Study on extraction and stability of dry altar laver pigment

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Abstract. This experiment was made of Porphyra haitanensis as raw material, extraction of pigment from Porphyra haitanensis by ultrasonic wave, the stability of the pigment was preliminarily studied, determine the best extractor and maximum absorption peak wavelength, the response surface analysis was used to optimize the four factors, the concentration of the extractant, the ratio of material to liquid, the temperature of extraction and the time of extraction. The results show that the optimum extraction agent for extraction of pigment from Porphyra haitanensis is ethanol, the maximum absorption wavelength is 580nm, the optimum extraction condition is 2% of the extractant concentration, the ratio of material to liquid is 1:50, the extraction temperature is 50°C, the extraction time is three hours. Under these conditions, the extraction rate of pigment from Porphyra haitanensis was 3.8%.

Laver is an alternate algae that grows on rocks in the sea, belonging to the bangiaceae of the flerideophyceae. It is brown green or purple, and the shape varies depending on the species. The reason why laver is used a natural food pigment resource of anthocyanins is because of mainly its strong adaptability and rich production. It includes porphyra yezoensis, porphyra tenera and porphyra haitanensis. Among them, the porphyra haitanensis is widely planted in the south of China, and it also belongs to flavonoids as anthocyanins, namely rich red pigments are contained. At present, the research on dry porphyra haitanensis is mainly limited to genetics, and there are few studies on pigments, which limits deep processing and added value promotion of the dry porphyra haitanensis. The purpose of this test is to extract pigments of the dry porphyra haitanensis with the ultrasonic assistance and analyze their stability to determine the stability conditions, providing the data reference for their further development and utilization.

1. Materials and Methods

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1.1. Test Materials
Dry porphyra haitanensis: produced by Shenyang Jiduoxing Food Co., Ltd. and purchased at Darunfa Supermarket, Jilin City; anhydrous ethanol, distilled water, ethyl acetate, citric acid, sodium chloride, and hydrochloric acid; provided by the Inorganic Chemistry Laboratory of Jilin Agriculture Science and Technology College;

1.2. Test Devices
FA1104N Electronic balance: Shanghai Precision Instruments Co., Ltd.; 752N UV-Vis Spectrophotometer: Shanghai Jinghua Technology Instrument Co. Ltd.; HH.S21-611 Digital Display Electrothermal Thermostatic Water Bath: Shanghai Yuejin Medical Instruments Co., Ltd.; DHG-9420A Electrothermal Thermostatic Blast Drying Oven: Shanghai Yiheng Scientific Instruments Co., Ltd.; KQ-100DE Ultrasonic Extractor: Kunshan Ultrasonic Instrument Co., Ltd.; RE-3000 Rotary Evaporator: Shanghai Yarong Biochemical Instrument Factory; FD-1-50 Vacuum Freeze Dryer: Beijing Boyikang Lab Instrument Co., Ltd.; PHB-4 Acidimeter: Shanghai Precision Instruments Co., Ltd.

1.3. Test Methods

1.3.1. Selection of the Best Extractant and Determination of the Maximum Absorption Wavelength
Five parts of 4g dry porphyra haitanensis powder were weighed, and put into the ultrasonic environment (50°C, 1h) for leaching after added with distilled water, anhydrous ethanol, ethyl acetate, 3% sodium chloride, 2% hydrochloric acid 100ml. Next, they were subject to filtration and rotary evaporation, and their respective extraction solvents were as their own reference solution. The absorbance value was measured in a spectrophotometer using a 1cm cuvette. The wavelength range was 400nm to 600nm. Through three times of parallel measurements, the best extractant and the maximum absorption peak wavelength were determined.

1.3.2. Process Flow
Cleaned dry porphyra haitanensis→ drying → griding → weighing → adding of extractant→ heating in ultrasonic environment and extracting → filtering → vacuum rotary evaporation → pigment concentrated solution → vacuum freeze-drying → dry porphyra haitanensis pigment obtained

1.3.3. Operating Procedures
The cleaned dry porphyra haitanensis was dried, ground, accurately weighed by 10g in a beaker with an electronic balance, and 80 ml anhydrous ethanol was added, and extracted after ultrasonic heating(T=50° C., t=3 h, ultrasonic power 100 Hz). Filtration was conducted with a funnel, and then the filtrate was put in a rotary evaporator for rotary evaporation (T = 65 °C, t = 20min) to obtain a pigment concentrate. The concentrate was poured into a petri dish in the freezer for complete freezing, and vacuum freeze-drying was done(T=-59°C , t=8h), thus the dry porphyra haitanensis pigment was obtained.
1.3.4. Pigment Extraction Test

1.3.4.1. Single Factor Test for Pigment Extraction

By pre-testing, the four factors affecting the extraction rate of pigment were determined as: extractant concentration, solid-liquid ratio, extraction temperature and extraction time, and the single factor tests were designed.

(1) Selection of extractant concentration: fixed solid-liquid ratio, extraction temperature and extraction time. Extractant concentration was controlled to 1%, 1.5%, 2%, 2.5%, and 3%, respectively.

(2) Selection of the solid -liquid ratio: fixed extractant concentration, extraction temperature and extraction time. The solid-liquid ratio was controlled to 1:10, 1:20, 1:30, 1:40, and 1:50, respectively.

(3) Selection of extraction temperature: fixed extractant concentration, solid-liquid ratio, and extraction time. The extraction was conducted with ultrasonic waves, and the extraction temperature was controlled to 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C, respectively.

(4) Selection of extraction time: fixed extractant concentration, solid-liquid ratio, and extraction temperature. The extraction time was controlled to 1h, 2h, 3h, 4h, and 5h, respectively.

1.3.4.2. Rotating Orthogonal Test for Pigment Extraction

According to Box-Behnken’s central composite test design principle, the three-level RSM analysis tests of the four factors were further conducted. The A, B, C, and D factors were used as the investigative variables, expressed by codes X1, X2, X3, and X4. Besides, the level variables of the test were represented by -1, 0, and +1, and the test results of the response surface were analyzed. The test factor level of the response surface is shown in Table 1, and the test results in Table 2.

| Factor                      | Code | Level |
|-----------------------------|------|-------|
| A  Extractant concentration (%) | X1   | -1    | 1.5  | 2    | 2.5  |
| B  Solid-liquid ratio       | X2   | 0     | 1:40 | 1:50 | 1:60 |
| C  Extraction temperature (℃) | X3   | +1    | 40   | 50   | 60   |
| D  Extraction time (h)      | X4   |       | 2    | 3    | 4    |

Pigment extraction rate = weight after drying (V) / initial pigment solution weight (V₀) × 100%

1.3.5. Research on Pigment Stability

1.3.5.1. Effect of temperature on the pigment of dry porphyra haitanensis: 0.05g pigment was taken, diluted by 100 times with water, heated in a water bath, and heated in a thermostatic water bath at 20°C, 40°C, 60°C, 80°C and 100°C respectively for 30min. And then, the absorbance value was measured.

1.3.5.2. Effect of light on the pigment of dry porphyra haitanensis: 0.5g pigment was taken, diluted by 100 times with water, placed in a dark environment indoors, a natural light environment indoors and a strong-light outdoors respectively for 1d and finally the absorbance value was measured.

1.3.5.3. Effect of oxidants on the pigment of dry porphyra haitanensis: 0.5g pigment was taken, diluted by 100 times with water, added with H₂O₂, and prepared into the solutions with the concentrations of
1%, 2%, 3%, 4% and 5% respectively. After the solutions were placed in the same conditions for 30 min, the absorbance value was measured.

1.3.5.4. Effect of reducing agent on the pigment of dry porphyra haitanensis: 0.5g pigment was taken, diluted by 100 times with water, added with Na₂SO₃, and prepared into the solutions with the concentrations of 1%, 2%, 3%, 4% and 5% respectively. After the solutions were placed in the same conditions for 30 min, the absorbance value was measured.

1.3.5.5. Effect of food additives on the pigment of dry porphyra haitanensis: 0.5g pigment was taken, diluted by 100 times with water, added with citric acid, and prepared into the solutions with the concentrations of 2%, 4%, 6%, 8% and 10% respectively. After the solutions were placed in the same conditions for 30 min, the absorbance value was measured.

2. Results and Analysis

2.1. Determination of the Best Extractant and the Maximum Absorption Wavelength

As can be seen from Fig.1, the ethanol was used as the extractant at the wavelength of 580nm and the measured absorbance value was the largest, indicating that the pigment content is the highest in the dry porphyra haitanensis. The maximum absorption peak wavelength was 580 nm.

![Fig.1 Absorption Curves](image-url)

2.2. Single factor Test on the Pigment Extraction of Dry Porphyra Haitanensis

2.2.1. Effect of Extractant Concentration on the Pigment Extraction Rate

![Fig.2 Effect of Extractant Concentration on the Pigment Extraction Rate](image-url)

2.2.2. Effect of Solid-liquid Ratio on the Pigment Extraction Rate

![Fig.3 Effect of Solid-liquid Ratio on the Pigment Extraction Rate](image-url)

2.2.3. Effect of Extraction Temperature on the Pigment Extraction Rate

2.2.4. Effect of Extraction Time on the Pigment Extraction Rate
2.3. Results of the Rotating Orthogonal Test

Table 2 Design and Results of the Response Surface Test

| Run | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Response Pigment extraction rate Y: % |
|-----|----------|----------|----------|----------|---------------------------------------|
| 1   | 0.00     | 0.00     | 0.00     | 0.00     | 3.8                                   |
| 2   | 0.00     | -1.00    | 1.00     | 0.00     | 3.62                                  |
| 3   | -1.00    | 1.00     | 0.00     | 0.00     | 3.5                                   |
| 4   | 0.00     | 0.00     | 0.00     | 0.00     | 3.8                                   |
| 5   | 1.00     | 1.00     | 0.00     | 0.00     | 3.78                                  |
| 6   | 0.00     | 0.00     | -1.00    | 1.00     | 3.46                                  |
| 7   | 0.00     | 0.00     | -1.00    | -1.00    | 2.55                                  |
| 8   | 0.00     | 1.00     | 1.00     | 0.00     | 3.54                                  |
| 9   | 0.00     | 0.00     | 1.00     | 1.00     | 3.68                                  |
| 10  | 0.00     | 0.00     | 0.00     | 0.00     | 3.8                                   |
| 11  | 0.00     | -0.00    | 0.00     | 0.00     | 3.8                                   |
| 12  | 0.00     | -1.00    | -1.00    | 0.00     | 2.55                                  |
| 13  | 1.00     | -1.00    | 0.00     | 0.00     | 3.0                                   |
| 14  | -1.00    | 0.00     | -1.00    | 0.00     | 2.48                                  |
| 15  | -1.00    | 0.00     | 1.00     | 0.00     | 2.97                                  |
| 16  | 0.00     | 1.00     | 0.00     | -1.00    | 3.13                                  |
| 17  | 0.00     | 0.00     | 0.00     | 0.00     | 3.8                                   |
| 18  | 1.00     | 0.00     | 0.00     | 1.00     | 3.52                                  |
| 19  | 0.00     | -1.00    | 0.00     | 1.00     | 3.16                                  |
| 20  | 0.00     | 1.00     | -1.00    | 0.00     | 3.25                                  |
| 21  | 1.00     | 0.00     | 1.00     | 0.00     | 3.78                                  |
| 22  | -1.00    | 0.00     | 0.00     | 1.00     | 2.85                                  |
| 23  | 1.00     | 0.00     | 0.00     | -1.00    | 3.12                                  |
| 24  | 0.00     | -1.00    | 0.00     | -1.00    | 2.43                                  |
| 25  | 0.00     | 0.00     | 1.00     | -1.00    | 3.16                                  |
| 26  | -1.00    | 0.00     | 0.00     | -1.00    | 2.25                                  |
| 27  | -1.00    | -1.00    | 0.00     | 0.00     | 2.38                                  |
| 28  | 1.00     | 0.00     | -1.00    | 0.00     | 2.98                                  |
| 29  | 0.00     | 1.00     | 0.00     | 1.00     | 3.62                                  |
The Box-Behken software was used to perform multivariate regression fitting of the test data, the quadratic regression model of the pigmentation extraction rate $Y$ in the dry porphyra haitanensis relative to extractant concentration $A$, solid-liquid ratio $B$, extraction temperature $C$, and extraction time $D$ was obtained:

$$Y=+3.80+0.31A+0.31B+0.29C+0.30D-0.085AB+0.077AC-0.050AD-0.20BC-0.060BD-0.098CD-0.44 A^2-0.27B^2-0.26C^2-0.40D^2$$

The variance analysis of the response surface test for this model was conducted, and the analysis results are shown in Table 3.

| source                | Sum of squares | df | Mean Square | F Value | p-value | Significance |
|-----------------------|----------------|----|-------------|---------|---------|--------------|
| Of variance           |                |    |             |         |         |              |
| Model                 | 6.82           | 14 | 0.49        | 17.48   | <0.0001 | ***          |
| A-concentration       | 1.17           | 1  | 1.17        | 42.04   | <0.0001 | ***          |
| B-Solid-liquid ratio  | 1.13           | 1  | 1.13        | 40.48   | <0.0001 | ***          |
| C-temperature         | 1.01           | 1  | 1.01        | 36.20   | <0.0001 | ***          |
| D-Extraction time     | 1.11           | 1  | 1.11        | 39.83   | <0.0001 | ***          |
| AB                    | 0.029          | 1  | 0.029       | 1.04    | 0.3259  |              |
| AC                    | 0.024          | 1  | 0.024       | 0.86    | 0.3689  |              |
| AD                    | 0.010          | 1  | 0.010       | 0.36    | 0.5588  |              |
| BC                    | 0.15           | 1  | 0.15        | 5.46    | 0.0349  | **           |
| BD                    | 0.014          | 1  | 0.014       | 0.52    | 0.4841  |              |
| CD                    | 0.038          | 1  | 0.038       | 1.36    | 0.2623  |              |
| A²                    | 1.25           | 1  | 1.25        | 44.79   | <0.0001 | ***          |
| B²                    | 0.47           | 1  | 0.47        | 16.96   | 0.0010  | **           |
| C²                    | 0.45           | 1  | 0.45        | 16.03   | 0.0013  | **           |
| D²                    | 1.03           | 1  | 1.03        | 37.00   | <0.0001 | ***          |
| Residual              | 0.39           | 14 | 0.028       |         |         |              |
| Lack of Fit           | 0.39           | 10 | 0.039       |         |         |              |
| Pure Error            | 0.000          | 4  | 0.000       |         |         |              |
| Cor Total             | 7.21           | 28 |             |         |         |              |
| $R^2$                 | 0.9459         |    |             | $R^2_{Adj}$ 0.8918 | $R^2_{Pred}$ 0.6883 |

Note: ** indicates the difference was highly significant (P<0.05); *** indicates the difference was extremely significant (P<0.0001).

From Table 3, we can see that the model had an extremely significant difference (P<0.0001) level, and the error term was not significant, indicating that the regression equation is consistent with the actual situation, and the experimental error was relatively small. The results of variance analysis showed that the items with extremely significant differences in the regression model were $A$, $B$, $C$, $D$, $A^2$, and $D^2$, and the items with highly significant differences were $BC$, $B^2$, and $C^2$. The correlation coefficient of the quadratic polynomial $R^2 = 94.59\%$, $R^2_{Adj} = 89.18\%$, and the correction determination coefficient $R^2_{Pred} = 68.83\%$, indicating it can be interpreted that the change of the response value was
up to 69% through this model, and the equation fitting degree was relatively good. The values of \( R^2_{\text{Adj}} \) and \( R^2_{\text{Pred}} \) were reasonable. From the P value and the F value, it can be seen that the priority of the four factors affecting the pigment extraction rate of the dry porphyra haitanensis is: extractant concentration > solid-liquid ratio > extraction time and > extraction temperature.

2.4. Curved Surface Analysis of Rotating Orthogonal Test

The curved surface analysis is as shown in Fig.6-11.

From Fig. 6-11, it can be seen that the extractant concentration has the most significant effect on the pigment extraction rate of the dry porphyra haitanensis; and its curve change amplitude was big;
followed by the solid-liquid ratio, extraction time, and extraction temperature, their curve change amplitudes were flat, which was consistent with the results of variance analysis. The strength of the interaction effect is mainly reflected by the shape of the contours. It can be seen that the significance of the interaction between the two factors is expressed by an ellipse and the insignificance of the interaction between the two factors is expressed by a circle. From the contours in Fig.9, it can be seen that the interaction between the solid-liquid ratio and extraction temperature shows that the contours are elliptical, indicating the significant interaction. In comparison, the interaction of extractant concentration and extraction time showed that the contour was a circle, indicating the insignificant interaction. According to the optimization results of the response surface software, it can be seen that the optimum process conditions are: extractant concentration 2%, solid-liquid ratio 1:50, extraction temperature 50 °C. Under these conditions, the pigment extraction rate of the dry porphyra haitanensis was 3.8%.

2.5. Results of the Stability Test

2.5.1. Effect of temperature on the pigment of dry porphyra haitanensis is shown in Table 4.

| Temperature/℃ | 20°C | 40°C | 60°C | 80°C | 100°C |
|---------------|------|------|------|------|-------|
| Absorbance    | 0.089| 0.082| 0.077| 0.071| 0.058 |

From Table 4, it can be seen that the higher the temperature was, the lower the absorbance value would be, indicating that the pigment is unstable to high temperatures.

2.5.2. Effect of light on the pigment of dry porphyra haitanensis is shown in Table 5.

| Illumination   | Dark environment | Natural light | Strong light |
|---------------|------------------|---------------|-------------|
| Absorbance    | 0.159            | 0.156         | 0.048       |

From Table 5, it can be seen that the absorbance value was basically not affected when the solution was placed in a dark environment for 1d; the absorbance value decreased and the color became lighter when the solution was placed in natural lights, and the loss was larger and the color was further lighter when the solution was placed in strong lights.

2.5.3. Effect of oxidants on the pigment of dry porphyra haitanensis is shown in Table 6.

| Oxidan (H₂O₂)% | 1   | 2   | 3   | 4   | 5   |
|----------------|-----|-----|-----|-----|-----|
| Absorbance     | 0.203| 0.185| 0.172| 0.095| 0.058|

It can be seen from Table 6 that the higher the oxidant concentration was, the lower the absorbance value would be when the solution was placed for 30 min, showing the obvious oxidation. The color became lighter and the extraction rate became lower.

2.5.4. Effect of reducing agent on the pigment of dry porphyra haitanensis is shown in Table 7.

| Reduction (Na₂SO₃)% | 1   | 2   | 3   | 4   | 5   |
|---------------------|-----|-----|-----|-----|-----|
| Absorbance          | 0.212| 0.183| 0.152| 0.078| 0.040|

It can be seen from Table 7 that the higher the absorbance value was, the lower the reducing agent concentration would be when the solution was placed for 30 min, indicating that the reducing agent can cause the loss of pigment.

2.5.5. Effect of citric acid on the pigment of dry porphyra haitanensis is shown in Table 8.

| Citric acid /% | 2   | 4   | 6   | 8   | 10  |
|---------------|-----|-----|-----|-----|-----|
| Absorbance    | 0.192| 0.186| 0.180| 0.068| 0.034|

It can be seen from Table 8 that the low concentration of citric acid caused the increase of the absorbance value, and the residual rate of pigment was high. The high concentration of citric acid caused the decrease of the absorbance value and the residual rate of the pigment was low.
3. Conclusion

Studies have shown that the pigment of laver belongs to the anthocyanin pigment, with many properties of anthocyanidin. The ethanol solution had a stable red, weak antioxidant and showed sensitive to light. In the process of applying this pigment and using laver to process food, therefore, various factors affecting its stability should be fully taken into account to minimize losses. The laver pigment belongs to a natural pigment and has high nutritional value. The extracted pigment can be applied in food technology.

For the extraction of the porphyra haitanensis pigment using ultrasonic wave, the best extractant was ethanol, the maximum absorption wavelength was 580 nm, the optimum extraction conditions were extractant concentration 2%, solid-liquid ratio 1:50, extraction temperature 50°C, and extraction time 3h. Under these conditions, the extraction rate of the dry porphyra haitanensis was 3.8%.

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