Composition and antioxidant activity of anthocyanins from Aronia melanocarpa extracted using an ultrasonic-microwave-assisted natural deep eutectic solvent extraction method

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ARTICLE INFO

Keywords:
Aronia melanocarpa anthocyanins
Ultrasonic-microwave-assisted natural deep eutectic solvent extraction
Response surface methodology
Composition identification
Antioxidant capacity

ABSTRACT

A time-saving, efficient, and environmentally friendly ultrasonic-microwave-assisted natural deep eutectic solvent (UMAE-NADES) extraction method was developed for the extraction of anthocyanins from Aronia melanocarpa. Eight different natural eutectic solvents were screened initially, and choline chloride-glycerol was selected as the extraction solvent. The extraction conditions were optimized using the response surface methodology, and the extraction rate of anthocyanins was higher than those achieved using the traditional ethanol method, natural deep eutectic solvent extraction method, and ultrasonic-microwave-assisted ethanol method. Six anthocyanins, including cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside, cyanidin-3-O-xyloside, cyanidin-3,5-O-dihexoside, and the dimer of cyanidin-hexoside were identified and extracted at a purity of 448.873 mg/g using high performance liquid chromatography-mass spectrometry (HPLC-MS). The compounds extracted using UMAE-NADES had higher antioxidant capacities than those extracted by the other three methods. The UMAE-NADES demonstrated significant efficiency toward the extraction of bioactive substances and has potential utility in the food and pharmaceutical industries.

1. Introduction

Aronia melanocarpa (A. melanocarpa) or black chokeberry is a shrub of the Rosaceae family which was introduced to China in the 1990s [1]. Over 10 species of A. melanocarpa have been discovered. The fruit of A. melanocarpa is rich in a variety of nutrients, among which polyphenols rank first in terms of content. Cyanidin, which is the principal polyphenol present in the fruit of A. melanocarpa, typically exists in the form of anthocyanins. In the past decades, anthocyanins have been popular because of their antioxidant effects [2]. Several studies have demonstrated the anti-cancer, anti-aging, anti-inflammatory, liver protective, and anti-obesity effects of anthocyanins [3,4,5]. Thus, it is necessary to effectively extract anthocyanins.

At present, the extraction of anthocyanins is dominated by organic solvent extraction. However, organic solvents are volatile, toxic, and harmful to the environment. In addition, waste liquid treatment is expensive [6]. Therefore, since the introduction of green chemistry in 1998, the demand for sustainable development has prompted an investigation on non-toxic, low-cost, pollution-free, and degradable green solvents as alternatives to organic solvents [7,8,9]. In 2003, Abbott et al. [10] first proposed the concept of deep eutectic solvents (DES), and Choi, et al. [11] proposed the concept of natural deep eutectic solvents (NADES) in 2011. In general, NADES are mixtures consisting of a hydrogen-bond acceptor, such as a quaternary ammonium salt, e.g., choline chloride (ChCl), and a natural hydrogen-bond donor (HBD), such as an amino acid, a carboxylic acid, or a sugar [12]. These mixtures of natural biocompatible compounds are characterized by intermolecular interactions, particularly hydrogen bonds, which confer excellent physicochemical properties to the NADES [13]. NADES have the advantages of being green, nonflammable, highly biodegradable, easy to prepare, and inexpensive. In terms of extraction, NADES offer high efficiency and can facilitate the extraction of products varying widely in polarity.

Ultrasound-microwave-assisted extraction (UMAE) is an emerging...
technology that combines ultrasonic and microwave methods [14], utilizing the high-energy effects of microwaves and ultrasonic cavitation, and compensates for the shortcomings of traditional, ultrasonic, and microwave extraction, such as long extraction times, difficulties related to temperature control, and uneven heating. A variety of active compounds have been extracted from plants using NADES and UMAE. In this study, UMAE-NADES was applied to extract higher amounts of anthocyanins from A. melanocarpa. The conditions of UMAE-NADES were optimized using the response surface methodology, and the effects of the extraction time, temperature, and microwave power on the quantity and quality of the extracted anthocyanins were investigated. The extraction rate, anthocyanin composition, and antioxidant capacity were studied by comparison with ethanol (EtOH), natural deep eutectic solvents (NADES), and ultrasonic-microwave-assisted ethanol (UMAE-EtOH) extraction methods. This study demonstrates the potential utility of UMAE-NADES in the food industry.

2. Materials and methods

2.1. Chemical and materials

ChCl, malic acid, glycerol, citric acid, glucose, lactic acid, sucrose, potassium chloride, sodium acetate anhydrous, absolute EtOH, hydrochloric acid, methanol, and cyanidin-3-O-glucoside used for extraction were of analytical reagent grade and were purchased from Dingguo Biological Technology Co., Ltd. (Shenyang, Liaoning, China). AB-8 macroporous resin was obtained from Anhui Sanxing Resin Technology Co., Ltd. (Anhui, China). T-AOC assay kit and ABTS assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), while potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), and centrifugal equipment were purchased from Shanghai Jing Hong Laboratory Instruments Co., Ltd. (Shanghai, China).

2.2. A. Melanocarpa samples

A. melanocarpa was obtained from Liaoning FuKangyuan Black Chokeberry Technology Co., Ltd. (Haicheng, Liaoning, China). A. melanocarpa without any mechanical damage were selected and squeezed. The pulp of A. melanocarpa was stored at −20 °C.

2.3. Preparation of NADES

ChCl, which was used as the HBA, was dried in an electric blast drying oven (DHG-9140A, Shanghai Jing Hong Laboratory Instruments Co., Ltd., Shanghai, China) at 40 °C for 1 h prior to use. ChCl was mixed with one or more HBAs at a certain molar ratio. The mixture was heated in a water bath at 80 °C and stirred continuously for 30–40 min, followed by dilution using 30 % (v/v) distilled water to obtain a uniform and transparent colorless solution that exhibited no crystallization after 12 h. The abbreviated names of the NADES and corresponding molar ratios are listed in Table 1.

2.4. UMAE-NADES and determination of anthocyanins

2.4.1. UMAE-NADES extraction

The pulp of A. melanocarpa (200 g) was mixed with NADES-6 (ChGly) at a solid–liquid ratio of 1:15 (w/v). UMAE (CW-2000, Shanghai Xtrust Analytical Instruments Co., Ltd., Shanghai, China) was performed, followed by filtration, and the filtrate was rotary evaporated at 40 °C. The resulting concentrate was collected and stored at 4 °C. The collected filtrate was purified using a chromatographic column equipped with AB-8 macroporous resin at a loading of 1/2. Adsorption was allowed to occur while standing for 12 h, and distilled water was used to elute glycerol and other impurities until there was no residual glycerol. The sample solution was eluted with 75% EtOH followed by rotary evaporation at 40 °C to remove the EtOH. The obtained concentrate was freeze-dried to a powder and stored at −20 °C for standby.

2.4.2. Determination of anthocyanins content and recovery

The anthocyanin content was determined using the pH-difference method [19]. The sample (0.5 mL) was mixed with 9.5 mL each of potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), and incubated at room temperature in the dark for 15–20 min. The absorbance values of the reaction mixture were measured at 520 and 700 nm.

The anthocyanins recovery was calculated according to the following equation:

\[
\text{Anthocyanins recovery} = \frac{C}{C_t} \times 100
\]

where C and C_t are the anthocyanins content in the crude extract obtained using UMAE-NADES (mg/g), and C is the total anthocyanins content (mg/g).

2.5. Extraction method optimization

Based on the single factor test, the Box-Behnken design (BBD) was used to select the microwave power (X_1), extraction time (X_2), and extraction temperature (X_3) as the independent variables, and the anthocyanin content (Y) extracted from A. melanocarpa was the dependent variable. The three-factor and three-level response surface analysis method was used to optimize the extraction conditions.

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**Table 1**

| NADES (30%, v/v) abbreviations and molar ratios | Natural deep eutectic solvents | Abbreviation | Molar ratio |
|-----------------------------------------------|--------------------------------|-------------|------------|
| NADES-1 Choline chloride: Citric acid         | ChCit                          | 1:1         |
| NADES-2 Choline chloride: Malic acid          | ChMa                           | 1:1         |
| NADES-3 Choline chloride:Lactic acid          | ChLa                           | 1:1         |
| NADES-4 Choline chloride: Glucose             | ChGlu                          | 1:1         |
| NADES-5 Choline chloride: Sucrose             | ChSuc                          | 1:1         |
| NADES-6 Choline chloride: Glycerol            | ChGly                          | 1:2         |
| NADES-7 Choline chloride:Citric acid: Glucose | ChCitGlu                       | 1:1:1       |
| NADES-8 Choline chloride:Citric acid: Glycerol| ChCitGly                       | 1:1:1       |

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**Table 2**

| Independent variables | Symbol | Coded factor |
|-----------------------|--------|-------------|
| Microwave power       | X₁(W)  | −1 0 1      |
| Extraction time       | X₂(s)  | 150 200 250 |
| Extraction temperature| X₃(°C)| 45 50 55   |

---

The independent variables and levels in the BBD of response surface.
used to optimize the extraction conditions of the UMAE-NADES method, and the independent variables and levels are shown in Table 2.

The ultrasonic power was held constant (50 W) in all of the experiments due to the instrumental setup. Seventeen experiments were performed with 5 center points per block to optimize the extraction method, and the responses were fitted to a second-order polynomial Eq. (3) [20].

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad i < j
\]  

(3)

where \(X_1, X_2, ..., X_k\) are the independent variables; \(Y\) is the dependent variable; \(\beta_0, \beta_i (i = 1, 2, ..., k), \) and \(\beta_{ij} (i = 1, 2, ..., k; j = 1, 2, ..., k)\) are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively; and \(k\) is the number of variables.

The statistical significance of the terms in the regression equations was examined by analysis of variance (ANOVA) for every response model. The response variable (\(Y\)), which was determined using Eq. (4), denotes the yield of anthocyanins (mg/g).

\[
Y(\text{mg/g}) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + Y(\text{mg/g})
\]

\[
= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3
\]  

(4)

The adequacy of the model accounting for \(R^2\) and \(R^2_{adj}\) and the absolute deviation PRESS was examined using Eqs. (5)–(7) [21]:

\[
R^2 = 1 - \frac{SS_{Rec\ model}}{SS_{Rec\ total} + SS_{Model}}
\]  

(5)

\[
R^2_{adj} = 1 - \frac{SS_{Rec\ model} / DF_{Rec\ model}}{(SS_{Rec\ total} / DF_{Rec\ total}) + (SS_{Model} / DF_{Model})}
\]  

(6)

\[
PRESS = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (Y_{Exp,i} - Y_{Pred,i})^2}
\]  

(7)

The degree of precision was calculated as the deviation of the predicted values of the test points relative to the predicted average value. It was defined using Eqs. (8) and (9).

\[
\bar{\gamma} = \frac{1}{n} \sum_{i=1}^{N} \gamma_i = \frac{P_{Exp}}{n}
\]  

(8)

\[
Adequate\ precision = \frac{\text{Max}(\gamma) - \text{Min}(\gamma)}{\sqrt{\bar{\gamma}}}
\]  

(9)

In the Eqs. (5)–(9), \(SS, DF, Y_{Exp,i}, Y_{Pred,i}\) \(Y, P, \sigma_2\) and \(n\) represent the quadratic sum, number of the degrees of freedom of the model, response value of the tests, predicted response value, predicted value, number of parameters of the model, residual error of the square mean sum acquired from ANOVA, and the number of tests, respectively [22].

The Design-Expert (Version 11) software was used for the ANOVA to obtain a quadratic polynomial mathematical model to describe the effects of the interaction between the process parameters on the extraction of the anthocyanins. The value of the determination (\(R^2\)) and the model P value were used to predict the model capability [23].

2.6. Extraction of A. Melanocarpa anthocyanins by other methods

To evaluate the efficiency of UMAE-NADES for the extraction of anthocyanins, solvent heating extraction (EtOH, NADES) and UMAE-EtOH were selected for comparison with UMAE-NADES. The following methods were obtained through single factors test optimization: (1) EtOH: 0.5 g A. melanocarpa pulp was weighed, mixed thoroughly with acidified EtOH (pH 2.5) to a concentration of 60% at a solid–liquid ratio of 1:36, and heated in a water bath at 60 °C for 72 min. (2) NADES: 0.5 g pulp was weighed, mixed with NADES-6 (ChGly) at a solid–liquid ratio of 1:26, and heated in a water bath at 51 °C for 51 min. (3) UMAE-EtOH: 0.5 g pulp was accurately weighed, mixed with acidified EtOH (pH 2.5) to a concentration of 60% at a solid–liquid ratio of 1:25, and extracted for 385 s at a temperature of 45 °C and microwave power of 226 W.

2.7. High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis

The freeze-dried powder (2 mg) obtained as described in sections 2.4.1 and 2.6, was dissolved in 2 mL chromatographic grade methanol, and purified via filtration through a 0.45 μm membrane. The anthocyanins were subsequently identified and quantified using HPLC-MS at 520 nm. Assuming that the area of each peak was the same on a molecular basis, the anthocyanins were identified with respect to the standard cyanidin-3-O-glucoside regardless of the chemical properties of the molecule, and quantified according to the calibration curve of cyanidin-3-O-glucoside. The standard concentrations were 6, 12, 60, 120, and 600 μg/mL, and the HPLC-MS conditions were the same as that described by Wang [24].

2.8. Antioxidant capacity assessment

The antioxidant capacities of the anthocyanins obtained using the different extraction methods, including UMAE-NADES, UMAE-EtOH, NADES, and EtOH, were evaluated using ABTS, FRAP, and T-AOC kits after purification using macroporous resin. The lyophilized anthocyanins extracted by the four methods were used to prepare test solutions of identical concentrations. The ABTS, FRAP, and T-AOC working solutions were prepared according to the manufacturer’s instructions. The sample (0.6 mg) was dissolved in EtOH (2 mL).

The ABTS working solution (200 μL) and 10 μL of the anthocyanin test solution were mixed thoroughly, incubated at room temperature for 6 min, and the absorbance was measured at 734 nm. The anthocyanin test solution was added to 180 μL FRAP reagent, followed by shaking and mixing. The mixture was incubated at 38 °C for 5 min, and the absorbance was measured at 593 nm. Trolox was used as the standard curve to calculate the ABTS, FRAP clearance capacity of the anthocyanin test solution (expressed as Trolox equivalent, TE/mM) [25].

The total antioxidant capacity of the anthocyanins was determined using a T-AOC kit based on colorimetry. The absorbance was measured at 520 nm. When the optical density (OD) of the reaction system increased by 0.01 per milligram of sample to be tested per minute at 37 °C, it was considered as a unit of total antioxidant capacity (U). The total antioxidant capacity was expressed as T-AOC (U/mg).

2.9. Statistical analysis

All the experiments were performed in triplicate, and the Origin 2021 software was used to arrange the data. Statistical analysis was performed using the software Design-Expert® 11, and the results are expressed as mean ± standard deviation. The data were analyzed by ANOVA (p < 0.05), different lowercase letters indicated significant differences between groups, and the averages were separated using Tukey’s test (SPSS 22.0 software).

3. Results and discussion

3.1. Selection of NADES system for extraction of A. Melanocarpa anthocyanins

3.1.1. Effect of different types of NADES

The NADES differed in polarity, solubility, viscosity, surface tension, and physicochemical interactions due to the use of different HBD and HBA species. Considering that the physicochemical properties and extraction rates were determined by the solvent structures of NADES, it was important to identify the most efficient NADES for anthocyanin
Fig. 1. Effect of NADES types (A), water content (B) and single factors of four methods(C,D,E,F) on anthocyanins content. The different letters show significant differences between groups ($p < 0.05$) through using Tukey’s test.
extraction. ChCl was used as an HBA, and organic acids (citric, malic, and lactic acid), sugars (glucose, sucrose), and glycerol were used as the HBDs to form eight binary and ternary NADES and their extraction efficiencies were compared. As shown in Fig. 1(A), among the eight NADES, the anthocyanin content extracted using ChGly was the highest, followed by ChLa, ChSuc, ChGlu, ChCitGly, ChMa, ChCitGlu, and ChCit. These results can be attributed to the correlation between the viscosity of the NADES and the yield of anthocyanins [26]. The high viscosity of the NADES is mainly attributed to the presence of hydrogen bonds, with intermolecular structure which hinders contact between the anthocyanin and NADES molecules. The addition of an appropriate amount of water weakens the interaction between the NADES molecules, facilitating the dissolution of the anthocyanin molecules in the NADES. To determine the optimal water content for anthocyanin extraction, the extraction was performed at different water contents (10–50%). As observed from Fig. 1(B), the anthocyanin content reached its highest value at a water content of 30% (v/v). However, a higher water content (≥50%) affects the extraction of anthocyanins. For water content ≥ 50%, the concentration of the NADES is reduced and the interaction between the NADES and the target compounds is weakened [29], thus reducing the extraction efficiency.

3.2. Single factors on the content of anthocyanins

The single-factor results of the four methods are shown in Fig. 1 (C, D, E, F). The optimum solid–liquid ratio, extraction time, temperature, and microwave power for UMAE-NADES were 1:15 g/mL, 300 s, 50 °C, and 200 W, respectively. The optimum solid–liquid ratio, time, temperature, and microwave power for UMAE-EtOH were 1:25 g/mL, 400 s, 40 °C, and 250 W, respectively. The optimum solid–liquid ratio, time, and temperature for NADES were 1:25 g/mL, 60 min, and 50 °C, respectively. The optimum solid–liquid ratio, time, and temperature for EOH were 1:35 g/mL, 90 min, and 60 °C, respectively. According to the overall trend of the single factors of the four methods, the anthocyanin

Table 3

| Source of Variation | Sum of Squares | df | Mean Square | F-value | P-value |
|---------------------|----------------|----|-------------|---------|---------|
| X₁                  | 0.7984         | 9  | 0.0887      |         |         |
| X₂                  | 0.0010         | 9  | 0.0002      | 2.17    | 0.1345  |
| X₃                  | 0.1925         | 9  | 0.0203      | 2.17    | 0.1345  |
| X₄                  | 0.0630         | 9  | 0.0060      | 2.17    | 0.1345  |
| X₁X₂                | 0.0802         | 9  | 0.0089      | 2.17    | 0.1345  |
| X₁X₃                | 0.0086         | 9  | 0.0009      | 2.17    | 0.1345  |
| X₁X₄                | 0.0096         | 9  | 0.0010      | 2.17    | 0.1345  |
| X₂²                  | 0.1562         | 9  | 0.0321      | 2.17    | 0.1345  |
| X₂X₄                | 0.1717         | 9  | 0.0177      | 2.17    | 0.1345  |
| Residual             | 0.0003         | 2  | 0.0001      | 2.17    | 0.1345  |
| Lack of Fit          | 0.0097         | 7  | 0.0013      | 2.17    | 0.1345  |
| Pure Error           | 0.0236         | 4  | 0.0060      | 2.17    | 0.1345  |
| Cor Total            | 0.8316         | 16 | 0.0519      | 2.17    | 0.1345  |
content first increased and then decreased.

Based on the significance analysis and the steepness of the broken line in Fig. 1(C–F), it was found that the significance of the solid-to-liquid ratio of UMAE-NADES and UMAE-EtOH was lower than those of temperature, time, and microwave. This was because high temperature increased the speed and diffusion rate of molecules, and the extraction time was long enough to ensure that the anthocyanins were fully dissolved into the solvent. The microwave irradiation provided local heating in the plant cells and facilitated the destruction of the cell walls, accelerating the solvent. The microwave irradiation provided local heating in the plant enough to ensure that the anthocyanins were fully dissolved into the

Table 5
Analysis of variance of anthocyanins content in NADES and EtOH.

| Source    | Sum of Squares | Df | Mean Square | F-value | p-value |
|-----------|----------------|----|-------------|---------|---------|
| Model     | 0.3659         | 9  | 0.0407      | 25.97   | 0.0001  |
| X1        | 0.0093         | 1  | 0.0009      | 5.92    | 0.0453  |
| X2        | 0.0253         | 1  | 0.0057      | 16.19   | 0.005   |
| X3        | 0.0014         | 1  | 0.0001      | 0.8743  | 0.3899  |
| X4        | 0.0121         | 1  | 0.0012      | 7.72    | 0.0274  |
| X5        | 0.0008         | 1  | 0.0008      | 0.5351  | 0.4882  |
| X6        | 0.0007         | 1  | 0.0007      | 4.45    | 0.0728  |
| X7        | 0.0673         | 1  | 0.0673      | 42.98   | 0.0003  |
| X8        | 0.0595         | 1  | 0.0595      | 38.03   | 0.0005  |
| X9        | 0.1525         | 1  | 0.1525      | 97.39   | 0.0001  |
| Residual  | 0.011          | 7  | 0.0016      | 0.566   | 0.666   |
| Lack of Fit| 0.0033         | 3  | 0.0011      | 0.0018  | 0.0001  |
| Pure Error| 0.0077         | 4  | 0.0019      | 0.0034  | 0.0001  |
| Cor Total | 0.3768         | 16 |             | 11.4815 | 11.4333 |

Table 6
Variance analysis of fitted model.

| Source          | UMAE-NADES | UMAE-EtOH | NADES | EtOH |
|-----------------|------------|-----------|-------|------|
| Std.Dev.        | 0.069      | 0.0834    | 0.0396| 0.0517|
| Mean            | 4.19       | 3.35      | 3.16  | 2.82 |
| R-squared       | 0.86       | 0.9514    | 0.9790| 0.9460|
| Adj R-squared   | 0.9085     | 0.8888    | 0.9335| 0.8766|
| Pred R-squared  | 0.7679     | 0.8675    | 0.8294| 0.6969|
| C.V.%           | 1.64       | 2.49      | 1.25  | 1.83 |
| PRESS           | 0.1915     | 0.1225    | 0.0643| 0.1052|
| Adeq precision  | 11.4815    | 11.8741   | 12.9885| 11.4333|

3.3. Optimization of anthocyanin extraction using BBD

3.3.1. Statistical analysis and modeling of extraction of anthocyanins

The process variables and experimental data related to the anthocyanin contents obtained by the different extraction methods are listed in Tables 3 and 4, and Table 5 summarizes the analysis results for the UMAE-NADES, UMAE-EtOH, NADES, and EtOH response surface experimental design and the adequacy and suitability of the model. As shown in Table 4, for the Y3 model, X2, X3, X1X2, X1’X2’1, X1’X3’, X2’X3’, and X1’X2’ were significant (p < 0.05); for the Y2 model, X3, X2X3, X2X3, X1’X2’1, and X1’X2’ were significant (p < 0.05). As shown in Table 5, for the Y3 model, X1, X2, X1X2, X1’X2’, X1’X3’, and X3’ were significant (p < 0.05), while for the Y4 model, X2, X1X3, X2X3, X1’X2’1, and X1’X2’ were significant. The other items had no significant difference (p > 0.05). A significant lack of fit (p < 0.05) indicates that the models failed to represent the data points in the experimental domain that were not included in the regression. As shown in Tables 4 and 5, the lack of fit of the four models was not significant (p > 0.05), indicating that the models represented the data satisfactorily. The determination coefficient (R2), modified coefficient of association (R2adj), predicted modified coefficient of association (R2pred), and coefficient of variation (CV) were calculated to check the adequacy of the models (Table 6).

The R2 value reflects the closeness of the generated response surface to the actual situation. A larger R2 value indicated that the response surface was closer to the actual value. At an R2 ≥ 0.90, the response surface was considered to adequately reflect the actual situation. The R2 values of the four models in this experiment were > 0.94, indicating that the test values were close to the actual values. However, a high R2 value does not indicate that the regression model is satisfactory. R2 can also increase upon the addition of a variable to the model if the contribution of the additional variable is statistically significant. Therefore, R2adj should be used simultaneously to evaluate the adequacy of the model. It can be seen from Table 6 that the R2adj values of the four models were slightly lower than the corresponding R2 values, and the differences between R2pred and R2adj were small, indicating that they were within a reasonable fluctuation range [31].

A low CV value (<0.10) indicates that the model is highly accurate and reliable [32]. The adequacy of precision (Adeq Precision) represents the signal-to-noise ratio, and a ratio > 4 is desirable [32,33]. As shown in Table 6, CV < 3, PRESS of the four models < 0.2, and Adeq Precision > 11, indicating that each point in the experimental design fitted the quadratic model satisfactorily, suggesting that the model fully explains the response surface. Therefore, the regression equation describes the actual relationship between each factor and the anthocyanin content, and can determine the optimum conditions for the extraction process, so that it can be used for the theoretical prediction of the test results.

According to the above analysis, the extracted anthocyanin content can be expressed by the quadratic multiple term regression Eq. (10):

\[
Y_1 = 4.44 + 0.0359X_1 + 0.1531X_2 + 0.0888X_3 + 0.1461X_4 + 0.0464X_5 + 0.0489X_6 + 0.1223X_7^2 - 0.1926X_8^2 - 0.2019X_9^2
\]

\[
Y_2 = 3.56 - 0.0531X_1 + 0.1022X_2 + 0.0661X_3 - 0.2057X_4 + 0.0827X_5 + 0.2295X_6 - 0.1526X_7^2 - 0.2519X_8^2 - 0.0459X_9^2
\]

\[
Y_3 = 3.37 - 0.0340X_1^2 - 0.0563X_2 + 0.0131X_3^2 + 0.0550X_4 - 0.0145X_5 - 0.0417X_6^2 - 0.1264X_7^2 - 0.0189X_8^2 - 0.1903X_9^2
\]

\[
Y_4 = 2.98 - 0.0086X_1^2 + 0.00820X_2^2 + 0.0218X_3^2 + 0.0487X_4^2 - 0.0769X_5^2 + 0.0734X_6^2 + 0.0667X_7^2 - 0.0772X_8^2 - 0.1894X_9^2
\]
Fig. 2. Three-dimensional and two-dimensional response surface diagrams of A) UMAE-NADES, B) UMAE-EtOH, C) NADES, and D) EtOH show the comprehensive effects of experimental factors on anthocyanins content.
Fig. 2. (continued).
where $Y_1$, $Y_2$, $Y_3$, and $Y_4$ represent the extracted anthocyanin contents using UMAE-NADES, UMAE-EtOH, NADES, and EtOH, respectively, $X_1$ and $X_1'$ represent the microwave power (W), $X_2$ and $X_2'$ represent the time (s), $X_3$, $X_3'$, $X_4$, and $X_4'$ denote the temperature (°C), $X_1$ and $X_1'$ are the solid–liquid ratios (g/mL), and $X_2$ and $X_2'$ represent the time (min).

### 3.3.2. Analysis of response surface and two-dimensional contour plots

By observing the changes in the response surface and contour in Fig. 2, we could intuitively determine the influence of the interaction between the factors in the four methods on the anthocyanins content extracted from A. melanocarpa. When the contour was largely circular, the interaction between the two factors was not significant. A greater degree of deviation from the circular contour corresponds to a more significant interaction between the two factors, and the steepness of the response surface 3D graph indicates the sensitivity of the extraction amount to the change in the extraction conditions. A change in the color from blue to red indicates an increase in the extraction.

As shown in Table 4, the interaction between microwave power and time in UMAE-NADES was significant, and the anthocyanins content increased significantly with the increase in microwave power. Fig. 2A shows that when the microwave power reached 150–200 W and time reached 200–300 s, the higher the microwave power and the higher the time, and the higher the anthocyanins content of UMAE-NADES, which was due to the significant positive interaction between microwave power and time. The contour line tilted sharply to the high and low levels of these two factors, and the two-dimensional contour line was oval. As shown in Table 4 and Fig. 2B, the interactions between microwave power and time, and between temperature and time in UMAE-EtOH were significant. The highest anthocyanins content was obtained when the microwave power reached 200–275 W, and the time and temperature were above 350 s and 41 °C, respectively.

Microwave power, time, and temperature were important factors affecting the anthocyanins content in UMAE-NADES and UMAE-EtOH. As shown in Fig. 2A and B, with the increase in these three factors, the content of anthocyanins in UMAE-NADES and UMAE-EtOH gradually increased. This was because at the initial stage of extraction, under fixed ultrasonic conditions, the cell wall was broken by shear and cavitation, so that anthocyanins begin to diffuse. At the same time, the solvent increases its temperature by absorbing microwave energy. This change accelerated the release of anthocyanins in plant cells and promoted their entry into solvents. With the extension of the extraction time, higher temperature accelerated molecular motion and changed the conductivity of extraction solvent and plant [34]. In addition, the increase in microwave power enhanced the penetration of the solvent into plant cells. At the same time, microwave irradiation accelerated cell rupture through the sudden rise of temperature and internal pressure in plant cells, so as to promote the destruction of cell wall matrix and epidermal tissue, and then accelerated the exudation of anthocyanins into the solvent [35].

As shown in Table 5, the interaction between time and solid–liquid ratio in NADES was significant. Fig. 2C shows that when the time and solid–liquid ratio reached 30–70 min and 1:22–1:33 g/mL, respectively, the anthocyanins content of NADES was the highest. As shown in Table 5 and Fig. 2D, the interactions between temperature and time, temperature and solid–liquid ratio in EtOH were very significant. The highest anthocyanins content was obtained when the temperature, time, and solid–liquid ratio reached 58–64 °C, 60–105 min and 1:30–1:40 g/mL.

The anthocyanins content first increased with an increase in the solid–liquid ratio, time, and temperature because the increase in the proportion of the solvent facilitated the diffusion and mass transfer of the anthocyanins, and the increased temperature and time increased the diffusion rate of anthocyanins. The anthocyanins content decreased as the solid–liquid ratio, time, temperature exceeded the threshold value, indicating that an increase in the solid–liquid ratio increased the proportion of the extraction solvent and reduced the concentration of the extraction solution; this led not only to a reduction in the anthocyanins content, but also wastage of the solvent. On the other hand, the excessive increase in the temperature and time led to the degradation of anthocyanins.

### 3.3.3. Optimization of extraction parameters and validation of the optimized conditions

Combined with the regression mathematical analysis model, the optimized conditions of four methods were obtained using the BBD. The optimum process conditions for extracting anthocyanins from A. melanocarpa were as Table 7.

In the actual operation, the parameters in UMAE-NADES, UMAE-

| Method | Extraction conditions | Anthocyanins content (mg/g) | Extraction rate (%) |
|--------|-----------------------|-----------------------------|---------------------|
|        | Solid-liquid ratio    | time | temperature | concentration | microwave power | | |
| EtOH   | 1:36 g/mL             | 72 min | 60 °C | 60%          | –         | 2.996 ± 0.013d | 82.25 ± 0.80d |
| NADES  | 1:26 g/mL             | 51 min | 51 °C | 1:2          | –         | 3.311 ± 0.015d | 87.66 ± 0.67d |
| UMAE-EtOH | 1:25 g/mL          | 385 s | 45 °C | 60%          | 226w | 3.599 ± 0.093d | 93.09 ± 0.75d |
| UMAE-NADES | 1:15 g/mL         | 367 s | 52 °C | 1:2          | 230w | 4.456 ± 0.032d | 97.05 ± 0.85d |
Fig. 4. HPLC chromatogram of A) UMAE-NADES, B) UMAE-EtOH, C) NADES, and D) EtOH, and the mass spectra of UMAE-NADES: a) dimer of cyanidin-hexoside, b) cyanidin-3-O-galactoside, c) cyanidin-3-O-glucoside, d) cyanidin-3-O-arabinoside, e) cyanidin-3-O-xyloside, and f) cyanidin-3,5-O-dihexoside purified using AB-8 resin at 520 nm.
EtOH, NADES, EtOH were adjusted. Their microwave powers were 230 W and 226 W, respectively. Extraction time were 367 s, 485 s, 51 min and 72 min, respectively. Extraction temperature were 52 °C, 45 °C, 51 °C, and 60 °C, respectively. Solid-liquid ratios were 1:26 and 1:36, respectively. Under these conditions, a validation test was performed, and the obtained extraction amounts were 4.456 ± 0.032, 3.599 ±
0.093, 3.311 ± 0.018, and 2.996 ± 0.013 mg/g. The actual extraction values for the four methods were close to the predicted values. Therefore, the process conditions optimized by the BBD analysis method are reliable.

3.4. Comparison of UMAE-NADES with other extraction methods

The extraction parameters of the anthocyanins from *A. melanocarpa* by UMAE-NADES, UMAE-EtOH, NADES, and EtOH are shown in Table 8 and Fig. 3. The results showed that the dosage, extraction time, and temperature of the NADES extraction method were lower than those of the EtOH method, and the anthocyanins content and extraction rate

Table 9

| Peak | Anthocyanin            | Retention time (min) | Molecular/Fragment (m/z) | Anthocyanin content (mg/g) |
|------|------------------------|----------------------|--------------------------|---------------------------|
|      |                        |                      |                          | A  | B  | C  | D  |
| 1    | Dimer of cyanidin-hexoside | 15.403–15.508        | 896.5/735,573,287        | 7.862 | 7.403 | 6.450 | 6.002 |
| 2    | Cyanidin-3-O-galactoside | 16.217–16.611        | 448.8/287                | 280.042 | 242.530 | 206.571 | 179.239 |
| 3    | Cyanidin-3-O-glucoside | 16.815–17.191        | 448.9/287                | 16.142 | 10.649 | 15.356 | 14.224 |
| 4    | Cyanidin-3-O-arabinoside | 17.903–18.126        | 418.9/287                | 123.801 | 90.755 | 91.361 | 82.216 |
| 5    | Cyanidin-3-O-xyloside | 19.660–19.670        | 418.9/287                | 21.024 | 13.827 | 17.063 | 17.063 |
| 6    | Cyanidin-3,5-O-dihexoside | 15.085–15.183        | 610.9/448.8,287          | – | – | – | – |
| Total anthocyanin content (mg/g) | – | – | 448.873 | 365.163 | 328.764 | 299.873 |
was superior to that of UMAE-EtOH. This is attributed to the fact that were significantly reduced, and the extraction effect of UMAE-NADES were higher than those obtained using EtOH. This is attributed to the lower volatility of NADES compared to EtOH which results in the improved dissolution of anthocyanins in the NADES. After auxiliary extraction, the amount of solution, time, content, and extraction rate were significantly reduced, and the extraction effect of UMAE-NADES was superior to that of UMAE-EtOH. This is attributed to the fact that while ultrasound and microwaves were used in both cases to accelerate the diffusion and dissolution of the anthocyanins, the capacity of the NADES to dissolve the anthocyanins was higher than that of EtOH. Therefore, UMAE-NADES can be used for the efficient extraction of anthocyanins, and it has been proven that this hybrid technology has significant potential for reducing energy consumption and achieving high efficiency [36]. Additionally, the UMAE-NADES method offered significantly higher extraction rates than the traditional and UMAE-EtOH methods ($p < 0.05$). Therefore, it is an effective technology for the extraction of anthocyanins from A. melanocarpa.

3.5. HPLC-MS analysis

After purification using AB-8 macroporous resin, the types of anthocyanins extracted by the four methods were identified by HPLC-MS, as shown in Fig. 4. At a wavelength of 520 nm, five peaks were identified from the HPLC retention time, elution sequence, spectral characteristics, and fragmentation mode; the sixth peak, which was not detected in the liquid chromatogram, appeared in the mass spectrum of the anthocyanins (Table 9). The anthocyanins follow a general retention order based on the degree of polarity of the molecular structure that is mainly affected by the anthocyanin components in the HPLC system. The different anthocyanins followed the elution series (from the shortest to the longest retention time): delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. The elution series (from the shortest to the longest retention time) differed only in the number and type of glycosides: 3,5-diglucoside, 3-diglucoside, galactoside, sambubioside, glucoside, arabinoside, rutinoside, and xyloside [37]. Therefore, peak 1 is assigned as the dimer of cyanidin-hexoside, and the sugar substituents of peaks 2 and 3 are the isomers, galactose and glucose, respectively. Therefore, peaks 2 and 3 are assigned as cyanidin-3-O-galactoside and cyanidin-3-O-glucoside, respectively, peak 4 is assigned as cyanidin-3-O-arabinoside, and peaks 5 and 6 are assigned as cyanidin-3-O-xyloside and cyanidin-3,5-O-dihexoside, respectively. Depending on the content of the attached glycosides, the order was consistent with those reported in previous studies [38,39,40,41].

Cyanidin-3-O-glucoside was used to quantify the anthocyanins of A. melanocarpa purified by HPLC using an external standard. Five concentration gradients of 6, 12, 60, 120, and 600 μg/mL were set, and a standard curve was drawn ($Y = 70.299X + 318.5, R^2 = 0.9997$). The total anthocyanin contents (Table 9) of A. melanocarpa after HPLC purification were 448.873 mg/g (A: UMAE-NADES), 365.163 mg/g (B: UMAE-EtOH), 328.764 mg/g (C: NADES), and 299.873 mg/g (D:EtOH). The order of the content of each anthocyanin from high to low was cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside, cyanidin-3-O-xyloside, cyanidin-3-O-glucoside, and the dimer of cyanidin-hexoside. The main anthocyanin of A. melanocarpa was cyanidin-3-O-galactoside, and the content of A was the highest. The contents of cyanidin-3-O-glucoside and cyanidin-3-O-xyloside in D (EtOH) were higher than those in the other three methods, which showed that the extraction methods were different. The total anthocyanin contents and proportions of each anthocyanin were different.

Six main anthocyanins were identified in this study, and a larger number of anthocyanin types were extracted in comparison with previous reports [38,39,40,41]. Meng et al., 2019 [42] identified seven anthocyanins. However, the total amount of anthocyanins extracted in this study was 1.6 times higher than that reported by Meng et al., and was 2.5 times higher than that reported by Panic et al., 2019 [23]. Although the different extraction methods had a negligible effect on the types of anthocyanins extracted (the main structures of the anthocyanins are determined by genetic information, and the variations in the anthocyanins caused by geographical location and environmental factors were accounted for), the content and purity of the extracted anthocyanins were affected. Thus, from the perspective of extraction volume, UMAE-NADES was established to be a rapid, simple, and green extraction method that can increase the content of anthocyanins.
3.6. Determination of antioxidant activity

As shown in Fig. 5, ABTS can be oxidized to a blue-green free radical ABTS·+. The color of an ABTS·+ solution fades in the presence of a substance with anti-oxidant properties [43]. According to the results, UMAE-NADES (0.401 mM) had the highest ABTS·+ scavenging ability. The scavenging abilities of UMAE-EtOH (0.339 mM), NADES (0.307 mM), EtOH (0.291 mM) were less than that of UMAE-NADES (p < 0.05). This showed that the anthocyanins extracted by UMAE-NADES had good antioxidant capacity that was 1.2–1.4 times higher than those of the other three methods.

In the FRAP assay, the ability of different species to reduce ferric ions is measured. According to the results, the reducing ability of UMAE-NADES (4.371 mM) was significantly higher than those of UMAE-EtOH (3.832 mM), NADES (3.369 mM), EtOH (2.618 mM) (p < 0.05), and the antioxidant capacity of UMAE-NADES was 1.1–1.7 times that of the other methods.

In the T-AOC assay, anthocyanins can reduce Fe³⁺ to Fe²⁺ and form stable complexes with phenanthrolines. Therefore, UMAE-NADES had the highest T-AOC value, reaching 44.329 U/mg, which was significantly higher than those of UMAE-EtOH (38.135 U/mg), NADES (28.617 U/mg), EtOH (19.861 U/mg) (p < 0.05), and the antioxidant capacity of UMAE-NADES was 1.2–2.2 times that of the other three methods.

4. Conclusion

In this study, anthocyanins were extracted from A. melanocarpa using UMAE-NADES, and the three-factor and three-level response surface design method was used to optimize the extraction conditions. According to the results, the optimum extraction conditions for UMAE-NADES were a solid–liquid ratio of 1:15, microwave power of 230 W, extraction time of 367 s, and extraction temperature of 52 °C. Under these conditions, the extraction rate of anthocyanins reached 97.05 ± 0.85%. Compared with UMAE-EtOH, NADES, and traditional EtOH, UMAE-NADES has a higher extraction efficiency, shorter extraction time, and lower dosage. Through HPLC-MS analysis, six anthocyanins were identified and extracted from A. melanocarpa, and UMAE-NADES was found to have significantly improved the total anthocyanin content and purity of each anthocyanin. In addition, ABTS, FRAP, and T-AOC assays were performed to study the antioxidant capacities of the different extraction methods. The results showed that the different extraction methods resulted in different extraction contents of anthocyanins. The antioxidant activity was found to increase significantly with increasing anthocyanin content. The UMAE-NADES technology, which employs highly efficient, selective, and environmentally friendly NADES instead of the traditional organic solvents, in conjunction with UMAE, enables the extraction of anthocyanins from A. melanocarpa with significantly increased yields. This technology is simple and fast, and conforms to the principles of green analytical chemistry. Therefore, UMAE-NADES is a rapid, safe, and green extraction technology that is highly suitable for the extraction of biologically active substances, and has potential for extensive use in the manufacture of foods, supplements, medicines, and cosmetics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors gratefully acknowledge the financial support from the Graduate Innovation Cultivation Project of Shenyang Agricultural University (2021YCXS12).

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