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
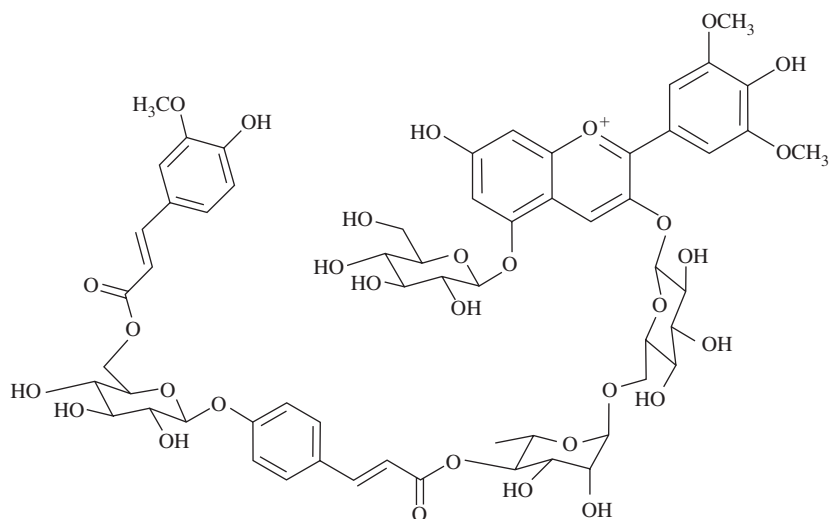

Fig. 1.1 Structure of one of the numerous anthocyanins isolated from violet petals of *Petunia hybrida* cv. Festival (Gonzalez *et al.*, 2001).

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′.

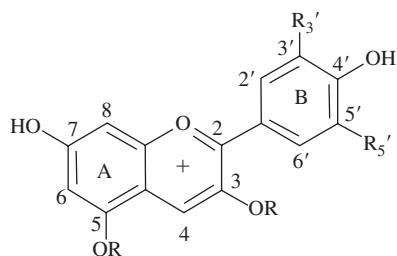| Anthocyanidin common name	3′ and 5′ substituents
Pelargonidin	H/H
Cyanidin	OH/H
Peonidin	OCH_3/H
Delphinidin	OH/OH
Petunidin	OH/OCH_3
Malvidin	OCH_3/OCH_3

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.
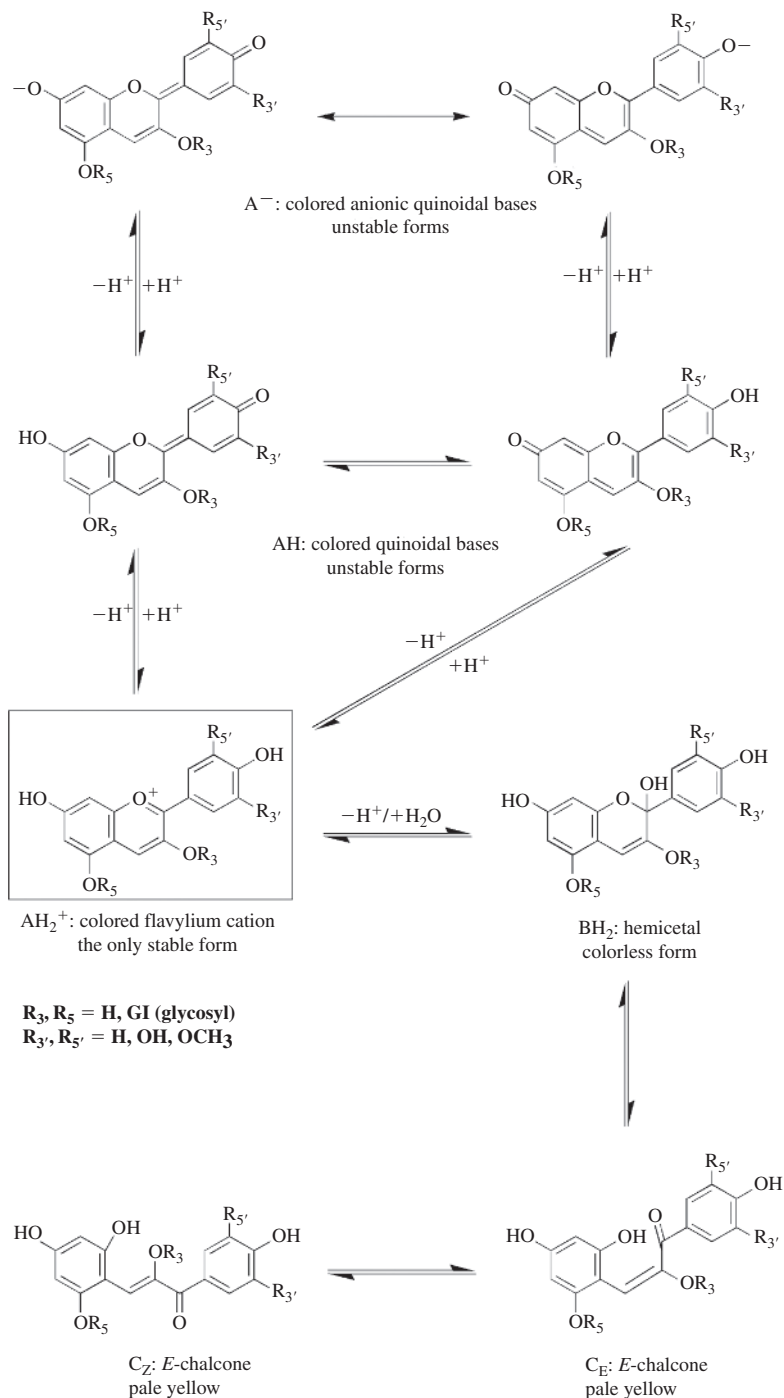
Fig. 1.3 Anthocyanin equilibria in aqueous solution and the corresponding structural transformations. AH_2^+ represents the flavylium cation that predominates at acidic pH values; AH represents the two tautomeric quinonoid bases; A^- depicts the anionic quinonoid bases that appears in alkaline solutions; 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

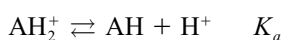**Table 1.2** Relative energies (kcal mol^{-1}) of quinonoid (AH) and anionic quinonoid (A$^-$) bases of **S3**.

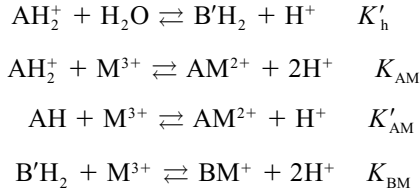| AH(7)	AH(4′)	A$^-$(7/4)/A$^-$(4′,7)	A$^-$(7,3′)	A$^-$(4′,3′)
0	1.4	0	12.7	9.1

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.

$$\text{AH}_2^+ \rightleftarrows \text{AH} + \text{H}^+ \qquad K_a$$

$$AH_2^+ + H_2O \rightleftarrows B'H_2 + H^+ \qquad K_h'$$

$$AH_2^+ + M^{3+} \rightleftarrows AM^{2+} + 2H^+ \qquad K_{AM}$$

$$AH + M^{3+} \rightleftarrows AM^{2+} + H^+ \qquad K_{AM}'$$

$$B'H_2 + M^{3+} \rightleftarrows BM^+ + 2H^+ \qquad K_{BM}$$

K_h' includes the hydration step, the very fast ring opening that transforms the hemiketal into the *E*-chalcone and the isomerization of this form into the *Z*-chalcone (Brouillard & Lang, 1990; Santos *et al.*, 1993). For most natural anthocyanins C_Z is formed in only minor quantities (Santos *et al.*, 1993) and thus disregarded. AM^{2+} represents the 1:1 metal complexes formed by the colored forms of the pigment and BM^+ represents the ones formed by the colorless hemiketal and/or chalcones. As the work was performed at a pH below 5, the ionized quinonoid bases, which are very minor species in this pH range, and the equilibrium constant K_{AM}' can both be neglected. Although the trivalent metal cations studied possess an octahedral structure that allows the coordination of 1, 2, or 3 bidentate ligands, and thus, the formation of 1:1, 1:2, and 1:3 complexes, in the acidic media used throughout these studies only 1:1 complexes are formed, and for that reason, all other stoichiometries were neglected in the calculations.

The values of K_h' and K_a are easily obtained from spectrophotometric measurements (Dangles *et al.*, 1993a,b; Figueiredo *et al.*, 1996a). The values for the complexation constants K_{AM} and K_{BM} can also be obtained in a similar way, through a simple mathematical model that takes into account the fact that, at a given visible wavelength, the absorbance can be expressed as

$$D = \varepsilon_{AH_2^+}[AH_2^+] + \varepsilon_{AH}[AH] + \varepsilon_{AM^{2+}}[AM^{2+}] \tag{1}$$

where ε denotes the molar absorption coefficients. The total concentration of pigment is written as

$$C_T = [AH_2^+] + [AH] + [AM^{2+}] + [B'H_2] + [BM^+] \tag{2}$$

Equations (1) and (2) are then combined with the thermodynamic constants K_a, K_h', K_{AM}, and K_{BM} to give

$$D = \frac{D_0 + D_1(K_a/[H^+]) + D_2(K_{AM}[M^{3+}]/[H^+]^2)}{1 + \{(K_a + K_h')/[H^+]\} + (K_{AM}[M^{3+}]/[H^+]^2) + (K_h' K_{BM}[M^{3+}]/[H^+]^3)} \tag{3}$$

where D_0, D_1, and D_2 stand for $\varepsilon_{AH_2^+} C_T$, $\varepsilon_{AH} C_T$, and $\varepsilon_{AH^{2+}} M C_T$, respectively. D_0 can be determined in a pH < 1 anthocyanin solution where AM_{2+} is the sole existing species, and D_1 and D_2 are additional floating parameters in the calculations, as none can be obtained through direct spectroscopic measurements. By varying the pH in aqueous solutions of anthocyanin and metal at fixed concentrations, one can obtain a curve fitting eq. (3)

that yields the values for K_{AM} and K_{BM}. To cross-check the value of K_{AM}, experiments are performed at variable metal concentrations and fixed pH (around 3.5–4.5, depending on the pigment concerned), where both AH_2^+ and BM^+ can be neglected. Under these conditions, eq. (3) becomes:

$$\frac{1}{D} = \frac{1}{D_2} + \frac{K'_h}{D_2(K_{AM}[M^{3+}]/[H^+])} \tag{4}$$

Additional simplification arises from $K'_h/[H^+] \gg 1$. A $1/D$ versus $1/[M^{3+}]$ plot gives a straight line from which K_{AM} can be readily obtained (Dangles *et al.*, 1994a).

The pK_{AM} values obtained for the present series of pigments, with different types of substituents, increase as one moves from the more complex acylated and malonylated anthocyanins to the simpler synthetic ones. This is caused by the distinctive molecular characteristics of the complex natural anthocyanins. On the one hand, pigment acylation selectively leads to complex formation between the small metal cations and the colored forms of anthocyanin (flavylium and quinonoid bases) that are preferentially stabilized through intramolecular copigmentation. On the other hand, the malonyl groups covalently bonded to a sugar unit of the pigment seem to participate in the deprotonation of the hydroxyl at position 7 of the chromophore, through the formation of a hydrogen bond, leading to quinonoid base formation at a pH lower (2.01 for **N4** and 2.26 for **N5**) than the one currently found for the majority of flavylium cations (Figueiredo *et al.*, 1996b; Elhajji *et al.*, 1997). This assumption is supported by MM+ (Allinger, 1977) molecular orbital calculations, performed in a computer-simulated water solution, as this is the medium predominantly found in plant vacuoles. The computed interatomic distances between the malonyl group and the hydroxyl at position 7 range from 280 to 320 pm, consistent with the existence of a hydrogen bond, which generally has a bond length of ca. 300 pm (Rice, 1975; Franks, 1984; Scheiner, 1994).

Among the three tested metals, we found that gallium (III) is the one that produces the most spectacular color modifications and that magnesium (II) seems to preferentially coordinate colorless forms of the pigments, independently of the pH used.

Although a variety of colors ranging from reddish to bluish may be obtained through the methodology used in this work to achieve metal complexation of anthocyanins, a pure blue color could not be attained with these binary complexes. As some authors (Brouillard & Dangles, 1993; Kondo *et al.*, 1994a,b) postulated that ternary complexes anthocyanin:metal: copigment are necessary to produce blue colors, addition of adenosine 5′-monophosphate (AMP) to a solution containing the anthocyanin **S3** and Al^{3+} was performed (Dangles *et al.*, 1994b; Elhabiri, 1997). AMP was chosen owing to its ability to both form an intermolecular association with the pigment through its purine system and chelate the metal cation through the phosphate group. At pH 3.75, a spectacular gain of color (hyperchromic and bathochromic shifts) is observed, which can be interpreted only in terms of an intramolecular copigmentation within the ternary molecular complex formed, the metal being chelated to both the pigment and the copigment.

To further stress the above-mentioned assumption, tests were performed by adding the nonphosphated purine or the ribose phosphate to the metal–anthocyanin mixture. In both

cases, the color changes were not of the same magnitude as those observed with the previous system. This brief study shows that simultaneous metal complexation of a pigment and a copigment may originate a covalent link capable of strengthening the copigmentation effect. Thus, the results obtained seem to confirm those brought forth by other authors (Takeda *et al.*, 1984, 1990), suggesting that a combination of metal complexation and copigmentation is necessary to express the blue color.

1.6 *Z*-Chalcones: unexpected open cavities for the ferric cation

Anthocyanins reacting with aluminum (III) always possess a catechol group in their B-ring (Asen *et al.*, 1969; Takeda, 1977; Harborne, 1989; Elhabiri *et al.*, 1997). Formation of aluminum–anthocyanin complexes provides color variation and stabilization to the pigment-containing media. Anthocyanins with a catechol moiety do not behave the same way in the presence of iron (III) as they do in the presence of aluminum (III). The difference is really striking: instead of the strong color stabilization and variation seen with Al^{3+}, loss of color occurs! Moreover, the iron (III) bleaching effect was also observed with the anthocyanins devoid of a catechol group like malvin (George, 1998; George *et al.*, 1999). It was concluded that the ferric cation bleaching effect had nothing to do with the presence or the absence of an α-dihydroxy moiety in the anthocyanin structure. A few results in the case of malvin chloride are given in the following text.

Fig. 1.4 represents the UV-visible spectra of a pH 1 aqueous solution containing malvin chloride with ferric chloride in excess. Three characteristic features had emerged from that experiment: (1) the equilibrium state is reached only after 17 h (25 °C); (2) two isosbestic points appear at 284 and 362 nm; and (3) during the kinetic course, the flavylium
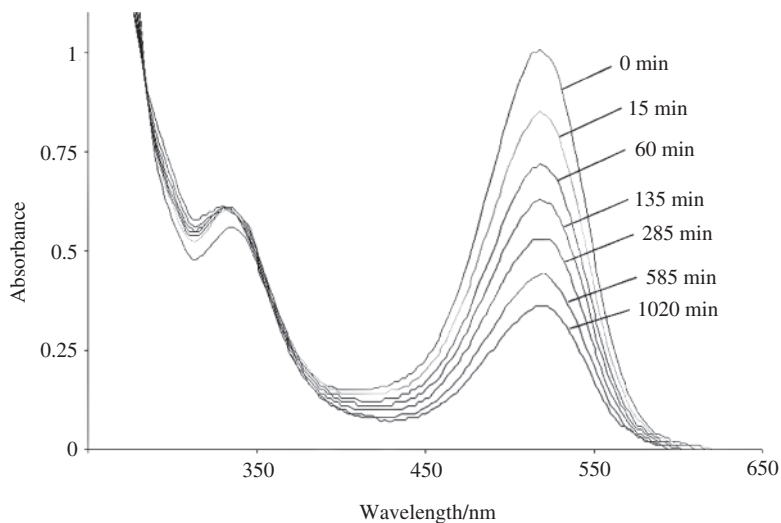

Fig. 1.4   Time evolution of the UV-visible absorption of malvin chloride at pH 1, in the presence of an excess of ferric chloride (George *et al.*, 1999).
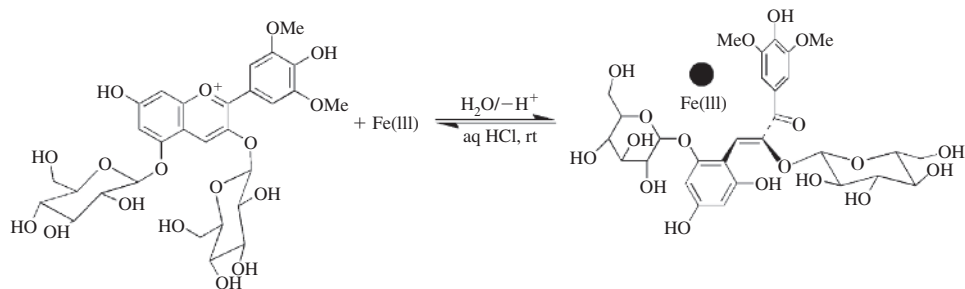
Fig. 1.5 The flexible open cavity of the malvin *Z*-chalcone best fits the steric and electronic requirements of the ferric ion in water (George *et al.*, 1999).

chromophore visible band remains *unaffected both in shape and in position*. In the presence of Fe^{3+} a new species is formed. It is not easily visible in the overall spectra, but after removing the absorption contributions from those spectra of the flavylium and iron chlorides, the absorption of the new species emerges. It corresponds to the iron-complexed *Z*-chalcone of malvin. This rare malvin structural form provides the open cavity that hosts iron (III) in a water-protected environment (Fig. 1.5).

It is remarkable that on addition of a strong copigment, chlorogenic acid for instance (Mazza & Brouillard, 1990), the metal complexation reaction does not take place. This signifies that this copigment fully protects the flavylium form from hydrating, and therefore, the *Z*-chalcone with its open cavity does not exist at all. Note that the covalent hydration reaction of the flavylium system appears again as a key step in the overall iron (III) complexation of natural and artificial anthocyanins. The characteristic absorption spectrum of the new metal complex between malvin and Fe^{3+} is in agreement with reported spectra for flavonoids in general (Markham, 1982).

Fig. 1.6 shows NMR spectral features characteristic of malvin-free forms (a), some malvin-free forms plus the iron complex (b), and finally, the iron (III)–*Z*-chalcone complex alone (c). A large excess of ferric chloride was added to the pH 1.5 heavy water solution maintained at 25 °C throughout the experiment. The chemical shifts of the malvin-free forms are in agreement with those that have been reported in the literature (Cheminat & Brouillard, 1986; Santos *et al.*, 1993). After complete evolution of the system toward equilibrium the only malvin species remaining in the solution is the *Z*-chalcone–iron edifice. ^{1}H NMR and UV-visible data are in good agreement: Fe^{3+} associates specifically with only one of the malvin forms and this is the *Z*-chalcone (a retrochalcone). The significant NMR features are as follows: H-2′ and H-6′ are no more equivalent; H-6 and H-8 are still observed, whereas H-4, under the iron effect, has probably been enlarged to a point that makes it impossible to be recorded under our experimental conditions (George *et al.*, 1999). An interesting result is brought by molecular modelization in the ZINDO/1 semiempirical mode, associated with crystallographic data concerning flavylium ions available from the literature (Ueno & Saito, 1977a,b). It demonstrates that, within the *Z*-chalcone open cavity, the ferric cation is located between the B-ring and the glucosyl residue at C-5 of the aglycone (George *et al.*, 1999). Moreover, two hydrogen bonds are formed in the complex, one between the hydroxyl at C-9 and the
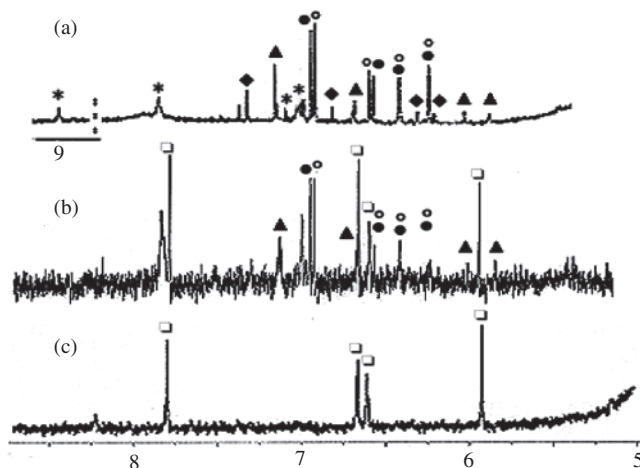
Fig. 1.6 ^{1}H NMR spectra of malvin chloride at 25 °C: (a) pure malvin chloride, (b) malvin with ferric chloride after 1 h, and (c) at equilibrium after a full day. Symbols indicate the different forms (see Fig. 1.3): (∗) flavylium cation, (•/○) hemiketal, (▲) *E*-retrochalcone, (♦) *Z*-retrochalcone, and (□) *Z*-chalcone–iron complex.

oxygen at C-3 and the other between the oxygen at C-9 and the hydroxyl at C-2 of the 3-sugar residue.

Almost all natural anthocyanins we tested demonstrated a large affinity for iron. This brings about the intriguing question of the biological role that could be played by the many anthocyanins encountered in the interior of fruits and tubers, and also in roots, leaves, and so on. Could biological effects of anthocyanins be related to the presence of *Z*-chalcone–iron complexes that may help in the regulation of iron uptake and activity? (Rhodes, 1998). This opens the door to a new era of research on natural anthocyanins, which looks for their function outside the only one always envisaged in the case of flowering plants, that is, to provide color to plant organs, even if, in the case of flowers, that function remains the main one. A direct application to humans' nutrition concerns the consumption of red fruits and their derivatives. This kind of food is to become more and more popular because of the good amounts of anthocyanins it provides in a balanced and intelligent human diet. Moreover, with rare exceptions, like the rose jelly, we have lost the habit to include flowers in our diet. It could well be that we should eat (again?) colored petals of those edible plants because they are excellent sources of the more structurally evolved anthocyanins that are not to be found in the other plant parts. Flowers are also interesting as they are low in fat and proteins if not in sugar, and they also synthesize many different types of flavonoids, which are the now well-known and cheap to acquire antioxidant molecules.

What is the best type of anthocyanin for a good association with Fe^{3+}? The survey shows that a 3-oxygen as well as a 5-*O*-glycosyl or a 5-hydroxyl group is necessary. Such structural elements are featured in almost all natural anthocyanins. For the metal complexation to occur some hydration of the flavylium salt is also necessary, which immediately gives way to the central ring opening with the formation of *E*-retrochalcones and *Z*-retrochalcones. The copigment effect is capable of successfully competing with the flavylium hydration

reaction. In that connection, it is interesting to note that copigmentation was never firmly demonstrated to occur within fruits, although it has been frequently shown to exist within epidermal flower tissues. This again points to the existence of different biological functions for the structurally sophisticated pigments of flowers on the one side as compared to the much more simple pigments of red fruits, leaves, tubers, and grains on the other side. Fig. 1.4 demonstrates that when good amounts of the metal complex are present, large changes in the color of the malvin solution take place: the red disappears to the benefit of the pale yellow brought by the stabilization of the *Z*-chalcone in the iron complex. Consequently, it would be worth looking for anthocyanins in yellow, if not white, flowering species, especially if they are known to contain good quantities of iron.

1.7 Anthocyanin biological activity

Stimulated by the existence of the "French paradox," a generation of researchers have turned their investigations toward the family of natural substances called polyphenols, stilbenes, and flavonoids (St Léger *et al.*, 1979; Fougerousse *et al.*, 1996; Brouillard *et al.*, 1997, 2003; Fougerousse & Brouillard, 2001; Quideau *et al.*, 2005; Cheynier, 2006). It seems well established that some of these molecules behave as radical scavengers and antioxidants (Bors *et al.*, 1996). For instance, they are thought to protect cholesterol in the low-density lipoprotein (LDL) from oxidation (Frankel *et al.*, 1995). Nevertheless, it should be kept in mind that nowadays phytochemists have identified about 5,000 natural flavonoids and only a handful of them have been tested for their antioxidant capacity, making rutin, quercetin, and catechins the most popular, if not the most potent, flavonoid types of antioxidants. Several years ago, Fougerousse *et al.* (1996) proposed the first mechanism accounting for the flavonol antioxidant effect, which was based on a structural analogy between ascorbic acid and flavonols. It was also concluded that it urges to prepare pure flavonoids by convenient chemical synthesis; elegant examples of which are given in the recent works by Chassaing (2006), Isorez (2007), Gaudrel-Grosay (2007), and Kueny-Stotz (2008). Also, analytical tools have improved and techniques like capillary electrophoresis are now accessible for the structural elucidation of flavonoids, including acidic conditions in the case of anthocyanins (Bicard *et al.*, 1999). Vegetable, more or less processed, samples are also good sources of unusual anthocyanin structures (Andersen & Jordheim, 2006; Gonzalez-Paramas *et al.*, 2006); the best examples being the specific wine pigments (Cheynier, 2006). For instance, a novel wine pigment formed by covalent association of the ellagitannin vescalagin and oenin, the more abundant *vinifera* anthocyanin, was fully elucidated (Quideau *et al.*, 2005).

Some easy to oxidize flavonoids, like dihydroflavonols, proanthocyanidins, and even anthocyanins, may be turned into active flavonols extremely efficient in radical deactivation. For the first two cited flavonoid subgroups (dihydroflavonols and proanthocyanidins), oxidation to a flavonol is a well-documented field of research. For anthocyanins, the situation is less obvious and a reasonable interpretation of how anthocyanin might be oxidized to flavonols is as follows. In aqueous acidic media, a water 4-adduct forms from the stable flavylium species by a decrease in the free acidity. This water 4-adduct has the

oxygen atom at the right position in the anthocyanin structure that can then be oxidized to a flavonol with simultaneous loss of a proton. However, note that in Fig. 1.3 the water 4-adduct is not present; this is because that species is usually only a very minor one being trapped between natural anthocyanin kinetic products or quinonoid bases and the water 2-adduct thermodynamic product much more stable than its 4-isomeric analog (Brouillard & Cheminat, 1988). Nevertheless, under peculiar conditions, that minor species might play an important role in the antioxidant effect observed with anthocyanins. At this stage, we should stress how fundamental it is to gain a good knowledge of the type of flavonoid and, in the case of anthocyanins, of the active monomeric structure as these anthocyanins seem capable of featuring the behavior of any of the many subgroups of the large flavonoid family. A very recent and fascinating example is the transformation of flavylium derivatives into *trans*-retrochalcones having low nanomolar affinity to benzodiazepine receptors (Kueny-Stotz *et al.*, 2008).

In vitro anticancer tests have been performed using aglycones and natural anthocyanins (Meiers *et al.*, 2001; Chen *et al.*, 2006). The most striking result is reported by Feng *et al.* (2007). Cyanidin 3-*O*-rutinoside can kill selectively leukemia cells by a mechanism suggested to be an anthocyanin prooxidant effect active on the malignant cells.

1.8 Some thoughts on applications

A large domain of applications from advanced fields of research to more applied industrial ones is suggested. But first, the following comments are to be made. The use of anthocyanins as food colors and as fabric dyes is everlasting (Cardon, 1998; Mompon *et al.*, 1998). Their presence in wines was reported in relation to epidemiological health studies (Hertog, 1998). The review by Suschetet *et al.* (1998), on the anticarcinogenic properties of flavonoids, signals an almost complete lack of data concerning this important group of flavonoids. Nevertheless, according to the Ames test, no mutagenic effect was detected for those natural anthocyanins investigated. In preventive nutrition, anthocyanin-rich red fruits appear as good dietary sources (Rémésy *et al.*, 1998). With so little information, anthocyanin users might easily conclude that there is not much to do with that kind of molecules. Our opinion is, of course, just at the opposite. The following text lists some foreseeable trends, which can eventually take a growing scientific and/or economic importance, owing to the fact that scientists, as well as public in developed countries, are more and more aware of the benefits of the consumption or use of anthocyanins.

One of the most interesting and probably the less used application of anthocyanins comes from the works by Merlin *et al.* (1985, 1994) and Birembaut *et al.* (1998). They used laser Raman spectroscopy, in association with electronic spectroscopy (in the absorption and emission modes), to investigate anthocyanins inside vacuoles of "Pinot noir" mature red berries and of many other "intact" living plant tissues. By comparing their results with the results gained from model experiments, they were able to accurately give the free acidity values of physiologically intact vacuoles of the epidermal cells of the investigated berries or of other plant tissues. Nowadays, this type of work could be fundamental for plant genetic studies, not only because it gives access to accurate, *in planta* pH values, without needing

an external colorant, but also because the flavylium chromophore has the unique property to be sensitive to any biophysicochemical factor present in its chemical structure or in its immediate microenvironment. From confrontation to model systems, the entire supramolecular edifice around the anthocyanins within "intact" vacuoles could be elucidated.

Another fascinating use of flavylium salts is in photoelectrochemical cells in which they are incorporated in the form of a dye adsorbed on a TiO_2 nanocrystalline film. Cherepy *et al.* (1997) first reported this type of application using cyanin. However, poor photoelectric yield was observed from the sunlight conversion, probably owing to inadaptation of cyanin to the many requirements of the rewarding "high-tech" technology. We value the opinion that a much better, synthetic, flavylium dye could be tailor made in connection with the rest of the sophisticated devices needed in this kind of experiment (Graetzel, 2000; Polo & Murakami, 2006).

Anthocyanin antioxidant activity can now be seen from two perspectives: one is the scavenging of radicals and the other the prevention of radical formation by the chelation of metals, especially iron (Dangles *et al.*, 2000). Gould *et al.* (2002) and Gould (2008) compared antioxidant activities within red (anthocyanins) and green (lack of anthocyanins?) leaves. Red leaves were better protected against the solar-light photochemical damaging reactive oxygen species (singlet oxygen).

An interesting point can be made in connection with the "French paradox." Big and small European cities are getting more and more polluted owing to the production of the strong oxidant, ozone. To prevent part of the long-term health problems that will arise from a too frequent exposure to that gas, people in the polluted areas, mainly cities, may drink moderate quantities of red wine on a regular basis. However, a scientific research scheme needs to be put in place to look for a model capable of measuring the amount of damage caused by ozone to human health, and if wine consumption is really beneficial to those drinking it!

The recent discovery that *Z*-retrochalcones (natural anthocyanin retrochalcones) encapsulate iron (III) points out that they can play a nutritional role. Owing to this property, anthocyanins may take part in the uptake and regulation of this vital metal in the human body. If it is the case, it would be wise to consume red fruits (edible berries) with good amounts of structurally simple glycosylated anthocyanins whose *Z*-chalcones are perfectly sized for complexing iron (III).

Another suggestion comes from the observation that there exist, in quantity, many more anthocyanins than that are visible to the human and animal eyes; this is so because they are located either under the opaque surface of aerial plant organs or in organs that normally develop underground. There seems to be no reason to have colors when they cannot be seen them. Therefore, it is worth asking what is the role of anthocyanins inside red fruits, in the tubers of the blue potato, and in general in the many red, blue, and almost black colored fruits and grains of the many varieties of edible plants like cereals (rice, corn, wheat, etc.), tomatoes, carrots, and so on. Unfortunately, these densely colored crops seem presently to be the less cultivated in the world. We think that they should be put again on the market and made available to those who would like to have them grown. Of course, this is probably not a good marketing strategy. For us, human health is more important than financial profits. Hoping that both can go the same way?

The understanding of the basic chemical principles lying behind the phenomenon of generating diversified colors from a single chromophore (think of the large Orchid family) could result in identifying more possible uses of anthocyanins in human activities. Color is a vast subject! Many things could be done with those colors. For instance, they can be used in the dyeing of textiles, in cosmetics, for vegetable food presentation and preservation, for ornamental purpose (e.g., keeping cut, fresh, and dried flowers), and for photoprotection against sunlight. Reversible color modifications under varied physicochemical changes are also known, for example, solvatochromism, thermochromism, and photochromism. All these processes require water, without which life is impossible. There is a unique relation between water and the flavylium chromophore (Brouillard *et al.*, 1989). What happens to the structure of water, in the solid and liquid states, is immediately seen in the "colored" response of the chromophore (for this, copigmentation provides many examples). Life has started and goes on in water. Was it that at the origin water was essential for the development of life or was water the best and *only* carrier to have living creatures expand all over the Earth? Undoubtedly, no other substance could have been a better carrier. Nowadays, that formidable property to have organic chemistry elements put together and start the chemistry building the very first living organisms may itself turn against life owing to the transport and dilution of pollutants generating from human activities into the waters all around the globe. For the ongoing century, this is probably the greatest threat to life on our planet!

There is a field in chemistry that remains completely open to research and the interactions established between anthocyanins and the large bioorganic and inorganic molecules. Good representatives would be proteins; for instance, members of the lectin family and also the human insulin with its 51 amino acids.

Finally, let us end this section with a few words on the origin of wine color, whether it is red or white. Call it the unifying theory. Everybody knows that red is given by the grape anthocyanins (Brouillard *et al.*, 1997). In our opinion, the pale yellow color of white wines could arise from the presence of iron–chalcone complexes (see Fig. 1.4), and the balance between iron and anthocyanins would determine the final aspect of a white wine. Thus, anthocyanins may be stored in living tissues under almost colorless or yellow combinations. If it is the case, phytochemists looking for new anthocyanin natural structures may turn their attention toward yellow and even white flowering plant species.

Another very interesting and less explored field is the color changes observed when anthocyanins are in contact with colorless substances, which are not necessarily copigments, for instance, surfactants, emulsions, gels, proteins, DNA, cotton, wool, hair and so on (Bicard, 1999). For hair coloring, anthocyanins could be alternatives to existing synthetic systems and generate new direct dyes for hair (cationic dyes).

1.9 References

Allinger, N.L. (1977). Conformational analysis. 130. MM2. A hydrocarbon force field utilizing V1 and V2 torsional terms. *Journal of the American Chemical Society*, **99**, 8127–8134.

Amic, D., Baranac, E. & Vukadinovic, V. (1990). Reactivity of some flavylium cations and corresponding anhydrobases. *Journal of Agriculture and Food Chemistry*, **38**, 936–940.

Andersen, O.M. & Jordheim, M. (2006). The anthocyanins. In: *Flavonoids: Chemistry, Biochemistry and Applications* (eds O.M. Andersen & K.R. Markham), pp. 471–551. CRC Press, Taylor & Francis Group, Boca Raton.

Asen, S., Norris, K.H. & Stewart, R.N. (1969). Absorption spectra and colour of aluminium–cyanidin 3-glucoside complexes as influenced by pH. *Phytochemistry*, **8**, 653–659.

Asen, S., Stewart, R.N. & Norris, K.H. (1972). Copigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry*, **11**, 1139–1144.

Bayer, E., Egeter, H., Fink, A., Nether, K. & Wegmann, K. (1966). Komplexbildung und blütenfarben. *Angewandte Chemie International Edition*, **78**, 834–841.

Bicard, V. (1999). Influence des tensioactifs sur la couleur des anthocyanes en solution aqueuse et détection des anthocyanes par électrophorèse capillaire. PhD thesis, University Strasbourg 1.

Bicard, V., Fougerousse, A. & Brouillard, R. (1999). Analysis of natural anthocyanins by capillary zone electrophoresis in acidic media. *Journal of Liquid Chromatography & Related Technol*ogies, **22**, 541–550.

Birembaut, F., Elhabiri, M., Cornard, J.P., Figueiredo, P., Merlin, J.C. & Brouillard, R. (1998). Resonance Raman characterisation of the structure of anthocyanin aluminium complexes. In: *Polyphenols Communications 98*, Vol. 1 (eds C. Rolando, A. Scalbert & R. Ibrahim), pp. 209–210. INRA Editions, Paris.

Bors, W., Heller, W., Michel, C. & Stettmaier, K. (1996). Flavonoids and polyphenols: chemistry and biology. In: *Handbook of Antioxidants* (eds E. Cadenas & L. Packer), pp. 409–466. Marcel Dekker, New York.

Britton, G. (1983). *The Biochemistry of Natural Pigments*. Cambridge University Press, Cambridge.

Brouillard, R. (1981). Origin of the exceptional colour stability of the *Zebrina* anthocyanin. *Phytochemistry*, **20**, 143–145.

Brouillard, R. (1983). The *in vivo* expression of anthocyanin colour in plants. *Phytochemistry*, **22**, 1311–1323.

Brouillard, R. & Cheminat, A. (1988). Flavonoids and plant colour. In: *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties* (eds V. Cody, E. Middleton Jr, J.B. Harborne & A. Beretz), pp. 93–106. Alan R. Liss, New York.

Brouillard, R. & Dangles, O. (1993). Flavonoids and flower colours. In: *The Flavonoids. Advances in Research Since 1986* (ed. J.B. Harborne), pp. 565–586. Chapman & Hall, London.

Brouillard, R. & Lang, J. (1990). The hemiketal-*cis*-chalcone equilibrium of malvin, a natural anthocyanin. *Canadian Journal of Chemistry*, **68**, 755–761.

Brouillard, R., Mazza, G., Saad, Z., Albrecht-Gary, A.M. & Cheminat, A. (1989). The copigmentation reaction of anthocyanins. A microprobe for the structural study of aqueous solutions. *Journal of the American Chemical Society*, **111**, 2604–2610.

Brouillard, R., Wigand, M.C., Dangles, O. & Cheminat, A. (1991). pH and solvent effects on the copigmentation reaction of malvin with polyphenols, purine and pyrimidine derivatives. *Journal of the Chemical Society, Perkin Transactions 2*, 1235–1241.

Brouillard, R., George, F. & Fougerousse, A. (1997). Polyphenols produced during red wine ageing. *BioFactors*, **6**, 403–410.

Brouillard, R., Chassaing, S. & Fougerousse, A. (2003). Why are grape/fresh wine anthocyanins so simple and why is it that red wine color lasts so long? *Phytochemistry*, **64**, 1179–1186.

Cardon, D. (1998). Polyphenols and natural dyes: a historian contemplates chemistry. In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze & J. Triaud), pp. 45–79. INRA Editions, Paris.

Chassaing, S. (2006). Contribution à la chimie des flavonoïdes. Voies d'accès aux dihydroflavonols: vers une synthèse biomimétique de flavonols et une synthèse de tanins condensés. Vers une synthèse de pigments anthocyaniques sophistiqués. PhD thesis, University Strasbourg 1.

Chassaing, S., Kueny-Stotz, M., Isorez, G. & Brouillard, R. (2007). Rapid preparation of 3-deoxyanthocyanidins and novel dicationic derivatives: new insight into an old procedure. *European Journal of Organic Chemistry*, 2438–2448.

Cheminat, A. & Brouillard, R. (1986). PMR investigation of 3-*O*-(β-D-glucosyl) malvidin structural transformations in aqueous solutions. *Tetrahedron Letters*, **37**, 4457–4460.

Chen, P.N., Chu, S.C., Chiou, H.L., Kuo, W.H. Chiang, C.L. & Hsieh, Y.S. (2006). Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Letters*, **235**, 248–259.

Cherepy, N.J., Smestad, G.P., Graetzel, M. & Zhang, J.Z. (1997). Ultrafast electron injection: implications for a photoelectrochemical cell utilizing an anthocyanin dye-sensitized TiO_2 nanocrystalline electrode. *Journal of Physical Chemistry B*, **101**, 9342–9351.

Cheynier, V. (2006). Flavonoids in wine. In: *Flavonoids: Chemistry, Biochemistry and Applications* (eds O.M. Andersen & K.R. Markham), pp. 263–318. CRC Press, Taylor & Francis Group, Boca Raton.

Dangles, O. & Brouillard, R. (1992a). Polyphenol interactions. The copigmentation case: thermodynamic data from temperature variation and relaxation kinetics. Medium effect. *Canadian Journal of Chemistry*, **70**, 2174–2189.

Dangles, O. & Brouillard, R. (1992b). A spectroscopic method based on the anthocyanin copigmentation interaction and applied to the quantitative study of molecular complexes. *Journal of the Chemical Society Perkin Transactions 2*, 247–257.

Dangles, O. & Brouillard, R. (1992c). Inclusion of natural pigments into cyclodextrins. Natural pigments in competitive spectrophotometric studies of host–guest systems. In: *Minutes of the Sixth International Symposium on Cyclodextrins* (ed. A.R. Hedges), pp. 117–122. Editions de Santé, Paris.

Dangles, O. & Elhajji, H. (1994). Synthesis of 3-methoxy- and 3-(beta-D-glucopyranosyloxy) flavylium ions. Influence of the flavylium substitution pattern on the reactivity of anthocyanins in aqueous solution. *Helvetica Chimica Acta*, **77**, 1595–1610.

Dangles, O., Stoeckel, C., Wigand, M.C. & Brouillard, R. (1992a). Two very distinct types of anthocyanin complexation: copigmentation and inclusion. *Tetrahedron Letters*, **33**, 5227–5230.

Dangles, O., Wigand, M.C. & Brouillard, R. (1992b). Anthocyanin anti-copigment effect. *Phytochemistry*, **31**, 3811–3812.

Dangles, O., Saito, N. & Brouillard, R. (1993a). Kinetic and thermodynamic control of flavylium hydration in the pelargonidin–cinnamic acid complexation. Origin of the extraordinary flower colour diversity of *Pharbitis nil*. *Journal of the American Chemical Society*, **115**, 3125–3132.

Dangles, O., Saito, N. & Brouillard, R. (1993b). Anthocyanin intramolecular copigment effect. *Phytochemistry*, **34**, 119–124.

Dangles, O., Elhabiri, M. & Brouillard, R. (1994a). Kinetic and thermodynamic investigation of the aluminium–anthocyanin complexation in aqueous solution. *Journal of the Chemical Society Perkin Transactions 2*, 2587–2596.

Dangles, O., Elhabiri, M. & Brouillard, R. (1994b). A quantitative investigation of metal–anthocyanin complexes in aqueous solution. In: *Polyphenols 94* (eds R. Brouillard, M. Jay & A. Scalbert), pp. 363–364. INRA Editions, Paris.

Dangles, O., Fargeix, G. & Dufour, C. (2000). Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *Journal of the Chemical Society Perkin Transactions 2*, 1653–1663.

Elhabiri, M. (1997). Synthèse de chlorures de flavylium à structure catéchol en vue de la modélisation de la complexation métallique des anthocyanes en solution aqueuse par des ions métalliques fortement oxophiles comme Al^{3+}, Ga^{3+}. PhD thesis. Université Louis Pasteur, Strasbourg.

Elhabiri, M., Figueiredo, P., Fougerousse, A. & Brouillard, R. (1995a). A convenient method for conversion of flavonols into anthocyanins. *Tetrahedron Letters*, **36**, 4611–4614.

Elhabiri, M., Figueiredo, P., Fougerousse, A., Dangles, O. & Brouillard, R. (1995b). Anthocyanin chemical synthesis: an important access to natural and synthetic pigments. *Polyphénols Actualités*, **13**, 11–13.

Elhabiri, M., Figueiredo, P., George, F. *et al.* (1996). Ground- and excited-state properties of some naphthoflavyliums. *Canadian Journal of Chemistry*, **74**, 697–706.

Elhabiri, M., Figueiredo, P., Toki, K., Saito, N. & Brouillard, R. (1997). Anthocyanin aluminium and gallium complexes in aqueous solution. *Journal of the Chemical Society Perkin Transactions 2*, 355–362.

Elhajji, H., Dangles, O., Figueiredo, P. & Brouillard, R. (1997). 3′-(β-D-Glycopyranosyloxy)flavylium ions: synthesis and investigation of their properties in aqueous solution. Hydrogen bonding as a mean of colour variation. *Helvetica Chimica Acta*, **80**, 398–413.

Feng, R., Ni, H.M., Wang, S.Y., Tourkova, I.L., Shurin, M.R., Harada, H. & Yin, X.M. (2007). Cyanidin 3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. *Journal of Biological Chemistry*, **282**, 13468–13476.

Figueiredo, P. (1994). Estudos sobre a estabilizaçao térmica e fotoquimica das propriedades corantes das antocianinas. PhD thesis, Universidade Nova de Lisboa, Lisboa.

Figueiredo, P. & Pina, F. (1994). Formation of anthocyanin ion-pairs. A copigmentation effect. *Journal of the Chemical Society Perkin Transactions 2*, 775–778.

Figueiredo, P., Elhabiri, M., Toki, K., Saito, N., Dangles, O. & Brouillard, R. (1996a). New aspects of anthocyanin complexation. Intramolecular copigmentation as a means for colour loss? *Phytochemistry*, **41**, 301–308.

Figueiredo, P., Elhabiri, M., Saito, N. & Brouillard, R. (1996b). Anthocyanin intramolecular interactions. A new mathematical approach to account for the remarkable colorant properties of the pigments extracted from *Matthiola incana*. *Journal of the American Chemical Society*, **118**, 4788–4793.

Fougerousse, A. & Brouillard, R. (2001). Le pouvoir antioxydant des flavonoïdes contenus dans le vin. In: *Vins et Santé*, Vol. 6 (ed. S. Azria), pp. 26–28. Editions du Voyage, Montpellier.

Fougerousse, A., Elhabiri, M., George, F. & Brouillard R. (1996). Les propriétés anti-oxydantes des flavonoïdes. Eléments structuraux en relation avec leur capacité à piéger des radicaux. In: *Polyphenols Communications 96* (eds J. Vercauteren, C. Cheze, M.C. Dumon & J.F. Weber), pp. 529–530. Université Bordeaux 2, Bordeaux.

Frankel, E.N., Waterhouse, A.L. & Teissedre, P.L. (1995). Principal phenolic phytochemicals in selected Californian wines and their antioxidant activity in inhibiting oxidation of human low density lipoproteins. *Journal of the Agriculture and Food Chem*istry, **43**, 890–894.

Franks, F. (1984). *Water*. The Royal Society of Chemistry, London.

Furtado, P., Figueiredo, P., Neves, H.C. & Pina, F. (1993). Photochemical and thermal degradation of anthocyanidins. *Journal of Photochemistry and Photobiology A: Chemistry*, **75**, 113–118.

Gaudrel-Grosay, S. (2007). Synthèse de flavan-3–4-diols sur support solide pour la détection d'enzymes impliquées dans la formation des anthocyanes et des tannins dans la baie de raisin. PhD thesis, Université Bordeaux 1.

George, F. (1998). Relations structure-réactivité des chlorures de flavylium synthétiques et des anthocyanes naturelles en solution aqueuse: application à la complexation métallique par Fe(III). PhD thesis, Université Louis Pasteur, Strasbourg.

George, F., Figueiredo, P. & Brouillard, R. (1999). Malvin *Z*-chalcone: an unexpected new open cavity for the ferric cation. *Phytochemistry*, **50**, 1391–1394.

Gonzalez, E., Fougerousse, A. & Brouillard, R. (2001). Two diacylated malvidin glycosides from *Petunia hybrida* flowers. *Phytochemistry*, **58**, 1257–1262.

Gonzalez-Paramas, A.M., Lopes da Silva, F., Martín-López, P. *et al.* (2006). Flavanol-anthocyanin condensed pigments in plant extracts. *Food Chemistry*, **94**, 428–436.

Goto, T. & Kondo, T. (1991). Structure and molecular stacking of anthocyanins. Flower color variation. *Angewandte Chemie International Edition in English*, **30**, 17–33.

Goto, T., Hoshino, T. & Ohba, M. (1976). Stabilization effect of neutral salts on anthocyanins, flavylium salts, anhydrobases and genuine anthocyanins. *Agricultural and Biological Chemistry*, **40**, 1593–1596.

Gould, K.S. (2008). Anthocyanin function in plants: a rediscovery. Paper given at XXIV International Conference on Polyphenols, Salamanca, Spain, July 8–11, 2008.

Gould, K.S., Mckelvie, J. & Markham, K.R. (2002). Do anthocyanins function as antioxidants in leaves? Imaging of hydrogenperoxide in red and green leaves after mechanical injury. *Plant Cell and Environment*, **25**, 1261–1269.

Graetzel, M. (2000). Conversion of light to electricity by anthocyanin dyes in nanocrystalline solar cells. In: *Proceedings of the First France/Europe–Japan Conference on Anthocyanins* (ed. R. Brouillard), pp. 13–14. Région Alsace, le Bischenberg.

Harborne, J.B. (1989). Plant phenolics. In: *Methods in Plant Biochemistry*, Vol. 1 (ed. J.B. Harborne), pp. 1–28. Academic Press, London.

Haslam, E. (2001). Natural polyphenols as drugs and medicines: potential modes of action. In: *Polyphenols, Wine and Health* (eds C. Chèze, J. Vercauteren & R. Verpoorte), pp. 1–47. Kluwer Academic Publishers, Dordrecht.

Hertog, M.G.L. (1998). Flavonols in wine and tea and prevention of coronary heart disease. In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze & J. Triaud), pp. 117–131. INRA Editions, Paris.

Isorez, G. (2007). Contribution à la chimie des flavonoïdes. Accès à des analogues de pigments du vin rouge. PhD thesis, University Strasbourg 1.

Kondo, T., Ueda, M., Tamura, H., Yoshida, K., Isobe, M. & Goto, T. (1994a). Composition of protocyanin, a self-assembled supramolecular pigment from the blue cornflower *Centaurea cyanus*. *Angewandte Chemie International Edition in English*, **33**, 978–979.

Kondo, T., Ueda, M., Yoshida, K., Titani, K., Isobe, M. & Goto, T. (1994b). Direct observation of a small molecule associated supramolecular pigment, commelinin, by electrospray ionization mass spectrometry. *Journal of the American Chemical Society*, **116**, 7457–7458.

Kueny-Stotz, M. (2008). Contribution à la chimie des flavonoïdes: (i) Elaboration de squelettes flavylium sophistiqués; (ii) Nouvelles voies d'accès aux flavan-3-ols et procyanidines. PhD thesis, University Strasbourg 1.

Kueny-Stotz, M., Isorez, G., Chassaing, S. & Brouillard, R. (2007). Straightforward synthesis of highly hydroxylated phloroglucinol-type 3-deoxyanthocyanidins. *Synlett*, **8**, 1223–1226.

Kueny-Stotz, M., Chassaing, S., Brouillard, R., Nielsen, M. & Goeldner M. (2008). Flavylium salts as in vitro precursors of potent ligands to brain GABA-A receptors. *Bioorganic & Medicinal Chemistry Letters*, **18**, 4864–4867.

Lu, T.S., Saito, N., Yokoi, M., Shigihara, A. & Honda, T. (1992). Acylated pelargonidin glycosides in the red-purple flowers of *Pharbitis nil*. *Phytochemistry*, **31**, 289–295.

Markham, K.R. (1982). *Techniques of Flavonoid Identification*. Academic Press, London.

Mazza, G., & Brouillard, R. (1990). The mechanism of copigmentation of anthocyanins in aqueous solutions. *Phytochemistry*, **29**, 1097–1102.

Meiers, S., Kemény, M., Weyand, U., Gastpar, R., von Angerer, E. & Marko, D. (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *Journal of Agriculture and Food Chemistry*, **49**, 958–962.

Merlin, J.C., Statoua, A. & Brouillard, R. (1985). Investigation of the *in vivo* organization of anthocyanins using resonance Raman microspectrometry. *Phytochemistry*, **24**, 1575–1581.

Merlin, J.C., Cornard, J.P., Statoua, A., Saidi-Idrissi, M., Lautie, M.F. & Brouillard, R. (1994). Vibrational analysis of hydroxyflavylium derivatives by IR, Fourier transform Raman and resonance Raman spectroscopies. *Spectrochimica Acta*, **50**, 703–712.

Mompon, B., Lemaire, B., Mengal, P. & Surbled, M. (1998). Extraction des polyphénols : du laboratoire à la production industrielle. In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze, J. Triaud), pp. 31–43. INRA Editions, Paris.

Polo, A.S. & Murakami Iha, N.Y. (2006). Blue sensitizers for solar cells: natural dyes from Calafate and Jaboticaba. *Solar Energy Materials and Solar Cells*, **90**, 1936–1944.

Quideau, S., Jourdes, M., Lefeuvre, D. *et al.* (2005). The chemistry of wine polyphenolic *C*-glycosidic ellagitannins targeting human topoisomerase II. *Chemistry European Journal*, **11**, 6503–6513.

Rémésy, C., Manach, C., Demigne, C., Texier, O. & Regerat, F. (1998). Interest of polyphenols in preventive nutrition In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze & J. Triaud), pp. 251–265. INRA Editions, Paris.

Rhodes, M.J.C. (1998). Physiological roles of phenolic compounds in plants and their interactions with microorganisms and humans. In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze & J. Triaud), pp. 13–20. INRA Editions, Paris.

Rice, S.A. (1975). *Topics in Current Chemistry, Structure of Liquids*, Vol. 60. Springer-Verlag, Berlin.

Robinson, G.M. & Robinson, R. (1931). Color tests are given and the constituents present in various anthocyanins are listed. *Biochemical Journal*, **25**, 1687–1705.

Saito, N., Lu, T.S., Yokoi, M., Shigihara, A. & Honda, T. (1993). An acylated cyanidin 3-sophoroside-5-glucoside in the violet-blue flowers of *Pharbitis nil*. *Phytochemistry*, **33**, 245–247.

Santos, H., Turner, D.L., Lima, J.C., Figueiredo, P., Pina, F.S. & Macanita, A. (1993). Elucidation of the multiple equilibria of malvin in aqueous solution by one- and two-dimensional NMR. *Phytochemistry*, **33**, 1227–1232.

Scheiner, S. (1994). *Ab initio* studies of hydrogen bonds: the water dimer paradigm. *Annual Review of Physical Chemistry*, **45**, 23–56.

St Leger, A.S., Cochrane, A.L. & Moore, F. (1979). Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *The Lancet*, **1**, 1017–1020.

Stewart, R.N., Norris, K.H. & Asen, S. (1975). Microspectrophotometric measurement of pH and pH effect on color of petal epidermal cells. *Phytochemistry*, **14**, 937–942.

Suschetet, M., Siess, M.H., Le Bon, A.M. & Canivenc-Lavier, M.C. (1998). Anticarcinogenic properties of some flavonoids. In: *Polyphenols 96* (eds J. Vercauteren, C. Cheze & J. Triaud), pp. 165–204. INRA Editions, Paris.

Takeda, K. (1977). Metallo-anthocyanins. II. Further experiments of synthesizing crystalline blue metallo-anthocyanins using various kinds of bivalent metals. *Proceedings of the Japan Academy, Series B*, 257–261.

Takeda, K., Futjii, T. & Iida, M. (1984). Magnesium in the blue pigment complex commelinin. *Phytochemistry*, **23**, 879–881.

Takeda, K., Yamashita, T., Takahashi, A. & Timberlake, C.F. (1990). Stable blue complexes of anthocyanin-aluminium-3-*p*-coumaroyl- or 3-caffeoyl-quinic acid involved in the blueing of *Hydrangea* flower. *Phytochemistry*, **29**, 1089–1091.

Toki, K., Saito, N., Imura, K., Suzuki, T. & Honda, T. (1994a). (Delphinidin 3-gentiobiosyl)(apigenin 7-glucosyl) malonate from the flowers of *Eichhornia crassipes*. *Phytochemistry*, **36**, 1181–1183.

Toki, K., Saito, N., Kawano, K., Lu, T.S., Shigihara, A. & Honda, T. (1994b). An acylated delphinidin glycoside in the blue flowers of *Evolvulus pilosus*. *Phytochemistry*, **36**, 609–612.

Ueno, K. & Saito, N. (1977a). 4′,6,7-Trihydroxyflavylium chloride monohydrate, a synthetic anthocyanidin. *Acta Crystallographica, Section B*, **33**, 111–113.

Ueno, K. & Saito, N. (1977b). Cyanidin bromide monohydrate (3,5,7,3′,4′-pentahydroxyflavylium bromide monohydrate). *Acta Crystallographica, Section B*, **33**, 114–116.

Wigand, M.C., Dangles, O. & Brouillard, R. (1992). Complexation of a fluorescent anthocyanin with purines and polyphenols. *Phytochemistry*, **31**, 4317–4324.

Yoshida, K., Kondo, T. & Goto, T. (1992). Intramolecular stacking conformation of gentiodelphin, a diacylated anthocyanin from *Gentiana* Makinoi. *Tetrahedron*, **48**, 4313–4326.