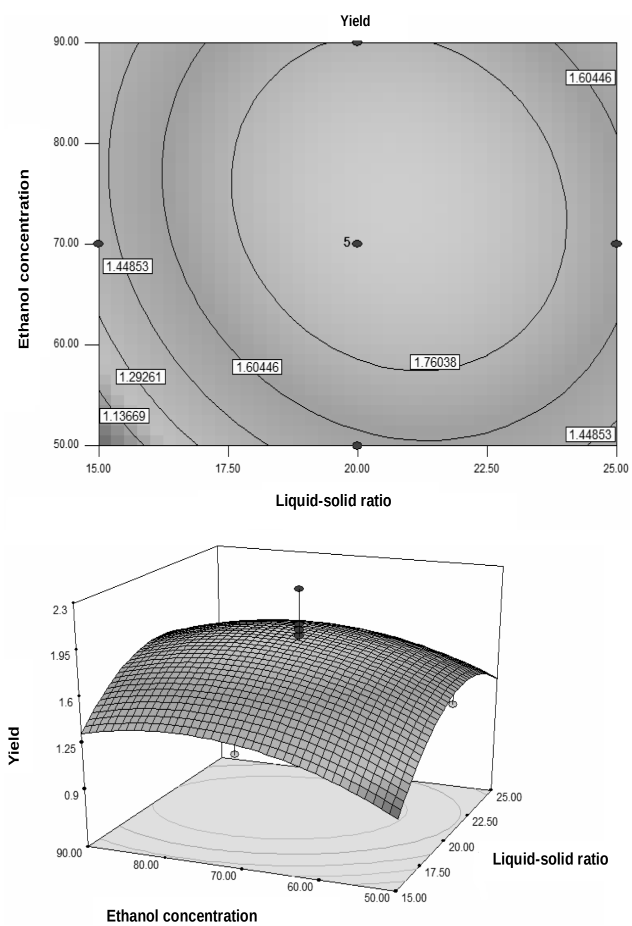

Figure 1. Effect of liquid-solid ratio and ethanol concentration on flavonoid yield. Extracting time = 20 min; Extracting temperature = 70°C.

proposed model is adequate, as revealed by the diagnostic checking provided by an ANOVA and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum.

ANOVA for the regression was performed to assess the “goodness of fit”. The model is as follow:

$$Y=+6.47253-0.028585\times X_1+0.72116\times X_2-0.47859\times X_3+0.11619\times X_4-0.00385\times X_1X_2+0.00148\times X_1X_3+0.00079\times X_1X_4+0.00050\times X_2X_3-0.00070\times X_2X_4-0.00048\times X_3X_4-0.00142\times X_1^2-0.015079\times X_2^2+0.00353\times X_3^2-0.00057\times X_4^2.$$

The result of ANOVA is shown in Table 3. The model F-value of 5.52 implies the model was significant. There was only a 2.26% chance that a “Model F-Value” this large could occur due to noise. Values of “Prob > F” less than 0.05 indicated model terms were significant. In this study, X_3, X_2^2, and X_3^2 were significant model terms.

Response surface plotting

The effect of liquid-solid ratio and ethanol concentration on flavonoid yield is as shown in Figure 1. Another two factors, extracting time and extracting temperature were set at 20 min and 70°C, respectively. As shown in the regression model, the flavonoid yield is depended upon liquid-solid ratio and ethanol concentration, which resulted in a curvilinear change.

Optimization of the extraction

The model is useful in indicating the direction in which to change variables in order to maximize the flavonoids content (Y). By using Design Expert 7.0 software, the optimum extraction conditions were determined as follows:

24 min extracting time, 20 liquid-solid ratio, 80°C extracting temperature, and 72% ethanol concentration could be recommended as a practical optimum. The estimated values for Y were 2.49%. A verification experiment at the optimum condition, consisting of 3 runs, was performed and the practical Y was 2.29%.

Conclusion

Optimum extraction of flavonoids from clover with water extraction could be achieved by 1 part of clover with 20 parts 72% ethanol concentration at 80°C for 24 min. Such conditions resulted in extraction of 2.29% flavonoids from clover.

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