Optimization of ultrasound-assisted extraction of flavonoids compounds from *Chenopodium hybridum* L. stem with response surface methodology

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Abstract. Ultrasound-assisted extraction (UAE) of flavonoids compounds (FC) from the stem of *Chenopodium hybridum* L.(C.hybridum L.) was investigated in this paper. Significant technological parameters were screened and optimized by using Plackett-Burman (PB) design, Steepest ascent method and Box-Behnken (BB) design, respectively. A mathematical model with high correlation coefficient ($R^2=0.9896$) was developed and showed good consistency between the experimental and predicted values. The optimum conditions for UAE were obtained by response surface methodology (RSM) as follows: volume fraction of ethanol 76.62 %, extractive temperature 78.69 °C, and liquid to solid ratio 58.43 for 30 min. Under these conditions, total flavonoid content (TFC) of 9.4701 mg RE/100g were gained and it was closely related with predicted value (9.4640 mg RE/100g) and indicated the suitability of the developed model.

1. Introduction

Genus *Chenopodium* belonging to the family Chenopodiaceae includes herbaceous, strongly fragrant annual plants and spreads widely worldwide, mainly in the moderate and subtropical zone [1]. In China, there are 19 species of *Chenopodium*. Many species of *Chenopodium* were reported to possess numerous medicinal properties used as folk medicine. Modern pharmaceutical research has also confirmed potent antipruritic, antibacterial and anticancer activities of these plants [2, 3]. Recently, The plants belonging to *Chenopodium* were known to be a rich source of flavonoids, phenolic acids and terpenoids attracted special attention[4,5], among which flavonoids was one of the most effective and important components, because of their antioxidant, free radical scavenging properties and other
biological activities[6,7]. Several studies have been carried out to evaluate C. hybridum L. which was rich in flavonoids in our previous work [8,9].

Ultrasound-assisted extraction promised to extract the bioactive components at lower temperature in a shorter time, and its operation more easily than other extraction technologies and methods [10,11]. These features could be due to the cavitation phenomenon generated in the ultrasonic propagation. To our knowledge, none of the published study reported on chemical composition and extraction flavonoids compounds (FC) from C. hybridum L.. Therefore, the purpose of present study was to establish optimum ultrasonic-assisted extraction technology for flavonoids from the stem of C. hybridum L.. In this paper, UAE of FC from the stem of C. hybridum L. was investigated and the significant technological parameters were screened and optimized by using Plackett-Burman (PB) design, Steepest ascent method and Box-Behnken (BB) design.

2. Material and method

2.1. Plant material

The stems of C. hybridum L. were collected from a local region (Qingyang, Gansu, China) in July 2014, and authenticated by the corresponding author. The stems were dried naturally and ground in a domestic grinder (Beijing ZTE Albert Instrument Co., Ltd., model FZ102) to pass through a 100-mesh screen, and then put in the dryer for experiment.

2.2. Chemicals and reagents

Potassium hydroxide (analytical grade) and anhydrous ethanol (analytical grade) were purchased from Xian Chemical Reagent Company (Xian, China).

2.3. Ultrasound-assisted extraction of FC

A certain amount of plant material was placed into Erlenmeyer flasks (250 mL), and mixed with different concentrations of ethanol aqueous solution. The extraction process was performed with a domestic ultrasonic device (Kunshan Hechuang Ultrasonic Instrument Co., Ltd., model KH-250E) equipped with timer and temperature controller. After ultrasonic extraction, the mixture was filtered under diminished pressure and the supernatants were collected and put in the refrigerator until further analysis.

2.4. Determination of total flavonoid content (TFC)

TFC of the extracting solution was determined by KOH assay method. A known volume of supernatants gained from “2.3” was mixed with 70 % ethanol aqueous solution to make 6 mL solution, and 0.5 mL 10 % KOH was added. After 5 min, the total volume was made up to 10 mL with 70 % ethanol aqueous solution. The solution was mixed well and the absorbance was measured at 403 nm on UV-Vis spectrophotometer (Analytik Jena, model SPECORD-50). TFC was calculated by the calibration curve of rutin mentioned in our previous work [9] and expressed as rutin equivalents (mg RE/ 100g). All samples were analyzed in triplicate and the average values were calculated.

2.5. Experiment design and data analysis
2.5.1. Plackett-Burman (PB) design for screening of significant variables. PB design performed in 12 runs was used in this study to screen the important variables that significantly influenced UAE. Each variable was detected at the low and high levels which were denoted -1 and +1, respectively. The variables and their levels were presented in table 1, two levels of variables were set by the single factor experimental results. PB design assumes that the main effects of the variables without interaction and first-order polynomial model can be set up.

The Plackett-Burman design matrix and the experimental results were shown in table 2. The significant variables \( p < 0.05 \) were considered to have an obvious impacts on the UAE of FC and would be selected and optimized. Minitab version 17.1.0 software (Minitab Inc., PA, USA) was used for the experimental design and data analysis.

### Table 1. Variables and levels tested in PB design.

| Variables                     | Coded Variables | Levels |
|-------------------------------|-----------------|--------|
| volume fraction of ethanol (%) | \( x_1 \)       | -1 50  |
| extraction time (min)         | \( x_2 \)       | 20 30  |
| extractive temperature (°C)  | \( x_3 \)       | 50 70  |
| liquid to solid ratio (mL/g)  | \( x_4 \)       | 30 40  |

### Table 2. PB design matrix of variables with TFC as response.

| No. | \( x_1 \) | \( x_2 \) | \( x_3 \) | \( x_4 \) | \( y = \text{TFC (mgRE/100 g)} \) |
|-----|----------|----------|----------|----------|----------------------------------|
| 1   | +1       | -1       | +1       | -1       | 6.400                            |
| 2   | +1       | +1       | -1       | +1       | 6.538                            |
| 3   | -1       | +1       | +1       | -1       | 4.516                            |
| 4   | +1       | -1       | +1       | +1       | 6.259                            |
| 5   | +1       | +1       | -1       | +1       | 6.587                            |
| 6   | +1       | +1       | +1       | -1       | 6.260                            |
| 7   | -1       | +1       | +1       | +1       | 5.361                            |
| 8   | -1       | -1       | +1       | +1       | 5.173                            |
| 9   | -1       | -1       | -1       | +1       | 4.817                            |
| 10  | +1       | -1       | -1       | -1       | 5.718                            |
| 11  | -1       | +1       | -1       | -1       | 4.012                            |
| 12  | -1       | -1       | -1       | -1       | 3.761                            |

2.5.2. Steepest ascent method. Steepest ascent method is a process moving along the maximum increase of the response value[12]. In this study, the direction of steepest ascent was determined according to that the response value increase most rapidly by increasing or decreasing the values of significant variables, and the step size in the direction was determined by the estimated coefficient from equation (1) and practical experience.
2.5.3. Box-Behnken (BB) design for optimization of UAE conditions. For BB design, a total of 17 experiments including five repetitions at the center point were adopted to optimize the variables and their interactions that were screened from PB experiment results. The chosen variables were volumn fraction of ethanol, extractive temperature and liquid to solid ratio coded as $z_1$, $z_2$, and $z_3$ in this design, respectively. Independent variables and corresponding levels were shown in table 3. In this part, Design-Expert version 8.0.6.1 software (Stat-Ease, Inc., Minneapolis, MN, USA) was adopted to design experiment matrix, drawing and data analysis. The experimental matrix and results of BB design were present in table 4. Using response surface regression analysis, BB design experimental results were fitted out a second-order polynomial equation. Meanwhile, contour plots and 3 d surface graphs were used to reflect the effects of coded variables to the response value. The correctness of the model was obtained by comparing the experimental results with predicted values under the optimal conditions.

**Table 3.** Significant variables and corresponding levels in BB design.

| Independent variables | Coded variables | levels of independent variables |
|-----------------------|----------------|---------------------------------|
| volumn fraction of ethanol(%) | $z_1$ | -1 0 +1 |
| extractive temperature(℃) | $z_2$ | 70 80 90 |
| liquid to solid ratio (mL/g) | $z_3$ | 65 75 85 |

**Table 4.** The experimental matrix and results of BB design.

| Run No. | $z_1$ | $z_2$ | $z_3$ | TFC(mgRE/100g) |
|---------|-------|-------|-------|----------------|
| 1       | 0     | 0     | 0     | 9.295          |
| 2       | -1    | 0     | +1    | 9.014          |
| 3       | +1    | 0     | +1    | 7.931          |
| 4       | 0     | -1    | -1    | 8.423          |
| 5       | -1    | +1    | 0     | 8.784          |
| 6       | +1    | -1    | 0     | 8.152          |
| 7       | -1    | -1    | 0     | 8.934          |
| 8       | 0     | +1    | -1    | 8.623          |
| 9       | -1    | 0     | -1    | 8.452          |
| 10      | +1    | 0     | -1    | 7.683          |
| 11      | 0     | 0     | 0     | 9.382          |
| 12      | 0     | -1    | +1    | 9.134          |
| 13      | 0     | 0     | 0     | 9.457          |
| 14      | 0     | 0     | 0     | 9.189          |
| 15      | 0     | +1    | +1    | 8.742          |
| 16      | 0     | 0     | 0     | 9.401          |
| 17      | +1    | +1    | 0     | 7.902          |

3. Results and discussion
3.1. PB design and variables screening

In PB design, a coded linear equation was obtained by data analysis, as follow:

\[ y = 5.4502 + 0.8435 x_1 + 0.0955 x_2 + 0.2113 x_3 + 0.3390 x_4 \quad (1) \]

According to the analysis of variance (ANOVA) presented in table 5, the model F-value of 38.72 indicated the model to be significant (p < 0.05). Meanwhile, the lack of fit (p=0.094, p > 0.05) and regression coefficient R^2=0.9568 also implied that the resulting model could accurately predict the response variable and was adequate for the extraction of FC from C.hybridum L. stem.

In general, variables with a p-value less than 0.05 are considered to be significant factors. Our results implied that the volume fraction of ethanol (x_1), extraction temperature (x_3) and liquid to solid ratio (x_4) were significant for TFC measured by spectrophotometric method.

Further analysis using Pareto charts of the standardized effect (Figure 1) confirmed that the terms beyond the vertical red line were considered to have significant impacts on response value, meanwhile, the similar results were gained by the Normal plot (Figure 2) that factors of A (x_1), C (x_3), D (x_4) were the most effective factors. Main effects plot (Figure 3) demonstrated that specific variables had positive or negative effects on response value [13].

Table 5. ANOVA results of PB design.

| Source                  | DF  | Adj SS  | Adj MS  | F-value | P-value |
|-------------------------|-----|---------|---------|---------|---------|
| Model                   | 4   | 10.5623 | 2.64059 | 38.72   | 0.000   |
| Linear                  | 4   | 10.5623 | 2.64059 | 38.72   | 0.000   |
| volum fraction of ethanol | 1  | 8.5379  | 8.53791 | 125.21  | 0.000   |
| extraction time         | 1   | 0.1094  | 0.10944 | 1.60    | 0.246   |
| extractive temperature  | 1   | 0.5359  | 0.53594 | 7.86    | 0.026   |
| liquid to solid ratio   | 7   | 1.3791  | 1.37905 | 20.22   | 0.003   |
| Error                   |     |         |         |         |         |
| Lack-of-Fit             | 6   | 0.07936 |         | 66.10   | 0.094   |
| Pure Error              | 1   | 0.4761  | 0.0012  |         |         |
| Total                   | 11  | 0.0012  | 11.0397 |         |         |

S          0.261133  R^2(adjusted)    0.9321
R^2        0.9568    R^2(Predicted)  0.8729
3.2. Steepest ascent method

According to the results of PB design, experimental points in this section moved along the direction in which volume fraction of ethanol, extractive temperature and liquid to solid ratio increased. The experimental design and results were presented in Table 6. The peak response value was up to 9.189 mg RE/100g with volume fraction of ethanol 80%, temperature 80°C, liquid to solid ratio was 55 and extraction time kept unchanged for 30 minutes. This point was considered to be near the optimal point and was selected for further optimization.

| Run No. | Volume fraction of ethanol (%) | Extractive temperature (°C) | Liquid to solid ratio | TFC (mg RE/100g) |
|---------|-------------------------------|-----------------------------|----------------------|------------------|
| 1       | 40                            | 60                          | 35                   | 5.443            |
| 2       | 50                            | 65                          | 40                   | 5.507            |
| 3       | 60                            | 70                          | 45                   | 6.052            |
| 4       | 70                            | 75                          | 50                   | 6.556            |
| 5       | 80                            | 80                          | 55                   | 9.189            |
| 6       | 90                            | 85                          | 60                   | 7.455            |
3.3. Optimization by Response Surface Methodology (RSM)

3.3.1. Second-order polynomial model. In BB design, a quadratic polynomial equation containing only significant terms was derived from multiple regression analysis to the experimental data represented in table 4, as follow:

\[ y = 9.34 - 0.44z_1 - 0.074z_2 - 0.15z_3 - 0.68z_1z_3 - 0.22z_1^2 - 0.39z_2^2 \]  

The results of the ANOVA shown in table 7 were used to check the adequacy of the quadratic polynomial equation. According to table 7, the quadratic model was highly significant (\( F = 73.86, P < 0.0001 \); “Lack of Fit P-value” of 0.8249, \( P > 0.05 \)), and the correlation coefficient (\( R^2 \)) was 0.9896, indicating good consistency between the experimental and predicted values. Meanwhile, the lower value of CV (1.00) denoted that the experimental results were highly reliable. Thus the quadratic model was adequate for prediction within the range of variables employed.

| source       | Sum of squares | df | Mean square | F-value | P-value a |
|--------------|----------------|----|-------------|---------|-----------|
| Model        | 5.11           | 9  | 0.57        | 73.86   | <0.0001   |
| \( z_1 \)    | 1.55           | 1  | 1.55        | 201.21  | <0.0001   |
| \( z_2 \)    | 0.044          | 1  | 0.044       | 5.70    | 0.0483    |
| \( z_3 \)    | 0.34           | 1  | 0.34        | 43.78   | 0.0003    |
| \( z_1z_2 \) | 2.500 \times 10^{-3} | 1 | 2.500 \times 10^{-3} | 0.33 | 0.5682 |
| \( z_1z_3 \) | 0.025          | 1  | 0.025       | 3.21    | 0.1163    |
| \( z_2z_3 \) | 0.088          | 1  | 0.088       | 11.41   | 0.0118    |
| \( z_1^2 \)  | 1.95           | 1  | 1.95        | 254.36  | <0.0001   |
| \( z_2^2 \)  | 0.20           | 1  | 0.20        | 26.69   | 0.0013    |
| \( z_3^2 \)  | 0.65           | 1  | 0.65        | 84.96   | <0.0001   |
| Residual     | 0.054          | 7  | 7.680 \times 10^{-3} |   |   |
| Lack of fit  | 9.876 \times 10^{-3} | 3 | 3.292 \times 10^{-3} | 0.30 | 0.8249 |
| Pure error   | 0.044          | 4  | 0.011       |         |           |
| Cor total    | 5.16           | 16 |             |         |           |
| \( R^2 \)    | 0.9896         |   |             |         |           |
| Adj \( R^2 \)| 0.9762         |   |             |         |           |

a \( P > 0.05 \) not significant, \( 0.01 < P < 0.05 \) significant, \( P < 0.01 \) highly significant

3.3.2. RSM analysis. The P-values gained by Design-expert 8.0.6.1 software were used to distinguish the significance of the variables on UAE. The results from table 7 revealed that \( z_1, z_2, z_3, z_1z_3, z_1^2, z_2^2, z_3^2 \) were significant terms (\( P < 0.05 \)) for UAE of FC from the stem of \( C. hybridum \) L.. Response surface graph and contour plot for TFC as a response value of volume fraction of ethanol and extraction temperature with a fixed liquid to solid ratio at zero level were gained and depicted in figure 4.
Figure 4. Response surface graph and contour plot for TFC as a response value of volum fraction of ethanol and extraction temperature with a fixed liquid to solid ratio at zero level.

The response surface graph of TFC referring to volume fraction of ethanol and extractive temperature is presented in figure 4, with liquid to solid ratio kept at zero level (55). An increasing in the extractive temperature resulted in the TFC of extraction solution increased firstly and was up to a maximum and then decreased. Liquid to solid ratio and volume fraction of ethanol had similar effects on TFC were describeld by the corresponding figures, and maximal TFC could be obtained at optimal value between every two variables.

The optimal conditions from each variable and the maximum predictive value of TFC were all gained by Design-expert software; the results were consistent with the figures analyzed above. The optimal conditions were listed as follows: volume fraction of ethanol 76.62 %, extractive temperature 78.69 ℃ and liquid to solid ratio 58.43, meanwhile, the predictive value of TFC was gained as 9.4640. Further trials were conducted under the optimal conditions to confirm the model by comparison between the predictive value of TFC and the practical result. An average value of 9.4701 (n=3) obtained from real experiments validated the suitability of the developed model.

4. Conclusion
The results of this study suggested that UAE was effective, low-cost and feasible for extracting flavonoids compounds from stem of C. hybridum L.. PB design showed that volume fraction of ethanol, extractive temperature and liquid to solid ratio were the significant variables in the UAE of FC. In addition, the optimal conditions were obtained by response surface methodology: volume fraction of ethanol 76.62 %, extractive temperature 78.69 ℃, liquid to solid ratio 58.43 and 30 min of extraction time.

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