Optimization of ultrasound-assisted extraction of phenolic compounds from *Cimicifugae rhizoma* with response surface methodology

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**ABSTRACT**

**Background:** *Cimicifugae rhizoma* was a Ranunculaceae herb belonging to the composite family, and the roots of *C. rhizoma* have been widely used in tradition Chinese medicine.

**Materials and Methods:** Ultrasound-assisted extraction (UAE) of phenolic compounds from *C. rhizoma*. Caffeic acid (CA), isoferulic acid (IA), ferulic acid (FA), and total phenols were quantified by high-performance liquid chromatography-diode array detection and ultraviolet-visible spectrophotometry. Effects of several experimental parameters, such as ultrasonic power (W), extraction temperature (°C), and ethanol concentration (%) on extraction efficiencies of phenolic compounds from *C. rhizoma* were evaluated.

**Results:** The results showed that the optimal UAE condition was obtained with ultrasonic power of 377.35 W, extraction temperature of 70°C, and ethanol concentration of 58.37% for total phenols, and ultrasonic power of 318.28 W, extraction temperature of 59.65°C, and ethanol concentration of 64.43% for combination of CA, IA, FA.

**Conclusions:** The experimental values under optimal conditions were in good consistent with the predicted values, which suggested UAE is more efficient for the extraction of phenolic compounds from plant materials.

**Key words:** *Cimicifugae Rhizome*, response surface methodology, phenolic compounds, ultrasound-assisted extraction

**INTRODUCTION**

“Shengma” (the rhizomes of *Cimicifuga dahurica* (Turcz.), *Cimicifugae heracleifolia*, *Cimicifugae foetida* L.), the genus *Cimicifuga*, belonging to the Ranunculaceae family, which has a long and diverse history of medicinal use. Currently, the rhizome, which encompasses three species namely *C. dahurica*, *C. foetida*, and *C. heracleifolia*, and known in Chinese pharmacopeia as “sheng‑ma.”[1] During a series of chemical investigations of *Cimicifuga* species, some cyclolanostanol glycosides, fukiic acid esters, piscidic acid esters, caffeic acid (CA) derivatives, phenolic acid derivatives, and chromones have been isolated.[2] Extensive studies have indicated that major bioactive components of *Cimicifugae rhizoma* are phenotype derivatives and oxidized cycloartane-type, which show various positive biological effects, including the ability to act as anti-human immunodeficiency virus, anti-inflammatory, antipyretic, antidiabetes, and antimalaria.[3] Moreover, it has been used in combination with other herbs in the ancient Kampo medicine in Japan as anti-inflammatory drugs.[4]

Conventional extractions such as reflux extraction, boiling, heating, and soxhlet extraction, have been used for extraction of phenolic compounds. However, these extraction methods require a large amount of solvents; long run times and lose a few phenolics, which constrain their industrial applications. Recently, various new extraction techniques have been developed for the extraction of the target compounds from plants, including ultrasound- and microwave-assisted extraction (UAE and MAE),[5,6] supercritical CO₂ fluid extraction,[7,8] pressurized liquid extraction.[9] Among these, UAE is an inexpensive, simple, and efficient alternative to conventional extraction techniques. It has higher extraction efficiency, lower energy, and solvents consumption.[10] The mechanism of UAE is

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attributed to mechanical and cavitation efficacies which can result in disruption of the cell wall, particle size reduction, and enhanced mass transfer across the cell membrane.\[13\]

In order to optimize the extraction conditions, including ultrasonic power, extraction temperature, and concentration solvents, response surface methodology (RSM) has been widely used. By establishing a mathematical model, RSM evaluates multiple parameters and their interactions using quantitative data, effectively optimizing complex extraction procedures in a statistical way, thus reducing the number of experimental trials required.\[13\] In this methodology, mathematical and statistical techniques are combined for designing experiments, building models, evaluating the effects of factors, and searching optimum condition of factors for desirable responses. Unfortunately, little data are available in literatures about the method used in the optimization for the phenolic compounds from C. rhizoma. The most common designs, such as central composite design and Box–Behnken design (BBD), of the principal RSM have been widely used in various experiments. Box–Behnken, a spherical and revolving design, has been applied in the optimization of chemical and physical processes because of its reasoning design and excellent outcomes.\[13, 19\]

In the present work, we used RSM to optimize the UAE extraction of phenolic compounds from C. rhizoma. The aim of our work was to establish the optimized parameters of UAE and to measure the phenolic compounds with the use of high-performance liquid chromatography-diode array detection (HPLC-DAD) and ultraviolet-visible (UV-Vis) spectrophotometer.

**MATERIALS AND METHODS**

**Plant materials**
The standardized “C. rhizoma” was collected in Heilongjiang province in October 2012 and identified by Prof. Chen Jianwei (Nanjing University of Chinese Medicine, Jiangsu, China). The sample was dried at 60°C until the moisture content remained constant. Dried sample was ground to powders using an electric grinder and passed through a 40-mesh sieve, and stored at 4°C until required.

**Chemicals and reagents**
CA, isoferulic acid (IA), ferulic acid (FA) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, formic acid, and ethanol which used were of analytical reagent grade were purchased from Tedia (Fairfield, USA). Deionized water for the HPLC analysis was purchased from Hangzhou Wahaha Group Co., Ltd. The other chemicals (analytical grade) were from Nanjing Chemical Co., Ltd. (Jiangsu Province, China).

**Ultrasound-assisted extraction**
Five grams of C. rhizoma power were placed in a capper tube and mixed with ethanol. The extraction process was performed with the ultrasonic device (KQ5200DE, 25 KHz, Kunshan Ultrasonic Instrument Co., Jiangsu, China) equipped with a digital timer and a temperature controller. The solvent used in the extraction was ethanol solution. This is due to the result from a preliminary study which showed that 80% ethanol (v/v, absolute ethanol/distilled water) yielded the higher content of phenolics compared to water and 80% ethyl acetate (v/v). After ultrasonic extraction, the sample was centrifuged at 4000 rpm for 15 min to collect the supernatant. Samples were stored at −20°C prior to analysis. After being diluted twice with the extraction solution, HPLC analysis was performed. The samples were filtered through a 0.45-µm microfiltration membrane before HPLC analysis. The ethanol concentration, ultrasonic power, and extraction temperature were assessed as shown in the results.

**High-performance liquid chromatography analysis of different phenolic compounds**
High-performance liquid chromatography was performed with a Shimadzu LC-20AB series instrument (Tokyo, Japan) composed of a double quaternary gradient system, column oven, DAD detector, and CLASS-VP was used for data collection. Chromatographic separation was performed on a Kromasil C18 column (250 mm × 4.6 mm i.d., 5 µm particles). The mobile phase was acetonitrile-0.1% formic acid (16:84, v/v). Samples (10 µL) were injected into HPLC instrument at a flow rate of 1.0 mL/min. The UV spectra wavelength set at 254 nm. The column temperature was maintained at 35°C. Quantification was performed by using standard curves, and the final concentrations were calculated in μg/g dry weight.

**Ultraviolet visible analysis of total phenolic compounds**
Total phenolic compounds (TPC) was measured according to the method of Gan and Latiff\[19\] with slight modification. Briefly, 0.5 mL extract was mixed with 0.5 mL of Folin–Ciocalteu reagent (prediluted at a ratio of 1:10) and 0.5 mL of sodium bicarbonate (7.5%, w/v) was added to the mixture. And then it was made up 10 mL by adding distilled water. After standing for 60 min at room temperature, the absorbance was measured at 765 nm. The regression equation of IA standard curve was obtained as Y = −0.1708+10.51×(R² = 0.9992).

**Experimental design**
Response surface methodology was used for investigating the influence of three independent variables on TPC
and different phenolic compounds (DPC), such as CA, IA, FA.

In this study, the experiment was performed on the BBD, which is a widely used form of RSM. The main factors affecting extraction efficiency, including ultrasonic power, extraction temperature, and ethanol concentration were selected as independent variables that should be optimized for the extraction of phenolic compounds. The independent variables were coded at three levels, and the complete design consisted of 17 experimental points including four replications of center points [Tables 1 and 2]. In detail, ultrasonic power (200W, 300W, 400W), extraction temperature (50°C, 60°C, 70°C), and ethanol concentration (40%, 60%, 80%) were investigated.

The data from BBD were analyzed by multiple regression to fit the following quadratic polynomial model:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i^2 X_i^2 + \sum_{i=1}^{3} \beta_{ij} X_i X_j \]  

(1)

Where \( Y \) is the response, \( \beta_0 \) is the constant coefficient, \( \beta_i \), \( \beta_i^2 \), and \( \beta_{ij} \) are the coefficients for the linear, quadratic, and interaction effect, and \( X_i \) and \( X_j \) represent the level of the independent variables, respectively.

### Statistical analysis

The experimental results of the response surface design were analyzed using Design-Expert 8.5 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA.), \( P < 0.05 \) was considered to be statistically significant. All experiments were conducted in triplicate unless otherwise noted in the text.

### RESULTS AND DISCUSSION

#### High-performance liquid chromatography analysis

Identification of the target compounds was accomplished by comparing the retention times against those of known standards. To confirm the validity of the statistical experimental strategies, a confirmation experiment with a duplicate set was performed at the selected conditions. For example, HPLC chromatograms of the samples are shown in Figure 1. As expected, three peaks indicated CA, IA, and FA, respectively, were identified in *C. rhizoma* after extraction.

#### Fitting the response surface models

Response surface methodology approach was used to determine the optimum extraction process parameters that yield higher phenolic compounds from *C. rhizoma*. A RSM design with 17 experiments was employed to optimize parameters including ultrasonic power, extraction temperature, and ethanol concentration. The statistical significance of regression equation was checked by \( F \)-test. Table 3 summarized the response surface quadratic polynomial model performed using analysis of variance (ANOVA) to determine whether the models were fit.

To determine whether or not the quadratic model was significant, the statistical significance of regression equation was checked by \( F \)-test and ANOVA for the response surface quadratic polynomial model was summarized in Table 3. The \( P \) value was used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The corresponding variables would be more significant if the absolute \( F \)-value becomes greater and the \( P \) value becomes smaller. Lack of fit was also given in Table 3. The “fitness” of the model was investigated through the lack-of-fit test (\( P > 0.05 \)), which indicated the suitability of models to accurately predict the variation.

The data shown in Table 3 indicate that the TPC yield and the extraction parameters were quadratic with a good regression coefficient \( R^2 = 0.9436 \). The large the
magnitude of the $F$-value and smaller the $P$ value, the more significant the corresponding coefficient. (ultrasonic power) was the most significant parameter ($P < 0.05$) on the UAE for TPC values.

In the case of DPC, the regression coefficients, and the corresponding $P$ value were presented in Table 3. From the $P$ value of each model term, it could be concluded that the independent variables studied ($X_i$) and three quadratic terms ($X_1^2$, $X_2^2$, $X_3^2$) significantly affected the yield of DPC. It could be seen from Table 3 that the $P$ value of TPC, CA, IA, and FA for lack-of-fit were 0.0528, 0.5096, 0.1153, and 0.1710 ($P > 0.05$).

### Interpretation of response surface models

Three-dimensional (3D) response surface plots, as presented in Figures 2-5, were very useful to see interaction effects of the factors on the responses, since the former one illustrated the sensitiveness of response value toward the change of variable and the latter one described significant coefficients between different variables.$^{[15,18,19]}$

### Effect of extraction parameters on total phenolic compounds

For TPC, the ethanol concentration, ultrasonic power, and extraction temperature were significant ($P < 0.05$) in Table 3. The predicted model obtained for is given as below:

### Table 3: Regression coefficients and ANOVA results

| Response variables | Source | Model | Residual | Lack of fit | Pure error | Total |
|--------------------|--------|-------|----------|-------------|------------|-------|
| $C_{TPC}$ (μg/g) $R^2=0.9525$ | SS | 2.968E+005 | 17,736.02 | 14,667.16 | 3068.86 | 3.146E+005 |
| | df | 9 | 7 | 3 | 4 | 16 |
| | MS | 32,981.32 | 2533.72 | 4889.05 | 767.21 |
| | $F$ | 13.02 | 6.37 |
| | $P$ | 0.0014 | 0.0528 |
| $C_{CA}$ (μg/g) $R^2=0.9200$ | SS | 417.95 | 4.28 | 4.28 | 6.24 | 428.46 |
| | df | 9 | 7 | 3 | 4 | 16 |
| | MS | 46.44 | 1.50 | 1.43 | 1.56 |
| | $F$ | 30.91 | 0.91 |
| | $P$ | <0.0001 | 0.5096 |
| $C_{IA}$ (μg/g) $R^2=0.9294$ | SS | 176.02 | 14.10 | 10.43 | 3.67 | 190.12 |
| | df | 9 | 7 | 3 | 4 | 16 |
| | MS | 19.56 | 2.01 | 3.48 | 0.92 |
| | $F$ | 9.71 | 3.76 |
| | $P$ | 0.0034 | 0.1153 |
| $C_{FA}$ (μg/g) $R^2=0.8847$ | SS | 53.50 | 3.09 | 2.10 | 0.99 | 56.59 |
| | df | 9 | 7 | 3 | 4 | 16 |
| | MS | 5.94 | 0.44 | 0.70 | 0.25 |
| | $F$ | 13.46 | 2.82 |
| | $P$ | 0.0012 | 0.1710 |

SS: Sum of squares; df: Degree of freedom; MS: Mean square; ANOVA: Analysis of variance; TPC: Total phenolic compounds; CA: Caffeic acid; IA: Isoferulic acid; FA: Ferulic acid
Figure 2: Response surface and contour plots for the effect of independent variables on total phenolic compounds yield

Figure 3: Response surface and contour plots for the effect of independent variables on caffeic acid yield
To determine the optimal levels of variables for the UAE of TPC, 3D surface plots [Figure 2] were established on the basis of equation 2. Figure 2 implied that the TPC increased with increasing ultrasonic power when extraction temperature was fixed, with further increase in ultrasonic power, a decline in TPC content was observed. The ultrasonic power could facilitate the disruption of plants’ cell walls as well as enhance the contact between solvents and targeted compounds. Researchers reported that at a higher temperature, the dielectric constant of water decreased and solvent property changed, leading to a better extraction of phenolics. Furthermore, the higher temperature could increase phenolic compounds solubility and diffusion rate as well as reduce surface tension and solvent viscosity. The results showed that the ultrasonic power of 377.35W, extraction temperature of 70°C, ethanol concentration of 58.43% resulted in the maximum TPC yield.

Effect of extraction parameters on different phenolic compounds

Figure 1 shows the HPLC chromatograms of standards and C. Rhizoma extract. Three phenolic compounds, including CA, IA, FA, were identified by comparing relative retention time and UV-Vis spectra with those of reference standards. The extraction of phenolic compounds depended largely on the polarity of solvents and the compounds, a single solvent might not be effective for the isolation of a
bioactive compound. Therefore, a combination of the pure solvent with water was more effective in extracting phenolic compounds than the solvent alone. This result was in accordance with the literature reported by Zhou and Yu.\cite{23} The 3D response surfaces and contour plot for CA, IA, FA as a function of ethanol concentration and the solvent-to-material ratio are given in Figures 3 and 5.

The ethanol concentration, ultrasonic power, and extraction temperature were significant ($P < 0.05$). The predicted model obtained for $Y_2$, $Y_3$, $Y_4$ is given as below:

$$Y_2 = 36.94 + 1.97 X_1 - 0.96 X_2 + 0.091 X_3 - 1.52 X_1X_2 - 0.50 X_1X_3 - 0.82 X_2X_3 - 8.85 X_1^2 - 2.23 X_2^2 - 0.62 X_3^2$$

$$Y_3 = 27.92 + 2.39 X_1 + 1.00 X_2 + 0.96 X_1 - 0.70 X_1X_2$$

$$Y_4 = 25.35 + 0.74 X_1 - 0.098 X_2 + 0.95 X_3 + 0.41 X_1X_2 - 0.45 X_1X_3 - 0.22 X_2X_3 - 1.62 X_1^2 - 2.35 X_2^2$$

For the CA, the response surfaces and contour plots shown in Figure 3 demonstrated the changes in the CA content as a function of three variables. The results showed that 65.22% of ethanol concentration, higher proportion of the ultrasonic power (200–300 W), and extraction temperature (50–60°C) would give a higher CA content. For the IA, the response surfaces and contour plots are shown on Figure 4, which demonstrate an increase in the IA extraction yield with increased ultrasonic power (200–330 W) and extraction temperature (50–65°C). However, further increases in ultrasonic power resulted in a reversal of this trend. The results showed that ethanol concentration of 64.42% resulted in the maximum IA yield. In the case of FA, as shown in Figure 5, it demonstrated the changes in the FA yield as a function of three variables. As the ethanol concentration increased in the range from 40% to 65.39%, FA yield increased. The ratio curve started to level off at 65.39%, which indicated that a ratio of ethanol concentration of 65.39% was required to achieve maximum increase.

**Verification of predictive models**

Based on the above findings, an optimized study was performed to evaluate the optimal operating conditions for an individual response as well as a combination of all responses. The target was to obtain the maximum yield of TPC and DPC within the extraction parameters, where consideration of the energy conservation, and the feasibility of the experiment were taken into account. Table 4 showed the optimal conditions for the two responses with the predicted and experimental values. Optimal conditions were established: (1) For a combination of TPC: Ultrasonic power of 377.35 W, extraction temperature of 70°C, and ethanol concentration of 58.37%. (2) For DPC: Ultrasonic power of 318.28 W, extraction temperature of 59.65°C, and ethanol concentration of 64.43%. The results are shown in Table 4 with total phenolic compounds and three phenolic compounds under the optimal conditions and solvent extraction conditions. No significant different ($P > 0.05$) was found between the experimental and predicted values of total phenolic and three phenolic compounds. Hence, the models can be used to optimize the process of phenolic compounds extraction from *C. Rhizome*.

**CONCLUSIONS**

The present study was used to determine optimum process parameters that could obtain a high yield of TPC and DPC. The use of multivariate optimization was of paramount importance in order to select the optimal operating conditions of interrelated variables, avoid or minimize degradation, and achieve the best yields in the extraction process. The optimal conditions determined were: Ultrasonic power of 377.35 W, extraction temperature of 70°C, ethanol concentration of 58.37% for TPC and ultrasonic power of 318.28 W, extraction temperature of 59.65°C, ethanol concentration of 64.43% for DPC.

This work clearly shows that the extraction of phenolic compounds from *C. Rhizome* can be improved by optimizing several key extraction parameters. This standardized processing technology was suitable for large-scale production of processed *C. Rhizome* and could provide valuable information for industry purposes since it allowed simplified handling and the quantity of targeted extracts.

**Table 4: Predicted and experimental values of phenolic compounds obtained under the optimal extraction conditions and solvent extraction**

| Process variables | Parameter | Predicted value | Experimental value |
|-------------------|-----------|-----------------|--------------------|
| $X_1$ (W)         | $X_2$ (°C) | $X_3$ (%)       |                    |
| 377.35            | 70        | 58.37           | 876.42             | 876.17±0.43        |
| 318.28            | 59.65     | 64.43           | 37.02              | 36.86±0.27         |
|                   |           |                 | 28.26              | 28.13±0.38         |
|                   |           |                 | Ferulic acid       | 25.55              | 25.17±0.63         |
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