A Spectroscopic Method Based on the Anthocyanin Copigmentation Interaction and Applied to the Quantitative Study of Molecular Complexes

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When monitored by visible absorption spectrometry, anthocyanin copigmentation appears to be of wide application in the field of quantitative investigation of molecular complexes in general. This is made possible by the strong hyperchromic effect in the visible range involving the influence of the copigment in slightly acidic aqueous solutions. For instance, when we add to a solution containing both malvin (the pigment) and chlorogenic acid (the copigment) a third species able to interact, in a non-covalent way, with the copigment, the effect produced by the presence of the new complex (chlorogenic acid–third species), on the copigmentation reaction itself, is significant. Two types of chlorogenic acid molecular associations have been investigated. The first one corresponds to the inclusion of chlorogenic acid into α- and β-cyclodextrins, both macrocycles being inert with respect to malvin. Concerning the study of inclusion complexes, our method remains valid when the guest molecule is not a copigment, provided that malvin is replaced by an anthocyanic pigment capable of entering the macrocycle cavity. Callistephin and chrysanthemin, two very abundant naturally occurring anthocyanins, fit this requirement. Inclusion of the latter pigments into β-cyclodextrin is characterized by a much better inclusion of their colourless forms than of their coloured flavylium cations, leading to what may be called an 'anti-copigment' effect. The second type of chlorogenic acid molecular association, investigated with our method, corresponds to the stacking of chlorogenic acid, the copigment, with the third species, which is now also a copigment. The two interacting copigments are chlorogenic acid and caffeine. The chlorogenic acid–caffeine stability constant was measured as 36 (±9) dm^3 mol^{-1} at 25 °C, a value in good agreement with that reported in the literature.

Many factors are known to influence the colour of anthocyanin pigments;[1–4] for instance, the pigment chemical structure and its concentration, the acidity of the solution, the presence of metallic cations as well as the presence of copigments. Among these factors, one of the most important is the presence of copigments. Anthocyanins are natural pigments which are responsible for many flower and fruit colours. In aqueous solutions, they exist as various coloured and colourless forms in chemical equilibrium.[5] The anthocyanin copigmentation reaction has a stabilizing effect on the pigment's coloured forms, a phenomenon leading to an increase in the colour intensity of the solution and also, frequently, to a blue shift.[6,7] These two effects are easily observed by means of UV–VIS absorption spectrometry giving rise to two spectral features which are as follows: a positive shift in the absorbance intensity in the visible range (hyperchromic effect) and a positive shift in the wavelength of the maximum of absorption of the visible band (bathochromic effect). Known since 1931,[8] the copigment effect has received much attention during the past twenty years. In particular, UV–VIS absorption investigations of the copigmentation reaction[9–16] have resulted in a better understanding of its main factors: temperature, medium acidity, role played by the solvent, and chemical nature and concentration of both pigment and copigment. However, in these studies, experimental parameters such as, for instance, the change in the pigment absorption maximum produced by the addition of the copigment, were usually not interpreted within the framework of a theoretical treatment. Indeed, it is only recently that the thermodynamic constants characteristic of the copigmentation reaction of malvin (**1**) (malvidin 3,5-diglucoside) have been measured using UV–VIS and NMR spectrometry.[17–19] Thus, it has been demonstrated[17] that the copigmentation of malvin, the pigment, by chlorogenic acid (**2**), the copigment, gives, in slightly acidic aqueous solution, a 1:1 molecular complex between the malvin flavylium cation and chlorogenic acid. The corresponding stability constant has been estimated to be as much as 390 dm^3 mol^{-1} at 20 °C. In that study, the authors also pointed out the crucial role played by the highly associated aqueous solvent in providing the driving force for the copigmentation interaction to occur (hydrophobic effect).

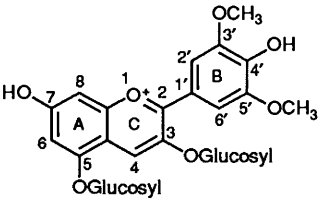
One should note that, in UV–VIS spectrometry, the copigment effect gives rise to a characteristic and highly sensitive signal which is largely due to the structure of the pigment and, in particular, to its structural transformations in a given aqueous medium (Scheme 1). In the present work, our aim is not so much to study the copigmentation reaction itself but, rather, to use it as a tool for investigating molecular complexes of the copigment, other than the copigment-to-pigment complex. For instance, adding to a copigmented anthocyanin solution a third species, which forms a new molecular complex with the copigment induces, under suitable conditions, a large modification in the pigment UV–VIS absorption. Our method permits one to deduce, from the observed spectral change, the value of the stability constant of the newly formed complex. Therefore, the copigmentation interaction serves as the indicator which reveals the existence of the new complex. This is an example of competitive spectrometry[20] since the anthocyanin (the indicator) and the third species compete in their association with the copigment.

Our method offers several advantages which are worth describing here. As previously stated, its high sensitivity is due, in part, to large values of the molar absorption coefficients of the anthocyanin coloured forms (typical values are of the order of 30 000–40 000 dm^3 mol^{-1} cm^{-1}). It is also a general method for studying the stability of molecular complexes present in aqueous solutions and the limiting condition, that one molecule

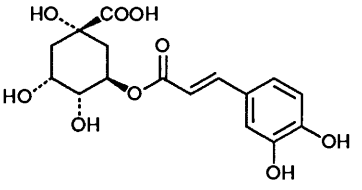
should be a copigment, is in fact not so stringent since so many structurally different molecules, in particular naturally occurring molecules like polyphenols, flavonoids, alkaloids, nucleic bases, amino acids *etc.* have been shown to act as copigments.[9] It also seems particularly well-adapted to the study of dilute solutions in which case alternative processes, like self-association reactions and formation of high stoichiometry complexes, can be avoided. Finally, molecular interactions of poorly water-soluble compounds may be investigated.

The pigment chosen is malvin and the copigment is chlorogenic acid, a very abundant polyphenol in the plant kingdom. Our work has been performed in slightly acidic aqueous solutions (pH 3.50). These conditions are often found in plant vacuoles which are the organelles where such molecules are stored. In particular, using micro-UV–VIS spectrometry, Asen and his co-workers[21] were able to demonstrate that pH values for the vacuolar cell sap of fresh flower coloured epidermal tissues, range from *ca.* 2.5 to 7.5. Chlorogenic acid being the copigment, this method is therefore illustrated by the study of its molecular complexes. Two typical cases have to be considered: the first one corresponds to a molecule which associates with the copigment but which does not interact with malvin; such a situation is encountered with natural macrocycles like α- and β-cyclodextrins (cyclohexa- and cyclohepta-amyloses, respectively), now well-known to form inclusion complexes with a large variety of organic molecules. In this case, inclusion complexes involving guest molecules, which are not anthocyanin copigments, cannot be investigated by the method described above if malvin is the indicator. However, the competitive spectrophotometric method, with an anthocyanin as the indicator, can still be used provided that the new anthocyanin also interacts with cyclodextrin; for this purpose, we found callistephin (**3**) (pelargonidin 3-glucoside) and chrysanthemin (**4**) (cyanidin 3-glucoside) to be suitable pigments. In the second case, the molecule which associates with the copigment is itself a copigment of malvin; such a situation is illustrated by caffeine (**5**), a cheap, naturally abundant, highly water-soluble molecule of great interest to the food industry.

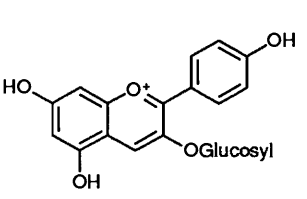
The following notations will be used throughout the text: I, L and S stand for the anthocyanin indicator, the chlorogenic acid ligand and the substrate, respectively. The substrate is considered to interact with chlorogenic acid and, eventually, with the anthocyanin. Unlike I, neither L nor S absorb light in the visible range and therefore, their spectral features are of no importance in the present work



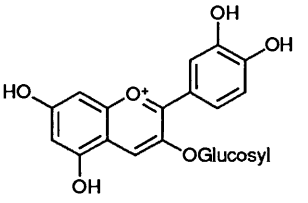
Malvin **1**



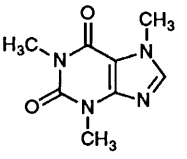
Chlorogenic acid **2**



Callistephin **3**



Chrysanthemin **4**



Caffeine **5**

Experimental

Solutions.—Malvin chloride, chlorogenic acid, α- and β-cyclodextrins, caffeine, callistephin and chrysanthemin were purchased from Roth. Their purity was checked by UV–VIS and ^{1}H NMR spectroscopy. The following titrated solutions were prepared in a pH 3.50 buffer (Na_2HPO_4 0.2 mol dm^{-3} and citric acid 0.1 mol dm^{-3}): malvin (2.5×10^{-3} mol dm^{-3}); chlorogenic acid (5×10^{-2} mol dm^{-3}); α- and β-cyclodextrins (2×10^{-2} mol dm^{-3}) and caffeine (2.5×10^{-2} mol dm^{-3}). Complete dissolution (even in the case of cyclodextrins) was achieved by sonication. Absorbance measurements (D) were performed on equilibrated solutions which were prepared as follows: 1 cm^3 of the malvin solution was mixed with X cm^3 of the chlorogenic acid solution and Y cm^3 of either the caffeine solution or one of the two cyclodextrin solutions. The final volume was made up to 5 cm^3 using the buffer solution. One takes for the X value 1, if S is α- or β-cyclodextrin, and 0.5 if S is caffeine. Y values were taken as: 0, 1, 1.5, 2, 2.5 and 3. Thus, overall concentrations of indicator, ligand and substrate are: 5×10^{-4} ($[I]_t$), 10^{-2} ($[L]_t$) and $4Y \times 10^{-3}$ mol dm^{-3} ($[S]_t$), when S represents α- or β-cyclodextrin and 5×10^{-4} ($[I]_t$), 5×10^{-3} ($[L]_t$) and $5Y \times 10^{-3}$ mol dm^{-3} ($[S]_t$), when S represents caffeine. The reference (D_0) for absorbance measurements was obtained from a malvin solution diluted, with the buffer solution, to the same $[I]_t$ value (5×10^{-4} mol dm^{-3}). pH values were measured by means of a Metrohm model 654 pH meter. When pH values of the investigated solutions were slightly different from 3.50, very small volumes of concentrated H_3PO_4 or NaOH solutions were added for correction.

Absorption Spectra.—Visible absorption spectra were recorded *ca.* 30 min after the solutions had been prepared. Molecular associations (inclusion, copigmentation *etc.*) are virtually instantaneous processes which induce in the malvin hydration equilibrium a shift which governs the malvin spectral changes (compared to the reference) in the visible range. Since the malvin hydration reaction has been demonstrated to be rather slow,[22,23] a delay of 30 min is a very good guarantee that all equilibria present are fully established. Spectra were recorded with a Hewlett-Packard diode-array spectrometer fitted with a quartz cell (d = 1 cm) with a stirring magnet. A constant temperature in the cell was obtained by use of a Lauda thermostatted water-bath; temperature measurements were made with a Comark thermocouple and the temperature was always found to range within 25 ± 0.2 °C. When necessary, spectra of cyclodextrin-containing solutions were corrected for a weak light scattering effect. For this purpose, the spectrometer software automatically deduces from the overall spectrum, the quantity of light scattered by the sample after it has been calculated within a spectral range where no absorption takes place at all; in our case, this range extends from 720 to 820 nm.

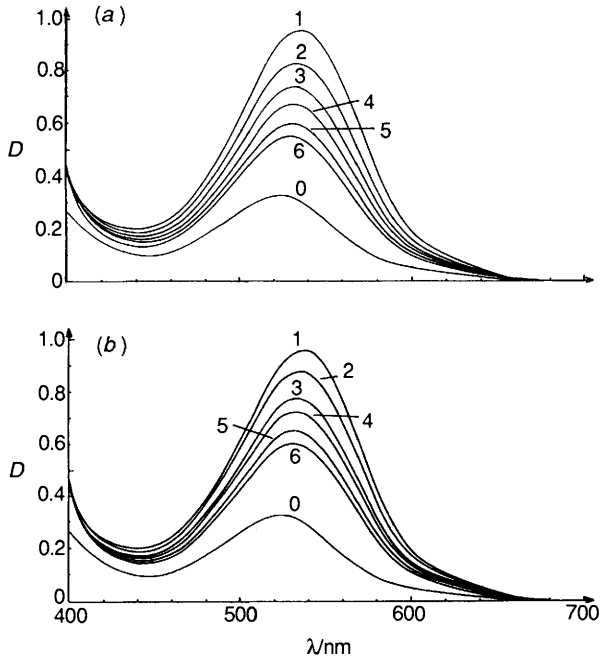


Fig. 1 Changes in the absorption in the visible range of the flavylium chromophore when cyclodextrins (S) are added to solutions of malvin (I) and chlorogenic acid (L). $[I]_t = 5 \times 10^{-4}$; $[L]_t = 10^{-2}$; $10^3[S]_t$ = 0(1), 4(2), 6(3), 8(4), 10(5), 12(6) mol dm^{-3}; malvin alone: spectrum 0; malvin with chlorogenic acid: spectrum 1; malvin with chlorogenic acid and either α-cyclodextrin (*a*) or β-cyclodextrin (*b*): spectra 2–6. pH = 3.50; T = 25 °C.

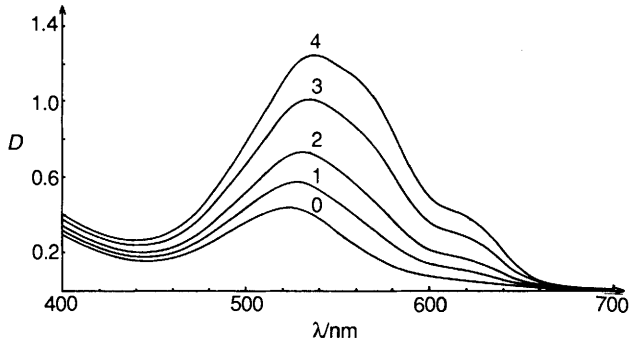


Fig. 2 Visible spectra of malvin (I) and caffeine (S) at different caffeine concentrations. $[I]_t = 6 \times 10^{-4}$; $10^3[S]_t$ = 0(0), 3(1), 6(2), 12(3), 18(4) mol dm^{-3}. pH = 3.50; T = 25 °C.

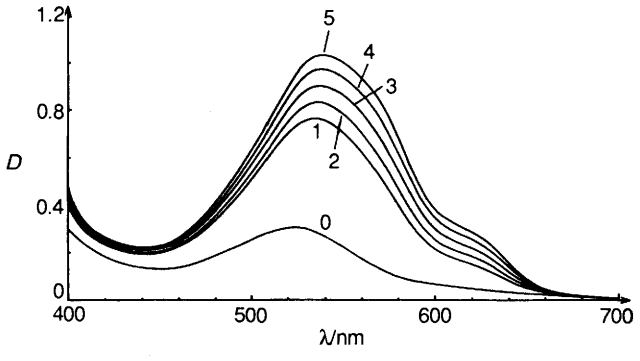


Fig. 3 Visible spectra of malvin (I) with chlorogenic acid (L) and caffeine (S) at different caffeine concentrations. $[I]_t = 5 \times 10^{-4}$; $[L]_t = 5 \times 10^{-3}$; $10^3[S]_t$ = 5(1), 7.5(2), 10(3), 12.5(4), 15(5) mol dm^{-3}; malvin alone: spectrum 0; malvin with chlorogenic acid and caffeine: spectra 1–5. pH = 3.50; T = 25 °C.

Results

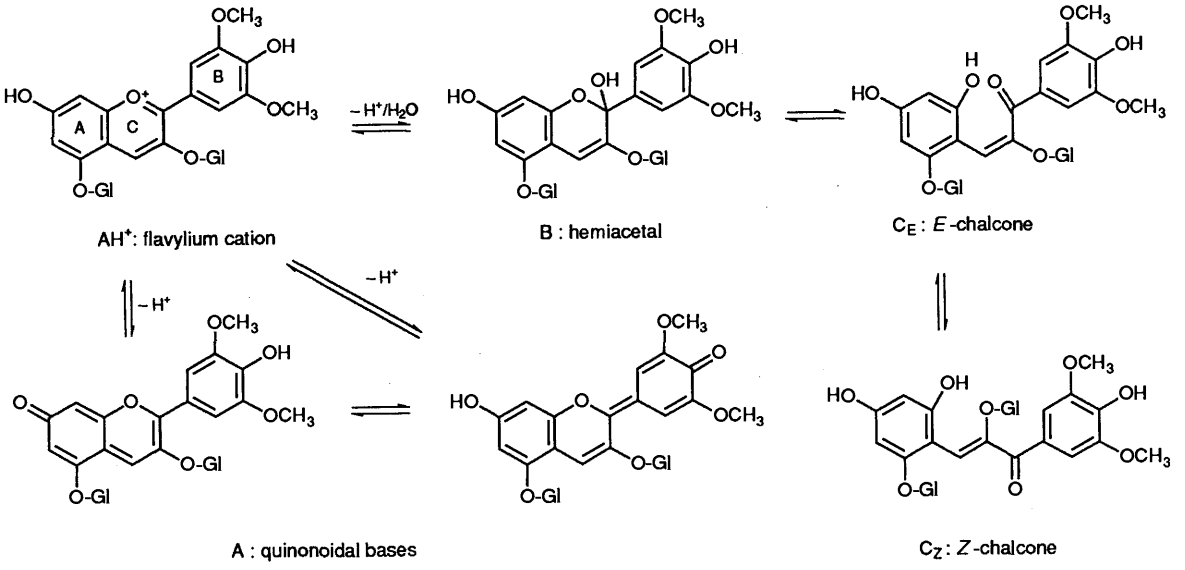
The pH value, used throughout this work, has been carefully selected for the following reasons: in aqueous solution, the pigment is present as several coloured forms in acid–base equilibrium and, among these coloured forms, the flavylium cation gives rise to the similarly pH-dependent hydration equilibrium (Scheme 1). Consequently, the copigmentation molecular interaction, in which the coloured pigment forms are stabilized by complexation, is a process strongly related to the acidity of the solution. We recently demonstrated,[24] in a study on the influence of pH on the copigmentation phenomenon, at fixed malvin and chlorogenic acid concentrations, that the hyperchromic effect reaches its maximum at pH values close to 3.50 for this pigment-to-copigment couple. Thus, the highest sensitivity in our method is obtained when the pH is of the order of 3.50. We have also shown in our laboratory that this maximum depends on both the pigment chemical structure and the copigment chemical structure. For instance, pigments with high hydration constant values give large copigment effects. As for copigments, they associate, more or less strongly, with the various coloured pigment forms, which are the flavylium cation, the quinonoidal bases and the anionic quinonoidal bases, the latter only appearing in neutral media.

Chlorogenic Acid–Cyclodextrin Complex.—Let us first point out the fact that a UV–VIS spectrophotometric study of the inclusion of chlorogenic acid into cyclodextrin seems to be quite impossible without use of an indicator. Indeed, we have observed that the strong absorption at 325 nm characteristic of chlorogenic acid hardly changes when cyclodextrin is added to the solution; we detected only a very small bathochromic shift. This result indicates that the molar absorption coefficients of free chlorogenic acid and chlorogenic acid in the cyclodextrin cavity are very similar. On the other hand, adding cyclodextrin to a malvin solution gives no appreciable change in the absorption band characteristic of the flavylium chromophore. In contrast, when one first adds to a malvin solution a given amount of chlorogenic acid (the copigment) and, after that, increasing amounts of α- or β-cyclodextrin, this absorption band is greatly modified (Fig. 1). Such a modification occurs according to the following two steps. There is first a strong increase in the absorbance as soon as chlorogenic acid is added to the malvin solution. It corresponds to the hyperchromic effect characteristic of copigmentation. Next, a steady decrease in the absorbance appears just after addition of increasing amounts of α- or β-cyclodextrin. Such a decrease tends to reduce the absorbance to its initial value in the absence of the copigment. These spectral features will be discussed further.

Chlorogenic Acid–Caffeine Complex.—When cyclodextrin is replaced by caffeine, experiments similar to the previous ones give opposite results. While adding α- or β-cyclodextrin does not produce any effect on the visible region of the malvin spectrum, adding caffeine gives rise to a strong hyperchromic effect on the flavylium chromophore absorption band (Fig. 2), a fact which permits one to conclude that caffeine is also a copigment. Therefore, if one adds caffeine to a solution containing both malvin and chlorogenic acid, the modifications observed in the visible range are not only due to the association between caffeine and chlorogenic acid but also to the association between caffeine and malvin. As shown in Fig. 3, these modifications result overall in a steady increase in the absorbance of the flavylium chromophore.

Discussion

Upper limits of the concentrations used in the present work are *ca.* 10^{-2} mol dm^{-3} for the species L and S and 5×10^{-4} mol dm^{-3} for I. Under such conditions, we have assumed that complexes, whose stoichiometry is different from 1:1, can be neglected. On the other hand, no evidence for the existence of interactions



Scheme 1

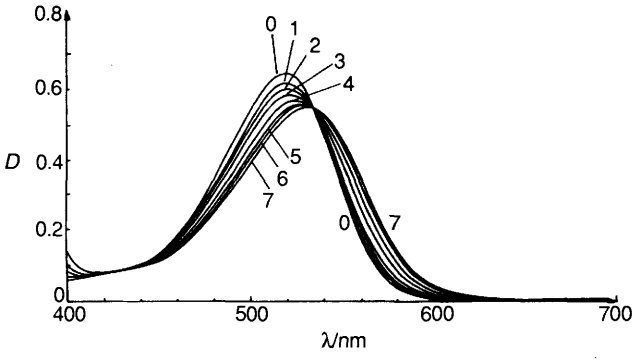


Fig. 4 Visible spectra of malvin (I) and chlorogenic acid (L) at different chlorogenic acid concentrations. $[I]_t = 3 \times 10^{-5}$; $10^4[L]_t$ = 0(0), 3(1), 6(2), 12(3), 24(4), 36(5), 48(6), 60(7) mol dm^{-3}. pH = 0.65; T = 25 °C (taken from Fig. 1 in ref. 24).

taking place between a given copigment and any of the colourless malvin forms was found. The lack of experimental proof for such associations is probably related to the fact that, for the colourless malvin forms, no extended electronic delocalization can occur. On the contrary, the malvin coloured forms are characterized by almost planar chromophores, which therefore correspond to highly conjugated species favouring interactions by vertical stacking. The presence of a C-2 tetrahedral carbon atom (hemiacetalic carbon) in the hemiacetal form B and a less rigid chalcone structure make such forms less likely to associate with a copigment. Self-association of any of the diverse pigment structures is not considered here due to the low concentration of malvin used throughout this work. In fact, only the coloured malvin forms (whose ability to form molecular complexes is well-known and sustains the copigmentation phenomenon) are liable to self-associate. At pH 3.50 however, their equilibrium concentration does not exceed a few percent of the overall pigment concentration. At such a low concentration (of the order of 10^{-5} mol dm^{-3}), circular dichroism [25] and ^{1}H NMR [26] measurements show that the coloured malvin forms are essentially monomeric. However, in a recent study,[27] it has been demonstrated that regular chalcones (compounds where the carbonyl group is next to the A-ring whereas, in our chalcones, the same group is adjacent to the B-ring) are able to act as copigments. Consequently, the chalcone derived from the hydration of the flavylium cation followed by the opening of the C-ring (Scheme 1), may well form a copigmentation complex with its own flavylium cation (hetero-self-association). In an equilibrated malvin solution however, the chalcone is never more than 30% of the overall malvin concentration at room temperature.[28] Therefore, in the presence of a large excess of an 'external' copigment (copigment-to-pigment molar ratios greater than ten), the latter hetero-self-association phenomenon can be neglected.

For slightly acidic copigmented malvin solutions, whose pH ranges from *ca.* 3 to 6, the main chemical equilibria are those characteristic of the structural transformations of malvin and also the copigmentation equilibria, which, as stated above, only affect the coloured malvin forms (see Scheme 2). With the exception of the relatively slow hydration of the flavylium cation, the equilibria shown in Scheme 2 are instantaneously established.[22,23] The hydrated B and C_E forms are extremely quick to equilibrate[28] and, therefore, have been written together in what is an overall hydration equilibrium. The very minor C_Z-chalcone has been neglected.

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

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

$$AH^+ + L \overset{K_1}{\rightleftharpoons} AHL^+$$

$$A + L \overset{K_2}{\rightleftharpoons} AL$$

Scheme 2

The different equilibrium constants are expressed as follows: $K_a = a_{H^+}[A]/[AH^+]$; $K_h = a_{H^+}([B] + [C_E])/[AH^+]$ where a_{H^+} is 10^{-pH}; $K_1 = [AHL^+]/([AH^+][L])$; $K_2 = [AL]/([A][L])$. The K_a and K_h constants have been previously found [29] to be 10^{-4} and 10^{-2}, respectively at 25 °C.

One can see from the K_a and K_h values that, in the more acidic solutions (pH < 1), and in the absence of L, the flavylium cation is the only form present in measurable amounts; such solutions are intensely coloured. Now if one adds chlorogenic acid (the copigment) to those solutions, one can simultaneously observe a bathochromic shift and a small hypochromic shift in the visible absorption band of the flavylium chromophore (Fig. 4). This is

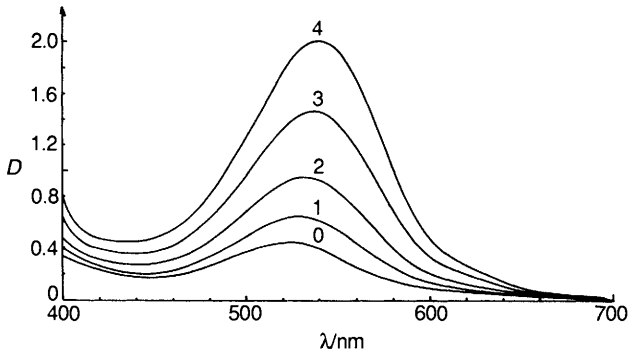


Fig. 5 Visible spectra of malvin (I) and chlorogenic acid (L) at different chlorogenic acid concentrations. $[I]_t = 6 \times 10^{-4}$; $10^3[L]_t$ = 0(0), 3(1), 6(2), 12(3), 18(4) mol dm^{-3}. pH = 3.50; T = 25 °C.

really not a copigmentation process; it features instead a classical type of complexation between the flavylium cation and chlorogenic acid. The weak hypochromic shift simply reveals that the maximum of the molar absorption coefficient of the flavylium cation in the complex is slightly lower than the maximum of the absorption coefficient of the free flavylium cation. At pH 3.50, the situation is completely different since the hydration equilibrium prevails and the colourless structures are now abundant. Thus, in the absence of any copigment, the solution is poorly coloured. Under such conditions, formation of molecular complexes between chlorogenic acid and the very minor coloured pigment forms, *i.e.* the flavylium cation AH^+ and the quinonoidal bases A, results in a shift of the hydration equilibrium toward the flavylium cation. Since the latter equilibrium connects the strongly coloured flavylium cation and the colourless forms, its displacement leads to spectacular changes in the visible absorbance which well explain the intense hyperchromic effect characteristic of the copigmentation reaction (Fig. 5).

The stability of the flavylium cation is greater in the complex than in the free state. When no copigment is present, the flavylium cation is strongly solvated and this intense solvation effect may well eventually transform into a true chemical reaction *i.e.* the nucleophilic attack of water at C-2 leading to the hemiacetal B. When a copigment is present, water molecules and copigment molecules are competing for their association with the flavylium cation. Thus, the vertical stacking of the copigment on the flavylium cation produces a relative desolvation of the flavylium cation which, consequently, becomes less vulnerable to nucleophilic attack by water molecules. In contrast, any association, involving only the copigment to the exclusion of the pigment, brings about a partial dissociation of the previously formed copigmentation complexes which, in turn, induces a shift in the hydration equilibrium toward the colourless forms resulting in an hypochromic effect. At this stage, emphasis should be laid on the following point: malvin is a sensitive indicator at pH 3.50 precisely because it is so largely hydrated (more than 95%) and, consequently, poorly coloured. Of course, that exceptional sensitivity no longer applies at pH < 1 where the hydration process becomes negligible. However, in the case of pH values higher than *ca.* 3, the amount of the colourless forms is more than ten times larger than the amount of the flavylium cation AH^+, as shown by the K_h expression. Concerning the quinonoidal bases A, the value of the $[A]/([B] + [C_E])$ pH-independent ratio is given by K_a/K_h *ca.* 10^{-2}. Thus, for pH values > 3, the total concentration of the coloured forms remains lower than 10% of the overall malvin concentration; under such conditions, an anthocyanin pigment like malvin is quite suitable for use in the competitive spectrophotometric studies of molecular associations in aqueous solutions.

Chlorogenic Acid–Cyclodextrin Complex.—Inclusion of an organic molecule into the cyclodextrin cavity is of great theoretical and practical interest. Some examples are:[30–37] regiospecific aromatic substitutions, asymmetric synthesis, models for the enzyme–substrate interaction and for the association between polyphenols and polysaccharides,[38] separation of isomers using HPLC or GLC, stabilization of aromatic compounds and chemical dyes. Cyclodextrins are unique among the macrocycles since they are, at the same time, naturally occurring, cheap and non-toxic. They are widely used in the food industry, in particular for inclusion of volatile aromas (so-called encapsulation) in order to avoid, either their chemical degradation, or their loss during food processing.

We observed that adding α- or β-cyclodextrin to a solution of malvin does not noticeably affect the visible absorption spectrum of the flavylium chromophore. We have already pointed out that even a very small shift in the hydration equilibrium would induce some measurable change in the visible absorption band, in the case of slightly acidic malvin solutions. One can conclude therefore, that no inclusion into the cyclodextrin cavity of any of the different malvin forms takes place. Haslam and his co-workers[39] came to the same conclusion, not only with β-cyclodextrin but also with γ-cyclodextrin *i.e.* cyclooctaamylose, the largest macrocycle in the cyclodextrin series. We also observed that cyanin (cyanidin 3,5-diglucoside, another common anthocyanin possessing a catechol-like B-ring) interacts with neither cyclodextrin. Similar results have been reported in the literature[40] in the case of nasunin, another 3,5-diglycosylated anthocyanin with a B-ring bearing three OH groups at C-3′, C-4′ and C-5′. Finally, in the azo dyes series investigated by Cramer and his co-workers,[41] the compound possessing, on the terminal aromatic ring, two methoxy groups adjacent to the *p*-hydroxy group does not enter the α-cyclodextrin cavity. From a structural viewpoint, the lack of inclusion of malvin into the cyclodextrin cavity may be reasonably well explained by the steric hindrance brought about by the presence of both a heavily substituted B-ring and two large sugar residues. Therefore, the net hypochromic shift, which is recorded when cyclodextrin is added to a solution of malvin and chlorogenic acid, can only be explained by the selective inclusion of chlorogenic acid into cyclodextrin. Moreover, when equal amounts of either α- or β-cyclodextrin are used, this shift is more important for α-cyclodextrin; this clearly indicates that the chlorogenic acid–α-cyclodextrin complex is more stable than the corresponding complex involving the β-cyclodextrin macrocycle. We now give details of our theoretical treatment and it will be seen that it strongly supports the above mentioned qualitative views.

The total indicator concentration $[I]_t$ is given by $[I]_t = [AH^+]_0 + [A]_0 + [B]_0 + [C_E]_0 = [AH^+] + [A] + [B] + [C_E] + [AHL^+] + [AL]$ where the subscript 0 stands for the situation where no copigment is present. Using the equilibrium constant relationships, $[I_t]$ is given by eqn. (1), in the absence of

$$[I]_t = [AH^+]_0\{1 + (K_a + K_h)10^{pH}\} \quad (1)$$

copigment, and by eqn. (2) when a copigment is present. Finally,

$$[I]_t = [AH^+]\{1 + (K_a + K_h)10^{pH} + [L](K_1 + K_2K_a10^{pH})\} \quad (2)$$

one gets eqn. (3).

$$\frac{[AH^+]_0}{[AH^+]} = 1 + a[L];\ a = \frac{K_1 + K_2K_a10^{pH}}{1 + (K_a + K_h)10^{pH}} \quad (3)$$

Applying the Beer–Lambert law to the malvin solution gives eqns. (4) and (5) where d is the optical pathlength; D and D_0 are

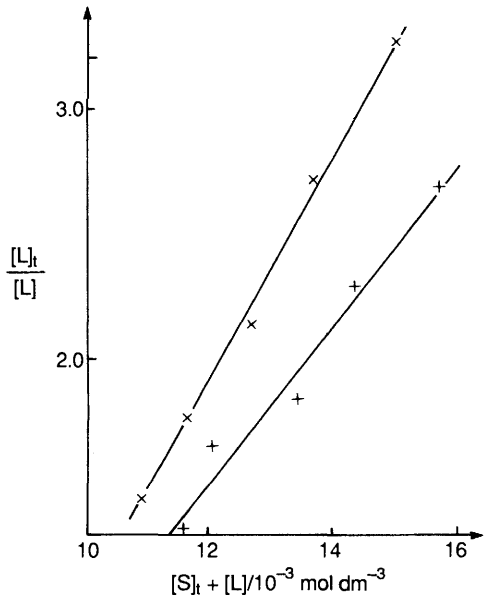


Fig. 6 The malvin–chlorogenic acid–cyclodextrin system. Plot of $[L]_t/[L]$ *versus* $([S]_t + [L])$; S is α-cyclodextrin (×) or β-cyclodextrin (+). Parameters for each equation are: $\rho = 0.998$; slope = 445 (±18) $dm^3\ mol^{-1}$; intercept = −3.41 (±0.23) (α-cyclodextrin) and $\rho = 0.986$; slope = 316 (±31) $dm^3\ mol^{-1}$; intercept = −2.29 (±0.42) (β-cyclodextrin). T = 25 °C.

$$D_0 = \varepsilon_{AH^+}[AH^+]_0 d + \varepsilon_A[A]_0 d = \varepsilon_{AH^+}[AH^+]_0 d\{1 + rK_a10^{pH}\} \quad (4)$$

$$D = \varepsilon_{AH^+}[AH^+]d + \varepsilon_A[A]d + \varepsilon_{AHL^+}[AHL^+]d + \varepsilon_{AL}[AL]d = \varepsilon_{AH^+}[AH^+]d\{1 + rK_a10^{pH} + [L](r_1K_1 + r_2K_2K_a10^{pH})\} \quad (5)$$

the absorbances of the solution with and without copigment, respectively. The epsilons represent the molar absorption coefficients of the quoted species; the r, r_1 and r_2 ratios are given by $\varepsilon_A/\varepsilon_{AH^+}$, $\varepsilon_{AHL^+}/\varepsilon_{AH^+}$ and $\varepsilon_{AL}/\varepsilon_{AH^+}$, respectively. Using eqn. (4) and eqn. (5), one obtains the D/D_0 ratio [eqn. (6)]. By

$$\frac{D}{D_0} = \frac{[AH^+]}{[AH^+]_0}(1 + b[L]);\ b = \frac{r_1K_1 + r_2K_2K_a10^{pH}}{1 + rK_a10^{pH}} \quad (6)$$

combining eqn. (3) with eqn. (6), the D/D_0 ratio may be transformed into eqn. (7). One should keep in mind that eqns.

$$\frac{D}{D_0} = \frac{1 + b[L]}{1 + a[L]} \quad (7)$$

(1)–(7) are valid in the case where the copigment is the only species interacting with the indicator (the pigment). At this stage, two different situations may be considered.

(i) *The pigment–copigment system.* In the absence of a third species S, the copigment total concentration is given by: $[L]_t = [L] + [AHL^+] + [AL]$. Since $[AHL^+] + [AL]$ is lower than $[I]_t$, which, in turn, is much lower than $[L]_t$ under the experimental conditions employed, the free copigment concentration [L] is approximately equal to the copigment overall concentration $[L]_t$. For given total concentrations of both the pigment and the copigment, eqn. (7) permits one to relate the magnitude of the copigment effect, defined as $(D - D_0)/D_0$ at a fixed wavelength,[17] to the pH of the medium. In another work, using this equation, we plotted $(D - D_0)/D_0$ *versus* pH, for different copigments, and also demonstrated that the theoretical curves fit the corresponding experimental curves.[24] For the malvin–chlorogenic acid system, we obtained: K_1 = 300 $dm^3\ mol^{-1}$ and K_2 = 120 $dm^3\ mol^{-1}$, at 25 °C with r_1 = 0.80 and r_2 = 0.45, at 520 nm. At the same wavelength, r was measured to be 0.50. It is known that the quinic moiety of chlorogenic acid bears a carboxylic group whose pK_a is 3.45, at 25 °C. In that paper, we also devised a more sophisticated treatment taking into account the copigmentation effects which would be produced, separately, by the acid and the base forms of chlorogenic acid; it was found that both copigmentation effects are identical and, therefore, one can conclude that, unlike the caffeoyl moiety, the quinic residue exerts a negligible influence on the chlorogenic acid copigmentation process.

(ii) *The pigment–copigment–cyclodextrin system.* In the presence of the substrate S, able to associate with the copigment L, there is one more equilibrium which leads to the complex SL. Therefore, Scheme 2 must be replaced by Scheme 3. Here, S

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

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

$$AH^+ + L \overset{K_t}{\rightleftharpoons} AHL^+$$

$$A + L \overset{K_2}{\rightleftharpoons} AL$$

$$S + L \overset{K_{SL}}{\rightleftharpoons} SL$$

Scheme 3

(cyclodextrin) is not a malvin copigment and therefore eqn. (7), which only involves the equilibria characteristic of malvin, remains valid. In fact, it gives access to the free copigment concentration by using a modified version of it [eqn. (8)].

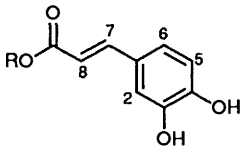
$$[L] = \frac{D - D_0}{bD_0 - aD} \quad (8)$$

Moreover, the total concentrations of both L and S are written as: $[L]_t = [L] + [SL] + [AHL^+] + [AL]$ and $[S]_t = [S] + [SL]$. K_{SL} is expressed as $[SL]/([S][L])$. Again, concentrations of the copigmentation complexes can be neglected and $[L]_t$ reduces to $[L] + [SL]$. By combining the latter relationships, one gets: $[L]_t/[L] = 1 + [SL]/[L]$ and, finally, one obtains eqn. (9).

$$[L]_t/[L] = 1 - K_{SL}[L]_t + K_{SL}([S]_t + [L]) \quad (9)$$

The value of K_{SL} was obtained as follows: in a typical experiment, for a given substrate (α- or β-cyclodextrin), $[L]_t$ was held constant and $[S]_t$ progressively increased; for each $[S]_t$ value, the corresponding ligand concentration [L] was estimated from the value of the absorbance at 520 nm [see eqn. (8)]. The plot of the $[L]_t/[L]$ ratio as a function of $([S]_t + [L])$ leads to a linear relationship whose slope corresponds to K_{SL} and the intercept to $1 - K_{SL}[L]_t$. In Fig. 6, plots corresponding to α- and β-cyclodextrins are displayed. At 25 °C, the K_{SL} values, deduced from both slope and intercept, were 440 (±23) $dm^3\ mol^{-1}$ and 330 (±44) $dm^3\ mol^{-1}$ for α- and β-cyclodextrins, respectively. These values are in good agreement with the qualitatively predictable greater stability of the α-cyclodextrin–chlorogenic acid inclusion complex as compared to the β-cyclodextrin–chlorogenic acid complex, although the difference is rather small. Indeed, with the exception of sterically hindered mole-

Table 1 Proton chemical shift displacements [D_2O; 200 MHz; Me_4Si as external reference; 25 °C; $\Delta\delta = \delta - \delta(S{=}O)$ in Hz] for chlorogenic acid (L) induced by α- or β-cyclodextrin (S). $[S]_t = 4 \times 10^{-3}$, $[L]_t = 8 \times 10^{-3}$ mol dm^{-3}. The values in parentheses are for $[S]_t = 12 \times 10^{-3}$ and $[L]_t = 6 \times 10^{-3}$ mol dm^{-3}



R = quinic moiety

	Δδ/Hz				
S	2-H	5-H	6-H	7-H	8-H
α-CD	−5.4	+5.8 (+16.2)	−6.4	+3.9 (+9.8)	−0.2 (−2.1)
β-CD	−5.3 (−10.5)	−3.4 (−8.3)	−6.9 (−15.4)	+1.2 (+5.1)	+1.0 (+3.7)

Table 2 Chemical shifts (D_2O; 200 MHz; Me_4Si as external reference; 25 °C; δ in ppm) for the cyclodextrin (S) protons 6-H at different chlorogenic acid (L) concentrations. $[S]_t = 4 \times 10^{-3}$ mol dm^{-3}

	δ	
$[L]_t/10^{-3}$ mol dm^{-3}	α-CD	β-CD
0	3.705	3.703
2	3.637	3.675
4	3.613	3.666
8	3.599	3.657
12	3.597	3.652

cules, the van der Waals contact between the included molecule and the inner wall of the macrocycle cavity has been shown to be more efficient with α-cyclodextrin. Thus, in a recent survey of inclusion into α- and β-cyclodextrins of alkyl-substituted hydroxyphenylazo derivatives of sulfanilic acid,[42] it was demonstrated that, for a given substrate, the α-cyclodextrin inclusion complex is more stable than the β-cyclodextrin one. However, the relative order of stability is reversed when the number and/or the size of the alkyl groups is increased. ^1H NMR measurements (200 MHz) not only confirm the inclusion of chlorogenic acid into cyclodextrins but also give some information about the way chlorogenic acid is encapsulated. The shifts of the peaks characteristic of the aromatic and vinylic protons of chlorogenic acid on adding α- or β-cyclodextrin clearly indicate that the caffeoyl moiety actually enters the macrocycle cavity in both cases (Table 1). In contrast, no evidence was found for the involvement of the quinic moiety in inclusion into the α-cyclodextrin cavity. Concerning β-cyclodextrin, appreciable downfield shifts of some quinic protons were observed suggesting a deeper penetration of chlorogenic acid into this larger cavity. However, potentiometric measurements on a half-neutralized chlorogenic acid solution containing increasing amounts of α- or β-cyclodextrin do not reveal significant changes in the pK_a value of the quinic carboxylic group. Finally, the upfield chemical shift displacements for the peak of the cyclodextrin 6-H methylene protons at different overall chlorogenic acid concentrations were recorded for α- and β-cyclodextrins (Table 2). They confirm that inclusion of chlorogenic acid is stronger into α-cyclodextrin than it is into β-cyclodextrin. Similar measurements carried out on the cyclodextrin 3-H and 5-H protons are qualitatively consistent with previously reported results:[43,44] the 3-H signal moves upfield in both cases whereas the 5-H signal moves upfield with β-cyclodextrin and downfield with α-cyclodextrin. However, because of unexpectedly low resolution in the case of the chlorogenic acid–α-cyclodextrin system, the corresponding values for the chemical shifts are not reported here.

As a preliminary result, we now very briefly report on the highly selective inclusion into the β-cyclodextrin cavity of the polyphenol (+)-catechin, a member of the proanthocyanidin family (condensed tannins). (+)-Catechin is a malvin co-pigment forming a very stable inclusion complex with β-cyclodextrin: we found K_{SL} of the order of 5000 dm^3 mol^{-1} at 25 °C and a previous estimate[38] gave 2908 (±87) dm^3 mol^{-1} at 45 °C. Surprisingly, no evidence could be found for the existence of an inclusion complex involving (+)-catechin and α-cyclodextrin. (+)-Catechin may be considered to possess a size and shape particularly well adapted to the β-cyclodextrin cavity giving, therefore, a highly stable inclusion complex with very intimate van der Waals contact. In this view, (+)-catechin would be sterically too hindered to enter the smaller α-cyclodextrin cavity.

This method is widely applicable to the study of the inclusion into cyclodextrins of molecules which are supposed to act as anthocyanin copigments. In such systems, the copigment provides the link between the macrocycle and the pigment since it was shown that the pigment (here malvin) does not interact with the macrocycle in any way. There is another possibility which we are now going to discuss; it concerns inclusion complexes involving molecules which are not copigments or are too poor copigments to allow a convenient determination of their inclusion complex stability constants when using malvin as the indicator. A good solution to this problem seems to choose as indicator an anthocyanin, less substituted than malvin, which would be capable of entering the cyclodextrin cavity, thus forming an anthocyanin–cyclodextrin inclusion complex. This time, the host macrocycle provides the link between the pigment and the organic molecule designated as the guest: the indicator (the anthocyanin) binds to the cyclodextrin cavity in competition with the guest whose addition could, therefore, lead to a shift in the indicator–cyclodextrin equilibrium giving measurable changes in the indicator absorption spectrum in the visible range. It has previously been reported[40] that two anthocyanin 3-monoglycosides, namely callistephin and chrysanthemin, possess the structural features necessary to form inclusion complexes with β-cyclodextrin. This statement is supported by the very strong decrease observed in the flavylium chromophore absorption band for these two anthocyanins when β-cyclodextrin is added to their solutions at pH 2.0 and 26 °C. Note that the corresponding effect when β-cyclodextrin is replaced by α-cyclodextrin is much smaller, pointing out that binding between each pigment and α-cyclodextrin is much weaker. Callistephin and chrysanthemin are characterized by a B-ring with fewer substituents than malvin: the callistephin B-ring bears only one OH group, in the 4′ position, and the chrysanthemin B-ring bears two OH groups, at the 3′ and 4′ positions. We explain the decrease in the visible absorption by the preferential inclusion of the colourless pigment forms into the β-cyclodextrin cavity as compared to the inclusion of the coloured forms, a fact leading to a shift in the hydration equilibrium toward the colourless forms which is at the origin of a hypochromic effect. Of course, this loss of colour is particularly impressive in a pH range where the pigment coloured forms are predominant ($pH < pK_h$). The Japanese authors' experiments[40] were performed again in our laboratory in order to elaborate a theoretical support required for estimating the values of the inclusion stability constants, K_3 for the flavylium cation complex and K_4 for the complexes involving the colourless hemiacetal and *E*-chalcone structures. At pH 2.0 no other anthocyanin forms have to be taken into consideration. For the purpose of clarity, the rapidly equilibrating B and C_E

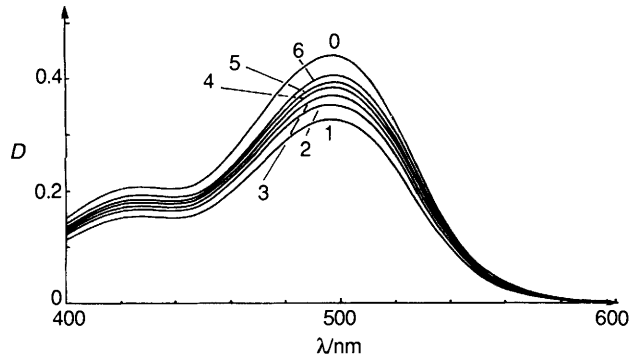


Fig. 7 Visible spectra of callistephin (I) with β-cyclodextrin (S) and *p*-bromophenol (G) at different *p*-bromophenol concentrations. $[I]_t$ = 3×10^{-5}; $[S]_t = 10^{-3}$; $10^3[G]_t$ = 0(1), 1(2), 2(3), 3(4). 4(5), 6(6) mol dm^{-3}; callistephin alone: spectrum 0; callistephin with β-cyclodextrin: spectrum 1; callistephin with β-cyclodextrin and *p*-bromophenol: spectra 2–6. pH = 2.00; T = 25 °C.

ring-chain tautomers are represented by B only. S still stands for cyclodextrin. The anthocyanin total concentration is given by $[AH^+]_0 + [B]_0$, in the absence of cyclodextrin and by $[AH^+] + [B] + [AHS^+] + [BS]$, in the presence of cyclodextrin. The mass action law is applied to the hydration equilibrium and to the equilibria forming the inclusion complexes AHS^+ and BS: $K_h = a_{H^+}[B]/[AH^+]$, $K_3 = [AHS^+]/([AH^+][S])$ and $K_4 = [BS]/([B][S])$. The Beer–Lambert law expresses the absorbance of the solution in the absence (D_0) and in the presence (D) of cyclodextrin: $D_0 = \varepsilon_{AH^+}[AH^+]_0 d$ and $D = \varepsilon_{AH^+}[AH^+]d + \varepsilon_{AHS^+}[AHS^+]d$. D_0 and D were measured for callistephin at 500 nm and for chrysanthemin at 510 nm. Both wavelength values are close to the maximum of absorption of the corresponding flavylium chromophores. By combining the previous relations, one gets $[AH^+]_0\{1 + K_h 10^{pH}\} = [AH^+]\{1 + K_h 10^{pH} + [S](K_3 + K_4 K_h 10^{pH})\}$ and $D/D_0 = (1 + r_3 K_3[S])[AH^+]/[AH^+]_0$ where $r_3 = \varepsilon_{AHS^+}/\varepsilon_{AH^+}$. These equations may be rearranged as shown to give eqn. (10). The total cyclodextrin concentration is given by:

$$\frac{[AH^+]_0}{[AH^+]} = 1 + c[S];\ c = \frac{K_3 + K_4 K_h 10^{pH}}{1 + K_h 10^{pH}}$$

$$\frac{D}{D_0} = \frac{1 + r_3 K_3 [S]}{1 + c[S]} \qquad (10)$$

$[S]_t = [S] + [AHS^+] + [BS]$ which is very close to $[S]$ in the case where $[S]_t \gg [I]_t$. Eqn. 10 may, therefore, be rewritten as eqn. (11).

$$\frac{D_0}{D_0 - D} = \frac{1}{c - r_3 K_3}\frac{1}{[S]_t} + \frac{c}{c - r_3 K_3} \qquad (11)$$

The plot of $D_0/(D_0 - D)$ *versus* $1/[S]_t$ should give a straight line with a slope equal to $1/(c - r_3 K_3)$ and an intercept expressed by $c/(c - r_3 K_3)$. The last two parameters permit one to estimate the values of the K_3 and K_4 constants, provided that the r_3 and K_h values of the investigated anthocyanin are known. In the case of callistephin, K_h has been reported[45] to be 10^{-3} at 25 °C; we found a very similar value for the chrysanthemin hydration constant. Owing to experiments performed in fairly acidic solutions (pH <1), where the hydration process no longer occurs to a significant extent, eqn. (11) simplifies into eqn. (12) from which estimates of the r_3 and K_3 values were obtained. Eqn. (12) is the well-known Benesi–Hildebrand relationship. The hypochromic shift produced on the flavylium cation absorption band by addition of increasing amounts of β-cyclodextrin to the anthocyanin solution is a relatively weak one at pH 0.6. For instance, in the case of a β-cyclodextrin-to-pigment molar ratio of 120, the decreases in the absorbance measured for chrysanthemin at 510 nm, and for callistephin at 500 nm, are 7% and 9%, respectively. One can conclude that the flavylium cations of both anthocyanins associate with β-cyclodextrin and also that their molar absorption coefficients are weaker when they are in the cavity. For chrysanthemin and callistephin, r_3 has been measured to be *ca.* 0.45 and 0.85, respectively. In contrast, at pH 2.0 where the hydration equilibrium is well established, the hypochromic shift becomes spectacular. For instance, in the case of a cyclodextrin-to-chrysanthemin molar ratio of 20, the drop in the absorbance at 510 nm is *ca.* 20% of its value in the absence of the macrocycle. A similar effect is observed at 500 nm in the case of callistephin with a cyclodextrin-to-callistephin molar ratio of only six. The strengthening of the hypochromic shift at pH 2.0 comes from the existence of the much stronger inclusion of the colourless anthocyanin forms into the cyclodextrin cavity as compared to the inclusion of the coloured flavylium cation (competitive inclusion). From the plot of $D_0/(D_0 - D)$ as a reciprocal of $[S]_t$, the following results were obtained: in the case of chrysanthemin, $D_0/(D_0 - D) = 7.4 \times 10^{-3}/[S]_t + 0.906$ (ρ = 0.999) which gives: K_3 = 30 (±20) and K_4 = 920 (±200) dm^3 mol^{-1} at 25 °C. In the case of callistephin, $D_0/(D_0 - D) = 2.7 \times 10^{-3}/[S]_t + 1.305$ (ρ = 0.999) which gives: K_3 = 130 (±30) and K_4 = 4000 (±400) dm^3 mol^{-1} at 25 °C.

The K_4 values are very large as compared to the K_3 ones. These results point to the selective inclusion of the colourless B and C_E forms of these less hindered anthocyanins into the cyclodextrin cavity. To our knowledge, this is the only example of a molecular association involving the colourless forms of an anthocyanin. One can say that β-cyclodextrin plays the role of an *anti-copigment*. The relationships relating the guest structure to its inclusion complex stability, which have been established by Connors and his co-workers[36,46–49] in series of 4-substituted benzoic acids, phenols, anilines and 4,4′-disubstituted biphenyls, are helpful to explain such a result. From these relationships, it appears that complex stability (at a binding site) increases when site electron density and/or site polarisability increase(s) since these parameters mainly contribute to attractive van der Waals forces between host and guest. In contrast, an increase in site polarity (in a polar solvent) produces a decrease in complex stability which could be explained as follows: the more polar a guest is, the more strongly solvated it is. Now, guest solvation is to be lost or much reduced upon insertion of the binding site into the apolar (relative to the solvent) cyclodextrin cavity, a phenomenon which makes a net destabilizing contribution to the overall complexation standard free enthalpy change. In this view, the very polar and charged anthocyanic flavylium cation is expected to give a less stable inclusion complex than the uncharged and much less polar hemiacetal and chalcone forms. Finally, possible formation of a hydrogen bond, between the hemiacetalic OH group of the B form and one of the glucose OH groups directed inside the β-cyclodextrin cavity, could be one more argument favouring the B form inclusion complex.

In Fig. 7 is shown an example of competitive inclusion in the β-cyclodextrin cavity between callistephin and a guest G, *p*-bromophenol, selected because it is not a callistephin copigment. Changes in the anthocyanin visible absorption band, upon addition to an acidic pigment solution (pH = 2.0) of a given amount of β-cyclodextrin followed by increasing amounts of G, are opposite to those observed in the case of the malvin–chlorogenic acid–β-cyclodextrin system: first, a strong

$$\frac{D_0}{D_0 - D} = \frac{1}{(1 - r_3)K_3}\frac{1}{[S]_t} + \frac{1}{1 - r_3} \qquad (12)$$

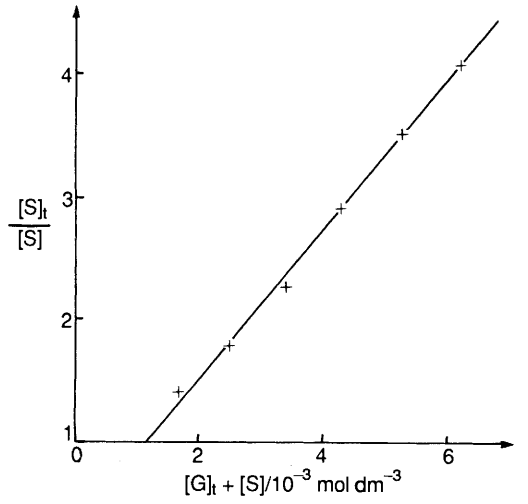


Fig. 8 The callistephin–β-cyclodextrin–*p*-bromophenol system. Plot of $[S]_t/[S]$ *versus* ($[G]_t$ + [S]); S is β-cyclodextrin. ρ = 0.998; slope = 596 (±15) $dm^3\ mol^{-1}$; intercept = 0.312 (±0.061). T = 25 °C.

decrease in the absorbance occurs when adding β-cyclodextrin ('anti-copigmentation') and, secondly, a steady increase in the absorbance when the concentration of G rises. This latter effect derives from the displacement of the colourless pigment forms by the new guest G in the β-cyclodextrin cavity, that is, a relative dissociation of the 'anti-copigmentation' complexes. The consequent release of the colourless free forms leads to a shift of the pigment hydration equilibrium toward the flavylium cation (hyperchromic effect). The various equilibria are summed up in Scheme 4. The stability constant of the new inclusion complex

$$AH^+ + H_2O \overset{K_h}{\rightleftharpoons} B + H^+$$

$$AH^+ + S \overset{K_3}{\rightleftharpoons} AHS^+$$

$$B + S \overset{K_4}{\rightleftharpoons} BS$$

$$G + S \overset{K_{GS}}{\rightleftharpoons} GS$$

Scheme 4

GS is computed in a very similar way to the one used for the chlorogenic acid–β-cyclodextrin complex. Eqn. (10) remains valid and gives access to the free β-cyclodextrin concentration [eqn. (13)]. Allowing for the new notations, eqn. (9) becomes:

$$[S] = \frac{D_0 - D}{cD - r_3K_3D_0} \qquad (13)$$

$[S]_t/[S] = 1 - K_{GS}[S]_t + K_{GS}/([G]_t + [S])$; it is used as already mentioned to estimate K_{GS}. The corresponding plot is shown on Fig. 8; it gives K_{GS} = 660 (±90) $dm^3\ mol^{-1}$ at 25 °C. By comparison, Lin and Connors [36] found a value of 704 (±32) $dm^3\ mol^{-1}$ for the stability constant of the *p*-bromophenol–α-cyclodextrin inclusion complex, at the same temperature.

Chlorogenic Acid–Caffeine Complex.—Our quantitative study of molecular interactions largely deals with molecules found in the plant kingdom and this is why caffeine has been chosen: it is a naturally abundant molecule, present in many beverages such as tea and coffee, and is also responsible for physiological effects which make it a very important material in the pharmaceutical and food industries.[50] Since it is a purine, caffeine has a structure which favours its molecular association by vertical stacking. Its much investigated self-association [51–54] has been taken as a model in the interactions between nucleic bases. Caffeine also forms molecular complexes with different organic molecules bearing aromatic rings,[55–58] a phenomenon which may explain some of its pharmacological effects. Finally, on the basis of structural similarities between caffeine and proline-rich proteins known to interact strongly with polyphenols, caffeine has been proposed as a protein model in such interactions.[38,59]

Potassium chlorogenate has been demonstrated to associate with caffeine. The molecular complex formed has been crystallized and analysed by means of X-ray diffraction.[57] Sondheimer and his co-workers,[58] using UV–VIS spectrometry, found for this complex a K_{SL} value of 44 (±4) $dm^3\ mol^{-1}$ in water at 25 °C. Moreover, the complexation reaction, which essentially involves the caffeic moiety of the chlorogenate anion, is pH independent as long as the phenolic OH groups are protonated (pH <6). Repeating Sondheimer's experiment in a pH 3.50 buffer, we effectively found the above-mentioned value for the stability constant in the case of the chlorogenic acid–caffeine pair. The validity of our competitive spectrometric method, with malvin as the indicator, has been checked against the latter value. As previously stated, both chlorogenic acid and caffeine are malvin copigments; therefore, adding caffeine to a malvin solution copigmented by chlorogenic acid (pH 3.50) results in two competitive processes: the first one involves the caffeine–chlorogenic acid complexation which tends to shift the copigmentation equilibrium between malvin and chlorogenic acid in such a way that dissociation of the malvin–chlorogenic acid complex increases. In the absence of any other effect, one would observe a hypochromic shift. The second one corresponds to the formation of the malvin–caffeine complex which stabilizes the flavylium cation. If this process is stronger than the first one, an apparent hyperchromic copigmentation shift should occur. We effectively observed a steady increase in the absorbance of the flavylium chromophore with caffeine concentration, as demonstrated in Fig. 3. This newly encountered situation, where the substrate S interacts simultaneously with malvin and the other copigment L, is described in Scheme 5 which not only takes into account the equilibria shown in Scheme 3 but also the copigmentation equilibria characteristic of S. The K'_1 and K'_2 association constants are expressed by $[AHS^+]/([AH^+][S])$ and $[AS]/([A][S])$, respectively. Although caffeine is known to self-associate (for instance, its dimerization constant [53] is 13 $dm^3\ mol^{-1}$ at 25 °C), the caffeine concentration used in this work permit one to neglect this phenomenon. A treatment analogous to the one used in establishing eqn. (7) but now taking into consideration all the equilibria appearing in Scheme 5 gives eqn. (14).

$$\frac{D}{D_0} = \frac{1 + b[L] + b'[S]}{1 + a[L] + a'[S]} \qquad (14)$$

The a' and b' parameters are deduced from the a and b parameters by replacing K_1, K_2, r_1 and r_2 by K'_1, K'_2, r'_1 and r'_2, respectively. As we did in the case of the malvin–chlorogenic acid system, investigation of the hyperchromic shift as a function of pH, in the case of the malvin–caffeine pair, gives the following values: K'_1 = 130; K'_2 = 220 $dm^3\ mol^{-1}$ at 25 °C and $r_1 = r'_1$ = 0.80; $r_2 = r'_2$ = 0.45, at 520 nm. Comparing the copigmentation stability constants shows that, unlike chlorogenic acid, caffeine stabilizes the quinonoidal bases of malvin better than its flavylium cation. It is still possible to neglect the amounts of the copigmentation complexes in the L and S total concentrations; thus, one obtains: $[L]_t = [L] + [SL]$; $[S]_t = [S] + [SL]$ from which one gets: $[S]_t - [L]_t = [S] - [L]$. The latter relationship combined with eqn. (14) enables one to measure the free chlorogenic acid concentration in solution

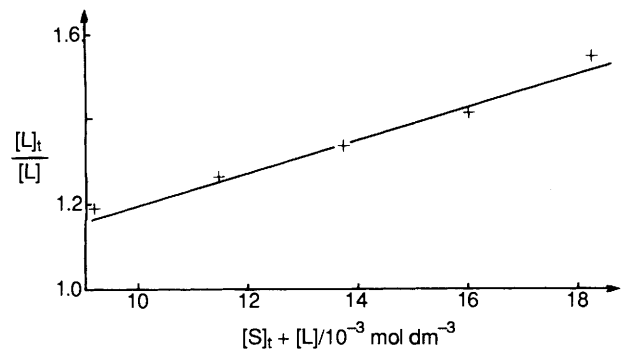


Fig. 9 The malvin–chlorogenic acid–caffeine system. Plot of $[L]_t/[L]$ *versus* ($[S]_t + [L]$); S is caffeine. $\rho = 0.991$; slope = 38 (± 3) dm^3 mol^{-1}; intercept = 0.822 (± 0.045). $T = 25$ °C.

$$[L] = \frac{D - D_0 + (b'D_0 - a'D)([L]_t - [S]_t)}{(b + b')D_0 - (a + a')D} \quad (15)$$

[eqn. (15)]. Eqn. (9) remains valid and we used it in the same way as previously to estimate the K_{SL} value which was found to be 36 (± 9) dm^3 mol^{-1} at 25 °C (see Fig. 9). This value is in good agreement with the value reported in the literature.[58]

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

$$AH^+ + H_2O \rightleftharpoons (B + C_E) + H^+$$

$$AH^+ + L \rightleftharpoons AHL^+$$

$$A + L \rightleftharpoons AL$$

$$S + L \rightleftharpoons SL$$

$$AH^+ + S \rightleftharpoons AHS^+$$

$$A + S \rightleftharpoons AS$$

Scheme 5

Conclusion

We have demonstrated in this work that the naturally occurring[60] copigmentation molecular interaction, which is responsible for so many flower and fruit colours, permits one to investigate, by means of visible absorption spectrometry, molecular complexes of the copigment other than the copigmentation complexes themselves.

The technique of competitive spectrometry has already been applied when the indicator is an azo dye (methyl orange or nitrazine yellow).[36,37] For these dyes, the study of α-cyclodextrin inclusion complexes was made possible by the fact that the molar absorption coefficients of the dye, in the free state and in the cyclodextrin cavity, are very different. Our competitive method applied to the investigation of chlorogenic acid molecular complexes, malvin being the indicator, exhibits a good sensitivity though ε_{AH^+} is close to ε_{AHL^+}. This sensitivity would remain even in the case where ε_{AH^+} equals ε_{AHL^+} since it depends mainly on the anthocyanin chemical structure and, in particular, on the existence of the colourless forms which are very abundant as long as the pH is higher than pK_h. Since pK_h values for ordinary anthocyanins range from *ca.* 2 to 3, this sensitivity is maintained for slightly acidic as well as slightly alkaline and, of course, neutral solutions. The colourless forms constitute a reservoir for the colour which can be readily modified whenever an association, implying either one of the indicator (pigment) forms or a copigment, appears. This outstanding feature makes our method widely applicable. Examples given in this paper are studies on molecular interactions as diverse as inclusion of molecules into cyclodextrins and the vertical stacking of chlorogenic acid with caffeine.

Another aspect of the present work is that measuring the stability of inclusion complexes is not limited to the cyclodextrins but may well be extended to other macrocycles like, for instance, calixarenes and cyclophanes whose cavities are good receptors for those organic compounds meeting the steric requirements appropriate to each case.[61] Since solubilities of the constituents of a complex are crucial factors, it is important to know that copigmentation still occurs, although to a lesser degree, in binary mixtures made of water and an organic solvent miscible with water such as alcohols, acetone, acetonitrile, formamide and *N,N*-dimethylformamide. Thus, in order to investigate the complexes formed by compounds which are insufficiently soluble in water, a large choice of mixed solvents is available.

Finally, the possibility of measuring the stability constants of the complex formed between two interacting copigments has been successfully demonstrated in the case of the chlorogenic acid–caffeine complex. Our final conclusion is that molecular interactions, exhibited by other biologically important materials, could be easily investigated by this method provided that such molecules are good copigments, a fact already demonstrated in the case of adenosine.[24,62]

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References

1 R. Brouillard, in *The Flavonoids, Advances in Research*, ed. J. B. Harborne, Chapman and Hall, London, 1988, p. 525.
2 G. Mazza and R. Brouillard, *Food Chemistry*, 1987, **25**, 207.
3 Y. Osawa, in *Anthocyanins as Food Colors*, ed. P. Markakis, Academic Press, New York, 1982, p. 41.
4 T. Goto and T. Kondo, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 17.
5 R. Brouillard and B. Delaporte, *J. Am. Chem. Soc.*, 1977, **99**, 8461.
6 J. B. Harborne, in *Chemistry and Biochemistry of Plant Pigments*, 2nd edn., ed. T. W. Goodwin, Academic Press, New York, 1976, vol. 1, p. 736.
7 C. F. Timberlake and P. Bridle, in *The Flavonoids*, eds. J. B. Harborne, T. J. Mabry and H. Mabry, Chapman and Hall, London, p. 214.
8 G. M. Robinson and R. Robinson, *Biochem. J.*, 1931, **25**, 1687.
9 S. Asen, R. N. Stewart and K. H. Norris, *Phytochemistry*, 1972, **11**, 1139.
10 S. Asen, R. N. Stewart and K. H. Norris, *Phytochemistry*, 1971, **10**, 171.
11 S. Asen, K. H. Norris and R. N. Stewart, *Phytochemistry*, 1972, **11**, 2739.
12 M. Williams and G. Hrazdina, *Food Science*, 1979, **44**, 66.
13 L. J. Chen and G. Hrazdina, *Phytochemistry*, 1981, **20**, 297.
14 K. Takeda, M. Kariuda and H. Itoi, *Phytochemistry*, 1985, **24**, 2251.
15 Y. Yasaki, *Bot. Mag.*, 1976, **89**, 45.
16 J. G. Sweeny, M. M. Wilkinson and G. A. Iacobucci, *J. Agric. Food Chem.*, 1981, **29**, 563.
17 R. Brouillard, G. Mazza, Z. Saad, A. M. Albrecht-Gary and A. Cheminat, *J. Am. Chem. Soc.*, 1989, **111**, 2604.
18 G. Mazza and R. Brouillard, *Phytochemistry*, 1990, **29**, 1097.
19 Y. Cai, T. H. Lilley and E. Haslam, *J. Chem. Soc., Chem. Commun.*, 1990, 380.

20 K. A. Connors, *Binding Constants, The Measurement of Molecular Complex Stability*, 1987, Wiley, New York.
21 R. N. Stewart, K. H. Norris and S. Asen, *Phytochemistry*, 1975, **14**, 937.
22 R. Brouillard and J. E. Dubois, *J. Am. Chem. Soc.*, 1977, **99**, 1359.
23 R. Brouillard, B. Delaporte and J. E. Dubois, *J. Am. Chem. Soc.*, 1978, **100**, 6202.
24 R. Brouillard, M. C. Wigand, O. Dangles and A. Cheminat, *J. Chem. Soc., Perkin Trans. 2*, 1991, 1235.
25 T. Hoshino and T. Goto, *Tetrahedron Lett.*, 1990, **31**, 1593.
26 T. Hoshino, *Phytochemistry*, 1991, **30**, 2049.
27 P. Rüedi and B. Hutter-Beda, *Bull. Liaison Groupe Polyphénols*, 1990, **15**, 332.
28 R. Brouillard and J. Lang, *Can. J. Chem.*, 1990, **68**, 755.
29 E. S. Sadlowski, Ph.D. Thesis, Colorado State University, Fort Collins, 1985.
30 M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer-Verlag, Heidelberg, 1978.
31 W. Saenger, *Angew. Chem., Int. Ed. Engl.*, 1980, **19**, 344.
32 W. Saenger, *Inclusion Compounds*, eds. J. L. Atwood, J. E. D. Davies and D. MacNicol, Academic Press, London, 1984, vol. 2, p. 231.
33 J. Szejtli, *Cyclodextrin Technology*, Kluwer Academik, Dordrecht, 1988.
34 D. Duchene, *Cyclodextrins and their Industrial Uses*, Editions de Santé, Paris, 1987.
35 For a comprehensive bibliography in a recent paper, see N. Yoshida, A. Seiyama and M. Fujimoto, *J. Phys. Chem.*, 1990, **94**, 4246.
36 For a comprehensive spectrophotometric study, see S. F. Lin and K. A. Connors, *J. Pharm. Sci.*, 1983, **72**, 1333.
37 D. D. Pendergast and K. A. Connors, *J. Pharm. Sci.*, 1984, **73**, 1779.
38 Y. Cai, S. H. Gaffney, T. H. Lilley, D. Magnolato, R. Martin, C. M. Spencer and E. Haslam, *J. Chem. Soc., Perkin Trans. 2*, 1990, 2197.
39 T. V. Mistry, T. Cai, T. H. Lilley and E. Haslam, *J. Chem. Soc., Perkin Trans. 2*, 1991, 1287.
40 T. Yamada, T. Komiya and M. Akaki, *Agric. Biol. Chem.*, 1980, **44**, 1411.
41 F. Cramer, W. Saenger and H. C. Spatz, *J. Am. Chem. Soc.*, 1967, **89**, 15.
42 N. Yoshida, A. Seiyama and M. Fujimoto, *J. Phys. Chem.*, 1990, **94**, 4254.
43 P. V. Demarco and A. L. Thakkar, *Chem. Commun.*, 1970, 2.
44 D. J. Wood, F. E. Hruska and W. Saenger, *J. Am. Chem. Soc.*, 1977, **99**, 1735.
45 E. Sondheimer, *J. Am. Chem. Soc.*, 1953, **75**, 1507.
46 K. A. Connors, S. Lin and A. B. Wong, *J. Pharm. Sci.*, 1981, **71**, 217.
47 A. B. Wong, S. Lin and K. A. Connors, *J. Pharm. Sci.*, 1983, **72**, 388.
48 K. A. Connors and D. D. Pendergast, *J. Am. Chem. Soc.*, 1984, **106**, 7607.
49 K. A. Connors, A. Paulson and D. Toledo-Velasquez, *J. Org. Chem.*, 1988, **53**, 2023.
50 S. N. Katz, *Proc. 9th Colloq. Sci. Int. Cafe*, 1980, 295.
51 P. O. P. Ts'O and S. I. Chan, *J. Am. Chem. Soc.*, 1963, **85**, 1289.
52 P. O. P. Ts'O, I. S. Malvin and A. C. Olson, *J. Am. Chem. Soc.*, 1964, **86**, 4176.
53 S. J. Gill, M. Downing and G. F. Sheats, *Biochemistry*, 1967, **6**, 272.
54 H. Bothe and H. K. Cammenga, *Thermochim. Acta*, 1983, **69**, 235.
55 T. Higuchi and D. A. Zuck, *J. Am. Pharm. Assoc.*, 1953, **42**, 132.
56 E. Shefter, *J. Pharm. Sci.*, 1968, **57**, 1163.
57 R. Martin, T. H. Lilley, C. P. Falshaw, E. Haslam, M. J. Begley and D. Magnolato, *Phytochemistry*, 1987, **26**, 273.
58 E. Sondheimer, F. Covitz and M. Marquisee, *J. Arch. Biochem. Biophys.*, 1961, **93**, 63.
59 C. M. Spencer, Y. Cai, R. Martin, S. H. Gaffney, P. N. Goulding, D. Magnolato, T. H. Lilley and E. Haslam, *Phytochemistry*, 1988, **27**, 2397.
60 S. Asen, R. N. Stewart and K. H. Norris, *Phytochemistry*, 1975, **14**, 2677.
61 F. Diederich, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 362.
62 R. Brouillard, M. C. Wigand and A. Cheminat, *Phytochemistry*, 1990, **29**, 3457.

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