Procedures for Extraction of Anthocyanins from Different Food

Subjects: Plant Sciences

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Anthocyanins are water-soluble pigments characterized by various intense colors found in fruits and vegetables. The extraction and separation of anthocyanins from plants is important, especially due to the instability of plant anthocyanins, selecting and optimizing. Anthocyanins are prone to degradation by several factors, including pH, temperature, oxygen, water activity, co-pigments and enzymes. Unwanted compounds, such as sugars, proteins, lipids, acids and other flavonoids, can also be removed from plant material by appropriate extraction methods. The most used method for anthocyanin extraction is the conventional one, solid–liquid extraction, also known as solvent extraction, during which anthocyanins can be dissolved in polar solvents (methanol/glycolic acid and acetone), followed by their quantification, achieved by using spectrophotometry, the differential pH method, which is a rapid and convenient quantitative assay. Starting from this point, it has developed and there are many anthocyanin-extraction methods, such as conventional solvent extraction (CSE), enzyme-assisted extraction (EAE), fermentation extraction (FE), supercritical fluid extraction (SFE) (CO_2) extraction, microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), high-hydrostatic-pressure extraction (HHPE) and pressurized-liquid extraction (PLE). One of the most frequent techniques for obtaining anthocyanins from plants is conventional solvent extraction. In order to meet the demands of safety and environmental sustainability, new extraction technologies with shorter extraction periods and higher yields have been developed (e.g., PLE, EFS, UAE, MAE, EAE, etc.).

Keywords: anthocyanins ; extraction ; compounds

1. Preliminary Treatments

Anthocyanins can be found inside the vacuoles of cells in several types of tissues, thus, influencing their accessibility, which depends on the integrity of plant tissues and the ability of solvents to penetrate tissues [1]. The preliminary treatments before the anthocyanin extraction increase the anthocyanin's accessibility from the plant matrix to the extraction medium and the stability of the anthocyanins in the extract [2]. According to prior research, there are three main pretreatment methods for anthocyanins: chemical, biological and physical [3].

Acid treatment and alkaline immersion are chemical techniques used to increase extraction efficiency. The most popular method is applying acid pretreatments, since it can enhance the stability of anthocyanins due to a more favorable extraction environment and the inactivation of enzymes that degrade anthocyanins. Alkaline treatments improve solvent penetration during extraction by removing the waxy outer layer in the case of food matrices containing wax bloom [4].

Enzymatic treatments are one biological technique that increases the effectiveness of anthocyanin extraction by destroying the cell wall of plant materials. Anthocyanins are found inside plant cells called vacuoles. Using enzymes to dissolve the cell wall, anthocyanins can be extracted from plant cells more easily, especially those with thicker cell walls and pectin [3].

Other methods for improving anthocyanins extraction are the physical methods, a breakdown of cell walls [2]. Commonly used preliminary treatments include non-thermal treatments (grinding, pulsed electric field treatment, freezing and homogenization) and heat treatments (hot-air drying). Obtaining powders from mechanical pretreatment is a conventional approach to reduce plant tissue particle size and increase solvents' permeability. The drawback of such treatments is that they make it easier for anthocyanins to be exposed to oxidizing chemicals, which leads to their destruction. Heat treatments can improve the efficiency of anthocyanin extraction by damaging the cells' membranes and have the disadvantage that they can cause the degradation of specific types of anthocyanins. Thus, new methods of thermal pretreatment have been developed, such as microwave heating. Alternative heat treatments can be used before or during extraction and can have a reduced rate of thermal degradation compared to conventional thermal methods.

2. Extraction

Sample preparation for anthocyanin analysis varies depending on the type of sample. Juices, syrups and wines are examples of liquid products requiring minimal preparation before analysis, while solids must be fractionated and/or homogenized before extraction [5]. Qualitative research involves extraction with a weakly acidified alcoholic solvent, concentrated under vacuum, purification and pigment separation. The procedure must recover the anthocyanins while avoiding their modification. Thus, acylated anthocyanins, which degrade in solvents containing mineral acids, such as sulphuric acid (H_2SO_4) or hydrochloric acid (HCl), must be extracted with acidified solvents with organic acids, such as acetic acid or formic acid (FA) [6]. Further, anthocyanins are sensitive to heat and avoiding high temperatures during extraction and concentration is recommended. The solvents most often used to prepare plant extracts are water (H_2O), methanol (MeOH), ethanol (EtOH), acetone, dichloromethane and hexane [7][8][9]. The extraction procedure also separates free sugars, organic acids, alcohols, proteins and amino acids from the plant in addition to anthocyanins [10][11]. Some of these, such as free sugars, cause the degradation of anthocyanins while being stored, becoming necessary for separating these compounds [10].

2.1. Procedures for the Extraction of Anthocyanins from Fruits

Several scientific papers report using various anthocyanin-extraction processes in food matrices, such as fruit extracts. For example, Chandra Singh et al. [12] proposed an extraction procedure for blueberry (*Vaccinium coroymbosum* L.) using a single-extraction method, ultrasound-assisted extraction (UAE) and combined-extraction methods (extraction method by grinding/UAE and grinding with a Dounce/UAE homogenizer). Therefore, twelve mixed solvents (MeOH/H_2O, EtOH/H_2O) were adjusted to pH 2.0 or pH 3.0 with concentrated HCl in different proportions (60:40, 70:30, 80:20, 70:30). The extraction methods included the following conditions: UAE extraction (30 min, at a temperature of 15 °C); extraction with sample grinding (using a system of 24 ceramic spheres (1,4 mm) to crush and shake the sample for 44 s) and for grinding with a Dounce homogenizer (sample was crushed to destroy cells and tissues by applying mechanical force in glass homogenizers). Further, Ultra-High-Performance Liquid Chromatography–Photodiode Array–Electrospray Ionization–Mass Spectrometry (UHPLC-PDA-ESI Chandra Singh -MS) was performed for anthocyanin quantification. The chromatographic profile of blueberry extract indicated the presence of three anthocyanin compounds by correlating them with the reference standards for anthocyanins: dpd-3-glu < ptd-3-glu < mvd-3-glu. The research suggests that using the UAE to quantify anthocyanins may be an effective method with acceptable performance, the reaction in which the mixture of MeOH solvents (80:20) produced the maximum extraction yield at pH 3.0. In another protocol, the anthocyanins from blueberry (*Vaccinium* spp.) were extracted using acidified glycerol coupled with the pulse ultrasound-assisted extraction (P-UAE) method and analyzed by UPLC-Triple-TOF/MS, which led to the identification of 10 anthocyanin compounds [13]. The research concluded that the glycerol extraction method was responsible for a better extraction and preservation of anthocyanins. Further, da Silva et al. [14] investigated the extraction of anthocyanins from blueberry with natural deep eutectic solvents (NADESs) based on choline chloride, glycerol and citric acid. Therefore, the extraction efficiency of NADES was compared with that of an organic solvent (methanol:water:formic acid 50:48.5:1.5 *v*/*v*.) and a 1% (*v*/*w*) citric acid aqueous solution. The blueberry extracts were analyzed using HPLC equipment coupled to a reverse phase Symmetry C-18 column and mass spectrometer (MS) detector. Natural deep eutectic solvent (NADES) based on choline chloride:glycerol:citric acid at a molar ratio of 0.5:2:0.5 was demonstrated to be equally efficient to the conventional organic solvent.

In the extraction procedure established by Wang et al. [15], the blueberry (*Vaccinium* spp.) fruit parts (pulp and peel samples) were finely chopped with liquid nitrogen. The ratio of the processed fruit powders to the solvents, as mixtures of various types and solvent concentrations, was 1:10. The samples were immersed in a water bath or an ultrasonic water bath of 30/40 kHz/185 W, using the following extraction parameters (temperature/time): CSE method (solvent type: MeOH, EtOH, acetone, solvent concentration: 60%, 70%, 80%, extraction temperature: 50 °C, 60 °C, 70 °C); UAE (solvent type: MeOH, EtOH, acetone; solvent concentration: 60%, 70%, 80%; extraction temperature: 20 °C, 40 °C, 60 °C). The optimized parameters were 60% MeOH, 50 °C, 1 h (CSE) or 70% MeOH, 30 °C, 20 min (UAE). The extracts' anthocyanins were detected by using an HPLC-PDA system (High-Performance Liquid Chromatography–Photodiode Array), which revealed the presence of 14 compounds for blueberries (**Figure 1**A).

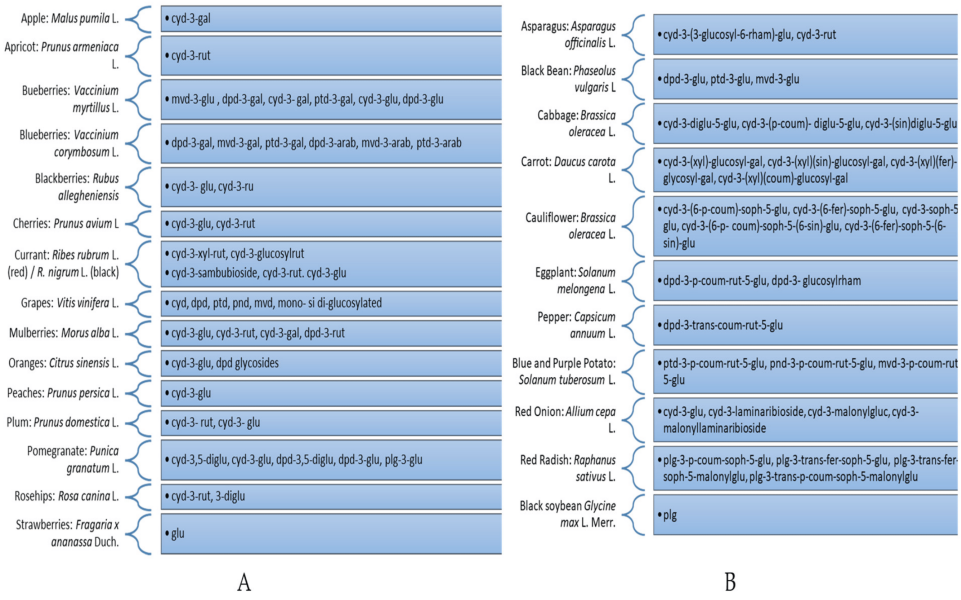

Figure 1. Majority of anthocyanin compounds in various fruits (**A**) and vegetables (**B**) [16] Note: cyanidin—cyd; delphinidin—dpd; petunidin—ptd; peonidin—pnd; pelargonidin—plg; malvidin—mvd; glu—glucoside; gal—galactoside; arabinoside—arab; rutinoside—rut; caffeoyl—caf; xylosyl—xyl; sinapoil—sin; feruloyl—fer; coumaroyl—coum; phenol—phen; p-hydroxybenzoyl—hydbenz; sophoroside—soph; rhamnosyl—rham.

The above-mentioned protocol of extraction was also applied to sweet cherries (*Prunus avium*) [15]. The results revealed the following optimized parameters: 60% EtOH, 70 °C, 1 h (CSE) or 80% EtOH, 30 °C, 20 min (UAE). The anthocyanins from the extracts were identified using an HPLC-PDA system and two compounds for red cherries were identified, dpd-3-gal and dpd-3-glu (**Figure 1**A). Blackhall et al. [17] extracted the anthocyanins from sweet cherries (*Prunus avium*) in acidified methanol and acidified ethanol (0.1% *v/v* 12 N HCl) at different solvent/solid ratios (2.5, 5, 7.5, 10 or 12.5 mL/g cherries). The quantitation of total anthocyanins was accomplished by ultra-performance liquid chromatography (UPLC) using a diode array UV/Vis detector. This study identified the optimum variables for the extraction of anthocyanins from cherries' extraction time of 90 min, temperature of 37 °C and a 10 mL/g solvent/solid ratio followed by 100% ethanol acidification.

In another protocol, the fresh sweet cherries in Early rivers (*Prunus avium* L.) [18] were subjected to four cycles of microwave irradiation at 1000 W for 45 s each. The extracts collected constituted the raw extract, centrifuged at 7000 rpm for 5 min at 10 °C. The anthocyanins were then purified via semipreparative liquid chromatography using an isocratic mobile phase consisting of a H_2O/EtOH/FA mixture circulating in a "closed-loop" system. HPLC-MS determined the anthocyanin content and the following compounds were identified and quantified: cyd-3-O-glu and cyd-3-O-rutinosides.

Karaaslan et al. [19] applied a procedure of extraction at room temperature with acidified solvent (5:1 ratio) to extract anthocyanins from cranberries (*Vaccinium oxycoccos)*. The extracts were centrifuged at 4000 rpm for 10 min and the supernatants were filtered (0.45 µm filter). The anthocyanins identified using an HPLC-ESI-MS system were: dpd-3-O-glu, cyd-3-O-glu, plg-3-O-glu and mvd-3-O-glu. The procedure developed by Alrugaibah et al. [20] involved the extraction of anthocyanins from cranberry pomace (*Vaccinium macrocarpon*) using different formulas of NADES and their efficiency was compared with that of 75% ethanol. The identification of anthocyanins was carried out using the HPLC system. The extraction with NADES had higher extraction efficiency and selectivity ethanol extraction. At NADES, an extraction mixture of glucose, lactic acid (1:5) and 20 mL/100 mL water produced the best yield, approximately 1.8-times higher than the yield with 75% ethanol. In the protocol developed by Saldana et al. [21], using a pressurized fluid reactor (40–160 °C and 50–200 bar) and water, 30–100% ethanol or 5% citric acid, anthocyanins were extracted from cranberry pomace (*Vaccinium macrocarpon*) and measured using an HPLC-UV system. According to this protocol, the optimal conditions for extracting anthocyanins from cranberries are: 70% ethanol at 120 °C and 50 bar and 5 mL/min flow rate.

The extraction procedures of grapes (*Vitis amurensis*) applied by Ji et al. [22] used lyophilized samples (Muscat Hamburg grape skin) extracted with 70% alcohol, for 24 h, in the dark. After 25 min of ultrasonication at 60 kHz, the samples were filtered and concentrated in a rotary evaporator at 35 °C in a vacuum. Before HPLC injection, the extracts were dissolved in MeOH and filtered with an 0.45 m filter. The HPLC-DAD-ESI-MS analysis identified 12 anthocyanin compounds.

Cvjetko Bubalo et al. [21] used lyophilized and ground samples (grape skin *Vitis vinifera* L. of the Plavac mali variety) for the extraction procedure. Conventional solvents, DES or three different extraction techniques (shaker, MAE and UAE)

were used. The samples were extracted and centrifuged at 5000 rpm for 15 min and the supernatant obtained was decanted and adjusted. The anthocyanin content was determined by HPLC-MS, with eight compounds identified and quantified. Further, Loarce et al. [23] used, in their protocol, freeze-dried under vacuum samples (grape pomace from *V. vinifera* L. cv. 'Tempranillo'), extracted by pressurized hot water with NADES. HPLC-DAD-ESI-MS/MS analysis of anthocyanins was applied, the best extraction results being recorded for 30% choline chloride and oxalic acid (ChOx) at 60 °C.

For the extraction procedure elaborated by Fang et al. [24], the plum (*Prunus salicina* Lindl.) peel was ground with liquid nitrogen obtaining a fine powder. The powders were extracted with 0.05% HCl in MeOH at 4 °C for 24 h and then centrifuged at 8000× *g* for 20 min.

2.2. Procedures for the Extraction of Anthocyanins from Vegetables

Wiczkowski et al. [25] used powder from red cabbage (*Brassica oleracea*) for the extraction procedure. The sample was extracted by sonication for 30 s with a mixture of MeOH/H_2O/TFA (0.58/0.38/0.04). Subsequently, the mixture was stirred for 30 s, sonicated and centrifuged for 10 min (13,200× *g* at 4 °C). HPLC-MS determined the anthocyanin content and twenty compounds were identified. Therefore, 2 identified cyd derivatives were non-oscillated, 11 monoacylated and 7 diacylates.

Strauch et al. [26] used 70% MeOH with 0.1% acetic acid in another extraction procedure for red cabbage (*Brassica oleracea*) anthocyanins. The mixture was stirred and sonicated for 10 min at room temperature, then centrifuged at 4000 rpm for 20 min. Before HPLC analysis of anthocyanins, the supernatant was filtered (0.2 µm polytetrafluoroethylene filters) and stored at −20 °C. UPLC-DAD-MSE determined the anthocyanin content and 29 anthocyanin compounds were identified. Among them, 27 were derivatives of glycosylate cyanidin in varying degrees and acylated with p-cumaryl, feruloyl or synapyl groups. Moreover, Yiğit et al. [27] used microwave-assisted extraction of red cabbage (*Brassica oleracea*) anthocyanins. For the extraction procedure, the authors used lyophilized red cabbage extracted via conventional extraction (maceration at 40 and 70 °C for 4 and 6 h) and microwave-assisted extraction MAE (solvents: water and 50/50 (*v*/*v*) ethanol–water mixture; microwave irradiation power: 200, 400 and 600 W) and for anthocyanin identification, HPLC-DAD-MS. The maximum extraction yield of MAE (220.0 mg cyanidin-3-glycoside/L) was recorded at 10 min using only part of the solvent compared to the amount used in the conventional extraction method.

For the extraction procedure [28][29], purple pulp sweet potato *(Ipomoea batatas)* powder was used, in which an internal standard, cyd-3,5-diglu, was introduced. The compounds were extracted with 5% FA using an orbital stirrer at a temperature of 40 °C for 12 h and centrifugated for 20 min (4000× *g* at 4 °C). The supernatant obtained by combining the two steps was purified using a C18 solid-phase extraction cartridge. The resulting eluent was evaporated dry and reconstituted in 5% FA. HPLC ESI/MS/MS was used in this research to identify anthocyanin compounds. Thus, fourteen anthocyanins were eluted and found in the roots of three varieties of sweet potatoes (**Figure 1**B). A recent study [30] demonstrated the superiority of applying the high-pressure carbon dioxide method (HPCD) on the purple sweet potato anthocyanin extraction compared to conventional aqueous- and ethanol-extraction methods (the extraction yield was over 25% higher compared to conventional methods).

The peels of mature green and ripe red tomatoes (*Solanum lycopersicum* L.) were used for the extraction procedure [31]. The lyophilized peel was extracted with a mixture of MeOH:H_2O: TFA (70:29.5:0.5), with a ratio of 1/20 at room temperature. After centrifugation at 3500× *g* for 10 min, the extraction was repeated for 1 h under the same condition. The supernatant was evaporated at 32 °C to 1/3 of the original volume, then freeze-dried. The extracts were analyzed using an HPLC-PDA system and two compounds were identified: petanin and negretein. The procedure described by Wang et al. [32] used Indigo Rose tomato powder (*Solanum lycopersicum*) for anthocyanin extraction. The extraction was conducted utilizing methanol/formic acid (9:1, *v*/*v*) and, for the quantification of anthocyanin content, was added to the samples peonidin-3-glucoside chloride as an internal standard. UPLC-QTOF-MS was used to analyze tomato extracts and 12 anthocyanins were identified.

2.3. Procedures for the Extraction of Anthocyanins from Cereals

Zhang et al. [33] used a barley powder (*Hordeum vulgare* L.) and 90% EtOH for the extraction procedure. Subsequently, the mixture extracted for 30 min at 50 °C in an ultrasonic water bath was centrifuged for 20 min (10,200× *g*). The extracts were filtered (0.45 µm filter) and concentrated at 40 °C in a rotary evaporator. They were then loaded onto a balanced AB-8 resin column and eluted with 1% FA in 80% MeOH. The effluent of the MeOH solution was concentrated at 40 °C, purified and freeze-dried. B UPLC-MS analysis identified anthocyanin compounds as cyd, plg and pnd.

For the extraction procedure [34][35], a black rice (*Oryza sativa* L. 'Violet Nori') powder and 40 mL EtOH/H_2O mixture (60:40) were used. The mixture was homogenized in two steps: 0.5 min at 8000 rpm and 1.5 min at 24,000 rpm. Direct sonication was used to achieve the final extraction for 5 min at an amplitude and pulse rate of 30% and 80%, respectively. The extracts were analyzed using an HPLC DAD system and the following compounds were identified: cyd-3,5-diglu, cyd-3-glu, cyd-3-rut and pnd-3-glu. Yi et al. [36] used their protocol, the nanobiocatalyst, to extract anthocyanins from black rice (*Oryza sativa* L.). The magnetic nanobiocatalyst was prepared by immobilizing cellulase and α-amylase on amino-functionalized magnetic nanoparticles. This procedure had a maximum yield at 30 °C compared to the anthocyanin extraction procedure using free enzymes, which recorded a maximum yield at 40 °C. Therefore, using the nanobiocatalyst can be advantageous because it could reduce energy costs during processing. Further, the enzymes immobilized on the nanobiocatalyst will be recovered at the end of the extraction process and reused in other extraction cycles.

3. Purification

Currently, the methods used to extract anthocyanins result in solutions containing amounts of unwanted elements, such as sugars, acids, amino acids and proteins, that require removal. In order to remove sugars, acids and other water-soluble substances, the crude extracts were purified using C18 cartridges that had been activated with MeOH, H_2O and HCl with 0.01% or 3% FA [37][38][39].

Purification of anthocyanins by adsorption is an effective and straightforward method. Chandrasekhar et al. [40] used silicone gel (Amberlite IRC 80, Amberlite IR 120, DOWEX 50WX8, Amberlite XAD-4 and Amberlite XAD-7HP) to purify anthocyanins from red cabbage extract. The Amberlite XAD-7HP proved to be the most effective. Desorption of anthocyanins was successfully carried out with EtOH, with a concentration of more than 60% vol.

The use of the same adsorbents was also investigated by Jampani et al. [11] to purify anthocyanins from Malabar plum extract (*Syzygium cumini*). The Amberlite XAD-7HP also proved to be the most effective.

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