Optimization of ultrasonic extraction of anthocyanin in mulberry residue by response surface methodology

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Abstract. Using local mulberry products in Chuxiong as raw material, skin residue was obtained after brewing mulberry wine, and the anthocyanin extraction process was optimized by ultrasonic assisted water extraction method. Firstly, the single factor experiment was used to study the effects of four different factors, namely, ratio of feed to liquid, temperature, time and ultrasonic power, on anthocyanin extraction from mulberries. On the basis of single factor test, the optimal design of response surface was analyzed. According to the analysis results and practical verification, the optimal ultrasound-assisted extraction conditions for anthocyanin in mulberries were determined as follows: the extraction time was 40 min, the extraction temperature was 45 °C, and the ultrasonic power was 305 W. Under these conditions, the anthocyanin content was 5.212 mg/g.

1. Introduction
Mulberry (*Morus alba* L.) is the fruit of the mulberry family, ripe fruit has a sweet and slightly sour taste [1-2]. It not only contains rich anthocyanin, organic acids, amino acids and other nutrients, but also has Yin nourishing blood, invigorating fluid and quenching thirst, tonifying liver and tonifying kidney, moistening intestines and relieving constipation, delaying aging, lowering blood sugar and lowering fat and other pharmacological effects, known as the best health care holy fruit [3-4]. Due to the strong seasonality of mulberry ripening, the fresh fruit tissue is tender and is not resistant to storage. As a result, a large number of mulberry fruits are likely to rot and deteriorate causing losses to fruit farmers and wasting resources [5].

In order to avoid the waste of mulberry resources, the fresh fruits of mulberries will be processed into mulberries wine, mulberry enzyme drinks and other products, which not only improves the added value of mulberries, but also avoids the waste caused by their intolerance storage [6-8]. However, after brewing mulberry wine and other products, it is accompanied by the production of mulberry skin
residue, which contains a large amount of anthocyanin. Anthocyanin, also known as anthocyanin, are flavonoid compounds, which are safe and non-toxic water-soluble natural pigments [9]. A number of studies have shown that anthocyanin have antioxidant, anti-aging, hypoglycemic, regulating blood lipids, anti-inflammatory sterilization, improving eyesight and other effects[10-13]. Therefore, the extraction and effective utilization of the effective components from the mulberry residue can not only increase the economic benefits, but also contribute to the realization of zero pollution and zero discharge. At present, there are many research literatures on the extraction of anthocyanin from mulberry, and the main methods are ultra-high pressure extraction[14], ultrasonic assisted extraction [12,15-16], supercritical CO₂ extraction [5], ethanol extraction [17], etc.

However, during the extraction and processing of anthocyanin from mulberry, other components of mulberry were lost. Only after the mulberry is processed into alcohol or non-alcoholic drinks, can the effective components in the remaining leather residue be extracted and utilized, so that the full value of mulberry can be maximized, economic benefits can be improved, and resource waste and environmental pollution can be reduced. Currently, there are few studies on the extraction of effective components from the mulberry residue, only a few studies on the extraction of total flavonoids [18], and even fewer studies on the extraction of anthocyanin from the mulberry residue. Considering the advantages of response surface method in optimizing the process of ultrasonic assisted extraction of substances and components [19-22], this paper took the optimization of ultrasonic assisted extraction of anthocyanin from mulberry skin residue as the research objective, and adopted response surface optimization, in order to provide theoretical support for anthocyanin extraction from mulberry skin residue.

2. Materials and methods

2.1. Materials and facility
Mulberry raw materials were purchased from Qinglonghe fruit wholesale market of Chuxiong city and produced locally.

2.2. Instruments and reagents
UV-5500 ultraviolet and visible spectrophotometer (Shanghai Yuanxi Instrument Co., LTD.), PB-10 pH meter (Satorius Scientific Instrument (Beijing) Co., LTD.), Quintix224-1CN electronic scales (Satorius Scientific Instrument (Beijing) Co., LTD.), HWS-12 electric-heated thermostatic water bath (Shanghai Yiheng Scientific Instrument Co., LTD.), DHG-9070A electrothermal blowing dry box (Shanghai Yiheng Scientific Instrument Co., LTD.), SHZ-III A circulating water pump (Gongyi Yuhua Instrument Co., LTD.), GPF-50 double-stage high efficiency fine pulverizer (Taizhou Guopinleji Machine Co., LTD).

Citric acid (analytical grade, Chengdu Cologne Chemical Co., LTD.), disodium hydrogen phosphate (analytical grade, Shandong Baiqian Chemical Co., LTD.), potassium chloride (analytical grade, Shandong Jiaying Chemical Technology Co., LTD.), anhydrous sodium acetate (analytical grade, Bengbu Jingcheng Chemical Co., LTD.), hydrochloric acid (analytical grade, Chongqing in east Sichuan Chemical Group Co., LTD., Chemical Reagent Factory), acetic acid (analytical grade, Shandong Baiqian Chemical Co., LTD.).

2.3. Test procedures

2.3.1. The acquisition of residue of mulberry wine skin. After the completion of the fermentation of mulberry wine, the residue of the skin of mulberry wine was obtained after separation. The residue was dried in an electric air-blast drying box at 105 °C until the moisture evaporated. After being crushed, the residue was stored for later use.
2.3.2. The anthocyanin content was calculated. Determination by pH differential method [23]. The 1.0ml extract was put in two test tubes, and the sample was diluted 20 times by pH 1.00 and pH 4.50 buffers, respectively. The sample was placed in the dark for 0.5 h to make it stable. Then the absorbance value was measured under ultraviolet spectrophotometer with wavelength of 520 nm and 700 nm, respectively, and the value was adjusted to zero with distilled water. Calculation methods of absorbance value and anthocyanin content of samples were presented in literature [12].

2.4. Single Factor Experiment

2.4.1. The effect of liquid to material ratio (skin slag mass: water mass) on anthocyanin extraction. In the 5 support plug colorimetric tubes, 1.0000g of leather slag powder was added respectively, and the mixture was extracted for 35 minutes at 250 W at 40 °C according to the liquid-material ratio of 1:15, 1:20, 1:25, 1:30 and 1:35. The content of anthocyanin was calculated and the water consumption was determined.

2.4.2. Effect of extraction time on anthocyanin extraction. In the 5 support plug colorimetric tubes, 1.0000g of leather slag powder was added, and extracted at 1:30, 40 °C and 250 W for 25 min, 30 min, 35 min, 40 min and 45 min, respectively. The anthocyanin content was calculated and the best time was selected.

2.4.3. Effect of extraction temperature on anthocyanin extraction. In the 5 support plug colorimetric tubes, 1.0000g of derm powder was added, the ratio of material to liquid was 1:30, and the ultrasonic power was 250 W. At 30 °C, 35 °C, 40 °C, 45 °C and 50 °C, the anthocyanin was extracted for 40 min, followed by filtration to determine the content of anthocyanin, and then the optimal extraction temperature was determined.

2.4.4. Effect of ultrasonic power on anthocyanin extraction. The ultrasonic power of 200w, 250w, 300w, 350w and 400w were set at 1:30 liquid-material ratio and constant temperature of 45 °C respectively in the 5 support plug colorimetric tubes, and the ultrasonic power of 200W, 250W, 300W, 350W and 400W were extracted for 40min, then filtered to determine the content of anthocyanin, and then the most suitable ultrasonic power was determined.

2.5. Optimization of the extraction process of anthocyanin from mulberry by response surface method

According to the results of the single-factor experiment, three factors, namely extraction time, extraction temperature and ultrasonic power, which have a great influence on the extraction of anthocyanin from mulberry, were selected for the response surface optimization experiment of three factors and three levels. Experimental factors and levels are shown in Table 1.

| Coding level | X1:Extraction time/min | X2:Extraction temperature/°C | X3:Ultrasonic power/W |
|--------------|------------------------|-----------------------------|-----------------------|
| -1           | 35                     | 40                          | 250                   |
| 0            | 40                     | 45                          | 300                   |
| 1            | 45                     | 50                          | 350                   |

2.6. Statistic analysis

The software design-expert 8.0 and Excel were used to process, analyze and plot the test data, and the software origin 8.5 was used to plot the single-factor test.
3. Result and analysis

3.1. Ultrasonic assisted water extraction

3.1.1. Determination of liquid to material ratio. The effect of solid-liquid ratio on anthocyanin extraction is shown in Fig. 1. As can be seen from Fig. 1, anthocyanin content generally shows a trend of first increasing and then decreasing. With the increase of extraction solvent, the anthocyanin content also increased. When the solid-liquid ratio was 1:15, the anthocyanin content was relatively low, only 3.556 mg/g. When the liquid-liquid ratio reached 1:30, the anthocyanin content reached the maximum value of 4.982 mg/g, 40.10% higher than the initial value. With the further increase of the extraction solvent ratio, the anthocyanin content changed from rising to falling. When the solid-liquid ratio was 1:35, the anthocyanin content was 4.769 mg/g. This may be due to an increase in the amount of extract that causes the anthocyanin that have been extracted to dissolve again in the solvent. Therefore, 1:30 is the best condition for anthocyanin extraction under the single factor of liquid-solid ratio.

![Figure 1. Effect of liquid-material ratio on anthocyanin extraction](image)

3.1.2. Determination of extraction time. The effect of extraction time on anthocyanin content is shown in Fig. 2. As shown in Fig. 2, within a certain range, increase with the increase of the anthocyanin content as the extraction time, extract from the time of 25 min to 4.750 mg/g gradually increased to 40 min 5.105 mg/g, and at the age of 40 min maximum, and then to rise further as extraction time, the content of anthocyanin is the phenomenon of decline, when the time is 45 min, anthocyanin content in only 5.071 mg/g. The reason for this phenomenon can be explained as follows: within a reasonable ultrasonic treatment time, ultrasonic treatment is conducive to the destruction of cell structure and the release of anthocyanin, while excessively long ultrasonic treatment time may cause the destruction and decomposition of anthocyanin, leading to the decrease of anthocyanin content. Therefore, the most appropriate ultrasonic processing time is 40 min.
3.1.3. Determination of extraction temperature. The effect of extraction temperature on anthocyanin content is shown in Fig. 3. As shown in Fig. 3, within the temperature range set by the experiment, anthocyanin content showed an overall trend of first increasing and then decreasing. In the early stage of the range of 30 °C ~ 45 °C, as the temperature rises, the anthocyanin content increasing, from 4.227 mg/g at 30 °C rapidly increased to 45 °C at 5.047 mg/g, increased 1.194 times, and reached the maximum when 45 °C, and then with the temperature rise further, anthocyanin content in the trend of decline, only 4.663 mg/g at 50 °C, is a maximum of 92.4%.

The reason for this situation is that within an appropriate temperature range, with the increase of temperature, the permeability of the cell membrane increases, resulting in the continuous release of anthocyanin. When the temperature is too high, anthocyanin in the leather residue will be unstable and easily degraded into chalcone. Therefore, the optimal extraction temperature was chosen to be 45 °C.
3.1.4. **Determination of ultrasonic power.** The influence of ultrasonic power treatment on anthocyanin extraction is shown in Fig. 4. It can be clearly seen from the Fig. that with the continuous increase of ultrasonic power, anthocyanin content first increases and then decreases, and the highest point appears at 300W.

The anthocyanin content in the extract from 200 W was 4.591 mg/g to 5.036 mg/g at 300 W, and reached the maximum value. Then, with the further increase of ultrasonic power, the anthocyanin content in the extract decreased continuously, until it decreased to 4.723 mg/g at 400 W, a decrease of 6.21%. Thus, ultrasound can destroy the cell wall to a certain extent, make free anthocyanin, and within a certain range increased with the increase of ultrasonic power manifest this effect, but when the ultrasonic power is too large, will cause damage to anthocyanin, decompose the anthocyanin due to instability, so as to reduce the amount of extracting anthocyanin. Therefore, the optimal ultrasonic power is determined to be 300 W.

![Figure 4. Effect of ultrasonic power on anthocyanin extraction](image)

3.2. **Response surface design and results**

The response surface test design results are shown in Table 2.

| Serial number | X1 | X2 | X3 | Y: Extraction amount(mg/g) |
|---------------|----|----|----|---------------------------|
| 1             | 0  | 0  | 0  | 5.539±0.012               |
| 2             | 0  | -1 | 1  | 4.039±0.016               |
| 3             | 0  | 0  | 0  | 5.227±0.020               |
| 4             | 0  | -1 | -1 | 4.355±0.009               |
| 5             | -1 | 0  | -1 | 4.397±0.021               |
| 6             | -1 | -1 | 0  | 4.542±0.014               |
| 7             | -1 | 0  | 1  | 4.719±0.016               |
| 8             | 0  | 0  | 0  | 5.207±0.016               |
| 9             | 0  | 1  | -1 | 4.365±0.053               |
| 10            | 0  | 1  | 1  | 4.484±0.008               |
| 11            | 1  | 0  | -1 | 4.168±0.030               |
| 12            | 1  | -1 | 0  | 4.959±0.004               |
| 13            | 0  | 0  | 0  | 5.256±0.005               |
| 14            | 1  | 1  | 0  | 4.504±0.025               |
| 15            | 0  | 0  | 0  | 5.050±0.020               |
| 16            | 1  | 0  | 1  | 4.513±0.008               |
| 17            | -1 | 1  | 0  | 4.626±0.032               |
3.3. Establishment of Mathematical Model and Significance Analysis

The content of anthocyanin in the extract of mulberry dregs was taken as the dependent variable (Y) and the three selected influencing factors as the independent variables. Regression modeling and analysis were conducted with design-expert 8.0 software, and the quadratic polynomial regression equation between anthocyanin content and the three influencing factors could be obtained:

\[ Y = 5.26 - 0.018X_1 + 0.011X_2^2 + 0.059X_3 - 0.13X_1X_2 + 0.11X_1X_3 - 0.23X_2X_3 - 0.37X_2^2 - 0.58X_3^2, \]

where \( X_1 \) represents extraction time (min), \( X_2 \) represents extraction temperature (°C) and \( X_3 \) represents ultrasonic power (W). The results of model variance analysis are shown in Table 3.

According to Table 3, \( P=0.0187<0.05 \) for the established model is significant. The out-of-fit value \( P=0.1893>0.05 \) showed no significant difference, indicating that the regression equation had a good fitting degree with the test. The model adjustment factor (0.8734) is not different from the model determination factor (0.7107), and the signal-to-noise ratio is greater than 4, indicating that the model is available.

Analysis of Table 3 shows that the degree of influence of various factors on anthocyanin extraction is \( X_2<X_1<X_3 \) (that is, extraction temperature < extraction time < ultrasonic power).

Table 3. Variance analysis results

| Source     | Sum of Squares | df | Mean Square | F Value | P value | Significance |
|------------|----------------|----|-------------|---------|---------|--------------|
| Model      | 2.56           | 9  | 0.28        | 5.37    | 0.0187  | Significant  |
| X1         | 2.450\times10^{-3} | 1  | 2.450\times10^{-3} | 0.046   | 0.8359  |
| X2         | 8.820\times10^{-4} | 1  | 8.820\times10^{-4} | 0.017   | 0.9010  |
| X3         | 0.028          | 1  | 0.028       | 0.52    | 0.4938  |
| X1X2       | 0.073          | 1  | 0.073       | 1.37    | 0.2800  |
| X1X3       | 1.322\times10^{-4} | 1  | 1.322\times10^{-4} | 2.495\times10^{-3} | 0.9616  |
| X2X3       | 0.047          | 1  | 0.047       | 0.89    | 0.3762  |
| X12        | 0.22           | 1  | 0.22        | 4.19    | 0.0798  |
| X22        | 0.57           | 1  | 0.57        | 10.78   | 0.0134  |
| X32        | 1.40           | 1  | 1.40        | 26.43   | 0.0013  |
| Residual   | 0.37           | 7  | 0.053       |         |         |
| Lack of fit| 0.25           | 3  | 0.082       | 2.60    | 0.1893  |
| Pure error | 0.13           | 4  | 0.031       |         |         |
| Cor total  | 2.93           | 16 |             |         |         |

3.4. The Result of the Interaction of Various Factors on the Response Surface

The effects of various factors on anthocyanin content were analyzed with design-expert 8.0 software, and the results were shown in Fig. 5 - 7. For response surface analysis from the shape and surface slope of the contour, contour shape when the response surface, the more tend to be large oval or surface slope of the eccentricity of the more steep, response value for processing condition change, the greater the sensitivity of the interaction between factors is more significant, the opposite interaction is not obvious\[^{24}\]. As can be seen from FIG. 5-7, the effects of extraction temperature, extraction time and ultrasonic power on anthocyanin in the extract all showed a trend of first increasing and then decreasing. According to contour line analysis, all contour lines in Fig. 5-7 were oval, indicating that any two interaction factors had significant influence on anthocyanin content in the extract, but the two interaction factors had different influence on anthocyanin content in the extract. In Fig. 5, the change of the surface of the temperature axis was more obvious, indicating that the influence of temperature on the content of anthocyanin in the extract was greater than that of the extraction time. In Fig. 6, the curved surface of ultrasonic power coordinate axis changes more obviously, indicating that the influence of ultrasonic power on the content of anthocyanin in the extract is greater than that of the extraction time. In Fig. 7, the curved surface of ultrasonic power coordinate axis changes more
obviously, indicating that the influence of ultrasonic power on the content of anthocyanin in the extract is greater than that of the extraction temperature. The response surface in FIG. 7 is relatively steep, indicating that the interaction between extraction temperature and ultrasonic power has a greater effect on the anthocyanin content in the extract than the other two interactive factors.

**Figure 5.** Effects of temperature and time on anthocyanin content of mulberry slag extract

**Figure 6.** Effects of time and ultrasonic power on anthocyanin content of mulberry slag extract

**Figure 7.** Effects of temperature and ultrasonic power on anthocyanin content of mulberry slag extract

According to the response surface analysis, the optimal extraction conditions were as follows: extraction time 39.77 min, extraction temperature 45.15 °C and ultrasonic power 302.70 W. Combining with the practical feasibility of the operation, make a little adjustment to the above parameters, adjust the process conditions of the: 40 min extraction time, extraction temperature 45 °C, ultrasonic power
305 W, under the condition of the three parallel tests, the result shows that anthocyanin content in the extract of 5.212 mg/g, slightly less than the model predicts the optimal value of 5.258 mg/g, also verified the validity of the model, model prediction and actual situation alignment is higher.

4. Conclusion
In this study, anthocyanin extraction from mulberry skin residue was taken as the research objective. On the basis of single-factor experiment, response surface method was used for optimization design, and a quadratic polynomial regression model for anthocyanin extraction from mulberry skin residue was established based on the obtained data. By comparing the optimized results with the actual test, it was verified that this method could optimize the optimal extraction conditions of anthocyanin from the mulberry skin residue and give the predicted value of anthocyanin content in the extract. Considering the actual operating condition, to determine the ultrasonic assisted extraction of mulberry anthocyanin in the skin slag: best condition for extracting time for 40 min, the extraction temperature 45 °C, the ultrasonic power is 305 W, under the condition of the mulberry anthocyanin in the skin slag of anthocyanin content in liquid extract of 5.212 mg/g, and the response surface optimization basic close to forecast, shows that response surface optimization method are used to get the mulberry anthocyanin in the skin residue extraction technology is reasonable and practical, can for the extraction of mulberry anthocyanin in the skin slag utilization to provide theoretical support.

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