Optimization of Extraction Process for Total Flavonoids of Lycium Barbarum by Response Surface Methodology

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Abstract. To optimize the Soxhlet extraction technology for total flavonoids of lycium barbarum by response surface methodology (RSM). Methods: On the base of single factor experiments, liquid to solid ratio, particle size and extraction time were selected as the main factors, with the extraction capacity as the response values, RSM was used to optimize the Soxhlet extraction process. Results: Optimal parameters were as follows: the particle size was 70 meshes, liquid to solid ratio was 17.59 ml/g, extraction time was 2.47 hours. Under these conditions, the extraction capacity was 14.57 mg/g on average, and the experimental values agreed with the predicted values. Conclusion: This optimized extraction technology for total flavonoids of lycium barbarum is stable, feasible and reasonable, and with high accuracy, which could guide the applications of production.

1. Introduction
Lycium barbarum, the dry fruit of Lycium barbarum L., is sweet in nature and mild in taste, and can nourish liver, kidney and eyes, which is commonly used in traditional Chinese medicine [1]. Recent pharmacological studies have shown that Lycium barbarum polysaccharide, Lycium barbarum total flavonoids, Lycium barbarum pigment and betaine are the main active components of Lycium barbarum. Among them, Lycium barbarum total flavonoids could scavenge free radicals, resist oxidation and aging, lower blood sugar and blood lipid, improve immunity and treat cardiovascular and cerebrovascular diseases [2-5].

The extraction of total flavonoids from Lycium barbarum mainly include organic solvent extraction, microwave-assisted extraction, ultrasonic-assisted extraction, enhanced magnetic extraction and high-pressure homogeneous extraction. There have been studies on Soxhlet-based extraction of total flavonoids from Lycium barbarum [6], but there are few reports on optimizing the Soxhlet extraction with ethanol as extraction solvent. This paper used ethanol, a solvent of lower cost and less harm, to study the extraction of total flavonoids from Lycium barbarum. Based on single factor experiment, the paper adopted response surface method to optimize the three factors of liquid-solid ratio, particle size and extraction time, which played a significant role in the extraction. The paper aimed to improve the extraction efficiency and provide references for the production, development and application of total flavonoids extracted from Lycium barbarum.

2. Material and Method
2.1. Material and instruments

2.1.1. Material and solvent
Lycium barbarum: dried fruit of Lycium barbarum from Zhongning, Ningxia; standard substance of Rutin from China Institute for the Control of Pharmaceutical and Biological Products; analytically pure anhydrous ethanol, sodium nitrite, sodium hydroxide and aluminum nitrate made in China.

2.1.2. Instruments and equipment
A11 crusher from Guangzhou IKA; SOX406 Soxhlet extractor from Jinan Haineng; UV-5900PC ultraviolet and visible spectrophotometer from Shanghai Yuanxiao; ME204E electronic balance from Mettler Toledo Instrument; RE2000A rotary evaporator from Shanghai Yarong Biochemical Instrument; C-MAG HS4 heating magnetic stirrer from Guangzhou IKA.

2.2. Method

2.2.1. Preparation of Rutin standard curve
Weigh 10mg of Rutin standard substance in a 100ml volumetric flask, dissolve it in 75% ethanol, fix the volume to obtain a Rutin standard solution at the concentration of 100μg/ml, and refrigerate it for later use. Accurately measure 0.00 ml, 1.00 ml, 2.00 ml, 3.00 ml, 4.00 ml, 5.00 ml, 6.00 ml and 7.00mL of Rutin standard solution respectively, place them in a 10ml volumetric flask, add 0.30 ml of 5% sodium nitrite solution, shake and let the solution stand for 6min. Then add 0.30 ml of 10% aluminum nitrate solution, shake and let the solution stand for 6 min. Then add 4.00 ml of 4% sodium hydroxide and ethanol to the scale, shake and let the solution stand for 20 minutes. The absorbance of each solution was measured at the wavelength of 510nm. Prepare a standard curve with concentration as abscissa and absorbance as ordinate, where the linear regression equation is obtained: y = 1.1052x-0.002, R2 = 0.9996 (x: mass concentration, mg/ml; y: absorbance value).

2.2.2. Extraction of total flavonoids
Weigh 1.00g of Lycium barbarum powder of a specified particle size, wrap it with filter paper, place it in Soxhlet extractor, add petroleum ether (30-60℃) for reflux degreasing for 3 hours, discard solvent, add in ethanol solution with different concentrations for reflux extraction for a particular period of time. The extractive solution is put into a 50ml volumetric flask, supplemented with ethanol at the same concentration to the scale, and shaken evenly to obtain the Lycium barbarum total flavone extractive solution for later use. Repeat each condition 3 times for parallel test.

2.2.3. Determining total flavonoids
Measure 1 mL of Lycium barbarum total flavonoids extract, transfer it to a 10 mL volumetric flask, add sodium nitrite, aluminum nitrate and sodium hydroxide in order based on the method described in Section 1.2.1, measure the absorbance at 510 nm after constant volume, calculate the concentration of total flavonoids according to regression equation, and calculate the amount (mg/g) of Lycium barbarum total flavonoids using dilution multiple (mg/g).

2.2.4. Single factor experiment of total flavonoids extraction
Provided the other conditions remain the same, this paper adopted various ethanol concentrations (40%, 50%, 60%, 70%, 80% and 90%), liquid-to-material ratios (5ml/g, 10ml/g, 15ml/g, 20ml/g, 25ml/g and 30ml/g), different period of time (1h, 1.5h, 2h, 2.5h, 3h, and 3.5h) and particle sizes (30 mesh, 40 mesh, 50 mesh, 60 mesh, 70 mesh and 80 mesh) were adopted as the single factor to investigate the impact of each factor on the amount of total flavonoids from Lycium barbarum based on the content of total flavonoids extracted and calculated in Section 1.2.2 and 1.2.3.

2.2.5. Determining the optimal extraction parameters
According to the results of single factor experiment where the amount of total flavonoids from Lycium barbarum served as the investigation indicator and the ratio of liquid to material, period of time for extraction and particle size as independent variables, this paper used Box-Behnken Design in Design-expert 8.0 software to design a three-factor and three-level experiment and optimize the extraction parameters. The factors and levels are shown in Table 1.

Table 1. Factors and levels in response surface design of extraction

| levels | Factors   | X1 Grain size (mesh) | X2 Liquid-material ratio (ml/g) | X3 extraction time (h) |
|--------|-----------|----------------------|---------------------------------|------------------------|
| -1     | X1        | 60                   | 15                              | 2                      |
| 0      | X2        | 70                   | 20                              | 2.5                    |
| 1      | X3        | 80                   | 25                              | 3                      |

3. Result and Analysis

3.1. Effect of ethanol concentration on extraction of total flavonoids from Lycium barbarum

Figure 1 shows that the amount of total flavonoids from Lycium barbarum increases with the ethanol concentration, with a significant growth when the ethanol concentration is lower than 70%. The ethanol concentration above 70% witnessed only slight changes in the amount of total flavonoids. To reduce the cost, conserve energy and improve efficiency, 70% ethanol was used for response surface test.

![Fig.1 Effects of ethanol concentration on flavonoid yield](image)

3.2. Effect of liquid-material ratio on extraction of total flavonoids from Lycium barbarum

Figure 2 shows that the amount of total flavonoids from Lycium barbarum increased significantly with the increase of solvent dosage, which peaks at the liquid to material ratio of 20ml/g and drops at higher ratios. Increase in the liquid to material ratio doesn't necessarily boost the amount of total flavonoids once the solution is saturated. Therefore, this paper adopted the ratio of 15ml/g, 20ml/g and 25ml/g for the response surface test.
3.3. Effect of period of time on the amount of total flavonoids from Lycium barbarum

Figure 3 shows that the extraction rate of total flavonoids from Lycium barbarum can be increased by prolonging the duration of extraction, which peaks at 2.5h. It takes a certain amount of time for total flavonoids to be dissolved, and the amount of dissolved particles increases with time. However, high temperature may cause total flavonoids to decompose, and long-time extraction will reduce the amount of extraction. Therefore, the duration of 2h, 2.5h and 3h was adopted for response surface test.

3.4. Effect of particle size on the amount of total flavonoids from Lycium barbarum

Figure 4 shows that decrease in the particle size would cause the extraction rate to increase before going down, and the 70 mesh particles feature the highest yields. The smaller the particle size, the better the diffusion and dissolution of total flavonoids. However, the polysaccharide in Lycium barbarum may cause adhesion between Lycium barbarum particles of smaller sizes, which means the
excessively small-sized particles would prevent the extraction. Therefore, Lycium barbarum powder with particle size of 60 mesh, 70 mesh and 80 mesh was used for response surface test.

![Fig.4 Effects of particle size on flavonoid yield](image)

3.5. Results of optimized response surface experiments

3.5.1. Design and result of response surface experiments

Based on the combined test scheme of Box-Benhnken, the response surface experiments were carried out, involving 17 test points with three factors at three levels. The design and results of the experiments are shown in Table 2.

| Test number | X1 grain size (mesh) | X2 liquid-material radio (ml/g) | X3 extraction time (h) | Extraction of total flavonoids (mg/g) |
|-------------|----------------------|---------------------------------|------------------------|--------------------------------------|
| 1           | 60                   | 15                              | 2.5                    | 13.52                                |
| 2           | 70                   | 25                              | 2                      | 12.91                                |
| 3           | 70                   | 15                              | 2                      | 13.37                                |
| 4           | 60                   | 20                              | 3                      | 12.35                                |
| 5           | 70                   | 25                              | 3                      | 12.67                                |
| 6           | 60                   | 20                              | 2                      | 12.38                                |
| 7           | 80                   | 25                              | 2.5                    | 13.22                                |
| 8           | 70                   | 20                              | 2.5                    | 14.34                                |
| 9           | 60                   | 25                              | 2.5                    | 12.33                                |
| 10          | 80                   | 15                              | 2.5                    | 13.56                                |
| 11          | 70                   | 20                              | 2.5                    | 14.31                                |
| 12          | 70                   | 20                              | 2.5                    | 14.21                                |
| 13          | 80                   | 20                              | 3                      | 12.51                                |
| 14          | 70                   | 20                              | 2.5                    | 14.37                                |
| 15          | 80                   | 20                              | 2                      | 12.56                                |
| 16          | 70                   | 20                              | 2.5                    | 14.10                                |
| 17          | 70                   | 15                              | 3                      | 12.96                                |
3.5.2 Establishment of the regression model and variance analysis

Multivariate linear regression and binomial fitting nonlinear regression were carried out on the above factors and levels, and the quadratic regression model equation of total flavonoids extraction (Y) of Lycium barbarum involving the three factors (X1, X2, X3) was established as follows:

\[ Y = 14.26 + 0.16X_1 - 0.28X_2 - 0.091X_3 + 0.21X_1X_2 - 0.005X_1X_3 + 0.42X_2X_3 - 0.82X_1^2 - 0.29X_2^2 - X_3^2 \]

The variance analysis in Table 3 shows that independent variables including X1 and X2, X1X2 and X2^2 are significant (P<0.05), while X1^2 and X3^2 are extremely significant (P<0.01). The influence of various factors on the amount of total flavonoids from Lycium barbarum was ranked as: ratio of liquid to material > particle size > period of time. In the quadratic equation model, P<0.001 shows that the model is highly significant. The correlation coefficient R^2 = 0.9775, R^2 adj = 0.9458, indicating good fitting. The mismatch error P value of 0.0976 > 0.05 means there is no mismatch factor, indicating that the response surface model fits well with the experiment. The higher the CV (coefficient of variation), the lower the precision and credibility of the model. The CV in the test model is at a lower level of 1.31%, suggesting accuracy and credibility of the test. In conclusion, the model and the regression equation generated reliable experimental results for analysis and prediction.

Table 3. ANOVA regression analysis

| Coefficient term | Sum of squares | Freedom | Mean square | F value | P value | Significance |
|------------------|---------------|---------|-------------|---------|---------|--------------|
| Model            | 9.128317      | 9       | 1.014257    | 33.76115| <0.0001 | **           |
| X1               | 0.201613      | 1       | 0.201613    | 6.710989| 0.0359  | *            |
| X2               | 0.6498        | 1       | 0.6498      | 21.62962| 0.0023  | *            |
| X3               | 0.066613      | 1       | 0.066613    | 2.217302| 0.1801  |              |
| X1X2             | 0.180625      | 1       | 0.180625    | 6.012387| 0.0440  | *            |
| X1X3             | 1.000E-004    | 1       | 1.000E-004  | 3.329E-003| 0.9556  |              |
| X2X3             | 7.225E-003    | 1       | 7.225E-003  | 0.240495| 0.6388  |              |
| X1^2             | 2.81048       | 1       | 2.81048     | 93.55125| <0.0001 | **           |
| X2^2             | 0.352885      | 1       | 0.352885    | 11.74634| 0.0110  | *            |
| X3^2             | 4.185301      | 1       | 4.185301    | 139.3143| <0.0001 | **           |
| Residual         | 0.210295      | 7       | 0.030042    |         |         |              |
| Mismatch error   | 0.160175      | 3       | 0.053392    | 4.261107| 0.0976  |              |
| Pure error       | 0.05012       | 4       | 0.01253     |         |         |              |
| The total        | 9.338612      | 16      |             |         |         |              |

*P<0.05, significant; **P<0.01, extremely significant

3.5.3 Response Surface Analysis and Optimization

Figure 5-7 offered three-dimensional spatial curved surface diagram of the interactions between period of time, liquid-material ratio and particle size working on the amount of total flavonoids from Lycium barbarum.
Fig. 5 Response surface graph of particle size and liquid to solid ratio to material vs the extraction capacity

Fig. 6 Response surface graph of liquid to solid ratio and extraction time to material vs the extraction capacity
The paper used the shape of response surface and Design expert 8.0.6 to obtain the optimal conditions of each factor, including particle size of 70.35 mesh, liquid-solid ratio of 17.59 ml/g, extraction duration of 2.47h, which was predicted to extract 14.39 mg/g of total flavonoids from Lycium barbarum. Considering the feasibility of production, the optimum particle size was modified to 70 mesh.

3.5.4. Verification test
The optimized result obtained by response surface analysis was the predicted value, which needed to be verified three times according to the optimal extraction conditions. 1.00g of Lycium barbarum was grinded into 70 mesh powder, wrapped with filter paper, placed in Soxhlet extractor, degreased with petroleum ether (30-60℃) for 3 hours. After the solvent was discarded, 17.59ml of 70% ethanol solution was added for reflux extraction for 2.47h hours. The amount of total flavonoids from Lycium barbarum measured in Section 1.2.3 was used for the calculation, which was determined to be 14.57 mg/g. The value was basically consistent with the predicted value of 14.39 mg/g.

4. Discussion
The experiment aims to optimize the extraction conditions of total flavonoids from Lycium barbarum by Soxhlet through response surface method, establish the prediction model of total flavonoids extraction, and obtain the best extraction conditions. The quadratic regression model equation determined in this experiment is:

$$ Y = 14.26 + 0.16X_1 - 0.28X_2 - 0.091X_3 + 0.21X_1X_2 + 0.005X_1X_3 + 0.42X_2X_3 - 0.82X_1^2 - 0.29X_2^2 - X_3^2 $$

Using Design-expert 8.0 software, we noticed that in the quadratic equation model, P<0.001 indicates difference of significance. The correlation coefficient $R^2 = 0.9775$ and $R_{adj}^2 = 0.9458$, indicating good fitting of the model. The P value of mismatch error is 0.0976 > 0.05, which means there is no mismatch factor, and the response surface model fits the experiment well. The optimal extraction conditions predicted by this model include the particle size of 70 mesh, the liquid to material ratio of 17.59 ml/g, and the extraction duration of 2.47h. The verification test showed that the amount of total flavonoids from Lycium barbarum under this condition was 14.57 mg/g, which was basically consistent with the predicted value of 14.39 mg/g. The results showed that the model was properly designed, which could be used to analyze and predict the extraction of total flavonoids from Lycium barbarum by Soxhlet.
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