Evaluation Study on Extraction of Anthocyanins from Red Cabbage Using High Pressure CO$_2$ + H$_2$O: A Fuzzy Logic Model and Metabolomic Analysis

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Abstract: In this work, a fuzzy logic model was developed to elucidate the extraction performance of high-pressure CO$_2$ + H$_2$O compared with traditional H$_2$O extraction and aqueous ethanol extraction. The high-pressure CO$_2$ + H$_2$O group acquired the highest comprehensive score considering yield, quality and stability. Both targeted and untargeted metabolomics results proved that the polarity of water was slightly modified; in particular, with the evidence from the untargeted metabolomics data, a higher proportion of water-insoluble compounds (2-methylindole, 3-formylindole, guanine, tryptamine and tryptophan) obtained by high-pressure CO$_2$ + H$_2$O extraction compared with traditional H$_2$O extraction has been reported for the first time. Finally, the “3I” extraction mechanism of high-pressure CO$_2$ + H$_2$O is proposed, which offers an improvement in the solid-liquid mass transfer efficiency of phytochemicals, improving the polarity of solution and the isolation of O$_2$.

Keywords: anthocyanins; high-pressure CO$_2$ + H$_2$O; a fuzzy logic model; metabolomics; extraction mechanism

1. Introduction

The addition of colorants can improve the appearance of products and attract consumers to buy [1]. Because of their bright color, strong coloring power and good stability, synthetic colorants are widely used in various food, cosmetics and other products [2]. The fatal disadvantage of synthetic colorant is its toxicity to humans [3]; previous studies have found that even if the intake dose of some colorants is low, long-term intake still carries a risk of teratogenicity, carcinogenesis and genotoxicity in the human population [4,5]. Currently, natural colorants obtained from various animals and plants are being discussed and explored for their physiological activities to alleviate harm caused by synthetic colorants [6,7].

Most natural colorants come from plants and have the characteristics of non-toxic side effects and high safety [8,9]. Anthocyanins are listed as a natural colorant for food adopted by the Codex Alimentarius Commission and approved by the European Union with the E-code E163 (EC/2003/822) [10]. The global anthocyanins market is growing at a CAGR of 4.5% for the forecast period 2020–2025 (https://www.mordorintelligence.com/industry-reports/anthocyanin-market, accessed on 15 September 2021). It concerns the polyphenolic phytochemicals belonging to the group of flavonoids, and these can give different colors, such as red, purple and blue [11]. They are soluble in water, and most are...
organic solvents but are not soluble in a polar organic solvent and not stable in alkaline or neutral solutions [12]. Anthocyanins are good alternatives to synthetic food dyes as they have health-promoting effects in the prevention of cancer, cardiovascular diseases, neurodegenerative diseases, obesity and diabetes [13,14].

At present, the industrial production of anthocyanins largely relies on H₂O or aqueous ethanol extraction and purification from anthocyanin-rich plants. The solvents most commonly used are acidified aqueous solutions [15]. Plenty of studies have shown that aqueous ethanol achieves a higher degree of extraction of anthocyanins than pure water or ethanol extraction but a lower stability of the anthocyanins extracted [11] because aqueous ethanol leads to the higher reactivity of anthocyanins, which lowers the lowest unoccupied molecular orbital energy of anthocyanins and accelerates the nucleophilic attack from water [12]. On the other hand, safety risks and less environmentally friendly processes have strongly stimulated the interest in developing green (environment-friendly) extraction technologies [14].

Water is frequently used as a green solvent for hydrophilic phytochemicals’ extraction, especially when combined with some assisted extraction techniques, such as microwave [16], ultrasound, pulsed electric field [17], high hydrostatic pressure (HHP) [18] and so on. High-pressure CO₂ is one promising novel non-thermal technique (its operating pressure does not exceed 50 MPa, and its temperature is below pasteurization temperature), with cell membrane permeabilizing features and minimal effects on the nutritional and sensory qualities of foods [19–21]. In our previous study, we proposed that high-pressure CO₂ + H₂O could be used as a novel extraction technology for anthocyanins for the first time [22]; however, the overall extraction efficiency and deep extraction mechanism are still unclear.

Recently, the field of metabolomics has achieved a very significant improvement in terms of its analytical capability, particularly MS technologies. Metabolomics based on liquid chromatography and ESI-QTOF MS (LC-ESI-QTOF MS), with a high selectivity, sensitivity, and accuracy, has been used to analyze metabolites in agricultural (and food) products in both targeted and untargeted ways [23–25].

In this study, fuzzy logic ranking based on the anthocyanins extraction data of yield (total phenolics and total monomeric anthocyanins), quality (Color (L*), Color (a*), Color (b*)) and stability (polymeric color and half-lives at 25 °C) was applied to compare the extraction performances of anthocyanins derived from red cabbage using H₂O, aqueous ethanol and high-pressure CO₂ + H₂O; UPLC-Q-TOF/MS combined with advanced data mining and chemometric tools was used to compare the metabolites obtained from different extraction methods and identify potential biomarkers in high-pressure CO₂ + H₂O and H₂O extractions for further clarification of the extraction mechanism of high-pressure CO₂ + H₂O.

2. Materials and Methods

2.1. Materials and Reagents

Fresh red cabbage was purchased from a local market in Beijing in January 2021. LC-MS grade acetonitrile and formic acid were purchased from Thermo Fisher scientific (Waltham, MA, USA). Folin–Ciocalteu’s phenol reagent, methanol, KCl, Na₂CO₃, sodium acetate, citric acid, ethanol and HCl were purchased from DiKMA Technologies (Beijing, China). Water was generated by the Milli-Q integral water purification system from Millipore Billerica (Billerica, MA, USA). The CO₂ gas (purity of 99.9%) in this study was purchased from Huanyu Jinghui Capital Gas Technology Co., Ltd. (Beijing, China).

2.2. Extraction Methods

Extractions were performed in the high-pressure CO₂ + H₂O system described by Liao et al. [26] with or without high-pressure CO₂ at a temperature of 60 °C, as in our previous work [22]. Briefly speaking, fresh red cabbage was cut into small pieces, then crushed in a blender (Midea Group Co., Ltd. WBL2501A, Foshan, China). Then 10 g
of chopped red cabbage was put into a nylon bag, tied tightly and placed in the vessel with 100 mL of preheated extraction solvent (1.7 g of citric acid was added to extraction solvents of H₂O and aqueous ethanol (70%, v/v), respectively, and the extraction solvents were preheated to 60 °C). For H₂O and aqueous ethanol extraction, the extraction time was 30 min; for the high-pressure CO₂ + H₂O extraction, a total 30 min extraction time with three stages was performed, with rising pressure (pressure rise to 10 MPa, 17 min), pressure holding (under 10 M²Pa CO₂ pressure for 10 min) and pressure dropping (pressure drop to atmospheric pressure, 3 min). After extraction, the anthocyanin-rich solution was automatically collected from the solid–liquid mixture through filtration of the nylon bag and collected into a sample bottle for further analysis.

We used the anthocyanin extraction method described by Zhang et al. [27] with some modification by stirring the mixture (10 g of red cabbage mash, 100 mL of 80% (v/v) aqueous methanol solution and acidified by citric acid (1.7 g) at 4 °C for 12 h), which can be used as a reference to detect the relative yield of anthocyanins in red cabbage. The experiments were repeated 12 times for each extraction method.

2.3. Traditional Quality Evaluation Using Targeted Analysis

2.3.1. Total Monomeric Anthocyanins, Relative Yield of Anthocyanins, Total Phenolics Content and Polymeric Color

A Shimadzu UV-1800 spectrophotometer (Shimadzu Co., Tokyo, Japan) was used to determine the total monomeric anthocyanins, total phenolics content and polymeric color of the anthocyanin-rich solution.

The pH differential method was used to calculate the total monomeric anthocyanins in the interfering substance; the method included two buffer systems (potassium chloride with a pH of 1.0 (0.025 mol/L) and sodium acetate with a pH of 4.5 (0.4 mol/L)). Total monomeric anthocyanins were reported as cyanidin-3-glucoside (C3G) equivalents, and the total monomeric anthocyanins content of each solution was calculated using the following Equation (1),

\[
\text{Anthocyanins (mg C3G/L)} = \frac{A \times M_w \times DF \times 1000}{E \times L}
\]

where \( A = (A_{520}-A_{700}) \) pH = 1.0–(A_{520}-A_{700}) pH = 4.5; \( M_w \) is the molecular weight of anthocyanin (449.2 g mol⁻¹), \( DF \) is the dilution factor (10), \( E \) is the extinction coefficient (26,900 L cm⁻¹ mol⁻¹) and \( L \) is the path length (1 cm).

The relative yield of anthocyanins in red cabbage by different methods was calculated based on the Equation (2),

\[
\text{Relative yield (\%)} = \frac{\text{Amount of total monomeric anthocyanins recovered by given method}}{\text{Amount of total monomeric anthocyanins recovered by aqueous methanol}}
\]

The method with slight modifications was used to quantify the total phenolics [28]. A total of 0.3 mL of the anthocyanin-rich solution or gallic acid standard was added into brown test tubes, followed by 2 mL of Folin–Ciocalteu reagent. The mixture was incubated in the dark at room temperature for 1 h, then 1.8 mL of 7.5% Na₂CO₃ solution was added and left in the dark for 15 min. Absorbance was measured in triplicate at 765 nm using the spectrophotometer. Total phenolics content was reported as mg of gallic acid equivalent (GAE) per quality (100 g) of sample (mg GAE/100 g), based on the standard curve \( y = 0.0046x - 0.0085, R^2 = 0.9977 \).

Compared with monomeric anthocyanins, polymerized anthocyanins were resistant to bleaching in bisulfite solution. Absorbance of the bisulfite-bleached and control (water diluted) samples were measured at 420 nm, 520 nm and 700 nm by the spectrophotometer. The percentage of polymer color was calculated according to the Equation (3) [29],

\[
\text{Polymeric color (\%)} = \frac{\left( A_{420} - A_{700} \right)_{\text{bleached}} + \left( A_{520} - A_{700} \right)_{\text{bleached}}}{\left( A_{420} - A_{700} \right)_{\text{control}} + \left( A_{520} - A_{700} \right)_{\text{control}}}
\]
2.3.2. Color Property, pH and Half-Life

Color was measured by ColorQuest XE (HunterLab, Inc., Reston, VA, USA), and the CIE color parameters L*, a* and b* were recorded. pH value of each anthocyanin-rich solution was measured by pH meter (FiveEasy Plus, METTLER TOLEDO, Shanghai, China). The classical first-order degradation reaction model Equation (4) was used for evaluating the stability of anthocyanin-rich solution. The anthocyanin-rich solution (12 mL) was stored in the dark at 25 ± 2 °C. The total monomeric anthocyanin content of the extract samples was measured every 7 days. The number of total monomeric anthocyanins in each matrix was fitted into Equation (5) to calculate its half-life, t1/2 [30],

\[
\ln \left(\frac{c_t}{c_0}\right) = -K_t \quad (4)
\]

\[
t_{1/2} = \ln 2 / K \quad (5)
\]

where \( c_0 \) is the total monomeric anthocyanins concentration at the starting point, \( c_t \) is the total monomeric anthocyanins concentration after storage for t days, and K is the reaction constant.

2.3.3. Statistical Analysis

Three replicates were performed for each quality character. The data are described as means ± SD (standard deviation). One-way ANOVA and Tukey’s test at \( \alpha = 0.05 \) were applied to evaluate the mean differences among different extraction methods. The significances of all terms were evaluated based on an analysis of variance (ANOVA) test with using the F-value at \( p \leq 0.05 \).

2.4. Fuzzy Logic-Based Ranking Function

Fuzzy logic defined by flexible rules can be used to solve complex problems that are hard to describe with clear logics. Fuzzy logic can present complex quality characteristics in the form of scores with the help of simple algebraic steps [31]. A fuzzy ranking function was applied to choose the best extraction system for recovering phenolics from flixweed seeds [32]. We used multiple dimensions to evaluate the red cabbage extract under different extraction methods, but it was difficult to find the best extraction system with a clear formula and value. Therefore, we considered using fuzzy ranking function to select the best extraction system of anthocyanin extract from red cabbage.

2.4.1. Fuzzification

The fuzzification step was obtained from information provided by 20 interviewed experts, including 8 with higher education, 4 senior managers in factories and 8 Ph.D. students in the field of natural colorant research or production. Fuzzification was the first step where crisp values of 7 input variables (Color (L*), Color (a*), Color (b*), total phenolics content, total monomeric anthocyanins, polymeric color and half-lives at 25 °C) and one output variable (score of extraction methods) that were used to rank the various extraction methods were transformed into fuzzy form using triangular-type membership. Triangular-type membership function was according to the Equation (6),

\[
f_\lambda(x_\lambda; a, b, c) = \begin{cases} 
0 & (x_\lambda < a) \\
\frac{x_\lambda - a}{b - a} & (a \leq x_\lambda \leq b) \\
\frac{c - x_\lambda}{c - b} & (b \leq x_\lambda \leq c) \\
0 & (x_\lambda > c)
\end{cases} \quad (6)
\]

The range of input variables was expressed as very high (VH), high (H), medium (M), and low (L). Figure 1 shows the membership functions for the inputs and flowchart of the fuzzy logic system.
2.4.2. Definition of Fuzzy Rules

According to the relevant knowledge of food science, some examples of fuzzy rules for ranking extraction systems are listed (Table 1) by using an “if then” fuzzy rule set, which are used as the basis of modeling.

Table 1. Some examples of fuzzy rules for ranking of extraction systems.

| Examples of Fuzzy Rules                                                                 |
|---------------------------------------------------------------------------------------|
| If (Color(L*) is L) and (Color(C*) is L) and (Color(h*) is L) and (Total Phenolic is L) and (Monomeric Anthocyanins is L) and (Polymeric Color is H) and (Half-lives at 25 °C is L) then (Score is VB) |
| If (Color(L*) is H) and (Color(C*) is H) and (Color(h*) is H) and (Total Phenolic is H) and (Monomeric Anthocyanins is H) and (Polymeric Color is H) and (Half-lives at 25 °C is L) then (Score is B)           |
| If (Color(L*) is H) and (Color(C*) is H) and (Color(h*) is H) and (Total Phenolic is M) and (Monomeric Anthocyanins is M) and (Polymeric Color is L) and (Half-lives at 25 °C is H) then (Score is M)                     |
| If (Color(L*) is H) and (Color(C*) is VH) and (Color(h*) is H) and (Total Phenolic is H) and (Monomeric Anthocyanins is H) and (Polymeric Color is L) and (Half-lives at 25 °C is VH) then (Score is VG)      |

B: bad; G: good; H: high; L: low; M: medium; VB: very bad; VG: very good; VH: very high.

2.4.3. Defuzzification of Output

The centroid method such as the following equation was used for defuzzification that converts the fuzzy output to a single clear number to help sort the extraction methods and make decisions [33].

\[
Y = \frac{\int_{-\infty}^{\infty} f \times \mu_{B_i}(f) df}{\int_{-\infty}^{\infty} \mu_{B_i}(f) df}
\]

\(\mu_{B_i}(f)\): a fuzzy set of output and \(Y\): crisp value of output.

The test environment for fuzzy logic was python3.7.0 with matplotlib and numpy (NumFOCUS, a 501(c) (3) nonprofit charity, Austin, TX, USA).
2.5. Metabolomics Analysis

2.5.1. Sample Pretreatment and Data Acquisition

A volume of 30 mL of anthocyanin-rich solution was put into a 50 mL brown test tube and stored in refrigerator at −20 °C for 24 h. The sample was then transferred to freeze dryer (Virtis Ultra 25 XL, New York, NY, USA) for 108 h to completely freeze dry. The temperature and vacuum of the freeze dryer were set as −60 °C and 10 Pa. Lyophilized samples were redissolved with 3 mL of extraction solvent and filtered with 0.22 μm filter membrane before they were placed in the vials UPLC-ESI-QTOF-MS (6600, Agilent, CA, USA) for analysis.

Chromatographic separation was achieved on an SB-C18 column (Poroshell 120 SB-C18 2.7 μm, 3.0 × 100 mm, Agilent, CA, USA) using a flow rate of 0.3 mL/min at 30 °C. The mobile phases consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient program was as follows: 5% B to 35% B (0–8 min); 35% B to 100% B (8–11 min); 100% B (12–15 min); 100% B to 5% B (15–15.01 min); 5% B (15.01–18 min). The mass data were acquired in negative and positive mode using parameters as follows: ion source gas 1 and gas 2 (50 arbitrary units); curtain gas (25 arbitrary units); drying gas temperature (500 °C); ion spray voltage floating, declustering potential and collision energy were 5500 V, 80 eV and 35 eV (for positive mode); mass ranges of TOF MS were m/z 100–1500 and product ion scans were m/z 50–1000. The LC-MS data were acquired using Analyst TF Version1.7.1 (SCIEX, Redwood City, CA, USA).

2.5.2. Individual Anthocyanins and Phenolics Identification

Firstly, the mass spectrum information of 23 anthocyanins [34,35] and 33 polyphenols [36,37] reported to be in red cabbage was sorted into a list according to existing literature reports. Import the list containing the relevant information (name, formula) of the known target compounds into the MasterView module of PeakView software (SCIEX, Redwood City, CA, USA), and then input the additive ions. At this time, the software automatically generated the accurate mass according to the molecular formula. The mass resolution was set to 1.0 m/z. The sample data collected by the instrument were also imported into the MasterView module.

According to the given molecular formula of the target, ionized adduct ions and retention time, MasterView determined whether there may be target ions in the MS1 data (MS1 tolerance < 10 ppm) and searched the MS2 (MS2 tolerance < 10 ppm) diagram corresponding to the target ions found in the sample in the spectrum library. Further, the MS2 of the sample target was calculated with the standard spectrum. Then, the system matched the presence and content of the sample according to the information in the import list.

2.5.3. Data Processing and Potential Marker Putative Annotation

The raw data were converted to mzXML format using MSConvert (Version 3.0, http://proteowizard.sourceforge.net, accessed on 28 September 2021). Then, the extracted peak table was generated using MS-DIAL Version 4.38 (Agilent, CA, USA) software after performing peak detection, peak alignment and primary identification. Principal component analysis (PCA), Student’s t-test, fold change (FC) and the latent structure discriminant analysis (OPLS-DA) were generated by MetaboAnalyst website (https://www.metaboanalyst.ca/, accessed on 30 September 2021). The MS1 and MS2 information of predefined potential markers was imported into MS-FINDER (Version 3.24) software for putative annotation by matching with the in-built databases. MS-FINDER calculates the possible molecular formula according to the accurate mass number of MS1 mass spectrum of the ion (MS1 tolerance < 10 ppm) and the isotope abundance ratio. At the same time, the molecular formula was verified by the accurate mass number of MS2 mass spectrum (MS2 tolerance < 15 ppm) according to a certain mass spectrum cleavage law. A list of potential markers for the discrimination of the different groups was obtained.
3. Results and Discussion

3.1. Traditional Quality Characters

In this study, relative yields of anthocyanins obtained by the three extraction methods are shown in Figure 2A. The relative yield of anthocyanins in the high-pressure CO$_2$ + H$_2$O extraction group was 1.62 and 1.56 times that of the pure H$_2$O and aqueous ethanol extraction groups, respectively. As shown in Table 2, the content of total monomeric anthocyanins was 157.71 ± 0.98 mg/100 g in high-pressure CO$_2$ + H$_2$O extraction, 92.10 ± 1.08 mg/100 g in aqueous ethanol extraction and 73.88 ± 0.54 mg/100 g in H$_2$O extraction. Similarly, the maximum yields of total phenols were observed in high-pressure CO$_2$ + H$_2$O extraction (355.32 ± 3.47 mg/100 g), followed by aqueous ethanol (314.26 ± 1.53 mg/100 g) and the lowest in H$_2$O (185.83 ± 0.77 mg/100 g).

![Figure 2](image-url)

**Figure 2.** The relative yields of anthocyanins (A) and fuzzy logic score (B) in the three groups. The three extraction methods are high-pressure CO$_2$ + H$_2$O, aqueous ethanol and H$_2$O extraction. Different letters (a, b, c) indicate significant differences ($p < 0.05$).

|                | H$_2$O          | Aqueous Ethanol | High-Pressure CO$_2$ + H$_2$O |
|----------------|-----------------|-----------------|-------------------------------|
| Total phenolics (mg GAE/100 g) | 185.83 ± 0.77 $^c$ | 314.26 ± 1.53 $^a$ | 355.32 ± 3.47 $^b$ |
| Total monomeric anthocyanins (mg C$_3$G/100 g) | 73.88 ± 0.54 $^a$ | 92.10 ± 1.08 $^a$ | 157.71 ± 0.98 $^b$ |
| The ratio of total phenolics to total monomeric anthocyanins | 2.52 | 3.41 | 2.25 |
| L$^*$          | 37.53 ± 0.55 $^b$ | 42.08 ± 0.72 $^a$ | 33.51 ± 0.26 $^c$ |
| a$^*$          | 1.43 ± 0.92 $^c$ | 1.01 ± 1.14 $^b$ | 1.78 ± 0.57 $^a$ |
| b$^*$          | 1.48 ± 1.16 $^a$ | −0.41 ± 1.54 $^b$ | 1.27 ± 0.77 $^a$ |
| Matrix pH      | 2.37 ± 0.02 $^a$ | 3.22 ± 0.08 $^a$ | 2.36 ± 0.04 $^b$ |
| Polymeric color (%) | 15.42 ± 2.72$^b$ | 32.93 ± 3.30$^b$ | 10.27 ± 0.04%$^b$ |
| Half-lives at 25 °C, dark (days) | 59 ($R^2 = 0.88$) | 46 ($R^2 = 0.93$) | 139 ($R^2 = 0.89$) |

Different letters (a, b, c) indicate significant differences at 0.05 level via ANOVA–Tukey comparison among different extraction matrices. $R^2$ was the linear regression coefficient for the anthocyanins first-order degradation model.

The finding that the high-pressure CO$_2$ + H$_2$O has great advantages in anthocyanin extraction is consistent with previous reports. Z. Xu et al. optimized the extraction condition and found that high-pressure CO$_2$ + H$_2$O produced 5–10% more anthocyanins from frozen red cabbage than H$_2$O [22]. F. Lao et al. found that high pressure CO$_2$ + H$_2$O (10 MPa, 60 °C) produced 50% and 25% more colorants from purple sweet potato than water and aqueous ethanol extraction [38]. Nunes et al. found that high-pressure CO$_2$ + H$_2$O (10 MPa 40 °C and 20% volume ratio) produced more 60% colorants than H$_2$O extraction from Opuntia spp. [39]. On the whole, the extraction yields of high-pressure CO$_2$ + H$_2$O were significantly higher than those of water and ethanol.

The total number of monomeric anthocyanins may be lost due to polymerization during extraction. As shown in Table 2, the polymeric color of high-pressure CO$_2$ + H$_2$O
extract was the lowest (10.27 ± 0.04%), followed by H$_2$O extract (15.42 ± 2.72%), and the color of aqueous ethanol extract was the highest (32.93 ± 3.30%). A high polymeric color of an aqueous ethanol extract may be associated with polymerization reactions. It has been demonstrated that the degradation of anthocyanins in purple rice bran can be delayed under limited oxygen [40]. The treatment of high-pressure CO$_2$ + H$_2$O could also protect anthocyanins against polymerization through removing oxygen from the solution [41]. Compared with water extraction, the content of O$_2$ in high-pressure CO$_2$ + H$_2$O extraction was lower, indicating that the treatment of high-pressure CO$_2$ + H$_2$O prevents polymers from forming through removing oxygen from the solution, which could inhibit the polymerization of anthocyanins.

The color of the anthocyanin-rich solution extracted by aqueous ethanol extraction was light red and had more blue tones. The L* value of extraction by high-pressure CO$_2$ + H$_2$O was the lowest (33.51 ± 0.26), followed by aqueous ethanol (37.53 ± 0.55), and the highest in H$_2$O (42.08 ± 0.72); the a* value of extraction by high-pressure CO$_2$ + H$_2$O was the highest (17.8 ± 0.57), followed by aqueous ethanol (14.3 ± 0.92), and the lowest in water (10.1 ± 1.14); the b* value of extraction by high-pressure CO$_2$ + H$_2$O was the highest (1.27 ± 0.77), followed by H$_2$O (1.48 ± 1.16), and the lowest in aqueous ethanol (0.41 ± 1.54) (Table 2). The most likely reason for the light color of the aqueous ethanol extract (3.41) is that its ratio of total phenols to total monomeric anthocyanins was much higher than that of the aqueous matrix (H$_2$O, 2.52; high-pressure CO$_2$ + H$_2$O, 2.25) (Table 2) [15]. In addition, the pH of the ethanol extract, which was higher than that of the aqueous matrix, also has a certain impact [40]. The color of the aqueous matrix extract was red, while the color of the high-pressure CO$_2$ + H$_2$O extract was darker. When the ratio of total phenols to total anthocyanins was similar, the increase in total anthocyanin concentration was the main reason for the darker color of the extract.

Further experiments confirmed this by using the classical first-order degradation reaction model for evaluating the degradation kinetics of anthocyanins described in 2.3.5; it was found that the half-life of the anthocyanin-rich solution extracted by high-pressure CO$_2$ + H$_2$O (139 days) was longer than that of traditional water extraction (59 days) and ethanol extraction (46 days). Studies showed that ethanol can reduce the stability of anthocyanins and accelerate their degradation, while high-pressure CO$_2$ + H$_2$O can improve the half-life of anthocyanins [42] and showed that although the number of anthocyanins extracted by ethanol was greater than that extracted by H$_2$O, the degradation rate of ethanol-extracted anthocyanins was much faster than that of anthocyanins extracted by H$_2$O.

Polyphenols are good co-pigments of anthocyanins, which was conducive to stabilizing the color of the high-pressure CO$_2$ + H$_2$O crude extract [43]. Palmira et al. demonstrated that there is a co-pigmentation phenomenon between cyanidin 3,5-diglucoside and ferulic acid by π-stacking interactions [44]. Moreover, condensation reactions of ferulic acids with anthocyanins have also been shown to occur in strawberry and raspberry juices [45]. As well as in combination with anthocyanins, their sole presence in the solution can prevent the invasion of light and heat and reduce the damage of anthocyanins [46]. It has been proved that the half-life of the anthocyanin-rich solution from purple sweet potato was prolonged by more than 3 times after using high-pressure CO$_2$ + H$_2$O technology [38]. Anthocyanins are vulnerable to degradation due to a nucleophilic attack by water, resulting in the partial destruction of molecular structure [47]. Acylated anthocyanins show higher stability through acyl accumulation reduces the probability of a water nucleophilic attack and generates steric hindrance [48]. However, with the degree of acylation and distribution uniformity increasing, a nucleophilic attack by water is no longer the main factor affecting the stability of anthocyanins [49]. Moreover, the foldable sugar chain of acylated anthocyanins can wrap and fix organic acids on the carbon skeleton, which shows that this accumulation is more resistant to the nucleophilic attack of water and has a certain defense against other types of degradation [50]. High-pressure CO$_2$ + H$_2$O was helpful in obtaining the anthocyanin-rich solution with higher stability at room temperature [51].
3.2. Fuzzy Logic Ranking

For a manufacturer, the extraction efficiency and the stability of anthocyanins are the most important criteria. The ranking of extraction methods is based on three dimensions including yield, quality and stability of anthocyanins. Thus, it is difficult to give a precise evaluation in a separate evaluation. The fuzzy logic evaluation comprehensively considered the three dimensions of the different extraction methods, making the evaluation results more scientific.

The results of the defuzzification score of each method by fuzzy rules are shown in Figure 2B. The high-pressure CO$_2$ + H$_2$O extraction method acquired the highest score (8.97), followed by aqueous ethanol extraction (4.36) and H$_2$O extraction (3.25) (a score > 8 can be considered as a “very good” extraction method).

The high-pressure CO$_2$ + H$_2$O method has the advantages of a high total phenolic content and total monomeric anthocyanin content, the longest half-life and the least polymer color, which have attracted the attention of experts in this evaluation. In general, high-pressure CO$_2$ + H$_2$O exhibited great advantages in anthocyanin extraction from plants compared to aqueous ethanol extraction and H$_2$O from a comprehensive perspective.

3.3. Targeted Metabolomics Analysis

As shown in Table 3, 19 anthocyanins were found in red cabbage. Compared with aqueous ethanol extraction, the contents of four non-acylated anthocyanins were higher in H$_2$O extraction, while the contents of five mono-acylated anthocyanins and nine di-acylated anthocyanins were higher in high-pressure CO$_2$ + H$_2$O extraction. Specifically, for mono-acylated anthocyanins, the extraction effect of high-pressure CO$_2$ + H$_2$O was significantly enhanced (about 30% higher than that of aqueous ethanol extraction), and the effect of H$_2$O extraction was about 10% lower than that of aqueous ethanol extraction; for di-acylated anthocyanins, the extraction effect of high-pressure CO$_2$ + H$_2$O was excellent (about 60% higher than that of aqueous ethanol extraction), and the performance of H$_2$O extraction was about 30% lower than that of aqueous ethanol extraction (Figure 3A).

![Figure 3.](image)

**Figure 3.** Relative peak area of anthocyanins (compared with aqueous ethanol group) in water and high-pressure CO$_2$ + H$_2$O groups (A) and relative peak area of phenolic compounds (compared with H$_2$O group) in aqueous ethanol and high-pressure CO$_2$ + H$_2$O groups (B). Abbreviation: Cy, cyanidin; glu, glucoside; caf, caffeic; p-cou, p−coumaric; fer, ferulic; sin, sinapic acid; K, kaempferol; Q, quercetin.
As shown in Table 3, 21 polyphenols were found in red cabbage. Aqueous ethanol has been known to be a good solvent for phenolic extraction. Based on Chem YQ (http://www.chemyq.com/xz.htm, accessed on 15 October 2021), the extracted phenolics are divided into the following three categories: (1) Substances insensitive to solvent polarity, such as kaempferol, quercetin and glycoside, can be well dissolved in water and ethanol. The increase in their content is mainly due to full contact with the extractant; (2) substances sensitive to solvent polarity, such as sinapic acid glucose, and the increase in content is mainly due to the change of solvent polarity. The content of strongly polar or non-polar substances such as phloretin and phlorizin remains basically unchanged because high-pressure CO$_2$ + H$_2$O treatment has little influence on solvent polarity; [52] and (3) substances sensitive to solvent temperature, such as 3-caffeoylquinic acid, which is greatly affected by temperature [53] and has high solubility in hot water and low solubility in cold water, decreased in content by high-pressure CO$_2$ + H$_2$O treatment, which may have an opposite or ineffective influence on the two main factors (solvent polarity and temperature).

| Tentative Annotation                      | RT (min) | m/z    |
|------------------------------------------|----------|--------|
| Cyanidin-3-galactoside                   | 7.97     | 449.1071 |
| Cyanidin-3,5-diglucoside                 | 8.23     | 611.1617 |
| Cyanidin-3-sambubioside-5-glucoside      | 8.43     | 743.2011 |
| Cyanidin 3-O-galloy-rutinoside           | 8.65     | 757.2101 |
| Cyanidin 3-diglucoside-5-glucoside       | 8.85     | 773.2106 |
| Cyanidin 3-(sinapoyl)glucoside-5-glucoside | 11.3   | 817.2172 |
| Cyanidin 3-(p-coumaroyl)diglucoside-5-glucoside | 10.87 | 919.249  |
| Cyanidin 3-(feruloyl)diglucoside-5-glucoside | 10.37 | 935.2488 |
| Cyanidin 3-(caffeoyl)diglucoside-5-glucoside | 10.99 | 949.2602 |
| Cyanidin 3-(sinapoyl)(feruloyl)diglucoside-5-glucoside | 11.48 | 1287.3596 |
| Cyanidin 3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside | 11.64 | 1347.3844 |
| 3-Caffeoylquinic acid                    | 5.6      | 355.1011 |
| Kaempferol-3-triglucoside                | 7.8      | 771.1981 |
| 5-Methoxysalicyclic acid                 | 8.1      | 169.0488 |
| Isoquercitrin                            | 8.7      | 465.1032 |
| Kaempferol-3-ferulic -triglucoside-7-glucoside | 9.3  | 1109.2278 |
| Quercetin-3-diglucoside-7-triglucoside   | 9.4      | 1111.313 |
| Sinapic acid glucoside                   | 9.6      | 385.1074 |
| Quercetin-3-diglucoside-7-glucoside      | 9.7      | 787.2181 |
| Kaempferol-3-diglucoside-7-glucoside     | 10.3     | 933.2577 |
| Sinapic acid diglucoside                 | 10.6     | 547.1658 |
| Quercetin-3-diglucoside                  | 10.7     | 629.1434 |
| Phloretin                                | 10.8     | 279.0922 |
| Quercetin-3-triglucoside-7-glucoside     | 10.9     | 494.2839 |
| Quercetin-3-diglucoside-7-sinapic-glucoside | 10.9  | 1095.3069 |
| Kaempferol-3- sinapic-diglucoside-7-glucoside | 11    | 977.2552 |
| Kaempferol-3-diglucoside                 | 11.2     | 609.1489 |
| Quercetin-3-diglucoside-7-ferulic-diglucoside | 11.6  | 1125.3225 |
| Phlorizin                                | 11.6     | 437.144 |
| Quercetin sinapic-3,7-di-O-glucoside-5-diglucoside | 11.7  | 1155.3181 |
| Sinapic acid                             | 12.4     | 223.058 |
| Ferulic acid                             | 12.5     | 193.0950 |
and the comprehensive effect is not obvious. Specifically, although CO₂ promotes the entry of the extractant into cells and fully extracts the substance, due to the evaporation and the endothermic heat of liquid CO₂ during high-pressure CO₂ + H₂O decompression, the solution temperature suddenly decreases [54,55], and the solubility decreases sharply.

3.4. Untargeted Metabolomics Analysis

3.4.1. PCA Analysis

In total, the three groups share 4394 compounds, the high-pressure CO₂ + H₂O and aqueous ethanol share 322 compounds, the high-pressure CO₂ + H₂O and H₂O share 2827 compounds and the aqueous ethanol and H₂O share 146 compounds, respectively (Figure 4A). PCA was carried out to determine whether the three extraction methods could be differentiated. The PCA score plots generated for the anthocyanin-rich solution by the three methods show a separation into distinctive clusters of two groups; one group was high-pressure CO₂ + H₂O and H₂O, and the other group was aqueous ethanol (Figure 4B). The results show that there were significant differences in the composition of anthocyanin-rich solutions with H₂O as a solvent and aqueous ethanol as a solvent.

Figure 4. Differential compounds analysis: Venn plots of high-pressure CO₂ + H₂O, H₂O and aqueous ethanol (A); PCA score plots of high-pressure CO₂ + H₂O, H₂O and aqueous ethanol (B); OPLS-DA score scatter plot of high-pressure CO₂ + H₂O and H₂O (C). Volcano plots of high-pressure CO₂ + H₂O and H₂O (D).

3.4.2. Differential Analysis and Potential Marker Annotation

Compounds with differential abundances are defined as compounds with a p less than 0.05 and an FC no less than 2 or no more than 0.5 (Figure 4D). By calculating FC, 217 compounds were defined. Considering the similarity of the extraction solvent between high-pressure CO₂ + H₂O and H₂O extraction, the orthogonal projection to latent structure discriminant analysis (OPLS-DA) model was built to find potential characteristic markers for these two groups (Figure 4C). Fifteen potential markers were selected based on VIP values (>2), including six alkaloids, one nucleotide, six amino acids and two fatty acids (Table 4).
Table 4. Annotation of differential compounds in red cabbage between high-pressure CO\(_2\) + H\(_2\)O and H\(_2\)O.

| Category       | Name               | Mass   | \(m/z\)   | RT (min) | Formula                  | Error (ppm) | \(p\)-Value (t) | FC (Pressure CO\(_2\) + H\(_2\)O/H\(_2\)O) |
|----------------|--------------------|--------|-----------|----------|--------------------------|-------------|-----------------|---------------------------------------------|
| Alkaloid       | Betaine            | 117.15 | 118.0857  | 1.99     | C\(_6\)H\(_{11}\)NO\(_2\) | 0.3558      | 0.0047          | 2.5988                                      |
|                | Phenylalanine betaine | 207.27 | 208.1328  | 8.71     | C\(_6\)H\(_{17}\)NO\(_2\) | -0.1996     | 0.1965          | 2.0554                                      |
|                | Lentin             | 246.30 | 247.1438  | 9.87     | C\(_{14}\)H\(_{18}\)N\(_2\)O\(_2\) | -0.2809     | 0.1025          | 2.7830                                      |
|                | Indole             | 117.15 | 118.0632  | 9.16     | C\(_3\)H\(_2\)N          | 0.8293      | 1.23 × 10\(^{-12}\) | 4.0135                                      |
|                | 2-Methylindole     | 131.17 | 132.0797  | 9.26     | C\(_3\)H\(_2\)N          | -0.7040     | 1.16 × 10\(^{-7}\) | 12.3634                                     |
|                | 3-Formylindole     | 145.16 | 146.0597  | 9.15     | C\(_3\)H\(_2\)NO         | 0.7957      | 2.33 × 10\(^{-13}\) | 3.6899                                      |
| Nucleic Acid   | Guanine            | 151.13 | 152.0550  | 6.64     | C\(_5\)H\(_5\)N\(_3\)O   | -0.5112     | 0.0006          | 3.2886                                      |
| Amino Acid     | Phenylalanine      | 165.19 | 166.0856  | 8.04     | C\(_6\)H\(_{11}\)NO\(_2\) | 0.7581      | 6.37 × 10\(^{-13}\) | 3.3084                                      |
|                | Tyrosine           | 181.20 | 182.0812  | 6.71     | C\(_6\)H\(_{13}\)NO\(_3\) | 0.7611      | 8.29 × 10\(^{-8}\)  | 3.1048                                      |
|                | Lentin             | 204.23 | 205.0965  | 9.15     | C\(_{11}\)H\(_{12}\)N\(_2\)O\(_2\) | 0.7147      | 3.21 × 10\(^{-9}\)  | 3.0079                                      |
|                | Lentin             | 115.13 | 116.0693  | 7.05     | C\(_3\)H\(_2\)N\(_2\)O\(_2\) | 0.7304      | 6.57 × 10\(^{-10}\) | 3.8139                                      |
|                | Isoleucine         | 131.17 | 132.1021  | 6.94     | C\(_3\)H\(_13\)NO\(_2\) | 0.7625      | 8.63 × 10\(^{-13}\) | 3.0268                                      |
|                | Triethyl phosphate | 182.15 | 183.0797  | 14.04    | (C\(_2\)H\(_3\)O\(_2\))PO | -0.3679     | 0.0262          | 2.3128                                      |
| Fatty Acid     | Triethylcitrate    | 276.28 | 277.1264  | 14.86    | C\(_{12}\)H\(_{20}\)O\(_7\) | -0.301      | 0.0406          | 2.3636                                      |
|                | Tri-              | 266.31 | 267.1718  | 16.5     | C\(_{12}\)H\(_{25}\)O\(_4\)P | 0.7346      | 7.17 × 10\(^{-6}\)  | 3.6693                                      |

These potential markers could also be divided into the three categories as described in Section 3.3: (1) soluble in water (betaine (Figure 5A), phenylalanine betaine (Figure 5B), lentin (Figure 5C), phenylalanine (Figure 5D), isoleucine (Figure 5E), triethyl phosphate (Figure 5F), triethylcitrate (Figure 5G) and tri-isobutylphosphate (Figure 5H); (2) insoluble in water (2-methylindole (Figure S1I), 3-formylindole (Figure S1J), phenylalanine betaine (Figure S1K), tyrosine (Figure S1L) and tryptophan (Figure S1M); and (3) sensitive to temperature (indole (Figure S1N) and proline (Figure S1O)).

The potential markers with good water solubility can be divided into two groups: the contents of phenylalanine betaine, lentin, triethyl phosphate, and triethylcitrate are higher in high-pressure CO\(_2\) + H\(_2\)O; and the contents of phenylalanine, betaine, isoleucine are higher in H\(_2\)O. During the pressure relief process, dissolved CO\(_2\) may impinge on and shear cells at gas-expanding velocity [56]. The “explosion” effect of high-pressure CO\(_2\) can enhance the damage to the cellular structure of the extracted substrate, reduce the mass transfer resistance of target materials and improve the extraction efficiency. This fact shows that the soluble compounds of phenylalanine betaine, lentin, triethyl phosphate, triethylcitrate and guanine are higher in high-pressure CO\(_2\) + H\(_2\)O extraction compared to H\(_2\)O extraction. This confirms that the explosion effect caused by high-pressure CO\(_2\) causes greater contact between the solvent and solute.

Generally, the polarity of water is strong, so it is difficult to extract substances with weak polarity. Surprisingly, in this work, the content of some insoluble compounds including 2-methylindole, 3-formylindole, guanine, tyrosine and tryptophan were higher, and the soluble compounds of phenylalanine, betaine, isoleucine were lower in high-pressure CO\(_2\) + H\(_2\)O extraction compared to H\(_2\)O extraction. The increase in the content of substances which are sensitive to solvent polarity was mainly due to the change in solvent polarity [57]. During high-pressure CO\(_2\) + H\(_2\)O extraction, CO\(_2\) gas is pressurized to a liquid, and the polarity of the solvent is changed. The weak polarity of CO\(_2\) fluid improves the extraction efficiency of weakly polar substances. Indole and proline are easily soluble in hot water and almost insoluble in cold water. We highly suspect that the reason for its lower content in the high-pressure CO\(_2\) + H\(_2\)O extraction is that the solution temperature decreases at that stage of pressure relief.
which changes the polarity of solution. That is why high-pressure CO$_2$ extraction is proposed. The “3I” extraction mechanism of pressure CO$_2$ is observed. (1) By improving the isolation of O$_2$, the existence of high-pressure CO$_2$ ensures the whole system is in a high-pressure, acidic and O$_2$-free environment, which effectively prevents the degradation of natural products and oxygen reactions during the extraction process [58]. It has been demonstrated that the degradation of anthocyanins can be delayed under the condition of limited oxygen. Additionally, high-pressure CO$_2$ + H$_2$O protects against polymerization of anthocyanins by removing oxygen from the solution [40,41]. (2) By improving the polarity of the solution, increased CO$_2$ solubility (and pH) in aqueous solutions under high pressure leads to the formation of more carbonic acid (H$_2$CO$_3$), which changes the polarity of solution. That is why high-pressure CO$_2$ + H$_2$O extraction is conducive to the extraction of acylated anthocyanins and water-insoluble substances (i.e., 2-methylindole, 3-formylindole, guanine, tyrosine and tryptophan); (3) By improving the solid–liquid mass transfer efficiency of phytochemicals, the “explosion” effect of high pressure can enhance the damage to the cellular structure and reduce the mass transfer resistance in target materials, thus improving the extraction efficiency. The higher content of water-soluble phenolics (kaempferol, quercetin and glycoside) and potential water-soluble markers (phenylalanine betaine, lentin, triethyl phosphate, triethylcitrurate and tri-isobutylphosphate. The blue bar represents high-pressure CO$_2$ + H$_2$O extraction and the red bar represents H$_2$O extraction.

3.5. Extraction Mechanism of High-Pressure CO$_2$ + H$_2$O

Based on our early experiments, the results of this paper and relevant literature, we propose the “3I” extraction mechanism of pressure CO$_2$ + H$_2$O as follows: (1) By improving the isolation of O$_2$, the existence of high-pressure CO$_2$ ensures the whole system is in a high-pressure, acidic and O$_2$-free environment, which effectively prevents the degradation of natural products and oxygen reactions during the extraction process [58]. (2) By improving the polarity of the solution, increased CO$_2$ solubility (and pH) in aqueous solutions under high pressure leads to the formation of more carbonic acid (H$_2$CO$_3$), which changes the polarity of solution. That is why high-pressure CO$_2$ + H$_2$O extraction is conducive to the extraction of acylated anthocyanins and water-insoluble substances (i.e., 2-methylindole, 3-formylindole, guanine, tyrosine and tryptophan); (3) By improving the solid–liquid mass transfer efficiency of phytochemicals, the “explosion” effect of high pressure can enhance the damage to the cellular structure and reduce the mass transfer resistance in target materials, thus improving the extraction efficiency. The higher content of water-soluble phenolics (kaempferol, quercetin and glycoside) and potential water-soluble markers (phenylalanine betaine, lentin, triethyl phosphate, triethylcitrurate) in high-pressure CO$_2$ + H$_2$O treatment can be attributed to the improvement in solid–liquid mass transfer efficiency.
4. Conclusions

The “3I” extraction mechanism of high-pressure CO₂ + H₂O is proposed. It is interesting to note that direct evidence for the modification of the polarity of H₂O combined with high-pressure CO₂ has been reported for the detection of the water-insoluble compounds (2-methylindole, 3-formylindole, guanine, tyrosine and tryptophan) in a anthocyanin-rich solution obtained by high-pressure CO₂ + H₂O, while less amounts of these compounds were found in the H₂O extract group. Additionally, direct evidence for reduced temperatures during the pressure relief process has also been provided by the discovery of a lower content of indole and proline (temperature-sensitive compounds) for the first time. Based on the above conclusion, we propose that high-pressure CO₂ + H₂O could be used as a promising technology for phytochemical extraction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14031369/s1, Figure S1. Mirror images, structures and content of quantified compounds. (I) 2-methylindole; (J) 3-formylindole; (K) guanine; (L) tyrosine; (M) tryptophan; (N) indole; (O) proline. The blue bar represents high pressure CO₂ + H₂O extraction and the red bar represents H₂O extraction.

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