Optimization of microwave-assisted extraction of silymarin from milk thistle seeds

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Abstract: Microwave-assisted extraction (MAE) technology was used to extract silymarin from milk thistle seeds. The single factor experiments and quadratic orthogonal rotation regression combination design were adopted to study the effects of such factors as extraction time, extraction temperature, ethanol concentration and solid-liquid ratio on silymarin yield. Ultraviolet-Visible (UV-Vis) spectrophotometry method was employed to measure the silybin content. The optimal technological conditions for extracting silymarin from milk thistle seeds by MAE were determined, and then the extraction regression mathematical model was established. The importance orders of factors that influenced the extraction yield within the experimental model were as follows: ethanol concentration, the ratio of solid to liquid, extraction temperature and extraction time. The optimum extraction parameters were obtained as follows: extraction time 50 min, extraction temperature 130℃, ethanol concentration 85% (V/V) , solid to liquid ratio 1∶40(g/mL), and under such conditions, experimental value of silymarin yield after being extracted three times is 59.33mg/g. The validation experiments verify that the established regression mathematical model can predict the extraction yield of silymarin accurately.

Keywords: microwave-assisted extraction, silymarin yield, optimum extraction parameters

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1 Introduction

Flavonoids are naturally bioactive substances with various pharmacological actions and therapeutic applications. Flavonoid antioxidants have been tested in clinical trials to treat different types of cancer in the USA[1] and Europe[2]. Silymarin, a flavonolignan from milk thistle (*Silybum marianum* L.) seeds, is used for traditional herbal and dietary supplement around the world including the USA for its strong antihepatotoxic activity (against almost every kind of human liver disease). It is a mixture, consisting chiefly of silybin, silydianin and silychristin. Silybin is a major biologically active compound of silymarin[3]. In view of these facts, production of silymarin has considerable importance with high potential commercial interests.

Extraction is the first step for the recovery and purification of bioactive phytochemicals from plant materials. The conventional methods used to extract the effective constituents from natural herb include reflux extraction (RE), Soxhlet extraction (SE), supercritical fluid extraction (SFE) and so on. Recently, microwave-assisted extraction (MAE) has been widely recognized as a simple, effective, and versatile extraction method. Extraction using microwave can result in a yield increase in shorter time at the same temperature using less solvent. Researches have been done for the extraction of biological compounds, such as extraction of lycopene from tomatoes[4], camptothecin from *Nothapodytes foetida*[5], flavonoids from *Radix Scutellariae*[6] , total phenolic of Longan peel[7] and Flaxseed [8].

RE has been employed to recover flavonoids from milk thistle seeds in some factories. However, microwave-assisted extraction of flavonoids from milk thistle seeds has not been reported yet. The objectives of the current study were to check the performance of MAE device for the extraction of flavonoids from milk thistle seeds and to determine the optimum extracting process parameters.

2 Materials and methods

2.1 Plant material and reagents

Milk thistle seeds were supplied by Jiayin countryside (Heilongjiang, China). Initial moisture content of milk thistle seed is 8.25%, which is average value by repeating three times(GB/5497-85). Moisture content of milk thistle

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seed after dried for 12h by vacuum freeze-drier is below 1%. Milk thistle seeds were milled in a household electrical grinder (SJ260C, Lanpu Electrical Equipment Factory, Guangdong, China) to fine powder. The material powder was passed through a 40 mesh sieve, and then they were treated with successive washes of petroleum ether (60−90℃) to remove the lipids. The residue was given an airing at room temperature (20−25℃) to remove the petroleum ether. Dried powder obtained was kept in an airtight desiccator and used in all experiments.

The standard sample of silybin was purchased from the Beijing Institute of Biologic Products (Beijing, China). Methanol, ethanol and petroleum ether (60−90℃) were of analytical grade（Tianjin Chemical Factory, China). Distilled water was purchased from a local distilled water company (Heilongjiang, China).

2.2 Equipment

Extractions were performed using an Advanced Microwave Digestion System (Ethos1, Milestone Inc., USA) and operated via a compact terminal touch screen display with Milestone Easy-Control software. A maximal power of 1600W was delivered to a chamber with a rotating sample tray of 10 Teflon vials of 100mL. One of the vials was used as reference vessel for monitoring temperature and pressure. Silymarin yield was determined by UV-Vis spectrophotometry (Cary50, Varian Inc, USA) with a variable wavelength UV detector. The solvents were removed from the extracts by using a rotavapour (RE-52AA, Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China). Water of milk thistle seeds was removed from the extracts by using a vacuum freeze-drier (Pudong Instrument Co. Ltd, Shanghai, China).

2.3 MAE procedures

A typical MAE sequence consists of loading the material in an extraction vial, filling the vial with extraction solvent according to ratio of solid to liquid, heating the cell to a preset temperature for five minutes followed by a static extraction process under microwave irradiation and cooling the samples(10 min). Some controlled factors, in terms of microwave power, extraction time, extraction temperature, ethanol concentration, ratio of solid to liquid were studied.

2.4 Experimental design

The response value was expressed as silymarin yield obtained by MAE, which is the ratio of the weight of the dry and defatted sample. A full second-order polynomial model of the design was given, which was used to evaluate the silymarin yield (response variable, *Y*) as a function of independent variables (*X*), namely extraction time (X_1), extraction temperature (X_2), ethanol concentration (X_3) and ratio of solid to liquid (X_4) and their significant interactions.

2.4.1 Experiment of flavonoids stability

The 20 mg silymarin extracts at room temperature were filled in the vial with 30 mL of 95% ethanol (V/V). The microwave power and extraction time were selected as 500 W and one hour, respectively. Stability of flavonoids was studied at 70, 90, 110, 130 and 150℃.

2.4.2 Effect of microwave power on extraction yield

The effect of microwave power on extraction yield was studied with two gram material and 30 mL of 95% ethanol at different microwave powers. The extraction temperature and time were selected as 110℃ and one hour, respectively.

2.4.3 Single factor experiment design

The two gram material was used at different extraction conditions (as shown in Table 1) except for the ratio of solid to liquid experiment with one gram material. And every extraction process was repeated three times.

Table 1 Factors and results of single factor experiment

Time（X_1）/min	Temperature（X_2）/℃	Ethanol concentration（X_3）/%(V/V)	Ratio of solid to liquid（X_4）/g • mL^{-1}
40	70	60	1:10
50	90	70	1:20
60	110	80	1:30
70	130	90	1:40
80	150	100	1:50

2.4.4 Quadratic orthogonal rotation regression combination design

For each factor, the experimental range was determined based on the results of single factor experimental. The four independent variables were coded according to the following equation. (1):

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad i = 1,2,3,4 \tag{1}$$

Where x_i and i are the dimensionless and the actual value of the independent variable *is* X_i, X_0 is the actual value of the independent variable i at the central point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value. The factors and their levels are shown in Table 2.

The behavior of system can be described by the following second-order polynomial equation. (2):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \tag{2}$$

Where Y is the response variable, β_0 is the constant

coefficient, β_i are the linear coefficients, β_{ii} are the quadratic coefficients, β_{ij} are the interaction coefficients, and x_i and x_j are the coded values of independent variables. The adequacy of proposed model is revealed by the diagnostic checking provided by an analysis of variance (ANOVA). MATLAB software was used to generate the response surfaces. Finally, the optimal conditions were obtained in DPS data processing system. The practical yield was obtained under the optimal conditions. The experimental and predicted yields of silymarin were compared in order to determine the validity of the model.

Table 2 Factors and levels of quadratic orthogonal rotation regression combination design

Levels	Factors			
	Time (X_1) /min	Temperature (X_2)/℃	Ethanol Concentration (X_3)/%	Ratio of solid to liquid (X_4)/g • mL^{-1}
-2	50	90	70	1:20
-1	55	100	75	1:25
0	60	110	80	1:30
1	65	120	85	1:35
2	70	130	90	1:40

2.5 Determination of silymarin yield by UV-Vis spectrophotometry

Silybin standard solution was prepared in methanol solvent. The concentration of the standard solution was limited within the range from 0.004 to 0.024 mg/mL. By considering the relationship between UV maximum absorbance and silybin concentration, a linear response range was found. The absorbance was measured at 287nm by UV-Vis spectrophotometry. A correlation equation (Eq. (3)) between the maximum UV absorbance (*A)* at 287nm and the mass fraction of silybin in methanol (*C*) was obtained.

$$C = 0.0232A - 0.0004 \quad \left(R^2 = 0.9998\right) \qquad (3)$$

Silymarin yield in the sample was calculated as silybin equivalents by using the standard curve. Before the determination of silymarin in the sample, the concentration of sample should be adjusted to be within the linear response range of the UV absorption. So firstly, extracts of MAE were diluted to 100 mL, and then 1 mL of them was diluted to 50mL to obtain the suitable absorbance. To eliminate the solvent effect on absorbance, the blank was filled with the identical solvent in the sample cell. In the end, response variable (*Y*) can be calculated.

$$Y = (0.0232A - 0.0004) * V * \frac{1}{m} \qquad (4)$$

Where Y is the response variable (silymarin yield in the sample), mg/g; A is the absorbance; V is the total volume of methanol solvent , mL; m is the mass of the dry and defatted milk thistle seeds powder , g.

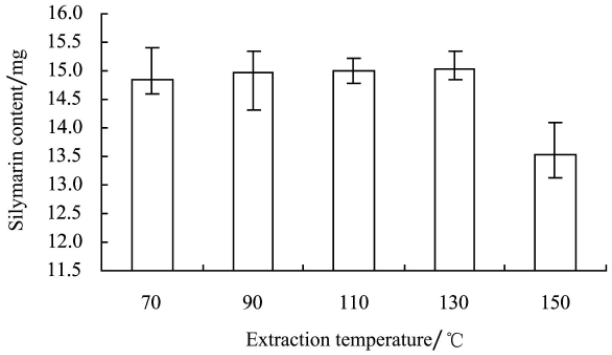
2.6 Statistical analysis

The analysis of variance (ANOVA) was carried out based on the experimental data using a DPS data processing system. A second-order polynomial was used to fit the data and obtain the regression equations. The statistical significance of the variables used in the regression equations was also examined.

3 Results and discussion

3.1 Stability of flavonoids at different extraction temperatures

As shown in Figure 1, the standard deviations of 0.8015, 0.5683, 0.2146, 0.2631, 0.5057 for the silymarin content were obtained under the extraction temperatures of 70, 90, 110, 130, 150℃, respectively. No significant difference at $\alpha = 0.01$ level was found at 70−130℃. Therefore, silymarin was not decomposed at 130℃. The investigation result of Cacace & Mazza (2003) matches with this experimental result [9].



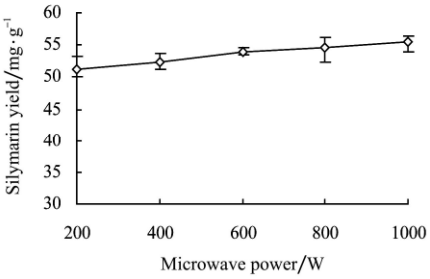
Fixed parameter level:
Extraction time: 60 min
Microwave power: 500 W
Ethanol concentration: 95%(V/V)
Ratio of solid to liquid: 1:30 g • mL^{-1}

Figure 1 Stability of flavonoids during extraction at different temperatures

3.2 Effect of microwave power on silymarin yield

In the Figure 2, the statistical analysis results showed that the standard deviations of silymarin yield were 0.0158, 0.0108, 0.0060, 0.0170, 0.0107 under microwave output powers of 200, 400, 600, 800, 1000 W, respectively. The microwave power showed light effect on the silymarin yield at $\alpha = 0.01$ level (Figure 2). So microwave power was selected according to requirement of equipment. A power setting of 500W was used for up to

three samples and 1000 W for more than three samples.



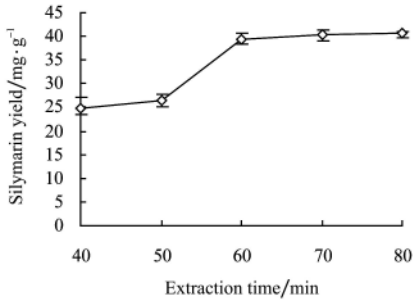
Fixed parameter level:
Extraction time: 60 min
Extraction temperature: 110 ℃
Ethanol concentration: 95%(V/V)
Ratio of solid to liquid: 1:30 g • mL^{-1}

Figure 2 Effect of microwave power on silymarin yield

3.3 Single factor experiment result

3.3.1 Effect of extraction time on silymarin yield

Extraction time was an important parameter in extraction procedure. To evaluate the robustness of MAE method, experiments were performed at 60℃ using 95% ethanol as extraction solvent for 40, 50, 60, 70 and 80 min. The power and solvent volume were selected at 500 W and 30mL, respectively. At the significant level α=0.01, the extraction yield of silymarin increased with the increase of extraction time up to a maximum and then presented a nearly constant (shown in Figure 3). The results indicated that 60 min was selected in the latter experiments.

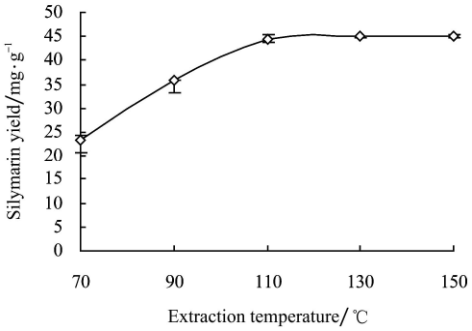


Fixed parameter level:
Extraction temperature: 60 ℃
Ethanol concentration: 95%(V/V)
Ratio of solid to liquid: 1:30 g • mL^{-1}
Microwave power: 500 W

Figure 3 Effect of extraction time on silymarin yield

3.3.2 Effect of extraction temperature on silymarin yield

The effect of extraction temperature on silymarin yield was studied at 70, 90, 110, 130 and 150℃ using 30mL of 95% ethanol as the extraction solvent. The result was shown in Fig.4. At the significant level α=0.01, an increase in the extraction yield was obtained with the increase of extraction temperature until 110℃. Elevated temperature may increase flavonoids solubility, accelerate diffusion rate, increase mass transfer and extraction rate and reduce solvent viscosity and surface tension [10]. The extraction yield of silymarin presented a nearly platform above 110℃. So 110℃ was selected as extraction temperature in the latter experiments.

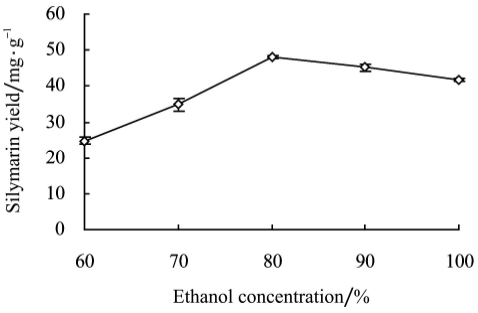


Fixed parameter level:
Extraction time: 60 min
Ethanol concentration: 95%(V/V)
Ratio of solid to liquid: 1:30 g • mL^{-1}
Microwave power: 500 W

Figure 4 Effect of extraction temperature on silymarin yield

3.3.3 Effect of ethanol concentration on silymarin yield

Ethanol was used as the extraction solvent of silymarin depending on preliminary experimental results. The result was shown in Figure 5. At the significant level α=0.01, extraction yields were increased with the increase of extraction solvent ratio of ethanol to water until 80%(V/V), and then decreased with further increase in ethanol concentration. Similarly, Wettasinghe and Shahidi (1999) found that the antioxidant activity in the borage meal increased with ethanol concentration up to a maximum of about 60% and then started to decline with the increase of extraction solvent concentration[11]. Therefore, ethanol with 80% (V/V) concentration was used as the extraction solvent for further experiments.

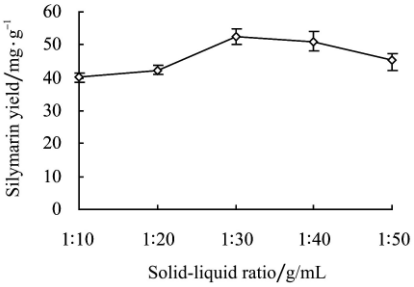


Fixed parameter level:
Extraction time: 60 min
Extraction temperature: 110 ℃
Ratio of solid to liquid: g • mL^{-1}
Microwave power: 500 W

Figure 5 Effect of ethanol concentration on silymarin yield

3.3.4 Effect of ratio of solid to liquid on silymarin yield

As shown in Figure 6, the ratio of solid to liquid also had a significant effect on extraction yield ($p<0.01$). The extraction yield increased unitl the ratio of solid to liquid was 1:30, then decreased with the ratio of solid to liquid. However, in conventional methods, an increase of the ratio of solid to liquid will lead to a higher extraction yield[12, 13].



Fixed parameter level:
Extraction time: 60 min
Extraction temperature: 110 ℃
Ethanol concentration: 80%(V/V)
Microwave power: 500W

Figure 6 Effect of solid-liquid ratio on silymarin yield

3.4 Optimization of MAE of silymarin from milk thistle seeds

Silymarin is composed of a large number of flavonoligans. The major component of silymarin is silybin (70%−80%). So silybin yield was determined as silymarin yield.

Table 3 Experimental design and results

Numbers	Factors				Silymarin yield Y/mg • g^{-1}
	x_1	x_2	x_3	x_4	
1	1	1	1	1	51.41
2	1	1	1	-1	44.81
3	1	1	-1	1	52.12
4	1	1	-1	-1	52.66
5	1	-1	1	1	50.88
6	1	-1	1	-1	46.48
7	1	-1	-1	1	56.14
8	1	-1	-1	-1	50.80
9	-1	1	1	1	54.59
10	-1	1	1	-1	48.55
11	-1	1	-1	1	51.36
12	-1	1	-1	-1	50.94
13	-1	-1	1	1	47.66
14	-1	-1	1	-1	41.93
15	-1	-1	-1	1	51.88
16	-1	-1	-1	-1	49.56
17	-2	0	0	0	50.94
18	2	0	0	0	52.71
19	0	-2	0	0	50.67
20	*0*	2	-2	0	52.23
21	0	0	2	0	48.34
22	0	0	0	0	43.45
23	0	0	0	-2	49.73
24	0	0	0	2	51.87
25	0	0	0	0	51.80
26	0	0	0	0	51.57
27	0	0	0	0	51.67
28	0	0	0	0	51.43
29	0	0	0	0	51.73
30	0	0	0	0	51.63
31	0	0	0	0	53.30
32	0	0	0	0	52.80
33	0	0	0	0	52.66
34	0	0	0	0	52.29
35	0	0	0	0	52.06
36	0	0	0	0	51.60

Table 4 Variance analysis of experimental results

Parameter	Sum of Squares	DF	Mean square	F Value	p-value
X_1	6.3829	1	6.3829	3.9054	0.0614
X_2	8.4413	1	8.4413	5.1649	0.0337
X_3	63.1848	1	63.1848	38.6604	0.0001
X_4	49.8229	1	49.8229	30.4847	0.0001
X_1^2	0.0403	1	0.0403	0.0246	0.8767
X_2^2	0.5307	1	0.5307	0.3247	0.5749
X_3^2	73.6318	1	73.6318	45.0525	0.0001
X_4^2	2.7039	1	2.7039	1.6544	0.2124
X_1X_2	19.5754	1	19.5754	11.9775	0.0023
X_1X_3	3.1809	1	3.1809	1.9463	0.1776
X_1X_4	0.0999	1	0.0999	0.0611	0.8071
X_2X_3	11.7697	1	11.7697	7.2014	0.0139
X_2X_4	1.7359	1	1.7359	1.0621	0.3145
X_3X_4	14.4840	1	14.4840	8.8622	0.0072
regression	255.5843	14	18.2560	F_2=11.17016	0.0001
residual	34.3215	21	1.6344		
fit	30.4676	10	3.0468	F_1=8.69616	0.0001
error	3.8539	11	0.3504		
total sum	289.9058	35			

Based on analysis result, the predictive equation (Eq. (5)) was obtained:

$$Y = 52.04343 + 0.51571\,x_1 + 0.59306\,x_2 - 1.62256\,x_3 + 1.44082\,x_4 - 0.03548\,x_1^2 - 0.12877\,x_2^2 - 1.51690\,x_3^2 - 0.29068\,x_4^2 - 1.10610\,x_1x_2 - 0.44588\,x_1x_3 + 0.07903\,x_1x_4 + 0.85768\,x_2x_3 - 0.32938\,x_2x_4 + 0.95145\,x_3x_4 \quad (5)$$

3.4.1 Test of the regression mathematical model

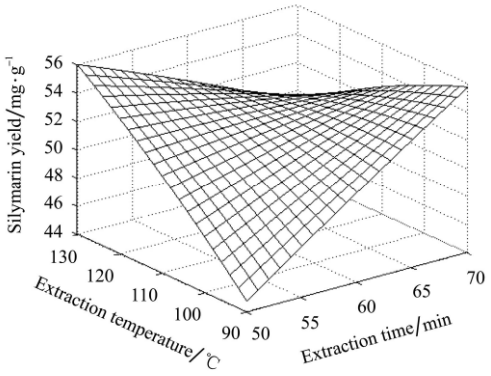
An analysis of variance (ANOVA) was carried out in order to test the model significance and suitability. Thus, various statistical data were given in ANOVA (Table 3). In table 3, F_1=8.69616>($F_{0.05}$(10,11)=2.85), the result showed that difference of fit was very significant;

F_2=11.17016＞($F_{0.01}$（14，21）=3.07), the results showed that the regression equation was very significant. The predictive equation (Eq. (6)) was acquired with the parameters that were not significant（p＞0.01）from the regression equation.

$$Y = 52.04343 + 0.51571x_1 + 0.59306x_2 - 1.62256x_3 + 1.44082x_4 - 1.51690x_3^2 - 1.10610x_1x_2 + 0.85768x_2x_3 + 0.95145x_3x_4 \quad (6)$$

3.4.2 Analysis of interaction between factors

Three dimensional response surfaces were used to show the changes of silymarin yield under different MAE conditions (Figs.7, 8 and 9) at α=0.05 level. Three-dimensional response surface plots were made with the vertical axes representing silymarin yield and each of the two horizontal axes representing two independent variables. For every plot, the other factors not represented by the two horizontal axes were fixed at zero levels.

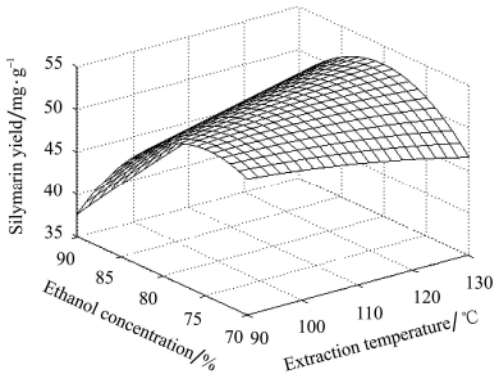


Fixed parameter level:
Ethanol concentration: 80% (V/V)
Ratio of solid to liquid: 1:30 g • mL^{-1}

Figure 7 Interaction of extraction time and temperature on silymarin yield

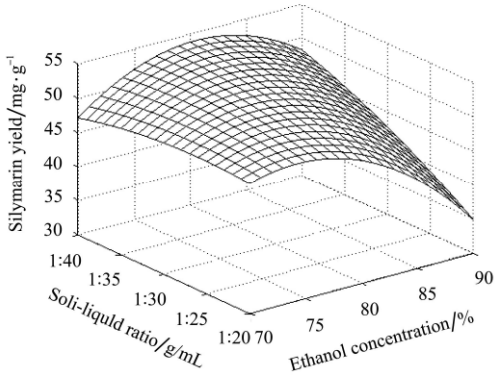
The result (Table 4) showed that extraction time did not have a significant effect on extraction yield (p＞0.05). The result was in agreement with reports that extraction time had no significant effect on the total ethanolic extraction yield of ginseng components when using MAE [14, 15]. However, there were significant interaction effects between extraction time and temperature on extraction yield（p＜0.01）in Figure 7. An increase of extraction yield was obtained with the increase of extraction temperature (below 130℃) when extraction time was less than 60 min.

A response surface plot for the interaction effect of extraction temperature and ethanol concentration was shown in Figure 8. Extraction temperature was found to be a significant factor affecting extraction yield (p＜0.05). The extraction yield of silymarin increased with the increase of ethanol concentration up to a maximum and then decreased at the same temperature.



Fixed parameter level:
Extraction time: 60 min
Ratio of solid to liquid: 1:30 g • mL^{-1}

Figure 8 Interaction of extraction temperature and ethanol concentration on silymarin yield



Fixed parameter level:
Extraction time: 60 min
Extraction time: 110℃

Figure 9 Interaction of ethanol concentration and ratio of solid to liquid on silymarin yield

The results showed that ethanol concentration had the most significant effect on extraction yield (p<0.01). A response surface plot for the effect of ethanol concentration and ratio of solid to liquid on extraction yield was shown in Figure 9. It was also observed that the interaction between ethanol concentration and ratio of solid to liquid was significantly related to extraction yield (p ＜ 0.01). The change of silymarin yield with the solid-liquid ratio and ethanol concentration in Figure 9 were the same as the change trend in Figure 5 and Figure 6.

2.4.3 Importance order of influence factors

Known from variance analysis of experimental results (Table 4): Fx_1=3.9054, Fx_2=5.1649, Fx_3=38.6604, Fx_4= 30.4847. Therefore, the importance order of factors that influence the extraction yield within the experimental

model was as follows: ethanol concentration, ratio of solid to liquid, extraction temperature, extraction time.

3.4.4 Optimization of extraction parameters

The combination of optimum extraction parameters was obtained by solving the Eq. (6) with DPS software (V7.05). The results were shown in Table 5.

Table 5 Combination of factors for the highest extraction yield

x_1	x_2	x_3	x_4	Y/mg · g^{-1}
-2	2	1	2	59.98

The optimum conditions predicted the highest extraction yield at extraction time 50 min, temperature 130℃, ethanol concentration 85%(V/V), and ratio of solid to liquid 1∶40 (g/mL), respectively. And under such conditions, average experimental value of silymarin yield after extracted three times was 59.33 mg/g. Predictive value of silymarin yield was 59.98 mg/g. The experimental data showed a good fit with the second -order polynomial equations.

4 Conclusions

An adequate quadratic polynomial model for predicting the values of silymarin yield was determined according to the optimization designs. Four independent variables involved in the model were ethanol concentration, ratio of solid to liquid, extraction temperature and extraction time. The *F*-test and *p* value indicated that the ethanol concentration had the most significant effect on the silymarin yield, and followed by the ratio of solid to liquid, extraction temperature and extraction time. And the optimal extraction conditions were obtained. Finally, MAE of silymarin from milk thistle seeds can be an alternative technique for the time- and energy-consuming reflux extraction in the factory.

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