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ORIGINAL ARTICLE

Optimization of Microwave-assisted Extraction of Silymarin from *Silybum marianum* Straws by Response Surface Methodology and Quantification by High-Performance Liquid Chromatograph Method

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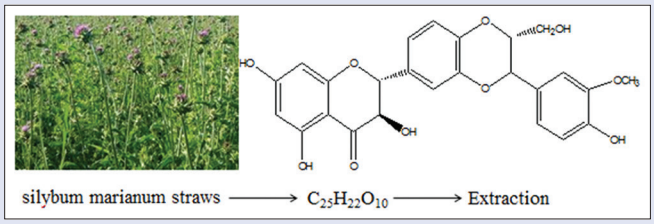
ABSTRACT

Background: *Silybum marianum*, a member of the Aster family, is a well-known Chinese herb and the source of a popular antioxidant that is extensively used in Asia. The abundant *S. marianum* straws are still underutilized and wastefully discarded to pollute the environment. **Objective:** To solve the above problem and better utilize *S. marianum* straws, the objective of this study was to optimize the conditions for extraction of silymarin from *S. marianum* straws. **Materials and Methods:** A combination of microwave-assisted extraction and response surface methodology (RSM) was used for silymarin from *S. marianum* straws and yield assessment by high-performance liquid chromatography method. The RSM was based on a five-level, four-variable central composite design (CCD). **Results:** The results indicated that the optimal conditions to obtain highest yields of silymarin were microwave power of 146 W, extraction time of 117 s, liquid-to-solid ratio of 16:1 mL/g, and ethanol concentration of 43% (v/v). Validation tests indicated that under the optimized conditions, the actual yield of silymarin was 6.83 ± 0.57 mg/g with relative standard deviation of 0.92% (n = 5), which was in good agreement with the predicted yield. **Conclusions:** The exploitation of the natural plant resources present very important impact for the economic development. The knowledge obtained from this work should be useful to further exploit and apply this material.

Key words: Microwave-assisted extraction, response surface methodology, *Silybum marianum*, silymarin

SUMMARY

- Silymarin has been isolated from *Silybum marianum* straws by microwave-assisted extraction and response surface methodology
- The results obtained are helpful for the full utilization of *S. marianum* straws
- The microwave-assisted extraction is a very useful method for the extraction of important phytochemicals from plant materials.



Abbreviations used: MAE: Microwave-assisted extraction, RSM: Response surface methodology, HPLC: High-performance liquid chromatography, CCD: Central composite design, ANOVA: Analysis of variance.

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INTRODUCTION

Silybum marianum is a member of the *S. marianum* (L.) Gaerth genus and the Aster family. Seeds of *S. marianum* (Shui Fei Ji in Chinese) are a famous medical herb. It is used for treating liver and gallbladder diseases.[1-3] The current research has focused on pharmacological efficacy and on component extraction processing of the seeds of *S. marianum* extracts.[4,5] Most of its hepatoprotective properties are attributed to the presence of silybin, which is the main constituent (60%–70%) of silymarin.[6,7] Silymarin is a complex mixture of polyphenolic molecules, including seven closely related flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin) and one flavonoid (taxifolin).[4,5] Recently, silymarin has been widely used in food, medicine, and health products.

As a new-type extraction technique, microwave-assisted extraction (MAE) has attracted interest as an alternative approach to the conventional extraction methods due to its unique heating mechanism, moderate cost, and good performance.[8] Later, MAE has been widely used in food, natural products, and traditional Chinese medicine extraction process.[8-11]

Response surface methodology (RSM) is an effective tool for optimizing the process.[12] With RSM, the number of experiments can be effectively reduced by a reasonable experimental design and multivariate quadratic regression equation to fit the function between factors and response. To date, RSM has been successfully applied to optimize complex processes used to extract compounds from plants.[13-15]

As an important traditional medicinal plant, *S. marianum* grows wild and is also being cultivated on large areas in some parts of the world for commercial production of silymarin complex.[16] Although

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there are bioactive and medicinal potentials in *S. marianum*, much attention had been paid to the silymarin extraction from *S. marianum* seeds. However, because of the lack of research on high value-added utilization of *S. marianum* straws, this abundant resource is discarded as useless residue after harvesting. Although some portion of these straws is consumed as animal feed, the majority of the processing wastes are thrown out. That is not only an environmental pollution but also a waste of bioresource. Therefore, the development of integrative utilization and high added-value products from *S. marianum* straws could benefit the rapid and sustainable development of *S. marianum* industry and present an additional source of income for farmers in the Chinese countryside.[17] To our knowledge, the extraction of silymarin from *S. marianum* straws with MAE method has not yet been reported. To solve above problem and better utilize *S. marianum* straws, MAE technology was used to extract silymarin from *S. marianum* straws and to optimize the extraction process. Central composite design (CCD) combined with RSM was applied to fit and exploit a mathematical model representing the relationship between the response (microwave power, extraction time, liquid-to-solid ratio, and ethanol concentration) and variables (silymarin yield). The results should be helpful in the further utilization of silymarin from *S. marianum* straws.

MATERIALS AND METHODS

Plant material

The samples of *S. marianum* straws were collected in Sunwu, Heihe, China. The plants were identified by Zhang Haifeng, and a voucher #151125 of the specimen was deposited at Tonghua Normal College. The content of silymarin from *S. marianum* straws was not <3.50 mg/g by high-performance liquid chromatography (HPLC) method. The obtained *S. marianum* straws were dried, ground, and then passed through the sieve screen. The powder obtained from the 20 and 40 mesh sieve screens was subjected to MAE extraction.

Chemicals

Silybin used as reference standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol was purchased from Tedia Company Incorporated (Ohio, USA). Ultrapure water was purified by a Milli-Q water purification system (Bedford, MA, USA). All reagents used in the study were of analytical grade.

Extraction procedure

MAE was carried out in a CW-2000 microwave preparation system (Xintuo Microwave Decomposition and Testing Technology Co. Ltd., Shanghai, China). *S. marianum* straw powder (10 g) was accurately weighted and placed into the extraction vessel in addition to a suitable amount of extraction solvent and subjected to set microwave power and extraction times for predefined irradiation time for two cycles. At the end of extraction, the extracts were allowed to cool to room temperature. Subsequently, the extract was filtered and the filtrate was collected for HPLC analysis.

Experimental design and statistical analysis

Specifically, data from the CCD were utilized to determine the optimum combination of variables. A fractional 5-level, 4-factor experimental design with three replicates at the center point was used to find effects of independent variables on the dependent variables. In the study, independent variables include microwave power (x_1), extraction time (x_2), liquid-to-solid ratio (x_3), and ethanol concentration (x_4) for *S. marianum* straws. Each factor was coded at five levels (−1.682, −1, 0, 1, and 1.682). The RSM experimental design is summarized in Table 1. The complete experimental design consisted of 30 points, including six replicates of the center point, were randomized to satisfy the statistical requirement of independence of observations, as shown in Table 2. A second-order polynomial regression model was used to express the yield as a function of the independent variables as follows:

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

Where y represents the response variables, β_0 is a constant, β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively, and x_i and x_j represent the coded independent variables. The adequacy of the model was determined by evaluating the lack of fit, coefficient of determination (R^2), and the Fisher test value (F-value) obtained from the analysis of variance (ANOVA) generated by the software Design-Expert version 7.0.(Stat-Ease Inc., Minneapolis, MN, USA). Three-dimensional (3D) response surface plots were generated by keeping two responses variable at its optimal level and plotting that against two factors (independent variables). Statistical significance was considered at $P < 0.05$.

Table 1 shows the code and levels of factors chosen for the experiments.

High-performance liquid chromatography analysis of extracts

Silybin was analyzed by a Shimadzu LC-2010 HT HPLC system (Shimadzu Corp., Kyoto, Japan) coupled with a UV detector. A Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm) was used. The mobile phase consisted of methanol and 1% acetic acid in water (48:52, v/v) at a flow rate of 1.0 mL/min.[18] The wavelength of detection was 287 nm, column temperature was 25°C, and injection volume was 10 μL.

RESULTS AND DISCUSSION

Extraction model and statistical analysis

The design matrix of the variables in coded units is given in Table 2 along with the predicted and experimental values of response. The silymarin yield ranged from 3.98 mg/g to 7.02 mg/g. 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:

$$Y = 6.73 + 0.44x_1 + 0.28x_2 - 0.084x_3 + 0.065x_4 - 0.2x_1x_2 + 0.058x_1x_3 - 0.29x_1x_4 + 0.12x_2x_3 - 0.019x_2x_4 + 0.025x_3x_4 - 0.22x_1^2 - 0.29x_2^2 - 0.19x_3^2 - 0.25x_4^2$$

Table 2 shows the CCD matrix four variables with experimental values of silymarin yield.

The significance of each coefficient was determined using the F-test and P values [Table 3]. It can be seen that the variables with the largest effect were the linear terms of microwave power (x_1), extraction time (x_2), and the quadratic term of microwave power (x_1^2), extraction time (x_2^2), liquid-to-solid ratio (x_3^2), and ethanol concentration (x_4^2), followed by the interaction effects of microwave power and extraction time (x_1x_2), microwave power and ethanol concentration (x_1x_4), and extraction time and liquid-to-solid ratio (x_2x_3). The results suggest that the change of microwave power and extraction time had highly significant effects on the yield of silymarin ($P < 0.0001$) from *S. marianum* straws.

ANOVA procedure was used to analyze the model for significance and suitability, and a statistical summary is given in Table 4. Values of probability (P) > F < 0.05 indicate model terms are significant. Values >0.10 indicate the model terms are not significant. The ANOVA showed that the model was highly significant ($P < 0.0001$) with F of 24.42. The value of 1.26 for lack of fit implied that it was not significant relative to the pure error. Nonsignificant lack of fit is good and indicates

Table 1: Code and levels of factors chosen for the experiments

Independent variable	Symbol		Levels				
	Uncoded	Coded	−1.682	−1	0	1	1.682
Microwave power (w)	X_1	x_1	100	140	200	260	300
Extraction time (s)	X_2	$x2$	30	54	90	126	150
Liquid-to-solid ratio (mL/g)	X_3	x_3	10:1	14:1	20:1	26:1	30:1
Ethanol concentration (V/V, %)	X_4	x_4	30	38.1	50	61.9	70

Table 2: Central composite design matrix four variables with experimental values of silymarin yield

Run	Coded variable levels				Silymarin yield (mg/g)	
	X_1	X_2	X_3	X_4	Observed (Y_1)	Predicted (Y_2)
1	−1	−1	−1	−1	4.71	4.767
2	1	−1	−1	−1	6.65	6.51
3	−1	1	−1	−1	5.55	5.527
4	1	1	−1	−1	6.51	6.465
5	−1	−1	1	−1	3.98	4.198
6	1	−1	1	−1	6.25	6.172
7	−1	1	1	−1	5.74	5.428
8	1	1	1	−1	6.41	6.597
9	−1	−1	−1	1	5.61	5.457
10	1	−1	−1	1	5.81	6.055
11	−1	1	−1	1	6.13	6.142
12	1	1	−1	1	6.12	5.935
13	−1	−1	1	1	5.01	4.988
14	1	−1	1	1	5.76	5.817
15	−1	1	1	1	5.97	6.143
16	1	1	1	1	6.29	6.167
17	−1.682	0	0	0	4.94	4.948
18	1.682	0	0	0	6.69	6.715
19	0	−1.682	0	0	5.12	5.012
20	0	1.682	0	0	5.98	6.122
21	0	0	−1.682	0	6.02	6.12
22	0	0	1.682	0	5.85	5.783
23	0	0	0	−1.682	5.56	5.612
24	0	0	0	1.682	5.89	5.872
25	0	0	0	0	6.6	6.732
26	0	0	0	0	6.48	6.732
27	0	0	0	0	7.02	6.732
28	0	0	0	0	6.79	6.732
29	0	0	0	0	6.78	6.732
30	0	0	0	0	6.72	6.732

Table 3: Estimated regression model of relationship between response variables (silymarin yield) and independent variables (x_1, x_2, x_3, x_4)

Variables	Sum of square	df	Mean square	*F*	*P* > *F*
x_1	4.68	1	4.68	117.51	<0.0001*
x_2	1.85	1	1.85	46.39	<0.0001*
x_3	0.17	1	0.17	4.27	0.0566
x_4	0.1	1	0.1	2.55	0.1315
x_1x_2	0.65	1	0.65	16.26	0.0011
x_1x_3	0.053	1	0.053	1.33	0.2672
x_1x_4	1.31	1	1.31	32.91	<0.0001*
x_2x_3	0.22	1	0.22	5.54	0.0326
x_2x_4	0.0056	1	0.0056	0.14	0.7124
x_3x_4	0.01	1	0.01	0.25	0.6237
x_1^2	1.39	1	1.39	34.85	<0.0001*
x_2^2	2.33	1	2.33	58.4	<0.0001*
x_3^2	1.04	1	1.04	26.18	0.0001
x_4^2	1.68	1	1.68	42.17	<0.0001*

*Values of "Prob.> F"<0.0001

that the model equation was adequate for predicting the silymarin yield under any combination of values of the variables. The determination of coefficient (R^2) of the model was 0.958, which indicated a relatively high degree of correlation between the observed and predicted values. The predicted R^2 of 0.8094 pointed to a good agreement between the experimental and predicted values for silymarin. The predicted R^2 of 0.8094 is also in reasonable agreement with the adjusted R^2 of 0.9187. An adequate precision of 17.949 for silymarin indicated an adequate signal. This model can be used to navigate the design space.

Optimization of the procedure by response surface methodology

Equation 1 allowed the prediction of the effects of the four factors on the silymarin yield. Four independent response surface plots are shown in Figure 1a-f. Two variables within the experimental rang were depicted in 3D surface plots while the other variable was kept constant at zero level. As shown in Figure 1, the increased microwave power (x_1), extraction time (x_2), liquid-to-solid ratio (x_3), and ethanol concentration (x_4) up to a threshold level led to increased silymarin yield. Beyond this level, the silymarin yield slightly decreased, which indicated that a greater yield could be achieved if the moderate microwave power (x_1), extraction time (x_2), liquid-to-solid ratio (x_3), and ethanol concentration (x_4) were selected. Therefore, it could be concluded that the optimal conditions for MAE of silymarin yield from S. *marianum* straws were a microwave power of 146 W, extraction time of 117 s, liquid-to-solid ratio of 16:1 mL/g, and ethanol concentration of 43% (v/v).

Validation of the model

Triplicates verification experiment was carried out under these conditions to validate the adequacy of the model. Under the optimal conditions, the maximum yield of predicted value was 6.97 mg/g. A mean value of 6.83 ± 0.57 mg/g with relative standard deviation of 0.92% (n = 5), obtained from actual experiments. The good agreement between the predicted and experimental results verified the validity of the model and also indirected that RSM was a powerful tool for searching the optimal values of the individual variables and the maximum response value.

CONCLUSIONS

In this work, an efficient MAE process has been developed for the extraction of silymarin from *S. marianum* straws. CCD was successfully employed to optimize the extraction parameters. The best conditions were shown to be microwave power of 146 W, extraction time of 117 s, liquid-to-solid ratio of 16:1 mL/g, and ethanol concentration of 43% (v/v). The maximum silymarin yield was 6.83 ± 0.57 mg/g (n = 5) under these optimal conditions. This study can be useful for the development of industrial extraction of silymarin from *S. marianum* straws, including further studies concerning the optimal number of sequential steps to enhance the efficacy of a potential large-scale extraction system. With all these merits, MAE should be considered for wider application in the extraction and purification of phytochemicals from plants. It was found that RMS could be used to optimize MAE process.

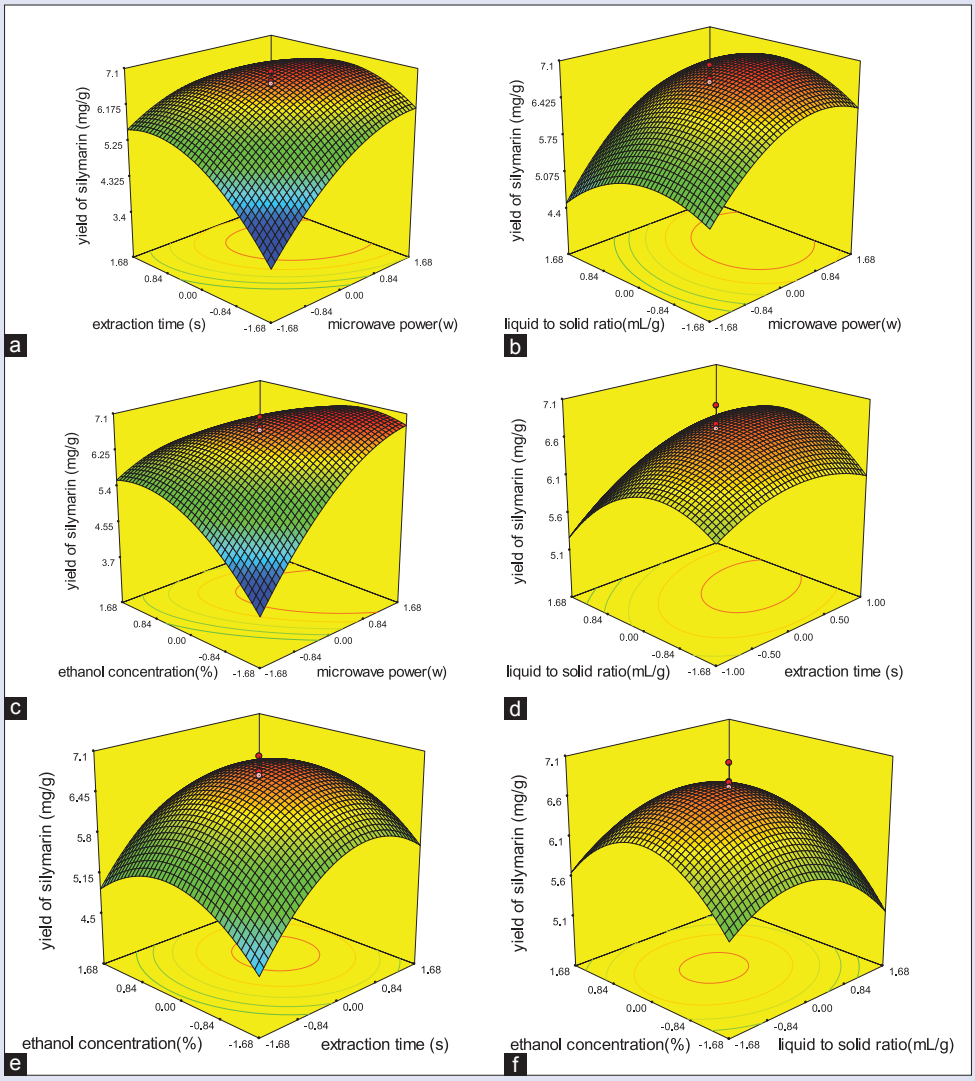

Figure 1: Response surface plots for the effects of (a) microwave power and extraction time; (b) microwave power and liquid-to-solid ratio; (c) microwave power and ethanol concentration; (d) extraction time and liquid-to-solid ratio; (e) extraction time and ethanol concentration; (f) liquid-to-solid ratio and ethanol concentration on the silymarin yield

Table 4: Variance analysis of the second-order regression model on silymarin yield

Source	Sum of square	df	Mean square	*F*	*P* > *F*
Model	113.62	14	0.97	24.42	<0.0001
Residual	0.60	15	0.04		
Lack of fit	0.43	10	0.043	1.26	0.4216
Pure error	0.17	5	0.034	0.00023	
Cor total	14.22	29			
R^2	0.958		Predicted-R^2	0.8094	
Adjusted-R^2	0.9187		Adequate precision	17.949	

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Conflicts of interest

There are no conflicts of interest.

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