Recent Advances in Polyphenol Research

Volume 2

Edited by

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Recent Advances in Polyphenol Research
A series for researchers and graduate students whose work is related to plant phenolics and polyphenols, as well as for individuals representing governments and industries with interest in this field. Each volume in this biennial series will focus on several important research topics in plant phenols and polyphenols, including chemistry, biosynthesis, metabolic engineering, ecology, physiology, food, nutrition, and health.

Volume 2 editors:
Celestino Santos-Buelga, Maria Teresa Escribano-Bailon, and Vincenzo Lattanzio

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Dedication

To Edwin Haslam – a very good friend of Groupe Polyphénols – whose studies of plant polyphenols (vegetable tannins) were "seminal" in the development of this area of science.

Acknowledgements

Contents

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Preface

Plant phenolics are secondary metabolites that constitute one of the most common and widespread group of substances in plants and that have been considered for a long time waste products of primary metabolism. Nowadays, plant phenols and polyphenols are considered to have a large and diverse array of beneficial effects on both plants and humans. The ability to synthesize secondary compounds has been selected throughout the course of evolution in different plant lineages when such compounds addressed specific needs. Secondary metabolites apparently act as defence (against herbivores, microbes, viruses, or competing plants) and signal compounds (to attract pollinating or seed-dispersing animals), as well as protect the plant from ultraviolet radiation and oxidants. Therefore, they represent adaptive characters that have been subjected to natural selection during evolution. In addition, biomedical research has revealed that dietary phenolics, because of their antioxidant and free radical scavenging properties, play important roles in the prevention of many of the major contemporary chronic diseases.

The diversity of structure and activity of phenolic compounds resulted in the multiplicity of research areas such as chemistry, biotechnology, ecology, physiology, nutrition, medicine, and cosmetics. The International Conference on Polyphenols, organized under the auspices of *Groupe Polyphénols*, is a unique opportunity for scientists in these and other fields to get together every other year and exchange their ideas and new findings.

The last edition of the conference (the 24th edition) was hosted by the University of Salamanca, Spain, from July 8 to 11, 2008, and covered five topics:

1. *Chemistry*: Structure, reactivity, physicochemical properties, analytical methods, synthesis ….
2. *Biosynthesis and metabolic engineering*: Molecular biology, omics, enzymology, gene expression and regulation, biotechnology ….
3. *Roles in Plant Ecophysiology and Environment*: Plant growth and development, biotic and abiotic stress, resistance, sustainable development, by-products valorization ….
4. *Food and Beverages***:** Composition, organoleptic properties, impact of processing and storage, functional foods, nutraceuticals ….
5. *Health and Disease*: Medicinal properties, mode of action, bioavailability and metabolism, cosmetics ….

Some 450 participants from 41 countries attended Salamanca's Conference, where over 370 presentations were made, including 330 posters, 31 selected oral communications,

and 12 invited lectures made by acknowledged experts. The present second volume in the series includes chapters from the guest speakers and some invited contributors.

The 24th International Conference on Polyphenols would not have been possible without the generous support of public and private donors such as the Spanish *Ministerio de Ciencia e Innovación*, *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA), *Junta de Castilla y León*, and *Caja Duero*. Furthermore, we are also indebted to the Natraceutical Group, Indena, "Viñas del Jaro" wine cellars, and Phytolab that also sponsored the conference. Our sincere thanks to all of them.

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Celestino Santos-Buelga,
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Chapter 1

The Visible Flavonoids or Anthocyanins: From Research to Applications

Raymond Brouillard, Stefan Chassaing, Géraldine Isorez,
Marie Kueny-Stotz, and Paulo Figueiredo

1.1 Introduction

Anthocyanins are polyphenolic pigments responsible for most of the color diversity found in plants. Here the *in vivo* color expression and the stability of anthocyanins are interpreted by extrapolation of the results acquired *in vitro* with model solutions of pigments obtained through plant extraction or laboratorial synthesis. Behavior of anthocyanins is explained in terms of molecular interactions of the chromophore units with parts of the pigments themselves and/or with some constituents of the plant cell. These include, among others, diverse polyphenols, metal cations, and inorganic salts. Attention is also given to the biophysicochemical environment found in plant vacuoles that plays a fundamental role on the intermolecular and intramolecular associations displayed by anthocyanins. For example, anthocyanin *Z*-chalcones (retrochalcones) provide an unexpected open cavity for the ferric cation. Medicinal, nutritional, and industrial applications of anthocyanins are proposed.

Colors are conferred to plants by chlorophylls, carotenoids, and flavonoids (Britton, 1983). Chlorophylls are responsible for the green colors displayed by the leaves, whereas carotenoids provide some of the red-orange hues often found in fruits, flowers, and other plant constituents. Flavonoids belong to a larger family, the polyphenols, and can be found in most flowers and fruits (Brouillard & Dangles, 1993; Andersen & Jordheim, 2006). They include the principal elements responsible for the color diversity found in the plant world, the anthocyanins (Fig. 1.1). In fact, these pigments are the only polyphenols that possess the ability to absorb light both in the ultra-violet and in all the visible range (from yellow-orange to bluish-green) (Goto & Kondo, 1991). It is well known that anthocyanins are at the origin of plants' most brilliant colors, a phenomenon particularly visible from flowers. Nevertheless, there exists only one chromophore – the flavylium nucleus – whose subtle interactions with vacuole biochemicals, including water, are capable of providing all kind of colors.

Anthocyanins are stored in an organized aqueous medium in the cell vacuoles. A slightly acidic environment (pH 3–5; Stewart *et al.*, 1975) rich in inorganic ions and other polyphenols is essential for the transformations in these pigments that enable the formation of molecular complexes and subsequent color changes and stabilization (Brouillard & Dangles, 1993).

The basic structure common to almost all anthocyanins is a 2-phenylbenzopyrylium (flavylium) heterocyclic skeleton bearing at least one sugar residue. Aliphatic or aromatic organic acids may esterify the sugar hydroxyls. Furthermore, OH and OCH_3 groups that bestow the characteristic names of the six basic anthocyanic structures (Table 1.1) typically substitute the B-ring of the aglycone moiety of these pigments. The existence of at least one free OH group is needed to produce the structural changes, described later, conducing to color variation. The structure presented in Fig. 1.2 depicts the positively charged flavylium cation, which is the dominant equilibrium form in strongly acidic aqueous solutions. The positive charge is delocalized through all the pyrylium moieties, although carbons 2 and 4 are the more positively charged atoms (Amić *et al*., 1990). The relative ease of deprotonation of the two OH groups at positions 4′ and 7 contributes to the color changes of the anthocyanin. One of these hydroxyls loses a proton at pH ∼ 4, producing the quinonoid bases AH (Fig. 1.3) that exhibit a chromatic deviation toward longer
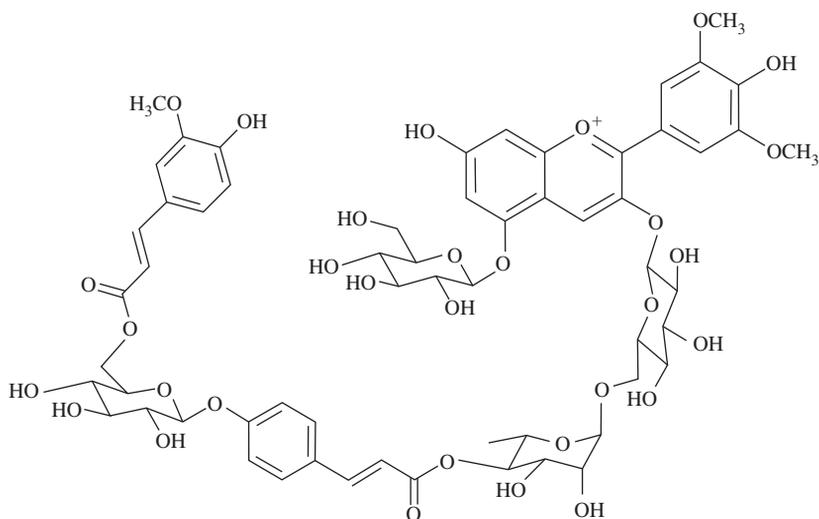
Fig. 1.1 Structure of one of the numerous anthocyanins isolated from violet petals of *Petunia hybrida* cv. Festival (Gonzalez *et al.*, 2001).

Table 1.1 Anthocyanins are glycosylated polyphenols with a basic C-15 skeleton hydroxylated at positions 4′ and 7 that can be divided in six basic structures according to the pattern of the substituents at positions 3′ and 5′.

Anthocyanidin common name	3′ and 5′ substituents
Pelargonidin	H/H
Cyanidin	OH/H
Peonidin	OCH_3/H
Delphinidin	OH/OH
Petunidin	OH/OCH_3
Malvidin	OCH_3/OCH_3
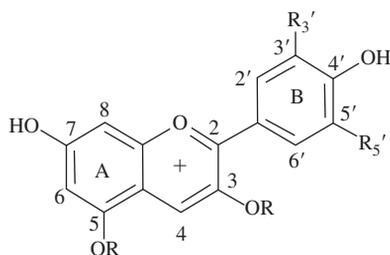
Fig. 1.2   The anthocyanin flavylium chromophore, a carboxonium cation stable in aqueous media. R is usually sugar or acylated sugar.

wavelengths relative to the flavylium cation (AH_2^+). At pH close to neutrality, a second deprotonation occurs leading to the formation of the anionic quinonoid bases (A^-), with another blue shift in the absorption spectrum. Moreover, the flavylium cation is susceptible to nucleophilic attack at the charge-defective positions 2 and/or 4, as evident from the strong electronic density calculated for the frontier lowest unoccupied molecular orbital (LUMO). When in an aqueous environment, the water molecules, available in large quantity, add to the flavylium form at pH values above 1.5–2.0, resulting in a loss of color owing to the formation of the colorless hemiketal adduct (BH_2) through a slow pseudo acid-base equilibrium. This may eventually be followed by a ring opening that leads to the formation of the retrochalcones (C_E and C_Z), which are also almost colorless. This loss of color can be reversed by a simple reacidification with complete recovery of the colored flavylium cation.

In the laboratory, aqueous solutions of anthocyanins, even kept under physicochemical conditions (temperature, pH, light, oxygen) similar to the ones found in plant vacuoles, tend to lose their bright colors either by formation of the colorless species or by degradation leading to the irreversible cleavage of the molecule (Furtado *et al.*, 1993; Figueiredo, 1994). However, *in planta*, the colorless forms BH_2, C_E, and C_Z are rarely found and the colors last for several days or even weeks, indicating the existence of vacuolar mechanisms that stabilize the colored species. Moreover, the same anthocyanin can be found in flowers of different tints, a fact that indicates the existence of diverse interactions of the pigment with the cellular environment. Among the stabilizing mechanisms found in the plant world, the most widespread are copigmentation and metal complexation or even combinations of the two (Goto & Kondo, 1991). The first one was found to be present in some flowers and its behavior in model solutions was thoroughly investigated (Robinson & Robinson, 1931; Brouillard, 1981, 1983; Brouillard *et al.*, 1989, 1991; Dangles & Brouillard, 1992a,b; Wigand *et al.*, 1992; Dangles *et al.*, 1993a,b; Dangles & Elhajji, 1994; Figueiredo *et al.*, 1996b), whereas the second is expected to occur between all anthocyanins possessing a catechol group in their B-ring and small divalent and trivalent metal cations (Dangles *et al.*, 1994a; Elhabiri *et al.*, 1997). In this chapter, we give more insight to these phenomena by means of an investigation on the interactions between several metals and a series of natural and synthetic anthocyanic pigments bearing different substitution patterns. New views on anthocyanin iron complexation, as well as some thoughts on possible applications, are also developed.
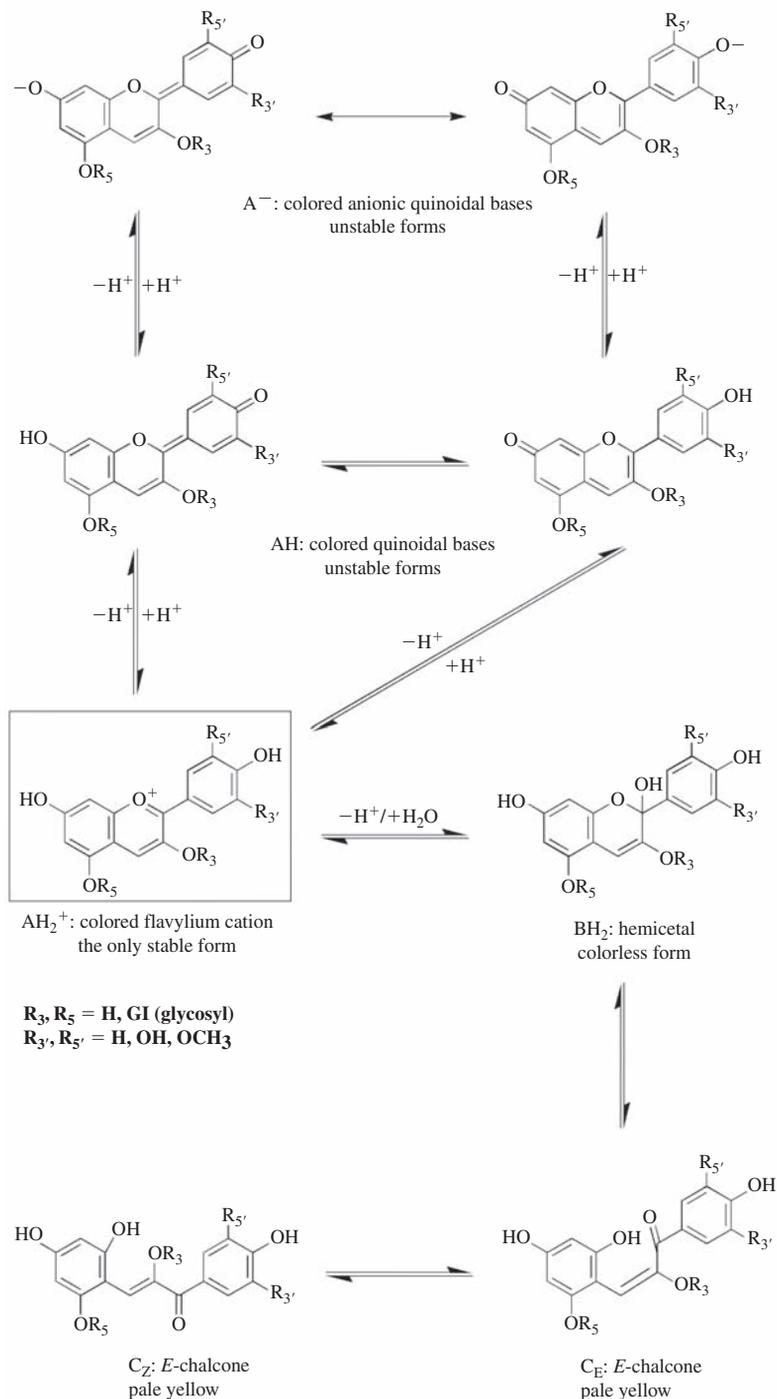
Fig. 1.3 Anthocyanin equilibria in aqueous solution and the corresponding structural transformations. AH_2^+ represents the flavylium cation that predominates at acidic pH values; AH represents the two tautomeric quinonoid bases; A^- depicts the anionic quinonoid bases that appears in alkaline solutions; BH_2 is the colorless hemiketal adduct; and C_E and C_Z are isomeric retrochalcones.

1.2 Copigmentation of anthocyanins

Copigmentation or anthocyanin color exaltation results from the presence of special molecules or copigments in an aqueous environment. This phenomenon is known for long, but even today, nothing comparable has been uncovered from the rest of the huge polyphenol family or any other class of organic molecules.

Copigmentation can be defined as a hydrophobic π–π molecular interaction, through a vertical stacking, between a planar anthocyanin structure (flavylium cation or quinonoid base) and another planar molecule possessing no color by itself, which results in an enhancement, and generally a modification, in the original color of the pigment-containing solution. Most polyphenols can act as copigments, their efficiency depending on their chemical structures. However, other families of molecules were also found to include good copigments, for example, purines and alkaloids (Elhabiri *et al.*, 1997), and several more will probably be uncovered as further investigations are on the way.

This loose association between the copigment and one of the colored forms of the anthocyanin, generally the flavylium cation, produces, in electronic absorption terms, both hyperchromic and bathochromic shifts (Asen *et al.*, 1972). Such spectral changes can be explained by (1) a partial desolvation of pigment and copigment molecules when the water molecules rearrange around the newly formed complex, allowing a closer contact between both structures (copigmentation generally originates 1:1 complexes) with the consequent formation of more chromophores owing to a more difficult access of the solvent molecules to the electrophilic site C-2 (hyperchromism) and (2) the change in polarity in the immediate vicinity of the anthocyanin brought about by the displacement of some water molecules by the less polar organic copigment (bathochromism).

The color enhancement effect is more spectacular in mildly acidic solutions than in very acidic solutions owing to the existence, at pH 3–4, of a large amount of colorless hemiketal and chalcone forms that may be turned into flavylium cations or quinonoid bases through the formation of copigmentation complexes, resulting in the striking color changes. By contrast, in strong acidic solutions all the anthocyanins are already in the colored flavylium form, therefore the copigmentation becomes an ordinary molecular association accompanied by a small hypochromic shift together with the always-present bathochromic shift (Dangles & Brouillard, 1992b).

In addition to UV-visible absorption spectroscopy, copigmentation can also be followed by[1] H NMR techniques, which provide further evidence of the formation of a 1:1 vertical stacking complex between the pigment and copigment molecules (Wigand *et al.*, 1992).

What is described earlier concerns a particular aspect of copigmentation – *intermolecular copigmentation* – that is, the interaction between two separate identities; however, a second type of association can also occur: *intramolecular copigmentation*. This type of molecular interaction can take place with only those anthocyanins that possess at least one copigment residue covalently bound to the pigment. Such residues are generally cinnamic ester derivatives attached to the chromophore through one or more sugar units that may act as "linkers" or "spacers" (see Fig. 1.1 for an example of such a molecule), allowing the interaction of its π-orbitals with the benzopyrylium nucleus (Goto & Kondo, 1991; Yoshida *et al.*, 1992; Dangles *et al.*, 1993a,b; Figueiredo *et al.*, 1996a).

Intramolecular copigmentation acts in a way similar to the one described for inter-molecular copigmentation, with the entropic advantage of the copigment being directly attached to the chromophore and consequently the nonrequirement of bringing together two molecules initially separated in solution. Those particular structures give rise, not so infrequently as one might imagine, to pigments that are continuously colored through a very wide range of pH values (Brouillard, 1981; Dangles *et al.*, 1993a,b; Figueiredo *et al.*, 1996a). Given the required number and flexibility of the linkers, some of these "internal" copigments can even adopt a sandwich-type conformation around the chromophore, pro-viding a very effective protection against hydration and subsequent loss of color (Dangles *et al.*, 1993b). In fact, while investigating the Orchidacea family, a group of anthocyanins that present no hydration at all, *in vitro*, was found. A natural pigment extracted from the blue-purple flowers of *Eichhornia crassipes* was found to covalently link a 7-glucosylapigenin (a flavone) to a 3-gentiobiosyldelphinidin (an anthocyanin) through a dimalonyl ester spacer (Toki *et al.*, 1994a; Figueiredo *et al.*, 1996a). Owing to the matching configuration of the two polyphenolic moieties, this molecule gives rise to a highly effective stacking complex, with a very low-value hydration constant, leading us to forecast the existence of a wider distribution of similar examples in nature.

Copigmentation is an exothermic process with unfavorable entropy changes. In aqueous solution, copigmentation increases with temperature diminution and decreases with tem-perature rise, becoming completely negligible when the temperature reaches close to the boiling point of water (Brouillard *et al.*, 1989; Dangles & Brouillard, 1992a). Formation constants not larger than 100–300 M^{-1} (25°C, in water) were found for this type of asso-ciation, indicating the existence of weak molecular interactions that permit the existence of a chemical equilibrium between the complexed and noncomplexed forms. Interaction of anthocyanins with proteins is of a different essence (Haslam, 2001), but it poses the inter-esting problem to know which of the numerous anthocyanin secondary structures is the reactive species.

1.3 Formation of inclusion complexes

A phenomenon until now observed only in the laboratory and that can still be included in the field of molecular interaction is the formation of inclusion complexes of anthocya-nins with the natural cyclodextrin macrocycles (Dangles & Brouillard, 1992c; Dangles *et al.*, 1992a,b). However, instead of leading to color stabilization, these complexes seem to decrease the anthocyanin visible absorption band. This is always the case with the small natural and synthetic anthocyanins studied up to the present, as the common α-, β-, and γ-cyclodextrins cannot accommodate bigger, highly substituted pigments. β-Cyclodextrin is the one that produces a more pronounced diminution of color inten-sity, a phenomenon that is known as anti-copigmentation (Dangles *et al.*, 1992a,b). This phenomenon is caused by selective inclusion and stabilization of the extremely flexible *Z*-chalcone into the macrocyclic cavity, with the consequence of shifting the pigment equilibria toward the formation of more colorless chalcone forms. Howbeit these results, it is not impossible to imagine that greater macrocycles will be able to preferentially

accommodate the colored flavylium or quinonoid forms, thus favoring their persistence in model solutions.

1.4 Ion-pair formation

Another aspect of molecular interactions that was verified in the laboratory and can also take place *in vivo* is the color enhancement of anthocyanin-containing solutions when molar quantities of ionic salts are added (Goto *et al.*, 1976; Figueiredo & Pina, 1994). This phenomenon is interpreted in terms of an ion-pair association between the mineral anion and the cationic flavylium form of the pigment that increases the production of this colored form, via the displacement of the equilibria depicted in Fig. 1.3. At the same time, through the proximity of the anion to the electrophilic C-2 atom of the chromophore (evidenced through ^{1}H NMR experiments; Figueiredo & Pina, 1994), it hinders the approach and attack of nucleophilic molecules. Very recently, a series of flavylium salts with the unusual hexafluorophosphate counterion have been prepared (Chassaing, 2006; Chassaing *et al.*, 2007; Kueny-Stotz *et al.*, 2007). The role of the anion, within the synthetic route, was also taken into consideration probably for the first time.

1.5 Metalloanthocyanins

All anthocyanins possessing a catechol structure in their B-ring, that is, all derivatives of cyanidin, delphinidin, and petunidin (cf. Table 1.1), are known to have the capacity of complex formation with several small divalent and trivalent metal cations. This type of association has been demonstrated to be at the origin of the blue color in some flowers (Goto & Kondo, 1991; Brouillard & Dangles, 1993; Kondo *et al.*, 1994a,b). Metals most commonly found in the formation of such metalloanthocyanins are iron (III), magnesium (II), and aluminum (III). Metal complexation was also observed between Al^{3+} or Ga^{3+} and anthocyanins possessing OH substituents at positions 7 and 8, whereas those with a catechol at positions 6 and 7 were shown not to form such complexes. The complexation results from an interaction between the metal center and the anionic quinonoid base that results from the deprotonation at positions 4′ and 7. Anionic bases resulting from deprotonation at position 3′ have higher energies than those that result from deprotonation at positions 4′ and 7 (Table 1.2). The introduction of a 6-oxygen diminishes the probability of hydration, and thus the formation of colorless forms, which favors the formation of the quinone at position 4′.

The color changes (bathochromic and hyperchromic shifts) observed when Al^{3+} is added to anthocyanin-containing solutions are known for a long time and used as a qualitative test for the presence of anthocyanins possessing the B-ring catechol group in plant extracts (Bayer *et al.*, 1966). A quantitative interpretation of this type of association, from the thermodynamic and kinetic points of view, was achieved by Dangles *et al.* (1994a). These authors demonstrated that the metal cation binds to the colored forms of the pigment and that there is a pH domain where the hyperchromic effect owing to the complexation is at a maximum. In the present work, we extended these experiments to a series of anthocyanic

Table 1.2   Relative energies (kcal mol^{-1}) of quinonoid (AH) and anionic quinonoid (A$^-$) bases of **S3**.

AH(7)	AH(4′)	A$^-$(7/4)/A$^-$(4′,7)	A$^-$(7,3′)	A$^-$(4′,3′)
0	1.4	0	12.7	9.1

The values were obtained through AM1 calculations.

pigments ranging from simpler synthetic ones to the more complex natural acylated pigments, including the following: 3′,4′,7-trihydroxyflavylium chloride (**S1**); 3′,4′-dihydroxy-7-methoxyflavylium chloride (**S2**); 3′,4′,7-trihydroxy-3-methoxyflavylium chloride (**S3**); 3′,4′-dihydroxy-3,7-dimethoxyflavylium chloride (**S4**); 2-((3′,4′-dihydroxy)-benzo)-3-*O*-methyl-naphto[2,1-b]pyrylium chloride (**S5**); 3-*O*-β-D-glucopyranosyl delphinidin (**N1**); 3-*O*-(6-*O*-(6-deoxy)-α-L-mannosyl)-β-D-glucopyranosyl cyanidin (**N2**); 3,5-di-*O*-β-D-glucopyranosyl cyanidin (**N3**); 3-*O*-(6-*O*-(*trans*-p-coumaryl)-2-*O*-(2-*O*-(*trans*-synapyl)-β-D-xylopyranosyl-β-D-glucopyranosyl)-5-*O*-(6-*O*-(malonyl)-β-D-glucopyranosyl   cyanidin (**N4**);   3-*O*-(6-*O*-(*trans*-caffeyl)-2-*O*-(2-*O*-(*trans*-synapyl)-β-D-xylopyranosyl-β-D-glucopyranosyl)-5-*O*-(6-*O*-(malonyl)-β-D-glucopyranosyl cyanidin (**N5**); 3-*O*-(6-*O*-(*trans*-coumaryl)-β-D-glucopyranosyl)-5-*O*-((6-*O*-malonyl)-β-D-glucopyranoside) delphinidin (**N6**); and 3-*O*-(6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-3-*O*-(β-glucopyranosyl)-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside)-5-*O*-((6-*O*-malonyl)-β-D-glucopyranoside) delphinidin (**N7**). **S** pigments were synthesized, whereas the seven **N** pigments were extracted from plant materials. Aluminum (III), gallium (III), and magnesium (II), as chloride salts, were the metals used to investigate the complexation abilities of these pigments. Pigments **N1**–**N7** were isolated according to published procedures (Lu *et al.*, 1992; Saito *et al.*, 1993; Toki *et al.*, 1994b). The synthetic pigments **S1**–**S5** were prepared according to procedures described elsewhere (Dangles & Elhajji, 1994; Elhabiri *et al.*, 1995a,b, 1996, 1997).

The strong affinity for the flavylium cation, in a pH range 2.0–4.0, shown by metal cations such as Al^{3+} and Ga^{3+}, comes from the exceptionally high acidity of the 4′-OH (or 7-OH). As a matter of fact, the conjugated base of AH$_2^+$ is not a simple phenolate ion but a quinonic structure, stabilized by its π electrons delocalization. This yields a pK_a of 3.5–5.0 for the pair AH$_2^+$/AH, which is lower than the one typically found for a catechol/catecholate pair (9.0). Thus, the complexation of AH$_2^+$ requires the substitution of only a slightly acidic proton (3′-OH) as opposed to the substitution of two slightly acidic protons on the colorless forms, a thermodynamically less favored process. In this way, metal complexation and hydration are two competitive processes, that is, the addition of a metal cation to a slightly acidic anthocyanin solution results in a bathochromic shift of the absorption spectrum, which reflects a displacement of the hydration equilibrium toward the flavylium cation. The anthocyanin adopts a quinonic structure when the complex is formed and it is this structure (analogous to that of form AH) that explains the strong bathochromic shift.

The following set of reactions expresses the equilibria involved when one of these metal cations (M^{3+}) is put into contact with a moderately acidic, anthocyanin-containing, aqueous solution. B′H$_2$ is a simplified representation of the ensemble of colorless forms.

$$AH_2^+ \rightleftarrows AH + H^+ \qquad K_a$$