Process Optimization of Microwave-Assisted Extraction of Flavonoids from *Salvia Plebeian* Using Response Surface Methodology

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Abstract. Flavonoids were extracted from *Salvia plebeian* by the microwave method, the process was optimized by Response Surface Methodology. On the basis of single factor experiment, ethanol concentration, microwave power, extraction time, material to liquid ratio were selected as independent variables, and the extraction content of homoplantaginin was selected as response value. The optimum extraction conditions of flavonoids from *S. plebeian* were determined by Box-Behnken response surface analysis. The optimum extraction conditions were that the ethanol concentration was 56%, the ratio of material to liquid was 1:30 g/mL, the extraction time was 5 minutes, the extraction power was 560 W, and the extraction content of flavonoids was 2.38 mg/g. The difference from the predicted value was 0.021%, which was not much different from the predicted value of the model, which fully verified the reliability of the model. The extraction parameters obtained by response surface method were accurate and reliable, and the extraction process was reasonable and feasible.

Keywords: *Salvia Plebeian*, Flavonoids, Response Surface Methodology

1. Introduction

*Salvia plebeian* a genus of *Salvia* in Labiatae, the whole grass is into medicine. It is widely distributed in east China, south China and southwest China. The main components of *S. plebeian* are flavonoids, terpenoids, phenylalanine compounds, volatile oil compounds, sterols and phenolic acids which are good for health, such as anti-inflammatory, antioxidant, bacteriostatic, and immunomodulatory effects [1]. The application of these chemical components in the chemical, food and pharmaceutical industries proves that people are more and more interested in developing and optimizing the extraction process of natural sources, especially when the content of flavonoids is known, the extraction conditions have a significant impact on its type and yield.

In recent years, the development direction of extraction technology is to find efficient and innovative techniques to extract natural active ingredients, in order to improve extraction rate, reduce solvent consumption and shorten extraction time. Microwave-assisted extraction (MAE) [2] usually up to these requirements. Compared with other techniques, MAE shows merits in that it has high
extraction efficiency, lower extraction temperature and time, shows relatively low cost, and can be marked as "green" in accordance with environmental standards [3]. Response surface methodology (RSM) is a statistical method to find the optimal process parameters and solve the multivariable problem by using the reasonable experimental design method and getting certain data through experiments, using multiple quadratic regression equation to fit the functional relationship between factors and response values, and analyzing the regression equation [4].

The purposes of the paper were to explore microwave-assisted extraction (MAE) as a means suitable for the extraction of flavonoids from *S. plebeian*, in order to evaluate the comprehensive influence of the main extraction parameters and optimize the operation parameters, so as to achieve a maximum possible content of extracting flavonoids by RSM.

2. Experimental

2.1. Materials

The sample was collected from Wenshang County, Jining City, China. The whole grass was washed and dried and milled immediately before extraction. After 40 mesh sieves, it was dried to constant weight under a 60 °C oven. And the powder was stored in darkness in a dry, cool place until treatment.

2.2. Experimental Design

A 3^4 full factorial experimental designs were used to estimate the process parameters for the yield of homoplantaginin (dependent parameter). The range of process parameters (independent variables) was investigated preliminarily: (45%, 60%, 75%, X₁), microwave power (400, 500, 600 W, X₂), solid-liquid ratio (1:10, 1:20, 1:30 g/mL, X₃) and extraction time (2, 4, 6 min, X₄). All the trial runs were performed in triplicate.

2.2.1. Response Surface Methodology

The optimal combination of process parameters are determined with RSM at the tree level. The output of homoplantaginin (related parameter Y) was measured in three times, and the average value was used for regression analysis. For predict the optimum conditions, the design expert software is used to analyze the experimental data and fit it to the empirical second-order polynomial regression model:

\[ Y = f(X₁, X₂, \ldots, X₄) + \varepsilon \]

Among them, Y is the observation value (the dry matter yield of homoplantaginin mg/g). Where \( f(X₁, X₂, \ldots, X₄) \) is the function of \( X₁, X₂, \ldots, X₄ \) (the independent variable), \( \varepsilon \) is the error term. In response surface analysis, the first step is to get the regression equation, and then through the reasonable value of the self cataplasm, to show the optimal value, which is the purpose of response surface design test.

The experimental design, regression and graphical analysis were expressed by the Design Expert software package (Trial version 8.0.0, USA). Variance analysis is used to assess the significance of independent parameters and their interaction, the sufficiency of the model and the statistical significance of regression coefficient.

2.3. Microwave Assisted Extraction

Microwave extraction was carried out by a Microwave Synthesizer (“Xiangwu”, XH-100A, China). The ground plant materials (1.00 g) and the solid-liquid ratio (1:10, 1:20, 1:30g/mL) of different concentrations (50%, 60%, 70%) were placed into beakerflasks (50 mL). The extraction was carried out at different microwave power (400, 500 and 600 W) for 2, 4 and 6 min. After the extraction cycle, the liquid extracts were condensed with a rotary evaporator at 45 °C. The concentrate solution was merged and shifted into a 50 mL volumetric flask and adjusted to the required volume with 60% ethanol. The sample solution was ultrasonicated for 10 min, filtered with a 0.45 μm filter for High Performance Liquid Chromatograph.
2.4. Determination of Homoplantaginin Content

2.4.1. Preparation of Control Solution
5 mg homoplantaginin was weighed accurately and placed a 10mL volumetric flask, added methanol to scale and shaked well, then obtained the stock solution. 2 mL the stock solution was absorbed, placed a 10 mL volume bottle, added methanol to the mark and shaked well, obtained 0.1mg/mL control solution and was kept in reserve in the refrigerator at 4 ℃ for available [5].

2.4.2. The HPLC Conditions
The chromatographic conditions were used by ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm), the mobile phase was consisted of methanol (A)-0.02% phosphoric acid solution (B), 0~30 min 20% A~80% A; 30~35 min 80% A~100% A; 35~50 min 100% A~20% A. The column temperature was 30 ℃, the injection volume was 1 μL, the flow rate was 1 mL/min, and the detection wavelength was 335 nm [6].

2.4.3. Determination of the Standard Curve
The above standard solution was drawn accurately and injected into 2, 4, 6, 8 and 10 μL respectively, and the chromatographic peak area was recorded according to the above chromatographic conditions “2.4.2”. The standard curve was drawn for linear regression with mass concentration (X) as transverse coordinate and chromatographic peak area (Y) as longitudinal coordinate. All measurements were made in triplicate. The obtained calibration curve of homoplantaginin was characterized by the equation y = 547.76x + 0.2457, (R²=0.9999), which was used for determining the total content of flavonoid compounds in samples.

3. Results and Discussion

3.1. RSM Modeling and Process Optimization
On the base of the combination design principle of Box-Behnken, 29 experiments were carried out in random order and made in three times to study affluence of different variable quantity on the yield of homoplantaginin. Table 1 showed the factors and their levels that affect the production of homoplantaginin, and the experimental and predicted the results.

| Table 1. Design and Results of Box-Behnken Test |
|-----------------------------------------------|
| Run  | Coded Factors | Uncoded factors | HY (mg/g) |
|------|---------------|-----------------|-----------|
|      | X1  | X2  | X3  | X4  | Microwave power W | ethanol concentrations | Solid-liquid g/mL (X1) | extraction time min (X4) | EV | PV |
| 1    | -1  | -1  | 0   | 0   | 400.00           | 50.00                  | 20.00                  | 4.00                     | 2.21 | 2.05 |
| 2    | 0   | -1  | 0   | 0   | 500.00           | 50.00                  | 20.00                  | 6.00                     | 2.12 | 2.10 |
| 3    | 0   | 1   | 0   | -1  | 500.00           | 70.00                  | 20.00                  | 2.00                     | 1.89 | 1.84 |
| 4    | 0   | 0   | 0   | 0   | 500.00           | 60.00                  | 20.00                  | 4.00                     | 2.17 | 2.10 |
| 5    | 0   | 0   | 1   | -1  | 500.00           | 50.00                  | 60.00                  | 20.00                    | 2.00 | 2.38 |
| 6    | 0   | 0   | -1  | -1  | 500.00           | 60.00                  | 10.00                  | 2.00                     | 2.16 | 2.17 |
| 7    | -1  | 1   | 0   | 0   | 400.00           | 70.00                  | 20.00                  | 4.00                     | 1.88 | 1.84 |
| 8    | 0   | 1   | -1  | 0   | 500.00           | 70.00                  | 10.00                  | 4.00                     | 1.71 | 1.81 |
| 9    | -1  | 0   | -1  | 0   | 400.00           | 60.00                  | 10.00                  | 4.00                     | 2.11 | 2.13 |
| 10   | 0   | 0   | 1   | 1   | 500.00           | 60.00                  | 30.00                  | 6.00                     | 2.34 | 2.33 |
| 11   | 1   | 0   | 1   | 0   | 600.00           | 60.00                  | 30.00                  | 4.00                     | 2.37 | 2.34 |
| 12   | 0   | 0   | -1  | 1   | 500.00           | 60.00                  | 10.00                  | 6.00                     | 2.16 | 2.17 |
| 13   | 0   | 0   | 0   | 0   | 500.00           | 60.00                  | 20.00                  | 4.00                     | 2.32 | 2.22 |
HY – homoplantaginin yield, EV – experimental values, PV – predicted values.

Based on the experimental data, a second-order polynomial model was established to predict the output of homoplantaginin from *S. plebeian; Y = + 2.35 + 0.038 X_1 - 0.089 X_2 + 0.085 X_3 + 0.033 X_4 + 0.091 X_1 X_2 - 0.076 X_1 X_3 + 0.093 X_1 X_4 + 0.055 X_2 X_3 - 0.028 X_2 X_4 + 0.088 X_3 X_4 - 0.032 X_1^2 - 0.11 X_2^2 - 0.087 X_3^2 - 0.041 X_4^2

Table 2 was given the summary results of ANOVA of quadratic model, indicating the importance and sufficiency of the selected model.

**Table 2.** Analysis of Variance (ANOVA) of Box-Behnken Test

| Source         | Sum of squares | df | Mean squares | F       | P value | Significant |
|----------------|----------------|----|--------------|---------|---------|-------------|
| Model          | 0.45           | 14 | 0.032        | 2.65    | 0.0391  | Significant |
| X_1            | 0.017          | 1  | 0.017        | 1.40    | 0.2567  |             |
| X_2            | 0.096          | 1  | 0.096        | 7.87    | 0.0140  | *           |
| X_3            | 0.087          | 1  | 0.087        | 7.12    | 0.0184  | *           |
| X_4            | 0.013          | 1  | 0.013        | 1.10    | 0.3127  |             |
| X_1X_2         | 0.033          | 1  | 0.033        | 2.73    | 0.1208  |             |
| X_1X_3         | 0.023          | 1  | 0.023        | 1.45    | 0.1914  |             |
| X_1X_4         | 0.035          | 1  | 0.035        | 2.10    | 0.1130  |             |
| X_2X_3         | 0.012          | 1  | 0.012        | 0.99    | 0.3360  |             |
| X_2X_4         | 3.026×10^{-3}  | 1  | 3.026×10^{-3}| 0.25    | 0.6261  |             |
| X_3X_4         | 0.031          | 1  | 0.031        | 2.54    | 0.1331  |             |
| X_1^2          | 6.803×10^{-3}  | 1  | 6.803×10^{-3}| 0.56    | 0.4674  |             |
| X_2^2          | 0.072          | 1  | 0.072        | 5.90    | 0.0292  | *           |
| X_3^2          | 0.049          | 1  | 0.049        | 3.99    | 0.0655  |             |
| X_4^2          | 0.011          | 1  | 0.011        | 0.89    | 0.3607  |             |
| Residual       | 0.17           | 14 | 0.012        | 2.24    | 0.2269  | Not significant |
| Lack of fit    | 0.14           | 10 | 0.014        | 2.24    | 0.2269  | Not significant |
| Error          | 0.026          | 4  | 6.460×10^{-3}|         |         |             |
| Total          | 0.62           | 28 |             |         |         |             |

CV = 4.92%  \ R^2=0.7264  \ AdjR^2=0.4527

*p<0.05 – significant, **p<0.01 – very significant.

The F value (2.65) and P value (0.0391 < 0.05) showed the established model had statistically significant effects. Adj. R^2 and R^2 were 0.7264 and 0.4527 respectively, suggesting that the mode was
in good fit of the experimental data. The low CV (4.92%) also confirmed the good reproducibility of the model. The lack of fit further verified the correctness of the model (0.2269 > 0.05). The effect of four independent variables on the yield of homoplantaginin were discussed through the significance (P < 0.05) coefficient of the second-order polynomial model, t. The lower values of the variable F and P mean greater the impact on the response. It can be seen from Table 2 that extraction concentration ($X_2$) and liquid-solid ratio ($X_3$) had a significant effect (p<0.05); microwave power ($X_1$) and extraction time ($X_4$) had no significant effect (p>0.05) on the response. In addition, $X_2^2$ had a significant effect on the response (P < 0.05), and the interaction of the two factors had a significant influence on the response (P < 0.05).

The three-dimensional (3D) response surface shows the significant (p<0.05) interact effect between independent variable quantity and the production of homoplantaginin. Figure 1. illustrated the interact effect of two factors at the same time. The third and fourth factors were fixed in the middle level.

The graphs in Figure 1. showed that with the increase of concentration of ethanol from 50% to 60%, the output of homoplantaginin added, but with the further increase of ethanol concentration, the output of homoplantaginin reduced. Compared with other analysis factors, the extraction concentration was the greatest, and the extraction time had the least effect on the yield of homoplantaginin.

![Figure 1. Three dimensional (3D) response surface for yield of homoplantaginin](image)

**Figure 1.** Three dimensional (3D) response surface for yield of homoplantaginin

The optimum extraction process of flavonoids by microwave with Design-Expert8.0.6 software was as follows: 56.17% ethanol concentration, 1:29.95g/mL solid to liquid ratio, 5 min extraction time, 561.44 W extraction power. Considering the application of ethanol concentration and material liquid ratio in the practical production, it was adjusted to 56%, 1:30 g / mL, 5 min and 560 W. The extraction content was 2.38 mg/g by three parallel confirmatory tests. The difference between the model and the predicted value was 0.021%, which was no difference from the predicted value of the model, which fully verified the reliability of the model.

**4. Conclusion**

In this paper, microwave-assisted extraction and RMS were used to optimize the extraction process of homoplantaginin from *S. plebeian*. It provided a relatively simple, economic and efficient way for the
extraction of homoplantaginin from *S. plebeian*, which can be used for the development of health food and functional products.

**Acknowledgments**
This work was financially supported by Innovation and entrepreneurship training program for college student (201811439006), Biology Key Construction Discipline of Jilin Agricultural Science and Technology University.

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