Preparing Glabridin-in-Water Nanoemulsions by High Pressure Homogenization with Response Surface Methodology

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Abstract: Glabridin is a pharmacological active hydrophobic pyranoisoflavan isolated from licorice. It has low bioavailability and solubility and therefore is difficult to apply for industry use. We investigated the effect of combining caprylic triglyceride with glabridin (2-6%, w/w), emulsifier (3-7%, w/w), and homogenization pressure (70-130 MPa) on the droplet size of glabridin nanoemulsions using response surface methodology by a 3-factor-3-level Box-Behnken design. Oil content, emulsifier content and pressure had a significant effect on droplet size (p < 0.05). The optimal conditions for preparing glabridin nanoemulsions were predicted to be caprylic triglyceride (oil content), 3.7%; emulsifier content, 5.3%, and homogenization pressure, 129 MPa.

Key words: Glabridin, nanoemulsions, response surface methodology, high pressure homogenization

1 INTRODUCTION

Glabridin is a pyranoisoflavan isolated from licorice. It has multiple pharmacological activities such as cytotoxic activity[1] and antimicrobial activity against *Helicobacter pylori* [2], and methicillin-resistant *Staphylococcus aureus* [3]. It has estrogenic and antiproliferative activity in human breast cancer cells [4,5] and inhibits melanogenesis [6], inflammation [7], low-density lipoprotein oxidation [8], human cytochrome P450s 3A4, 2B6 and 2C9 activities [9], and nephritis [10]. It protects mitochondrial functions against oxidative stress [11]. Glabridin is the main compound in the hydrophobic fraction of licorice extract but is susceptible to oxidative degradation, which results in low bioavailability and loss of pharmacological activities under normal storage conditions. It is also insoluble in water at neutral pH [12,13]. Therefore, emulsification technology maybe a good method to solubilize, encapsulate and protect this component.

Nanoemulsions are emulsion systems in the range of 50 to 200 nm. Because of their small droplet size, nanoemulsions appear transparent or translucent to the naked eye and possess good stability against sedimentation, coalescence, creaming and Ostwald ripening [14-16]. The character-
emulsion, as well as the texture, greatly depends on the droplet size distribution, which can be adjusted by controlling the rate of droplet breakage and coalescence during emulsion formation. This control requires knowledge of the effect of operating parameters and formulation of the emulsion on droplet breakage and coalescence. Furthermore, the emulsification conditions significantly affect the quality of the formed nanoemulsions and ought to be systematically studied. Several publications have explored the parameters of emulsification on preparing nanoemulsions. For instance, β-carotene nanoemulsions was formed with 10% Tween 20 by using high pressure homogenization, their results showed that parameters such as homogenization pressure can significantly affect droplet size and size distribution. Li and Chiang reported the preparation of D-limonene in water nanoemulsion by ultrasonic emulsification method using mixed emulsifiers of sorbitane trioleate and polyoxyethylene oleyl ether, and indicated that applied power, emulsification time and emulsifier concentration had significant effects on the droplet size of nanoemulsions. Liu et al. studied the paraffin oil in water nanoemulsions with mixed emulsifiers Tween 80/Span 80 by using the emulsion inversion point method at different emulsification temperatures. Their study showed that the emulsifier concentration significantly affects the droplet formation and stability of nanoemulsions.

We aimed to systematically investigate the effect of emulsifying conditions on the physicochemical properties of glabridin-in-water nanoemulsions with the smallest droplet size using response surface methodology (RSM).

2 EXPERIMENTAL

2.1 Materials

Polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan monooleate (Span 80) was from Katayama Chemical Co. (Japan), and were mixed at a ratio of 10:2 (w/w) for the emulsifier phase. Ultrapure water was from Milli-Q Plus system (Molsheim, France). Glabridin was from Propagate Trading Co. (Taipei). The oil phase consisted of 3% glabridin in capric triglyceride (First Chemical Works, Taiwan).

2.2 Nanoemulsions preparation

Nanoemulsions consisted of the oil phase, mixed emulsifier, and water phase. All emulsions were prepared in 2 stages. The coarse emulsions were prepared with use of a Polytron (PT-MR 3000, Kinematica AG, Littau, Switzerland), and then further emulsified by use of a high pressure homogenizer (EmulsiFlex-C3, AVÉSTIN inc., Ottawa, Canada). During emulsification, the difference in temperature from initial coarse emulsions to final emulsion was not more than 20°C. Each experiment was performed in triplicated.

2.3 Experimental design

Response surface methodology by a 3-factor-3-level Box-Behnken design was used to study the effect of the independent variables: oil phase concentration ($X_1$), emulsifier concentration ($X_2$) and homogenization pressure ($X_3$), on droplet size ($Y$) of the nanoemulsions. The independent variables used in the RSM design are listed in Table 1. The experiments were designed according to the central composite design (CCD) with a 15 factorial and star design and 3 central points as shown in Table 2. Individual experiments were carried out in a random order. A second-order polynomial equation was used to express the droplet size ($Y$) of the nanoemulsions as a function of the independent variables as follows:

\[
Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3
\]

where $a_0$ is a constant, and $a_1$, $a_2$ and $a_3$ are the linear, quadratic and interactive coefficients, respectively. The coefficients of the response surface equation were determined by use of Statgraphics Centurion XV (StatPoint, Inc., 2005).

2.4 Droplet size analysis

The droplet size of the nanoemulsions was determined by use of the dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). The measurement was carried out at a fixed angle of 90° with the samples diluted approximately 1000 fold with use of Milli-Q water. Emulsion droplet size was estimated as the mean diameter of the volume distribution (MV) from 3 measurements.

### Table 1 Independent variables used in RSM design.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Symbol</th>
<th>Coded variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil phase concentration (%)</td>
<td>$X_1$</td>
<td>-1 2 4 6</td>
</tr>
<tr>
<td>Surfactant concentration (%)</td>
<td>$X_2$</td>
<td>3 5 7</td>
</tr>
<tr>
<td>Homogenization pressure (MPa)</td>
<td>$X_3$</td>
<td>70 100 130</td>
</tr>
</tbody>
</table>
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\[ MV = \sum V_d_i / \sum V_i \]
where \( V_i \) is the volume fraction between droplet sizes and \( d_i \) is the diameter of droplets.

### Table 2
The experimental droplet size and predict droplet size obtained from central composite design.

<table>
<thead>
<tr>
<th>Experimental number</th>
<th>Oil content (%): ( X_1^b )</th>
<th>Surfactant content (%): ( X_2^b )</th>
<th>Pressure (MPa): ( X_3^b )</th>
<th>Experimental size (nm): ( Y_1^c )</th>
<th>Predicted size (nm): ( Y_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 (-1)</td>
<td>3 (-1)</td>
<td>100 (0)</td>
<td>87 ± 3</td>
<td>85.05</td>
</tr>
<tr>
<td>2</td>
<td>6 (1)</td>
<td>3 (-1)</td>
<td>100 (0)</td>
<td>110 ± 4</td>
<td>115.68</td>
</tr>
<tr>
<td>3</td>
<td>2 (-1)</td>
<td>7 (1)</td>
<td>100 (0)</td>
<td>87 ± 4</td>
<td>81.43</td>
</tr>
<tr>
<td>4</td>
<td>6 (1)</td>
<td>7 (1)</td>
<td>100 (0)</td>
<td>73 ± 1</td>
<td>76.05</td>
</tr>
<tr>
<td>5</td>
<td>2 (-1)</td>
<td>5 (0)</td>
<td>70 (-1)</td>
<td>104 ± 7</td>
<td>113.31</td>
</tr>
<tr>
<td>6</td>
<td>6 (1)</td>
<td>5 (0)</td>
<td>70 (-1)</td>
<td>122 ± 3</td>
<td>123.39</td>
</tr>
<tr>
<td>7</td>
<td>2 (-1)</td>
<td>5 (0)</td>
<td>130 (1)</td>
<td>52 ± 4</td>
<td>51.61</td>
</tr>
<tr>
<td>8</td>
<td>6 (1)</td>
<td>5 (0)</td>
<td>130 (1)</td>
<td>75 ± 5</td>
<td>66.79</td>
</tr>
<tr>
<td>9</td>
<td>4 (0)</td>
<td>3 (-1)</td>
<td>70 (-1)</td>
<td>138 ± 3</td>
<td>132.04</td>
</tr>
<tr>
<td>10</td>
<td>4 (0)</td>
<td>7 (1)</td>
<td>70 (-1)</td>
<td>103 ± 2</td>
<td>101.06</td>
</tr>
<tr>
<td>11</td>
<td>4 (0)</td>
<td>3 (-1)</td>
<td>130 (1)</td>
<td>60 ± 4</td>
<td>63.54</td>
</tr>
<tr>
<td>12</td>
<td>4 (0)</td>
<td>7 (1)</td>
<td>130 (1)</td>
<td>45 ± 7</td>
<td>51.26</td>
</tr>
<tr>
<td>13</td>
<td>4 (0)</td>
<td>5 (0)</td>
<td>100 (0)</td>
<td>56 ± 2</td>
<td>56.1</td>
</tr>
<tr>
<td>14</td>
<td>4 (0)</td>
<td>5 (0)</td>
<td>100 (0)</td>
<td>57 ± 1</td>
<td>56.1</td>
</tr>
<tr>
<td>15</td>
<td>4 (0)</td>
<td>5 (0)</td>
<td>100 (0)</td>
<td>56 ± 2</td>
<td>56.1</td>
</tr>
</tbody>
</table>

\( ^a \) The experiments were run in random order.
\( ^b \) The values (-1), (0), and (1) are independent variables used in response surface methodology design.
\( ^c \) The values are the mean and standard deviation (n=3).

### Table 3
Analysis by ANOVA of the regression coefficients of the quadratic equation on droplet size of glabridin nanoemulsions.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>F-Value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>3</td>
<td>8251.51</td>
<td>43.56</td>
<td>0.0005</td>
</tr>
<tr>
<td>Quadratic</td>
<td>3</td>
<td>2420.63</td>
<td>12.78</td>
<td>0.0080</td>
</tr>
<tr>
<td>Cross product</td>
<td>3</td>
<td>417.93</td>
<td>2.21</td>
<td>0.2055</td>
</tr>
<tr>
<td>Total model</td>
<td>9</td>
<td>11090</td>
<td>19.52</td>
<td>0.0022</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>3</td>
<td>314.69</td>
<td>212.63</td>
<td>0.0047</td>
</tr>
</tbody>
</table>

3 RESULTS AND DISCUSSION
3.1 Analysis of response surfaces
The droplet size of the glabridin nanoemulsions obtained from all the experiments are in Table 2. The experimental data was used to calculate the coefficients of the quadratic polynomial equation, and the derived equation was then used to predict the values of droplet size of the nanoemulsions. The predicted values agreed well with the experimental values obtained from the RSM design. ANOVA showed that the resulting quadratic polynomial models adequately represented the experimental data with a coefficient of multiple determinations\( (R^2) \) of 0.945.

The coefficient estimates and the corresponding P-values for the polynomial equation suggested that all of the independent variables had a significant effect on the formation of glabridin nanoemulsions (\( P < 0.05 \) \( Table 3 \)). The qua-
dratic term also had significant effect on the droplet size ($P < 0.05$). Therefore, the following second-order polynomial equation was used to predict the droplet size of glabridin nanoemulsions: $Y = 501.72269 - 22.96875X_1 - 42.92708X_2 - 0.47308X_3 + 4.32292X_1^2 + 3.87292X_2^2 + 0.00016352X_3^2$.

### 3.2 Optimization of conditions for preparing nanoemulsions

Response surface methodology is an empirical modeling tool consisting of a group of mathematical and statistical techniques that can be used to develop, improve and optimize processes in which the response is influenced by several variables. RSM allows researchers to conduct optimization study with ease as it helps to reduce the number of experimental trials required to as minimum as possible. We generated a surface response of the quadric polynomial model by varying 3 of the independent variables within the experimental range while holding another one constant at the central point. Thus, Fig. 1 was generated by varying the applied pressure and emulsifier content while holding oil content at 4%. Homogenization pressure can significantly influence the properties of emulsions, because pressure dependency of the shear forces and turbulence produced during homogenization, both pressure dependent, can affect the droplet size and size distribution. In this study, increasing the homogenization pressure resulted in a significant decrease in droplet sizes over the entire pressure ranges studied, which agreed with previous studies. The initial average radius of the droplets declines from about 130 to 60 nm as the emulsifier concentration ranged from 3.0 to 5.0 wt %. Increasing the emulsifier concentration from 5.5 to 7.0 wt % further lowers the droplet size to less than 50 nm in diameters. This can be explained by the added amount of emulsifier increases the total interfacial area and hence enables the stabilization of emulsion droplets at lower sizes.

**Figure 2** was generated by varying the applied pressure and oil content while maintaining the emulsifier content at 5%. In general, the droplet size increased with increasing concentration of oil. Among the pressure range provided, a desirable threshold oil content that produces lower range of droplet size was shown at range between 3.0 and 4.0 %. During high pressure homogenization emulsification, increasing of oil concentration can result in the increasing of amount of newly formed droplet, as well as increased extent of coalescence. It is likely that once the threshold oil content is reached, the additional oil phase promotes the disruption of interfacial layer that initially stabilized by limited emulsifiers.

**Figure 3** was generated by varying the emulsifier and oil content while maintaining the applied pressure at 100 MPa. The droplet size increased with increasing of concentration of emulsifier, in that the newly formed droplet had sufficient emulsifier to stabilize the emulsion system. Our result agreed with those from previous studies. When the concentration of emulsifier was <4.5 %, the size of the droplet increased. If the emulsifier concentration is low and the interfacial area generated during emulsification is large, a considerable amount of droplets will not have enough emulsifier to overcome coalescence. After emulsification, droplets will coalesce until the total interfa-

![Fig. 1](image1.png)  **Fig. 1** Response surface contour plot of the combined effects of pressure, emulsifier content and oil content on the formation of glabridin nanoemulsions: applied pressure and emulsifier content was processed at 4% oil contents.

![Fig. 2](image2.png)  **Fig. 2** Response surface contour plot of the combined effects of pressure, emulsifier content and oil content on the formation of glabridin nanoemulsions: applied pressure and oil content were processed at 5% emulsifier contents.
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The final size of the droplet is a collective result of the emulsifier adsorption time and the emulsifier concentration of the adjacent liquid\(^{30,31}\).

The condition for the preparing glabridin nanoemulsions would be optimal with the smallest size of nanoemulsions droplets. Therefore, from the results we described above, we chose the oil and emulsifier contents of 3.7% and 5.3%, respectively, as the optimal conditions, under homogenization pressure of 129 MPa. The droplet size distribution in the glabridin nanoemulsions at optimal conditions is in Fig. 4. The droplet size distributions were unimodal and typically extended from 20 to 120 nm. Therefore, high-pressure homogenization emulsification can be used to produce glabridin nanoemulsions with diameter in the nanometer range by RSM.

3.3 Appearance of glabridin nanoemulsions

The glabridin nanoemulsions were translucent to the naked eyes. TEM was used to observe glabridin droplets in the nanoemulsions. Figure 5 shows phosphotungstic acid stained glabridin droplets visible by TEM, and the droplet size agreed with the results of droplet size analysis. In addition, TEM images showed discrete dark spherical outline with monodispersed size distribution of the glabridin nanoemulsions. Due to the advantages of extremely small droplet sizes, translucent appearance and very large dispersed phase surface-to-volume ratios, glabridin nanoemulsions should be attractive for cosmetics, pharmaceutical and food industries, as they may have the potentials to be readily absorbed by the skin, easily sterilized by filtration, and can deliver high concentrations of glabridin to the targets of interests. Further investigations are currently undergoing to elucidate the applications of glabridin nanoemulsions.

4 CONCLUSION

Use of RSM with a second-order polynomial model was sufficient to describe and predict the response of the droplet of glabridin nanoemulsions. The independent variables of homogenization pressure, oil and emulsifier content and the quadrics of the 3 variables had a significant effect on the droplet size of glabridin nanoemulsions. We adopted an optimization method to determine the best emulsifying conditions. The optimal conditions for preparing glabridin nanoemulsions were predicted to be emulsifier (Tween 80 + Span 80) concentration 5.3%, caprylic triglycerides concentration 3.65%, and homogenization pressure 129 MPa.
pressure 129 MPa.

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References
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