Chapter

HPLC-MS$^{(n)}$ Applications in the Analysis of Anthocyanins in Fruits

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Abstract

Anthocyanins are water-soluble pink/red/blue/purple pigments found abundantly in the flesh and skin of fruits, flowers, and roots of different varieties of plants. Compared to vegetative tissues in many plants, fruits have much higher contents of anthocyanins. In general, anthocyanins have antioxidant, anti-inflammatory, antimutagenic, and antiapoptotic activities that benefit human health. To date, anthocyanins in many different fruits have gained intensive studies in structures, biosynthesis, genetics, and genomics. Despite this, difficulties exist in identifying anthocyanins with similar structures and precisely estimating contents within fruit matrices. To improve this challenge, high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) based metabolomics has been shown a powerful technology to distinguish structure-similar anthocyanins. This chapter reviews, summarizes, and discusses the application of HPLC-MS/MS in the annotation or identification of anthocyanins in fruits.

Keywords: anthocyanins, chromatography, HPLC, mass spectrometry, fruits

1. Introduction

Anthocyanins are a class of plant flavonoids belonging to polyphenolics. Anthocyanins are water-soluble pigments that give pink/red/purple/blue color to plant tissues. Anthocyanins are found in the majority of higher plant species except in plant species of Caryophyllales. Moreover, anthocyanins have been found in some lower plants, such as mosses and ferns [1]. Certainly, anthocyanins are important agronomical traits in many crops, particularly ornamental ones for flowers and fruits [2]. Anthocyanins are synthesized in the cytosol and mainly transported to the central vacuoles. Plant cells such as epidermal cells in the peel of fruits and flower petals are the main locations with the active biosynthesis of anthocyanins. Generally, anthocyanins are stored as the colored flavylium ion form due to the acidic conditions of the vacuoles [3]. The color changes of plant tissues are normally associated with pH value variations in the central vacuoles.

1.1 Structure

All anthocyanins are derived from a specific chromophore core, namely 2-phenyl-benzopyrylium or flavylium, which consists of two aromatic rings (A and B) and one heterocyclic pyran ring including three carbons (C) (**Figure 1**), thus is featured by C6-C3-C6. Seven positions (R_3, R_5, R_6, R_7, $R_{3'}$, $R_{4'}$, $R_{5'}$) are commonly subjected to modification of monosaccharides, methyl, or other groups *via* –OH or carbon [4, 5]. Past structural studies have shown that anthocyanins predominantly found in nature are glycosylated or galloylated 2-phenyl-benzopyrylium or flavylium salts in acidic conditions. The flavylium structures also are subjected to modification by the vacuolar pH values to give different hue features of plant tissues.

To date, more than 700 distinct anthocyanins have been identified in the plant kingdom. Structurally, anthocyanins are composed of an aglycone also called anthocyanidin and carbohydrate residue. All anthocyanins are derivatives of anthocyanidin aglycones. Although different reports have listed 19 anthocyanidins (6 major and 13 minor groups) (**Table 1**), based on the biosynthesis from phenylalanine and three malonic acids, pelargonidin, cyanidin, and delphinidin form the three basic ones. Other types such as peonidin, petunidin, and malvidin result from methylation of the B-ring of the three basic forms. Actually, methylated anthocyanidins have different physical and chemical features from their precursors. To date, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin are commonly accepted to represent six major groups of anthocyanidins [6–8]. The glycosylation or methylation, acylation, galloylation, and other modifications diversify anthocyanin structures. Based on the structures reported, glycosides of anthocyanidin form the most predominant structures in nature. Past investigations have reported that glycosylation mainly occurs at the –OH group of C_3, C_5, or C_7 of the core chromophore. The main monosaccharides involved in glycosylation include glucose, xylose, arabinose, rhamnose, rutinose, fructose, and galactose moieties. In addition, disaccharides are involved in glycosylation. The acylation, acetylation, and malonylation of anthocyanidins or anthocyanins lead to more diversity of structures. Based on structural and biosynthetic reports, the common acylation result from the addition of a coumaric, caffeic, ferulic, *p*-hydroxy benzoic, synaptic, malonic, acetic, succinic, oxalic, or malic acid to sugar moiety or moieties [7, 9]. The acylation can alter the physical or chemical features of anthocyanins, such as water solubility and color such as blue color. In addition, the hydroxyl and methoxyl groups as well as other modifications also lead to different colors and stability of anthocyanins [6, 8]. Furthermore, secondary, ternary, and more complicated modifications on sugars or acylate groups diversify

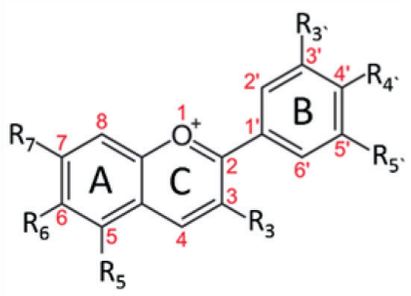

Figure 1.
The core chemical structure of anthocyanidins. A; aromatic ring, B; phenyl ring, C; Benzopyran ring. R; -H, -OH, or -CH_3.

Anthocyanidins	**Substitutions**						
	R_3	**R_5**	**R_6**	**R_7**	**$R_{3'}$**	**$R_{4'}$**	**$R_{5'}$**
Cyanidin (**Cy**)	OH	OH	H	OH	OH	OH	H
Delphinidin (**Dp**)	OH	OH	H	OH	OH	OH	OH
Pelargonidin (**Pg**)	OH	OH	H	OH	H	OH	H
Peonidin (**Pn**)	OH	OH	H	OH	OMe	OH	H
Petunidin (**Pt**)	OH	OH	H	OH	OMe	OH	OH
Malvidin (**Mv**)	OH	OH	H	OH	OMe	OH	OMe
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe
Rosinidin	OH	OH	H	OMe	OMe	OH	H
Aurantinidin	OH	OH	OH	OH	H	OH	H
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H
6-Hydroxydelphinidin	OH	OH	OH	OH	OH	OH	OH
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe
Europinidin	OH	OMe	H	OH	OMe	OH	OH
5-Methylcyanidin	OH	OMe	H	OH	OH	OH	H
Pulchellidin	OH	OMe	H	OH	OH	OH	OH
Luteolinidin	H	OH	H	OH	OH	OH	H
Apigenidin	H	OH	H	OH	H	OH	H
Tricetinidin	H	OH	H	OH	OH	OH	OH
Riccionidin A	H	H	OH	OH	H	OH	H

Table 1.
Basic and methylated or hydroxylated anthocyanidins (OMe: Methoxy group; OH: Hydroxyl group; H: Hydrogen) modified from ref. [6].

anthocyanin structures. Most of those modifications result from enzymatic reactions catalyzed by glycosyltransferases, methyltransferases, acyltransferases, malonyltransferases, and others [4, 10]. These modifications are associated with the color, chemical stability, bioavailability, biological activity, and diverse molecular structures of anthocyanins in plants.

1.2 Functions in plants and health benefits

Anthocyanins play important physiological functions associated with plant reproduction and defenses. Anthocyanins act as visual signals to attract pollinators for the pollination of flowers and dispersers to spread seeds or fruits [4]. Anthocyanins can play as warning signals to repulse birds and insects for protection of plant tissues from being consumed [11]. They serve as filters to absorb UV-B light and visible light for protecting plant tissues from being damaged by severe irradiation [12]. The accumulation of anthocyanins protects leaves from radiation-caused damage of photosynthesis by absorbing extra light [13]. Past studies have reported that many anthocyanin structures defend plants against diseases infected by various pathogens and damage caused by abiotic stresses, including cold shock, drought, osmotic and wounding, and

biotic stresses [5, 14, 15]. Furthermore, anthocyanidins are essential precursors of proanthocyanidins, which are powerful antioxidative, anti-pathogen, anti-radiative, and anti-pest flavonoids in most of the plant species [16].

Anthocyanins are of growing interest in beneficial values for human and animal health because of their antioxidative, antibacterial, and anticancer activities reported by *in vitro* experiments [17]. Some structures have been shown to scavenge free radicals such as reactive oxygen species generated in human cells [18]. More studies have shown that the antioxidative and anti-inflammation activities of anthocyanins help to improve visual acuity [19] and to protect from a heart attack [20, 21]. Metabolic studies have shown that certain anthocyanin structures can prevent obesity and diabetes by interfering the body weight gain and adipose tissue increase [22, 23]. Especially, different structures have been observed to associate with specific activities [24]. Accordingly, a general health fact is that regular consumption of high levels of dietary anthocyanin-rich plant sources, such as red, purple, and dark-colored berries, grapes, and vegetables, is considered to benefit human health [25].

2. Analysis of anthocyanins in fruits

Anthocyanins are the main pigments responsible for red, magenta, violet, blue, and dark blue colors of many fruits and berries. Therefore, fruit or seed anthocyanins have gained a large number of studies, which include the formation and stability of color, and color changes during ripening, processing and storage, isolation, and identification from fruits (**Table 2**). Many have been developed as colorants for food, pharmaceutical, and nutritional industries.

Fruits are one of the main sources of novel anthocyanins with valuable health benefits [8, 26, 27]. A large number of anthocyanins have been isolated from fruits (**Table 2**).

To date, methods for the identification of fruit anthocyanins have been appropriately established by numerous studies. In generation, three main sequential steps are sample preparation, separation and purification, and identification.

Sample preparation, as an initial step for anthocyanin determination, is highly variable depending on the fruit samples and the objectives of the analysis. Sample preparation mainly consists of collection, drying, powdering, and extraction of samples. Liquid samples of fruit, such as juices, wines, and syrups, need very little preparation before the analysis. However, solid or dried fruit materials require to be fractionated, homogenized, crushed, or pulverized. The most commonly used solvents for the extraction of anthocyanins from fruits are the mixtures of ethanol, methanol, acetonitrile, and acetone compositions of water. Depending on the aim of the research, various types and different compositions of solvents can be implemented. For example, aqueous acetone solvents can be mostly preferred for a higher yield, an efficient and more reproducible extraction of anthocyanin [57, 58]. There is no universal and simplified sample preparation method to extract anthocyanins from fruit samples. However, a variety of modern techniques, including solid-phase extraction (SPE), accelerated solvent extraction (ASE), microwave-assisted extraction (MWE), ultrasound-assisted extraction (UAE), pressurized hot water extraction, and supercritical fluid extraction (SFC), have been developed based on maximizing the highest recovery, minimizing the amount of non-anthocyanins and degradation or alteration of the native anthocyanins [59].

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Cranberry, • Highbush blueberry, • Lowbush blueberry • Tifblue blueberry (2001)	Material: Freeze-dried berries. Extraction: Acetone, water, and acetic acid (70:29.5:0.5, v/v). Sonication for 5 min in a water bath at 50 °C. Dilution with 20% methanol/ water (v/v). Purification through Sephadex LH-20 column. Anthocyanin elution with 40 mL of 60% methanol/water (v/v).	Dp-3-galactoside, Dp-3-glucoside, Cy-3-galactoside, Dp-3-arabinoside, Cy-3-glucoside, Pt-3-galactoside, Pt-3-glucoside, Cy-3-arabinoside, Pn-3-galactoside, Pt-3-arabinoside, Mv-3-galactoside, Pn-3-glucoside, Mv-3-glucoside, Pn-3-arabinoside, Mv-3-arabinoside, Dp-6-acetyl-3-glucoside, Cy-6-acetyl-3-glucoside, Mv-6-acetyl-3-galactoside, Pt-6-acetyl-3-glucoside, Pn-6-acetyl-3-glucoside, Mv-6-acetyl-3-glucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Zorbax C18 column (150 x 4.6 mm) Solvent A; 5% formate acid B; 100% methanol. Flow Rate: 1.0 mL/min	[28]
• Evergreen blackberry (2002)	Material: Frozen berries. Powdered by liquid nitrogen. Extraction: Acetone (1:1, w/v). Treatment with chloroform (1:2, v/v). Purification through C18 Sep-Pak cartridges. Anthocyanin elution with methanol containing concentrated formic acid (95:5, v/v).	Cy-3-glucoside, Cy-3-rutinoside, Cy-xylose, Cy-3-glucoside acylated with malonic acid	HPLC/PDA/ESI-MS/MS Positive ion mode. Reverse phase. Prodigy C18 column (250 x 4.6 mm, 5 μm) Solvent A; 100% acetonitrile B; A mixture of 1% phosphoric acid (85%), 10% acetic acid (glacial), and 5% acetonitrile (v/v/v) in water. Flow Rate: 1.0 mL/min	[29]
• Red raspberry (2002)	Material: Fresh fruits. Macerated. Extraction: Methanol containing 0.1% HCl	Cy-3-sophoroside, Cy-3-(2 -glucosylrutinoside), Cy-3-glucoside, Pg-3-sophoroside, Cy-3-rutinoside, Pg-3-(2 -glucosylrutinoside), Pg-3-glucoside, Pg-3-rutinoside	HPLC/UV-Vis/APCI-MS/MS Positive ion mode. Reverse phase. Novapac C18 column (250 x 4.6 mm, 5 μm) Solvent A; 100% acetonitrile B; 1% formic acid. Flow Rate: 0.8 mL/min	[30]
• Linden arrowwood (2003)	Material: Squeezed fresh fruit juice. Purification through Sephadex LH-20 column (700mm 25 mm). Anthocyanin elution with H2O, 20% methanol containing 0.1 N HCl acid.	Cy 3-sambubioside, Cy-3-O-,-D-glucopyranoside (namely kuromanin)	HPLC/PDA/ESI-MS/MS Positive ion mode. Reverse phase. Capcell Pak C18 column (150 x 2.0 mm) Solvent A; 5% formic acid B; acetonitrile. Flow Rate: 1.0 mL/min	[31]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Black, Green, Red and White currants (2003)	Material: Frozen fresh berries. Extraction: Removal of other phenolics by ethyl acetate. Acidification of the residue with hydrochloric acid (2 M, 2 mL), and anthocyanins extraction with methanol.	Dp 3-O-glucoside, Dp 3-O-rutinoside, Cy 3-O-sophoroside, Cy 3-O-(2G-glucosylrutinoside), Cy 3-O-sambubioside, Cy 3-O-glucoside, Cy 3-O-(2G-xylosylrutinoside), Cy 3-O-rutinoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse phase. Purospher RP-18e column (125 x 3 mm, 5 μm) Solvent A; 5% formic acid B; acetonitrile. Flow Rate: 0.5 mL/min	[32]
• Bilberry, • Rab-biteye blueberry, • Black currant (2004)	Material: Frozen fruits. Extraction: 450 mL of 90% ethanol (0.1% H2SO4) at RT. Purification through a column of non-ionic polymeric absorbent (Amberlite XAD-7). Anthocyanin elution with aqueous ethanol (0.05% citric acid).	Dp 3-galactoside, Dp 3-glucoside, Dp 3-rutinoside, Cy 3-galactoside, Dp 3-arabinoside, Cy 3-glucoside, Pt 3-galactoside, Cy 3-rutinoside, Cy 3-arabinoside, Pt 3-glucoside, Pt 3-rutinoside, Pn 3-galactoside, Pt 3-arabinoside, Pn 3-glucoside, Mv 3-galactoside, Pn 3-rutinoside, Mv 3-arabinoside	HPLC/PDA/ESI-MS/MS Positive ion mode. Reverse phase. Capcell Pak C18 column (150 x 4.6 mm, 5 μm) Solvent A; 0.1% TFA/H_2O B; 50% CH_3CN/0.1% TFA/50% H2O. Flow Rate: 0.5 mL/min	[33]
• Red and White grape cultivars (2004)	Material: Frozen grape pomace. Lyophilized and finely grounded. Extraction: 100 mL of methanol/0.1% HCl (v/v) for 2 h.	Dp 3-O-glucoside, Cy 3-O-glucoside, Pt 3-O-glucoside, Pn 3-O-glucoside, Mv 3-O-glucoside, Dp 3-O-acetylglucoside, Pt 3-O-acetylglucoside, Pn 3-O-acetylglucoside, Mv 3-O-acetylglucoside, Cy 3-O-p-coumaroylglucoside, Pt 3-O-p-coumaroylglucoside, Pn 3-O-p-coumaroylglucoside, Mv 3-O-p-coumaroylglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse phase. Aqua C18 column (250 x 4.6 mm, 5 μm) Solvent A; water/formicacid/acetonitrile (87:10:3, v/v/v) B; water/formic acid/acetonitrile (40:10:50, v/v/v). Flow Rate: 0.8 mL/min	[34]
• Raspberry, • Arctic bramble, • Cloudberry, • Strawberry (2004)	Material: Frozen berries. Extraction: Ethyl acetate (410 mL), acidification of the berry residue with HCl (2 M, 2 mL), and anthocyanins extraction as flavylium cations with methanol.	Cy 3-hexoside-deoxyhexoside, Cy 3-sophoroside, Cy 3-(2G-glucosylrutinoside), Cy 3-glucoside, Cy 3-rutinoside, Cy glycoside, Pg 3-glucoside, Pg 3-rutinoside, Pg 3-malonylglucoside, Pg 3-succinylglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse phase. Purospher RP-18e column (125 x 3 mm, 5 μm) Solvent A; 5% formic acid B; acetonitrile. Flow Rate: 0.5 mL/min	[35]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Butcher's broom (2005)	Material: Frozen berries. Extraction: 0.1% HCl (v/v) in methanol for 20 h at RT, in the dark. Purification through C-18 Sep-Pak cartridge. Anthocyanin elution with methanol containing 0.01% HCl (v/v).	Pg-3-glucoside, Pg-3-rutinoside, Pg-3-cis-p-coumarylglucoside, Pg-3-trans-p-coumarylglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse phase. Wakosil C18 column (150 x 4.6 mm, 5 μm) Solvent A; 0.1% TFA in water B; 0.1% TFA in acetonitrile. Flow Rate: 1.0 mL/min	[36]
• Buckthorn (2005)	Material: Frozen berries. Extraction: 100 mL of 0.1% HCl (v/v) in methanol for 20 h at RT in the dark. Purification throug C-18 Sep-Pak cartridge. Anthocyanin elution with methanol containing 0.01% HCl (v/v).	Dp 3-rutinoside, Cy 3-rutinoside, Pt 3-rutinoside, Pg 3-rutinoside, Pn 3-rutinoside, Mv 3-rutinoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse phase. Wakosil C18 column (150 x 4.6 mm, 5 μm) Solvent A; 0.1% TFA in water B; 0.1% TFA in acetonitrile. Flow Rate: 1.0 mL/min	[37]
• Red Kiwi cultivars (2005)	Material: Fruit pericarp Extraction: 5 times volume (v/w) of ethanol/H2O/acetic acid (80:20:1 v/v/v) in an Ultra-Turrax homogenizer for 2 days at 4°C in the dark.	Cy 3-O-xylo (1–2)-galactoside, Cy 3-O-galactoside, Cy 3-O-glucoside, Cy 3-O-arabinoside	HPLC/PDA/ESI-MS/MS Positive ion mode. Reverse phase. LiChroCart Superpher 100 RP-18 end-capped column (250 x 2 mm) Solvent A; methanol/formic acid/water (v/v/v, 15:3.75:81.25) B; 100% methanol. Flow Rate: 0.25 mL/min	[38]
• Camu-camu (2005)	Material: Fruit peel. Freeze-dried. Extraction: 1000 mL of 0.5% HCl in methanol overnight at 5°C, in darkness. Purification through an Amberlite XAD-7 resin open column.	Dp-3-glucoside, Cy-3-glucoside	HPLC/Uv-Vis/ESI-MS/MS. Positive ion mode. Reverse Phase. RP-12 Synergi MaxRO column (250 x 4.6 mm, 5 μm) Solvent A; water/acetonitrile/ formic acid (87:3:10, v/v/v) B; water/ acetonitrile/formic acid (40:50:10, v/v/v). Flow Rate: 0.5 mL/min	[39]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Plum, • Black plum, • Nectarine, • Peach, • Marionberry (2005)	Material: Freeze-dried fruits. Powdered. Extraction: Methanol/water/acetic acid (85:15:0.5, v/v, MeOH/H2O/AcOH).	Cy 3-galactoside, Cy 3-xyloside, Cy 3-glucoside, Cy 3-(6″-acetoyl) glucoside, Cy 3-rutinoside, Cy 3-glucoside, Pg 3-glucoside, Cy 3-(maloyl) glucoside, Pn 3-glucoside, Cy 3-(6″-acetoyl) glucoside, Pn 3-rutinoside, Cy 3-(6″-malonoyl) glucoside, Cy 3-dioxaloylglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Zorbax Stablebond Analytical SB-C18 column (4.6 x 250 mm, 5 μm) Solvent A; aqueous 5% formic acid B; methanol. Flow Rate: 1 mL/min	[40]
• Lowbush blueberries (2006)	Material: Fresh fruits. Extraction by preparative techniques (data not shown).	Dp 3-O-galactoside, Dp 3-O-glucoside, Dp 3–O-arabinoside, Cy 3-O-galactoside, Cy 3-O-glucoside, Cy 3-O-arabinoside, Pt 3-O-galactoside, Pt 3-O-glucoside, Pt 3-O-arabinoside, Pn 3-O-galactoside, Pn 3-O-glucoside, Mv 3-O-galactoside, Mv 3-O-glucoside, Mv 3-O-arabinoside	HPLC/PDA/ESI-MS/MS Positive ion mode. Reverse Phase. Zorbax SB-C8 column (150 x 62.1 mm, 3.5 μm) Solvent A; aqueous 10% formic acid B; Acetonitrile. Flow Rate; 0.35 mL/min	[41]
• Wild blueberries (2007)	Material: Frozen berries. Grounded and homogenized. Extraction: Ethanol at 7.7°C, 26°C, or 79°C without acid (pH 5.4) or acidified with HCl (pH 4.1), citric acid (pH 4.9), tartaric acid (pH 5.0), lactic acid (pH 4.8), or phosphoric acid (pH 4.6; 0.02%, v/v).	Dp 3-galactoside, Dp 3-glucoside, Dp 3-hexose, Cy 3-galactoside, Pt 3-galactoside, Mv 3-galactoside, Dp 3-arabinoside, Pt 3- glucoside, Cy 3-glucoside, Mv 3- glucoside, Pt hexose, Cy 3-arabinoside, Pn 3-galactoside, Mv 3-hexose, Pn 3-glucoside, Mv hexose, Pt propionyl-hexose, Cy malonyl-hexose, Mv 3-arabinose, Dp -oxalyl-hexose, Cy 3-propionyl-galactoside, Cy -malonyl-hexose, Cy 3-malonyl-galactoside, Pt -malonyl-pentose, Cy malyl-pentose, Dp succinyl-pentose, Pt -propionyl-hexose, Pt 3-propionyl-glucoside/-hexose, Pt 3-acetyl-glucoside, Pn 3-succinyl-arabinoside, Pn 3-oxalyl-galactoside, Mv 3-acetyl-hexose, Mv oxalyl-pentose, Mv 3-acetyl-glucoside, Mv 3-acetyl-galactoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Zorbax Stablebond Analytical SB-C18 column (4.6 x 250 mm, 5 μm) Solvent A; aqueous 5% formic acid B; methanol. Flow Rate; 1 mL/min	[42]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Lycium ruthenicum fruit (2015)	Material: Fresh fruits. Extraction: 70% ethanol for 2 h at pH 2.5 (adjusted with HCl). Extraction: Purification through AB-8 macroporous resin. Anthocyanin elution with 70% ethanol.	Pt-3-O-rutinoside-5-O-glucoside, Mv-3-O-rutinoside-5-O-glucoside, Dp-3-O-rutinoside(glucosyl-trans-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(glucosyl-trans-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(glucosyl-cis-p-coumaroyl)-5-O-glucoside, Mv-3-O-rutinoside(glucosyl-trans-p-coumaroyl)-5-O-glucoside, Dp-3-O-rutinoside(cis-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(trans-caffeoyl)-5-O-glucoside, Mv-3-O-rutinoside(glucosyl-cis-p-coumaroyl)-5-O-glucoside, Dp-3-O-rutinoside(trans-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(cis-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(trans-p-coumaroyl)-5-O-glucoside, Pt-3-O-rutinoside(feruloyl)-5-O-glucoside, Mv-3-O-rutinoside(cis-p-coumaroyl)-5-O-glucoside, Mv-3-O-rutinoside(trans-p-coumaroyl)-5-O-glucoside, Malvidin-3-rutinoside(feruloyl)-5-O-glucoside	HPLC/DAD/ESI-QTOF-MS/MS Positive ion mode. Reverse Phase. XUnion C8 column (4.6 x 150 mm, 5 μm) Solvent A; acetonitrile B; 0.5% (v/v) formic acid/water. Flow Rate; 0.2 mL/min	[43]
• Indian blackberry (2015)	Material: Fresh fruit. Pulp filtration through a sieve. Extraction: Acidified (0.05% HCl) aqueous methanol (80% methanol) using an ultra sonicator. Liquid-liquid partitioning with n-hexane, chloroform, ethyl acetate, n-butanol and aqueous methanol.	Cy-3-glucoside, Cy-3,5-diglucoside, Cy-3-diglucoside-5-glucoside, Cy-3-(p-hydroxybenzoyl) (oxaloyl) diglucoside-5-glucoside, Cy-3-(sinapoyl) glucoside-5-glucoside, Dp-3-glucoside, Dp-3,5-diglucoside, Dp acetyl-diglucoside, Mv-3-glucoside, Mv-3,5-diglucoside, Mv-3-(6″-acetoyl)glucoside, Mv-3-(6″-acetoyl) glucoside-5-glucoside, Pn-3-glucoside, Pn-3,5-diglucoside, Pt-3-glucoside, Pt-3,5-diglucoside	HPLC/PDA/MALDI-ESI-MS/MS Positive ion mode. Reverse Phase. Phenomenx C18 colum (4.6 x 250 mm, 5 μm) Solvent A; 5% HCOOH in water B; 100% acetonitrile. Flow Rate; 1 mL/min	[44]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Highbush blueberries, • Murta, • Calafate, • Arrayán, • Chequén, • Meli (2015)	Material: Fresh fruits. Homogenized in a blender and freeze-dried and pulverized. Defatted thrice with 100 ml of n-hexane. Extraction: 100 ml of 0.1% HCl in MeOH in the dark in an ultra-sonic bath for one hour. Purification through XAD-7 (100 g) column. Anthocyanin elution with 100 ml of MeOH acidified with 0.1% HCl.	Pt-3-O-di-hexoside, Cy-3-O-di-hexoside, Dp 3-O-galactoside, Dp 3-O-glucoside, Cy 3-O-rutinose, Cy-3-O-galactoside, Cy-3-O-glucoside, Pt-3-O-rutinoside, Mv-3-O-rutinose, Pt-3-O-glucoside, Pt-3-O-galactoside, Pn-3-O-rutinose, Pn-3-O-galactoside, Mv-3-O-galactoside, Dp-3-O-arabinoside, Pn-3-O-glucoside, Mv-3-O-glucoside, Pn-3-O-arabinoside, Pt-3-O-arabinoside, Cy-3-O-arabinoside, Mv 3-O-(6″coumaroyl) glucoside, Mv-3-O-arabinose, Cy-3-O-(6″succinoyl)-glucose, Pn 3-O-di hexoside, Dp-3-O-rutinose (6″-p-coumaroyl)-2″-O-glucose, Dp 3-O-(6″acetyl) glucoside, Cy 3-O-(6″acetyl) glucoside, Pt 3-O-(6″acetyl) glucoside, Mv 3-O-(6″acetyl) galactoside, Dp-3-O-(6″caffeoyl)-glucose, Mv 3-O-(6″acetyl) glucoside	HPLC/PDA/ESI-TOF-MS/MS Positive ion mode. Reverse Phase. Luna C-18 column (4.6 x 250 mm, 5 μm) Solvent A; 0.1% aqueous formic acid B; acetonitrile 0.1% formic acid. Flow Rate; 0.5 mL/min	[45]
• Pomegranate cultivars (2016)	Material: Fresh fruits. Pressurized to make juice. Extraction: 5 mL of MeOH/water (80:20 v/v) containing 2 mM NaF and then centrifuged at 15,000g for 15 min.	Dp 3,5-O-diglucoside, Cy 3,5-O-diglucoside, Dp 3-O-diglucoside, Cy 3-O-diglucoside, Pg 3-O-diglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. XRs 5 C18 column (250 x 4.6 mm, 5 μm) Solvent A; water-formic acid (95:5, v/v) B; acetonitrile. Flow Rate; 0.8 mL/min.	[46]
• Chokeberry (2016)	Material: Fresh fruits and pomace. Powdered. Extraction: 50 mL of methanol acidified with 2.0% formic acid. Sonication for 20 min with occasional shaking. Centrifugation at 19,000×g for 10 min.	Cy-3-hexoside-(epi) catechin, Cy-3-pentoside-(epi) catechin, Cy-3-hexoside-(epi) cat-(epi) ca, Cy-3-O-galactoside, Cy-3-O-glucoside, Cy-3-O-arabinoside, Cy-3-O-xyloside	UHPLC/PDA/ESI-MS/MS Positive ion mode. Reverse Phase. BEH C18 column (2.1 x 100 mm, 1.7 μm) Solvent A; 2.0% formic acid, v/v) B; 100% acetonitrile. Flow Rate; 0.45 mL/min	[47]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Myrtus Berries (2017)	Material: Fresh berries. Extraction: 50 mL of 0.1% HCl (v/v) in methanol for 24 h in the dark.	Dp 3-O-glucoside, Pt 3-O-glucoside, Mv 3-O-glucoside Dp-pentose, Dp-pentose, Cy 3-O-glucoside, Pt-pentose. Pn 3-O-glucoside	HPLC/DAD/ESI-QTOF-MS/MS Positive ion mode. Reverse Phase. Gemini-NX C18 column (250 x 4.6 mm, 5 ɥm) Solvent A; 0.1% formic acid in water B; 0.1% formic acid in acetonitrile. Flow Rate; 0.3 mL/min	[48]
• Mulberry (2017)	Material: Fresh fruits. Extraction: Acetone-water-acetic acid (75:14.9:0.1, v/v/v), through homogenization (900Kr/min, 10min) and centrifugation (10,000 rpm, 10min). Purification by a polyamide resin column (4×100 cm). Anthocyanin elution with 10%, 30%, 50%, or 80% (v/v) ethanol solutions containing 0.1% HCl.	Cy 3-sophoroside, Cy 3,5-diglucoside, Pn 3-rutinoside Cy 3-laminaribioside, Pt 3-arabinoside, Dp 3-rutinoside, Cy 3-O-(diglucoside)-glucosylrutinoside, Dp 3-galactoside, Cy 3-glucoside, Cy 3-rutinoside, Dp 3,5-diglucoside, Dp 3-rutinoside-5-glucoside, Cy 3-(glucosyl) rhamnoside, Pg 3-glucoside, Cy 3-galactoside, Pg 3-rutinoside	UHPLC/DAD/HR-ESI-TOF-MS/MS Positive ion mode. Reverse Phase. ZORBAX SB-C18 column (2.1 x 50 mm, 1.8 ɥm) Solvent A; 0.1% (v/v) formic acid in water B; acetonitrile. Flow Rate; 0.2 mL/min	[49]
• Nitraria tangutorun fruit (2017)	Material: Dried fruits. Grounded into powder with liquid nitrogen in a grinder Extraction: 15 mL of aqueous ethanol (0.1% HCl, v/v). Purification through a column (16 mm × 50 mm) contained X-5 resin (Bed volume of 60 mL). Anthocyanin elution with 120 mL of ethanol–water–HCl (80:19:1, v/v/v; pH 1) at a flow rate of 2 mL/min for 1 h.	Mv-3-O-diglucoside-5-O-arabinose, Cy-3-O-diglucoside, Cy-3-O-galactoside, Pg-3-O-diglucoside, Pn-3-O-diglucoside, Cy-3-O-(caffeoyl)-diglucoside, Dp-3-O-(p-coumaroyl)-diglucoside, Cy-3-O-(cis-p-coumaroyl)-diglucoside, Cy-3-O-(feruloyl)-diglucoside, Cy-3-O-(trans-p-coumaroyl)-diglucoside, Pg-3-O-(p-coumaroyl)-diglucoside, Dp-3-O-(p-coumaroyl)-hexose, Cy-3-O-(p-coumaroyl)-diglucoside, Cy-3-O-(p-coumaroyl)-hexose	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Hedera ODS-2 column (150 x 2.1 mm, 3 µm) Solvent A; aqueous formic acid (0.1%, v/v) B; acetonitrile. Flow Rate; 0.2 mL/min	[50]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Muscadine grape (2017)	Material: Freeze-dried berries. Grounded into powder. Extraction: 50 mL 80:20 (v/v) methanol–water solution containing 0.1 mL/L HCl. Shaking of the extract in water-bath at 30 C in the dark for 16 h. Sonication for 15 min, and centrifugation at 2,000g for 10 min. Purification through C18 Solid phase extraction cartridge. Anthocyanin elution with 10 mL methanol.	Dp-3,5-diglucoside, Cy-3,5-diglucoside, Pt-3,5-diclucoside, Mv-3,5-diglucoside, Pn-3,5-diglucoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Ultrasphere Cl8 column (250 x 4.6 mm) Solvent A; O-phosphoric acid/methanol/ water (5:10:85, v/v/v) B; acetonitrile. Flow Rate; 0.5 mL/min	[51]
• Chinese dwarf cherry (2019)	Material: Freeze-dried berries. Pulverized in liquid nitrogen. Extraction: 2 mL of 2% formic acid methanol (v/v). Sonication for 20 min, and centrifugation at 10,000g for 10 min.	Cy 3-O-glucoside, Cy 3-O-rhamnosyl-hexoside, Pg 3-O-hexoside, Pg 3-O-rhamnosyl-hexoside, Cy 3-O-acetyl-hexoside, Pg 3-O-acetyl-hexoside	HPLC/PDA/ESI-QTOF-MS/MS Positive ion mode. Reverse Phase. ODS-80Ts QA C18 column (250 x 4.6 mm) Solvent A; 10% formic acid in water (v/v) B; 1% formic acid in acetonitrile (v/v). Flow Rate; 0.8 mL/min	[52]
• Red-fleshed apple fruit (2020)	Material: Fresh fruits. Grounded into a fine powder in liquid nitrogen. Extraction: 60 mL chilled 80% methanol (1:6, w/v) at 4°C for 4 hr.	Cy-3-galactoside, Cy-3-arabinoside	UHPLC/ESI-MS/MS Positive ion mode. Reverse Phase. Acquity BEH C18 column (100 x 2.1 mm, 1.7 μm) Solvent A; 0.1% formic acid (v/v) B; acetonitrile. Flow Rate; 0.3 mL/min	[53]
• Goji berry (2021)	Material: Frozen and homogenized fruits. Treatment with solid NaF (2 mM) to inactivate polyphenol oxidases and prevent phenolic degradation. Extraction: Methanol, acetone or water with hydrochloric, acetic, citric and ascorbic acid for 30 min, at 25 °C. Sonication for 30 min. Centrifugation for 15 min at 3000 rpm at RT.	Pt-3-O-(glucosyl-trans-p- coumaroyl)-rutinoside-5-O-glucoside, Pt-3-O-(glucosyl-cis-p-coumaroyl)-rutinoside-5-O-glucoside, Pt-3-O-(caffeoyl)-rutinoside-5-O-glucoside, Pt-3-O-(trans-p-coumaroyl)-rutinoside-5-O-glucoside,Mv-3-O-(p-coumaroyl)-rutinoside-5-O-glucoside, Pt-3-O-(p-coumaroyl)-rutinoside	HPLC/DAD/ESI-MS/MS Positive ion mode. Reverse Phase. Supelco C18 column (250 x 4.6 mm, 5 qm) Solvent A; water-formic acid (2%, v/v) B; acetonitrile. Flow Rate; 0.4 mL/min	[54]

Fruit types (Year)	Sample treatment	Anthocyanin type	HPLC/MS features, stationary and mobile phases	Ref. #
• Northern highbush blueberry cultivars (2022)	Material: Frozen fresh berries. Grounded in liquid nitrogen. Extraction: 1.25 mL of 80 % methanol containing 0.1 % of formic acid (v/v) for 15 min at RT. Centrifugation for 10 min at 3500 rpm, RT. The pellet re-extracted by shaking with 1.25 mL of 65 % acetone containing 0.1 % of formic acid (v/v) for 30 min at RT. Purification: Removal of sugars and proteins by solid-phase extraction (SPE). Anthocyanin elution with 2.5 mL of water containing 2 % formic acid (v/v), and 2.5 mL of methanol containing 0.1 % formic acid (v/v).	Dp-3-O-galactoside, Dp-3-O- glucoside, Cy-3-O-glucoside, Pt-3-O-glucoside, Pn-3-O-glucoside, Mv-3-O-galactoside, Mv-3-O-glucoside	HPLC/DAD/ESI-QTOF-MS/MS Positive ion mode. Reverse Phase. Zorbax SB-C18 column (50 x 4.6 mm) Solvent A; water with 2 % formic acid (v/v), B; acetonitrile with 0.1 % formic acid (v/ v)	[55]
• Mulberry (2022)	Material: Fresh berries. Freeze-dried and fine powdered. Extraction: acidified 95% ethanol (0.1% trifluoroacetic acid v/v). The concentrated extract was dissolved in the acidified methanol (7% acetic acid v/v) to stabilize the anthocyanins, converting anthocyanins to flavylium cations. Purification: 001X7 cation-exchange column. Anthcoyanin elution with a mixture of methanol/NaCl solution (1 M) 1:1 (v/v)	Cy-3-*O*-glucoside and Cy-3-*O*-rutinoside	HPLC-/DAD/ESI-MS Positive ion mode. Reverse Phase. Acclaim TM C18 column (4.6 mm × 250 mm, 5 μm) Solvent A; formic acid and water (1:100 v/v), B; acetonitrile. Flow rate; 0.2 mL/min	[56]

Table 2.
Fruit anthocyanin examples annotated or identified by HPLC-MS/MS-based profiling. Cy, cyanidine; Dp, delphinidin; Pg, pelargonidin; mv, malvidin; Pn, peonidin; Pt, petunidin.

Because of the structural diversity of anthocyanins and their instability in light, high temperature and other conditions, it is necessary to avoid degradation during the sample preparation. In aqueous solutions, anthocyanins exist in four major forms, including the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone depending on pH. At pH below 2, anthocyanins are found primarily in the form of the red flavylium cation. Hydration of the flavylium cation gives the colorless carbinol pseudobase at pH values from 3 to 6, and the colorless chalcone pseudobase at pH values higher than 6. Since the flavylium cation form of anthocyanins is stable in a highly acidic medium, extraction solvents are required to be acidified by acetic, formic, hydrochloric, or sulfuric acids to prevent the degradation of the non-acylated anthocyanin pigments. However, the excessive acidic condition may have various effects during the extractions such as degradation or partial hydrolysis of the acylated anthocyanins as well. Solvents acidified with 0.1% hydrochloric acid (HCl, v/v) is the most commonly used for the extraction of anthocyanins from fruit samples [57, 58]. Anthocyanins can undergo a structural transformation during the sample treatment under a high temperature. When the temperature is increased, anthocyanins can turn into unstable chalcone formation, and even further degrade to brown products, giving a reduction in the concentration of the major anthocyanins. Therefore, using a lower temperature can improve the stability of anthocyanins during the sample treatment [58]. Temperature values between 30 and 50°C have been mostly used for the extraction of anthocyanins from fruit samples. Light is also destructive to anthocyanins. To minimize the degradation of anthocyanins, it is advisable to perform sample treatment and storage of extracts in darkness [58, 60]. An investigation provided parameters of the sample preparation based on the objectives of the study [61].

Due to the diversity and complexity of plant secondary metabolites, extractions methods for anthocyanins are nonselective and result in solutions with a lot of undesirable substances, such as sugars, proteins, fats, acids, and other water-soluble compounds. Based on this, a further efficient purification and separation method is normally required to remove other substances and elute anthocyanins from the extracts. These processes can normally lead to the loss of minor anthocyanin components, which result from heat, pH, metal complexes, and copigmentation [8, 62]. Undesired components, such as sugars, acids, and other water-soluble compounds, in the crude fruit extracts have been removed with C18 solid-phase extraction (SPE) cartridges containing octadecyl silica and Sephadex LH-20 containing cross-linked dextran resin. In order to purify anthocyanins by adsorption, silicone gels, such as Amberlite IRC 80, Amberlite IR 120, DOWEX50WX8, Amberlite XAD-4, and Amberlite XAD-7HP, have been used. Among the silicone gels, the Amberlite XAD-7HP has been proven to be the most effective resin for anthocyanin purification. Furthermore, other less polar polyphenolics or nonpolar compounds can be removed from the extracts by washing with ethyl acetate, chloroform, butanol, or acetonitrile in acidic conditions. After removal of other undesirable substances, anthocyanin fraction can be eluted with organic solvents, acidified with formic or hydrochloric acids (0.1%, v/v), containing water, ethanol, methanol or their composition in different ratios [57, 59, 63].

Separation of anthocyanins can be carried out by different chromatography techniques that have been developed in the past. Common methods include column chromatography, (CC), counter-current chromatography (CCC), paper chromatography (PC), thin-layer chromatography (TLC), capillary electrophoresis (CE), and high-performance liquid chromatography (HPLC) [61, 62].

A series of methods have also been developed for the characterization of anthocyanin structures. In brief, these methods include UV-visible spectrophotometry (UV-vis), HPLC facilitated with diode array detection or UV-detection, mass spectrometry (MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance (NMR) [64, 65]. Since anthocyanins have a specific absorption in the visible wavelengths from 515 to 540 nm, spectrometry has been the main approach for quantification. Unfortunately, this is a measurement of total anthocyanins because it cannot measure specific components in crude extracts [26]. MS and MS/MS technologies are powerful in fragmenting anthocyanin molecules to generate featured fingerprints, which allow the annotation of unknown or known structures. NMR is a powerful technology for assigning hydrogen and carbon. There are different NMR tests such as homo and heteronuclear 2D and 3D techniques [66, 67].

2.1 Chromatographic separation of anthocyanins

Chromatography is a separation technique for the isolation of different compounds in a particular matrix. This type of technique is composed of a stationary phase formed from different materials, and a mobile phase (solvent). The separation of compounds of interest is based on their affinities for the stationary phase. The stationary phase retains the desired compounds, while the mobile phase elutes or migrates undesired substances. Based on the stationary phases, common chromatography techniques include column, paper, and thin-layer chromatography. Based on the mobile phase, the most used technique is liquid chromatography utilizing the physical and chemical features of analytes [68].

2.1.1 Column chromatography (CC) and counter-current chromatography (CCC)

Column chromatography (CC) is an effective method to fractionate and purify anthocyanins. This separation method is based on the different distribution coefficients of anthocyanins in solid and mobile phases. Common materials used for the solid phase packed in the column include macroporous, polyamide, and sephadex resins, which do not contain ion exchange groups. Macroporous resins (MRs) have multiple advantages, such as fast, stronger, and large capacity of adsorption and desorption potential for anthocyanin purification. MRs are useful for the first step of isolation to obtain fractions. Polyamide and sephadex resins are normally used to separate anthocyanins. To date, although the CC is a favorable method for anthocyanin purification in the laboratory, it is a challenge for scale-up purification [62, 69].

Counter-current chromatography (CCC) has an industry-scale technology for the separation and purification of bioactive anthocyanins from a large amount of plant materials. CC is a support-free liquid-liquid chromatography. Its development is based on the fractionation of compounds between immiscible stationary and mobile liquid phases of a biphasic solvent system. In the separation of active anthocyanins, important factors that need to be considered are acidic solvents in the absence of oxygen, linear elution or gradient elution, pH zone refining, and strong ion exchange. The main drawback is that certain organic solvents used are toxic to human health [63, 70].

2.1.2 Paper chromatography (PC) and thin layer chromatography (TLC)

Paper and thin layer chromatography are two simple techniques used for the separation of anthocyanins. PC was one of the earliest methods. It depends on specific

samples, different mobile phases, and papers used. The advantage of PC is simple and fast to examine anthocyanins. The disadvantage of PC is the limited capacity to separate scale-up samples and the separation of metabolites is relatively poor. TLC uses silica or cellulose gel or both. The separation capacity depends on silica and cellulose size as well as developing solvents. In comparison, TLC can overcome poor separation problems occurred in PC. To date, these two methods are used in common for anthocyanin research in the laboratory because of simple, fast, and low-cost advantages [6, 65].

2.1.3 Capillary electrophoresis (CE)

Analysis of anthocyanins is difficult because they can undergo structural degradation under alkaline pH, light, and high temperature. Therefore, it is required to perform analytical methods as rapidly as possible for preventing their degradation and to avoid using more solvents during analysis. For this reason, CE is a promising separation technique due to being more rapid than other techniques and using only minor amounts of solvents [71]. CE is a separation method based on the electrophoretic motility of metabolites. This technique has excellent mass sensitivity, high resolution, low sample requirement, and decreased solvent waste. When a sample is introduced into the capillary tube at the anode, the basic or acidic mobile phase migrates some components of the sample toward the cathode while others are stuck at the anode. Because anthocyanins are not stable in basic solvents, acidic solvents are used to maintain protonated the flavylium cation form. In addition, CE is configured from cathode to anode. Based on its charge-to-size ratio, particular anthocyanins or other compounds are migrated in the CE system. The migration time of compounds with higher charge-to-size ratio takes a longer time. Detection of compounds is achieved by the UV-vis spectrophotometry coupled to CE, which records the spectra from 200 to 599 nm for each peak [72–74].

The separation of anthocyanins by the CE method includes the use of fused-silica capillaries non-coated or coated with Bare or poly-LA 313 (polycationic amine-containing polymer). Non-coated capillaries are rarely used because anthocyanins cannot be excluded due to interactions between negatively charged silica surfaces and positively charged anthocyanins. Therefore, the coated capillaries are the most suitable for anthocyanin separation. Several background electrolyte (BGE) buffers, such as ammonium formate, ammonium acetate, borate, acetic acid, formic acid, and mixtures of formic acid and hydrochloric acid, are applied both basic and acidic. In order to get highly efficient separations, MS-compatible volatile BGE buffers are used. Non-volatile borate or phosphate buffers can be also applied but these buffers are not compatible enough with MS. Furthermore, the alkaline pH of borate buffers could cause the degradation of anthocyanins. However, an acidic BGE buffer helps to prevent anthocyanin degradation [71, 75].

2.1.4 High-performance liquid chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a separation technique where the mobile phase is pressurized so that it can flow through the column much more efficiently. HPLC is the most convenient for components that cannot withstand high temperatures. Thus, HPLC is widely used for the qualitative and quantitative analysis of anthocyanins. There are two main types of columns of HPLC depending on the aim of the study, including analytical columns for analysis and preparative

columns for isolating and refining specific compounds from samples. HPLC columns are packed with inert materials to form the stationary phase and vary in length and internal diameter. Analytical and preparative columns are normally designed for microgram-scale and milligram-to-gram-scale separation of compounds, and adjusted to the characteristics of each analyte. HPLC utilizes different separation modes depending on the primary characteristics of compounds such as polarity and electrical charge [6, 68, 76].

Depending on polarity, there are two types of HPLC separation modes, normal-phase mode (NP) and reverse-phase mode (RP). The basic principle of these separation modes is that compounds with similar polarity will show much more attraction to each other. Furthermore, the separation result and accuracy will be depending on the retention time and the speed flow rate, respectively. The normal phase system consists of the non-polar mobile phase and polar stationary phase. When the sample enters the column, the metabolites (polar) with similar polarity to the stationary phase are retained, resulting in longer retention time while other metabolites (non-polar) with similar polarity to the mobile phase move along the column with shorter retention time. The retention time differences allow appropriate separation of anthocyanins and other metabolites. Past studies have reported that the NP system is effective only for the separation of proanthocyanidins but not for anthocyanins due to retaining in the NP's polar stationary phase. By contrast, the reverse-phase system uses a polar mobile phase and nonpolar stationary phase. Therefore, RP chromatography is effective for the separation of anthocyanins due to having a similar polarity with the mobile phase. In RP chromatography, compounds with higher polarity elutes earlier than non-polar compounds. For flavonoid analysis, there are different RP separation phases using C8, C12, C18, phenyl or phenyl-hexyl, pentafluorophenyl (PFP), and polar and polymeric embedded RP columns. In general, C8 and C18 columns, which are filled with particles of silica bonded with alkyl chains, have been used to separate anthocyanins in RP chromatography. The majority of anthocyanin separations in fruits have been performed using C18 columns with the column particle size mainly ranging from 1.7 μm to 5.0 μm [68, 76].

Multiple organic solvents have been used as mobile phases to elute anthocyanins from the columns. Commonly used ones include methanol, acetonitrile, isopropanol, or ethanol, which are mixed with water supplemented with acetic acid, formic acid, ammonium acetate, or trifluoroacetic acid to form aqueous/organic elution solvents. In many separation experiments, organic solvents and acidic water are used to develop a gradient binary solvent system for the ideal separation of different structures. Furthermore, the acidic water or solvents allow for maintaining the flavylium cationic species, thus increasing chromatographic performances [61, 76].

RP chromatography is effective for the separation of anthocyanins and anthocyanidin aglycones. This is associated with the solubility of the compounds in the mobile phase solvents. In general, an optimized gradient solvent system such as acetonitrile-water or methanol-water solutions supplemented with 0.11% acetic acid or formic acid are appropriate to elute anthocyanins. The elution order of anthocyanins through RP chromatography is normally a function of the number of hydroxyl groups and their degree of methoxylation. A general rule is that diglycosylated or more glycosylated anthocyanins are eluted earlier in the column, followed by monoglycosylated anthocyanins (in an order of galactosides, glucosides, arabinosides, xylosides, and rhamnosides) and aglycones [76]. For example, the elution order of anthocyanins in grapes follows a trend; delphinidin < cyanidin < pelargonidin < peonidin < malvidin, along with the number of glucosides and their acylation pattern

following the order; diglucosylated < monoglucosylated < monoglucosylated–acetic acid < diglucosylated-coumaric acid < monoglucosylated-caffeic acid < monoglucosylated-coumaric acid [57].

Another separation mode being applied in the HPLC is hydrophilic interaction in liquid chromatography (HILIC). The development of this technique is based on polarity and hydrophilicity. HILIC uses a combination of NP's polar stationary phase, RP's aqueous-organic mobile phase, and the net surface charge of compounds (ion exchange), thus is a better separating method for small polar compounds. In HILIC, stationary phases usually consist of polymer-based, bare silica, or modified silica gels (Accucore HILIC and Acclaim HILIC 10). For the mobile phase, high organic water-miscible polar organic solvents and acetonitrile are used, giving a better polar component separation and an optimal retention time. When samples enter the column and move along the stationary phase, an interaction between water and silica will occur while acetonitrile will form layers above, giving a gradient of mobile phase, and retention caused by partitioning. More hydrophilic molecules retain more in the stationary phase. The preference of HILIC for polar metabolites has allowed its application to separate highly polar anthocyanins that cannot be separated by RP. To date, a column packed with ethylene bridged hybrid (BEH) amide (2.5 μm particle size) has been shown to provide efficient separation of diverse structures of glycosylated and acylated anthocyanins in fruits. However, HILIC may not be appropriate for the separation of isomeric anthocyanidin-hexosides and cis/trans acylated anthocyanin isomers [61, 68, 77, 78].

The polarity of anthocyanins can be reduced by acylation occurring at –OH or carbon on A, B, and C rings. Common acylation groups include malic, acetic, malonic, succinic, gallic, protocatechuic, hydroxybenzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, and synaptic acids. Acylated anthocyanins are usually eluted later than glycosylated ones. In addition, the anthocyanidin elution order of RP mode follows this trend; delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin. This is in contrast with the elution order reported from HILIC (hydrophilic interaction in liquid chromatography); malvidin, followed by peonidin, petunidin, cyanidin, and lastly delphinidin. Therefore, a combination of HILIC and RP-LC separation modes is useful for the comprehensive 2-dimensional liquid chromatographic (LC × LC) analysis of anthocyanins [76, 78, 79].

Because of the structural complexity of anthocyanin content, the polarities of different anthocyanin subgroups may yield peaks overlapped, causing unresolved chromatographic peaks. For instance, for a given aglycone base, the molecular masses for the 3,5-diglucoside and the caffeoyl glucoside are identical, resulting in a limitation for precise identification. Furthermore, anthocyanins are not easy to effectively separate from copigments such as phenolic acid and flavanols due to their similar structure as well. To alleviate these limitations associated with conventional C18 reversed-phase methods, an ion-exchange mode has been applied for anthocyanin separation. The stationary phase of ion-exchange columns consists of anion-exchange (AV-17-8, AV-17-2P, and EDE-10P) and cation-exchange (KU-2-8, Primesep B2, SCX, and 001X7) resins, which are having polar fixed groups. While the anion-exchange resins are positively charged, the cation-exchange resins have a negative charge. Depending on the net surface charge of analyte's, the cation-exchange and anion-exchange stationary phases bind with negatively and positively charged compounds, respectively. In the case of the anion-exchange resins, the adsorption capacity increases, while pH of external solutions raises from acidic to neutral and alkaline values. However, anthocyanins can undergo partial degradation and lose their

biological functions in alkaline solutions. Therefore, to ensure maximum adsorption and separation, cation-exchange resins are mostly preferred for anthocyanin separation. Depending on the pH of environment, anthocyanins alternate between the cationic flavylium ion and the neutrally charged carbinol or quinoidal forms. In highly acidic conditions, anthocyanins would convert to positively charged flavylium cations because of the hydroxyl group in the 3-position, resulting in retarding on the negatively charged cation-exchange resins through the ionic interaction, and flushing other phenolic compounds that are not likely adsorbed by the resin. The mobile phase of ion-exchange mode is a solution that has counterions in general [56, 80, 81].

The separation mechanism for the ion-exchange is a mode based on the net charge contained in samples and their pH. It starts with an application of the counterion mobile phase including ion charges (Na^+ and Cl^-). After loading of the sample, molecules with different net charges from the stationary phase bind to the resin while other unbound molecules are washed out by increasing the concentration of counterions or pH value of the mobile phase. For example, a cation exchange chromatography with 001X7 resin has been developed for copigments-free anthocyanins isolation both on a small and large scale from mulberry extracts. In this study, acidifed anthocyanin fractions were eluted with a mixture of methanol/NaCl solution. Cation-exchange 001X7 resin has been reported to be more advantageous with more than 95% purity compared to the macroporous adsorbent and strong cation exchange resins for the purification of anthocyanins [56, 81].

A novel separation mode combining both ion-exchange and reversed-phase separation mechanisms has been also developed, called mixed-mode ion-exchange reversed-phase chromatography. For example, for the mixed-mode separation method, Primesep B2 columns with embedded basic ion-pairing groups have been used for grapes, giving a significant improvement for chromatographic separation, purification, and detection of anthocyanidin diglucosides and acylated anthocyanins. However, the identification of anthocyanins is hard to predict by comparing with previously published data, because the ion-exchange elution mechanism significantly affects the retention orders of anthocyanins [56, 80].

The results from HPLC not only depend on column and separation mode but also on instruments and conditions used. Although instruments from different companies are comparable, the separation efficiency is always different among instruments. Once a new instrument is set up, it is better to optimize conditions and protocols for anthocyanins or other compounds. As such, results from the same instrument are highly reproducible. The detector can also provide informative characteristics of metabolites [82].

2.2 Detection, annotation, and identification

Ultraviolent (UV) and visible (Vis) detectors are commonly used in HPLC. The detectors measure the absorbance intensity of UV and Vis spectra between the 190 and 900 nanometer (nm) wavelengths. There are two types of UV-vis detectors, including tunable and photodiode array (PDA), also known as diode array (DAD) detectors. The tunable UV-vis detectors can measure the maximum absorption of each analyte of a sample at one or more discrete wavelengths during the analysis. PDA detectors can measure the absorbance of each analyte at the entire wavelength range or a fixed wavelength in real time (at intervals of 1 second or less) during separation by HPLC with continuous eluate delivery [83]. The use of a detector is dependent on the metabolites analyzed. To detect and/or identify anthocyanins, detectors mostly

coupled with HPLC include UV-vis, /DAD, and PDA. These detector systems allow a few to full spectrophotometric scans on each peak as it elutes and provides a unique chromatogram for each anthocyanin that is used to compare with others for identification aims [6, 57]. In an acidic condition, the flavylium cation can maintain its red color and have absorption at a maximum between 510 and 545 nm (depending on the number of hydroxylated carbon atoms on the B-ring). The unique and maximum absorption wavelengths allow for distinguishing anthocyanins from other flavonoids for identification and quantification [6, 72]. PDA detector provides a spectral profile that can assist in detecting unknown peaks in the chromatograms, and provides characteristic spectra that give information about acylation and glycosylation patterns of anthocyanins [82, 84–86]. However, these detectors cannot distinguish anthocyanins with similar retention times and similar spectral characteristics. Also, the identification of anthocyanins with UV-vis dectors requires authentic standards, many of which are not commercially available [31, 82, 84, 87, 88].

Mass spectrometry (MS), an analytical technique, is used to measure the mass-to-charge ratio of ions. Mass spectrometry (MS) is completed on the mass spectrometer, which has MS detector. A mass spectrometer ionizes molecules and ionized molecules are sent to the mass analyzer, which is an electromagnetic field sorting and separating ions according to their mass and charge. Then, the mass detector detects and measures separated ions, and the results are displayed on a chart. HPLC coupled with a mass spectrometer has been used to effectively analyze anthocyanins (**Table 2**) and other plant secondary metabolites. Multiple accomplishments have been made to understand anthocyanins during fruit development [61, 89, 90]. In particular, HPLC-MS/MS or ultra-performance liquid chromatography (UPLC)-MS/MS is powerful to annotate unknown anthocyanins or identify known anthocyanins in fruits [61, 91]. These successes enhanced the understanding of anthocyanin biosynthesis and structures in fruits and other plant samples.

2.2.1 Electrospray ionization (ESI) mass spectrometry (MS): ESI-MS

The analysis of specific types of individual compounds by HPLC-MS requires an appropriate ionization interface between the physical coupling of LC and MS. Detection and quantification of an individual compound in MS-based measurements is determined by the level of ionization that generates intact molecular ions and/or a few fragments in MS1. Soft ionization techniques by desorption have been developed for nongaseous or thermally unstable natural compounds, for example, anthocyanins. These techniques cause a direct formation of gaseous ions by supplying power to solid or liquid sample, giving a little fragmentation and a simple mass spectrum for accurate molecular weight determination of the molecules. The most suitable ionization techniques for the chemical structure of anthocyanins are continuous-flow fast-atom bombardment (CF-FAB), desorption electrospray ionization (DESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI) [61, 82, 92–94].

In general, as an ionization mode, ESI has been mainly used, but some studies have reported the use of MALDI coupled with a time-of-flight (TOF) mass analyzer (MALDI-TOF) as an alternative. The main advantage of MALDI-TOF is the speed of analysis (a few minutes per sample). Also, MALDI-TOF mass spectrometry prevents the unwanted fragmentation of the molecules, giving a fingerprint mass spectrum for the desired molecules. Furthermore, this technique provides direct use of complex

sample mixture without prior separation. For example, anthocyanin profiling of the crude aqueous-methanolic extract of the pulp of Jamun fruit was performed by MALDI-TOF mass spectrometry operating in positive ion mode and using sodium chloride and 2, 5-dihydroxybenzoic acid as the matrix. However, MALDI-TOF is not capable of generating MS/MS data compared to ESI-MS/MS systems [95, 96]. MALDI-TOF mass spectrometer was applied for analysis of anthocyanins from blueberries, and found to be quicker and to give nontargeted quantitative estimates compared to HPLC-PDA-MS method, but unable to distinguish between anthocyanins and other flavonoids, which generate ions of the same m/z value, giving an inherent limitation of the method [95, 97].

For MALDI mode, 2,4,6-trihydroxyacetophenon (THAP) and cyano-4-hydroxycinnamic acid (CHCA) are used as a potential matrix for the flavonoids, but these matrixes tend to be fragmented and decomposed under the most instrumental conditions, resulting in a complicated mass spectra and difficulties to analyze flavonoids with a small molecular mass. However, the surfactant cetyltrimethylammonium bromide (CTAB) has been introduced as a MALDI matrix-ion suppressor and reported to yield a higher resolution and greater reproducibility than those without surfactant for qualitatively identifying anthocyanins from multiple berry extracts in a few minutes. Because of the specificity of the matrix-ion suppression, the method is called "surfactant-mediated" MALDI, and demonstrated as a complementary rapid screening technique for anthocyanins [98].

ESI is an appropriate method to generate ions in a positive or negative mode. The only prerequisite that ESI ionization needs are that the sample of interest must be soluble in appropriate solvents, and introduced to a mass spectrometer in the form of a solution. In addition, ESI is a common interface between LC/MS because of avoiding many problems seen with other LC/MS ionization interfaces. ESI-MS is a powerful versatile ionization for thermally labile, nonvolatile, and polar compounds because this soft ionization technique can produce intact ions from large and complex compounds. Past studies have shown that ESI is effective for anthocyanin ionization that produces gaseous ions from highly charged evaporating liquid droplets. To date, ESI-MS has been described and used as a powerful technology to identify the molecular structure and contents of anthocyanins in fruits. Although both positive and negative ionization modes have been generally reported for analyses of fruit anthocyanins, the positive ionization mode has been more commonly preferred by researchers [61, 86, 99–101].

Atmospheric pressure chemical ionization (APCI) is another ionization interface that has been used particularly for the broad class of flavonoids such as aglycons and glycosides. Because APCI produces a single charged product, the molecular mass spectrum of the product can be directly observed. In contrast to ESI mode, LC coupled to positive or negative ion mode APCI is more suitable for the analysis of weakly polar or nonpolar compounds due to the sample vaporization [102, 103]. For identification of anthocyanins with the same molecular mass, either sample treatment such as acid hydrolysis must be performed to release the anthocyanidin aglycons or MS fragmentation data must be obtained. The mass spectrometric data of LC-APCI-MS method provides information on the fragmentation of the anthocyanins, allowing the identification of the conjugate and the aglycone moiety. Therefore, LC-APCI-MS method allows the characterization of anthocyanins in samples without the need for sample preparation. For example, anthocyanins from red raspberries have been identified from the methanolic extract by reversed-phase HPLC through an atmospheric pressure chemical ionization probe operating in positive ion mode [30]. The combination

of both LC-APCI-MS and LC-ESI-MS methods was also reported to overcome the disadvantages of each ion source when applied individually, as well [103].

2.2.2 Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS or MS^n) is powerful to characterize individual compounds and identify the structure of compounds by separate ionization and fragmentation steps. MS/MS allows for the formation of the fragments of each individual molecule by collision-induced dissociation (CID). Individual compounds are detected by the first quadrupole mass detector and then fragmented in the collision cell *via* a suitable gas, usually argon or nitrogen, and their fragments are detected by the second quadrupole mass analyzer [82]. In the last decade, the improvements in resolving power, selectivity, and sensitivity have accelerated the use of HPLC-MS/MS to identify known or annotate unknown anthocyanins [91].

2.3 HPLC coupled with ESI-quantitative time-of-flight MS/MS: HPLC/ESI-qTOF-MS/MS

For LC-MS interfaces, there are different types of mass analyzers available, such as magnetic sectors, time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole ion traps, and ion cyclotron resonance. Mass analyzers can broadly be divided into two main groups including high- and low-resolution analyzers depending on their ability to distinguish ions with small mass-to-charge (m/z) differences. The high-resolution analyzers are useful in the structural annotation of anthocyanins. They can provide accurate m/z values of fragments, which allows to predict the location of structural fissions in MS/MS fragmentations. The resulting fragments can be useful to annotate anthocyanin structures.

Time-of-flight mass analyzers work on the principle that lighter ions travel faster than heavier ions following an initial acceleration by an electric field. All ions acquire the same kinetic energy during this initial acceleration period and are separated in the field-free flight tube, according to their different velocities. The physical property that is measured is flight time, which is directly related to the mass-to-charge ratio of the ion. Due to this mode of operation, TOF instruments offer very high mass ranges, very high acquisition rates, relatively high resolving power, and good sensitivity [6, 76, 104].

Ion trap analyzers enable the true MS^n operation by allowing selective trapping and fragmentation of parent and/or daughter ions as a function of time. In general, ion trap analyzers are for qualitative analysis. The quadrupole or triple quadrupole ion traps are for quantitation purposes. For structure elucidation or annotation of an individual compound, a triple quadrupole ion trap has high specificity and sensitivity, thus it is an effective mass analyzer to filter the ion of choice. In the triple quadrupoles, the first quadrupole (Q1) and the third quadrupole (Q3) function as mass filters to isolate parent ions (precursor-ion) and to monitor selected daughter ions (fragment or product-ion), respectively. The second quadrupole (Q2) serves as a collision cell, where parent ions are fragmented by ionization [6, 76, 105–107]. For example, anthocyanins from northern highbush blueberry extracts were identified by performing purification of solid-phase extraction, elution with acidified water and methanol, separation with the gradient mix of acidified water and acetonitrile through Zorbax SB-C18 column, and detected by reversed-phase HPLC coupled with electrospray ionization probe operating in positive ion mode, and time-of-flight tandem mass spectrometer [55].

3. Conclusion

Anthocyanins are water-soluble glycosides acquiring different colors, from red to blue or violet. They are naturally the most occurring flavonoids containing sugar moiety and are synthesized abundantly in many fruits, particularly berries. Anthocyanins from fruits have high significance for food, cosmetic, and pharmaceutical industries. To obtain high-quality fruit anthocyanins, many different approaches, methods, and techniques have been created for extraction, structural characterization, and profiling. In this chapter, we introduced multiple methods for sample treatments, including extraction, chromatographic separation and purification, and for detection, annotation, and identification of fruit anthocyanins. We also discussed the use of HPLC-DAD in combination with mass spectrometry (MS) as an outstanding tool providing the chromatographic and spectral characteristics of the LC system and the resolution and separation by mass fragmentation. Especially, HPLC and MS/MS technologies were highlighted as powerful to understand anthocyanin profiles in fruits. As a result, the combination of the liquid chromatography (LC) method with electrospray ionization (ESI) and mass spectrometry (MS) or quadrupole time-of-flight (QTOF) with mass spectrometry (MS) was evaluated as the most popular and reliable methods for analyzing these compounds in fruit samples.

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Conflict of interest

The authors declare no conflict of interest.

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