

Study on Optimization of Ethanol Reflux Extraction of Phenolic Acids from *Salvia miltiorrhiza*

Ying Gao, Jinlin Wu

College of Biomedicine and Chemical Engineering, Liaoning Institute of Science and Technology, Benxi, China
Email: 984149951@qq.com

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Abstract

The extraction technology of phenolic acid compounds from *Salvia miltiorrhiza* by ethanol reflux was studied. In this experiment, salvianolic acid B standard was used to make the standard curve. Single factor experiment and orthogonal experiment were used to study the extraction of different ethanol concentrations, reflux times and material-to-liquid ratios. The OD value of salvianolic acid compounds was measured with a spectrophotometer. The extraction rate of phenolic acid compounds under different extraction conditions was calculated through a regression equation, so as to obtain the optimal conditions for the ethanol reflux extraction process of *Salvia miltiorrhiza*. The experimental data can provide a reference for the ethanol reflux extraction process of salvianolic acids in the industry. According to the experiment, the extraction rate of phenolic acids in *Salvia miltiorrhiza* was the highest when the ethanol concentration was 60%, the reflux time was 1.5 hours, and the ratio of material-to-liquid was 1:10.

Keywords

Salvia miltiorrhiza, Phenolic Acid Compounds, Salvianolic Acid B, Ethanol Reflux, Orthogonal Experiment

1. Introduction

1.1. *Salvia miltiorrhiza* and Its Efficacy

Salvia miltiorrhiza is the name of traditional Chinese medicine. It is the dry root and rhizome of *Salvia miltiorrhiza* Bge. It is distributed in most regions of the country [1]. The main effects of *salvia miltiorrhiza* are to promote blood circulation and remove blood stasis, smooth menstruation and relieve pain, clear the

heart and remove annoyance, cool blood and eliminate carbuncle [2].

1.2. Main Chemical Components of *Salvia miltiorrhiza*

The main chemical components of *Salvia miltiorrhiza* can be divided into fat-soluble components and water-soluble components. The fat-soluble components are mainly tanshinones, and the water-soluble components are mainly phenolic acids [3]. Fat-soluble components belong to quinone and ketone structure, including tanshinone, cryptotanshinone, isotanshinone, isocryptotanshinone, hydroxytanshinone, methyl tanshinone, methylenetanshinone, dihydrotanshinone, etc. The water-soluble phenolic acid compounds are: salvia acid A, B, C; Salvianolic acid A, B, C, D, E, G; Rosmarinic acid, methyl rosemary acid, methyl shikonate, dimethyl shikonate, ethyl shikonate, shikonate B, protocatechuic aldehyde, etc. [4].

1.3. Main Extraction Methods of *Salvia miltiorrhiza*

The extraction methods of the main components of *Salvia miltiorrhiza* include water decoction, ethanol reflux, gradient percolation, ultrasound, CO₂ supercritical extraction, etc. [5]. Among them, the gradient percolation method has the advantages of a large concentration gradient, good leaching effect and less solvent consumption [6]. The ethanol reflux method is a classical extraction method for extracting the main components of *Salvia miltiorrhiza*, which is widely used in industrial extraction. In this experiment, the ethanol reflux method was used to extract the main components of *Salvia miltiorrhiza*, and the ethanol concentration, reflux time, and material-to-liquid ratio were optimized, and the optimal extraction process was studied [7].

2. Experimental Contents and Methods

2.1. Experimental Design

2.1.1. Experimental Drugs

Salvia miltiorrhiza slices, absolute ethanol, deionized water, sodium nitrite, aluminum nitrate, sodium hydroxide, etc.

2.1.2. Equipment Required for Experiment

Electronic balance, centrifuge, thermostatic water bath, spectrophotometer, thermostatic drying instrument, pulverizer, etc.

2.1.3. Instruments Required for the Experiment

Conical flask, condensate return pipe, beaker, round bottom flask, pipette, filter paper, absorbent paper, volumetric flask, gauze, iron stand, centrifuge tube, cuvette, etc.

2.1.4. Test Steps

1) Standard curve is made with salvianolic acid B standard. 2) The temperature of thermostatic water bath is set at about 80°C. 3) Weigh Danshen powder. 4) The ethanol solution for reflux extraction was prepared according to the expe-

rimental design (60%, 80%, 100%). 5) According to the experimental design, the ratio of material-to-liquid is 1:10, 1:15, 1:20 respectively, and *Salvia miltiorrhiza* powder and ethanol are mixed evenly. 6) The extraction time was different according to the experimental design (1 h, 1.5 h, 2 h). 7) After the reflux is completed, the mixed solution is preliminarily filtered with 4 layers of gauze. 8) Take the filtrate for centrifugation (centrifuge parameters: speed 6000 r/min, time 10 min). 9) Use a pipette to absorb 0.4 mL of the centrifuged extract and put it into a clean test tube. Repeat the operation twice and label it. Then use a pipette to absorb 0.4 mL of deionized water for blank adjustment. 10) Add the reagent components of the color developing agent in order, and conduct dark treatment. a) Add 2.5 mL of 1% sodium nitrite solution, shake well, and conduct dark treatment for 5 min. b) Add 0.25 mL of 20% aluminum nitrate solution, shake well, and conduct dark treatment for 5 min. c) Add 4 mL of 1 mol of sodium hydroxide solution, shake well, add sterile distilled water to 10 mL of liquid in the test tube, and conduct dark treatment for 10 min. 11) Measure the OD value and record the original data.

2.2. Experimental Process

2.2.1. Treatment and Preparation of Experimental Materials

1) *Salvia miltiorrhiza* slices are crushed into powder, dried in a thermostatic dryer, sealed and stored away from light. 2) Prepare color developing agent: 1% sodium nitrite solution, 20% aluminum nitrate solution, 1 mol/L sodium hydroxide solution.

2.2.2. Selection of Measurement Wavelength

Take salvianolic acid B standard sample and add color developing agent for treatment. Use a spectrophotometer to detect it at the wavelength of 300 - 500 nm. It is found that the absorption value at 493 nm is the most obvious. It is decided to use 493 nm as the wavelength for determination.

2.2.3. Draw Standard Curve of Salvianolic Acid B

1) Preparation of standard solution: Accurately weigh 2 g of reference substance of salvianolic acid B, fix the volume with a 10 mL volumetric flask, and obtain 0.2 g/mL of standard solution of salvianolic acid B. 2) Drawing of standard curve: Take 0, 0.4, 0.8, 1.2, 1.6, 2.0 mL of standard solution, and dilute to 4 mL. Carry out spectrophotometer measurement and record the OD value [7]. With the sampling amount of salvianolic acid B as the abscissa and the OD value as the ordinate, the standard curve is drawn, and the regression equation is $Y = 0.4958X - 0.0261$, $r = 0.9973$. The results were recorded in **Table 1**.

Table 1. OD value of salvianolic acid B standard.

Test tube No	1	2	3	4	5	6
Sample volume/mL	0	0.4	0.8	1.2	1.6	2
OD value	0	0.143	0.346	0.547	0.713	0.879

2.2.4. Single Factor Experiment

1) Effect of reflux time on the extraction rate of effective components of *Salvia miltiorrhiza*

Take the ethanol solution with the concentration of 60%, mix the *Salvia miltiorrhiza* powder and ethanol solution according to the ratio of material-to-liquid 1:20, and set the reflux time as 1 h, 1.5 h and 2 h respectively. Other operations were performed according to the experimental procedures. Measure the corresponding OD values, and record the data in **Table 2** [8].

2) Effect of material liquid ratio on the extraction rate of effective components of *Salvia miltiorrhiza*

Accurately weigh three parts of dried *Salvia miltiorrhiza* powder, 6 g, 8 g and 12 g respectively, and add 120 ml of ethanol solution with a concentration of 60%. The ratio of material-to-liquid is 1:20; 1:15; 1:10. Other operations were performed according to the experimental procedures. Measure the corresponding OD values, and record the data in **Table 3**.

3) Effect of ethanol concentration on the extraction rate of effective components of *Salvia miltiorrhiza*

Use deionized water to prepare ethanol solutions with ethanol concentration of 60%, 80% and 100%, take 120 mL each, and mix them with 6 g Danshen powder evenly. Other operations were performed according to the experimental procedures. Measure the corresponding OD values, and record the data in **Table 4**.

2.2.5. Orthogonal Test

On the basis of single factor experiment, the technological conditions of ethanol reflux extraction of *Salvia miltiorrhiza* were further optimized. Parameter conditions: reflux time, material liquid ratio, ethanol concentration. The orthogonal test and results were recorded in **Table 5** and **Table 6**.

Table 2. OD values of salvianolic acids extracted by ethanol reflux at different reflux times.

Return time/h	1	1.5	2
OD value of test tube 1	0.384	0.383	0.356
OD value of test tube 2	0.403	0.393	0.406
OD value of test tube 3	0.363	0.377	0.386
Average OD value	0.383	0.384	0.382

Note: Control variables: material liquid ratio 1:20, ethanol concentration 60%.

Table 3. OD Values of *Salvia miltiorrhiza* phenolic acid compounds extracted by ethanol reflux at different material-to-liquid ratios.

Material liquid ratio	1:10	1:15	1:20
OD value of test tube 1	0.794	0.533	0.446
OD value of test tube 2	0.745	0.601	0.480
OD value of test tube 3	0.841	0.533	0.402
Average OD value	0.793	0.556	0.443

Note: Control variable: reflux time 1 h, ethanol concentration 60%.

Table 4. OD values of salvianolic acid compounds extracted by ethanol reflux at different ethanol concentrations.

Ethanol concentration/%	60	80	100
OD value of test tube 1	0.446	0.405	0.161
OD value of test tube 2	0.480	0.453	0.134
OD value of test tube 3	0.402	0.465	0.142
Average OD value	0.443	0.441	0.146

Note: Control variable: reflux time 1 h, material liquid ratio 1:20.

Table 5. Orthogonal test factor level table.

level	A Return time (h)	B Material liquid ratio	C Ethanol concentration (%)
1	1	1:10	60
2	1.5	1:15	80
3	2	1:20	100

Table 6. Orthogonal experiment results.

Test No	A	B	C	OD value		
1	1	1	1	0.794	0.745	0.841
2	1	2	2	0.509	0.484	0.496
3	1	3	3	0.161	0.134	0.142
4	2	1	2	0.442	0.403	0.432
5	2	2	3	0.324	0.298	0.347
6	2	3	1	0.383	0.393	0.377
7	3	1	3	0.568	0.579	0.544
8	3	2	2	0.478	0.532	0.463
9	3	3	1	0.487	0.468	0.445

2.3. Original Record of Experimental Data

The OD values of *Salvia miltiorrhiza* extract at different reflux time, ratio of solid to liquid and ethanol concentration were recorded in **Tables 2-4** respectively.

2.4. Experimental Data Processing and Analysis

According to the average value of OD data obtained in **Tables 2-4**, histograms were made in **Figures 1-3**. The effects of reflux time, ratio of solid to liquid and concentration of ethanol on the absorbance of *Salvia miltiorrhiza* extract were shown respectively. On the basis of single factor experiment, the orthogonal design was carried out for the extraction of phenolic acids from *Salvia miltiorrhiza*. The results of the orthogonal design were recorded in **Table 6**.

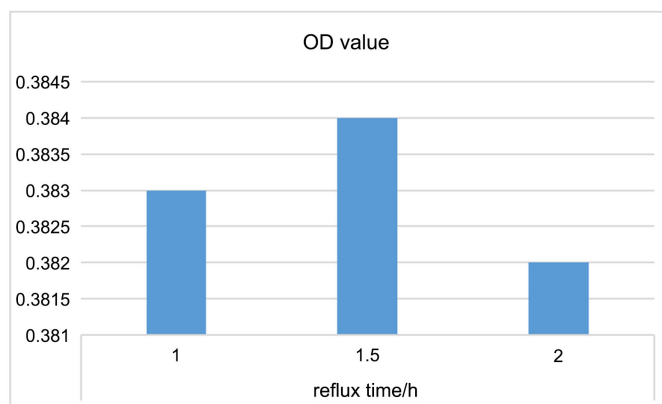


Figure 1. Broken line diagram of influence of reflux time on extraction rate of salviolic acids.

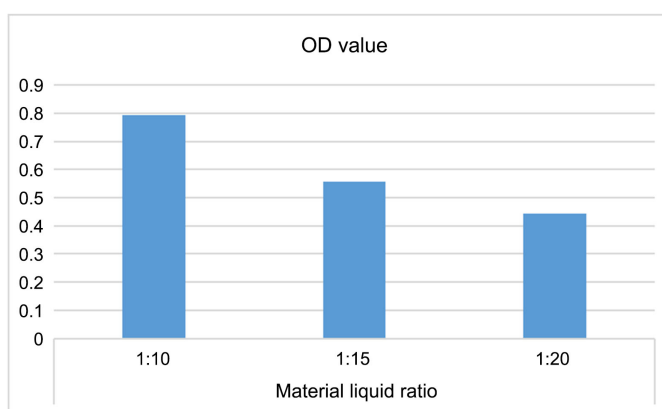


Figure 2. Broken line diagram of the influence of material liquid ratio on the extraction rate of phenolic acid compounds in *Salvia miltiorrhiza*.

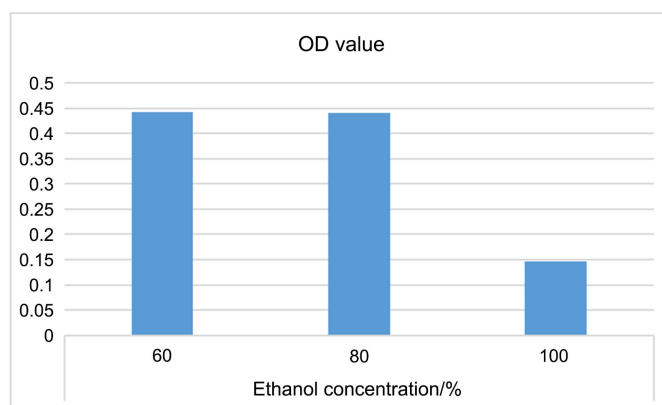


Figure 3. Broken line diagram of the influence of ethanol concentration on the extraction rate of phenolic acids in *Salvia miltiorrhiza*.

3. Result and Discussion

3.1. Results

1) From the analysis of experimental data in Experiment 1, it is concluded that

when other experimental conditions are the same, the longer the refluxing time is, the better. Under the extraction condition of 1.5 h refluxing time, the content of salvianolic acid compounds in the extraction solution is the highest. 2) From the analysis of experimental data in Experiment 2, it can be concluded that when other experimental conditions are the same, the higher the multiple of the material-to-liquid ratio is, the better. Under the extraction condition of the material-to-liquid ratio of 1:10, the content of salvianolic acid compounds in the extraction solution is the highest. 3) From the analysis of experimental data in Experiment 3, when other experimental conditions are the same, the higher the ethanol concentration is the better. Under the extraction condition of 60% ethanol concentration, the content of salvianolic acid compounds in the extraction solution is the highest. This should be related to the fact that the salvianolic acids in *Salvia miltiorrhiza* are water-soluble substances. When the water content is relatively high, the contact probability between the phenolic acids in *Salvia miltiorrhiza* and water molecules is increased. 4) According to the orthogonal test analysis, the main factor affecting the extraction rate of salvianolic acid compounds is the ratio of material-to-liquid, and the other two factors have weak effects on the extraction rate of salvianolic acid compounds.

3.2. Analysis and Discussion

In this experiment, the ethanol reflux method was selected to extract phenolic acid compounds, the main components of *Salvia miltiorrhiza*, and the process was optimized. The main reason was that the ethanol reflux method was widely used in the extraction of traditional Chinese medicine, with simple operation, low cost and higher research significance. The conclusions obtained in this experiment can provide a certain reference for the ethanol reflux extraction process of salvianolic acids in industry, and can also provide data reference for other researchers.

4. Conclusion

In summary, salvianolic acids have poor stability in water, but good stability in ethanol and other organic solutions. Therefore, with a longer extraction time, salvianolic acid will not decompose. The optimal scheme of ethanol reflux extraction process optimization of phenolic acid compounds in *Salvia miltiorrhiza* in this experiment is as follows: reflux time is 1.5 h, material liquid ratio is 1:10, and ethanol concentration is 60%.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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