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Optimization of ultrasonic extraction of total flavonoids from *Tussilago farfara L.* using response surface methodology

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Received September 2, 2013, accepted October 9, 2013

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Pharmazie 69: 311–315 (2014) *doi: 10.1691/ph.2014.3190*

In this work, ultrasound technology was used for the extraction of total flavonoids from *Tussilago farfara* L. Response surface methodology (RSM), based on a Box–Behnken design (BBD), was used to optimize the effects of processing parameters on total flavonoids yields. The parameters were ultrasonic frequency (X_1), extraction time (X_2) and ratio of liquid to solid (X_3). The statistical analysis indicated that the parameters of ultrasonic power and ratio of liquid to solid, and the square effects among them had significant effects on the yield of flavonoids from *Tussilago farfara* L. The interaction between ultrasonic power and extraction time, and ultrasonic power and ratio of liquid to solid also caused significant effects on the yields. The optimum extraction conditions were determined as follows: ultrasonic power 420 W, extraction time 30 min, ratio of liquid to solid 25 mL/g. Under these conditions, the experimental yield 6.59 ± 0.061% agreed closely with the predicted yield (6.64%).

1. Introduction

Tussilago farfara L. is a member of the Asteraceae family. The flower buds of this plant commonly known as coltsfoot or "Kwandong Hwa" in China and Korea, have been used as a traditional Chinese herbal medicine for the treatment of bronchitic and asthmatic conditions (Namba 1980). Major chemical components of *Tussilago farfara* L. are terpenoids (Zhi et al. 2012), flavonoids (Kaloshina and Konopleva 1971), alkaloids (Roeder 2000) and essential oils (Suzuki and Kikuchi 1992). It has been reported that flavonoids are important contributors to the biological activity of *Tussilago farfara* L., such as antimicrobial activity (Kokoska et al. 2002), inhibitory activity against nitric oxide synthase (Hwang et al. 1987), antioxidant and anti-inflammatory activities (Kim et al. 2006; Ravipati et al. 2012).

Conventional methods, including refluxing, boiling and heating, have been used for the extraction of flavonoids, however, the disadvantages of these methods are the loss of flavonoids due to hydrolysis, oxidation and ionisation during extraction as well as the long extraction time (Li et al. 2005). In recent years, ultrasonic treatment has been employed for preparing flavonoids from different plant materials (Huang et al. 2009; Veličković et al. 2007; Wang et al. 2012) and showed good extraction efficiency, proved to be a simple, inexpensive and efficient alternative to conventional extraction techniques (Wang et al. 2008).

Response surface methodology (RSM) is an effective statistical technique for optimizing complex extraction procedures. It has been widely used for optimizing the media for production of flavonoids (Pan et al. 2012; Zhang et al. 2011), polysaccharides (Li et al. 2011) and phenolic compounds (Liyana-Pathirana and Shahidi 2005) from various plants. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Chen et al. 2005; Gunawan et al. 2005). Therefore, it is less laborious and time-consuming than other approaches required to optimize a process (Giovanni 1983). Box–Behnken design (BBD) with three levels, is more efficient and easier to arrange to interpret experiments than other RSMS (Ferreira et al. 2007).

In this study, the aim was to optimize ultrasonic technology conditions for the extraction of flavonoids from *Tussilago farfara* L. BBD was used to study the effects of extraction parameters (ultrasonic power, extraction time and ratio of solid to liquid) on the yield of flavonoids and their interactions.

Table 1: Analysis of variance for the fitted quadratic polynomial model of extraction of flavonoids

Source	SS	DF	MS	F- value	P- value
Model	5.55	9	0.62	565.00	< 0.0001
Residual	7.645×10^{-3}	7	1.092×10^{-3}		
Lack of fit	6.325×10^{-3}	3	2.108×10^{-3}	6.39	0.0526
Pure error	1.320×10^{-3}	4	3.3×10^{-4}		
Cor Total	5.56	16			
$R^2 = 0.9986$	CV = 0.55		$R^2_{Adj} = 0.9969$		

SS = Sum of squares, DF = Degrees of freedom, MS = Mean squares, F = Fishers variance ratio, P = Probability

2. Investigations, results and discussion

2.1. Fitting the model

A regression analysis (Table 1) was carried out to fit mathematical models to the experimental data aiming at an optimal region for the responses studied. The

Table 2: Estimated regression model of relationship between response variables (yield of flavonoids) and independent variables (X1, X2, X3)

Variables	SS	DF	MS	F- value	P- value
X_1	0.014	1	0.014	12.46	< 0.0001
X_2	5.513×10^{-3}	1	5.513×10^{-3}	5.50	0.0595
X_3	0.013	1	0.013	11.72	0.0111
X_1X_2	0.11	1	0.11	96.71	< 0.0001
X_1X_3	0.036	1	0.036	33.05	0.0007
X_2X_3	8.10×10^{-3}	1	8.10×10^{-3}	7.42	0.0296
$X_1{}^2$	4.93	1	4.93	4511.39	< 0.0001
$X_2{}^2$	3.36×10^{-3}	1	3.36×10^{-3}	3.08	0.1229
$X_3{}^2$	0.29	1	0.29	269.21	< 0.0001

second-order polynomial equation (in terms of coded values) for predicting yield(Y) was given as below:(1)$Y = 6.58 + 0.041\,X_1 - 0.026\,X_2 - 0.040\,X_3 - 0.16\,X_1\,X_2 - 0.095\,X_1\,X_3 - 0.045\,X_2\,X_3 - 1.08\,X_1^2 + 0.028\,X_2^2 - 0.26$where X1, X2 and X3 represent ultrasonic frequency, extraction time and ratio of liquid to solid, respectively.

The analysis of variance was required to test the significance and adequacy of the model, as given in Table 1. F-test suggested that the model was highly significant as evident from a very high F-value (F = 565.00) and a very low p-value ($p < 0.0001$) (Cai et al. 2008). The lack of fit F-value (6.39) and p-value (0.0526) implied that the lack of fit was not significant relative to the pure error and indicated that the model equation was adequate for predicting the yield of flavonoids under any combination of values of the variables. The R^2 (coefficient of determination) of 0.9986 for the predicted mode indicated that the response model could explain 99.86% of the total variations, suggesting the goodness of fit of the mode (Haaland 1989). Furthermore, the R^2_{adj} (adjusted determination coefficient) of 0.9969 was also high enough to indicate the significance of this model. The value of CV (Coefficient of variation) was 0.55, showing a better precision and reliability of the experiments.

The P-values were used as a tool to check the significance of each of the coefficients, which indicated the patterns of the interaction among the variables (Muralidhar et al. 2001). The corresponding variables would be more significant at greater F-value and smaller p-value (Atkinson and Donev 1992). The data in Table 2 indicated that the variables (X1, X3,) and two quadratic terms ($X1^2$, $X3^2$) were significant model terms and the interactive effects of X1X2 and X2X3 were also significantly affected the yield of flavonoids.

2.2. *Analysis of response surface*

To provide a better visualization of the statistically significant factors derived from the statistical analysis, the 3D response surface and 2D contour plots for the effects of independent variables on the extraction of flavonoids were depicted in Figs. 1–3. The plots were obtained by plotting the response on the Z-axis against any two variables while keeping the third variable at zero level.

As shown in Fig. 1, ultrasonic power (X1) showed a quadratic effect on the yield, while extraction time (X2) showed a slightly linear increase. The yield was increased with increases of ultrasonic power (X1) and extraction time (X2) and then decreased with a further increase in ultrasonic power (X1). It is evident from Fig. 1 that ultrasonic power(X1) and the interaction between ultrasonic power and extraction time (X1X2) had a significant effect on the yield. Fig. 2 indicates that both ultrasonic

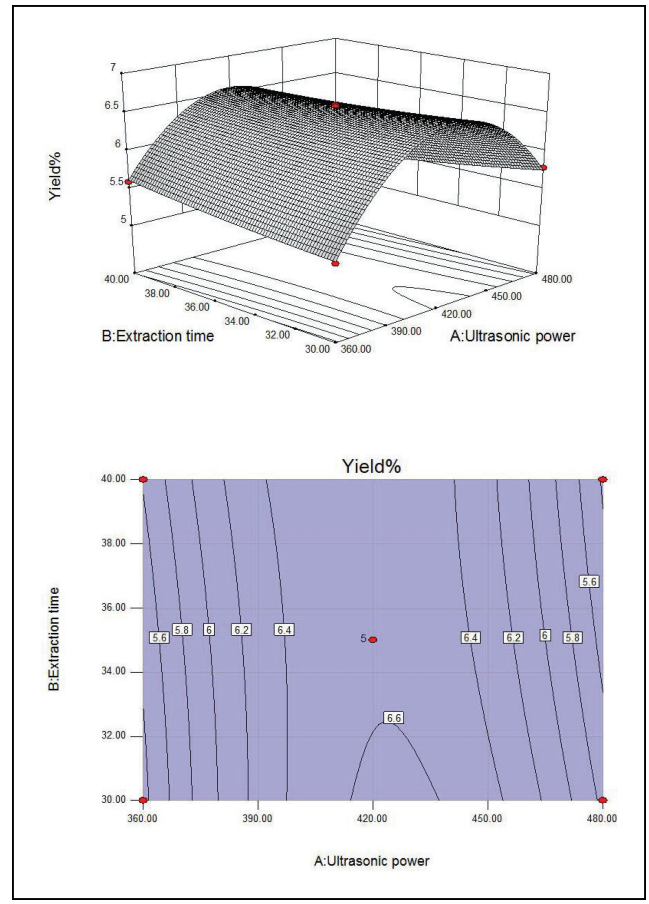

Fig. 1: Response surface plot and contour plot of ultrasonic power and extraction time and their mutual interactions on the yield of flavonoids.

power (X1) and ratio of liquid to solid (X3) had a quadratic effect on the yield. The extraction ratio was increased with an increase in the ratio of liquid to solid (X3). A further increase in ultrasonic power (X1) resulted in reversal of this trend. As shown in Fig. 2, the interaction between ultrasonic power and ratio of liquid to solid (X1X3) had a significant effect on the extraction ratio. Figure 3 shows that the yield changed slightly with an increase of extraction times, indicating a smaller effect on the yields. Considering all the responses, it is evident that ultrasonic power, ratio of liquid to solid and the square effects among them had a significant effect on the yield. It also indicated that the interactions between ultrasonic power and extraction time, and ultrasonic power and ratio of liquid to solid impacted the yield significantly. The results above agree well with Table 2.

2.3. *Optimization of extracting parameters and validation of the model*

The optimal conditions were used to test the suitability of the quadratic model for achieving maximal yield. The predicted optimal extraction conditions were X1 = 425.67 W, X2 = 30 min and X3 = 24.96 mL/g, and the predicted optimal yield was 6.64%. To confirm the goodness of the model for predicting maximal yield, an additional experiment was performed using this modified optimal conditions: X1 = 420 W, X2 = 30 min and X3 = 25 mL/g. The mean value of experimental yield was $6.59 \pm 0.061\%$. This value was higher than that reported elsewhere, whilst extracting total flavonoids from *Tussilago farfara* L. by water bath extraction (Ravipati et al. 2012). This indicated that ultrasonic extraction was an effective method for the extraction of flavonoids from *Tussilago farfara* L. The maximum predicted yield and experimental yield are shown in Table 3. No

Table 3: Optimum conditions and the predicted and experimental value of response at the optimum conditions

	Ultrasonic power (W)	Extraction time (min)	ratio of liquid to solid (mL/g)	Yield%
Optimum conditions	425.67	30	24.96	6.64 (predicted)
Modified conditions	420	30	25	6.59 ± 0.061(actual)

significant difference ($p > 0.05$) was found between the experimental and predicted values of total flavonoids. Hence, the models could be used to optimise the process of total flavonoids extraction from *Tussilago farfara* L.

3. Experimental

3.1. Materials and chemicals

Flower buds of *Tussilago farfara* L. were purchased from Taian Pharmacy in Shandong Province, China. The samples of *Tussilago farfara* L. were air-dried at 50 °C and crushed up (20 mesh), then collected and stored in a desiccator at room temperature (15–20 °C) until used (less than one month). Rutin was purchased from national institutes for food and drug control. Other chemicals and solvents were of analytical grade and purchased from China National Medicine Group Shanghai Corporation (Shanghai, China). Water was prepared doubly distilled.

Ultrasonic cleaner (SB5200DTD, Ultrasonic Instrument Co. Kun-shan, Jiangsu, China) was used for ultrasonic extraction of flavonoids. UV-1700 spectrophotometer (Shimadzu Corporation, Japan) for total flavonoids analysis of sample.

3.2. Extraction of total flavonoids

The extraction was performed in a conical flask with cover by ultrasonic treatment. The samples were extracted with 70% ethanol for different times at various ultrasonic power. One gram of dried *Tussilago farfara* L. powder was used for each treatment. In the whole extraction processing, the temperature of solution was held below 50 °C by ice bathing. The supernatant was collected for the determination of total flavonoid content.

3.3. Determination of total flavonoids

The total flavonoid content was determined using a colorimetric method described by Zhu H. et al. (2010) and slightly modified in our laboratory. Briefly, 2 mL of the sample solution and 0.6 mL of $NaNO_2$ (5%) solution were mixed in a volumetric flask (10 mL), standing for 6 min. And then 0.5 mL of the $Al(NO_3)_3$(10%) solution was added to the volumetric flask, shaken, and was left to stand for 6 min. At last, 3.0 mL of the NaOH (4.0%) solution was added, followed by addition of distilled water to the scale. The mixture was allowed to stand for 15 min and the absorption was measured at 506 nm against the same mixture, without the sample as a blank. The content of the total flavonoids was expressed as rutin equivalents (mg rutin/g sample) through the calibration curve of rutin.

3.4. Determination of flavonoid yield

The flavonoid yield (%) was calculated as follows:

$$yield(\%) = \frac{m_{\,flavonoids}}{m_{T.\,farfara}} \times 100 \qquad (2)$$

where $m_{\,flavonoids}$ is the total flavonoids extract mass (g) and the $m_{T.\,farfara}$ is the extracted *Tussilago farfara* L. powder mass (g).

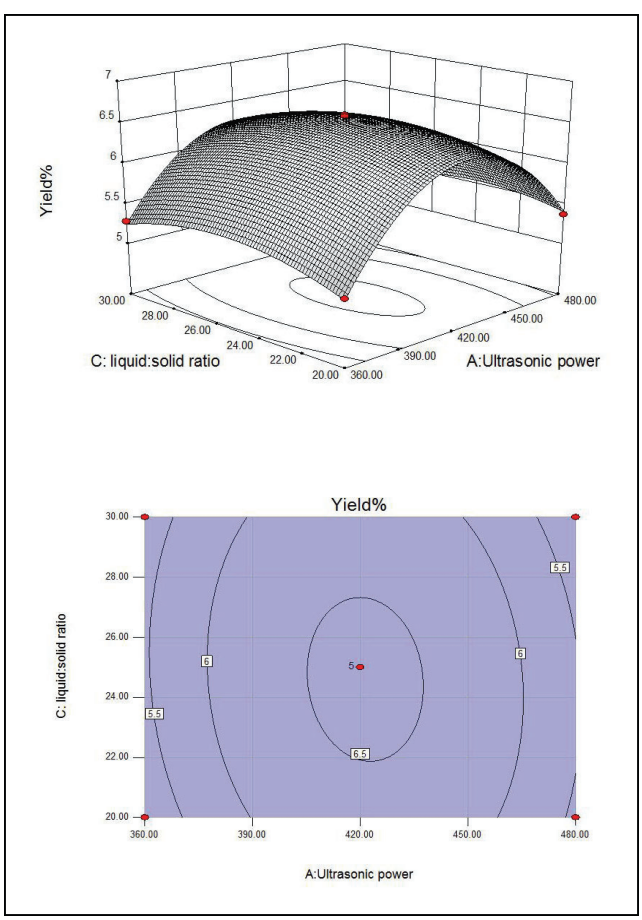

Fig. 2: Response surface plot and contour plot of ultrasonic power and the ratio of liquid to solid and their mutual interactions on the yield of flavonoids.

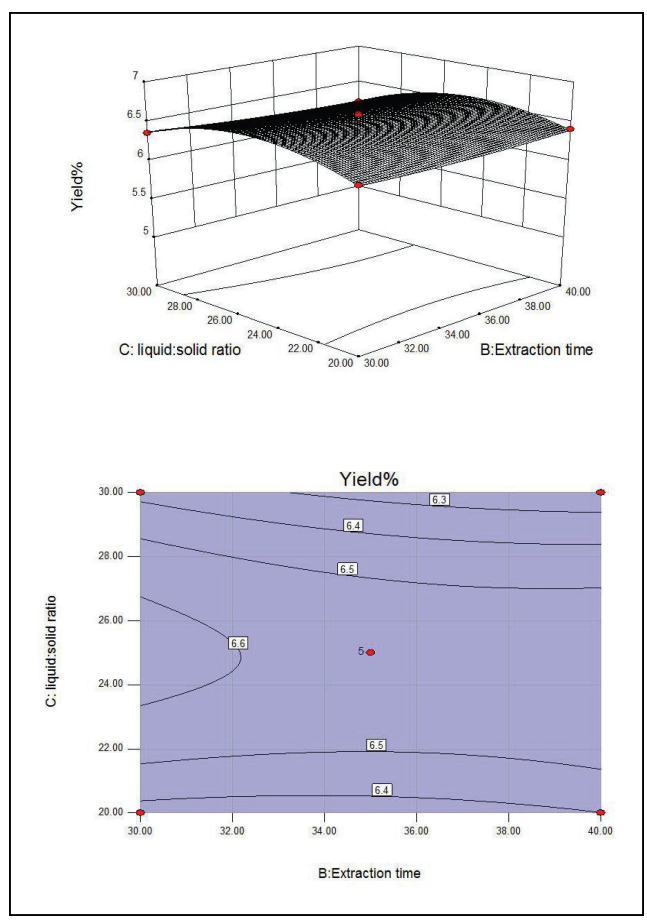

Fig. 3: Response surface plot and contour plot of extraction time and the ratio of liquid to solid and their mutual interactions on the yield of flavonoids.

Table 4: Factors and levels

Factors	Low	Center	High
Ultrasonic power (w, X1)	−1(360)	0(420)	+ 1(480)
Extraction time (min, X2)	−1(30)	0(35)	+ 1 (40)
Ratio of liquid to solid (mL/g, X3)	−1(20)	0(25)	+ 1 (30)

3.5. Experimental design

A three-level-three-factor, Box–Behnken factorial design (BBD) was employed in this optimization study. Three extraction variables considered for this research were X_1 (ultrasonic power), X_2 (extraction time), and X_3 (ratio of liquid to solid), which were determined on the basis of a single-factor experiment for the flavonoid production. Extraction yield (Y) was taken as the response of the design experiments. The coded and uncoded (actual) levels of the independent variables are given in Table 4. Seventeen experiments (Table 5) augmented with five replications (treatment 13–17) were carried out at the center of the design to evaluate the pure error sum of squares. The triplicates were performed at all design points in randomized order.

Experimental data were fitted to a quadratic polynomial model and regression coefficients were obtained. The general form of quadratic model was as follows:

$$\Upsilon = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j=2}^{n} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_{ii} {x_i}^2 \qquad (3)$$

where Y is the response (dependent variables), β_0 is the constant coefficient, β_i, β_{ii} and β_{ij} represent the coefficient for the linear, quadratic and interaction effect, and x_i and x_j are the independent variables.

3.6. Statistical analyses

Data were expressed as means ± standard errors (SE) of three replicated determinations. The responses obtained from each set of experimental design (Table 5) were subjected to multiple non-linear regressions using the Design Expert software (Trial Version 8.0.6, Stat-Ease Inc., Minneapolis, MN). The significance of the regression coefficient was checked by F-test and P-value. The fitted polynomial equation was expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimal conditions (Triveni et al. 2001).

Table 5: Box–Behnken experimental design with the independent variables

Run	Coded variable levels			Yield of flavonoids (%)	
	X_1	X_2	X_3	Actual values	Predicted values
1	−1	−1	0	5.32	5.34
2	1	−1	0	5.78	5.75
3	−1	1	0	5.59	5.62
4	1	1	0	5.40	5.38
5	−1	0	−1	5.15	5.13
6	1	0	−1	5.37	5.41
7	−1	0	1	5.28	5.24
8	1	0	1	5.12	5.14
9	0	−1	−1	6.37	6.36
10	0	1	−1	6.41	6.40
11	0	−1	1	6.36	6.37
12	0	1	1	6.22	6.23
13	0	0	0	6.55	6.58
14	0	0	0	6.58	6.58
15	0	0	0	6.60	6.58
16	0	0	0	6.58	6.58
17	0	0	0	6.57	6.58

Acknowledgement: This work was supported by the Shandong Province traditional Chinese medicine Technology Development Program (No. 2013–258).

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