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NEW ASPECTS OF ANTHOCYANIN COMPLEXATION. INTRAMOLECULAR COPIGMENTATION AS A MEANS FOR COLOUR LOSS?

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Key Word Index—*Eichhornia crassipes*; Pontederiaceae; *Evolvulus pilosus*; Convolvulaceae; intramolecular copigmentation; disaccharide hydrophilic effect; acylated anthocyanins.

Abstract—Two series of structurally related anthocyanins, extracted from the blue flowers of *Evolvulus pilosus* cv. Blue Daze and from the blue–purple flowers of *Eichhornia crassipes*, exhibit remarkable colour stabilities in aqueous solution at mildly acidic pH values. All the pigments possess the same chromophore (delphinidin), but a different pattern of glycosylation and acylation. Moreover, one of the pigments has an apigenin 7-glucoside molecule (a flavone) attached to the glycosidic chain by two ester bonds with malonic acid, instead of an aromatic acid and is the only known anthocyanin with such a structure. All the molecules studied, except one which has only a 3-gentiobioside (a disaccharide) as substituent, denote an effect of reduction in the hydration constant when compared with the parent delphinidin 3-glucoside or 3,5-diglucoside molecules, which supports the existence of intramolecular hydrophobic interactions between the chromophoric skeleton and the acyl or flavonoid groups. The role played by the sugar units on the hydrophobicity/hydrophilicity of the pigments is also discussed.

INTRODUCTION

It has long been known that anthocyanins bearing the same chromophoric structure can give rise to different colours [1, 2], depending on several chemical and physical factors [3], such as temperature, pH, solvent, the structure of the pigment itself and the presence of other molecules which can be generally described as copigments. The last few years have brought to knowledge one new group of anthocyanin molecules possessing aliphatic and/or aromatic acyl residues linked to the glycosyl units [4–12]. Such pigments, especially those with planar aromatic substituents, have the remarkable capacity of producing rather stable colours when kept in mildly acidic aqueous solutions, unlike the well-known mono- and diglucosylated anthocyanins, which are almost colourless under similar conditions, due to the formation of hydrated species according to the equilibria depicted in Scheme 1 [13–15]. Some authors [2, 16–18] thus theorized that the colour stability of the acylated molecules is caused by a phenomenon of intramolecular copigmentation by the aromatic acyl moiety which folds over and interacts with the π-system of the planar pyrylium core protecting the coloured forms against the nucleophilic water attack which is known to occur in positions 2 and 4 of the chromophore [19–21]. This assumption is supported by NMR data which demonstrate the existence of long distance NOEs between the aromatic acyl protons and those of the chromophore [22, 23].

The present paper reports the spectroscopic determination of thermodynamic and kinetic data obtained from mildly acidic aqueous solutions of two series of anthocyanins which have the delphinidin aglycone in common. The pigments delphinidin 3-gentiobioside (**A-2**) and (6'''-(delphinidin 3-gentiobiosyl)) (6''-(apigenin 7-glucosyl)) malonate (**A**) were extracted from the blue–purple flowers of *Eichhornia crassipes* [12] (Scheme 2), and delphinidin 3,5-diglucoside (**B-1**), delphinidin 3,5-diacetylglucoside (**B-2**), delphinidin 3-*p*-coumarylglucoside-5-malonylglucoside (**B-3**), delphinidin 3-glucosylcaffeylglucosylcaffeylglucoside-5-glucoside (**B-4**) and delphinidin 3-glucosylcaffeylglucosylcaffeylglucoside-5-malonylglucoside (**B**) from the blue flowers of *Evolvulus pilosus* cv Blue Daze [10] (Scheme 3) their structures being thoroughly elucidated by ^{1}H NMR and FABMS [10, 12]. Delphinidin 3-glucoside and cyanidin 3-rutinoside (also known as antirrhinin) were studied for purposes of comparison.

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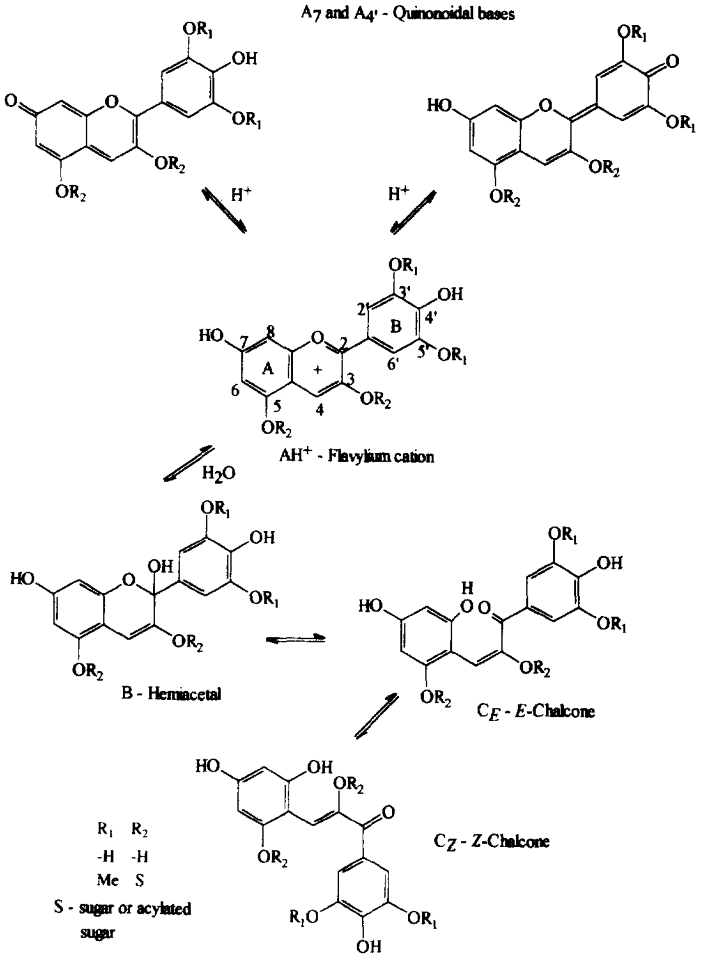
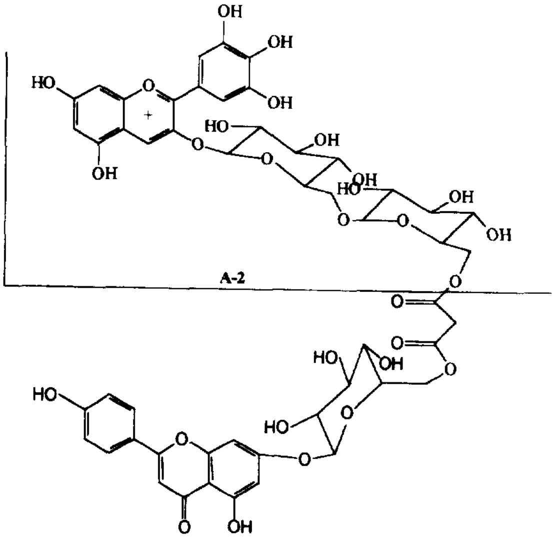


Scheme 1.



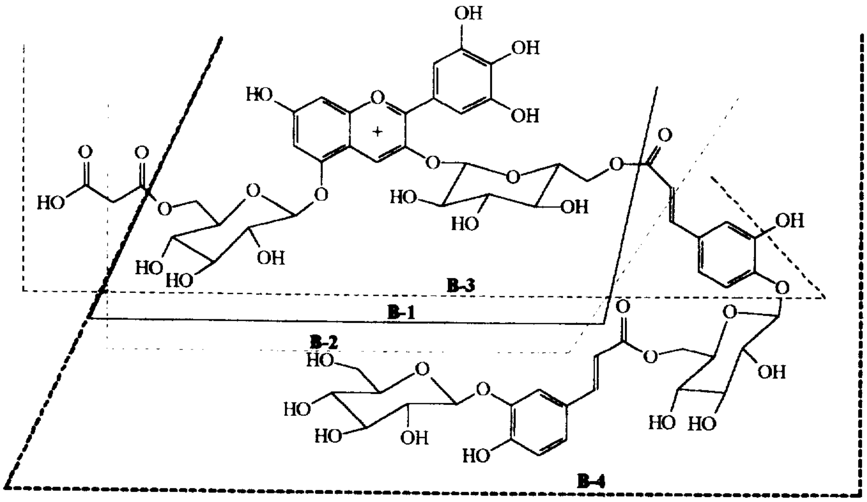
Pigment A

Scheme 2.

RESULTS AND DISCUSSION

The chemical structure of the pigments is one of the main factors affecting the colours of anthocyanins. For example, by simply changing the hydroxylation or methoxylation pattern of ring B a shift of some nanometers in the maximum of the visible absorption band can be verified [19, 24]. In the present case, where all the pigments studied share the same chromophore, a net effect of colour change results from the different pattern of substitution in positions 3 and/or 5 of these molecules. (Cyanidin 3-rutinoside, which presents a different chromophore, is here included only for comparison purposes with **A-2** since it is also an anthocyanin 3-bioside. A more comprehensive study regarding its physico-chemical properties will be reported elsewhere.) From inspection of Fig. 1 it can be stated that the larger the degree of substitution the larger the bathochromic shift observed, for the same pH value. Another general characteristic of such highly substituted molecules, that was also verified throughout this work, is an increase in the molar extinction coefficient (ε) of the flavylium cation form AH^+, when compared with their parent mono- and diglucosides.

Aqueous solutions of anthocyanins are typically colourless or only slightly coloured at mildly acidic pH values ($\sim$ 2–4), due to the displacement of the hydration equilibrium towards the hemiacetal and chalcone forms (Scheme 1). This pH-dependent reaction is also affected



Scheme 3.

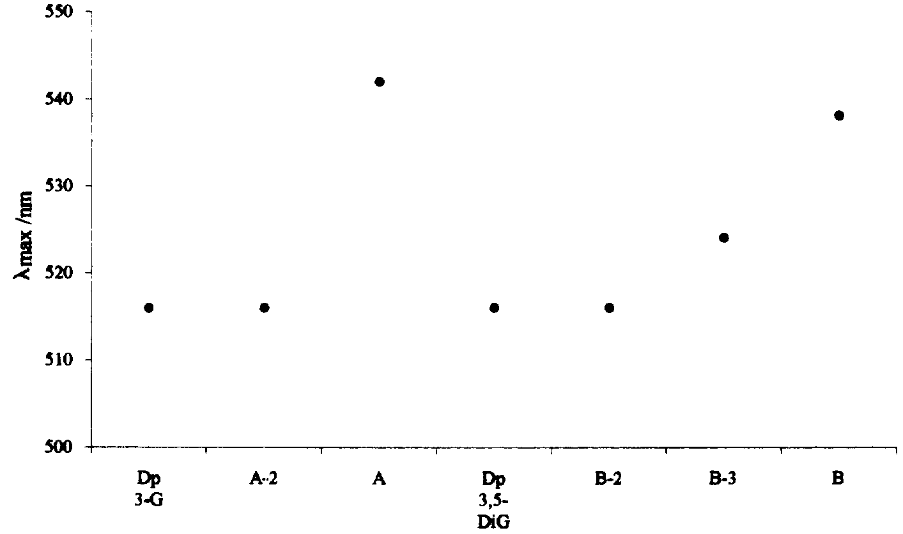


Fig. 1. Maximum visible wavelengths for the flavylium cation forms; pH = 0.9.

by the type and number of substituent groups attached to the aglycone [2, 17, 18, 25]. When the substituent groups are long enough to adopt a folded conformation over the pyrylium ring, this should protect the reactive sites (C-2 and C-4) against the nucleophilic water attack, thus favouring the existence of the coloured forms.

Let us define the following scheme to account for the transformations undergone by anthocyanin molecules in mildly acidic aqueous solutions:

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \tag{1}$$

$$AH^+ + H_2O \overset{K_h}{\rightleftharpoons} (B + C_E) + H^+ \tag{2}$$

Since the ring opening of B to form the E-chalcone occurs in a very fast step, we write the equilibrium as a whole for the sake of clarity; C_Z, which is usually formed in very small amounts [26] is neglected.

Moreover, if we express the hydration equilibrium constant K_h as k_1/k_2, where k_1 stands for the hydration rate constant and k_2 is the rate constant for the reverse process, through the manipulation of the spectrophotometric data and by applying equations (3) and (4), deduced as described elsewhere [18],

$$\frac{A_0}{A_0 - A} = \frac{K_h + K_a}{K_h + K_a(1 - r_A)} + \frac{[H^+]}{K_h + K_a(1 - r_A)} \tag{3}$$

$$\frac{K_a + K_h + [H^+]}{k} = \frac{1}{k_2} + \frac{K_a}{[H^+]k_2} \tag{4}$$

where A represents the absorbance of the anthocyanin solution at a given pH, A_0 the absorption of the flavylium cation (pH < 1.0), K_a the deprotonation equilibrium constant, r_A is the following ratio of molar absorption coefficients $\varepsilon_A/\varepsilon_{AH^+}$, and k is the first-order apparent rate constant of the hydration reaction delivered by the spectrophotometer, we verified an increase in the

Table 1. Hydration and deprotonation reaction values, obtained from the thermodynamic and kinetic data, for the eight delphinidin-based pigments (T = 25°)

Anthocyanin	k_1 (min^{-1})	pK_h	pK_a
Dp 3,5-Digl. (**B-1**)	1159.9 (± 9.3)	1.79 (± 0.02)	3.87 (± 0.04)
B-2	204.7 (± 1.6)	1.925 (± 0.02)	4.63 (± 0.05)
B-3	96.5 (± 0.8)	2.30 (± 0.02)	4.52 (± 0.05)
B-4	232.1 (± 4.6)*	3.05 (± 0.06)	4.20 (± 0.08)
B	36.5 (± 0.3)	3.23 (± 0.06)	4.46 (± 0.04)
Dp 3-Gl.	6.9 (± 0.1)	2.36 (± 0.05)	3.62 (± 0.07)
A-2	24123.3 (± 193)	1.30 (± 0.05)	3.52 (± 0.07)
A	91.3 (± 0.7)	2.86 (± 0.03)	3.61 (± 0.04)

*Purity = 75%.

value of pK_h when going from the smaller to the larger anthocyanins in each series (**A** and **B**), Table 1, with the exception of pigment **A-2** which presents the opposite effect. This intriguing behaviour will be discussed later. The k_1 values (Table 1), which denote the stability of the pigments toward hydration, show, for the **B** series, a decrease when going from the less to the more substituted pigments, in good conformity with the assumption that the degree of acylation is a synonym of higher protection against nucleophilic attack. The value for **B-4** does not follow this behaviour, which should be explained by its lower purity; hence the value of k_1 can also be of use in the determination of the purity of this type of pigments. In the other series of anthocyanins noticeable is the very high k_1 value obtained for **A-2**, in good agreement with its poor colour stability. On the other hand, delphinidin 3-glucoside and **A** have smaller k_1 values, reflecting the high hydrophobicity of both compounds. However, the lower value found for delphinidin 3-glucoside does not reflect its higher K_h value, when compared with **A**. This may be explained by the also lower k_2 value obtained, which means that despite its hydration process having a small direct rate constant, the reverse action (dehydration) is less effective than in the case of **A**.

The verified increase in pK_h denotes the existence of an intramolecular interaction that stabilizes the coloured cationic form at the expense of the colourless forms. This interaction will be stronger for the anthocyanins with longer and more linear chains which allows a greater flexibility, possibly due to a weak potential energy barrier between the different conformations of the linker, for the folding of the planar ring(s) of the aromatic acids (or the flavone, in the case of **A**) over the also planar pyrylium ring, thus allowing the existence of an intramolecular complex through the formation of π–π hydrophobic interactions between the two parts of the molecule. This assumption is supported by AM1 [27] molecular orbital calculations which, albeit performed *in vacuo*, and thus not directly comparable to the molecular behaviour in aqueous solution, forecast a folded conformation as thermodynamically favoured over a more elongated structure. The formation of hydrogen bonds between the phenolic OH groups in positions 4′ and 7 of the chromophore and the other moiety of the molecule, as the driving force of the intramolecular copigmentation, seems to be excluded, since the molecules retain the ability to form quinonoidal bases, as denoted by the pK_a values, obtained from spectroscopic data (Table 1), which do not greatly change when compared to the parent anthocyanins. This means that the hydroxyl groups of the aglycone should be free in order to perform the proton transfer reactions that lead to the formation of the quinonoidal bases. This assumption is in good agreement with the data published for a similar series of compounds [17, 18].

This phenomenon of intramolecular association does not, however, totally preclude the formation of the colourless forms but, as the value of pK_h approaches that of pK_a, for example, respectively, 2.86 and 3.61 for compound **A**, it leads to the existence of coloured solutions through a more extended pH range, since, contrary to the verified for the simpler anthocyanins, where the quinonoidal bases rapidly yield hemiacetal and chalcone, here they are also stabilized by intramolecular interactions (Fig. 2).

Anthocyanins and anthocyanidins undergo thermal degradation, its rate and extension depending on the structure of the molecule [28, 29]. Thus, it will be interesting to verify if the formation of intramolecular interactions has any influence on this property. Our preliminary results indicate that for the most complex anthocyanins, that is **A**, **B-3**, **B-4** and **B**, there is at least an effect of slowing down on the rate of thermal degradation, since in solutions of pH $\approx$ 3.5 kept in the dark for a period of *ca* 24 hr, no appreciable change in the electronic absorption spectra occurs, whereas for the simpler pigments of the series studied the inverse happens, i.e. there is already a significant decrease in the intensity of the visible absorption band. This thermal stability suports the existence of a folded conformation of the pigments since this would diminish the extent of chalcone formation.

As stated above, the only molecule of the two series here reported that follows a different behaviour is **A-2**, which possesses only a gentiobioside (a linear disaccharide) as substituent in position 3. The electronic absorption spectrum of its flavylium form presents a sharp drop in the molar absorption coefficient (ε) value, when compared with delphinidin 3-glucoside. This contrasts with the small hypochromic shift usually found for the anthocyanin complexed forms, either resulting from inter- or intramolecular associations [3]. Nevertheless, this

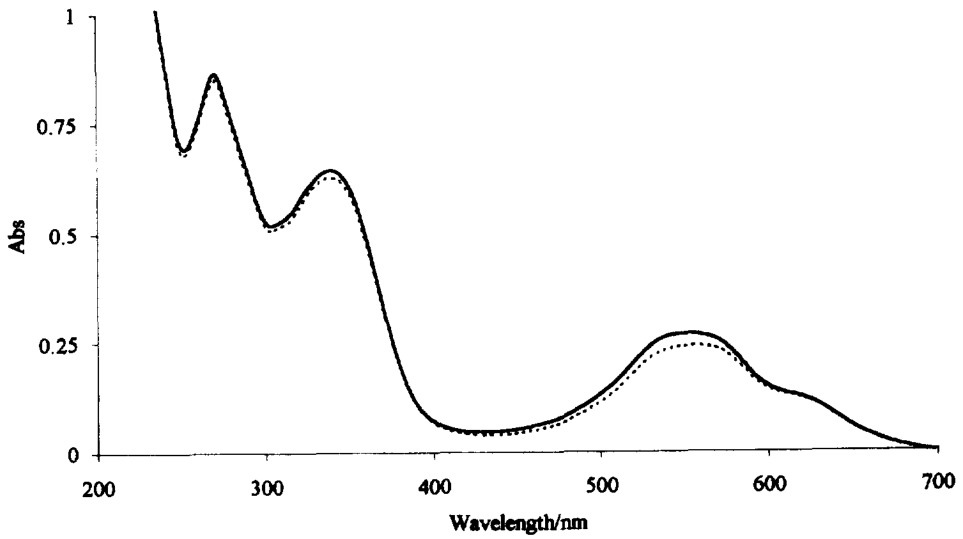


Fig. 2. Comparison of the absorption spectra of pigment A. (——) 2 h, (---) 28 h, pH = 3.63; T = 25°.

Table 2. Interatomic distances (in Å) for cyanidin 3-rutinoside (antirrhinin) and **A-2** calculated by computer modelling*

Anthocyanin	Equilibrium form	*in vacuo*		Water box	
		C^2-O^G	O^{2^H}-O^G	C^2-O^G	O^{2^H}-O^G
Antirrhinin	Flavylium	5.877	—	5.999	—
	Hemiacetal	5.041	5.012	4.975	4.822
A-2	Flavylium	3.760	—	3.433	—
	Hemiacetal	4.316	3.260	3.878	2.870

*C^2 represents the carbon atom in position 2, O^G the nearest OH oxygen atom attached to the terminal sugar (attached to carbon 4 in cyanidin 3-rutinoside and to carbon 6 in **A-2**) and O^{2^H} the hemiacetal oxygen atom.

large drop in the value of the absorption coefficient for **A-2** seems to be a general characteristic of the molecules possessing a disaccharide as sole substituent group in position 3 of the chromophore, since a similar effect is also verified in two other anthocyanins possessing also 3-biosidic residues (cyanidin 3-sambubioside-5-glucoside (Figueiredo, P. *et al.* unpublished results) and cyanidin 3-rutinoside).

Furthermore, although its pK_a value does not appreciably change when compared with the one obtained for delphinidin 3-glucoside (Table 1) the calculated pK_h value for **A-2** denotes a remarkable decrease of one unit relative to the value for the parent molecule. This may be caused by a particular stereochemical structure of the pigment in which the sugar moiety will be folded over the pyrylium system. Such a conformation would allow the formation of a hydrogen bond between the OH in position 2 of the hemiacetal form and the free OH in position 6 of the second sugar. Thus, the hemiacetal form would be stabilized and favoured over the flavylium cation at very acidic pH values and over the chalcones at milder ones, which is confirmed by the absorption spectra at pH 2.2–3.3 where no evidence for chalcone existence is found after subtraction of the flavylium contribution [30]. To assess the validity of this model, molecular mechanics calculations of both **A-2** and cyanidin 3-rutinoside, which has a CH_3 terminal group in its second sugar instead of an OH, were performed both *in vacuo* (AM1 [27]) and with a periodic box of 552 molecules of water (MM^+ [31]). For **A-2**, a folding of the gentiobioside towards the chromophore either in the flavylium or the hemiacetal forms was observed. The values obtained for the interatomic distances (Table 2) support the above stated hypothesis, since in a typical hydrogen bond the distance between the adjacent oxygen atoms ranges from 2.5 to 3.3 Å [32–34]. The value of 2.870 Å is consistent with the existence of a hydrogen bond between the sugar and the chromophore. Moreover, both types of theoretical calculations performed with cyanidin 3-rutinoside have produced interatomic distances between the hemiacetal oxygen and its closest neighbour largely superior to the ones generally accepted as capable of accommodating hydrogen bonds (Table 2).

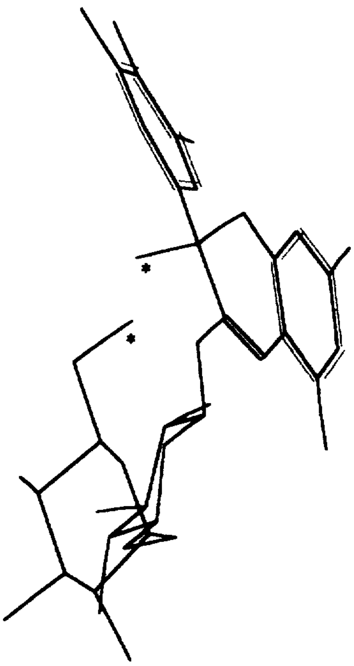


Fig. 3. AM1 calculated structure for the hemiacetal form of pigment A-2. The asterisks denote the two oxygens that may form a hydrogen bond. Hydrogen atoms are not represented for the sake of clarity.

The formation of intramolecular complexes by the hemiacetal form of an anthocyanin could also constitute a way to discriminate between the two isomers (*R* and *S*) of this species, since the molecule must be in its *R* conformation in order to form the hydrogen bond. The computational model gives in fact an *R* conformation for **A-2** as the sole existing isomer (Fig. 3) and an *S* for cyanidin 3-rutinoside as the most energetically favoured.

The results obtained with the present two sets of natural anthocyanins confirm also the known higher resistance of 3-monoglucosides to nucleophilic attack when compared to 3,5-diglucosides [3] (Table 1). This effect is attributed to a delocalization of the electron-density of the *exo*-anomeric oxygen towards the pyranose ring, resulting in a reduction of its electron-donating ability towards the pyrylium ring, when replacing an OH group by a β-D-glucopyranosyloxy group [35–37]. This substitution is expected to enhance the electrophilic character of C-2 or/and C-4, resulting in the formation of larger amounts of hemiacetal at equilibrium. In order to try to obtain some further explanations for this phenomenon, *in vacuo* AM1 molecular orbital calculations were performed on three pairs of 3-monoglucosides and 3,5-diglucosides (pelargonidin, cyanidin and delphinidin). The results obtained (Table 3) indicate a two-fold increase of the positive charge density on the carbon 4 of the chromophore when going from the mono- to the diglucosides, whereas the charge density on C-2 remains almost unchanged. We thus theorize that it is the increase of the electrophilic character of C-4 with the consequent formation of greater amounts of the C-4 OH adduct, by the diglucosides, which is responsible for the increase in K_h.

Table 3. Comparison of the data obtained through molecular orbital calculations (AM1) for a series of 3-mono- and 3,5-diglucosides

	Atomic charges		
Anthocyanin	C^2	C^4	C^5
Pg 3-gl.	0.278	0.023	0.207
Pg 3,5-digl.	0.273	0.047	0.193
Cy 3-gl.	0.271	0.022	0.207
Cy 3,5-digl.	0.264	0.056	0.183
Dp 3-gl.	0.268	0.023	0.208
Dp 3,5-digl.	0.259	0.056	0.184

The results above reported support the existence of an intramolecular association between the planar pyrylium ring and the aromatic acids of complex acylated anthocyanins that block the formation of the hydrated species. This work demonstrates also that a similar type of association could likewise occur, given the necessary conditions, i.e. the presence of a disaccharide residue in position 3, to produce the opposite effect, a stabilization of the colourless hemiacetal form.

Moreover, for the first time, is reported an intramolecular copigmentation effect on a different type of anthocyanin, one which bears a flavone moiety instead of an acidic one attached to the sugar linkers. Given the more similar structure of this type of compounds with the pyrylium ring they should constitute excellent natural sources for the stabilization of the colourant properties of anthocyanins in their natural milieu and, with the recent developments in extraction and identification techniques, it is possible that more molecules bearing this type of substituent will be discovered.

EXPERIMENTAL

Materials. Delphinidin 3-glucoside was a kind gift of Prof. Sam Asen and used without further purifications. Cyanidin 3-rutinoside was synthesized and purified according to a procedure described elsewhere [38]. All the other pigments were isolated according to published procedures [9, 11]. Their purity was checked by ^{1}H NMR spectroscopy and the results are all very good ($\geqslant$ 98%), except for the cases of pigments **B-4** (75%), which is contaminated by smaller anthocyanins resulting from acid hydrolysis and **B-2** which is a mixture of 65% of delphinidin 3,5-diacetylglucoside and 35% of both delphinidin 3-acetylglucoside-5-glucoside and delphinidin 3-glucoside-5-acetylglucoside. All other reagents were of analytical grade.

Absorption spectra. Spectra were recorded with a diode-array spectrometer fitted with a quartz cell ($d = 1$ cm) equipped with a stirring magnet. A constant temp. of 25 (± 0.1)° was obtained by use of a water-thermostated bath. Temp. was measured with a thermocouple.

Thermodynamic measurements. Stock solns of *ca* 5×10^{-4} M of the nine anthocyanins were prepared in 0.1 M HCl and left to equilibrate, in the dark, for about two hr. Then, for each pigment, 10 solns were prepared by 1:10 dilutions of the stock solns with different volumes of a NaOH 0.1 M soln and H_2O so that the final pH covered a range of 1.0 to 3.5. After equilibration in the dark, the UV-Vis spectra of these solns were recorded. The values of the global equilibrium constants ($K' = K_h + K_a$) are gained from measuring the relative hyperchromic shift at the visible absorption maxima of the flavylium cation, for all the pigments studied, as a function of pH.

Kinetic measurements. 1 ml of each equilibrated aq. soln of anthocyanin, at different pH values (from 1.3 to 2.6, depending on the pigment) is magnetically stirred in the spectrophotometer cell. The concentrations of the pigments are: 4.3×10^{-5} M for **A**; 1.4×10^{-5} M for **A-2**; 3.6×10^{-5} M for **B**; 5.0×10^{-5} M for **B-1**; 2.7×10^{-5} M for **B-2**; 3.1×10^{-5} M for **B-3**, 2.3×10^{-5} M for **B-4**, 5.0×10^{-5} M for delphinidin 3-glucoside and 6.3×10^{-5} M for cyanidin 3-rutinoside. To these solns 1 ml of phosphate buffer solns, ranging in pH from 4.3 to 7.4, was quickly added, and the visible absorbance at 520 nm (near the visible absorption maxima for all the pigments) is immediately recorded every sec over 120 sec, to guarantee that the hydration equilibrium is attained. The final pH was then measured and ranged from 2.6 to 4.4. The spectrophotometer software automatically computes the first-order apparent rate constant of the hydration reaction (k).

Molecular mechanics calculations. Molecular mechanics, both *in vacuo* and inside a simulated box of *ca* 500 water molecules, and *in vacuo* semi-empirical quantum mechanical calculations were performed on a PC using a computer program in the MM^+ [31] and AM1 [27] parametrizations, respectively.

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