Optimization of enzymatic hydrolysis assisted ultrasonic extraction of polyphenols from *Chaenomeles sinensis* (Thouin) koehne

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Abstract. In this paper, the optimal extraction process of polyphenols from *Chaenomeles sinensis* (Thouin) koehne was studied by single factor test and response surface test. Research results showed that the optimum extraction conditions were as follows: enzymatic time 55 min, ethanol concentration 59%, enzymatic liquid-material ratio 28:1 mL/g, pectinase amount 20 μL/g, enzymatic pH 3.4, enzymatic temperature 65°C, ultrasonic liquid-material ratio 30:1 mL/g, ultrasonic time 40 min, ultrasonic temperature 55°C. Under these conditions, the extracted content of *Chaenomeles sinensis* (Thouin) koehne polyphenols was 62.07 mg/g.

1. Introduction

*Chaenomeles sinensis* (Thouin) koehne (*C. sinensis*) is a fruit belonging to the family of Rosacea, and has the functions of benefiting stomach, relaxing tendons, relieve rheumatic pains, eliminating phlegm and relieving cough[1]. *C. sinensis* is native to China and has a history of more than 2500 years of cultivation. It is rich in functional components such as polyphenols, flavonoids, oleanolic acid, triterpenes and glycosides, which has become a rare fruit with health care, edible, ornamental, and medicinal functions, and has great development potential[2].

The extraction methods of polyphenols mainly include solvent reflux method, ultrasonic-assisted extraction method, microwave-assisted extraction method, supercritical fluid extraction method, enzyme-assisted extraction method, et al.[3]. According to the recent studies, the ultrasonic-assisted extraction method has the advantages of fast extraction speed, high yield, and low damage to heat-sensitive substances. The enzyme-assisted extraction method has the advantages of mild conditions, high extraction rate and no pollution. In this study, the method of combining enzymes and ultrasound was used to extract polyphenols from *C. sinensis*, which had theoretical and practical significance for promoting its deep processing and utilization.

2. Materials and methods

2.1. Materials

*C. sinensis* were picked from Heze City, Shandong Province, sliced and dried at 45°C, then ground
into powder of 60 mesh. Pectinase (Pectinex XXL, 10,000 pectin-transelminase units per mL [PECTU/mL]) were purchased from Novozymes (Beijing, China).

2.2. Extraction processes
The *C. sinensis* powder was treated with enzymatic hydrolysis according to an experimental design. After the end of enzymatic treatment, the enzyme was inactivated at 90°C for 5 min. The mixture was centrifuged at 4000 rpm for 5 min and collected the supernatant. Then the precipitate was added with ethanol solution for ultrasonic treatment. After centrifugation, the supernatant was collected. Two supernatants were combined to determine the polyphenol concentration and calculate the yield.

2.3. Determination of total phenolic (TP) content
The Folin-Ciocalteu method was used to determine the *C. sinensis* TP content. Pipette 1.0 mL of the extract into a 20 mL test tube with stopper, dilute to 10 mL with deionized water. Next, add 0.5 mL of Folin-Ciocalteu reagent, and dilute to 20 mL with 7.5% sodium carbonate solution. After shaking well, the absorbance was measured at 765 nm using a spectrophotometer after standing for 30 min. The TP content was shown as gallic acid equivalent (mg/g) using the following equation according to the standard curve: $y=4.6272x+0.018$, $R^2=0.999$.

2.4. Single factor experiment design
The effect of the enzyme amount (0-50 μL/g (Volume of Pectinase/Quality of *C. sinensis* Powder)), enzymatic time (20-70 min), enzymatic pH (2.8-5.8), enzymatic liquid-solid ratio (15:1-40:1 mL/g), enzymatic temperature (35-85°C), ethanol concentration (0-100%), ultrasound time (10-60 min), ultrasonic liquid-to-material ratio (15:1-40:1 mL/g), ultrasonic temperature (25-75°C) on the TP yield were investigated by the single factor experiment.

2.5. Screening key factors by Plackett-Burman design (PBD)
PBD is usually used as a basis for the selection of important variables during optimization [4]. Based on the results of the single-factor experiments, PBD was applied to evaluate twelve combinations of nine factors (coded as A-I) estimated at low (−) or high (+) levels.

2.6. Box-Behnken design (BBD)
On the basis of single factor test and PBD test, the factors with greater influence were screened out and the BBD test was carried out.

2.7. Statistical analysis
We used the Design Expert 10.0 software for the PBD and BBD approaches. The experiments were carried out in triplicates and the results were expressed as the mean ± standard deviation (SD).

3. Results and Discussion
3.1. Single Factor Analysis
The influences of the enzyme amount, enzymatic time, enzymatic pH, enzymatic liquid-material ratio and enzymatic temperature on the TP yield were showed in Fig. 1a–e. As shown in Fig. 1a, with the increasing enzyme amount from 0 to 20 μL/g, the TP yield also increased and reached the peak values of 54.43 ± 0.01 mg/g at 20 μL/g. *C. sinensis* powder comes into contact with enzymes and reacts to destroy their structures, making it easier for the polyphenols to be released[2]. However, when the amount of enzyme added exceeds 20 μL/g, the substrate was already saturated, and the polyphenol yield was not significant as the enzyme increasing. The enzymatic time (20-70 min) was investigated during the single factor experiment (Fig. 1b) and the results showed that the TP yield increased with the increasing enzymatic time and reached the peak values of 56.72 ± 0.15 mg/g at 50 min. With the exceeding of enzymatic time, pectic substances was decomposed and cells were destroyed, which was
conducive to the dissolution of polyphenols [5]. As Fig. 1c shown that the TP yield increased with the pH increased from 2.8 to 3.4 and reached the peak values of 55.86 ± 0.44 mg/g at pH 3.4. This was because the acidity and alkalinity of the solution can affect the activity of the enzyme. The enzyme has the highest activity and the strongest catalytic activity at the most suitable pH value. When deviating from the most suitable pH, the activity center or activity related groups of the enzyme changes, thereby being affected the viability of the enzyme[5]. The effect of enzymatic liquid-material ratio to the C. sinensis TP yield was shown in Fig. 1d. The C. sinensis TP yield reached the peak values of 56.61 ± 0.45 mg/g at 25:1 mL/g. When the liquid-material ratio was less than 25:1 mL/g, the TP yield increased with the increase of the liquid-material ratio. The reason was that the less solute in the extraction solvent, the better the solute dissolution. When the liquid-material ratio was greater than 25:1 mL/g, the contact between the enzyme and the substrate would be less, which was not conducive to the destruction of cells and the dissolution of C. sinensis polyphenols, resulting in a decrease in the yield of polyphenols[6]. The effect of the enzymatic temperature (35–85°C) on the TP yield was showed in Fig. 1e, where 65°C was the optimum temperature of the pectinase. When the enzymatic temperature was lower or higher than the optimum temperature, the enzyme activity would decrease which lead to the decrease of polyphenol yield. If the temperature exceeded the 65°C, that might change the structure of enzyme protein and decrease the activity of enzyme[5].

![Fig. 1](image-url)

Fig. 1 Effects of main factors on the extraction yield of TP
(a) Enzyme amount, (b) Enzymatic time, (c) Enzymatic pH, (d) Enzymatic liquid-material ratio, (e) Enzymatic temperature, (f) Ethanol concentration, (g) Ultrasonic liquid-material ratio, (h) Ultrasonic time, (i) Ultrasonic temperature.
The effect of different ethanol concentration (0%-100%) on the TP extraction from *C. sinensis* was shown in Fig. 1f. The TP yield increased with the increasing of ethanol concentration from 0% to 60% and reached the highest values of 56.04 ± 0.18 mg/g. The polyphenols in *C. sinensis* are mixtures. The polarity and chemical structure of these monomeric phenols were different, resulting in different solubility in extraction solutions with different polarities. According to the principle of similar compatibility, when the polarity of *C. sinensis* polyphenols was the same as that of the ethanol solution, it had the maximum solubility [7]. Therefore, in this experiment, it was determined that the ethanol concentration of the *C. sinensis* polyphenols was 60%.

We also investigated the ultrasonic liquid-material ratio, ultrasound time and ultrasonic temperature. The influence of the liquid-material ratio on the TP yield was presented in Fig. 1g, the TP yield increased with the liquid-material ratio increasing from 15:1 mL/g to 30:1 mL/g and reached the highest values of 54.47 ± 0.26 mg/g. When the ultrasonic liquid was greater than 30:1, the concentration of solute in the extracted solution degreased, which did not affect the dissolution of the polyphenol material [7]. The ultrasonic time (10–60 min) was also investigated (Fig. 1h). The TP yield varied irregularly with different ultrasonic times and reached a maximum at 40 min. With the increasing of ultrasonic time, the more polyphenols were dissolved out, which lead to the increase of the extraction rate of polyphenols. Excessively long ultrasonic time would also destroy the other active ingredients and release impurities, resulting in TP yield reduce[8]. The effect of different ultrasonic temperature (25-75℃) on the TP extraction from *C. sinensis* was shown in Fig. 1i. The TP yield increased with the increasing ultrasonic temperature from 25℃ to 55℃ and reached the highest values of 57.82 ± 0.22 mg/g. When the temperature was lower than 55℃, the TP yield was increased with the increasing of temperature, because the increase of temperature can enhance the solubility of solvents and accelerate the penetration and diffusion of polyphenols. However, polyphenols are easily oxidized at higher temperature, which reduces the yield of polyphenols [7].

### 3.2. The screening of key factors using PBD

According to the single factor experiment, PBD was used to screen the important factors that influence the extraction yield [4]. The TP yield (mg/g) of the extraction process was selected as the response of the design experiments. The experimental design of PBD (factors and tested range) is shown in Table 1, and the test analysis was shown in Table 2. The important impacts were represented by the lower P-value and the higher F-value in the experiment. As shown in Table 2, the enzymatic time, enzymatic liquid-material ratio, and ethanol concentration had significant effects on the TP by enzymatic hydrolysis assisted ultrasonic extraction. The effects of enzyme amount, enzymatic pH, enzymatic temperature, ultrasonic liquid-material ratio, ultrasound time, and ultrasonic temperature were not significant. Therefore, according to the result of PBD, the insignificant effect parameters were determined as follows: enzyme amount 20 μL/g, enzymatic pH 3.4, enzymatic temperature 65℃, ultrasonic liquid-material ratio 30:1 mL/g, ultrasound time 40 min, and ultrasonic temperature 55℃.

#### Table 1 Experimental design and response value of Plackett-Burman

| Run | A   | B   | C   | D   | E   | F   | G   | H   | I   | TP (mg/g) |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------|
| 1   | 1(30)| -1(40)| 1(4.0)| 1(30:1)| 1(75)| 1(80)| 1(35:1)| -1(30)| -1(45)| 62.01     |
| 2   | 1(30)| 1(60)| 1(4.0)| -1(20:1)| -1(55)| -1(40)| 1(35:1)| -1(30)| 1(65)| 55.88     |
| 3   | -1(10)| -1(40)| -1(2.8)| -1(20:1)| -1(55)| -1(40)| -1(25:1)| -1(30)| -1(45)| 56.46     |
| 4   | 1(30)| 1(60)| -1(2.8)| 1(30:1)| -1(55)| 1(80)| -1(25:1)| 1(50)| -1(45)| 52.46     |
| 5   | -1(10)| 1(60)| -1(2.8)| -1(20:1)| 1(75)| 1(80)| 1(35:1)| 1(50)| 1(65)| 49.68     |
| 6   | -1(10)| -1(40)| 1(4.0)| 1(30:1)| 1(75)| -1(40)| -1(25:1)| 1(50)| 1(65)| 63.18     |
| 7   | -1(10)| -1(40)| -1(2.8)| 1(30:1)| -1(55)| 1(80)| 1(35:1)| -1(30)| 1(65)| 63.41     |
| 8   | 1(30)| -1(40)| 1(4.0)| -1(20:1)| -1(55)| 1(80)| -1(25:1)| 1(50)| 1(65)| 52.55     |
9 1(30) 1(60) 1(2.8) 1(30:1) 1(75) 1(40) 1(25:1) 1(75) 1(40) 1(35:1) 1(50) 1(45) 63.65
10 1(30) 1(40) 1(2.8) 1(20:1) 1(75) 1(40) 1(35:1) 1(50) 1(45) 61.68
11 1(10) 1(60) 1(4.0) 1(20:1) 1(75) 1(80) 1(25:1) 1(30) 1(45) 46.90
12 1(10) 1(60) 1(4.0) 1(30:1) 1(55) 1(40) 1(35:1) 1(75) 1(40) 1(35:1) 1(35:1) 1(50) 1(45) 55.25

Note: A-enzyme amount (μL/g), B-enzymatic pH, C-enzymatic time (min), D-enzymatic liquid-material ratio (mL/g), E-enzymatic temperature (℃), F-ethanol concentration (%), G-ultrasonic temperature (℃), H-ultrasound time (min), I-ultrasonic liquid-material ratio (mL/g).

Table 2 ANOVA of Plackett-Burman

| Factor | Level | Effect Coefficient | F-value | P-value | Significance |
|--------|-------|--------------------|---------|---------|--------------|
| A      | -1    | 2.22               | 12.6    | 0.0739  | 6            |
| B      | 1     | -2.96              | 85.22   | 0.0115  | 2            |
| C      | -1    | -0.96              | 9.07    | 0.0948  | 8            |
| D      | 1     | 3.07               | 91.84   | 0.0107  | 1            |
| E      | 1     | 0.92               | 8.31    | 0.1022  | 9            |
| F      | -1    | -2.42              | 57.31   | 0.017   | 3            |
| G      | 1     | 1.06               | 10.95   | 0.0805  | 7            |
| H      | -1    | -1.13              | 12.38   | 0.0721  | 5            |
| I      | 1     | 1.13               | 12.52   | 0.0714  | 4            |

Note: P<0.05 has significant difference, P>0.05 is not significant.

3.3. Optimization of significant factors by BBD

3.3.1. Fitting the model

Based on the PBD results, three independent variables were chosen, namely enzymatic time (A), ethanol concentration (B), and enzymatic temperature (C). The TP yield (mg/g) of the extraction process was selected as the response of the design experiments. The Box–Behnken design and result were presented in Table 3. The analysis of variance (ANOVA) for BBD was shown in Table 4. The fitted model was explained by the second-order polynomial equation as follows:

\[ Y=62.14+0.39A-0.38B+0.91C+0.54AB+0.51AC-0.17BC-0.63A^2-1.90B^2-1.02C^2 \]  

(1)

It could be found that the model F-value of 32.05 and the low probability value (P < 0.0001) demonstrating that the model was of great significance for the reasonable prediction of the TP yield. Besides, the value of R^2 was 0.9763, which confirmed that the response model did not explain only 2.37% of the total variation. The P-value for lack-of-fit (0.3301) was higher than 0.05, which indicated the model had good reliability. Thus, this response model was adequate to reflect the expected optimization.

Table 3 Experimental design and response value of Box-Behnken

| Run | A    | B    | C    | TP (mg/g) |
|-----|------|------|------|-----------|
| 1   | -1(40)| 0(60)| 1(30:1)| 60.79     |
| 2   | 0(50)| -1(40)| 1(30:1)| 60.65     |
| 3   | 0(50)| 0(60)| 0(25:1)| 62.54     |
| 4   | -1(40)| 1(40)| 0(25:1)| 59.86     |
| 5   | 0(50)| 0(60)| 0(25:1)| 62.41     |
| 6   | 0(50)| 1(80)| 1(30:1)| 59.34     |
| 7   | 0(50)| 1(80)| -1(20:1)| 58.12    |
Table 4 ANOVA of Box-Behnken

| ANOVA Source | Sum of Squares | df | Mean Square | F-Value | P-Value |
|--------------|----------------|----|-------------|---------|---------|
| Model        | 34.6           | 9  | 3.84        | 32.05   | < 0.0001|
| A            | 1.22           | 1  | 1.22        | 10.21   | 0.0152  |
| B            | 1.14           | 1  | 1.14        | 9.51    | 0.0177  |
| C            | 6.61           | 1  | 6.61        | 55.08   | 0.0001  |
| AB           | 1.19           | 1  | 1.19        | 9.91    | 0.0162  |
| AC           | 1.05           | 1  | 1.05        | 8.76    | 0.0211  |
| BC           | 0.12           | 1  | 0.12        | 0.96    | 0.3589  |
| A²           | 1.68           | 1  | 1.68        | 14.03   | 0.0072  |
| B²           | 15.28          | 1  | 15.28       | 127.37  | < 0.0001|
| C²           | 4.4            | 1  | 4.4         | 36.69   | 0.0005  |
| Residual     | 0.84           | 7  | 0.12        |         |         |
| Lack of Fit  | 0.45           | 3  | 0.15        | 1.56    | 0.3301  |
| Pure Error   | 0.39           | 4  | 0.097       |         |         |
| Cor Total    | 35.44          | 16 |             |         |         |

R²=0.9763    R²_adj=0.9458    R²_pred=0.7785

Note: A-enzymatic time (min), B-ethanol concentration (%), C-enzymatic liquid-material ratio (mL/g).

3.3.2. Analysis of the response surface

As depicted in Fig. 2, the response 3D plots were provided to visualize the relationship between the responses and interactions of two variables. They visually demonstrated the effects of parameters
(enzymatic time, ethanol concentration, enzymatic liquid-material ratio) on TP. The round shape of contour indicated the interaction between the two factors was not significant, if the contour was ellipse shape, the interaction between the two factors was significant. Therefore, the interactions between enzymatic time to ethanol concentration and enzymatic time to enzymatic liquid-material ratio were significant.

3.3.3. Model validation
By the optimization analysis of the response surface, the optimal extraction conditions of TP were followed: enzyme time 55.23 min, ethanol concentration 58.99%, enzymatic liquid-material ratio 27.90:1 mL/g, and the maximum predictive yield was 62.52 mg/g. Considering operation practicability, the enzymatic time, ethanol concentration, enzymatic liquid-material ratio were revised to 55 min, 59%, and 28:1 (mL/g), respectively. In order to assess the validity of the model, the verification experiments were performed under the modified optimal condition. The actual yield was 62.07 mg/g, which was close to the predicted value, and the relative error was 0.72%. Therefore, these results confirm the predictability of the model.

4. Conclusion
In this study, the polyphenols from \emph{C. sinensis} were extracted by enzymatic hydrolysis and ultrasonic method. Based on the single factor experiment and optimization experiment, the optimal process parameters were as follows: enzyme time 55 min, ethanol concentration 59%, enzyme liquid-material ratio 28:1, enzyme amount 20 μL/g, enzyme pH 3.4, enzyme temperature 65°C, ultrasonic liquid-material ratio 30:1, ultrasonic time 40 min, ultrasonic temperature 55°C. Under these conditions, the TP yield was 62.07 mg/g. The results will provide a theoretical basis for the deep processing of \emph{C. sinensis}.

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