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Optimization of ultrasonic-assisted extraction of phillyrin from *Forsythia suspensa* using response surface methodology

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Phillyrin is an abundant naturally occurring lignan, which makes it a potentially important raw material for a precursor of biologically active compounds. The objective of the current study was to optimize the extraction conditions for phillyrin from *Forsythia suspensa*. The optimum conditions were evaluated with preliminary experiments and optimized using response surface methodology. Statistical analysis of results showed that the linear and quadric terms of these four variables had significant effects, and evident interaction existing between extraction time and extraction temperature was also found to contribute to the response at a significant level. The extraction conditions were further investigated with Box-Behnken design. The fitted second-order model revealed that the optimal conditions consisted of methanol concentration of 21.17%, extraction time 85.43 min, extraction temperature 66.81°C and ratio of liquid to material 11.2. Under the optimized condition, the experimental value of 0.801±0.012 mg/g was well in close agreement with the value predicted by the model. Moreover, the yield of phillyrin in the condition was higher than that of the non-optimized condition. These results help in designing the process of optimal phillyrin from *F. suspensa*.

Key words: *Forsythia suspensa*, phillyrin, optimum conditions, response surface methodology; box-behnken design.

INTRODUCTION

*Forsythia suspensa* (Thunb.) Vahl is widely distributed in China, Korea and Japan. Its fruits have long been used in Chinese and Japanese folk medicines to treat gonorrhea, erysipelas, inflammation, pyrexia and ulcer (Li and Chen, 2005; Piao et al., 2008; Qu et al., 2008; Wang et al., 2008). Moreover, it also has been shown that the extract of the fruits exhibits antioxidant activity, as well as antibacterial, antiviral, choleretic and antiemetic effects (Dai et al., 2009; Hao et al., 2010; Lu et al., 2010; Li et al., 2011). Phillyrin is one of the major effective constituents of *F. suspensa*. It plays an important role in the effect of anti-inflammatory (Ozaki et al., 1997; Ozaki et al., 2000; Li et al., 2011), antibacterial (Qu et al., 2008; Li et al., 2009), antiviral (Chattopadhyay, 2006; Chattopadhyay and Naik, 2007), antioxidant (Zhao et al., 2005; Gül et al., 2006; Qu et al., 2008), antiobesity (Hasani-Ranjbar et al., 2009) and inhibiting activity of phosphodiesterases (Li et al., 2009). Thus, phillyrin is commonly used as a chemical marker for quality control of the *F. suspensa* raw materials (Qu et al., 2008) or the derivable preparations (Cao et al., 2006). Therefore, nowadays more and more attention was cast on extraction of phillyrin in pharmaceutical, biochemical, food and cosmetic industry and so on. Extracting phillyrin from *F. suspensa* can be carried out in various ways, such as maceration extraction and supercritical fluid extraction. However, this conventional extraction technique is not always interesting for an industrial extraction or production while the process is slow or demand costly technological aids. As well-known, ultrasound extraction is the new technology that attracts much more attention in the department of separation and extraction in recent years (Dong et al., 2010; Heo and...
Kim, 2010; Riera et al., 2010; Yue et al., 2010). The application of ultrasound-assisted extraction offers many advantages, including the reduction of solvents, temperature and the time for extraction (Toma et al., 2001; Wu et al., 2001), which is very useful for the extraction of thermolabile and in unstable compounds. Ultrasonic cavitation creates shear forces that break cell walls mechanically and improve material transfer (Wu et al., 2001). So the uses of power ultrasound are considered as a potential energy assistance that can give significant effects on the rate of various chemicals and/or physical processes. Ultrasound-assisted extraction is influenced by many extraction conditions and the reported methods have a wide range of optimized conditions. For example, studies have used the frequency and power combinations of 40 kHz and 600 W, temperatures from 25 to 70°C, ration of liquid to material from 5 to 40, concentration of methanol from 0 to 80% and extraction times from 10 to 60 min (Xia et al., 2011).

However, the optimum parameters of the extraction process could not be gained by the single-factor experiment only. Thus, it is necessary to optimize the extraction process of phillyrin based on the single-factor experiments. When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process (Fu et al., 2006; Rodrigues et al., 2008; Chen et al., 2009; Lieu and Le, 2010; Virot et al., 2010). RSM is a collection of statistical and mathematical techniques that are based on the fit of the empirical model to the experimental data obtained in relation to experimental design. The optimization process of this methodology involves studying the response of the statistically designed combinations, estimating the coefficients by fitting it in a mathematical model that best fits the experimental conditions, predicting the response of the fitted model and checking the adequacy of the mode.

The common design that is, central composite design (CCD) and Box-Behnken design (BBD), of the principal RSM have been successfully applied for optimizing conditions in food and pharmaceutical research (Babar, 2007; Bezerra et al., 2008; Yang et al., 2009; Yang et al., 2010; Bazhdanzadeh et al., 2011). The main advantage of RSM is to reduce the number of experimental trials needed to evaluate multiple variables and their interactions. So it is less laborious and time-consuming than other approaches required optimizing a process. Meantime, the experimental data obtained by RSM is fitted with a second-order polynomial equation and the model gained is fitted with the experimental data. In the present study, the optimization of phillyrin production was conducted using RSM. In the preliminary experiments (Xia et al., 2011), four significant variables were further investigated by Box-benhken design in details, lastly the experimental results were analyzed and the optimal levels of the important variables were determined by using RSM compared to original extraction conditions, the optimal conditions strongly enhanced phillyrin production.

MATERIALS AND METHODS
Dried fruits of *F. suspensa* were collected from Shanxi province (China) and verified by Professor YanBing Li (Heilongjiang University of traditional Chinese Medicine, Harbin, China). Phillyrin was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was of high performance liquid chromatography (HPLC) grade and purchased from Merck (German). Methanol was of analytical grade and purchased from Hangzhou Reagent Company (Hangzhou, PR China). Water was purified by a Milli-Q academic water purification system (Millipore, Bedford, MA, USA).

Phillyrin extraction
The ultrasound-assisted extraction was carried out in a KQ-600E ultrasonic device (Changzhou Nuoj Instrument Company, China) with an ultrasound power of 600 W, heating power of 800 W and frequencies of 40 kHz, equipped with a digital timer and a temperature controller. Dried fruits of *F. suspensa* (2000 g) were ground to the particle size through a standard mesh sieve No.10. Each dried pretreated sample (20 g) was extracted by extraction solution in a designed temperature, extraction time and the ratio of liquid to material and concentration of methanol. The extraction solutions were separated from the insoluble residue by centrifugation (3000 rpm for 5 min), and then diluted into a triple volume with the extraction solution for HPLC analysis.

Chromatographic conditions
A Waters Alliance 2695 HPLC pump separation module with a Waters 2996 photodiode array detector was used. A Diamonsil C18 column (250 mm×4.6 mm, 5 μm) and an auto-injector (10 μL) were used. The mobile phase was acetonitrile and water (25:75, v/v) with a flow-rate of 1 mL/min. The Ultraviolet (UV) spectra were recorded between 190 and 400 nm for peak characterization and the detection wavelength was set at 280 nm. The column temperature was kept at 30°C. The peak area was used to calculate the amount of phillyrin from the standard curve. The chromatograms of phillyrin in standard solution and in the sample were shown in Figure 1. All the experiments were conducted in triplicate and the average values were reported.

Experimental design and statistical analysis
The effects of extraction conditions on yield of phillyrin such as extraction temperature, extraction time, the ratio of liquid to raw material and methanol concentration have been investigated by the single-factor method (Xia et al., 2011). Based on the preliminary results, the four major influence factors were confirmed and then a RSM was conducted to design experimental project. As shown in Table 1, the four factors chosen for this study were designated as $X_1$, $X_2$, $X_3$ and $X_4$ and prescribed into three levels, coded $+1$, $0$, $-1$ for high, intermediate and low value, respectively. The four variables were coded according to the following equation:

$$X_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1 - 4$$

(1)
Figure 1. Chromatograms of phillyrin in standard solution and in the sample.

Table 1. Variables and experimental design levels for response surface.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Coded symbols</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of methanol</td>
<td>$X_1$</td>
<td>-1</td>
</tr>
<tr>
<td>Extraction time</td>
<td>$X_2$</td>
<td>-1</td>
</tr>
<tr>
<td>Extraction temperature</td>
<td>$X_3$</td>
<td>-1</td>
</tr>
<tr>
<td>Ratio of liquid to material</td>
<td>$X_4$</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
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<td>60</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

where $X_i$ was a coded value of the variable; $x_i$ was the actual value of the variable; $x_0$ was the actual value of the independent variable at the center point; and $\Delta x$ was the step change of the variable. A second-order polynomial model corresponding to the BBD was fitted to correlate the relationship between the independent variables and the response (yield of phillyrin) to predict the optimized conditions. The non-linear computer-generated quadratic model was given as:
Where \( Y \) was the measured response associated with each factor level combination; \( \beta_0 \) was an intercept; \( \beta_i \) was regression coefficients computed from the observed experimental values of \( Y \); and \( X_i \) was the coded levels of independent variables. The terms \( X_i X_j \) and \( X_i^2 \) represented the interaction and quadratic terms, respectively. Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (version 7.0, Stat-Ease, Inc., Minneapolis, USA). Design-Expert 7.0, trial version was used for the analysis of variance (ANOVA) of the experimental data obtained.

The quality of the fit of the polynomial model equation was expressed by the coefficient of determination \( R^2 \) and the values of adjusted-\( R^2 \) of models were evaluated to check the model adequacies. The significance of each term in the equation was to estimate the goodness of fit in each case. The analysis of variance tables was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined.

To determine whether or not the quadratic model was significant, the experimental data were statistically analyzed using the Design-expert 7.0 package for ANOVA and the results were shown in Table 3. The P-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the P-values were, the bigger the significance of the corresponding coefficient (Li et al., 2011). Here, the P-value of the model was smaller than 0.0001, which indicated that the model was suitable for use in this experiment. The P-value of "lack of fit" was 0.0765 (P>0.05), which indicated that "lack of fit" was insignificant relative to the pure error. The values of the determination coefficient and the adjusted determination coefficient showed the model was significant. At the same time, a relatively lower value of the coefficient of variation indicated a better precision and reliability of the experiments carried out. The regression coefficients and the corresponding P-values were presented in Table 4. From the P-values of each model term, it could be concluded that all the independent variables studied (\( X_1, X_2, X_3, X_4 \)) and four quadratic terms (\( X_1^2, X_2^2, X_3^2, X_4^2 \)) significantly affected the phillyrin yield. The analysis showed there were significant interactions between extraction temperature and time too. The results of the study also showed that the extraction temperature and time were the most significant parameters which influenced phillyrin yield followed by concentration of methanol and ratio of liquid to raw material.

The p-values of less than 0.05 were considered to be statistically significant. The regression coefficients were then used to make statistical calculations to generate contour and dimensional maps from the regression models.

**RESULTS AND DISCUSSION**

**The model fitting and statistical analysis**

Response surface optimization is more advantageous than the traditional single parameter optimization is that it saves time, space and raw material. The 27 designed experiments were conducted for optimizing the four individual parameters in the current Box-Behnken design. Table 2 showed the experimental conditions and the results of phillyrin yield according to the factorial design. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation:

\[
Y = \beta_0 + \sum_{i=0}^{4} \beta_i X_i + \sum_{j=0}^{4} \beta_{ij} X_i^2 + \sum_{i=0}^{4} \sum_{j=0}^{4} \beta_{ij} X_i X_j
\]

(2)

\[
Y=-0.18792+2.51831E-003* X_1+1.89875E-003* X_2+4.18517E-003* X_3+3.49170E-003* X_4-2.80287E-006* X_1* X_2-1.54446E-005* X_1* X_3-1.89875E-003* X_2* X_3+3.63402E-006* X_2* X_4+4.56150E-006* X_3* X_4-2.99241E-005* X_1^2-7.38810E-006* X_2^2-2.41281E-005* X_3^2-1.57376E-004* X_4^2
\]

(3)

**Analysis of response surface**

Response surfaces were plotted using Design-expert version 7.0 software to study the effects of parameters and their interactions on phillyrin yield. Three-dimensional response surface plots, as presented in Figure 2 to 7, were very useful to see interaction effects of the factors on the responses. These types of plots showed effects of two factors on the response at a time. In all the presented figures, the other two factors were kept at level zero. As shown in Figure 2a, it can be concluded the three-dimensional plot of the response surface for the phillyrin yield as related to concentration of methanol and extraction time with extraction temperature and ratio of liquid to raw material set at 60°C and 10%, respectively. Extraction time exhibited an important effect on the phillyrin yield, which did not continue to increase significantly until the extraction time was over 80 min. Although prolonging the contact time led to a slight increase in the phillyrin yield, further increases in extraction time to 90 min, resulted in little change in the yield of phillyrin. With respect to concentration of methanol, the influence of this parameter was not as significant as that of extraction time. As shown in this figure, when the concentration of methanol was increased, the amount of phillyrin extracted increased at first, but began to decrease when the concentration of methanol surpassed 21%. Additional, Figure 2b represented that the contour lines were parallel with the
Table 2. The Box-Behnken experimental design with four independent variables.

<table>
<thead>
<tr>
<th>No.</th>
<th>X₁ (concentration of methanol)</th>
<th>X₂ (extraction time)</th>
<th>X₃ (extraction temperature)</th>
<th>X₄ (ratio of liquid to material)</th>
<th>Y (extraction yield mg/g)</th>
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<tr>
<td>1</td>
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<td>30</td>
<td>60</td>
<td>10</td>
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<td>90</td>
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<td>5</td>
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<td>0.70137</td>
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<td>60</td>
<td>60</td>
<td>10</td>
<td>0.716</td>
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</table>

Table 3. Analysis of variance for the fitted quadratic polynomial model of optimization of phillyrin extraction from F. suspense.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-Value</th>
<th>Probability (P)&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>2.852E-003</td>
<td>14</td>
<td>2.037E-004</td>
<td>56.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>4.283E-005</td>
<td>10</td>
<td>4.283E-006</td>
<td>12.47</td>
<td>0.0765</td>
</tr>
<tr>
<td>Pure error</td>
<td>6.867E-007</td>
<td>2</td>
<td>3.433E-007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation total</td>
<td>2.896E-003</td>
<td>26</td>
<td></td>
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</tr>
<tr>
<td>R²</td>
<td>0.9850</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Adj R²</td>
<td>0.9674</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

axis of concentration of methanol, which indicated that insignificant interaction exists between these two variables. As expected, a greater increase in phillyrin yield resulted when the extraction temperature was increased in the range from 30 to 90°C. The temperature curve did not level off at 90°C, which may indicate that a slightly higher temperature is required to achieve the maximum increase (Figure 3b). However, when the extraction temperature was unchanged, the extraction rate of phillyrin rose and then declined as the methanol concentration increased in the range from 10 to 30% (Figure 3a). It was insignificant that the increase of methanol concentration affected the extraction rate of phillyrin at certain extraction temperature. In addition, the contour curves directly exhibited little interactions exist between the extraction temperature and methanol.
Table 4. Regression coefficients and their significance of the quadratic model of optimization of phillyrin extraction from *F. suspense*.

<table>
<thead>
<tr>
<th>Model term</th>
<th>Coefficient estimate</th>
<th>Degree of freedom</th>
<th>Standard error</th>
<th>Probability (P)&gt;F</th>
</tr>
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<tbody>
<tr>
<td>Intercept</td>
<td>0.071</td>
<td>1</td>
<td>1.099E-003</td>
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</tr>
<tr>
<td>X(_1)</td>
<td>2.445E-003</td>
<td>1</td>
<td>5.497E-004</td>
<td>0.0008***</td>
</tr>
<tr>
<td>X(_2)</td>
<td>0.013</td>
<td>1</td>
<td>5.497E-004</td>
<td>&lt; 0.0001***</td>
</tr>
<tr>
<td>X(_3)</td>
<td>5.451E-003</td>
<td>1</td>
<td>5.497E-004</td>
<td>&lt; 0.0001***</td>
</tr>
<tr>
<td>X(_4)</td>
<td>2.180E-003</td>
<td>1</td>
<td>5.497E-004</td>
<td>0.0019**</td>
</tr>
<tr>
<td>X(_1)X(_2)</td>
<td>-8.409E-004</td>
<td>1</td>
<td>9.521E-004</td>
<td>0.3945</td>
</tr>
<tr>
<td>X(_1)X(_3)</td>
<td>-1.544E-003</td>
<td>1</td>
<td>9.521E-004</td>
<td>0.1307</td>
</tr>
<tr>
<td>X(_1)X(_4)</td>
<td>9.022E-005</td>
<td>1</td>
<td>9.521E-004</td>
<td>0.9261</td>
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<tr>
<td>X(_2)X(_3)</td>
<td>-2.407E-003</td>
<td>1</td>
<td>9.521E-004</td>
<td>0.0265*</td>
</tr>
<tr>
<td>X(_2)X(_4)</td>
<td>-5.451E-004</td>
<td>1</td>
<td>9.521E-004</td>
<td>0.5775</td>
</tr>
<tr>
<td>X(_3)X(_4)</td>
<td>2.281E-004</td>
<td>1</td>
<td>9.521E-004</td>
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</tr>
<tr>
<td>X(_1)^2</td>
<td>-2.992E-003</td>
<td>1</td>
<td>8.246E-004</td>
<td>0.0035**</td>
</tr>
<tr>
<td>X(_2)^2</td>
<td>-6.649E-003</td>
<td>1</td>
<td>8.246E-004</td>
<td>&lt; 0.0001***</td>
</tr>
<tr>
<td>X(_3)^2</td>
<td>-2.413E-003</td>
<td>1</td>
<td>8.246E-004</td>
<td>0.0127*</td>
</tr>
<tr>
<td>X(_4)^2</td>
<td>-3.934E-003</td>
<td>1</td>
<td>8.246E-004</td>
<td>0.0005***</td>
</tr>
</tbody>
</table>

*Significant at p<0.05; **significant at p<0.01; *** significant at p<0.001.

Figure 2. Response surface plot and contour plot of concentration of methanol and extraction time and their mutual interactions on the yield of phillyrin.

Moreover, It is shown in Figure 4a that the extraction rate of phillyrin increased with the methanol concentration and ratio of liquid to material increasing in the beginning. However, when they were at a certain value, the extraction rate of phillyrin dropped. The best point of balance should be sought for the
maximum extraction rate of phillyrin between methanol concentration and ratio of liquid to material. The interactions between methanol concentration and ratio of liquid to material are shown in Figure 4b. The contour curves were parallel with either of the axes, which implied there were no interactions between the two variables. Figure 5a showed the effects of extraction time and extraction temperature on the phillyrin yield at the fixed methanol concentration of 20% and ratio of liquid to material of 10. It was observed that the yield of phillyrin significantly increased with increasing extraction time at a given temperature, especially at low time and temperature. If the given temperature was higher than a certain value, while time was extended, the phillyrin yield increased at low-time levels, but once the time reached the high levels, the phillyrin yield slightly decreased. This can be explained that it may induce more chemical reactions on the longer extraction time and then cause oxidative conversion of phillyrin. In addition, elliptic contours in Figure 5b indicated there were striking interactions between the two variables. It is shown in Figure 6a that when the ratio of liquid to material was at a certain value, the extraction rate of phillyrin obviously increased with the extraction
time added from 30 to 90 min. In addition, when the extraction time was 30 min, the extraction rate of phillyrin increased as the ratio of liquid to material increased. However, when the extraction time was 90 min, the extraction rate of phillyrin did not increase and slightly fell as the ratio of liquid to material increased. It was concluded that extraction time affected significantly on extraction rate of phillyrin again. Additional, Figure 6b showed the contour lines were parallel with the axis of the ratio of liquid to material, which indicated that no interaction existed between these two variables. Figure 7a presented the effects of temperature and ratio of liquid to material on phillyrin yield. At a fixed ratio of liquid to material, the phillyrin yield increased rapidly when the temperature was raised, which also implied that phillyrin yield was significantly influenced by temperature. The phillyrin yield increased when the ratio of liquid to material was rapidly increased from 5 to 11, but not increased when the ratio of liquid to material continued to be increased. This could be explained that most of phillyrin had been excessively dissolved when the ratio was 11 and only a small portion retained in the fruiting bodies of *F. suspensa*. Therefore, the yield of phillyrin could not greatly change with the
ratio of liquid to material increased from 11 to 15. Furthermore, the contour lines were parallel with the ratio of liquid to material of axis (Figure 7b); it revealed that little interaction existed between the two variables.

Optimization of extracting parameters and validation of the model

In this study, the aim of optimization was to find the conditions, which gave the maximum yield of phillyrin. By solving the regression equation and analyzing the response surface plots, the optimum extraction conditions (concentration of methanol 21.16%, extraction time 85.45 min, extraction temperature 66.81°C and ratio of liquid to raw material 11.19) were estimated. The predicted extraction yield was given by the Design Expert 7.0 software under the above conditions was 0.792 mg/g. However, considering the operability in actual production, the optimal conditions can be modified as follows: concentration of methanol 21%, extraction time 85 min, extraction temperature 67°C and ratio of liquid to raw material 11. Under the modified conditions, the experimental yield of phillyrin was 0.801±0.012 mg/g (N = 3), which was close to the predicted value, indicating that the model was adequate for the extraction process (Table 5). Compared with the single-factor test (Xia et al., 2011), the yield of phillyrin increased from 0.713±0.009 to 0.801±0.012 mg/g. Meantime, the extraction time changed from 60 to 85 min whereas other three variables were no significant different. From the perspective of extraction yield, the extraction conditions gained

Figure 5. Response surface plot and contour plot of extraction time and extraction temperature and their mutual interactions on the yield of phillyrin.
from RSM were more attractive. In comparison with the conventional reflux extraction method (Hu and Yao, 2005), the extraction yield with the ultrasonic method with 85 min was higher than that of reflux extraction with 120 min, indicating the higher efficiency of the ultrasound-assisted extraction. These results showed the optimization of ultrasonic extraction technique by RSM was one of the suitable methods to optimize the operating conditions and maximize the yield of phillyrin.

In summary, RSM proved to be accurate in predictive modeling and optimization of conditions for the yield of phillyrin and the yield under the extraction conditions was great higher than that of the non-optimized condition. In addition, ultrasound had the potential to be used in phillyrin extraction to improve efficiency and reduce time. Therefore, this process can be considered as a sustainable alternative for the industry since it allowed simplified handling, time reduction and the quantity of targeted extracts to be improved.

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Figure 7. Response surface plot and contour plot of extraction temperature and ratio of liquid to material and their mutual interactions on the yield of phyllrin.

Table 5. Predicted and experimental values of the responses at optimum conditions.

<table>
<thead>
<tr>
<th>Concentration of methanol (%)</th>
<th>Extraction time (min)</th>
<th>Extraction temperature(°C)</th>
<th>Ratio of liquid to raw material</th>
<th>Extraction yield (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.17</td>
<td>85.43</td>
<td>66.81</td>
<td>11.20</td>
<td>0.801±0.012</td>
</tr>
</tbody>
</table>

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REFERENCES


