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Chapter 5

Anthocyanin Pigments: Importance, Sample Preparation and Extraction

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Additional information is available at the end of the chapter

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Abstract

Anthocyanins are naturally occuring pigments belonging to the group of flavonoids, a subclass of the polyphenol family. They are common components of the human diet, as they are present in many foods, fruits and vegetables, especially in berries and red wine. There were more studies conducted on effect of processing and storage on changes and stability of colors of anthocyanins in foods such as fruits and also for their use as natural colorants. Besides, the interest on anthocyanins is still growing also owing to their strong antioxidant activity against many chronic diseases, numerous studies about their medicinal, therapeutical and nutritional value were also conducted. There are pieces of evidence regarding the positive association of their intake with healthy biological effects. They act as antioxidants both in the foodstuffs in which they are found and in the organism that take in foods rich in anthocyanins. Many efforts have been carried out to develop new analytical techniques for identification and quantification of anthocyanins in plant materials, as well as their effects *in vivo* and *in vitro*. With this in mind, an overview to general considerations concerning (i) polyphenol and flavonoid history; (ii) chemical structure, color and intake of anthocyanins and (iii) sample preparation and extraction methods are presented in this chapter.

Keywords: anthocyanins, pigments, sample preparation, extraction

1. Introduction

The progress in the last years in the interdisciplinary fields of chemistry, separation science, biology and pharmacy has boosted the natural product chemistry research [1–4], providing a valuable information about many classes of naturally occurring dietary phytochemicals. Among these phytochemicals, polyphenols are the worldwide redox-active secondary

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metabolites of a phenolic nature [5–12]. The importance of secondary metabolites and their crucial role in many important functional aspects of plant life was recognized for the first time in the second-half of the ninetieth century by Julius Sachs (1873) [13, 14]. Polyphenols are natural compounds occurring in plants [15–18], including foods such as fruits, vegetables, cereals, tea, coffee and wine.

The study mainly focuses on organoleptical properties of polyphenols [19] and their physiological importance to plants [20]. Later, polyphenols are found to be recognized by their nutritional value, since they may help reduce the risk of chronic diseases [1, 21–24] and, in general, have a positive effect on health, because of their free radical scavenging capacity [25–27], which, among other biological effects, increases antioxidant activity and prevents cellular oxidation.

The research on phenolic compounds is mainly focused on anthocyanins [28–29], natural pigments and common components of the human diet (foods, fruits and vegetables, especially in berries and in red wine), As they provide for much of the red to blue pigmentation of flowers and fruits and have physiological functions in vegetative tissues. Their biosynthetic pathway has been the subject of much research and the associated biosynthetic and regulatory genes are well defined. Besides considerable interest in coloring properties of anthocyanins, they have also attracted attention due to their antioxidant activity [30–34] and their property is closely related, to a large extent, with their chemical structure. The pH-dependent ground-state chemistry of anthocyanins is extremely rich. In the past 20 years, the health benefits of anthocyanins have become the subject of intensive research.

Analytical chemistry plays an importance role in this context [35–37] which determines the identity and quantities of anthocyanins in natural products, as well as their effects *in vivo* and *in vitro*.

This chapter intends to reflect the interdisciplinary nature of the research that is currently carried out in anthocyanin pigments through an update of the state-of-art of a series of previously published reviews on this field in the year 2012 [28, 29, 38, 39]. First, general considerations concerning polyphenols with emphasis on their role as secondary metabolites are made. Flavonoid classification, structure, biological activities, databases, intake and dietary sources are also contemplated. Second, aspects of anthocyanin concerning its early history and chemical structure, color and intake are dealt. It should be noted that anthocyanins are readily distinguished from other flavonoids as they undergo rearrangements in response to pH. The antioxidant activity of anthocyanins is depending on their chemical structure. Finally, special attention is paid to analytical methodologies involved in the isolation, determination and characterization of bioactive polyphenols in plants, fruits and vegetables, herbal drugs, medicinal plants and wines, including sample-handling strategies, a feature of analysis often ignored. The use of nonthermal technologies in the assisted extraction of anthocyanins will be covered in future reports.

2. Polyphenols

Plant phenols are among the most abundant and widely represented class of existing plant natural products [40] thanks to the continuous evolution of new genes brought about by gene duplication and mutation and subsequent recruitment and adaptation to specific functions.

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The amino acids phenylalanine and tyrosine (derived from the shikimic acid pathway) are the most common origin of polyphenols [41, 42]. Chemically, polyphenols belong to four main classes (**Figure 1**): flavonoids, phenolic acids (hydroxy derivatives of benzoic acid and cinnamic acid, i.e., p-hydroxybenzoic, protocatechuic, vanillic and syringic acids) and their esters (chlorogenic, caftaric, coutaric and fertaric acids), stilbenes (resveratrol, pterostilbene, piceatannol), characterized by a double bond (1,2-diarylethene) connecting the phenolic rings and lignans (pinoresinol, podophyllotoxin, steganacin), characterized by a 1,4-diarylbutane structure, i.e., having 2-phenylpropane units. Flavonoids and phenolic acids account for 60 and 30%, respectively, of the total dietary polyphenols [43].

When the phenolic molecules are not attached to sugar moieties are known as the aglycone form; while those molecules conjugated with one or more sugar residues are called glycosides. Most phenolic compounds are found in nature associated with mono- or polysaccharides or functional derivatives [44] such as esters or methyl esters, varying widely in their hydroxylation pattern and can be glycosylated or acylated.

Despite the fact that most of the literature on phenolic compounds focuses mainly on those found in fruits, vegetables, wines and teas. However, many phenolic compounds present in fruits and vegetables (phenolic acids and flavonoids) are also found in cereals [45, 46].

For decades, this family of compounds has attracted the attention [47] since three British scientists open the door to understand separation, structural elucidation and taxonomical distribution of phenolic compounds. As mentioned above, traditionally, the interest was focused on organoleptical properties of polyphenol such as color, astringency, bitterness, astringency and a range of other tactile or "mouth feel" characteristics [48, 49], as well as their physiological role in plants in the reproduction, pathogenesis and symbiosis. In last decades, polyphenols

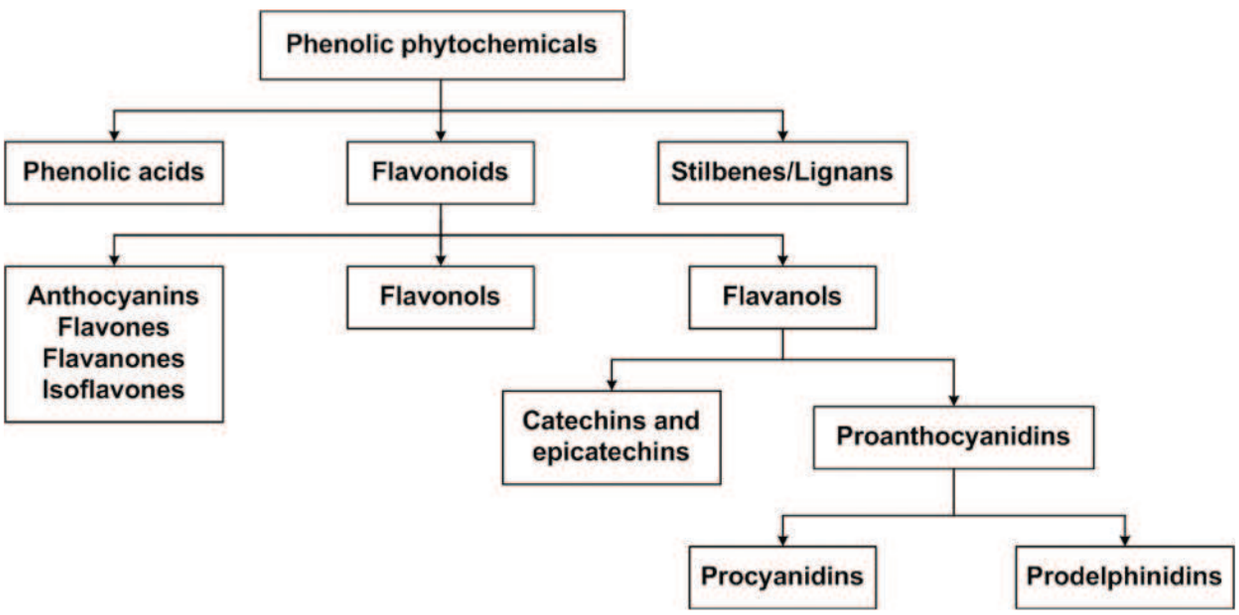


Figure 1. Types of phytochemicals [38].

are increasingly recognized due their nutritional value, since they may help reduce the risk of chronic diseases [50–53]. The capacity of phenolic compounds to trap free radicals depends upon their structure, in particular of the hydrogen atoms of the aromatic group that can be transferred to the free radicals [54, 55] and of the capacity of the aromatic compound to cope with the uncoupling of electrons as a result of the surrounding displacement of the electron-π system [27]. The polyphenols are still gaining attention.

Although the percentage of absorbed natural polyphenols is usually quite low [56], researchers have seen a large quantity of metabolites of polyphenols in the form of simple phenolic acids in the blood. The amount and form in which plant phenolic substances are administered influence greatly the physiological effects connected with their consumption [57]. About 1 g of polyphenols per day is commonly ingested with foods, being the most abundant antioxidant in the diet (about 10 times higher than the intake of vitamin C and 100 times that of vitamin E) [28, 29, 58]. The daily intake of polyphenols is difficult to estimate and depend on several factors. In the literature there are about 1000 peer-reviewed publications [28] concerning the polyphenol content in food. Recently, the construction and application of a database with polyphenols content in foods has facilitated this task [59]. The Institute Nationale de la Recherche Agronomique [60] has developed a new Phenol-Explorer database [59] covering over 60,000 foods useful to epidemiologists, food scientists and food manufacturers. The content of polyphenols of the 100 richest dietary sources can range from 15,000 mg per 100 g in cloves to 10 mg per 100 mL in rose wine [61]. EuroFir [62] is another database to build national food composition in different countries within the framework of the European Food Information Resources Network (i.e., Spanish [63] and Irish [64] databases).

3. Flavonoids

The flavonoids constitute the largest group of polyphenols (of low molecular weight) and are considered to be responsible for the color and taste of many fruits and vegetables [65, 66]. Since first identified in the mid-1800s, more than 9000 flavonoid structures have been described, with formulas, references and biological information [67, 68] and the list is constantly increase. These include over 600 different naturally occurring anthocyanins [69] that are widely distributed among at least 27 families, 73 genera and innumerable species.

Many of these compounds are yellow in color, as the Latin root suggests. They are present particularly in the epidermis of leaves and the skin of fruits [9, 70]. Flavonoids can accumulate in vacuolar compartments, or be secreted, for example, as part of root exudates. Most attracting is the accumulation of flavonoids on the surface of leaves and flowers [70]. Flavonoids play roles in many facets of plant physiology [71] and their influence on the transport of the plant hormone auxin is one of their most important roles.

Around 5000 of the flavonoids studied have antioxidant activity. Because the number of phytochemicals already identified is only a small part of those that exist in nature, there is

considerable interest in new methods [37, 72–78] of separation, isolation and characterization of polyphenol structures from foods.

Concerning the chemical structures of flavonoids in which two aromatic rings are present that linked by three carbons in an oxygenated heterocycle, i.e., a flavan (2-phenyl-benzo-γ pyran) nucleus consisting of two benzene rings combined with an oxygen-containing pyran ring, the parent compound bearing a tricyclic (C6-C3-C6) skeleton. The heterocyclic benzopyran ring is known as the C ring, the fused aromatic ring as the A ring and the phenyl constituent as the B ring. The structural differences in each flavonoid family result from variations in the number/substitution pattern of the hydroxyl and methoxy groups [28, 79, 80], as well as different glycosylation patterns and the presence of a C2-C3 double bond in the heterocycle pyran ring.

Compounds are classified according to differences in their heterocycle (C ring): flavonols, flavones (catechins), flavanones, chalcones, dihydrochalcones and dihidroflavonols, anthocyanins and isoflavonoids (isoflavones) [29, 55], varying in the oxidation state (degree of saturation) of the heterocyclic central pyran ring. When unsaturation is present, the geometry of the molecule is planar, as in the case of anthocyanins, flavones and flavonols.

Flavonoids usually occur as glycosides in plants, reflecting a biological strategy increasing their polarity and necessary for storage in the plant cell vacuoles and decreasing their reactivity to interact with macromolecules [81–83]. While flavan-3-ols (catechins and theaflavins) are present in either a free form or as gallic acid esters (e.g., in tea). The glycosidic linkages appear to be important for the absorption of flavonoids [44].

Williams and Grayer [84] have stated: "Flavonoids continue to capture the interest of scientists from many different disciplines because of their structural diversity, biological and ecological significance (e.g. the colored pigments in many flower petals) and health promoting and anticancer properties." Recent advances in genomics, proteomics and metabolomics provide new approaches in the field of flavonoids in plant: protection against oxidative diseases, ability to modulate the activity of various enzymes and interactions with specific receptors are among the most significant health benefits [67, 85].

Flavonoids of dietary significance are present in edible plants in widely varying combinations [86, 87]. Unlike traditional vitamins, flavonoids are not essential for short-term well-being. The daily intake is almost at the same level as the sum of other antioxidants, including carotene, vitamin C and vitamin E, can range from several hundred mg up to 1–2 g [88] and although flavonoids are not essential for short-term may exhibit potential health benefits at modest long-term intake [85, 89, 90]. **Table 1** [91–98] shows the dietary sources of flavonoids. Great differences in flavonoid intake and food sources were observed between a large Mediterranean cohort and non-Mediterranean populations (U.S. and Finland as non-Mediterranean countries) [99]. The mean intake for a Spanish population was 313 mg/day [99]. Estimated per capita daily flavonoid intake is 182 and 177 mg for the UK and Ireland, respectively [100].

Flavonoid subclass	Prominent food flavonoids	Typical rich food sources
Anthocyanins	Cyanidin Delphinidin	Bilberries, black and red currants, blueberries, cherries, chokeberries, elderberries, grapes, strawberries, pomegranate
Chalcones	Cinnamon Methylhydroxychalcone	Apples, pears, strawberries, tomatoes, cinnamon
Flavanols	Catechin Epigallocatechin Epigallicatechingallate	Apples, blueberries, grapes, onions, lettuces, red wine, tea, chocolate, apricots, sour cherries, grape juice, mint
Flavanonols	Taxifolin or dihydroquercetin Aromaderin or dihydrokaempherol	Grapes, red onion, açai palm
Flavanones	Hesperetin Naringenin Eriodiclyol	Citrus fruits and juices, peppermint
Flavonols	Quercetine Kaempferol Myricetin	Apples, bean, blueberries, buck wheat, cranberries, endive, leeks, broccoli, lettuces, onions, olive, pepper, tomatoes
Flavones	Apigenin Luteolin	Citrus fruits, celery, parsley, spinachs, rutin, olives, artichoke
Isoflavones	Genistein Daidzein Glyatein	Soybeans, grape seed/skin, chick peas, black beans, green peas
Xanthones	Mangostin	Mango, mangosteen, bark of pear, apples, cherries

Table 1. Dietary sources of flavonoids [28, 91–98].

4. Anthocyanins

Anthocyanins are the largest group of phenolic pigments and the most important group of water-soluble pigments in plants [68, 69, 101–104], responsible for the red, purple and blue colors found in many fruits, vegetables, cereal grains and flowers, being odorless and nearly flavorless and contributing to taste as a moderately astringent sensation. Anthocyanins are almost universally found in higher plants (occurring in about 30 families), but in general anthocyanins seem to be absent [103] in the liverworts, algae and other lower plants, although some of them have been identified in mosses and ferns. **Figure 2** shows a picture of plant species rich in anthocyanins [105–107].

Anthocyanins are found mainly in the skin, except for certain types of red fruit [94], in which they also occur in the flesh (cherries and strawberries).

Anthocyanin biosynthesis was one of the first branches of the general propanoid metabolism [41, 108, 109], for which biosynthetic enzymes and corresponding transcription factors were identified, given the ease of visualization and control of mutants and genetic imbalances.

Anthocyanins have characteristic physicochemical properties that confer them its unique color and stability [30, 32, 110–112]. They are highly reactive molecules and thus sensitive to

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Figure 2. Natural sources of anthocyanin pigments [105–107].

degradation reactions. Oxygen, temperature, light, enzymes and pH are among the factors that may affect anthocyanins chemistry and, consequently, their stability and color [113]. In the following, aspects of anthocyanins concerning its chemical structure, color, antioxidant activity and intake are dealt.

4.1. Chemical structure

Chemically, anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrilium [68, 103, 114], usually with molecular weights ranging from 400 to 1200 (medium-size biomolecules) and containing two benzyl rings (A and B). Anthocyanins usually contain a single glucoside unit, but many anthocyanins contain two, three, or more sugars attached at multiple positions [79], or occurring as oligosaccharide side chains. Intensity and type of the color of anthocyanins is affected by the number of hydroxyl and methoxyl groups [68, 115]: if more hydroxyl groups are present then the color goes toward a more bluish shade; and redness is increased if more methoxyl groups are present. The major anthocyanins are shown [116, 117] in **Figure 3** and **Table 2**.

Anthocyanins mainly exist in glycosidic forms in fruits and with the exception of blueberries, fruits usually contain anthocyanin derived from only one or two of the aglycone bases. Grapes offer a richer anthocyanin profile than many other fruits [118] (red grapes may contain a mixture of more than 20 pigments [85, 119]. Various berries and black currants are the anthocyanin-richest fruits [120–122]. The eggplant is only one common vegetable [123] that contains a high level of anthocyanins.

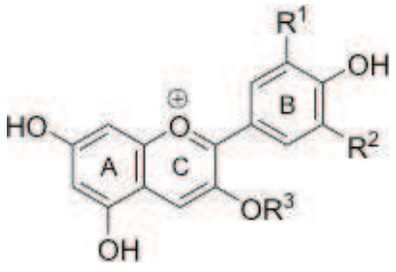


Figure 3. Structure of the major anthocyanins-3-O-glucoside present in fruits [29, 116, 117].

Anthocyanidin	Abbrev.	R_1	R_2	λ_{max} (nm)*		Some of the produced colors
				R_3 = H	R_3 = gluc	
Delphinidin	Dp	OH	OH	546	541	Purple, mauve and blue
Petunidin	Pt	OH	OCH_3	543	540	Purple
Malvidin	Mv	OCH_3	OCH_3	542	538	Purple
Cyanidin	Cy	OH	H	535	530	Magenta and crimson
Peonidin	Pn	OCH_3	H	532	528	Magenta
Pelargonidin	Pg	H	H	520	516	Orange salmon

Source: Ref. [29].

Table 2. Major anthocyanins-3-O-glucoside present in fruits [29].

The anthocyanins are all amphoteric [32, 34] forming salts with either acids or bases. In addition, anthocyanins occur in plants as salts (indicated by the positive charges on the heterocyclic ring) and their color in plant cells depends mainly upon their mode of combination. The conjugated bonds in their structures (light-conjugated double bonds carrying a positive charge), which absorb light at about 500 nm, are the basis of the bright red, blue and purple color of fruits and vegetables [124] as well as the autumn foliage of deciduous trees. Every color except green has been observed (either natural or synthetic), depending on aspects such as a kind of substituent present in the B-ring, the local pH, the state of aggregation of the anthocyanins, complexation by organic molecules, or, as in the case of blue color [125], complexation by metal cations.

In spite of the increasingly large number of structures, they are derived from only about 30 different anthocyanidins [69, 126], most of them are based on cyanidin (31%), delphinidin (22%), or pelargonidin (18%). The other common anthocyanidins (peonidin, malvidin and petunidin), which contain methoxy group(s) on their B-ring (**Figure 4**), represent together 21% of the isolated anthocyanins. One new methylated anthocyanidin, 7-O-methylcyanidin, five new desoxyanthocyanidins and a novel type of anthocyanidin called pyroanthocyanidin [11, 127] have also been reported. In spite of the structural diversity of anthocyanins, the three nonmethylated anthocyanidins are the most widespread in nature [128], which are present in 80% of pigmented leaves, 69% of fruits and 50% of flowers.

4.2. Antioxidant activity

The relationship between diet and health has been known since ancient times and recent studies demonstrated the relevance of many food components in modulating health [1]. Due to anthocyanin's positive charge (**Figure 3**), the number and arrangement of aromatic hydroxyl groups, the extent of structural conjugation and the presence of electron-donating and electron-withdrawing substituents in the ring structure made anthocyanins very effective donors of hydrogen to highly reactive free radicals (such as superoxide (O_2^-), singlet oxygen ($^1O_2$), peroxide (RCOO·), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) and reactive nitrogen species in a terminator reaction) and, thereby preventing further radical formation

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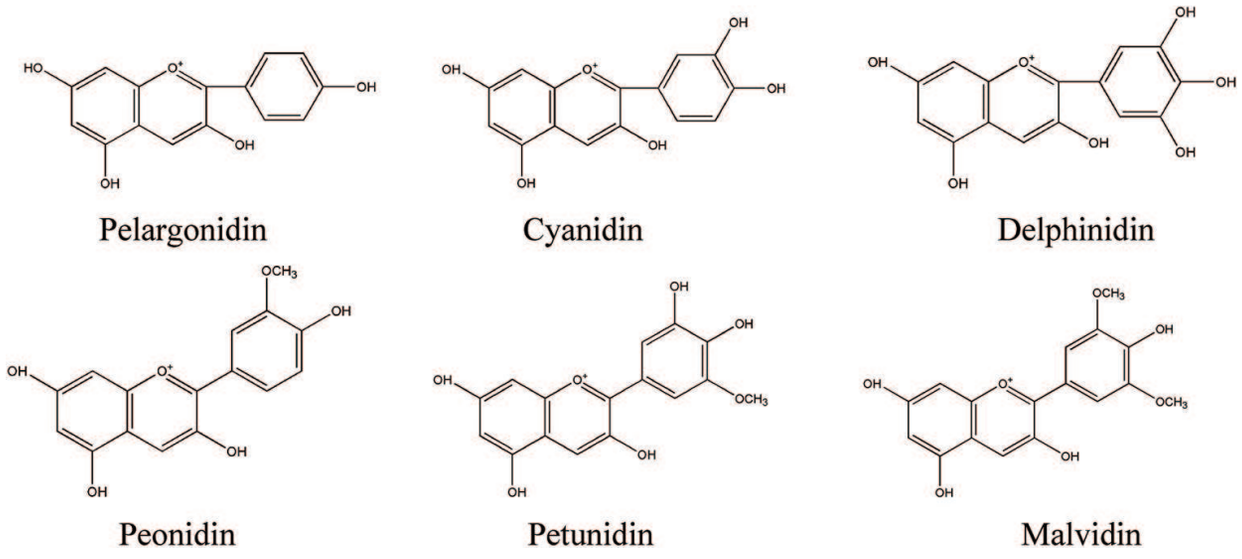


Figure 4. Structure of the main anthocyanins.

[55, 91, 129]. This effect protect cells from oxidative damage, which leads to aging and various diseases [102, 130–133], such as cancer, neurological and cardiovascular diseases, inflammation, diabetes and bacterial infections. The antioxidant capacity of phenolic compounds is also attributed to chelate metal ions involving in the production of free radicals [134], thereby reducing metal-induced peroxidation. Anthocyanin bioavailability has been the subject [135–138] of recent reviews.

Laboratory-based evidence was provided (the potential health benefits of anthocyanins [139]. Consumption of diets rich in natural bioactive components (i.e., fruits and vegetables) as an alternative to pharmaceutical medication has been a subject [104, 140, 141] of considerable interest to researchers. In recent investigations carried out in population-based anthocyanins have been linked to a decrease in the incidence of several diseases, such as diabetes mellitus, cancer and cardiovascular diseases. *In vivo* studies have reported evidence regarding the positive association of their intake with healthy biological effects. However, much work remains to achieve definitive conclusions [139, 142] and the need for additional basic and applied research in this area is evident.

4.3. Color

Anthocyanins are the pigment compounds responsible for pale yellow, orange, red, magenta, violet and blue colors [143]. Carotenoids and betalains confer yellow and red colors [144], although only the families of Caryophyllales (except for Caryophyllaceae and Molluginaceae) produce betalains. Up to now, no plants producing both anthocyanins and betalains [109] have been discovered. Anthocyanins, carotenoids and other pigments contribute to the UV patterns that are visible to insects and serve [145] to signal flowers that are attractive to pollinators. **Figure 5** shows a schematic representation of the biochemical process involving anthocyanins in color plants [146].

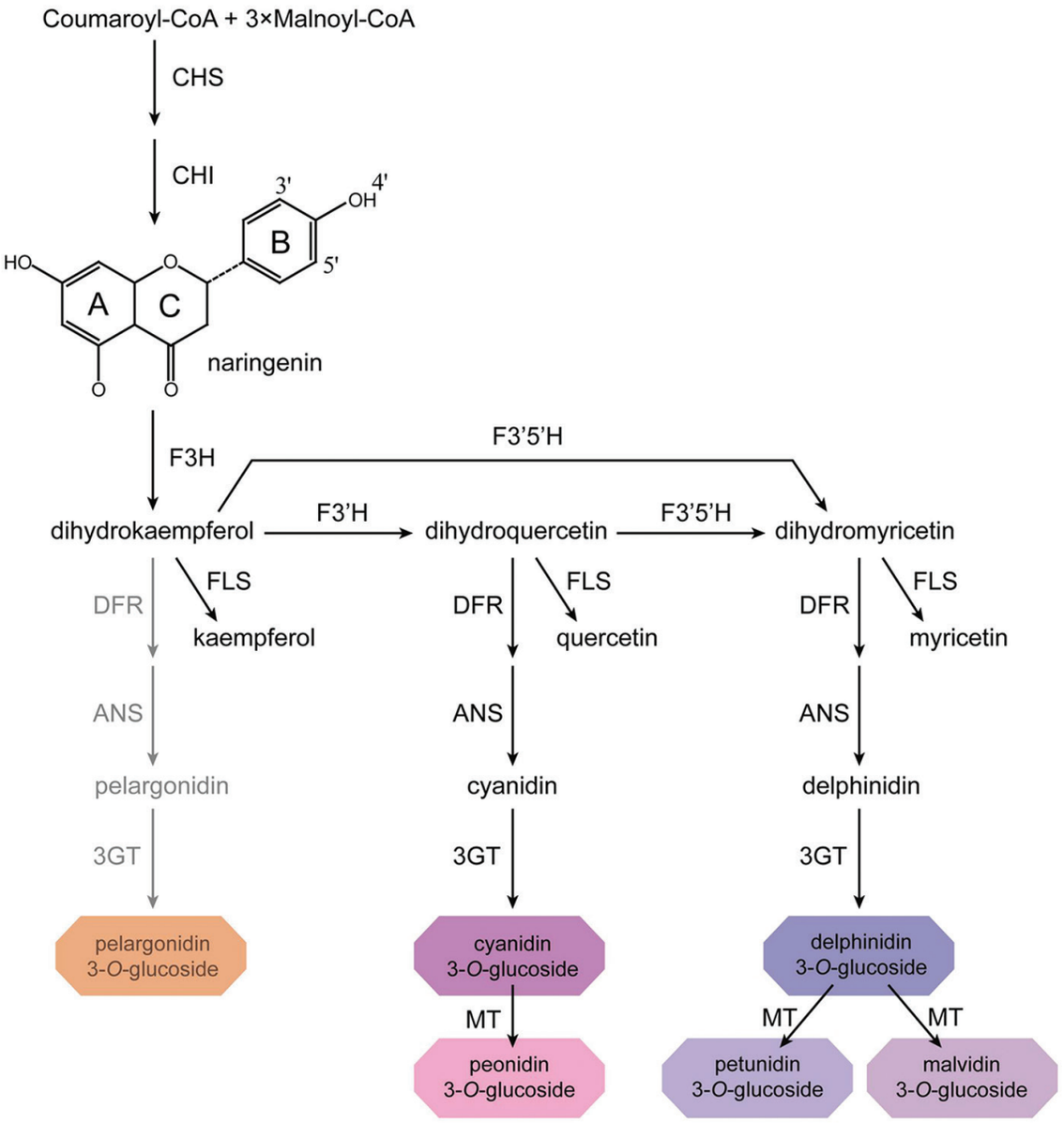


Figure 5. Schematic of the general flavonoid biosynthetic pathway relevant to flower color (ANS: anthocyanidin synthase; CHI: chalcone isomerase; CHS: chalcone synthase; DFR: dihydroflavonol 4-reductase; F3H: flavonoid-3′ hydroxylase; F3′5H: flavonoid-3′5′hydroxylase FLS: flavonole synthase; 3GT: flavonoid 3-O-glucosyltransferase; MT: Malonyl transferase) [146].

In food chemistry, anthocyanins have been studied [68, 147, 148] in relation to changes and stability of colors in foods such as fruits during processing and storage and also for their use as natural colorants. Indeed, many types of anthocyanin food colorants have been developed and are now available to customize the appearance of foods. In horticulture, color conversion of flower pigments has become possible by new findings of anthocyanin research. Creation of flowers in new colors enriches our life; for example, the creation of blue roses [109] is a noteworthy achievement. Genetic engineering is the key technology for converting flower color and it became possible after the discovery of genes involved in anthocyanin biosynthesis and elucidation of their expression mechanisms.

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Additionally, color may act as a "fingerprint" of a food product, being related to its flavor and at the same time [149] an estimate of its overall quality. In this sense, special attention is paid to the application of anthocyanin analysis in classification of wine [150]. Anthocyanins can be used as markers to classify wines according to the grape variety [49, 151], although this requires a complex separation with very high chromatographic efficiency, together with advanced statistical methods, especially when dealing with aged red wines, because of the formation [152] of pyranoanthocyanins (formed through the reaction of anthocyanins with small molecules).

4.4. Copigmentation

Copigmentation is almost always a variation toward blueness. This phenomenon induced by the presence of substances [153, 154] that are themselves colorless or only slightly colored in wine has received considerable attention [155, 156]. The basic role of copigments is to protect [125] the colored flavylium cation from the nucleophilic attack of the water molecule. The copigmentation complexes are easily disrupted by dilution returning to the pH-dependent equilibria among the structural forms of anthocyanins. It has been suggested that in acylated anthocyanins, the acyl groups interact with the basic anthocyanin structure, thus avoiding the formation of the hydrated species. This is the basis of characterizing [157] the color due to copigmentation. Copigmentation of flavonoids other than anthocyanins is also possible [158], but it is either a rare or an understudied phenomenon.

4.5. Intake

Anthocyanins are widely ingested by humans, mainly due to consumption [159] of red fruits (like berries and red grapes), vegetables such as red cabbage, red wines, cereals and purple corn. Accurate estimation of anthocyanin contents in foods and daily intake is critical in food science, nutrition and other related research fields. The type and concentration of anthocyanins differ widely among different fruits and vegetables. Intake levels of anthocyanins varies widely with region, season and among individuals with different social, cultural and educational backgrounds. High intake levels of anthocyanins can be achieved with the regular consumption of fruits (blueberries, blackberries, raspberries, strawberries, red grapes and saskatoon berries). Depending on nutritional habits, the daily intake has been estimated in the range [58] from several milligrams to hundreds of milligrams per person, while the consumption of other phytonutrients such as carotenoids, vitamin E and vitamin C are estimated at 5, 12 and 90 mg/day, respectively.

Regular consumers of red wine are likely to have [149] significantly higher intakes. A glass of red wine provides around 115 mg of polyphenols, contributing toward a total intake [58] of phenolic compounds of 1171 mg/person/day. In the United States, an average daily intake of anthocyanins has been estimated [119] at 215 mg during the summer and 180 mg during the winter. Wu et al. [160] estimated that the mean daily intake of anthocyanins is 12.5 mg/person in the United States; such a huge difference of the total anthocyanin daily intake estimation must result from different food intake data. The influence of methodological differences in the assessment, as well as nutritional, social and cultural differences of the investigated

populations, may also explain the wide range of anthocyanin consumption estimated by different authors. Anthocyanin intake in the German young shows differences between girls and boys [161], decreasing from young childhood to adolescence.

Up to now, anthocyanins have not been detected in processed food such as canned food, bread, or cereals. Also, although prepared baby food containing blueberries, rich in anthocyanins, are expected to find these compounds, analyses have hardly detected them [162]. In young infants [163] the anthocyanidin intake was found to be zero.

Due to antioxidant and other potential beneficial properties, grapes, various berries, red cabbage and other anthocyanin-rich foods are becoming more popular. Berry extracts are also being commercialized as nutraceuticals and dietary supplements [164] to meet consumer demands.

Currently, there is no recommended intake level of anthocyanins for optimal health or to avoid adverse effects; however, future research and continued consumer interest will undoubtedly present opportunities for pursing dietary guidance recommendations.

5. Sample preparation and extraction of anthocyanins

The presence of phytochemical bioactive compounds in food and dietary supplements poses difficult problems in connection with the optimization of their extraction process and determination. Aspects related to Ref. [165] the complexity of sample matrices, the presence of varying forms of bioactive substances and interaction with other components need to be solved. A number of factors including pH, metal ions, complex formation, light, temperature, enzymes, sugars, oxygen and ascorbic acid exert influence [166] on the stability of anthocyanins. The role of analytical chemistry is vital in this context, e.g., [34, 76, 91, 167–171] promoting advances in separation science.

Most phenolic compounds are made up of only C, H and O, differing in some cases even by only one atom and in many others by constitutional or stereochemical isomers. The identification of species proves thus be difficult because of subtle structural changes, being necessary for this purpose to apply [38] complementary techniques. Anthocyanins may be forming part of complexes, may be present in matrices of a complex nature and may appear in distinct equilibrium forms [30, 32, 34], depending of the pH of the medium. Acid dissociation, rate constant and tautomerization constants are of great importance in the analysis of bioactive compounds and in the interpretation of their mechanisms of action.

Time-consuming processes are involved [172] in the isolation, purification and determination of the structures of anthocyanins, which must be accomplished with care. However, there is no universal sample pretreatment technique applicable to all kind of samples. The primary steps required, sampling, sample preservation and sample preparation, are not always properly documented [173] in the analytical literature.

5.1. Sample preparation

A number of strategies are used [37, 174, 175] for the characterization of phenolic samples in plant materials. In any case extraction techniques and semipreparative isolation methods are

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usually applied prior to the separation and quantification steps. After the first operations of drying and of powdering the plant material, it follows [176]: (i) a previous extraction step of the plant materials as well as a preliminary consequent purification step; (ii) fractionation of the mixture in order to isolate pure pigments and (iii) the final characterization and identification of pure anthocyanins compounds (**Table 3**).

In order to avoid sample oxidation, thermal degradation, chemical and biochemical changes under mild extraction conditions [177] are recommended and drying, lyophilized, or frozen samples should be used. The deterioration processes of the compounds may be avoided by the

Sample pretreatment	Air-drying, freeze drying
	Milling
	Grinding
	Homogenization
	Filtration
	Centrifugation
Extraction	Direct
	Solid extraction (SE)
	Liquid-liquid extraction (LL)
	Soxhlet extraction
	Microwave-assisted extraction (MAE)
	Ultrasound-assisted extraction (UAE)
	Pressurized fluid extraction (PFE)
	- pressurized liquid extraction (PLE/ASE)
	- subcritical water extraction (SWE)
	- supercritical fluid extraction (SPE)
	Enzyme-assisted extraction (EAE)
	Solid phase microextraction (SPME)
	Membrane extraction (ME)
	High hydrostatic pressure (HHP)
	Electric fields (EF)
Purification	Solid phase extraction (SPE)
	Column chromatography (CC)
	Countercurrent chromatography (CCC)
Analysis	Spectrophotometric assays
	Gas chromatography (GC)
	Liquid chromatography techniques (LC)
	Mass spectrometry (MS)

Nuclear magnetic resonance (NMR)	
Capillary electrophoresis (CE)	
Thin layer chromatography (TLC)	
Voltammetry	
Others	
Hyphenated techniques	GC-MS, LC-MS, LC-DAD-ES-MS/MS, CE-MS, LC-NMR, others

Table 3. Strategies for preparation and characterization of anthocyanin samples from plant materials [38].

addition of antioxidants compounds, by using inert atmospheres or working in the absence of light. However, no definitive procedure for storage and collection has been established.

To determine either the target analytes (in their various conjugated forms) or the aglycones is an important question [175, 178] to answer. When dealing with plant, food products and biological matrices, the instant conjugates are usually search, whereas in the other instances it is necessary to carry out a preliminary hydrolysis, e.g., an enzymatic or chemical (acidic or alkaline) treatment. Intentional hydrolysis for obtaining the aglycones of some flavonoid or derivatization of some fatty acids to esters is sometimes intentionally incorporated [79] to the extraction process.

Extraction represents an important phase [34, 38, 39, 79, 169] in the isolation, identification and utilization of anthocyanins. Anthocyanins are usually recovered by mean of a solvent extraction procedure. Parameters such as solvent-extraction kind and its composition, liquid-to-solid ratio, extraction time and temperature require [179] proper optimization. The flavylium cation form of anthocyanins is red and stable in a highly acidic medium. Thus, the extraction solution should be enough (slightly) acid to maintain the flavylium cation form [180], but not so much as to cause partial hydrolysis of the acyl moieties in acylated anthocyanins. Protocols of extraction and analysis of plant materials and biological fluids are, however, difficult to accomplish because of [181] the structural diversity of anthocyanins and their susceptibility to heat, pH, metal complexes and copigmentation.

In the same way as flavonoids, in general, anthocyanins possess aromatic rings that contains polar substituent groups (—OH, —C═O, or —OCH_3) and glucosyl residues, which altogether [28, 29] constitute a polar molecule. Flavonoid glycosides are of a more polar nature, whereas aglicones are extracted either with alcohols or alcohol-water mixtures. Cold acidified solvents (polar organic solvents, water) under mild conditions [167, 182] are used for the extraction of anthocyanins. The organic solvent usually used is methanol. However, solvents such as acetone, ethanol, or acetonitrile may be used. These solvents system denature the cell membranes [93] also dissolving and stabilizing the anthocyanins. Acetic acid at about 7% or trifluoroacetic acid at about 3% are usually used; the organic solvent content [183] varying from 50 to 100%

in the mixture. When a mineral acid is used it may assist [174] to the loss of the attached acyl group. Sulfurous water also allows [181] the reduction of organic solvent and cost extraction.

Phytochemical recovery of a good antioxidant from various sources may be achieved by using solvent extraction. The conventional solvent extraction procedure suffers from the drawback of requiring subsequent extraction and cleanup prior analysis. In addition, health and safety risks are associated with the use of large amounts of organic solvents, being on the other hand environmentally unfriendly. A modern trend toward [184]: (1) the use of samples smaller in size, volume, or organic solvent content; (2) an extraction with increasing selectivity or specificity; (3) improved recoveries and reproducibility; (4) greater automation facilities. A variety of modern techniques have been developed for this purpose, including solid phase extraction (SPE), countercurrent chromatography (CCC), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized hot water extraction (PHWE) and high hydrostatic pressure extraction (HHP), among others. Selected applications of sample preparation techniques on anthocyanin compounds are listed in **Table 4** [185–213], an extension of applications previously published by the authors in [39]. The applications of other novel nonthermal techniques will be the subject of further study. **Figure 6** shows a schematic representation of a highly separation and purification methodology based on a macroporus polymeric adsorbent for the determination of anthocyanins in bilberry [198].

Solid phase extraction				
Type of matrix/analyte	**Extraction/cleanup technique**	**Sorbent**	**Final analysis**	**References**
Petals of the oboshibana plant (*Commelina communis*)/anthocyanidin 3,5-diglucosides and their related chemicals	Sonication with 10% TFA aqueous solution/SPE	Discovery DPA-6S and DSC-SCX	UPLC-DAD	[185]
Urine, serum and feces/ anthocyanin metabolites	Addition of a preservative (10% w/v ascorbate in 0.5 mM EDTA) prior to SPE	Strata-X SPE cartridges	LC-MS/MS	[186]
Grapes/anthocyanins	SPE	Vinylbenzene-based cartridges	UPLC-DAD	[187]
Red wines/anthocyanins and derived pigments	SPE	Zip-Tip® pipette tips filled with C18 stationary phase	ToF-MS	[188]
Chinese black rice wine/ phenolic constituents	Acidification at pH 2 with HCl/SPE	Oasis HLB	LC-MS/MS	[189]
Wine lees/anthocyanidins, proanthocyanidins and anthocyanins	Liquid phase: SPE with filtration previous. Solid residue: MAE with a mixture 60:40 (v/v) ethanol-water	HySpere C8 EC cartridges (end-capped silica-based octyl phase, particle size 10 μm, 10 × 2 mm i.d.)	LC-MS/MS	[190]
Hybrid grape/monomeric, nonanthocyanins, condensed tannins and anthocyanins	Acidification with HCl 0.01 M and centrifugation/SPE	Oasis HLB	LC-DAD	[191]

Countercurrent chromatographic methods			
Type of matrix/analyte	**Solvent system**	**Elution mode/comment**	**References**
Crude mulberry/ cyanidin-3-glucoside and cyanidin-3-rutinoside	MTBE/n-butanol/ acetonitrile/0.01% TFA (1:3:1:5, v/v)	The upper phase was used as the stationary phase and the lower phase as the mobile phase	[192]
Blue honeysuckle fruits/ cyanidin 3-glucoside	MTBE/n-butanol/acetonitrile/ water/TFA (2:2:1:5:0.01, v/v). HSCCC	Head–tail elution mode with the upper organic phase as stationary phase	[193]
Mulberry fruit/anthocyanins	MTBE, 1-butanol, acetonitrile, water and TFA (10:30:10:50:0.05; %, v/v)	Stationary phase: upper organic phase. Mobile phase: lower aqueous phase. The elution was in "head to tail" mode	[194]
Petals of *Chaenomeles sinensis*/ Anthocyanidins	n-butanol/MTBE/ acetonitrile/0.1% aqueous TFA (0.715:1.0:0.134:1.592, v/v/v/v). HSCCC	The lower phase in the solvent separator was pumped into the mobile phase bottle, the pumps for pumping MTBE, n-butanol, acetonitrile and 0.1% TFA aqueous were set to a certain flow rate, the solvents were mixed and separated into two layers; and after rinsing the solvent separator, the upper phase flowed into the stationary phase bottle	[195]

Adsorbents and eluting agents			
Solution (target compounds)	**Adsorbent**	**Eluting and/or regenerating agent**	**References**
Purple-fleshed potato/ anthocyanins	Amberlite XAD-7HP	75 vol.% acidic (7 vol.% acetic acid) aqueous ethanol	[196]
Blackberries/anthocyanins	Polyamide resin	Deionized water and ethanol (0.1% v/v HCl, pH = 3)	[197]
Bilberry based/anthocyanins	Copolymerization of divinylbenzene and ethylene glycol dimethyl acrylate	Ethanol	[198]
Blueberries/anthocyanins and polyphenols	FPX66 resin	3 bed volumes of 95% ethanol	[199]
Jamun (*Syzygium cumini* L.)/ anthocyanins	Amberlite XAD7HP	Aqueous acidified ethanol (above 40%, v/v)	[200]
Aronia melanocarpa berries/ antioxidant phenolics	XAD 7HP resin	Ethanol-water mixtures	[201]
Strawberry/aroma compounds and Anthocyanins	Cross-linked acrylonitrile-co-divinylbenzene (AN/DVB)	Methanol	[202]
Muscadine (*Vitis rotundifolia*) juice pomace/anthocyanins	FPX-66 resin	Three bed volumes of aqueous ethanol (70%)	[203]
Black carrot/anthocyanins	T-10-coded	1% acetic acid solution and methanol	[204]

Pressurized fluid extraction						
Matrix/compounds	**Solvent**	**T (°C)**	**Pressure/ cycles**	**Extraction time**	**Technique**	**References**
Blackberry (*Rubus fruticosus* L.)/anthocyanins	Ethanol:water 50% v/v	100	7.5 Mpa	55 min	UPLC-QToF-MS and UPLC-UV-Vis	[205]
Red grape pomace/ anthocyanins	Ethanol:water 50% v/v	120	90 bar	90 min	LC-Uv-vis	[206]
Solanum stenotomun peel/ antioxidants	Ethanol in water acidified to pH 2.6	80	100 bar	3 h	LC-Uv-vis	[207]
Jabuticaba skins/ anthocyanins	Ethanol	553 K	5 Mpa	31 min	Uv-vis	[208]
Purple-fleshed sweet potato genotypes/ anthocyanins	Acetic acid:methanol:water mixture of 7:75:18% (v/v)	100	1500 psi	20 min	LC-Uv-vis	[209]

Microwave assisted extraction				
Sample/analyte	**Comment**	**Result**	**Technique**	**References**
Blackberry/ anthocyanins	Ethanol was used as solvent. Microwave power of 469 W, a solvent concentration of 52%, a liquid–solid ratio of 25 g mL^{-1} and a time of 4 min	MAE was found to be the most effective method for improving the yield and antioxidant capacity among the other methods tested	Vis	[210]
Blueberry leaves/ anthocyanins	80 mL of solvent consisting of 30% ethanol and 1.5 M citric acid combination in a ratio of 97:3 (v/v)	Time extraction and microwave power level were observed to be significant factors affecting the extraction of phenolic compounds. MAE was the best extraction method among MAE, ultrasonic extraction and 24-h room temperature extraction	Folin-Ciocalteu reagent (spectrophotometric method)	[211]
Black currant marc/ anthocyanins	Maximum yields of anthocyanins were achieved at pH2 with an extraction time of 10 min with a microwave power of 700 W.	A significant reduction of extraction time was achieved using MAE and the final anthocyanin concentration in the solvent phase of MAE increased by 20 % compared to the conventional extraction	LC-DAD	[212]
Mulberry/ anthocyanins	59.6% acidified methanol, 425 W power, 25 (v/w) liquid-to-solid ratio and 132 s time	In comparison with conventional extraction, MAE is more rapid and efficient for extracting anthocyanins from mulberry	LC-MS	[213]

Source: Ampliation from Ref. [39].

Table 4. Selected applications of extraction techniques applied to anthocyanins.

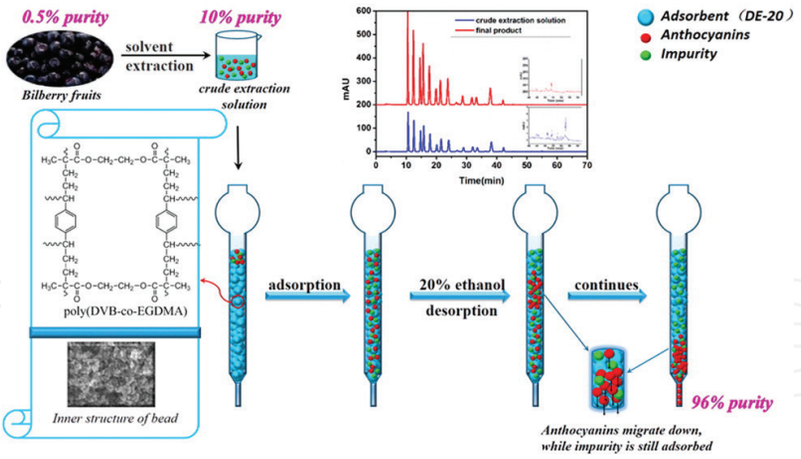


Figure 6. Schematic representation of a highly separation and purification of anthocyanins from bilberry based on macroporus polymeric adsorbent [198].

6. Identification and quantification: a primer

The identification of anthocyanins has a critical role in taxonomic [214] and adulteration [215] studies, besides anthocyanins might replay synthetic days. HPLC, especially in the reversed phase, is the most widely used separation technique. Due to pH-dependent interconversions among various molecular forms of the anthocyanins, a highly acidic mobile phase (pH < 2) is required to ensure that they are maintained predominantly in the flavylium cationic form for maximum chromatographic efficiency. However, even at low pH [31, 32, 35, 110] some interconversion between the anthocyanin flavylium cationic and carbinol pseudobasic forms occurs.

Regarding chromatographic detection techniques for the study of anthocyanins we have [168, 180, 216, 217] diode array detection (DAD) and MS or tandem mass spectrometry (MS/MS) among the most widely used. Spectroscopy is the main technique used due to its simplicity and low cost providing very useful qualitative and quantitative information (anthocyanins have a specific and intense absorbance band in the range of 520–560 nm) [218–220], however the difficulty in obtaining reference compounds and the spectral similarities of the anthocyanins represent important drawbacks.

Various MS instruments, as well as the advances in nuclear magnetic resonance (NMR) have given a fresh impetus to anthocyanin analysis [78, 83, 221]. MS/MS is particularly suited for structure elucidation and compound identification [217, 222, 223] since information pertaining to the aglycone moiety, type and number of sugars and other substituents can be obtained and many of the previously proposed reaction mechanisms for the formation of polymeric anthocyanins and other new pigments have been verified. NMR identification of anthocyanin compounds [78] offers new promising approaches for analysis of complex phenolic mixtures. NMR is based primarily on the analysis of ^{1}H NMR spectra but important structural information can also be provided by ^{13}C NMR [170] and, especially for compounds that have many quaternary carbons, by combining homo and

heteronuclear 2D and 3D techniques. However, the relatively high capital costs are still an impediment [218] to their routine use in enforcement laboratories, a fact that must be taken into consideration.

The almost universal distribution of anthocyanins in flowering plants makes them also suitable for chemotaxonomic considerations [224] both at the family and genus level. Differential anthocyanins profiles may be used [164] for the detection and adulteration in specific commodities of berry fruit products. In the last few years, special attention has been paid [225] to the isolation and characterization of compounds that may delay the onset of aging, as occurs with some berry phenolics. The extremely low levels of anthocyanins usually present in biological samples [57, 168, 181, 186] (blood plasma and body tissues) possess further challenges to their identification and quantification, together with the lack of commercially available anthocyanin standards.

7. Final comments

In last decades, polyphenol chemistry has experienced an explosion of knowledge, being anthocyanins one of the most widely studied groups [27, 226, 227], due to its great potential for practical applications in various fields, contributing in addition this to obtain a better understanding of the chemistry of life.

Anthocyanins occur in all plant tissues including leaves, stems, roots, flowers and fruits imparting color. Anthocyanins are responsible for the red, purple and dark blue colors of many fruits and berries [68, 228–230]. Anthocyanins have antioxidant activity [55, 129, 231–233] preventing radical formation. These nontoxic natural pigments have received considerable attention from such as food, pharmaceutical and nutritional industries due to their potential applications in color-processed food and medicines [31, 33, 147, 234] which may replace synthetic dyes.

It was only a few decades ago that anthocyanins were regarded as highly degradable compounds and the research studies mainly were focused on their chemical structures, color stability, use as food constituents and changes in foods during storage. Anthocyanins are now recognized as food constituents with potential health benefits [102, 131] and research related to these properties has markedly progressed at the molecular level. Anthocyanins will continue to attract researchers across various disciplines, including those involved in the creation of new flower varieties with novel colors. Research on the health benefits of anthocyanins will provide information [172] on underlying molecular mechanisms and absorption and metabolism. Moreover, once these benefits are proven in humans, development of foods and dietary supplements in a capsule form [164, 235, 236] can be accelerated to promote the proven functions, i.e. berry extracts are being commercialized as nutraceuticals and as dietary supplements to fulfill consumer demands.

The development of analytical techniques to determine the identity and quantities of anthocyanins in natural products, as well as their effects *in vivo* and *in vitro*, is challenging. Up to

date, there is no universal extraction procedure suitable for extraction of all plant phenolics. The choice of an extraction method should maximize pigment recovery [237] with a minimal amount of adjuncts and minimal degradation or alteration of the natural state.

Solvent extraction involving the use of acidic solvents has been the most commonly used method [36, 79] for the recovery of diverse compounds found in flavonoids, including anthocyanins. The traditional solid-liquid or liquid-liquid extraction offers good recovery. Nevertheless, they are often described as laborious, time and solvent consuming and prone to errors. However, in recent years there are trends toward other environmentally and economically friendlier extraction techniques [38, 39, 110, 167, 184, 206, 238] using a smaller amount of (nontoxic) solvents and sample sizes, reducing working time and increasing selectivity, specificity, recovery and potential of automation. MAE, SFE, PLE, or PHWE are among the greener techniques that have experienced a large increase in recent years to extract anthocyanins from plant material and other samples.

Abbreviations

CCC	Countercurrent chromatography
DAD	Diode array detection
EDTA	Ethylenediaminetetraacetic acid
HHP	High hydrostatic pressure extraction
HPLC	High-performance liquid chromatography
HSCCC	High-speed countercurrent chromatography
MAE	Microwave-assisted extraction
MS	Mass spectrometry
MS/MS	Triple quadrupole mass spectrometer
MTBE	Methyl tert-butyl ether
NMR	Nuclear magnetic resonance
PHWE	Pressurized hot water extraction
PLE	Pressurized liquid extraction
QToF	Quadrupole-time of flight
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
UPLC	Ultra performance liquid chromatography
Uv-Vis	Ultraviolet-visible spectroscopy

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