pH and Solvent Effects on the Copigmentation Reaction of Malvin with Polyphenols, Purine and Pyrimidine Derivatives

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The influence of pH on the copigmentation reaction of malvin has been investigated from an experimental and theoretical viewpoint. The general equation for the copigment effect, when monitored by visible absorption spectrometry, is derived and it is shown to be in good agreement with results obtained in the case of three different copigments, namely chlorogenic acid, caffeine and adenosine. In particular, we demonstrate that association of malvin with the copigment occurs for all coloured malvin species and the corresponding stability constants are given. A few tannins and a few purine or pyrimidine derivatives have also been tested for their ability to act as copigments; some were shown to associate quite strongly with malvin. On the effect of the solvent on the extent of the copigmentation phenomenon, we reached an interesting conclusion: no solvent is better than water. Such a result seems to indicate that the strength of the copigment effect parallels the cohesion of the hydrogen-bonded tetrahedral network of water molecules.

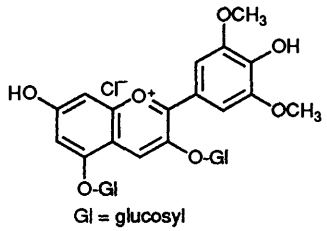
Copigmentation is one of the most important factors involved in plant pigmentation due to the presence of anthocyanins.[1] It is now well-established that, in aqueous solutions, common anthocyanins exist as a mixture of several different structures in chemical equilibrium.[2] Only a few of these structures are coloured. When a colourless copigment is added to a previously equilibrated, weakly coloured, aqueous anthocyanin solution, a fast increase in the intensity of the colour of the solution occurs.[3] Copigmentation gives rise to two UV–VIS absorption features characteristic of the anthocyanin chromophore: a positive shift in the absorbance in the visible range (hyperchromism), and a positive shift in the wavelength of the absorption maximum of the visible band (bathochromism). It is generally assumed that non-covalent complex formation, between the anthocyanin coloured structures and the copigment, is responsible for the intensification and change in the solution initial colour.[4] Copigmentation is a spectacular type of interaction which one may include in the more frequently encountered polyphenol molecular interactions.[5] Nevertheless, copigmentation is not just one more molecular interaction among polyphenols; for instance, it possesses unique features, among which is its unusual and extraordinary sensitivity, not only to the nature and the concentration of both pigment and copigment, but also to the temperature and to the composition of the medium in which it takes place.[6] Copigmentation is also of importance for the food industry in preparing anthocyanin-based food colourants and in the colour stabilization processes of many fruit-juice-based beverages.[7]

In a recent paper,[6] we demonstrated that chlorogenic acid, a naturally very abundant polyphenol, associates with the flavylium cation of malvin to give a 1:1 molecular complex. In that work, copigmentation was studied at only one pH value (3.6), which corresponds to the domain where the copigment effect exerted by chlorogenic acid on the coloured malvin molecules reaches its maximum. In the present paper, we extend our knowledge of the copigmentation reaction, by investigating the full pH range within which copigmentation can occur. In particular, the general equation for the copigment effect is derived. Experimental results, in the case of three different pigment-to-copigment couples, are shown to be in satisfactory agreement with their theoretically predictable behaviour. The pigment chosen is malvin (**1**), a common anthocyanin known to be very sensitive to the copigment effect.[6] As copigments, in the pH effect investigation, three different naturally occurring chemicals, namely chlorogenic acid (**2**), caffeine (**3**) and adenosine (**4**) have been selected, two of them mainly for their importance in the food industry, and the third one for its importance in molecular biology. In this paper we also report results on the influence of the nature of the medium on the extent of the copigmentation reaction. For this, various amounts of ten different cosolvents were mixed with water and the corresponding solvent effects on the copigmentation of malvin were measured. Finally, a few more copigments, belonging either to the family of purine and pyrimidine derivatives, or to the family of tannins, were tested at two pH values. Interestingly, two epimers, (+)-catechin (**5**) and (−)-epicatechin (**6**), were found to produce significantly different copigment effects with malvin.

Experimental

Materials.—Malvin chloride, chlorogenic acid, caffeine, adenosine, (+)-Catechin, (−)-epicatechin and gallic acid (**7**) were purchased from Roth. The purity of the above mentioned polyphenols was checked by chromatography according to previously described procedures.[8] ^{1}H NMR spectra of compounds **1**–**7** were found to agree with published spectra. With the exception of adenosine, purine and pyrimidine derivatives appearing in Table 3 were provided by Sigma and used as such. β-1,2,3,4,6-Pentagalloylglucose (**8**) was kindly supplied by Dr. Moutounet from *Institut des Produits de la Vigne* at Montpellier and used without further purification. Spectrophotometric grade, or equivalent, cosolvents were obtained either from Merck (methanol, ethanol, formamide, *N*-methylformamide) or Fluka (acetic acid, *N*,*N*-dimethylformamide, acetonitrile, ethylene glycol) or Prolabo (formic acid) or Carlo Erba (acetone). Buffer components were purchased from Prolabo (hydrochloric acid, disodium hydrogenphosphate) and Lancaster (citric acid).

Solutions.—Solutions, with and without copigment, were prepared according to the following procedure. A known amount of malvin chloride was dissolved in either pure water or in a mixture of water and an organic cosolvent (solution S). To an aliquot of solution S, citric acid was added to the concentration of 0.1 mol dm^{-3} (solution S_1). Solution S_2,



Gl = glucosyl

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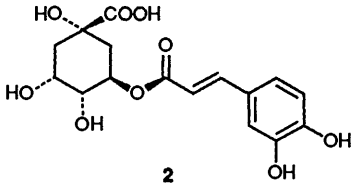

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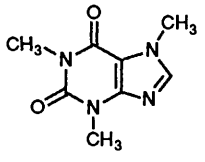

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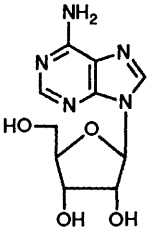

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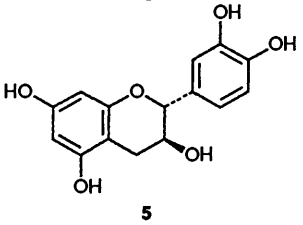

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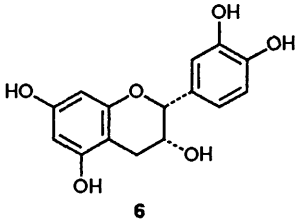

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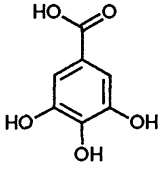

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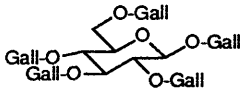


(Gall = gallic acid)

8

containing disodium hydrogenphosphate (0.2 mol dm^{-3}), was also prepared from solution S. The next step was to mix x cm^3 of S_1 with y cm^3 of S_2 resulting in a solution whose absorbance D_0 is taken as the reference in the absence of a copigment. Solutions with a copigment were prepared in the same way, except that a given amount of the copigment was dissolved in solution S, prior to the introduction of the buffer components. Thus, besides water and eventually a cosolvent, solutions S'_1 and S'_2 contained, respectively, malvin, copigment and citric acid (S'_1), or malvin, copigment and disodium hydrogenphosphate (S'_2). x cm^3 of S'_1 added to y cm^3 of S'_2 resulted in a final copigmented solution whose absorbance D corresponds to the equilibrium absorbance of malvin in the presence of a copigment. Thus couples of D and D_0 absorbances were recorded, at the same wavelength, for the same medium and at 20 (±0.2) °C. Whenever necessary, the small differences in pH values, in the final copigmented solution relative to the corresponding solution without copigment, were corrected by addition of a few mm^3 of concentrated HCl or NaOH solutions. As the pH was changed, slight variations in the ionic strength of the solutions occurred; no attempt, however, was made to maintain the ionic strength either, rigorously constant, or buffered. Solutions S_1, S_2, S'_1 and S'_2 were used immediately after they had reached equilibrium. Care was taken to avoid pigment decomposition, especially among the slightly alkaline S_2 and S'_2 solutions. By changing the volumes x and y, buffered pH solutions, ready for spectral measurements (D_0 and D), could be prepared with pH values in the range *ca.* 3–8. For the more acidic solutions (pH ⩽ 2.5), instead of the citric acid–disodium hydrogenphosphate buffer, hydrochloric acid was used to adjust the pH to the desired value.

Spectrophotometric Measurements.—Visible absorption spectra of equilibrated and buffered malvin solutions, with and without copigment, were recorded with a Hewlett-Packard diode-array spectrophotometer fitted with a thermostatted 1.0 cm pathlength quartz cell. If not otherwise stated, D_0 and D absorbance values were recorded at 520 nm. At this wavelength no interference in the pigment absorption by a copigment or a cosolvent occurs. Whenever necessary, a small correction for the scattering of light was made according to a procedure already incorporated in the available spectrophotometer software.

pH *Measurements.*—The pH of the solutions was recorded with a Metrohm model 654 pH meter fitted with a small combined glass electrode (Metrohm EA 125). The buffers used to calibrate the pH meter were pH 7 and pH 4 NBS standards, and the temperature of each solution was kept at 20 (±0.2) °C using a thermostatted water-bath. In the case of the solutions containing a cosolvent added to water no correction of the pH value read on the pH meter, for the presence of the cosolvent, was made.

Results and Discussion

pH *Effect: Experimental Evidence.*—The magnitude of the copigment effect has been demonstrated to be dependent upon the type and the concentration of both the anthocyanin and the copigment,[9] the temperature and the composition and pH of the aqueous medium.[10] In particular, the copigmentation reaction takes place in the whole acidic pH range and even extends slightly into the alkaline range.[11] Part of the present paper is devoted to the study of the copigmentation complexation of malvin by three different copigments from pH 0.5 to pH 8.

The effect produced by various amounts of chlorogenic acid, on the absorption band, in the visible range, of malvin at pH 0.65 is shown in Fig. 1. Adding large quantities of chlorogenic acid to the acidic malvin solution gives, at the same time, a bathochromic shift and a hypochromic shift. At this pH, malvin exists only in the flavylium form (see Scheme 1), and its association with chlorogenic acid only leads to a blue shift with a simultaneous slight decrease in the molar absorption coefficient maximum value. As the pH rises, the bathochromic shift remains more or less constant, whereas the hypochromic

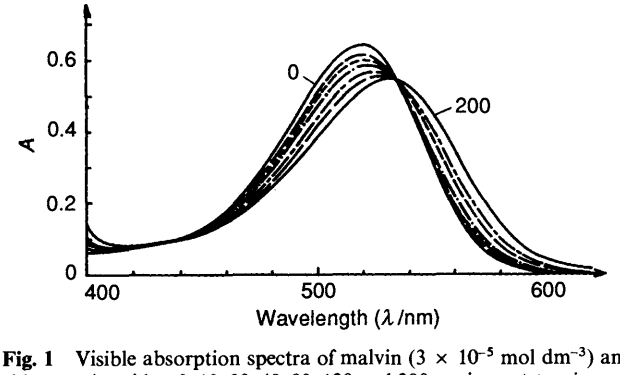


Fig. 1 Visible absorption spectra of malvin (3×10^{-5} mol dm^{-3}) and chlorogenic acid at 0, 10, 20, 40, 80, 120 and 200 copigment-to-pigment molar ratios. pH = 0.65; T = 25 °C; d = 1 cm; solvent: aqueous hydrochloric acid.

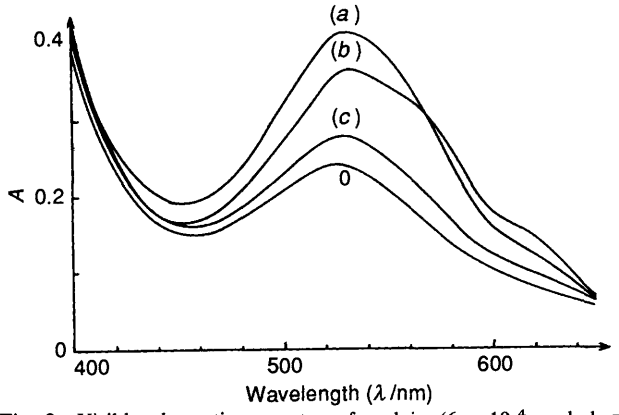
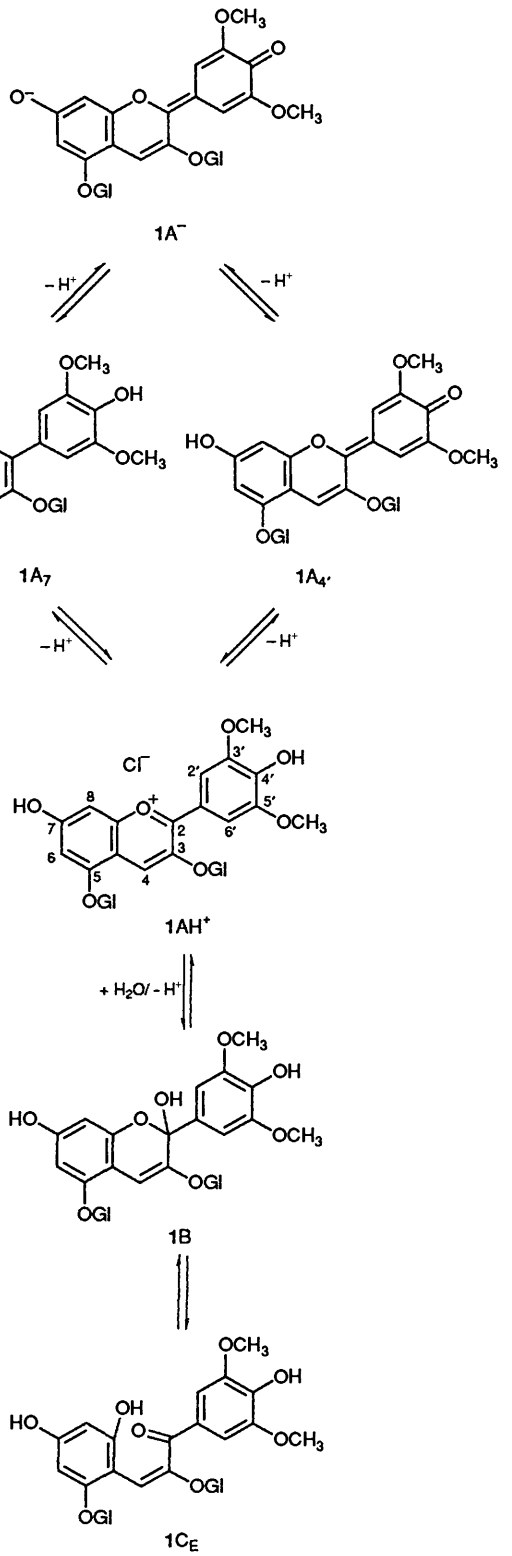


Fig. 2 Visible absorption spectra of malvin (6×10^{-4} mol dm^{-3}) without copigment (0), and with, either chlorogenate ion (*a*), caffeine (*b*) or adenosine (*c*). pH = 4.00; T = 20 °C; d = 1 cm. Copigment-to-pigment molar ratio: 7. Solvent: aqueous citric acid–disodium hydrogenphosphate buffer.

shift is replaced by the reverse effect, that is a strong increase in the absorption at any wavelength in the visible range (hyperchromism). The latter phenomenon represents what is usually called the copigment effect (bathochromism and hyperchromism). Examples are given in Fig. 2, where three different copigments, namely the chlorogenate anion, caffeine and adenosine have been used. It can be seen that, at pH 4, the chlorogenate anion appears as the more powerful copigment, caffeine being second while adenosine seems to act as a rather poor copigment. Nevertheless, caffeine and adenosine qualitatively behave in the same manner, producing, at pH 4, larger increases in the absorption at the higher wavelengths. The difference between the chlorogenate type and the caffeine type of copigment will be explained further on the basis of the possible interactions between a given copigment and the various, pH-dependent, coloured malvin structures (see Scheme 1). Now we would like to point out a major difference between copigmentation in strongly acidic solutions (Fig. 1) and copigmentation in slightly acidic solutions (Fig. 2). In the former case (pH 0.65), even in the presence of a very large excess of the copigment, there is only a small effect on the absorption of the flavylium cation in the visible range; the absorbance remains roughly of the same order of magnitude, with only a slight decrease, below *ca.* 530 nm, and a corresponding slight increase above this value. In contrast, at pH 4 (Fig. 2), the increase in the absorption occurs at all wavelengths in the visible domain and, moreover, this increase is much more sensitive to the amount of copigment present in the solution. For instance, for the low pH and at 520 nm, with a chlorogenic acid to malvin molar ratio of 200, a 0.12 decrease in the absorbance is observed, while at pH 4 and a molar ratio of chlorogenate to malvin of only 7, a 0.16 increase in the 520 nm absorbance is recorded. It has been previously demonstrated[6] that, in the case of the chlorogenic acid–malvin system at pH close to 3.6, and in the presence of a large excess of the copigment, the final absorbance can be as much as 20–30 times its initial value in the absence of the copigment. Of course, chlorogenic acid, caffeine and adenosine are colourless molecules and, therefore, they do not contribute by themselves to the absorption increase in the visible range. On the basis of the Beer–Lambert law and the law of mass action, and taking into account the structural transformations of malvin, we are now able to establish the general equation of the copigmentation reaction when monitored by means of visible absorption spectrometry.

pH *Effect: the General Equation of the Copigment Effect.*— For the sake of clarity, we decided to divide our theoretical treatment into two parts. The first part concerns solutions whose pH lies in the range 0.5 to 5–6; the second part deals with solutions having pH values from 5–6 to 8. In Scheme 1 are drawn the various, pH-dependent, malvin structures appearing in strongly acidic to slightly alkaline aqueous media. Structures A^-, A, AH^+, B and C_E are, respectively, the anionic quinonoidal base, the neutral base, the flavylium cation, the hemiacetal and the (*E*)-chalcone.[12] Very minor species have been omitted from Scheme 1; these are an isomeric form of B (water 4-adduct), a (*Z*)-chalcone and, as far as slightly basic solutions are concerned, the ionized (*E*)-chalcone. Species A^-, A and



Scheme 1

$$AH^+ \rightleftharpoons (B + C_E) + H^+ \qquad K_h$$

$$AH^+ \rightleftharpoons A + H^+ \qquad K_a$$

$$AH^+ + CP \rightleftharpoons AHCP^+ \qquad K_1$$

$$A + CP \rightleftharpoons ACP \qquad K_2$$

Scheme 2

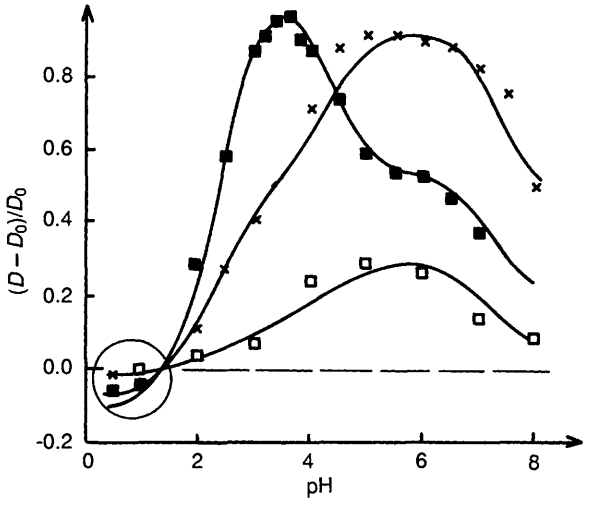


Fig. 3 Plot of $(D\text{-}D_0)/D_0$ *vs.* pH for 6×10^{-4} mol dm^{-3} malvin solutions copigmented by chlorogenic acid (■), caffeine (×) and adenosine □). Solvent: aqueous citric acid–disodium hydrogenphosphate buffer or aqueous hydrochloric acid. Copigment-to-pigment molar ratio: 7. T = 20 °C; λ = 520 nm. The solid lines were drawn according to the values calculated from eqns. (4) or (7), depending on the investigated pH range. For each copigment-to-pigment couple, they represent the best fit to the corresponding experimental values. The nine derived values for the stability constants K_1, K_2 and K_3 are listed in Table 1. The specific malvin equilibrium constants have been taken from ref. 13 and are as follows: $K_h = 10^{-2.0}$; $K_a = 10^{-3.9}$; $K_A = 10^{-7.0}$. At 520 nm the listed r values were measured as: $r_1 = 0.80$; $r_2 = 0.50$; $r_3 = 0.45$; $r_4 = 0.90$; $r_5 = r_6 = 0.55$. The latter set of values was used throughout this work, although small changes may occur according to different types of copigment. Measurements reported inside the circled area show magnitudes representative of a classical molecular interaction. Such values should be compared to those in the pH range 2–8 where the amplifying factor related to the large reservoir of colourless B and C_E forms is at work.

AH^+ strongly absorb visible light. AH^+ is stable in the more acidic solutions, A and A^- are unstable and only appear in the slightly acidic to neutral solutions. At equilibrium, and in the absence of any copigment, A and A^- are never more than a few percent of the total malvin concentration. Finally, neither B nor C_E absorb light in the visible range.

(i) pH *Range from* 0.5 *to* 5–6. The copigmentation reactions of AH^+, A and A^- have been demonstrated by us to be bimolecular processes. Scheme 2 represents the reactions taking place in the presence of a copigment (CP) for the pH range mentioned above. $AHCP^+$ and ACP are molecular complexes whose stabilities are pH-dependent as will be seen later.

Eqns. (1)–(3) may be written in which D_0 is the absorbance, in the visible range, in the absence of copigment, D is the

$$D_0 = d(\varepsilon_{AH^+}[AH^+]_0 + \varepsilon_A[A]_0) \qquad (1)$$

$$D = d(\varepsilon_{AH^+}[AH^+] + \varepsilon_A[A] + \varepsilon_{AHCP^+}[AHCP^+] + \varepsilon_{ACP}[ACP]) \qquad (2)$$

$$C_0 = [AH^+]_0 + [A]_0 + [B]_0 + [C_E]_0 = [AH^+] + [A] + [B] + [C_E] + [AHCP^+] + [ACP] \qquad (3)$$

absorbance of the solution, at the same wavelength and at the same pH, in the presence of copigment, C_0 is the overall concentration of malvin and d the optical pathlength. The epsilons are molar absorption coefficients. It has been previously demonstrated,[6] that the ratio $(D - D_0)/D_0$ is a good measure of the copigment effect. By use of eqns. (1), (2) and (3), and expressing K_a, K_h, K_1 and K_2 as functions of the equilibrium concentrations of the species shown in Scheme 2, one obtains eqn. (4). This

$$(D - D_0)/D_0 = \{(1 + K_S[CP])/(1 + K_T[CP])\} - 1 \qquad (4)$$

$$K_S = (r_1K_1[H^+] + r_3K_2K_a)/([H^+] + r_2K_a)$$

$$K_T = (K_1[H^+] + K_aK_2)/([H^+] + K_a + K_h)$$

equation permits one to calculate the magnitude of the copigment effect in the pH range 0.5 to *ca.* 6. r_1, r_2 and r_3 are given by $\varepsilon_{AHCP^+}/\varepsilon_{AH^+}$, $\varepsilon_A/\varepsilon_{AH^+}$ and $\varepsilon_{ACP}/\varepsilon_{AH^+}$, respectively, and [CP] is the equilibrium concentration of the free copigment. Very often [CP] may be approximated to the overall copigment concentration. At given temperature and pH, K_S is a constant which depends not only on the system itself, but also on the method of investigation, presently electronic absorption spectrometry. In contrast, K_T is a thermodynamic constant independent of the method of analysis.

(ii) pH *Range from* 5–6 *to* 8. At pH values in the range 5–6, the flavylium cation completely disappears from solution and is replaced by tiny amounts of the ionized quinonoidal base A^-. In this pH range, therefore, one has to substitute Scheme 3 for

$$A \rightleftharpoons (B + C_E) \qquad K_H = K_h/K_a$$

$$A \rightleftharpoons A^- + H^+ \qquad K_A$$

$$A + CP \rightleftharpoons ACP \qquad K_2$$

$$A^- + CP \rightleftharpoons ACP^- \qquad K_3$$

Scheme 3

Scheme 2. ACP^- is the molecular complex formed between the ionized quinonoidal base A^- and the copigment molecule CP. This complex is characterized by the stability constant K_3. D_0 and D are now expressed by eqns. (5) and (6). In eqn. (3), AH^+

$$D_0 = d(\varepsilon_A[A]_0 + \varepsilon_{A^-}[A^-]_0) \qquad (5)$$

$$D = d(\varepsilon_A[A] + \varepsilon_{A^-}[A^-] + \varepsilon_{ACP}[ACP] + \varepsilon_{ACP^-}[ACP^-]) \qquad (6)$$

and $AHCP^+$ should be replaced by A^- and ACP^-, respectively. Combining eqns. (5) and (6) with the modified version of eqn. (3), gives eqn. (7) which is valid within the pH range 5–6 to

$$(D\text{-}D_0)/D_0 = \{(1 + K'_S[CP])/(1 + K'_T[CP])\} - 1 \qquad (7)$$

$$K'_S = (r_4K_2[H^+] + r_6K_3K_A)/([H^+] + r_5K_A)$$

$$K'_T = (K_2[H^+] + K_3K_A)/\{K_A + [H^+](1 + K_H)\}$$

8. r_4, r_5 and r_6 are defined as $\varepsilon_{ACP}/\varepsilon_A$, $\varepsilon_{A^-}/\varepsilon_A$ and $\varepsilon_{AHCP^+}/\varepsilon_A$, respectively, where again the epsilons are molar absorption coefficients of the quoted species.

(iii) pH *Range* 0.5 *to* 8. In Fig. 3 are reported both experimental and calculated values for the $(D - D_0)/D_0$ ratio in the case of three different copigments: chlorogenic acid, caffeine and adenosine. The Figure clearly shows that copigmentation takes place over the entire acidic pH range and even extends

Table 1 Stability constants, in water at 20 °C, for the molecular association between malvin and three different copigments. The reported values are those which best fit eqns. (4) and (7) to the experimental values (see Fig. 3).

Copigment	K_1/mol^{-1} dm^3	K_2/mol^{-1} dm^3	K_3/mol^{-1} dm^3
Chlorogenic acid	350[a]	140	40
Caffeine	150	250	110
Adenosine	30	80	5

[a] At 20 °C and a 0.20 mol dm^{-3} ionic strength, a value of 390 (±50) mol^{-1} dm^3 has been previously measured.[6]

into the alkaline range. In the more acidic media (pH ≤ 1.5), the copigment effect always gives rise to an hypochromic shift as indicated by the negative values of the $(D - D_0)/D_0$ ratio. This means that, at 520 nm and pH < 1.5, D becomes less than D_0, a case previously seen in Fig. 1. One should remember that, at such low pH values, malvin exists exclusively in the AH^+ flavylium form and that in these media the copigment effect, as measured by the absolute value of $(D - D_0)/D_0$, is a small one. In contrast, for pH values larger than 2, the $(D - D_0)/D_0$ ratio always remains positive and, in the case of chlorogenic acid as well as in the case of caffeine, large values of this ratio are obtained, even when the copigment-to-pigment molar ratio is small, as shown in Fig. 3.[14] Such behaviour is representative of the copigment effect, whose magnitude is, therefore, much greater than the magnitudes usually observed in molecular interaction absorption spectrometry investigations.[15] In the more acidic solutions (Fig. 3, circled area), there is little difference in the three investigated copigments, whereas in the less acidic solutions distinct differences appear. For instance the chlorogenic acid curve reaches its maximum close to pH 3.5, a fact already pointed out,[6] whereas for caffeine and adenosine there is a flat summit extending, approximately, from pH 4.5 to 6.5. In the latter pH domain, the chlorogenic acid curve is characterized by a shoulder. Under alkaline conditions, the copigment effect is reduced in all cases, but it remains large for caffeine. One could also note that caffeine and adenosine are characterized by curves similar in shape, with the copigment effect for caffeine being simply three to four times that for adenosine.

As far as plant pigmentation related to the presence of anthocyanins is concerned, it appears that chlorogenic acid, caffeine and adenosine have a stabilizing influence on the colour of malvin. However, at a given copigment-to-pigment molar ratio and a given pH, the colour will be characteristic, not only of malvin, but also of the type of associated copigment. Indeed, an infinite variety of colours may be produced by only one anthocyanin associated with various copigments. The list of potential copigments is extremely long, even if one only considers those present in the cell vacuoles where pigments and copigments are stored.[16] For instance, very recently Haslam and his co-workers,[17] have tested a series of tannins (polyphenols), which all behave as copigments when mixed with malvin. When more than one copigment is present, a case frequently encountered in cell vacuoles, one should be cautious not to add copigment effects, since possible interactions between copigments will reduce their ability to act as copigments (O. Dangles and R. Brouillard, in preparation).

Values of K_1, K_2 and K_3, for each of the investigated copigments (Table 1), indicate that chlorogenic acid associates more strongly with the flavylium cation AH^+ than with the neutral base A, which, in turn, interacts more strongly with the chlorogenate anion than does the anionic quinonoidal base A^-. Chlorogenic acid bears on its quinic moiety a carboxylic group whose pK_a is close to 3.5.[18] In order to take into account a possible effect of the chlorogenate anion negative charge on the strength of the complexation, we devised a more sophisticated theoretical treatment from which we concluded that the carboxylic group does not contribute to the copigment effect. This view is supported by the fact that caffeic acid, which lacks the quinic acid moiety present in chlorogenic acid, gives copigment effects almost identical to those of chlorogenic acid. One can conclude that the conjugated system in chlorogenic acid is the structural element interacting with the various visible-light-absorbing malvin species. In the pH range investigated in the present work, caffeine does not change its structure,[19] except for the more acidic solutions (pH < 1) where protonation occurs, and its three complexation constants K_1, K_2 and K_3 are characteristic of the neutral caffeine molecule. Caffeine binds more strongly to the neutral base A than to the AH^+ cation or the A^- anion. The maximum for the $(D - D_0)/D_0$ ratio, in the case of the caffeine molecule, coincides with the pH range 4.5–6.5 where the neutral base A is the only coloured malvin species present in the solution. It should also be noted that, even in neutral solutions, caffeine's copigment effect remains important. Adenosine belongs to the caffeine-type class of copigment, showing again a maximum of its complexation ability with the neutral base A. In the 0.5–8 pH range, adenosine changes its structure twice, going from a dication at pH < 1, to a monocation between 1 and *ca.* 3.5 and to a neutral base at higher pH values.[20] Despite such important variations in the electric charge borne by the adenosine molecule, its copigment effect remains qualitatively similar to its neutral analogue, caffeine. One can then conclude that gain or loss of charge in the adenosine molecule has little influence on the strength of its binding to malvin, at least as far as aqueous solutions are concerned. The latter assumption may be seen by many as risqué, but it clearly points out the fact that the main driving force in the copigment-to-pigment association is not related to either coulombic repulsion or coulombic attraction between the molecules. For instance, the binding constant K_1 of the association between the two malvin and adenosine cations is *ca.* six times larger than the binding constant K_3 of the association between the malvin anion and the neutral adenosine form.

Solvent Effect.—In a previous work[6] we reported that adding methanol or formamide to an aqueous copigmented malvin solution considerably reduces the copigment effect. Our conclusion was that copigmentation probably occurs only when sufficient water is present. In this work ten different cosolvents, including methanol and formamide, were mixed with water and the extent of the copigmentation reaction was measured in these ten different media. For instance, in Fig. 4, the reduction of the copigmentation effect between malvin and chlorogenic acid brought about by three cosolvents, namely methanol, ethanol and acetone is shown. Methanol is less damaging to the copigmentation reaction than are ethanol and acetone. Further increase in the amounts of the latter cosolvents makes the copigment effect vanish. We also plotted the relative copigment effect against the static dielectric permittivity and against the solvent polarity parameter $E_T(30)$.[21] $E_T(30)$ values for binary liquid mixtures were taken from ref. 22. Both plots are very similar to the ones shown in Fig. 4. In particular, good linear correlations, between the relative copigment effect and the percentage of water (Fig. 4), and also between the relative copigment effect and the dielectric permittivity as well as the solvent polarity $E_T(30)$ parameter, were found in the case of methanol and ethanol, whereas in the case of the acetone–water mixtures, no linear correlations were obtained. Interestingly, and perhaps more surprisingly, we always found the relative copigment effect to be lower than 100% whatever the cosolvent used (see Table 2). This result should be compared with the $E_T(30)$ scale, which, with one exception among hundreds of examples, also reaches its maximum in the case of pure water.[21]

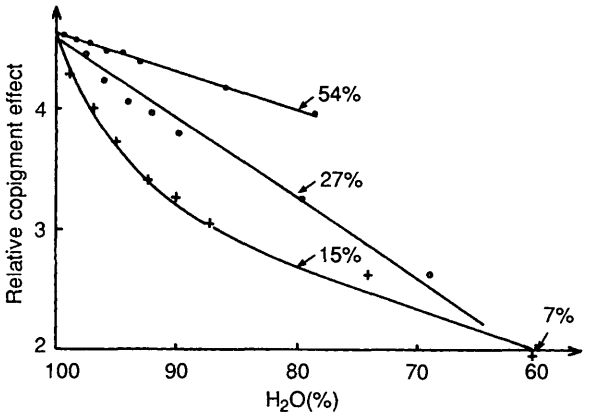


Fig. 4 Plot of the relative magnitude of the copigment effect for 6×10^{-4} mol dm^{-3} malvin solutions copigmented by chlorogenic acid *vs.* the mass percent of water. Copigment-to-pigment molar ratio: 12.8; $T = 20$ °C; $\lambda = 520$ nm; pH = 5. Cosolvent added: methanol (●); ethanol (○) and acetone (+). All reported measurements have been referred to the corresponding $(D - D_0)/D_0$ value in pure water under the same experimental conditions with the exception of the composition of the solvent. Therefore the relative copigment effect is expressed as $\ln\{\{[(D - D_0)/D_0]_{mix}/[(D - D_0)/D_0]_{water}\} \times 100\}$, and the three curves drawn on the Figure theoretically met at ln 100 = 4.60, which is the pure water reference value.

Table 2 Relative copigment effects for 6×10^{-4} mol dm^{-3} malvin solutions copigmented by caffeine. Values correspond to the following quantity: $[\{(D - D_0)/D_0\}_{mix}/\{(D - D_0)/D_0\}_{water}] \times 100\%$. Copigment-to-pigment molar ratio: 12.8. $T = 20$ °C; pH = 5; $\lambda = 520$ nm.

	Concentration/mol dm^{-3}	
Cosolvent	2	4
Protic cosolvents		
Ethylene glycol	90	85
Methanol	81	70
Formic acid	74	*a*
Ethanol	72	47
Formamide	56	34
Acetic acid	27	*a*
N-Methylformamide	21	*a*
Aprotic cosolvents		
Acetonitrile	52	26
Acetone	33	13
N,*N*-Dimethylformamide	13	9

[a] No values are given because of poor experimental accuracy, related to dilution effects.

The $E_T(30)$ scale has been based on the solvatochromic effect of an exceptional dye.[21] Our coloured species, for instance AH^+, also exhibit a solvatochromic effect when the nature of the solvent is changed.[13] However, the way we measure the copigment effect eliminates the solvatochromic effect produced by changing the solvent composition and the relative copigment effect only reflects the strength of the copigment-to-pigment association. In this instance, the copigmentation reaction could constitute the basis for a very sensitive, new solvent polarity scale, which would be capable of handling a previously rather ignored physical parameter when dealing with solvent polarity scales, *i.e.* the temperature. We previously pointed out,[6] that the extent of the copigmentation process is to be related, not only to the polarity of the water molecule itself, but probably more importantly to the hydrogen-bonded network of water molecules. In this regard, the partial disruption of this hydrogen-bonded network of water molecules is more readily achieved by acetone or ethanol than by methanol. Another remark is, that though less intense than in pure water, copigmentation still takes place in binary mixtures providing that water remains the main component of the investigated medium.

In Table 2 measurements have been gathered on the regression produced by different cosolvents on the magnitude of the copigmentation of malvin by caffeine. A tremendous influence is observed in the case of acetic acid, acetone, *N*-methylformamide and *N*,*N*-dimethylformamide, and it does not seem to matter too much whether the cosolvent is protic or aprotic. If one excepts acetic acid, the less damaging cosolvents are those which bear an hydroxy group. For instance, ethylene glycol with its two hydroxy groups produces the smallest decrease in the copigment effect. Methanol, formic acid (in fact the formate anion) and ethanol show a pronounced, but not too strong, influence. Polarity of the cosolvent molecule is apparently not a decisive factor in the regression of the copigmentation molecular interaction. For instance, *N*-methylformamide, with its high dielectric permittivity value (182), has an influence similar to that of acetic acid whose dielectric permittivity in the pure state is only 6.2. Recently, we also demonstrated that the copigment effect is extremely sensitive to temperature, and the lower the temperature, the stronger the association between the pigment and the copigment.[6] Such a result prompted us to see what would be the influence, on the magnitude of the copigment effect, of a change in the physical state of the medium without modification of its overall chemical composition. Thus, we observed that, at *ca.* 0 °C and under atmospheric pressure, more copigment-to-pigment complex is formed in the solid state (ice I), than in the liquid state at the same temperature. It is known that ice is a quasi-crystalline, well organized medium, whereas in liquid water only patches of this organization remain, especially at low temperature.[23] As stated previously, once a given copigment-to-pigment couple has been selected, the most important structural parameter to be considered, for the copigmentation interaction to take place to a large extent, is the unique hydrogen-bonded network of the water molecules in the liquid or in the solid state. Therefore the increase in the amount of complex in the ice, as compared to the amount in the liquid, is in agreement with the higher degree of order in the ice compared to that in the liquid. Such a crucial influence of the highly associated aqueous solvent is characteristic of a hydrophobic effect, which, in addition to other contributions to the stability of copigmentation complexes (van der Waals interactions, possible hydrogen-bonding between pigment and copigment) certainly provides the major driving force for copigmentation. Returning to the influence of a cosolvent on copigmentation, one can conclude that the order appearing in Table 2 is representative of the disrupting effect, related to the presence of a cosolvent, on the approximately tetrahedral hydrogen-bonded aggregates of water molecules. Those cosolvents bearing at least one hydroxy group, more or less successfully mimic, in some way, water molecules in their ability to hydrogen bond to their immediate neighbours. It is also remarkable that, with one exception, cosolvents possessing a CO or a CN group largely inhibit the formation of the complex between malvin and caffeine.

Copigmentation of Malvin by Purine and Pyrimidine Derivatives and by Polyphenols.—Although in more acidic solutions (pH $\leqslant$ 1), the copigment effect produced by adenosine is hard to detect (Fig. 3), in the less acidic pH range, the interaction between the adenine nucleoside and malvin can be measured without difficulty, owing to the large amplifying factor in the copigment effect brought about by the reservoir of the malvin hemiacetal and chalcone forms. In order to see if this interaction occurs generally among purine and pyrimidine derivatives, we investigated a few of these compounds. Results are reported

Table 3 $[(D - D_0)/D_0] \times 100\%$ ratios for the copigmentation of malvin in water by purine and pyrimidine derivatives, and by polyphenols. Malvin: 6×10^{-4} mol dm^{-3}; copigment-to-pigment molar ratio: 10; $T = 20$ °C; $\lambda = 520$ nm.

Copigment	pH 3.6	pH 6.0
Adenine	14	43
Adenosine (A: **4**)	15	42
A-5′-monophosphate (AMP)	21	35
A-5′-diphosphate (ADP)	29	29
A-5′-triphosphate (ATP)	32	24
A-3′:5′-cyclicmonophosphate (cyclicAMP)	28	70
P^1,P^2-di(A-5′) diphosphate	68	52
P^1,P^4-di(A-5′) tetraphosphate	63	63
Guanosine 5′-monophosphate (GMP)	41	30
Cytosine	*a*	8
Cytidine 5′-monophosphate (CMP)	*a*	12
Cytidine 5′-triphosphate (CTP)	*a*	12
Uracil	*a*	4
Uridine 5′-monophosphate (UMP)	*a*	5
(+)-catechin (**5**)[b]	50[c]	40[d]
(−)-epicatechin (**6**)[b]	71[c]	47[d]
Gallic acid (**7**)[e]	8[f]	10[d]
β-1,2,3,4,6-Pentagalloylglucose (**8**)[e]	67[f]	69[d]

[a] No values are given owing to too small $D - D_0$ differences, almost similar to the experimental uncertainty. [b] Malvin: 3×10^{-4} mol dm^{-3}; copigment-to-pigment ratio: 7. [c] Measured at pH 3.0. [d] Measured at pH 5.0. [e] Malvin: 4×10^{-4} mol dm^{-3}; copigment-to-pigment ratio: 1. [f] Measured at pH 3.5.

in Table 3. Although the purine derivatives all appear to be rather good copigments, and therefore increase anthocyanins colour, our investigation of these molecules was essentially prompted by the fact that, if a molecule acts as a copigment, its molecular interactions with any other species can be quantitatively determined with the help of its copigmentation reaction.[24] This is clearly an extension of the usefulness of the copigmentation phenomenon. As far as plant pigmentation is concerned, there is little chance for the latter biochemicals to intervene in the *in vivo* copigmentation of anthocyanins in coloured plant cell vacuoles although purines and pyrimidines, as well as their nucleosides and nucleotides, have been reported to occur in plant tissues in the free state.[25]

Adenine- and guanine-based nucleosides and nucleotides copigment with malvin under both slightly acidic and neutral conditions. These copigments, in their different states of protonation,[20] all associate, more or less readily, with coloured malvin species AH^+, A and A^-. At low pH values, the base cations complex with the malvin cation, and near neutrality the base anions complex with the malvin anion. Intermediate situations are encountered where the copigment is neutral, zwitterionic or not, and the pigment is in the neutral A form. It is difficult to say whether the small changes in the copigment effect, when passing from AMP to ADP and finally to ATP, are related to the presence of one more possible negative charge or to the presence of one more phosphate group. The copigment effects produced by pyrimidines and their derivatives are much weaker than those of the corresponding purine derivatives. For instance, at pH 3.6 no copigmentation could be detected in the case of the pyrimidines. Nevertheless, at pH 6 a small, but significant, effect could be recorded for cytosine, CMP, CTP, uracil and UMP. One can conclude that the purine type of heterocycle binds more readily to the coloured malvin forms than does the pyrimidine type of heterocycle.

We also investigated the copigment effect produced by two epimeric flavan-3-ols (Table 3). Their oligomers are the condensed tannins (polyphenols), long known to interact with proteins.[26] Though not much different, the two couples of values for (+)-catechin and (−)-epicatechin clearly indicate that (−)-epicatechin associates more strongly with malvin, both in the flavylium and the quinonoidal base forms, than does its 3-stereoisomer (+)-catechin. A closer approach of (−)-epicatechin to the malvin forms is probably provided by a 3-hydroxy group and a catechol group situated on the same side of the A-ring plane, while in the (+)-catechin molecule both sides of that plane are occupied by either a catechol or a hydroxy group. Once pH, temperature and concentration differences have been taken into consideration, the (+)-catechin and the β-pentagalloylglucose copigment effects measured by us (see Table 3), are shown to be in good agreement with the values reported by Haslam and his co-workers[17] at pH 3.65 and 22 °C.

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