Separation and Purification Technology 70 (2009) 34–40

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

# Application of response surface methodology to optimize microwave-assisted extraction of silymarin from milk thistle seeds

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ARTICLE INFO

*Article history:*
Received 6 August 2008
Received in revised form 10 August 2009
Accepted 15 August 2009

*Keywords:*
Microwave-assisted extraction
Silymarin yield
Milk thistle seeds
Response surface methodology

ABSTRACT

Milk thistle (*Silybum marianum* L.) is an excellent source of silymarin used as an antioxidant. Microwave-assisted extraction (MAE) was employed to extract silymarin from milk thistle seeds. The effects of four independents variables in terms of extraction time, temperature, ethanol concentration, and solid–liquid ratio on the silymarin yield were determined and the optimal conditions for silymarin were evaluated by means of response surface methodology. Correlation analysis of the mathematical regression model indicated that a quadratic polynomial model could be employed to characterize MAE process for the silymarin. Response surface plots showed that these independent variables, except for extraction time, and interactions significantly influenced the extraction yield of silymarin. The optimal extraction parameters to obtain the highest silymarin yield were time duration of 60 min, temperature of 112 °C, ethanol concentration of 81.5% (*v*/*v*), and a solid–liquid ratio of 1:38 (g/mL). The average experimental silymarin yield under the optimum conditions was found to be 56.67 ± 1.36 mg/g, which agree with the predicted value of 57.40 mg/g. MAE method was applied successfully to extract silymarin from milk thistle seeds.


## 1. Introduction

Milk thistle (*Silybum marianum* L.) is a well-known Chinese herb and the source of a popular antioxidant that is extensively used in Asia. Milk thistle seed extracts contain silymarin complex, including silybin and other flavanolignans (isosilybin, silychristin, silydianin, etc) [1], structures molecular as shown in Fig. 1, and it is used for multiple medicinal purposes, due to its various physiological characteristics. Research has confirmed that silymarin extracted from milk thistle seeds can protect healthy liver cells from deterioration, helping cleansing and detoxification, as well as contributing to regeneration of damaged cells [2–4]. Silybin, with a molecular formula of $C_{25}H_{22}O_{10}$ and a molecular weight of 482, is the most active component in the silymarin group.

Reflux extraction (RE), Soxhlet extraction (SE), and supercritical fluid extraction (SFE) treatments have classically been used for the extraction of botanical materials. However, these extraction processes are associated with long extraction times and high temperatures. Therefore, it is desirable to develop a new extraction process to improve upon the inherent limitations of conventional approaches [5]. Microwave-assisted extraction (MAE) was used for the extraction of biologically active compounds from different materials [6,7]. Recent research studies have shown the development of MAE methods for the extraction of biological compounds, such as the extraction of flavonoids from Radix Herba [8] and Epimedii [9], plant phenolic compounds [10], bioactive compounds in Gastrodia elata Blume, and active pharmaceutical ingredients from solid dosage forms [11,12].

Compared with conventional methods, MAE has many merits with shorter time, less solvent, higher extraction rate, and superior products quality at lower cost. The mechanism of MAE is based on the impact of microwaves on molecules by ionic conduction and dipole rotation inside target materials. Ionic conduction is the electrophoretic migration of ions caused by an electromagnetic field applied. And solution will be heated due to the resistance friction of the solution to the ions flow. Dipole rotation leads to the realignment of polar molecules under an electromagnetic field applied. At the commercial frequency 2.45 GHz, the dipoles align randomize $4.9 \times 10^9$ times per second, which results in quickly heating. Thus, the microwaves heat the solvent or solvent mixture directly, and interact directly with the free water molecules presents inside the materials, resulting in a rapid buildup of pressure within cells, and a pressure-driven enhanced mass transfer of target compounds out of the source material, which causes rupture of the plant tissue and release of the active compounds into the organic solvent [13,14]. A preliminary research showed also that the transfer rate of silymarin from milk thistle seeds increased with the microwave output power level and temperature during MAE processing, which could

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doi:10.1016/j.seppur.2009.08.008

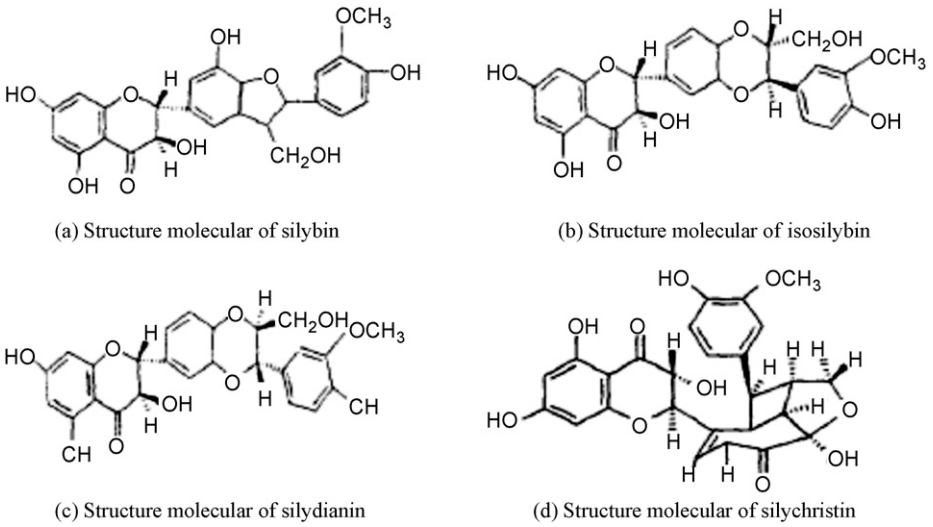


**Fig. 1.** The structures molecular of the main flavones in milk thistle seed: (a) structure molecular of silybin; (b) structure molecular of isosilybin; (c) structure molecular of silydianin; (d) structure molecular of silychristin.

be attributed mainly to the subsequent micro-structure change resulting from superheating effects during MAE [15]. Therefore, MAE has the potential to be an alternative to conventional extraction methods, especially in the case of plant material extraction. However, limited information has been published on the use of microwave technology for the extraction of silymarin from milk thistle seeds. The objective of this study was to employ response surface methodology to study the effects of extraction time, temperature, ethanol concentration, and solid–liquid ratio on silymarin yield, and to determine the optimum parameters to maximize silymarin yield.

## 2. Materials and methods

### 2.1. Preparation of sample and chemicals

Milk thistle seeds were collected from the Jiayin region, Heilongjiang Province, China. The raw material was dried in a vacuum freeze dryer (GLZY-B, Shanghai Pudong freeze dryer equipment Co., Ltd., China) for 12 h, and was then ground using a grinder (SJ260C, from Lanpu Electrical Equipment Factory, Guangdong, China) to a fine powder and sieved with a 40-meshs. A deracination treatment was performed successively that the raw sample powder was immersed in 50 mL petroleum ether over a temperature range of 60–90 °C for duration of 30 min to remove the lipids. The residue was filtered followed dried at room temperature (20–25 °C) to evaporate the remaining petroleum ether. The dried sample powder was stored in airtight desiccators as the practical samples. A standard for silybin (isomer) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Chemical reagents were used as these: HPLC-grade acetic acid, methanol (Fisher Scientific, Pittsburgh, PA, USA) and analytical-grade ethanol, and petroleum ether (Tianjin Chemical Reagent Factory, China).

### 2.2. Equipment

MAE experiments were performed using an Ethos-1 Advanced multimode microwave extraction system (EAMS, Milestone Inc., USA) with maximum output power 1600 W, schematic diagram as shown in Fig. 2. The rotating sample fixed frame inside the extraction system chamber holds 10 Teflon vials, each with a volume of 100 mL. The Ethos-1 instrument has an internal temperature control system with a fiber temperature probe and a pressure sensor inserted into a designated vial, which monitors the temperature and the pressure, respectively inside the vessel as reference value. In all of the experiments, the pressure was set under 200 kPa to prevent the dissolution of the target compound. The EAMS digestion system embodying the Milestone Easy-Control software is operated via a compact terminal touch screen to set microwave power settings and extraction times for the extraction process.

Silymarin yield was determined by an UV–vis spectrophotometer (Cary 50, Varian Inc., USA) with a wavelength of 287 nm. Gas chromatography (Agilent 6890 GC with autosampler and capillary GC column using IP innowax 30 mm × 0.25 mm × 0.25 μm, Agilent Technologies, USA) was used to accurately measure the silybin content in silymarin. The extracting liquids were removed from the extracts by using a rotavapour (RE-52AA, Shanghai Yarong Biochemistry Instrument Factory, China).

### 2.3. Microwave-assisted extraction procedures

One gram of dried sample powders was dissolved in 20 mL of ethanol solution (volume concentration: 85%). Then the dissolved solution was transferred equally into 10 vessels fixing the extraction system. All 10 vessels were closed to avoid volatilization losses during the extraction process and were placed in the sample tray. The extraction process was performed at impendent variables as

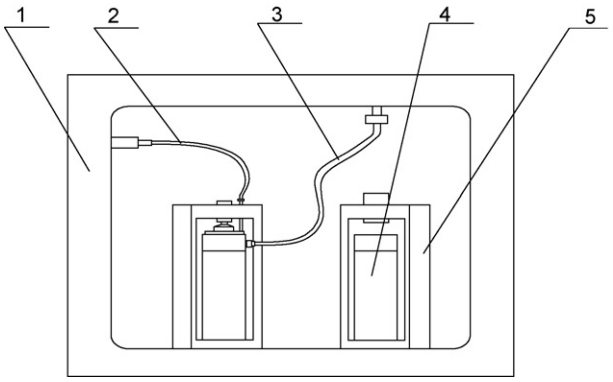


**Fig. 2.** Schematic microwave cavity with layout of reactor and sensor: (1) cavity inner wall; (2) temperature sensor; (3) pressure sensor; (4) Teflon vial; (5) container fixing-frame.

**Table 1**
Coded and un-coded levels of the four variables used in MAE of silymarin.

| Factor | Symbols | | Levels[a] | | | | |
|---|---|---|---|---|---|---|---|
| | | | −2 | −1 | 0 | 1 | 2 |
| Time (min) | $x_1$ | $X_1$ | 50 | 55 | 60 | 65 | 70 |
| Temperature (°C) | $x_2$ | $X_2$ | 90 | 100 | 110 | 120 | 130 |
| Ethanol concentration (%) | $x_3$ | $X_3$ | 70 | 75 | 80 | 85 | 90 |
| Solid–liquid ratio (g/mL) | $x_4$ | $X_4$ | 1:20 | 1:25 | 1:30 | 1:35 | 1:40 |

[a] $x_1 = (X_1 - 60)/5$; $x_2 = (X_2 - 110)/10$; $x_3 = (X_3 - 80)/5$; $x_4 = (X_4 - 30)/5$.

shown in Table 1. Then the vessels were allowed to cool at room temperature until the temperature was lower than 45 °C. The vessels were taken out and washed successively twice using ethanol solution to obtain the extracted mixtures. The mixtures were dried by evaporating the liquid using a rotary evaporator at 65 °C under vacuum pressure of 70 kPa to get the dehydrated materials. These materials were dissolved followed in methyl alcohol to a constant volume of 100 mL to get the stock solutions. One milliliter of stock solution was taken and diluted to 50 mL in a conical flask. The absorbencies of the diluents were measured at 287 nm wavelengths to determine the yield of silymarin in extracts. Each experiment was replicated thrice.

### 2.4. Preparation of calibration standard solutions and calibration of standard curve

A comparison was conducted between gas chromatography (GC) and UV–vis spectrophotometry in a preliminary experiment (three replications) to measure the silybin content in silymarin [15]. The results showed that the total flavonoid content in the silymarin sample appeared to be 55.3 ± 0.4%, and 54.0 ± 0.3% for the two detection systems, respectively. Thus, the relative detection rate of UV–vis spectrophotometer was 97.6% of that reported by GC method. Considering the experimental operation convenience and lower cost, the UV–vis spectrophotometer was selected to measure the mass fraction of silybin in silymarin.

Twenty milligrams of the dried silybin standard sample was immersed in moderate methanol solvent in a round bottom flask. Then the solution was placed into a volumetric flask. After the solute was dissolved in the volumetric flask in a warm water bath, the mixtures were diluted to 50 mL by adding methanol, and thoroughly mixed. Thus, the stock solution with a silybin concentration of 0.4 mg/mL was prepared. Then 0.5–5.0 mL (intervals: 0.5 mL) of standard stock solution containing 0.4 mg/mL silybin was pipetted into 10 flasks, respectively. Methanol solution was added into each flask to a constant 50 mL volume. Thus, 10 diluted silybin solutions with concentration ($C$) ranging from 4 to 40 mg/L were obtained.

An UV–vis spectrophotometer set at a wavelength of 287 nm was used to measure the absorbance of each diluted silybin solution. Five replicate assays were performed for each silybin at each concentration level. The mean absorbance value ($A$) deviation was obtained corresponding each concentration of silybin in methanol over the range given above at 4 mg/L steps was as follows: 0.1901, 0.3622, 0.5241, 0.7064, 0.8783, 1.0504, 1.225, 1.3963, 1.5681, and 1.7417, respectively. A blank test was prepared by substituting distilled water for the silybin solution. A standard curve for the UV absorbance ($A$) at maximum wavelength and the concentration of silybin in methanol ($C$) was developed as following the linear regression equation:

$$C = 0.0232A - 0.0004 \quad (1)$$

The statistical indices of Eq. (1), including recovery, linearity, and standard deviation (SD), and the coefficient of variation (CV), were calculated as these: 98.5%, 0.9998, ±0.0001 mg/mL, and 0.8%, respectively. Therefore, high reproducibility exists in Eq. (1), which can be used to determine the silybin content in the aqueous extract of milk thistle seeds with high reliability. Two milliliters of silybin standard sample solutions were subjected to the above procedure, and silybin content of samples calculated by the linear regression equation from standard curves. All analyses were carried out in triplicate to minimize errors.

### 2.5. Determination of silymarin yield by UV–vis spectrophotometry

The content of silybin is higher than 80% ($v/v$) in the silymarin of milk thistle seeds; the silymarin yield in the sample was calculated as silybin equivalents by using the standard curve [15]. For the determination of silymarin yield in the sample using Eq. (1), the concentration of the extract liquid was adjusted within the linear response range of the UV absorption. Aqueous extracts of MAE were diluted to 100 mL, and then 1 mL of the diluent was diluted again to 50 mL achieving a suitable absorbance range. The silymarin yield (mg/g) was calculated using the following equation:

$$Y = (0.0232A - 0.0004) \times V \times \frac{1}{m} \quad (2)$$

where $Y$ is the silymarin yield in the sample (mg/g), $A$ is the absorbance, $V$ is the total volume of aqueous extract (mL), and $m$ is the mass of the dry and defatted milk thistle seed powder (g).

### 2.6. Experimental design

The basic principle behind response surface methodology (RSM) analysis is to relate the observed value (dependent variables) to process parameters (independent variables) using statistical methods, yielding a multivariate regression equation, often of second-order. RSM takes interactions into consideration and optimizes the process parameters to reasonable range, with the advantage of less the number of replicates and the total time required to perform the experiments [16]. Therefore, it is an effective method to optimize the conditions for silymarin extraction from milk thistle seeds. A central composite rotatable design (CCRD) [17] was used in this study. The design consists of a four-factored ($n = 4$) factorial design with five levels. The single factor experimental data from preliminary studies became the guiding factors for establishing the range to be used for the factors in the experiments. In the present study, the ranges of experimental parameters were selected, based on preliminary trials. The matrix for the CCRD optimization experiment is summarized in Table 2. The CCRD has 16 experimental points (run nos. 1–16) and ($2n$) eight star points with an axial distance of 2 ($\alpha = 2n/4$) (run nos. 17–24), replicated eight times at the central point of the design (run nos. 25–32) to control for experimental error.

The response values were expressed as silymarin yield obtained by MAE relative to the weight of the dry defatted sample. A full second-order polynomial model of the design was used to evaluate the extraction yield as the response variable ($Y$), as a function of the four independent variables ($X_i$), namely extraction time ($X_1$), extraction temperature ($X_2$), ethanol concentration ($X_3$), and solid–liquid ratio ($X_4$), and their interactions. The ranges for the variables are shown in Table 1. Finally, the level values ($x_i$) of the independent variables were obtained through solving for the prediction equation by least square regression methods.

The four independent variables were coded according to the following Eq. (3):

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

where $x_i$ and $X_i$ are the dimensionless and the actual value of the independent variable $i$, respectively, $X_0$ is the actual value of the

**Table 2**
Experimental designs using CCED and results.

| Experiments | Coded levels | | | | Silymarin yield $Y$ (mg/g) |
|---|---|---|---|---|---|
| | $x_1$ | $x_2$ | $x_3$ | $x_4$ | |
| 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 | 0 | 0 | 52.23 |
| 21 | 0 | 0 | −2 | 0 | 48.34 |
| 22 | 0 | 0 | 2 | 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 |

independent variable $i$ at the central point, and $\Delta X_i$ is the step change of $X_i$ corresponding to a unit variation of the dimensionless value.

The behavior of the system can be described by the following second-order polynomial Eq. (4):

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

where $Y$ is the response, $\beta_0$ is the constant coefficient, $\beta_i$s are the linear coefficients, $\beta_{ii}$s are the quadratic coefficients, $\beta_{ij}$s are the interaction coefficients, and $x_i$ and $x_j$ are the coded values of the independent variables.

### 2.7. Data processing

Numerical and graphical optimization was carried out for the process parameters for microwave-assisted extraction to obtain the highest yield of silymarin. To perform this operation, the Design Expert software program (V6.0.4) (Stat-Ease, Inc., Minneapolis, MN, USA) was utilized to analyze the statistical characteristics of the data and to develop a regression equation between process variables in terms of extraction time, extraction temperature, ethanol concentration, and solid–liquid ratio, and silymarin yield. According to the experimental data, the fitting model represented by Eq. (4) was constructed and the statistical significance of the model terms was examined by regression analysis and analysis of variance (ANOVA). The reliabilities of the models were determined by model analysis, lack of fit tests, and $R^2$ (coefficient of determination) analysis. The $R^2$ is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit. The coefficient of variation (CV) indicates the relative dispersion of the experimental points from the model prediction. Finally, the optimal conditions were obtained. 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 3**
Analysis of variance for the response surface quadratic model for silymarin.

| Source | *df* | Sum of squares | Mean squares | *F* value | *P* value |
|---|---|---|---|---|---|
| Model | 14 | 253.24 | 18.09 | 9.28 | <.0001 |
| Residual | 17 | 33.15 | 1.95 | | |
| *Lack of fit* | 10 | 30.47 | 3.05 | 7.93 | 0.11 |
| *Pure error* | 7 | 2.69 | 0.38 | | |
| Cor total | 31 | 266.38 | | | |
| *R*-squared 0.8843 | | | Pred. *R*-squared 0.3751 | | |
| Adj-*R*-squared 0.7889 | | | Adeq precision 12.756 | | |

## 3. Result and discussion

### 3.1. A regression equation describing the processing of microwave-assisted extraction of silymarin from milk thistle milk

Experiments were randomized as detailed in Table 2, to maximize the effects of unexplained variability in the observed responses. Thirty six tests were studied; five replicates at the centre of the design were used to allow for estimation of a pure error sum of squares. Multiple regression analysis of the experimental data yielded the following second-order polynomial stepwise equation:

$$\begin{aligned} Y = {} & 52.26 + 5.52x_1 + 0.59x_2 - 1.62x_3 + 1.44x_4 - 1.11x_1x_2 \\ & - 0.45x_1x_3 + 0.08x_1x_4 + 0.86x_2x_3 - 0.33x_2x_4 + 0.96x_3x_4 \\ & - 0.09x_1^2 - 0.18x_2^2 - 1.57x_3^2 - 0.35x_4^2 \end{aligned} \quad (5)$$

An analysis of variance (ANOVA) procedure was used to analyze the model for significance and suitability, and a statistical summary is given in Table 3.

The correlation measures for testing the goodness of fit of the regression equation are the multiple correlation coefficients $R$. The value of $R^2$ (0.8843) for Eq. (5) indicates a relatively high degree of correlation between the observed and predicted values. The value of the determination coefficient, Adj-$R^2$ (0.7889) suggests that only about 21.11% of the total variations are not explained by the model. Statistical testing of the model was done in the form of analysis of variance, which is required to test the significance and adequacy of the model. Here the ANOVA of the regression model demonstrates that the model is relatively highly significant, as is evident from the calculated $F$ value (11.7) and very low probability value ($p < 0.0001$). The computed $F$ value (9.28) indicates that the treatment differences are highly significant. The model also showed the "*Lack of Fit F value*" of 7.93 implies the lack of fit is not significant relative to the pure error even at 0.05 levels. The model was found to be adequate for prediction (12.756) within the range of variables employed. However, the "*pred. R-squared*" of 0.3751 is not as close to the "Adj-*R*-squared" of 0.7889 as one might normally expect. This may indicate a large block effect. The values of the coefficients in Eq. (5) were calculated and tested for their significance listed in Table 4. It can be seen that two linear coefficients, all the quadratic terms, and the interactive terms were significant.

The $F$ value in this table is the ratio of the mean-squared error to the pure error obtained from the replicates at the design centre. The experimental data showed a good fit with the second-order polynomial equations, which were statistically acceptable at the $p < 0.05$ level. The statistical analysis data (Table 4) shows that the linear and quadratic terms are more significant while some of the interactions are less so. The response variable depends more upon

**Table 4**
Regression coefficients and analysis of variance of the CCRD model for silymarin with MAE.

| Model term | *df* | Coefficient estimate | Standard error | 95% CI Low | 95% CI High | *F* value | Prob > *F* | Significant |
|---|---|---|---|---|---|---|---|---|
| Intercept | 1 | 52.26 | 0.49 | 0.49 | 51.22 | 9.28 | <.0001 | ** |
| $x_1$ | 1 | 0.52 | 0.29 | −0.086 | 1.12 | 3.27 | 0.0883 | NS |
| $x_2$ | 1 | 0.59 | 0.29 | 0 | 1.19 | 4.33 | 0.0470 | * |
| $x_3$ | 1 | −1.62 | 0.29 | −2.22 | −1.02 | 32.29 | <.0001 | ** |
| $x_4$ | 1 | 1.44 | 0.29 | 0.84 | 2.04 | 25.57 | <.0001 | ** |
| $x_1x_2$ | 1 | −1.11 | 0.35 | −1.84 | −.037 | 10.05 | 0.0056 | ** |
| $x_1x_3$ | 1 | −0.45 | 0.35 | −1.18 | 0.29 | 1.63 | 0.2189 | NS |
| $x_2x_3$ | 1 | 0.86 | 0.35 | 0.12 | 1.59 | 6.02 | 0.0252 | * |
| $x_1x_4$ | 1 | 0.08 | 0.35 | −0.66 | 0.82 | 0.053 | 0.8201 | NS |
| $x_2x_4$ | 1 | −0.33 | 0.35 | −1.07 | 0.41 | 0.89 | 0.3586 | NS |
| $x_3x_4$ | 1 | 0.96 | 0.35 | 0.22 | 1.69 | 7.43 | 0.0144 | ** |
| $x_1^2$ | 1 | −0.09 | 0.26 | −0.63 | 0.45 | 0.12 | 0.7340 | NS |
| $x_2^2$ | 1 | −0.18 | 0.26 | −0.72 | 0.36 | 0.50 | 0.4871 | NS |
| $x_3^2$ | 1 | −1.57 | 0.26 | −2.11 | −1.03 | 37.40 | <.0001 | ** |
| $x_4^2$ | 1 | −0.35 | 0.26 | −0.89 | 0.20 | 1.80 | 0.1970 | NS |

* Significant at $p < 0.05$; NS, not significant.
** Very significant at $p < 0.01$.

the individual change of the independent variables rather than their interactions.

### 3.2. Effects of extraction variables on silymarin yield

Three-dimensional response surfaces presented in Figs. 3–8 for the independent variables (extraction time, temperature, ethanol concentration, and solid–liquid ratio) were obtained by keeping two of the variables constant, which indicated the changes in silymarin yield under different MAE conditions.

The effects of extraction time and temperature on the extraction yield of silymarin are shown in Fig. 3. The other two factors, ethanol concentration and solid–liquid ratio, were set at 80% (*v*/*v*) and 1:30 g/mL, respectively. It was concluded from Table 4 that the silymarin yield in milk thistle has a positive linear relationship with extraction temperature and with the interaction of extraction time and temperature. During the initial extraction period (lower times), the silymarin yield increased significantly as the temperature increased. At higher extraction time periods, the silymarin yield does not change with an increase in temperature. These results were in agreement with reports that extraction time had no significant effect on the total ethanol extraction yield of ginseng components when using MAE [18,19].

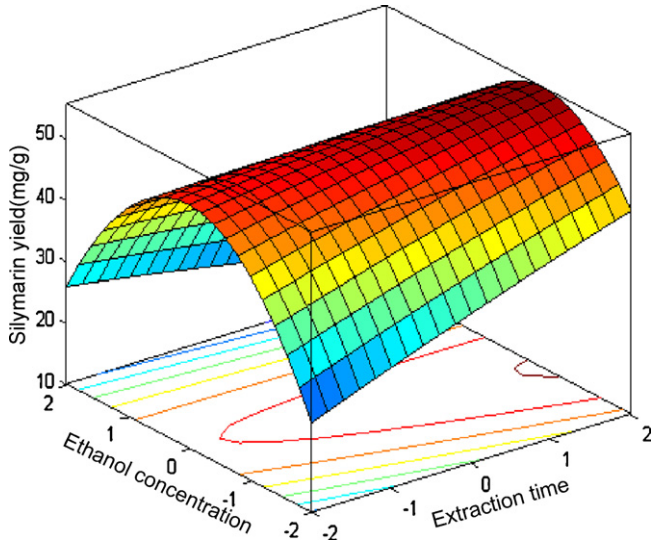


**Fig. 4.** Response surface for the effect of extraction time and ethanol concentration on extraction yield.

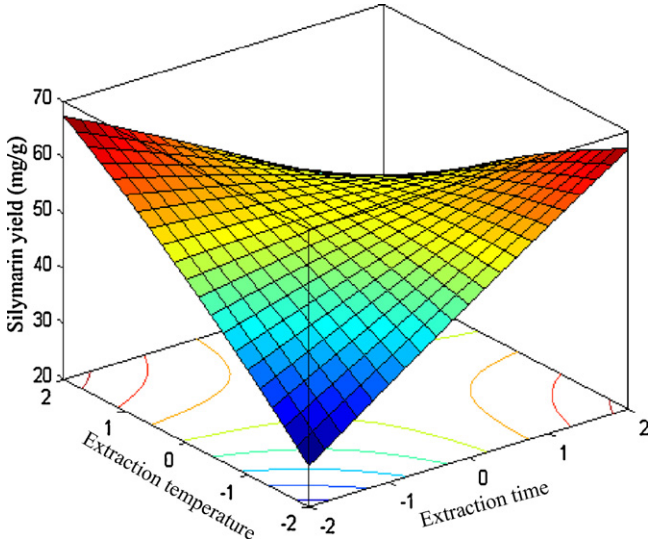


**Fig. 3.** Response surface for the effect of extraction time and temperature on extraction yield.

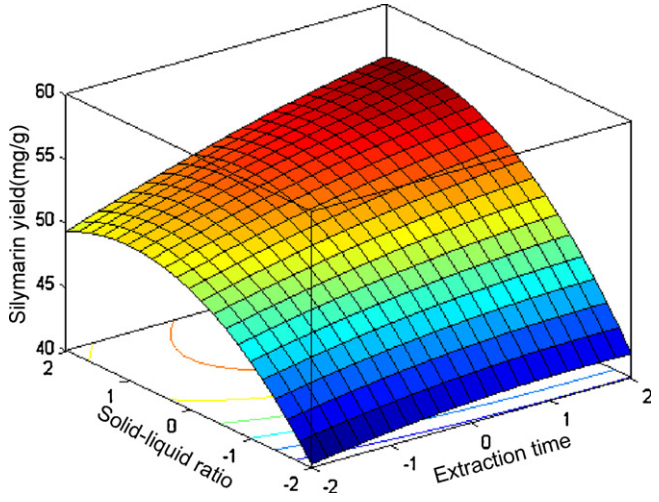


**Fig. 5.** Response surface for the effect of extraction time and solid–liquid ratio on extraction yield.

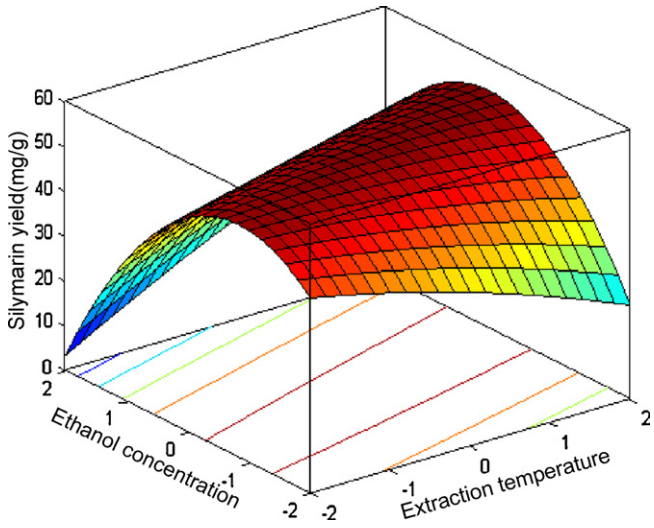


**Fig. 6.** Response surface for the effect of extraction temperature and ethanol concentration on extraction yield.

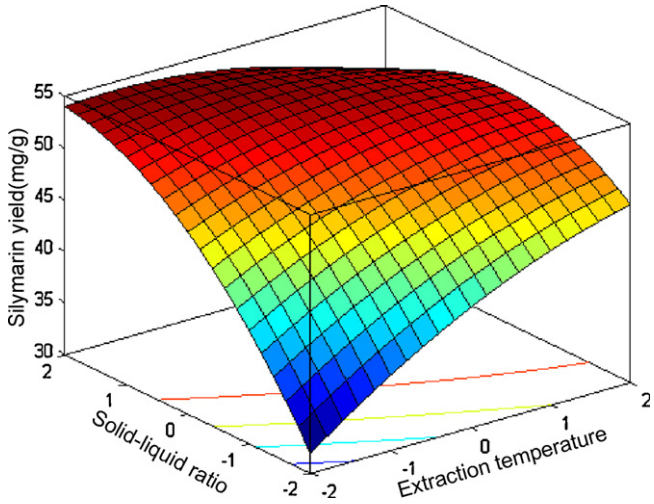


**Fig. 7.** Response surface for the effect of extraction temperature and solid–liquid ratio on extraction yield.

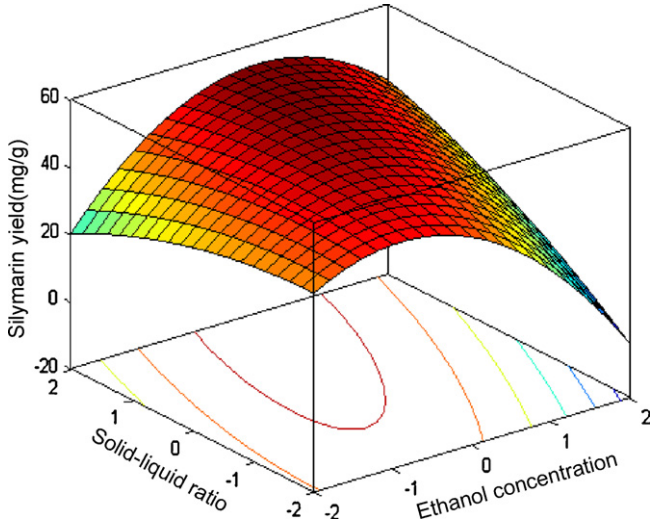


**Fig. 8.** Response surface for the effect of ethanol concentration and solid–liquid ratio on extraction yield.

The interactive influences of the extraction time and ethanol concentration on extraction yield are shown in Fig. 4. The other two factors, extraction temperature and solid–liquid ratio, were set at 110 °C and 1:30 g/mL, respectively. The silymarin yield from milk thistle seeds mainly depends upon ethanol concentration, as its linear effect ($p < 0.01$) was significant, and results in a curvilinear increase until zero level 80% ($v/v$), and then to decreases in silymarin yield.

Fig. 5 demonstrates the effect of the extraction time and solid–liquid ratio on extraction yield at a constant ethanol concentration of 80% and an extraction temperature of 110 °C. As shown in Table 4, the silymarin yield was found to be a function of the positive linear effect of the solid–liquid ratio ($p < 0.01$) and the negative interaction effect between solid–liquid ratio and extraction time ($p < 0.01$). The result was a curvilinear increase in silymarin yield for all extraction times employed.

The mutual influence of extraction temperature and ethanol concentration on extraction yield is shown in Fig. 6. The other two factors, extraction time and solid–liquid ratio, were set at 60 min and 1:30 g/mL, respectively. According to results of ANOVA from Table 4, the interaction between the extraction temperature and ethanol concentration was insignificant. The positive influence of the linear terms for ethanol concentration ($p < 0.01$) and temperature ($p < 0.05$) were found. It is shown in Fig. 7 that the effect of the extraction temperature on the silymarin yield was linear. At low-ethanol concentrations, the silymarin yield increased with an increase in temperature. However, at the high-ethanol concentration, the silymarin yield decreased significantly with an increase in temperature. The flavonoids' solubility, mass transfer, and extraction rate increases, and the solvent viscosity and surface tension decrease with increasing temperature [19]. These results are similar to the research findings by Richter et al. [20]. However, preliminary experimental results showed that silymarin begins to decompose above 130 °C extraction temperature. Cacace and Mazza also reported that temperature affected the extraction of anthocyanins and that increasing the temperature beyond 30–35 °C resulted in the degradation of anthocyanins [21].

An increase in the extraction yield was noted with increased extraction temperatures (below 130 °C). The extraction yield of silymarin initially increased, and then gradually decreased, with the increases of ethanol concentration and solid–liquid ratio. The response surface plots that were developed (Fig. 7) indicate that the extraction temperature and solid–liquid ratio had significant effects on silymarin extraction yields. The other two factors, ethanol concentration and extraction time, were held at 80% ($v/v$) and 60 min, respectively. It may be concluded from Table 4 that both extraction temperature ($p < 0.05$) and solid–liquid ratio ($p < 0.001$) indicate significantly positive effects on silymarin yield. However, the mutual influence on silymarin yield between extraction temperature and solid–liquid ratio was insignificant. At the low-extraction temperature, the silymarin yield increased significantly when the solid–liquid ratio increased. At high-extraction temperatures, the silymarin yield changed slightly with an increase in the solid–liquid ratio.

In Fig. 8, the effects of solid–liquid ratio and ethanol concentration on the silymarin yield are shown for a constant extraction temperature of 110 °C and extraction time of 60 min. The interaction between the solid–liquid ratio and ethanol concentration was very significant ($p < 0.01$) as shown in Table 4. The influence of the solid–liquid ratio linear term ($p < 0.01$) was positive and the influence of the ethanol concentration was negative, which may be observed in the curve shown in Fig. 8. With an increase in ethanol concentration, the silymarin yield increased when the ethanol concentration was kept at levels under 80%, but it decreased when the ethanol concentration was kept at levels higher than 80%. This shows the similarity of Wettasinghe and Shahidi's study on borage

**Table 5**
Optimization criteria for different factors and responses.

| Indendpent variables/dendpent variable | Goal | Lower limit | Upper limit | Weight | Total | Significant |
|---|---|---|---|---|---|---|
| Time (min) | In the range | 50 | 70 | 1 | 1 | 3 |
| Temperature (°C) | In the range | 90 | 130 | 1 | 1 | 3 |
| Ethanol concentration (%) | In the range | 70 | 90 | 1 | 1 | 3 |
| Solid–liquid ratio (g/mL) | In the range | 1:20 | 1:40 | 1 | 1 | 3 |
| Silymarin yield | Maximize | 41.93 | 54.59 | | | |

meal [22]. An increase in the solid–liquid ratio resulted in a higher extraction yield, while the yield reached a maximum value when the solid–liquid ratio reached a certain value (nearly the 2 levels). Already existing researches verified the result [23–25].

The experimental results showed that the extraction temperature, ethanol concentration, and solid–liquid ratio had significant effects on the silymarin yield, while the extraction times did not have a significant effect on the silymarin yield.

### 3.3. Optimization of microwave-assisted extraction for silymarin and experimental validation

According to the desired goals, each factor and response was chosen to optimize MAE process conditions are shown in Table 5. In order to adjust the shape of its particular desirability function, different weights were assigned to each goal. The optimum conditions were obtained by running the program of Box-Behnken design. The optimum conditions for independent variables and the predicted values of the responses also are presented as follows: extraction time of 60 min, extraction temperature of 112 °C, ethanol concentration of 81.5% (*v*/*v*), and solid–liquid ratio of 1:38 (g/mL), respectively. Once the optimum conditions had been determined, they would be used to extract the silymarin using the MAE. According to the predicted optimum conditions, extracting experiments were performed and the yield of the final products was determined. A verification experiment was performed using the derived optimum extraction conditions, and the yields of the resulting products were determined. The experimental values (means of 5 measurements), as well as the predicted values for silymarin yields, were presented as $56.67 \pm 1.36$ and 57.40 mg/g, respectively. No significant differences ($p > 0.05$) between the actual and predicted values were found. Therefore, the verification experiment well demonstrates the goodness of fit for the curve, and the reproducibility of the results for an extraction performed with the optimum parameters.

## 4. Conclusions

During MAE of silymarin from milk thistle seeds, the independent variables, except for extraction time, significantly influenced the extraction yield of silymarin. The interactions among extraction time, extraction temperature, ethanol concentration, and solid–liquid ratio were also investigated by response surface analysis. The second-order polynomial equation predicted the extraction conditions for the highest yield at 60 min extraction time, 112 °C extraction temperature, 1:38 (g/mL) solid–liquid ratio and an ethanol concentration of 81.5% (*v*/*v*). The developed model predicted silymarin yield of 57.40 mg/g at the optimal conditions, and under such conditions, the experimental results showed an average silymarin yield of $56.67 \pm 1.36$ mg/g. MAE can be applied to silymarin extraction from milk thistle seeds, and can be an alternative technique to the more time- and energy-consuming traditional procedures.

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