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Evaluation Study on Extraction of Anthocyanins from Red Cabbage Using High Pressure CO_2 + H_2O: 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_2O compared with traditional H_2O extraction and aqueous ethanol extraction. The high-pressure CO_2 + H_2O 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, tyrosine and tryptophan) obtained by high-pressure CO_2 + H_2O extraction compared with traditional H_2O extraction has been reported for the first time. Finally, the "3I" extraction mechanism of high-pressure CO_2 + H_2O 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_2O; a fuzzy logic model; metabolomics; extraction mechanism

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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 genotoxicityon 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 phytochemiscals 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 H_2O 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 CO_2 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 CO_2 + H_2O 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 H_2O, aqueous ethanol and high-pressure CO_2 + H_2O; 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 CO_2 + H_2O and H_2O extractions for further clarification of the extraction mechanism of high-pressure CO_2 + H_2O.

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, Na_2CO_3, 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 CO_2 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 CO_2 + H_2O system described by Liao et al. [26] with or without high-pressure CO_2 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 H_2O and aqueous ethanol (70%, v/v), respectively, and the extraction solvents were preheated to 60 °C). For H_2O and aqueous ethanol extraction, the extraction time was 30 min; for the high-pressure CO_2 + H_2O 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 M°Pa CO_2 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),

$$Anthocyanins(\text{mg C3G/L}) = \frac{A \times Mw \times DF \times 1000}{E \times L} \quad (1)$$

where A = (A520-A700) pH = 1.0–(A520-A700) pH = 4.5; Mw 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),

$$Relative\ yield\ (\%) = \frac{Amount\ of\ total\ monomeric\ anthocyanins\ recovered\ by\ given\ method}{Amount\ of\ total\ monomeric\ anthocyanins\ recovered\ by\ aqueous\ methanol} \quad (2)$$

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% Na_2CO_3 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],

$$Polymeric\ color(\%) = \frac{(A_{420} - A_{700})_{bleached} + (A_{520} - A_{700})_{bleached}}{(A_{420} - A_{700})_{control} + (A_{520} - A_{700})_{control}} \quad (3)$$

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],

$$\ln\left(c_t/c_0\right) = -K_t \quad (4)$$

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

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 α = 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),

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

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.

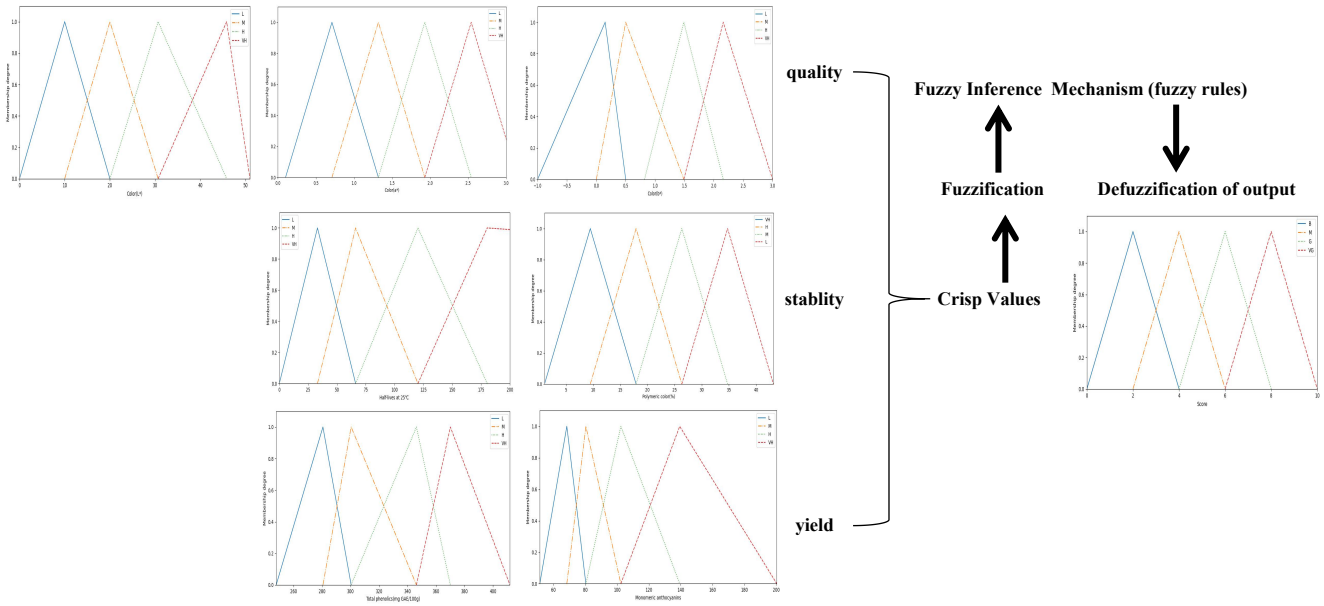

Figure 1. Flowchart of fuzzy logic system and membership functions for the values of input and output. Input variables including yield (total phenolics, total monomeric anthocyanins), quality (Color (L*), Color (a*), Color (b*)) and stability (polymeric color and half-lives at 25 °C); output is score of extraction system. Four fuzzy sets of input variables were used: low (L), medium high medium (HM), high (H), and very high (VH); Four fuzzy sets of output were used: bad (B), medium (M), good (G) and very good (VG).

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 (c) (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 (c) (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 (c) (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 H) and (c) (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 L) then (Score is G)
If (Color(L*) is H) and (Color(C*) is VH) and (c) (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].

$$Y = \frac{\int_f^i \sum_{i=1}^n f \times \mu_{Bi}(f) df}{\int_f^i \sum_{i=1}^n \mu_{Bi}(f) df}$$

$\mu_{Bi}(f)$: 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_2O extraction group was 1.62 and 1.56 times that of the pure H_2O 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_2O extraction, 92.10 ± 1.08 mg/100 g in aqueous ethanol extraction and 73.88 ± 0.54 mg/100 g in H_2O extraction. Similarly, the maximum yields of total phenols were observed in high-pressure CO_2 + H_2O extraction (355.32 ± 3.47 mg/100 g), followed by aqueous ethanol (314.26 ± 1.53 mg/100 g) and the lowest in H_2O (185.83 ± 0.77 mg/100 g).

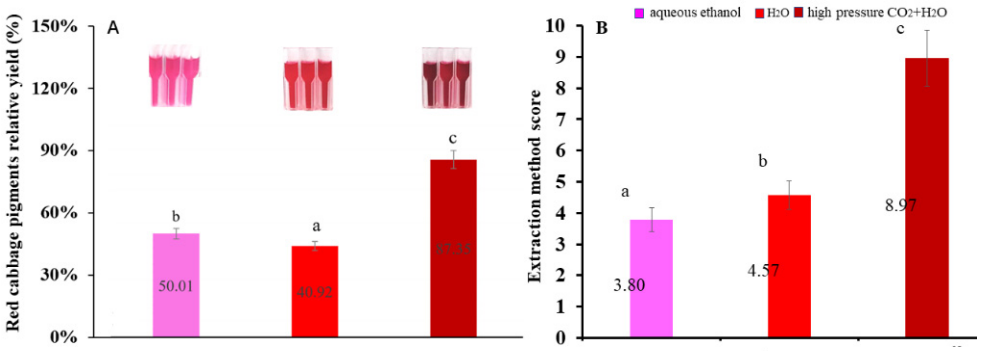

Figure 2. The relative yields of anthocyanins (**A**) and fuzzy logic score (**B**) in the three groups. The three extraction methods are high-pressure CO_2 + H_2O, aqueous ethanol and H_2O extraction. Different letters (a, b, c) indicate significant differences ($p < 0.05$).

Table 2. The color, quality and stability properties of red cabbage pigment extracts recovered.

		H_2O	Aqueous Ethanol	High-Pressure CO_2 + H_2O
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 C3G/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
Color	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% ^{a}	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_2O 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_2O produced 5–10% more anthocyanins from frozen red cabbage than H_2O [22]. F. Lao et al. found that high pressure CO_2 + H_2O (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_2O (10 MPa 40 °C and 20% volume ratio) produced more 60% colorants than H_2O extraction from Opuntia spp. [39]. On the whole, the extraction yields of high-pressure CO_2 + H_2O 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_2O

extract was the lowest (10.27 ± 0.04%), followed by H_2O 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_2O 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_2O extraction was lower, indicating that the treatment of high-pressure CO_2 + H_2O 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_2O was the lowest (33.51 ± 0.26), followed by aqueous ethanol (37.53 ± 0.55), and the highest in H_2O (42.08 ± 0.72); the a* value of extraction by high-pressure CO_2 + H_2O 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_2O was the highest (1.27 ± 0.77), followed by H_2O (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_2O, 2.52; high-pressure CO_2 + H_2O, 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_2O 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_2O (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_2O 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_2O, the degradation rate of ethanol-extracted anthocyanins was much faster than that of anthocyanins extracted by H_2O.

Polyphenols are good co-pigments of anthocyanins, which was conducive to stabilizing the color of the high-pressure CO_2 + H_2O 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_2O 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_2O 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_2O extraction method acquired the highest score (8.97), followed by aqueous ethanol extraction (4.36) and H_2O extraction (3.25) (a score > 8 can be considered as a "very good" extraction method).

The high-pressure CO_2 + H_2O 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_2O exhibited great advantages in anthocyanin extraction from plants compared to aqueous ethanol extraction and H_2O 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_2O extraction, while the contents of five mono-acylated anthocyanins and nine di-acylated anthocyanins were higher in high-pressure CO_2 + H_2O extraction. Specifically, for mono-acylated anthocyanins, the extraction effect of high-pressure CO_2 + H_2O was significantly enhanced (about 30% higher than that of aqueous ethanol extraction), and the effect of H_2O extraction was about 10% lower than that of aqueous ethanol extraction; for di-acylated anthocyanins, the extraction effect of high-pressure CO_2 + H_2O was excellent (about 60% higher than that of aqueous ethanol extraction), and the performance of H_2O extraction was about 30% lower than that of aqueous ethanol extraction (Figure 3A).

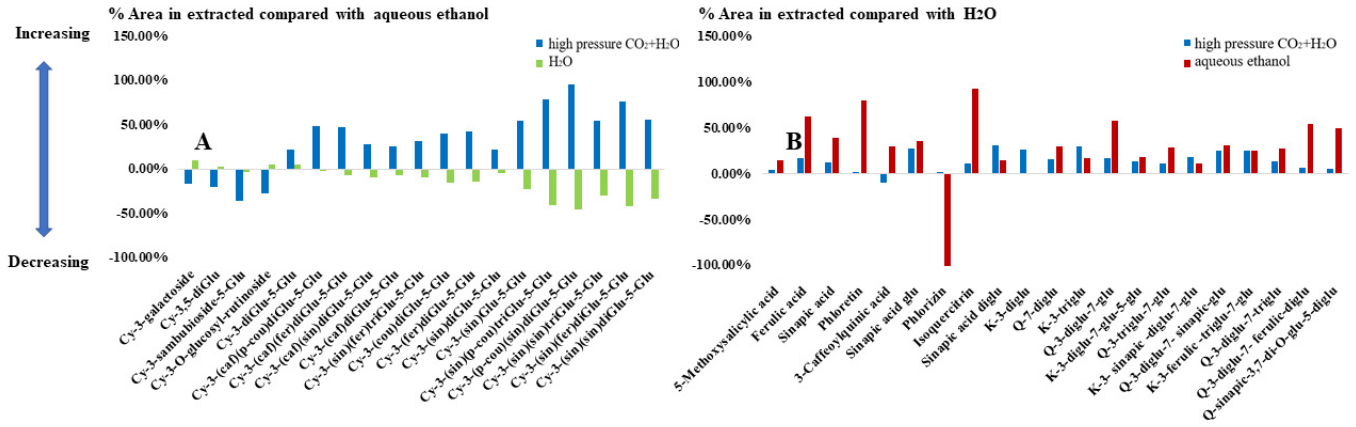

Figure 3. Relative peak area of anthocyanins (compared with aqueous ethanol group) in water and high-pressure CO_2 + H_2O groups (**A**) and relative peak area of phenolic compounds (compared with H_2O group) in aqueous ethanol and high-pressure CO_2 + H_2O groups (**B**). Abbreviation: Cy, cyanidin; glu, glucoside; caf, caffeic; *p*-cou, *p*−coumaric; fer, ferulic; sin, sinapic acid; K, kaempferol; Q, quercetin.

Table 3. The red cabbage anthocyanin and phenolic compositional profiles.

	Tentative Annotation	RT (min)	*m/z*		
			[M] $^+$	[M] $^+$ Fragments	
Anthocyanin	Cyanidin-3-galactoside	7.97	449.1071	344	287
	Cyanidin-3,5-diglucoside	8.23	611.1617	449	287
	Cyanidin-3-sambubioside-5-glucoside	8.43	743.2011	611	419
	Cyanidin 3-O-glucosyl-rutinoside	8.65	757.2101	611	443
	Cyanidin 3-diglucoside-5-glucoside	8.85	773.2106	611	449
	Cyanidin 3-(sinapoyl)glucoside-5-glucoside	11.3	817.2172	655	449
	Cyanidin 3-(*p*-coumaroyl)diglucoside-5-glucoside	10.87	919.249	757	449
	Cyanidin 3-(caffeoyl)diglucoside-5-glucosides	10.37	935.2488	773	287
	Cyanidin 3-(feruloyl)diglucoside-5-glucoside	10.99	949.2602	787	449
	Cyanidin 3-(sinapoyl)diglucoside-5-glucoside	11.02	979.2708	817	449
	Cyanidin 3-(caffeoyl)(*p*-coumaroyl)diglucoside-5-glucoside	9.29	1081.2835	919	752
	Cyanidin 3-(caffeoyl)(feruloyl)diglucoside-5-glucoside	9.39	1111.2956	949	787
	Cyanidin 3-(*p*-coumaroyl)(sinapoyl)diglucoside-5-glucoside	11.57	1125.3063	979	449
	Cyanidin 3-(caffeoyl)(sinapoyl)diglucoside-5-glucoside	9.47	1141.2498	979	817
	Cyanidin 3-(sinapoyl)(feruloyl)diglucoside-5-glucoside	11.69	1155.3173	993	449
	Cyanidin 3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside	11.73	1185.3325	1023	817
	Cyanidin 3-(sinapoyl)(*p*-coumaroyl) triglucoside-5-glucoside	11.48	1287.3596	1125	979
	Cyanidin 3-(sinapoyl)(feruloyl)triglucoside-5-glucoside	10.47	1317.3758	1155	979
	Cyanidin 3-(sinapoyl)(sinapoyl)triglucoside-5-glucoside	11.64	1347.3844	1155	963
Phenolic	3-Caffeoylquinic acid	5.6	355.1011	217	193
	Kaempferol-3-triglucoside	7.8	771.1981	447	284
	5-Methoxysalicylic acid	8.1	169.0488	109	64
	Isoquercitrin	8.7	465.1032	91	64
	Kaempferol-3-ferulic -triglucoside-7-glucoside	9.3	1109.2278	947	771
	Quercetin-3-diglucoside-7-triglucoside	9.4	1111.313	787	625
	Sinapic acid glucoside	9.6	385.1074	385	223
	Quercetin-3-diglucoside-7-glucoside	9.7	787.2181	625	463
	Kaempferol-3-diglucoside-7-glucoside-5-glucoside	10.3	933.2577	771	447
	Sinapic acid diglucoside	10.6	547.1658	385	223
	Quercetin-7-diglucoside	10.7	625.1434	625	301
	Phloretin	10.8	275.0922	259	109
	Quercetin-3-triglucoside-7-glucoside	10.9	949.2839	787	462
	Quercetin-3-diglucoside-7-sinapic-glucoside	10.9	1095.3069	771	609
	Kaempferol-3- sinapic-diglucoside-7-glucoside	11	977.2552	815	609
	Kaempferol-3-diglucoside	11.2	609.1489	609	284
	Quercetin-3-diglucoside-7-ferulic-diglucoside	11.6	1125.3225	949	625
	Phlorizin	11.6	437.144	275	259
	Quercetin sinapic-3,7-di-O-glucoside-5-diglucoside	11.7	1155.3181	993	787
	Sinapic acid	12.4	223.058	108	178
	Ferulic acid	12.5	193.0505	161	134

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_2O 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_2O treatment, which may have an opposite or ineffective influence on the two main factors (solvent polarity and temperature),

and the comprehensive effect is not obvious. Specifically, although CO_2 promotes the entry of the extractant into cells and fully extracts the substance, due to the evaporation and the endothermic heat of liquid CO_2 during high-pressure CO_2 + H_2O 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 CO_2 + H_2O and aqueous ethanol share 322 compounds, the high-pressure CO_2 + H_2O and H_2O share 2827 compounds and the aqueous ethanol and H_2O 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 CO_2 + H_2O and H_2O, 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 H_2O as a solvent and aqueous ethanol as a solvent.

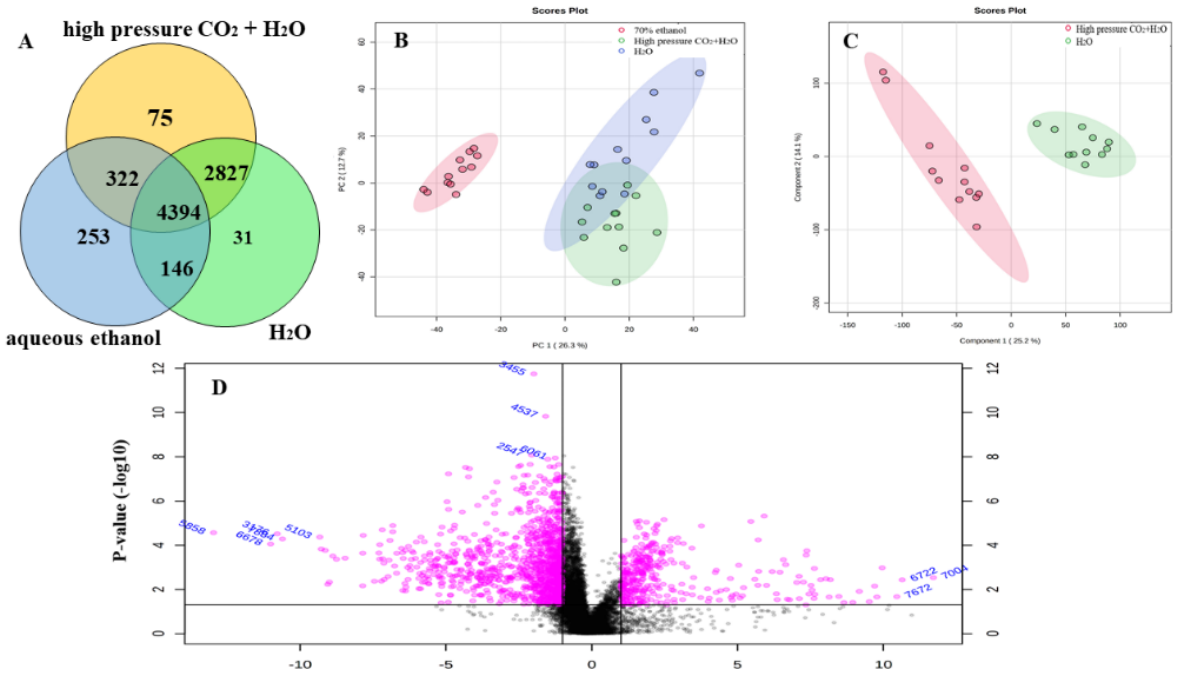

Figure 4. Differential compounds analysis: Venn plots of high-pressure CO_2 + H_2O, H_2O and aqueous ethanol **(A)**; PCA score plots of high-pressure CO_2 + H_2O, H_2O and aqueous ethanol (**B**); OPLS-DA score scatter plot of high-pressure CO_2 + H_2O and H_2O (**C**). Volcano plots of high-pressure CO_2 + H_2O and H_2O (**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 CO_2 + H_2O and H_2O 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_2O and H_2O.

Category	Name	Mass	*m*/*z*	RT (min)	Formula	Error (ppm)	*p*-Value (t)	FC (Pressure CO_2 + H_2O/H_2O)
Alkaloid	Betaine	117.15	118.0857	1.99	$C_5H_{11}NO_2$	0.3558	0.0047	2.5988
	Phenylalanine betaine	207.27	208.1328	8.71	C_{12} $H_{17}NO_2$	−0.1996	0.1965	2.0554
	Lenticin	246.30	247.1438	9.87	$C_{14}H_{18}N_2O_2$	−0.2809	0.1025	2.7830
	Indole	117.15	118.0632	9.16	C_8H_7N	0.8293	1.23×10^{-12}	4.0135
	2-Methylindole	131.17	132.0797	9.26	C_9H_9N	−0.7040	1.16×10^{-7}	12.3634
	3-Formylindole	145.16	146.0597	9.15	C_9H_7NO	0.7957	2.33×10^{-13}	3.6899
Nucleic Acid	Guanine	151.13	152.0550	6.64	$C_5H_5N_5O$	−0.5112	0.0006	3.2886
Amino Acid	Phenylalanine	165.19	166.0856	8.04	$C_9H_{11}NO_2$	0.7581	6.37×10^{-13}	3.3084
	Tyrosine	181.20	182.0812	6.71	$C_9H_{11}NO_3$	0.7611	8.29×10^{-8}	3.1048
	Tryptophan	204.23	205.0965	9.15	$C_{11}H_{12}N_2O_2$	0.7147	3.21×10^{-9}	3.0079
	Proline	115.13	116.0693	7.05	$C_5H_9NO_2$	0.7304	6.57×10^{-10}	3.8139
	Isoleucine	131.17	132.1021	6.94	$C_6H_{13}NO_2$	0.7625	8.63×10^{-13}	3.0268
	Triethyl phosphate	182.15	183.079	14.04	$(C_2H_5O)_3PO$	−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-isobutylphosphate	266.31	267.1718	16.5	$C_{12}H_{27}O_4P$	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), lenticin (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), guanine (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, lenticin, triethyl phosphate, and triethylcitrate are higher in high-pressure CO_2 + H_2O; and the contents of phenylalanine, betaine, isoleucine are higher in H_2O. 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, lenticin, triethyl phosphate, triethylcitrate and guanine are higher in high-pressure CO_2 + H_2O extraction compared to H_2O 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_2O extraction compared to H_2O 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_2O 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_2O extraction is that the solution temperature decreases at that stage of pressure relief.

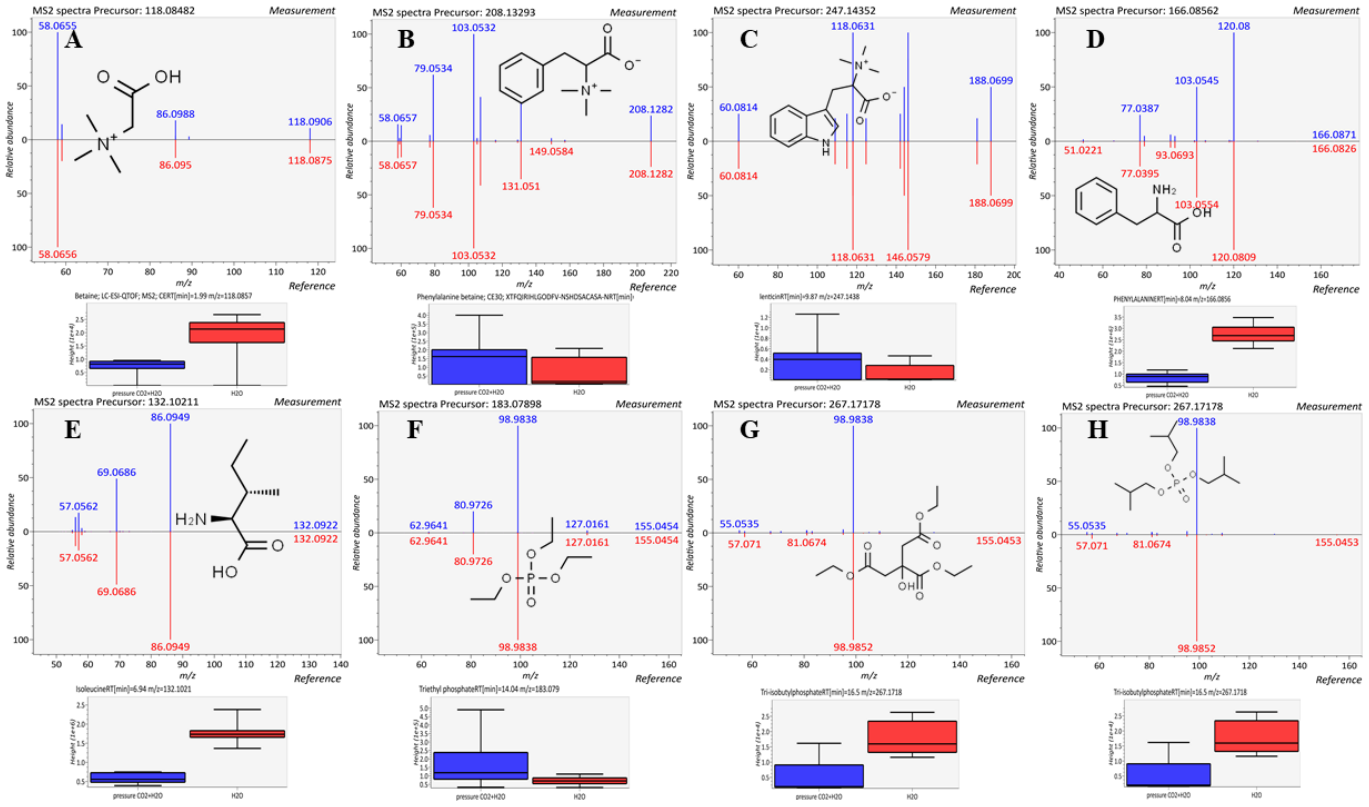

Figure 5. The boxplots of the abundance and structures of (**A**) betaine, (**B**) phenylalanine betaine, (**C**) lenticin, (**D**) phenylalanine, (**E**) isoleucine, (**F**) triethyl phosphate, (**G**) triethylcitrate and (**H**) tri-isobutylphosphate. The blue bar represents high-pressure CO_2 + H_2O extraction and the red bar represents H_2O extraction.

3.5. Extraction Mechanism of High-Pressure CO_2 + H_2O

Based on our early experiments, the results of this paper and relevant literature, we propose the "3I" extraction mechanism of pressure CO_2 + H_2O 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]. It has been demonstrated that the degradation of anthocyanins can be delayed under the condition of limited oxygen. Additionally, high-pressure CO_2 + H_2O 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_2CO_3), which changes the polarity of solution. That is why high-pressure CO_2 + H_2O 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, lenticin, triethyl phosphate, triethylcitrate) in high-pressure CO_2 + H_2O treatment can be attributed to the improvement in solid–liquid mass transfer efficiency.

4. Conclusions

The "3I" extraction mechanism of high-pressure CO_2 + H_2O is proposed. It is interesting to note that direct evidence for the modification of the polarity of H_2O combined with high-pressure CO_2 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 CO_2 + H_2O, while less amounts of these compounds were found in the H_2O 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 CO_2 + H_2O 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 CO_2 + H_2O extraction and the red bar represents H_2O extraction.

Author Contributions: Conceptualization, Z.X.; methodology, L.X.; software, B.W.; validation, B.W.; investigation, X.W.; data curation, B.W.; writing—original draft preparation, B.W.; writing—review and editing, S.Y.; data curation, L.M. and K.W.; funding acquisition, X.L. and Z.X. All authors have read and agreed to the published version of the manuscript.

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