Complexation of aluminium and gallium ions with synthetic anthocyanin models and natural anthocyanins extracted from the blue flowers of Evolvulus pilosus cv 'Blue Daze' and the violet flowers of Matthiola incana has been thoroughly investigated in aqueous solution. From UV–VIS spectroscopic data collected at pH 2–5, the presence of complexes, involving not only the coloured forms but also the colourless forms of the pigments is demonstrated. A theoretical treatment is developed for the calculation of the corresponding stability constants. The pigments studied throughout this work can be divided into two series, one sharing a cyanidin chromophore and the other a delphinidin one. Within both series, individual pigments are distinguished according to the degree and type of glycosylation and/or acylation. Intramolecular effects such as copigmentation of anthocyanin–aluminium complexes and the effect of the presence of a malonyl group on the formation of those complexes are discussed. These results are important to plant pigmentation and, for instance, a narrow pH domain in which colour amplification due to complexation is at a maximum has been found.

Introduction

The chromophore units of anthocyanins are hydroxy- and methoxy-derivatives of the 2-phenylbenzopyrylium (flavylium) structure. They occur as non-plastid, water soluble glycosylated pigments dissolved in the vacuolar cell sap, predominantly in the epithelial tissue of flowers and fruits, more rarely in stems and leaves. These hydroxy- and methoxy-flavylium derivatives exist as glycosides of hydroxy groups (mainly at the 3 and 5 positions) of the benzopyrylium core. The glycosylating sugars may be mono-, di- or tri-saccharides, and the aglycone forms are known as anthocyanidins (Scheme 1). Additionally, anthocyanins may be acylated through esterification of the sugar residues with one or more of a variety of aliphatic acids, phenolic benzoic acids or phenolic cinnamic acids.

Scheme 1 The flavylium form of common natural anthocyanins

The more common anthocyanins (3-monoglucosides and 3,5-diglucosides), rapidly fade when put in mildly acidic aqueous solutions. Indeed, the water molecule readily reacts at position 2 of the flavylium cation with the consequent formation of large amounts of colourless forms (hemiacetal and chalcones) according to a reversible process called the hydration reaction (Scheme 2). In vivo, these pigments may be found in association with metal ions, other flavonoids (which may themselves be glycosylated and acylated) and probably with polysaccharide macromolecular carriers. All these interactions affect the absorption spectra of the anthocyanins involved and thus they give access to the mechanisms explaining the great variations in plant colours. It is now well known that colourless polyphenols (flavanoids, flavonol, cinnamic and benzoic acid esters, tannins) are able to form molecular, non-covalent, stacking complexes with the large planar, π-electron rich flavylium nucleus. We have demonstrated that the hydrophobic interaction efficiently protects the chromophore against nucleophilic attack from water, displacing at the same time the overall equilibrium between the coloured and colourless forms towards the selectively complexed coloured forms. This phenomenon, called copigmentation, can also operate in an intramolecular way in more complex anthocyanins bearing cinnamic or benzoic acid residues on their glycosyl groups, leading to stable coloured solutions at mildly acidic pH values.

Unlike polyphenolic copigments, metal ions which could be present in the anthocyanin natural media seem more rarely involved in colour stabilization. However, small highly charged metal ions such as Al³⁺ and Mg²⁺ have been reported possibly to strengthen the pigment–copigment interaction leading to hyperchromic and bathochromic shifts. In particular, it has been proposed that the blue colour displayed by some flowers is the result of pigment–copigment–metal ion assemblies. The most remarkable achievement in this field is the X-ray structure of the commelinin pigment elucidated by Goto and Kondo, which shows up to six pigment and six copigment molecules packed around two magnesium ions in a crystalline state.

A first report on anthocyanin–aluminium complexation has provided information on the mechanism of complexation. From UV–VIS spectroscopic measurements on equilibrated solutions at different pH values and relaxation kinetics measurements (pH-jump), the binding constants have been calculated and the percentage of different free and complexed pigment forms plotted as a function of pH. ¹H NMR analysis in CD₃OD (in which complexation is much stronger than in water), has confirmed the conversion of the anthocyanin from the red flavylium form to the deep-purple quinonoidal forms upon coordination to A³⁺.
In this work, spectroscopic measurements on aluminium- and gallium–anthocyanin systems are interpreted according to a theoretical treatment so as to give information on the stability and relative abundance of metal complexes as a function of pH, and also on the mechanisms leading to their formation. Influence of malonyl and acyl groups, born by the glycosyl groups, on metal complexes production and stabilization is also discussed by comparing two series of acylated anthocyanins with either a cyanidin or a delphinidin aglycone, respectively extracted from the violet flowers of *Matthiola incana* and the blue flowers of *Evolvulus pilosus* cv ‘Blue Daze’. Their structures (Scheme 3), fully elucidated by $^1$H NMR and FAB-MS techniques, are: 3-O-{6-O-(2-O-(trans-caffeyl)-$\beta$-D-glucopyranosyl)-$\beta$-D-glucopyranosyl}-5-O-$\beta$-D-glucopyranosyl cyanidin 1, 3-O-{6-O-(trans-caffeyl)-2-O-[2-O-(trans-synapyl)-$\beta$-D-glucopyranosyl]-$\beta$-D-glucopyranosyl}-5-O-[6-O-(malonyl)]-$\beta$-D-glucopyranosyl] cyanidin 2, 3-O-{6-O-(trans-ferulyl)-2-O-[2-O-(trans-synapyl)-$\beta$-D-glucopyranosyl]-$\beta$-D-glucopyranosyl}-5-O-[6-O-(malonyl)]-$\beta$-D-glucopyranosyl] cyanidin 3, 3-O-{6-O-(trans-4-O-{6-O-(trans-3-O-[6-O-(6-deoxy)-$\alpha$-L-mannosyl]-$\beta$-D-glucopyranosyl]caffeoyl]}-$\beta$-D-glucopyranosyl}-caffeoyl-$\beta$-D-glucopyranosyl]-5-O-[6-O-(malonyl)]-$\beta$-D-glucopyranosyl] delphinidin B1, and 3-O-$\beta$-D-glucopyranosyl] delphinidin B2 (Scheme 3). 3,5-D-i-O-$\beta$-D-glucopyranosyl] cyanidin chloride (also known as cyanin chloride) 4, 3-O-[6-O-(6-deoxy)-$\alpha$-L-mannosyl]-$\beta$-D-glucopyranosyl] cyanidin chloride (also known as antirrhinin chloride) 5, 3',4',7-trihydroxy-3-methoxyflavylium chloride A1, 3',4',7-trihydroxyflavylium chloride A2, 3',4',7-trihydroxy-7-methoxyflavylium chloride A3, 3',4',7-dihydroxy-7-methoxyflavylium chloride A4 and 2-[3',4',7-di-hydroxy-3-phenyl]-3-O-methyl-naphtho[2,1-b]pyrylium chloride A5 were also studied for comparison (Scheme 4).
E xperimental

M aterials
The 3-0-β-D-glucopyranosylidophenidin B2 and cyanin chloride 4 were a kind gift from Professor Samuel Asen and were used without further purification. Compounds 5, A1, A3 and A5 were synthesized and purified according to procedures described elsewhere. All the other acylated pigments (Scheme 3) studied were isolated according to published procedures. Aluminium chloride hexahydrate (99% pure) was purchased from Aldrich and anhydrous gallium chloride from Strem, and were used as supplied.

The flavyl chloride A2 was synthesized according to Scheme 5. Reduction of 2'-chloro-3,4-dihydroxyacetophenone by zinc powder in acetic acid-tetrahydrofuran mixture led to 3,4-dihydroxyacetophenone. The corresponding enol ether was prepared in high yield (88%) via the in situ formation of trimethylsilyl iodide. M ethylation of the trimethylsilyl enol ether of 3,4-trimethylsilyloxyacetophenone was adapted from a procedure developed by Moriarty and co-workers, commercially available iodosobenzene diacetate replacing iodosobenzene. The yield was ca. 40% after chromatography on silica gel. In the, (1a) condensation step, gaseous hydrogen chloride (d) was gently bubbled (3 h) into an equimolar solution of 2'-methoxy-3,4-dihydroxyacetophenone and 2-hydroxy-4-methoxybenzaldehyde in distilled ethyl acetate at 0 °C. Red crystals of 3',4'-dihydroxy-3,7-dimethoxyflavylium chloride were formed and the reaction was completed by keeping the mixture at −20 °C for 3 d. The crystals were filtered off, thoroughly washed with ethyl acetate and dried under vacuum (yield: 65%). Compound A2 was characterized by electrospray mass spectrometry (positive mode, m/z = 290.73), UV–VIS spectroscopy (λmax = 276 and 498 nm in 0.1 mol dm−3 HCl) and 1H NMR, α [200 M Hz, (CD3)2SO–CF3CO2D; J in Hz]: 8.75 (s, H-4), 8.14 (dd, J 8.7 and 2.2, H-6), 8.10 (d, J 2.2, H-2'), 7.94 (d, J 9, H-5), 7.54 (d, J 2.2, H-8), 7.30 (dd, J 9 and 2.2, H-6), 6.98 (d, J 8.6, H-5'), 4.08 (s, CH3), 3.96 (s, CH2).

3',4'-Dihydroxy-7-methoxyflavylium chloride A4 was obtained by condensation under acidic conditions (gaseous hydrogen chloride-ethyl acetate) of 3,4-dihydroxyacetophenone and 2-hydroxy-4-methoxybenzaldehyde, and characterized by electrospray MS (positive mode, m/z 268), UV–VIS spectroscopy (λmax = 268, 275 and 498 nm in 0.1 mol dm−3 HCl) and 1H NMR, α [400 M Hz, (CD3)2SO–CF3CO2D; J in Hz]: 8.48 (d, J 9, H-3), 7.50 (dd, J 9 and 2.4, H-6), 7.67 (d, J 2.2, H-2'), 8.20 (d, J 10, H-5'), 7.94 (d, J 2.3, H-8), 8.00 (dd, J 8.7 and 2.4, H-6), 7.11 (d, J 8.6, H-5'), 4.06 (s, CH3).

The purity of A2 and A4 was checked by reverse-phase HPLC on a Merck C-8 column (5 μm, 125 mm × 4 mm) with a flow rate of 1 ml min−1 and a linear gradient elution for 30 min from 5–20% solvent A [5% HCO2H in CH3CN–H2O (1:1)] in solvent B (5% HCO2H in H2O) followed by a linear gradient elution for 30 min from 20% solvent A in solvent B to 100% solvent A. Chromatograms were recorded with a Spectra-Physics apparatus equipped with a Hewlett Packard diode-array detector monitoring at 260 and 500 nm.

A bsorption spectra
A bsorption spectra were recorded with a Hewlett Packard diode-array spectrophotometer fitted with a quartz cell (d = 1 cm) equipped with a stirring magnet. A constant temperature of 25 ± 0.1 °C, measured with a Comark thermocouple, was maintained in the spectrometer cell by use of a Lauda water-thermostatted bath. Water used in samples preparation was distilled, deionized and ultrafiltered to a resistance of ca. 18 MΩ using a Millipore Mili-Q apparatus. Methanol was spectroscopy grade (M erck).

P H M easurements
The pH of the solutions was recorded with a M etrohm model 654 pH meter fitted with a small combined glass electrode. The buffers used for calibration were pH 7 and pH 4 Aldrich standards.

D ata a nalysis
The curve fittings were carried out on a Macintosh IIsi computer using the K ALED IAG RAPH program. Standard deviations are reported.

S emi-empirical quantum mechanical calculations
S emi-empirical quantum mechanical calculations were performed on an Escom Pentium P100 PC using the HYPER-CHEM program (version 4, Hypercube, Inc., Ont., Canada) in the MM+ and AM1 parametrizations.

T hermodynamic measurements
S tructural transformations (general procedure). M other solutions of ca. 10−5–10−4 mol dm−3 of all the anthocyanins were prepared in 0.1 mol dm−3 HCl and left to equilibrate in the dark for 2 h. Then, for each pigment 10 solutions were prepared by 1:10 dilutions of the mother solutions with increasing volumes of NaOH (0.1 mol dm−3) so that the final pH covered was 1.0–5.0. The value of the thermodynamic constant K′h of the overall hydration equilibrium connecting the flavylium ion and the mixture of colourless forms, hemiacetal BH2 and chalcone forms, was gained from recording the visible absorbance at the maximum visible wavelength of flavylium absorption (D) on fully equilibrated solutions at different pH values (eqn. (1)); see later, D0 being the visible absorbance at the

$$\frac{D_0}{D_0 - D} = \frac{K_h + K_a + 10^{-pH}}{K_h + K_a + 10^{-\\phi_{AM}/\\epsilon_{AM}}} \tag{1}$$

The theoretical treatment that ensues for the obtention of the final pH plot of the apparent rate constant of hydration (first-order) of all anthocyanins was obtained from pH-jump experiments and curve-fitting of the plot of the apparent rate constant of hydration (first-order) vs. final pH [eqns. (2)]. Both procedures have been recently published with full details.10

\[
\frac{K_a + K + 10^{-pH}}{K_a} = \frac{1}{K_a + K + 10^{1-pH}} \quad (2)
\]

Complexation equilibria. M other solutions of ca. 10^{-5}–10^{-4} mol dm^{-3} of all anthocyanins were prepared in 0.1 mol dm^{-3} HCl (and 2–3% MeOH for anthocyanins of low solubility) and left to equilibrate in the dark for 2 h. A 0.1 mol dm^{-3} solution of AlCl₃·6H₂O was prepared in a 3.8 pH buffer. A range of solutions of pH 2.0–5.0 in CH₃CO₂H–CH₃CO₂Na buffers were prepared in the following way. 1 ml of a mother solution and 1 ml of the metal ion solution were mixed and diluted with different pH buffers so that the final volume was 10 ml and the final pH 2.0–5.0. The final solutions were allowed to equilibrate in the dark for ca. 3 h. Values of the complexation constants were gained from a curve-fitting of the visible absorbance at a given wavelength vs. pH plot according to eqns. (3) and (4) (see later).

Kinetic measurements

A 1 ml sample of each equilibrated aqueous solution of anthocyanin, at different pH (1.0–5.0), was magnetically stirred in the spectrophotometer cell. To these solutions 1 ml of phosphate buffer solutions, ranging in pH from 4.3 to 7.4, was added (mixing time of the order of 1 s), and the visible absorption maximum was recorded every second, over 120 s, to full completion of the hydration equilibrium. The final pH was then measured and shown to range from 2.3 to 5.0. The spectrometer software automatically computes the hydration first-order apparent rate constant, as well as the absorbance once the equilibrium state at the new pH value is finally attained. The solutions deviations were less than 2%. In the calculations the concentration of the hydronium ion is approximated to 10^{-5} mol dm^{-3}.

The theoretical treatment that ensues for the obtention of the rate constants is given in full detail in ref. 9.

Results

Anthocyanins having a catechol or a pyrogallol group in their structure are widespread in flowers. They usually appear as glycosylated derivatives of the cyanidin, delphinidin and peonidin chromophores. Reaction of metal ions like iron(II) and aluminium(III) with such flavilyum systems has been used for a long time as a qualitative test showing the presence of a catechol group in plant anthocyanins. The test is based on the observation of a colour change, or a bathochromic shift of the visible \(\lambda_{\text{max}}\) on addition of aluminium ion, usually as AlCl₃·6H₂O. Moreover, aluminium being relatively abundant in plants, its complexation with anthocyanins could be of biological relevance in the expression of the blue colour in flowers.

Anthocyanins structural transformations (exception is made for compounds 2 and 3)

The combination of thermodynamic and kinetic measurements allows us to estimate the values for the following parameters: the thermodynamic constant of the A₈H₉A₈ proton transfer reactions \(K_a\) is the modynamic constant of the overall hydration of A₈H₉A to the mixture of colourless hemiacetal and chalcone forms \(K_{1,a}\). The rate constants of the hydration process \(k_1\) and \(k_2\) for the forward and backward reactions, respectively, connecting A₈H₉A with the hemiacetal BH₇, which in our kinetic experiments is indistinguishable from the E-chalcone C₂, because of the very fast ring-chain tautomerism. The corresponding thermodynamic constant is denoted \(K_a\) and equals \(k_1/k_2\). \(K_a\) is defined as \(k_1/(k_1 + k_2)\). \(K_a\), \(K_{1,a}\), \(K_{2,a}\), \(K_{3,a}\), and \(K_{4,a}\) are respectively \(K_{[BH₇] + [C₂] + [C₃]}/[A₈H₉A]\), \(K_{[BH₇] + [C₃]}/[A₈H₉A]\), \(K_{[BH₇] + [C₂]}/[A₈H₉A]\), \(K_{[BH₇] + [C₃]}/[A₈H₉A]\), and \(K_{[BH₇] + [C₃]}/[A₈H₉A]\), respectively. \(K_{1,a}\) being 10^{-5} mol dm^{-3}, \(K_a\) is thus expressed as \(K_{1,a} = [BH₇] + [C₂]/[A₈H₉A]\). The thermodynamic constant of the E-isomerism \(K_a\) can be estimated from the relationship, \(K_a = K_{1,a}(1 + K_1)\) (Scheme 6). Values of \(K_a\), \(K_{1,a}\), \(K_{2,a}\), \(K_{3,a}\), and \(K_{4,a}\) are reported in Table 1.

\[
\begin{align*}
\text{A₈H₉A} + \text{H₂O} & \rightleftharpoons \text{BH₇} + \text{C₂} + \text{C₃} + \text{H}^+ \\
\text{A₈H₉A} & \rightleftharpoons \text{A} + \text{H}^+ \\
\text{C₂} & \rightleftharpoons \text{C₃}
\end{align*}
\]

Scheme 6

Structural transformations of compounds 2 and 3 (special cases)

For this kind of acylated pigments, it has been shown that at pH > 1 intramolecular copigmentation occurs giving rise, for the flavilyum cation, to a special conformational CP with a smaller \(c\) value than that of the flavilyum ion at pH < 1.1\textsuperscript{13} In these two particular cases \(K_{1,a}\) and \(K_{2,a}\) are denoted \(K_{1,a}\) and \(K_{2,a}\), and redefined as \(a_{[BH₇] + [C₂]} + [C₃]/[C₃][C₃][CP] + a_{[BH₇] + [C₃]}[CP] + K_{1,a}\), the equilibrium constant of the flavilyum cation conformational change, is given by \([CP][A₈H₉A]\) (Table 1). The theoretical treatment that follows for the obtention of the equilibrium rate constants is described in ref. 17 [see eqn. (10) and Fig. 3 in the reference].

Complexation equilibria

The experimental curves of Fig. 1 are best fitted by theoretical curves postulating formation of two metal complexes of 1:1 stoichiometry, one (A M) involving the coloured forms of the pigment and the other (B M) involving the colourless forms taken as a whole (Scheme 7). When complex formation is
Table 2  Thermodynamic constants of complexation of the flavylium cations with AIPB and GaA in acetate buffers at 25 °C (see text for definitions)

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKAM</th>
<th>pKBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.96 ± 0.04</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>2.66 ± 0.04</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>2.84 ± 0.05</td>
<td>b</td>
</tr>
<tr>
<td>4</td>
<td>3.13 ± 0.03</td>
<td>4.84 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>4.05 ± 0.05</td>
<td>5.22 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>2.39 ± 0.08</td>
<td>5.67 ± 0.04</td>
</tr>
<tr>
<td>B1</td>
<td>2.33 ± 0.06</td>
<td>7.05 ± 0.04</td>
</tr>
<tr>
<td>B2</td>
<td>3.53 ± 0.04</td>
<td>6.64 ± 0.08</td>
</tr>
<tr>
<td>A1</td>
<td>4.04 ± 0.06</td>
<td>6.39 ± 0.05</td>
</tr>
<tr>
<td>A2</td>
<td>3.17 ± 0.04</td>
<td>6.81 ± 0.06</td>
</tr>
<tr>
<td>A3</td>
<td>4.36 ± 0.02</td>
<td>6.17 ± 0.04</td>
</tr>
<tr>
<td>A4</td>
<td>4.12 ± 0.09</td>
<td>6.82 ± 0.12</td>
</tr>
<tr>
<td>A5</td>
<td>4.25 ± 0.02</td>
<td>5.84 ± 0.16</td>
</tr>
</tbody>
</table>

\[ \text{pK}_{\text{AM}}^* \text{ and } \text{pK}_{\text{BM}}^* \text{ values are not available due to very limited amounts of the corresponding pigments.} \]

Discussion

Thermodynamic investigation in water

In slightly acidic aqueous solutions, aluminium binds moderately to a given anthocyanin requiring large aluminium: pigment molar ratios to guarantee a complete complexation. In such conditions only 1:1 complexes should be formed. This is no longer true in methanol due to a much stronger complexation. \[^{10}\] AIPB and GaA are small highly charged metal ions. The weak binding ability of the acetate ions (buffer) is neglected for this kind of metal but would have to be considered for some other metals, such as Mg. \[^{2}\] The complexation equilibria are written from the completely protonated ligands AHB and BH. One should recall that BH notation relates to the mixture of colourless forms (hemiacetal and chalcones) at equilibrium and that BM represents the corresponding mixture of aluminium complexes.

\[
\begin{align*}
\text{AH}_2 + \text{H}_2 \text{O} & \rightleftharpoons \text{BH}_2 + \text{H}^+ + \text{K}^+ \\
\text{AH}_2 & \rightleftharpoons \text{AH} + \text{H}^+ + \text{K}_a \\
\text{AH} + \text{M} & \rightleftharpoons \text{AM} + 2\text{H}^+ + \text{K}_{\text{AM}} \\
\text{BH}_2 + \text{M} & \rightleftharpoons \text{BM} + 2\text{H}^+ + \text{K}_{\text{BM}} \\
\end{align*}
\]

Scheme 7

Expressed from the protonated ligands, the corresponding thermodynamic constants \( \text{K}_{\text{AM}} \) and \( \text{K}_{\text{BM}} \) in the coloured forms are also reported. The strong bathochromic shifts accompanying complexation point to the pigment having, after the loss of two protons in the complex, a form similar to A. According to the data collected from molecular calculation (M M * force field, followed by semi-empirical AM1 parametrization), it appears that for the synthetic 3-deoxyflavylium chlorides, the calculated charge density in position C-3 of the flavylium-AIB or -GaA complexes correlates with the \( \lambda_{\max} \) of these complexes in acidic methanolic solutions. It seems that the substituent in position 3 is important for anthocyanins to complex metals. Indeed, increasing the size and changing the nature of that substituent leads to rotation in the B-ring, which is certainly important in the evolution of the hydration and the complexation processes. Simulation of the electronic spectra using the CI method (AM1 parametrization) gives good results for the visible absorption maxima, showing that the calculated structures and charge density at C-3 reflect the behaviour of these pigments in solution (pigments A3 and A4).

\[
\begin{align*}
\text{AH}_2 & \rightleftharpoons \text{CP} + \text{K}_1 \\
\text{CP} + \text{H}_2 \text{O} & \rightleftharpoons \text{BH}_2 + \text{H}^+ + \text{K}^{\text{CP}}_1 \\
\text{CP} & \rightleftharpoons \text{AH} + \text{H}^+ + \text{K}^{\text{CP}}_a \\
\text{CP} + \text{M} & \rightleftharpoons \text{AM} + 2\text{H}^+ + \text{K}^{\text{CP}}_{\text{AM}} \\
\text{BH}_2 + \text{M} & \rightleftharpoons \text{BM} + 2\text{H}^+ + \text{K}^{\text{CP}}_{\text{BM}} \\
\end{align*}
\]

Scheme 8

\[ \text{At a fixed wavelength in the visible range, the absorbance may be expressed in terms of: } D = \varepsilon_{\text{AM}}[\text{AH}_2] + \varepsilon_{\text{AM}}[\text{AH}] + \varepsilon_{\text{AM}}[\text{A}] + \varepsilon_{\text{AM}}[\text{M}] , \text{ the } \varepsilon \text{ values being the molar absorption coefficients (Scheme 7). At pH lower than 5, the anionic quinonoidal base A is a very minor species and can be neglected. The total concentration of pigment can be written as: } c = [\text{AH}_2] + [\text{AH}] + [\text{A}] + [\text{M}] + [\text{BH}_2] + [\text{BM}] . \text{ These relations are combined with the thermodynamic constants } K_b, K_a, K_{\text{AM}}, K_{\text{BM}} \text{ and } K^{\text{CP}}_a, K^{\text{CP}}_1, K^{\text{CP}}_{\text{AM}}, K^{\text{CP}}_{\text{BM}} \text{ for the fitting.} \]

(see Results for their definitions) and $K_{a2} (K_{a3} = a_{w}[A]/[A\text{H}])$ to give eqn. (3). Generally we can consider $K_h >> K_a >> K_{a2}$.

$$D = \frac{D_2 + D_1K_{10^{0pH}} + D_2K_{a2}K_{10^{0pH}} + D_2K_{AM}M_{10^{2pH}}}{1 + (K_h + K_d)K_{10^{0pH}} + K_{a2}K_{10^{0pH}} + K_{AM}M_{10^{2pH}} + K_{BM}M_{10^{2pH}}} \tag{3}$$

which leads to the more simple eqn. (4). In these equations, $M_1$, $M_2$, and $M_3$ are the total metal ion concentration and can be approximated to the free metal ion concentration because of the large metal–pigment molar ratios used in the experiments in aqueous solution. Finally, $D_2$, $D_1$, $D_2$ and $D_3$ are $a_{w}C_2$, $a_{w}C_2$, $a_{w}C_2$ and $a_{w}C_2$, respectively. Eqn. (4) can be checked either by varying the pH in aqueous solutions of pigment and aluminium at fixed concentrations (Fig. 1) or by varying the metal ion concentration in aqueous solutions of pigment at fixed concentration, the pH being held constant. In the first case, a curve fitting yields the best values for $K_{AM}$ and $K_{BM}$, $D_1$ and $D_2$ being additional floating parameters. $D_3$ is determined in a solution at pH < 1, where neither hydration nor complexation take place. In the second case, for some anthocyanins, the pH is selected so that both $A\text{H}$ and BM can be neglected. In those conditions, eqn. (3) can be rearranged in eqn. (5) (with additional simplification due to $K'_{10^{0pH}} >> 1$) and a D vs. $M_1$ double reciprocal plot gives a straight line from which $K_{AM}$ can be readily estimated.

In the other cases, when $A\text{H}$ cannot be neglected, eqn. (6)

$$D = \frac{D_1K_{10^{0pH}} + D_2K_{AM}M_{10^{2pH}}}{1 + (K_h + K_d)K_{10^{0pH}} + K_{a2}K_{10^{0pH}} + K_{AM}M_{10^{2pH}}} \tag{6}$$

gives good results, fitting correctly the experimental points (acylated anthocyanins 2, 3, 8, B1).

For anthocyanins 2 and 3, a special treatment is necessary, and the absorbance can be expressed at a fixed wavelength in the visible range as:

$$D = a_{AM}[A\text{H}]_2 + c_{AM}[A\text{H}] + c_{AM}[A\text{M}]_2 + c_{AM}[A\text{M}]_3 + c_{AM}[A\text{M}]_4,$$

$A\text{M}$ being the anthocyan–metal complex with intramolecular complexation of the chromophore by the acyl groups (see Scheme 8). The total concentration of pigment can be expressed as:

$$c = [A\text{H}]_2 + [A\text{H}] + [A\text{M}]_2 + [A\text{M}]_3 + [A\text{M}]_4 + [B\text{H}] + [B\text{M}] + [CP]$$

The combination of these two relations with the constants $K_h$, $K_{a2}$ and $K_{a3}$ defined before leads to eqn. (7) (Figs 1 and 2).

$$D = \frac{D_1 + D_2K_{10^{0pH}} + D_2K_{AM}M_{10^{2pH}}}{1 + (K_h + K_d)K_{10^{0pH}} + K_{a2}K_{10^{0pH}} + K_{AM}M_{10^{2pH}}} \tag{7}$$

The values for $K_h$, $K_{a2}$, $K_{AM}$ and $K_{BM}$ given in Tables 1 and 2 allow us to plot the relative concentrations of coloured forms as a function of pH in the presence of aluminium. Figs 3 and 4 express the influence of the pH on metal–anthocyanin complexation and deserve a few comments.

![Fig. 2](view_online)

**Fig. 2** Plot of absorbance (580 nm) vs aluminium ion concentration of anthocyanin 3 in a 0.5 mol dm$^{-3}$ formate buffer at pH 4.00 (T 25°C). Pigment concentration: 9.62 × 10$^{-4}$ mol dm$^{-3}$. Aluminium molar ratio: 1:1000. pH: 2.42 (0); 2.74 (1); 3.00 (2); 3.51 (3); 3.82 (4); 4.16 (5).

![Fig. 3](view_online)

**Fig. 3** UV–VIS spectra of equilibrated solutions of anthocyanin 3 in a 0.5 mol dm$^{-3}$ formate buffer at different pH values in the presence of Al$^{3+}$ (T 25°C). The first transformation taking place is hydration, regardless of whether a metal is present or not. In both cases, large amounts of colourless forms (mainly the hemiacetal) are produced and most of the colour is lost at pH 2 to 3. When the values of $pK_{AM}$ and $pK_{BM}$ (Fig. 5) are clearly appears that metal complexation is dependent on the concentration of hydrated forms except for acylated anthocyanins 1, 2, 3, 8, B1 and B1. In the metal containing solutions, at pH 2–3, competition between protons and metal ions for the complexing sites of the pigment is in favour of the former and no significant complexation occurs. When the pH is increased above pH 3, A$^{1-}$ becomes able to remove the phenolic protons of the flavylium
ring and increasing amounts of the chelate appear as the pH is raised. This complexation process is in competition with hydration. The colour is not only strongly intensified but also very different. Indeed, in the pH domain where the flavylium ion is the dominant coloured form, complexation is accompanied by the loss of two phenolic protons so that the chromophore adopts a quinonoidal structure responsible for the deep-purple colour. From pH 4 to 5, the quinonoidal bases replace the flavylium ion; the proton activity becomes weak enough for the phenolic protons of the still abundant hemiacetal and chalcones to be replaced by aluminium and strong catechol-aluminium complexation begins to operate with those species. A consequence, the overall hydration equilibrium is shifted towards the hemiacetal and the chalcone and the colour begins to decay (Fig. 4).

It is important to emphasize that pigment acylation selectively stabilizes the metal–anthocyanin coloured complexes through intramolecular complexation. In fact, the non-availability of $K_{BM}$ values reflects the influence of these acyl groups via the overall conversion of the coloured forms into molecular complexes. The isosbestic point in Fig. 3 at 550 nm provides evidence for the equilibrium between the two forms CP and $\text{AM}^{+}$. The presence of malonyl groups attached to a glycosyl residue seems to participate in the deprotonation of the hydroxy group at position 7 of the chromophore, leading to quinonoidal base formation at a pH lower than usually observed for most of the flavylum cation. This assumption is supported by $\text{M}^{+}$ molecular orbital calculations.\(^{19}\) performed in a water periodic box simulating the aqueous solutions. The computed interatomic distances for anthocyanins 2 and 3, between the malonyl moieties and the hydroxyl at position 7 of the chromophore range from 280 to 320 pm. An interval of 300 pm is consistent with the existence of an hydrogen bond between the malonyl and the hydroxyl group of these chromophores.

**Photochromism of Some Metalloanthocyanins**

Another important aspect within the present series of pigments is the photochromic behaviour of some of our flavylium cations such as A3 and A4, for instance (Fig. 6). It is now well established\(^{20}\) that the colour enhancement observed upon irradiation of moderately acidic solutions of some synthetic anthocyanidins is due to a trans-to-cis-chalcone photoisomerization followed by ring closure to give the coloured flavylum cation. The same behaviour is here reported for A3 and A4 where irradiation of equilibrated solutions at different pH values, in the presence of Al\(^{3+}\) or Ga\(^{3+}\) leads to enhancement of the visible absorbance due to the metal-flavylium complex. The trans-to-cis-photoisomerization should thus in this particular case be immediately followed by the metalloanthocyanin ring closure.

**Conclusions**

Metallic complexation of anthocyanins bearing a catechol moiety is a process strong enough to induce impressive colour changes going from pale-red to deep-purple which are interpreted as a large conversion of colourless forms into a coloured chelate in which the pigment adopts a quinonoidal structure. The presence of acyl and malonyl groups seems to be fundamental for colour stabilization in weakly acidic solutions. Relative to previous reports on anthocyanin molecular interactions, this work shows the ability of not only synthetic but also natural anthocyanins to form stable complexes with small highly charged metal ions such as Al\(^{3+}\) and Ga\(^{3+}\). Moreover, aromatic and aliphatic acylations of these pigments is demonstrated to play a role in the stability and intensity of colours exhibited by the complexes formed. This indicates a more widespread in vivo occurrence of supramolecular edifices constituted by acylated anthocyanins and small cations such as Fe\(^{3+}\), Al\(^{3+}\) and/or Mg\(^{2+}\) and even other polyphenols that commonly occur in cell vacuoles.\(^{21}\)

**Acknowledgements**

P. F. wishes to thank the European Union for an ERBC-BICT941610 post-doctoral grant. The authors wish to express their gratitude to Professor Samuel Azen for the gift of delphinidin 3-glucoside and cyanidin 3,5-diglucoside samples and to Professor Georges Wipff and Dr Philippe Guilbault for their help in the molecular modelling of metalloanthocyanins.

**References**

12 N. Toki, N. Saito, K. Kawano, T. S. Liu, A. Shigihara and T. Honda, Phytochemistry, 1994, 36, 609.