Optimum Extraction of Flavonoids from Broccolini Leaves Using Response Surface Methodology

Bingfang WANG,1 Shang-Tian YANG1,2* and Xuewu ZHANG1*

1College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, China
2William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, 140 West 19th Avenue, Columbus, Ohio 43210 US

*To whom correspondence should be addressed: Prof. XW Zhang (snow_dance@sina.com) and Prof. ST Yang (yangst.ohio.state.university@gmail.com), College of Light Industry and Food Sciences, South China University of Technology, 381 Wushan Road, Guangzhou 510640, China. Tel: 86 20 87110840; Fax: 86 20 87110840.

(Received December 30, 2010; Accepted January 17, 2011)

Broccolini (Brassica oleracea Italica × Alboglabra) is a hybrid of broccoli and kai-lan, Chinese broccoli. To date, no information is available on the extraction of flavonoids from Broccolini. In this study, an efficient ultrasonic-assisted extraction technique was developed to extract flavonoids from Broccolini leaves. The operating parameters were optimized using Box-Behnken design combined with response surface method. The analysis of variance showed that the response surface model fits very well with the actual situation. Based on the response model, the optimal conditions were determined as extraction temperature 60 °C, ethanol concentration 70% and solid to liquid ratio 1:27. Under the optimal conditions, the maximum response value of yield (11.96 mg/g dry weight) was consistent with the experimental value (11.95 mg/g dry weight), demonstrating that large amounts of flavonoids can be efficiently extracted from Broccolini leaves using ultrasonic-assisted method although the process needs further optimization along with the development of purification method for food applications.

1. Introduction

Broccolini (Brassica oleracea Italica × Alboglabra) is a green vegetable similar to broccoli but with small florets and long, thin stalks. Although often misidentified as young broccoli, it is a cross between broccoli and kai-lan, Chinese broccoli. It is also known by the names Asparation, Bimi, Baby broccoli, Broccoletti and Tender Stem. Nutritionally, broccolini is high in some active ingredients such as vitamins (A, C), folate, flavonoids and gluosinolates.

Flavonoids are a large group of naturally occurring phenolic compounds ubiquitously distributed across the plant kingdom, and have many positive impacts on human health including antioxidation, anti-inflammatory, anti-cancer, preventing atherosclerosis and anti-microbial activity [1].

Conventional extraction as heating, boiling, or refluxing can be used to extract flavonoids, however, the disadvantages are the loss of flavonoids due to ionisation, hydrolysis and oxidation during extraction as well as the long extraction time [2]. In recent years, various novel extraction techniques have been developed for the extraction of nutraceuticals from plants, including ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction [3]. Among these, ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques [4]. Ultra-sonic cavitation creates shear forces that break cell walls mechanically and improve material
transfer. The application of ultrasound-assisted extraction offers many advantages including the reduction of solvents, temperature and the time for extraction, which is very useful for the extraction of thermolabile and unstable compounds [5].

In the present study, ultrasound-assisted extraction for the flavonoids from broccolini leaves were investigated and the Box-Behnken design combined with response surface methodology were conducted to optimize the operational parameters for further development and application of the resource.

2. Experimental

2.1 Materials and chemicals

Broccolini was bought from Guangzhou Research Institute of Vegetables (Guangzhou, China). The dried leaf was ground in a cutting mill to pass through 80-mesh sieve to obtain fine powder, which was stored in a well-closed container for further use. Rutin was purchased Sigma Chemicals Co. Other chemicals were purchased from China national Medicine Group Shanghai Corporation (Shanghai, China). All chemicals and solvents used were of analytical grade.

2.2 Extraction of Broccolini leaves flavonoids

The dried leaf powder samples (1.0 g) were extracted for flavonoids by ultrasonic-assistance under the designed conditions. When different solvents (water, ethanol and methanol) were used, other extraction conditions were fixed at: pH=4, solid to liquid ratio 1:30, ultrasonic power 180 W (25 Hz), water bath temperature 60 °C and extraction time 60 min. After ethanol was finally selected as extraction solvent, its concentration was changed from 40%-80%, other conditions were adjusted to ultrasonic power 180 W, pH=4, solid to liquid ratio 1/25, water bath temperature 60 °C and extraction time 60 min. Similarly, when varying solid to liquid ratio (1/10, 1/15, 1/20, 1/25, and 1/30) or extraction temperature (40, 50, 60, 70 and 80 °C), other conditions should be made corresponding alteration. Then, the extracted solution was vacuum filtrated through 0.45 μm microporous membrane, and the filtrates were combined and concentrated using a rotary evaporator at 40 °C.

Based on the single factor experiments above, three major factors were involved including temperature, ethanol concentration and solid to liquid ratio. Then, a Box-Behnken design was employed to fit a second order polynomial model which indicated 17 experiments to be required for this procedure. The general equation of the second degree polynomial equation is

\[ Y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2 \]

where \( Y \) is the predicted response; \( x_1, x_2 \) and \( x_3 \) correspond to temperature, ethanol concentration and solid to liquid ratio, respectively; \( B_i (i=1,2,3) \) is the ith linear coefficient; \( B_{ii} (i=1,2,3) \) is the quadratic coefficient and \( B_{ij} (i \neq j) \) is the linear-by-linear interaction between \( x_i \) and \( x_j \).

The test variables were transformed to range between −1 and 1 for the appraisals of factors. The variables were coded according to the equation:

\[ X_i = (X_i - X_0)/\Delta X_i \]

where \( x_i \) is the independent variable coded value. \( X_i \) is the independent variable real value. \( X_0 \) is the independent variable real value at the centre point and \( \Delta X_i \) is the step change of the real value of the variable ‘i’ corresponding to a variation of a unit for the dimensionless value of the variable ‘i’. The actual and coded levels of the independent variables used in the experimental design are shown in Table 1.

2.3 Determination of total flavonoids

The total flavonoids content in the extracts was determined using a colorimetric method (NaNO₂-Al(NO₃)₃-NaOH...
system) with minor modification [6]. Preparation of standard curve: 0,0.5,1.0,1.5,2.0 and 2.5 mL rutin standard solution (0.2mg/mL) were added into separate volumetric flask (10mL). Sodium nitrite of 0.3 mL 5% and 0.3 mL 10% aluminum nitrate were added, respectively. After fully mixing and standing for 6min, 4mL 4% sodium hydroxide was added. Each flask was finally diluted with ethanol (60%) to a defined volume (10mL). The absorbance of each flask was examined at 510 nm with 1mL blank solution as contrast. The regression equation of rutin standard curve obtained as Y = 0.087x – 0.0005 (R2 = 0.9991) (linear range 0.01~0.05 mg/mL), where Y is the absorbance, x is the flavonoid content (mg/mL). Using the method of standard curve preparation, 1mL of prepared sample was added to measure the flavonoids content of extract sample. The absorbance of the mixture a 510 nm was measured immediately in comparison to a standard curve prepared by rutin. The yield of flavonoids was expressed as mg rutin equivalents per g dry weight.

2.4 Statistical analysis

All experimental results were centered at using three parallel measurements of mean ±SD. Design Expert software package (version 5.0.7, State-Ease Inc., Minneapolis, MN, USA) was used to alalyze the experiemntal data. Analysis of variance was performe by ANOVA procedure. P values<0.05 were regarded as significant and P values<0.01 as very significant, i.e. the variables corresponding to the coefficients have significant or very significant effects on flavonoids extraction, respectively.

3. Results and Discussion

3.1 Single factor experiment

Firstly, we studied the effect of three solvents (water, methanol and ethanol) on extraction yield of flavonoids, while other extraction conditions were ultrasonic power 180 W, ratio of solid/liquid (g/mL) 1:30, extraction time 60 min and water bath temperature 60 °C. It can be seen from Figure 1A that the extraction yield of flavonoids is the minimum (<4 mg/g) using water as solvent, and more than 2-fold increase (>8 mg/g) in the extraction yield of flavonoids using ethanol or methanol as solvent. Probably this is due to the fact that flavonoids are presented primarily in the form of glycosides or agycone, which are easily dissolved by organic solvents [7]. In terms of non-toxicity and economy, ethanol was chose as extraction solvent in the subsequent experiments.

To investigate the effect of ethanol on extraction yield of flavonoids, different concentrations of ethanol v/v (40, 50, 60, 70 and 80%) were applied, other extraction conditions were ultrasonic power 180 W, ratio of solid/liquid (g/mL) 1:25, extraction time 60 min and water bath temperature 60 °C. Figure 1B showed that the extraction yield of flavonoids increased with the concentration of ethanol. In consideration of the fact that higher concentration of ethanol is more easily volatile (lower boiling point) and more impurities will be taken out together, a suitable concentration of ethanol 70% was used in late experiments.

The ratio of soil to liquid can influence the dissolution of flavonoids to aqueous ethanol. A suitable volume of ethanol solution will make flavonoids fully dissolved out from plants [8]. In present study, different solid to liquid ratios (g/mL) (1:10, 1:15, 1:20, 1:25 and 1:30) were applied to study their effects on extraction yield of flavonoids, while other extraction conditions were ultrasonic power 180 W, ethanol concentration 70% (v/v), extraction time 60 min and water bath temperature 60 °C. As shown in Figure 1C, when the solid to liquid ratio increased from 1:10 to 1:20 (g/mL), the extraction yield of flavonoids rose rapidly, and then rose steadily from 1:20 to 1:30 (g/mL). This is probably due to that with the increase of solid to liquid ratio, the contact area between material and solvent also increased, making flavonoid more fully dissolved out from material. However, higher solid to liquid ratio will enable more impurities like polysaccharide and proteins dissolved out, which will hinder the dissolution of flavonoids and take more time and energy for further
concentration [9]. Therefore, a moderate solid to liquid ratio 1:25 (g/mL) was selected for other experiments.

Figure 1. Effect of single factors on extraction yield of flavonoids, (A) different solvents, (B) ethanol concentrations, (C) solid to liquid ratio, and (D) temperature. The number of independent experiments n=3.

Extraction temperature is one of the important factors affecting extraction yield of flavonoids. High temperature can increase molecular movement, hence to make flavonoids more rapidly dissolved from plant cell. In order to investigate the effect of different extraction temperatures on the yield of flavonoids, five temperatures (40, 50, 60, 70 and 80 °C) were set, while other extraction conditions were ultrasonic power 180 W, ethanol concentration 70% (v/v), extraction time 60 min and solid to liquid ratio 1:25 (g/mL). Figure 1D indicated that the extraction yield increase rapidly with the increase of temperature from 40 to 60 °C, and the maximum yield was obtained at 60 °C. After this, the extraction yield dropped from 60 to 80 °C. This could be interpreted by the fact that molecular movement will accelerate as temperature rises, making the solubility rise and leading to the increase of yield. However, if the temperature is too high, it could oxidize flavonoids and dissolve out more impurities [10]. Thus, the optimal temperature was 60 °C.

3.2 Optimization of extraction process

The experimental screening performed was designed to assess the influence of three factors: the ethanol concentration, temperature and solid to liquid ratio. The extraction yield of flavonoids was employed as a response value. Based on a Box-Behnken design, all experimental data obtained from 17-run-experiment was shown in Table 1. By model fitting, the response surface analysis model was established as follows:

\[ Y = 8.71 + 0.40x_1 + 2.68x_2 + 0.43x_3 + 0.58x_1x_2 + 0.021x_1x_3 + 0.37x_2x_3 - 0.26x_1^2 - 0.28x_2^2 - 1.1x_3^2 \]
where $Y$ is the flavonoid yield of Broccolini leaves, and $x_1$, $x_2$ and $x_3$ are the coded variables for temperature, ethanol concentration and solid to liquid ratio, respectively. The predicted data from the response surface analysis model were also shown in Table 1.

Table 1. Coded and real levels of the operational parameters and observed and predicted values for different levels of experimental design

<table>
<thead>
<tr>
<th>Runs</th>
<th>Temperature ($^\circ$C) ($x_1$)</th>
<th>Ethanol concentration (%) ($x_2$)</th>
<th>Solid to liquid ratio (g/mL) ($x_3$)</th>
<th>Experimental yield (mg/g)</th>
<th>Predicted yield (mg/g) ($Y$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1(50)</td>
<td>0(60)</td>
<td>-1(1/20)</td>
<td>6.72</td>
<td>6.47</td>
</tr>
<tr>
<td>2</td>
<td>-1(50)</td>
<td>-1(50)</td>
<td>0(1/25)</td>
<td>5.40</td>
<td>5.65</td>
</tr>
<tr>
<td>3</td>
<td>-1(50)</td>
<td>1(70)</td>
<td>0(1/25)</td>
<td>9.80</td>
<td>9.86</td>
</tr>
<tr>
<td>4</td>
<td>-1(50)</td>
<td>0(60)</td>
<td>1(1/30)</td>
<td>7.36</td>
<td>7.29</td>
</tr>
<tr>
<td>5</td>
<td>1(70)</td>
<td>-1(50)</td>
<td>0(1/25)</td>
<td>5.38</td>
<td>5.31</td>
</tr>
<tr>
<td>6</td>
<td>1(70)</td>
<td>0(60)</td>
<td>-1(1/20)</td>
<td>7.17</td>
<td>6.56</td>
</tr>
<tr>
<td>7</td>
<td>1(70)</td>
<td>0(60)</td>
<td>1(1/30)</td>
<td>7.90</td>
<td>8.14</td>
</tr>
<tr>
<td>8</td>
<td>1(70)</td>
<td>1(70)</td>
<td>-1(1/20)</td>
<td>8.96</td>
<td>9.15</td>
</tr>
<tr>
<td>9</td>
<td>0(60)</td>
<td>1(70)</td>
<td>0(1/25)</td>
<td>12.07</td>
<td>11.82</td>
</tr>
<tr>
<td>10</td>
<td>0(60)</td>
<td>0(60)</td>
<td>0(1/25)</td>
<td>8.75</td>
<td>8.71</td>
</tr>
<tr>
<td>11</td>
<td>0(60)</td>
<td>0(60)</td>
<td>0(1/25)</td>
<td>8.64</td>
<td>8.71</td>
</tr>
<tr>
<td>12</td>
<td>0(60)</td>
<td>0(60)</td>
<td>0(1/25)</td>
<td>8.81</td>
<td>8.71</td>
</tr>
<tr>
<td>13</td>
<td>0(60)</td>
<td>-1(50)</td>
<td>-1(1/20)</td>
<td>4.54</td>
<td>4.54</td>
</tr>
<tr>
<td>14</td>
<td>0(60)</td>
<td>0(50)</td>
<td>0(1/25)</td>
<td>8.67</td>
<td>8.71</td>
</tr>
<tr>
<td>15</td>
<td>0(60)</td>
<td>1(70)</td>
<td>1(1/30)</td>
<td>10.75</td>
<td>10.76</td>
</tr>
<tr>
<td>16</td>
<td>0(60)</td>
<td>-1(50)</td>
<td>1(1/30)</td>
<td>4.83</td>
<td>4.65</td>
</tr>
<tr>
<td>17</td>
<td>0(60)</td>
<td>0(60)</td>
<td>0(1/25)</td>
<td>8.68</td>
<td>8.71</td>
</tr>
</tbody>
</table>

In general, exploration and optimisation of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit. A reliable way to evaluate the quality of the model fitted is the application of analysis of variance (ANOVA), which is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses [11]. It can be seen from Table 1 that the greater similarity of the experimental and predicted values indicates the accuracy of prediction of model for flavonoids extraction yield. The ANOVA results shows that experimental data for flavonoids yield has correlation coefficient ($R^2$) of 0.9949 with the calculated model. The calculated model is also able to explain 98.84% of the results in the case of flavonoids extraction yield. In addition, statistical analysis gave high significant level ($p<0.0001$), attesting the goodness of fit of the model. These suggest that the model could work well for the prediction of flavonoids extraction yield from Broccolini leaves by ultrasonic-assisted method.

The statistical analysis also indicated that some operational parameters are significant ($p<0.05$) for the extraction, such as the linear terms temperature $x_1$ ($p=0.0014$) and solid to liquid ratio $x_3$ ($p=0.001$), the quadratic terms temperature $x_1^2$ ($p=0.0455$) and ethanol concentration $x_2^2$ ($p=0.0379$), and the interactive terms between temperature and ethanol
concentration x1x2 (p=0.0013) and between ethanol concentration and solid to liquid ratio x2x3 (p=0.0124). In particular, the linear term ethanol concentration x2 (p<0.0001) and the quadratic term solid to liquid ratio x32 (p<0.0001) are highly significant. But the interactive temperature with solid to liquid ratio x1x3 (p=0.8570) is non-significant. The results suggest that the changes of ethanol concentration had a very significant effect on flavonoid extraction yield of Broccolini leaves.

To investigate how the operational parameters interactively affect flavonoids extraction, the two-dimensional contour and three-dimensional response surface plots of multiple non-linear regression model were depicted in Figure 2. In all the presented figures, the other independent variable was kept at its zero level. These types of plots reflected the effects of two variables on the response value at a time.

Figure 2A

Figure 2B
Figure 2C

Figure 2D
Figure 2. Response surface plots showing interaction effects of independent variables on extraction yield, (A)–(B) ethanol concentration and temperature, (C)–(D) temperature and solid to liquid ratio, (E)–(F) ethanol concentration and solid to liquid ratio. The number of independent experiments n=3.

The optimal conditions obtained using the response surface model were as follows: extraction temperature 60 °C, ethanol concentration 70% and solid to liquid ratio 1:26.80 (adjusted to 1:27). Under the optimal conditions, the maximum
response value of yield was predicted to be 11.96 mg/g by the model. To compare the predicted result with the practical value, experimental validation was performed using the optimized conditions. A mean value of 11.95±1.32 mg/g (n=3) was observed from real experiments, which is in close agreement with the predicted value and is higher than the value in the extraction without ultrasonication (9.83 mg/g). The good correlation between these results confirmed that the response surface model was adequate to reflect the expected optimization of flavonoids extraction process from Broccolini leaves. Similar results are obtained in the flavonoids extraction from other plants by ultrasonic-assisted method, such as 1.87% of flavonoids yield from the flower of Citrus aurantinum [5] and 12.09 mg flavonoids/g citrus peel [12].

4. Conclusion

An optimized ultrasonic-assisted extraction method of flavonoids from Broccolini leaves has been developed. This is the first report on the extraction of flavonoids from Broccolini leaves. Under the optimal conditions obtained by response surface method (extraction temperature 60 °C, ethanol concentration 70% and solid to liquid ratio 1:27), the predicted yield (11.96 mg/g) was consistent with the experimental yield (11.95 mg/g). In summary, this study demonstrated that large amounts of flavonoids can be efficiently extracted from Broccolini through ultrasonic-assisted extraction although the process needs further optimization along with the development of purification method for food applications.

References