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Analytical Methods

Optimisation of supercritical fluid extraction of flavonoids from *Pueraria lobata*

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Abstract

Supercritical carbon dioxide extraction was employed to extract flavonoids from *Pueraria lobata*. The optimal conditions for flavonoid extraction were determined by response surface methodology. Box–Behnken design was applied to evaluate the effects of three independent variables (pressure, temperature and co-solvent amount) on the flavonoid yield of *P. lobata*. Correlation analysis of the mathematical-regression model indicated that a quadratic polynomial model could be employed to optimise the supercritical carbon dioxide extraction of flavonoids. From response surface plots, pressure, temperature and co-solvent amount exhibited independent and interactive effects on the extraction of flavonoids. The optimal conditions to obtain the highest flavonoid yield of *P. lobata* were a pressure of 20.04 MPa, a temperature of 50.24 °C and a co-solvent amount of 181.24 ml. Under these optimal conditions, the experimental values agreed with the predicted values, using analysis of variance, indicating a high goodness of fit of the model used and the success of response surface methodology for optimising supercritical carbon dioxide extraction of flavonoids from *P. lobata*.

Keywords: *Pueraria lobata*; Flavonoid; Supercritical fluid; Response surface methodology

1. Introduction

Pueraria lobata is a famous traditional medicinal herb in China, which is also widely accepted by consumers over the world for its healthy effects. It has been used for the management of various diseases, including cardiovascular disorders (Yeung, Leung, Xu, Vanhoutte, & Man, 2006), or as an antimicrobial, pain-releasing and appetite-inducing agent (Keung & Vallee, 1998). It has also proven useful in the treatment of alcohol abuse and hypertension (Fan, O'Keefe, & Powell, 1985). *P. lobata* is abundant in flavonoids and other bioactive substances.

Several flavonoids are reported to be responsible for the broad therapeutic effects. Cherdshewasart, Subtang, and Dahlan (2007) have described the flavonoid compositions of *Pueraria mirifica* and *P. lobata*. Puerarin, daidzin, genistin, daidzein and genistein are the five flavonoids mainly identified in all the tested samples; their contents differ with changes in location and cultivar. Xu and He (2007) have indicated that puerarin (daidzein 8-C-glucoside), daidzein and rutin are the major flavonoids in *P. lobata*. Recent investigations demonstrate that they are effective antioxidants and show many physiological activities, such as anti-proliferative effects on human cancer cell lines, inhibiting alcohol dehydrogenase and xanthine oxidase, antigiardial activity and anti-diabetic activity (Chen, Zhang, & Ye, 2001; Guerra et al., 2000). Therefore, it is interesting to find an effective method to prepare flavonoids from *P. lobata*.

Supercritical fluid extraction has been applied in the food and medical industries extensively in recent years. Supercritical carbon dioxide is the most commonly-used solvent; it is non-toxic, non-flammable, non-explosive, cost-efficient, readily available, and easy to remove from the extracted materials (Chiu, Cheng, Chen, Chang, &

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Yang, 2002). The solvating power of supercritical carbon dioxide can be summarised by a few rules (Brunner, 2005): (i) it dissolves non-polar or slightly polar compounds; (ii) the solvent power for low molecular weight compounds is high and decreases with increasing molecular weight; (iii) free fatty acids and their glycerides exhibit low solubilities; (iv) pigments are even less soluble; (v) water has a low solubility (<0.5%, w/w) below 100 °C; (vi) proteins, polysaccharides and mineral salts are insoluble; and (vii) supercritical carbon dioxide is capable of extracting compounds that are less volatile, having a higher molecular weight and/or a higher polarity, as pressure increases.

Response surface methodology is a statistical method, which uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equations (Triveni, Shamala, & Rastogi, 2001). In addition, this experimental methodology can generate a mathematical model (Baş & Boyaci, 2007).

Supercritical fluid extraction has been documented as an effective method for preparing bioactive products from plant materials. However, few publications on flavonoid extraction from *P. lobata* by supercritical fluid extraction are available till now. In this study supercritical carbon dioxide was employed to extract flavonoids from *P. lobata*. The effects of pressure, temperature and co-solvent amount on flavonoid yield were investigated. Response surface methodology was used to build a model between the flavonoid yield and these independent factors, and to optimise the extraction conditions.

2. Materials and methods

2.1. Materials

P. lobata (Wild.) Ohwi roots were purchased from a commercial market in Lianyungang, China. Samples were pulverised in a knife mill and were passed through a 20-mesh sieve.

2.2. Chemicals

Rutin was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade.

2.3. Extraction by supercritical carbon dioxide

The extraction by supercritical carbon dioxide was conducted using a Speed SFE™ extraction unit (Applied Separation, Lehigh, PA). Forty grams of sample were weighed accurately and loaded into a 250-ml extraction vessel. Total extraction time was set for 90 min, with an initial static extraction period of 10 min, followed by a dynamic extraction for 80 min. The flow rate of supercritical carbon dioxide was 20 l/h. The conditions were set as follows: pressure (15–25 MPa), temperature (40–60 °C) and co-solvent amount (100–200 ml). Ethanol was chosen as co-solvent. The flavonoid content was determined by the aluminum nitrate method (Moreno, Isla, & Sampietro, 2000). Rutin was used to prepare a standard curve. The recovery of flavonoid was expressed as mg of rutin equivalents per gram of *P. lobata* on a dry weight basis.

2.4. Box–Behnken design

The software Design Expert (Trial Version 7.0.3, Stat-Ease Inc., Minneapolis, MN) was employed for experimental design, data analysis and model building. A Box–Behnken design with three variables (Box & Behnken, 1960) was used to determine the response pattern and then to establish a model. The three independent variables used in this study were pressure (X_1), temperature (X_2) and co-solvent amount (X_3), with three levels for each variable, while the dependent variable was the flavonoid yield. The symbols and levels are shown in Table 1. Five replicates at the centre of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomised, to maximise the effects of unexplained variability in the observed responses, due to extraneous factors. A full quadratic equation or the diminished form of this equation, shown as follows, was used for this model:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum_{i<j} \beta_{ij} X_i X_j \tag{1}$$

where Y is the estimated response, β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively, while X_i and X_j are the independent coded variables.

Table 1
Box–Behnken design and response for the flavonoid yield of *Pueraria lobata*

Experiments	Coded levels			Response
	X_1	X_2	X_3	Flavonoid yield (mg/g)
	Pressure (MPa)	Temperature (°C)	Co-solvent amount (ml)	
1	−1 (15)	−1 (40)	0 (150)	6.8
2	+1 (25)	−1 (40)	0 (150)	8.2
3	−1 (15)	+1 (60)	0 (150)	6.1
4	+1 (25)	+1 (60)	0 (150)	6.8
5	−1 (15)	0 (50)	−1 (100)	7.6
6	+1 (25)	0 (50)	−1 (100)	6.4
7	−1 (15)	0 (50)	+1 (200)	9.5
8	+1 (25)	0 (50)	+1 (200)	10.5
9	0 (20)	−1 (40)	−1 (100)	6.2
10	0 (20)	+1 (60)	−1 (100)	8.6
11	0 (20)	−1 (40)	+1 (200)	14.7
12	0 (20)	+1 (60)	+1 (200)	14.2
13	0 (20)	0 (50)	0 (150)	16.8
14	0 (20)	0 (50)	0 (150)	16.2
15	+1 (20)	−1 (50)	0 (150)	17.3
16	+1 (20)	+1 (50)	0 (150)	16.4
17	+1 (20)	0 (50)	−1 (150)	17.0

3. Results and discussion

3.1. Effects of pressure, temperature and co-solvent amount on the flavonoid yield

The effects of pressure, temperature and co-solvent amount on the flavonoid yield of *P. lobata,* as well as their interactions, are shown in Figs. 1–3. An increasing co-solvent amount resulted in a higher extraction yield, while the flavonoid yield reached a maximum when co-solvent amount was up to a certain value, with no significantly further improvement thereafter (Figs. 1 and 3). Different effects on the flavonoid yield were shown for pressure and temperature. As shown in Figs. 1 and 2, there was an optimal value for pressure to obtain the highest flavonoid yield. Lower or higher than this value will lead to a decreased flavonoid yield. This optimal value for pressure could vary when different temperatures and co-solvent amounts were employed. Temperature had a similar effect on the flavonoid yield to pressure. The flavonoid yield increased to a certain value with elevating temperature, and thereafter decreased (Figs. 2 and 3).

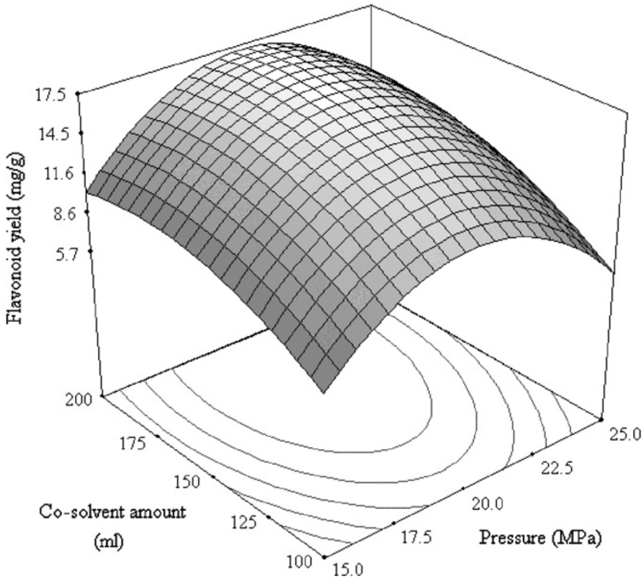

Fig. 1. Response surface plots showing effects of pressure and co-solvent amount on the flavonoid yield and their interaction. The temperature was constant at 50 °C.

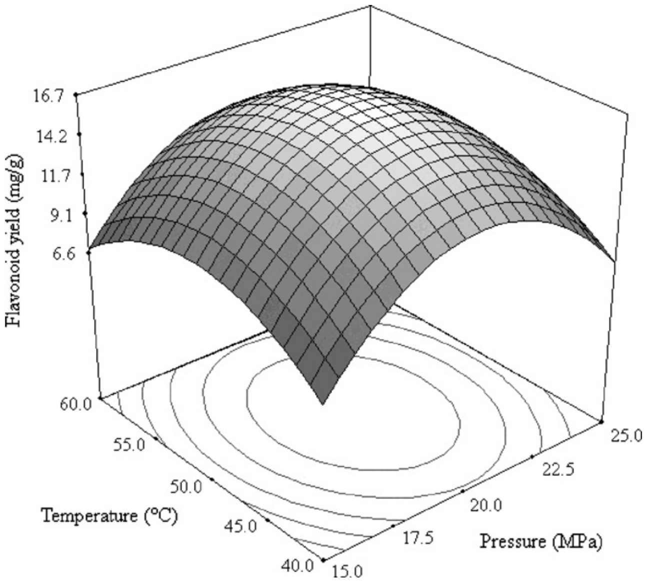

Fig. 2. Response surface plots showing effects of pressure and temperature on the flavonoid yield and their interaction. The co-solvent amount was constant at 150 ml.

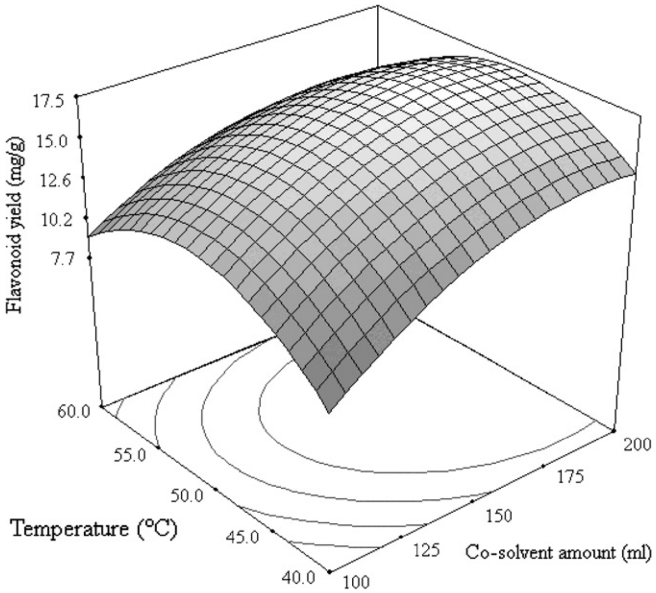

Fig. 3. Response surface plots showing effects of co-solvent amount and temperature on the flavonoid yield and their interaction. The pressure was constant at 20 MPa.

Supercritical fluid extraction has attracted a great deal of attention because this technique can considerably reduce sample preparation time and can provide analyte recovery from solid or semi-solid samples that is equal to or better than that of classical extraction techniques. A supercritical fluid is a gas-like compressible fluid at temperature and pressure greater than its critical temperature and pressure. In supercritical fluid extraction, extraction conditions are generally defined in terms of variables directly related to the relative solvent strength, which is primarily dependent on the density (Pitzer, 1955). The effect of temperature on solute solubility is different at pressures in the critical range. Near the system critical pressure, the fluid density is very sensitive to temperature. This might be the reason that flavonoid yield was changed significantly when temperature was changed over the range of 40–60 °C. A moderate increase in temperature can lead to a large decrease in fluid density, with a consequent reduction in solute solubility (Roop, Akgerman, Dexter, & Irvin, 1989). However, the increase in temperature will also accelerate mass transfer and improve the extraction yield. For a volatile solute, there is competition between its solubility in supercritical carbon dioxide and its volatility (Pourmortazavi &

Hajimirsadeghi, 2007). Therefore, it is difficult to predict the effect of temperature.

An increase of pressure can result in an increase in the fluid density, which alters solute solubility. Gomes, Mata, and Rodrigues (2007) have indicated that a higher recovery of volatile fractions and a lower recovery of non-volatile fractions are obtained at high pressure. Therefore, it is interesting to control the composition of the extract using pressure. In this study, the flavonoid yield increased with increasing pressure to a certain value. Over this range of pressure, increasing fluid density is presumably the main mechanism leading to a higher flavonoid yield. Above this range of pressure, a decreasing flavonoid yield with increasing pressure was observed. The volatility and polarity of extracted analytes might be responsible for the result.

The main requirement of a co-solvent is that it is a good solvent in its liquid state for the target analyte. The nature of the solute to be extracted is the critical basis for co-solvent choice (Walsh, Ikonomou, & Donohue, 1987). The co-solvent improves supercritical carbon dioxide extractability by increasing the polarity of the carbon dioxide. Choi, Chin, Kim, Jeon, and Yoo (1999) have observed that addition of methanol can drastically increase the extraction yield of hyoscyamine and scopolamine from plant matrices. In this study, a similar effect was shown for ethanol. It increased the polarity of the extraction fluid and greatly improved the flavonoid yield of *P. lobata*.

3.2. Model fitting

The mathematical model representing the flavonoid yield of *P. lobata* as a function of the independent variables within the region under investigation was expressed by the following equation:

$$Y = 16.54 + 0.24X_1 - 0.025X_2 + 2.51X_3 - 0.17X_1X_2 + 0.55X_1X_3 - 0.73X_2X_3 - 6.00X_1^2 - 3.57X_2^2 - 2.05X_3^2 \tag{2}$$

where Y is the flavonoid yield of *P. lobata*, and X_1, X_2 and X_3 are the coded variables for pressure, temperature and co-solvent amount, respectively.

In general, exploration and optimisation of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking the model adequacy essential (Liyana-Pathirana & Shahidi, 2005). The p-values of the model were 0.0007 (Table 2), which indicated that the model fitness was significant. Meanwhile, the lack of fit value of the model was 0.10, which was not significant.

Coefficient (r^2) of determination is defined as the ratio of the explained variation to the total variation, and is a measurement of the degree of fitness (Nath & Chattopadhyay, 2007). A small value of r^2 indicates a poor relevance of the dependent variables in the model. The model can fit well with the actual data when r^2 approaches unity (Sin, Yusof, Hamid, & Rahman, 2006). By analysis of variance, the r^2 value of this model was determined to be 0.954, which showed that the regression model defined well the true behaviour of the system.

Table 2
Analysis of variance for the response surface quadratic model for the flavonoid yield of *Pueraria lobata*

Source	Degrees of freedom	Sum of squares	Mean square	F-value	p-Value
The recovery					
Model	9	297.58	33.06	16.15	0.0007
Residual	7	14.33	2.05		
Lack of fit	3	10.86	3.62	4.17	0.1007
Pure error	4	3.47	0.87		
Total	16	311.91			

By computation, the optimal conditions to obtain the highest flavonoid yield of *P. lobata* were determined as follows: a pressure of 20.04 MPa, a temperature of 50.24 °C and 181.24 ml of co-solvent. After extraction under these optimal conditions, the flavonoid yield of *P. lobata* was 16.95 ± 0.43 mg/g, and this value was not significantly different to the predicted value of 17.30 mg/g, at the 95% confidence interval.

4. Conclusions

The high correlation of the mathematical model indicated that a quadratic polynomial model could be employed to optimise flavonoid preparation from *P. lobata* by supercritical carbon dioxide extraction. From response surface plots, three factors (pressure, temperature and co-solvent amount) significantly influenced the flavonoid yield of *P. lobata*, independently and interactively. The optimal conditions to obtain the highest flavonoid yield of *P. lobata* were determined to be 20.04 MPa, 50.24 °C and 181.24 ml of co-solvent. Under optimal conditions, the experimental values agreed with the predicted value. Thus, this methodology could provide a basis for a model to examine the non-linear nature between independent variables and response in a short-term experiment.

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