Charge-Transfer Complexation as a General Phenomenon in the Copigmentation of Anthocyanins

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Color intensification of anthocyanin solutions in the presence of natural polyphenols (copigmentation) is re-interpreted in terms of charge transfer from the copigment to the anthocyanin. Flavylium cations are shown to be excellent electron acceptors ($E_{red} \approx -0.3$ V vs SCE). It is also demonstrated, for a large series of anthocyanin−copigment pairs, that the standard Gibbs free energy of complex formation decreases linearly with $EA_{Anthoc} - IP_{Cop}$, the difference between the electron affinity of the anthocyanin, EA_{Anthoc}, and the ionization potential of the copigment, IP_{Cop}. Based on this correlation, copigmentation strengths of potential candidates for copigments can be predicted.

Introduction

Anthocyanins constitute the major red, blue, and purple pigments in plants and are particularly attractive candidates for safe colorants that are simultaneously health-promoting, disease-preventing dietary supplements (antioxidants).[1−7] Although widespread use of anthocyanins as food dyes has long been envisaged,[5] problems of chemical and color stability[5] still represent a significant barrier to this application. Thus, the red flavylium cation, AH^+, participates in a series of coupled ground-state equilibria in aqueous solution (Scheme 1) involving an initial hydration reaction to produce a colorless hemiacetal form, B, that in turn tautomerizes to give colorless isomeric chalcones (C_E and C_Z in Scheme 1).[8−11] Deprotonation of the cation, on the other hand, yields the purple quinonoidal base form, A.

Because B and/or the chalcones are usually the thermodynamically most stable species in aqueous solution at pH > 2−3, the red or purple colors generally start to disappear as the pH is raised above this range.

Nature has developed strategies for stabilizing the red color of anthocyanins at pH values around 5 (the pH of plant cell vacuoles in which anthocyanins are located in vivo).[12] In the vacuoles, complexation of the flavylium cation by other polyphenols (copigmentation)[13] or metals[14] prevents color bleaching by stabilizing the flavylium cation with respect to the uncolored hemiacetal and chalcone forms. The term copigmentation was introduced in the early works of Robinson[15] and Willstater[16] to designate the phenomenon of "color intensification of an anthocyanin as the result of interaction with an uncolored molecule called a copigment". Robinson, Brouillard,[13] and others[17−23] have shown that a great number of colorless natural compounds (such as hydroxylated benzoic and cinnamic acids, hydroxyflavones, etc.) are capable of inducing copigmentation effects.

In the past, the driving force for copigmentation has usually been assumed to be the so-called hydrophobic effect,[17−23] which would imply that an increase in the hydrophobicity of the anthocyanin cation and/or the copigment should result in a corresponding increase in the equilibrium constant of complex formation and the resulting copigmentation effect. We recently provided evidence, however, that charge transfer is an important factor in the stabilization of complexes of several polyphenol copigments with the 4-methyl-7-hydroxyflavylium ion (Scheme 2) and with the flavylium cation form of pelargonin[24] (Scheme 1).

Furthermore, electronic excitation of these complexes induces ultrafast (subpicosecond) electron transfer from the polyphenol to the anthocyanin that results in total quenching of the fluorescence of the complex.[24] Measurement of the extent of this static fluorescence quenching thus permits straightforward determination of the equilibrium constants for complex formation.[24]

In this work, we provide additional evidence, both experimental and theoretical, for the generality of charge transfer (strictly a charge-shift), from the copigment to the flavylium cation, as a major driving force for the stabilization of anthocyanin−copigment complexes. Unlike models based on hydrophobicity as the only driving force, the present charge transfer formulation of the copigmentation phenomenon nicely rationalizes the observed dependence of copigmentation constants on the redox properties of the anthocyanin−copigment pair. In particular, these correlations provide a straightforward means of estimating the relative copigmentation strengths of potential copigment molecules based on calculated values of their ionization potentials.

Experimental Section

Materials. The anthocyanins, peonidin-3-glucoside, pelargonidin-3,5-diglucoside (pelargonin), cyanidin-3,5-diglucoside (cyanin), malvidin-3-glucoside (oenin), and malvidin-3,5-diglucoside (malvin) chlorides (Extrasynthése, HPLC grade) were used without further purification. The synthetic flavylium salt,

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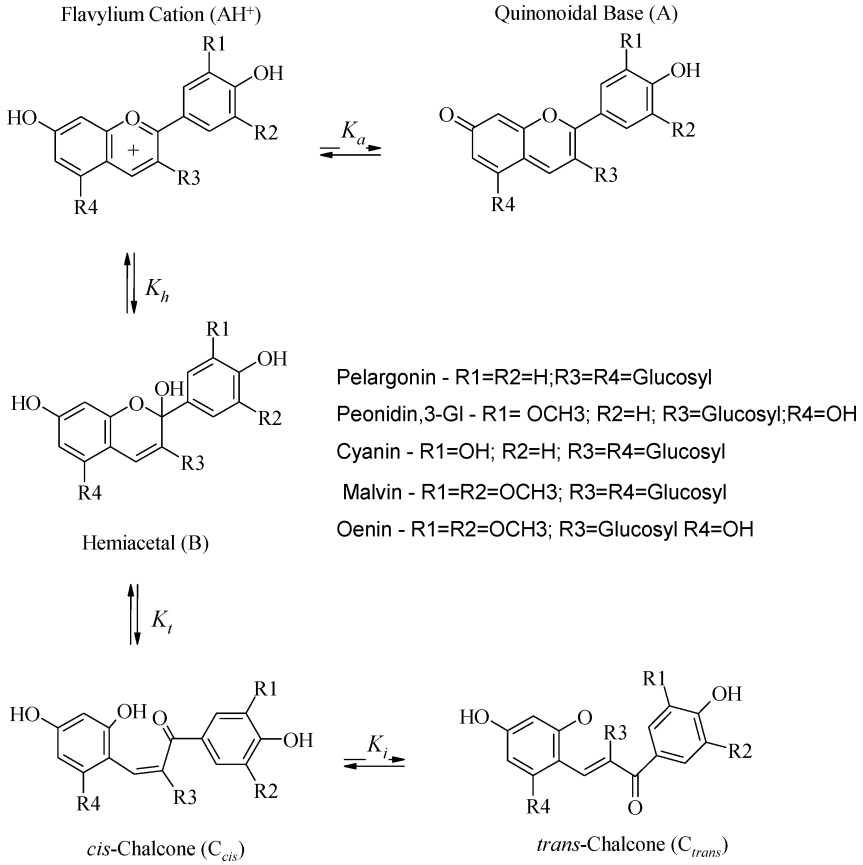
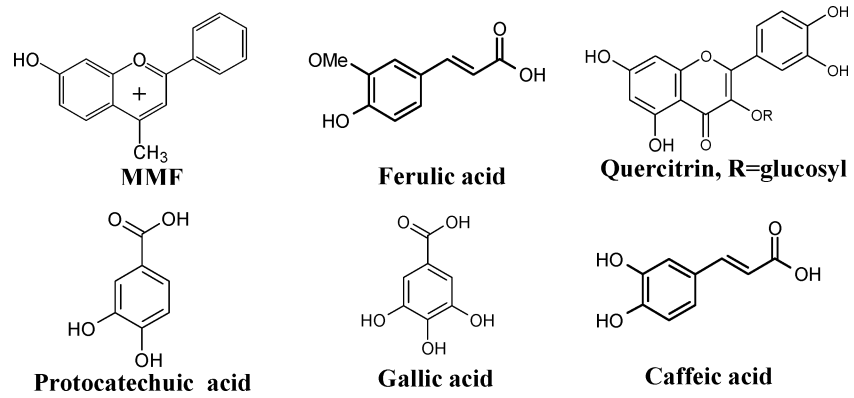
SCHEME 1



SCHEME 2



4-methyl-7-methoxyflavylium chloride (MMF) was synthesized and purified as described previously.[25] The polyphenolic compounds, protocatechuic acid (Aldrich, 97%), quercitrin (Extrasynthése, HPLC grade), isoquercitrin (Extrasynthése, HPLC grade), gallic acid (Aldrich, 97%), ferulic acid (Aldrich, 99%), and caffeic acid (Sigma, HPLC grade), were used as received. Water was twice distilled and deionized (Elgastat UHQ PS).

Measurements. The pH was measured at 20 °C with a Crison micropH 2002. UV−Vis absorption spectra were recorded on a Beckman DU-70 spectrophotometer.

Fluorescence spectra were measured using a SPEX Fluorolog 212I spectrofluorimeter. All spectra were collected in the S/R mode and corrected for optics and detector wavelength dependence.

Reduction potentials of anthocyanins were measured in acetonitrile (MeCN), using cyclic voltammetry, on an AUTOLAB PGSTAT12 potentiostat, as described.[24] The three-electrode system, used in the common triangular configuration, consisted of a Hg suspended drop working electrode, a Ag/AgCl/3.0 M KCl reference electrode, and a platinum wire as auxiliary electrode.[24]

Molecular Orbital Calculations. Molecular orbitals and states energies were computed by density functional theory (DFT) as implemented in the *Gaussian 03* package.[26] Gas-phase single point energies were obtained at the mPW1PW91 level with a 6-31+G(d,p) basis set and mPW1PW91/6-31+G(d,p) optimized geometries. Solvation free energies were computed by the polarizable continuum model (PCM) from geometries optimized in aqueous solution at the mPW1PW91/6-31+G(d) level. The absorption wavelengths and oscillator strengths were calculated by time dependent DFT (TD-DFT) and ZINDO employing the optimized geometries obtained in aqueous solution with mPW1PW91/6-31+G(d) and PCM.

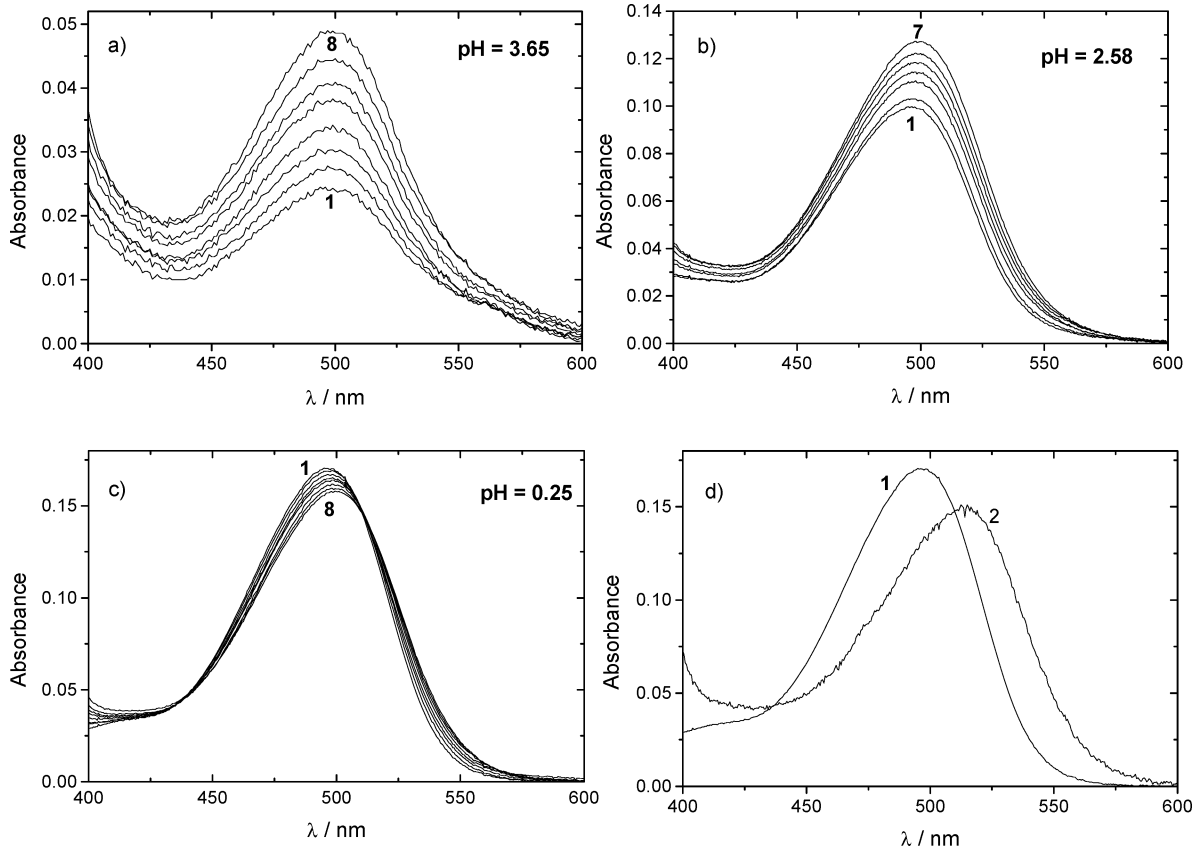


Figure 1. Absorption spectra of pelargonin (7.8×10^{-6} M) as a function of the concentration of ferulic acid (FRA). (a) In aqueous acetate buffer, pH 3.65: (1) no copigment, (2) 2.7 mM (3) 5.4 mM, (4) 8.2 mM, (5) 10.9 mM, (6) 13.6 mM, (7) 16.3 mM, (8) 19.1 mM. (b) In aqueous solution, pH 2.58: (1) no copigment, (2) 2.0 mM (3) 6.0 mM, (4) 8.0 mM, (5) 9.9 mM, (6) 11.9 mM, (7) 13.9 mM. (c) In aqueous solution, pH 0.25: (1) no copigment, (2) 2.8 mM (3) 5.6 mM, (4) 8.4 mM, (5) 11.2 mM, (6) 14.0 mM, (7) 16.8 mM, (8) 19.6 mM. (d) Absorption spectra of (1) the free flavylium ion of PLG and (2) the PLG-FRA complex obtained from global decomposition of the spectra in (c).

Gas-phase ionization energies (IE) and electron affinities (EA) were calculated from the formation enthalpies of the molecule and of its oxidized and reduced forms, respectively.[24] Molecular geometries were first calculated using the MM^+ molecular mechanics method and then optimized with the semiempirical AM1 method at the UHF level, using HyperChem version 5.0 software by Hypercube.[27] Enthalpies of formation were obtained by AM1 from single-point calculations on the optimized geometries. From a comparison of estimated vs experimental EA values in a large number of compounds it was found that the former are systematically larger than the experimental ones by a mean value of ca. 0.65 eV, with a deviation never greater than 1.15 eV.[24] In the case of ionization potentials, the agreement between calculated and experimental values is much better, with deviations always smaller than 0.5 eV.[24] Gas-phase polarizabilities were also calculated using the HyperChem QSAR module.

Results

1. Electronic Spectra of Pelargonin−Polyphenol Complexes. Figure 1 shows the effect of ferulic acid (FRA, see Scheme 2) on the absorption spectra of pelargonidin-3,5-diglucoside (pelargonin, PLG) chloride at three pH values: 3.65, 2.58, and 0.25. At the two highest pH values (Figure 1, panels a and b), the copigmentation effect is evidenced by the increase in the absorbance at 498 nm with increasing FRA concentration, which is noticeably larger at pH 3.65 (Figure 1a) than at pH 2.58 (Figure 1b). The increase in absorbance is accompanied by a red shift of the absorption maximum better seen in Figure 1b.

At pH 0.25 (Figure 1c), addition of FRA induces a clear red shift of the absorption maximum, but only a small decrease in absorbance, with an isosbestic point at 510 nm. Because only the AH^+ form of the anthocyanin exists at pH 0.25 ($pK_h = 1.88$), changes in the visible region of the absorption spectrum are due exclusively to differences between the spectra of the free (AH^+) and complexed (CP) flavylium cations.

Figure 1d presents the absorption spectra of the free flavylium ion of PLG (curve 1) and of the PLG−FRA complex (curve 2) obtained from global decomposition of the spectra in Figure 1c (see the Discussion). As previously proposed for a number of flavylium−polyphenol complexes,[17] complexation of PLG with FRA results in a slight decrease in the value of the molar extinction coefficient at the maximum of the first band ($\epsilon_{513\,nm} = 1.7 \times 10^4$ M^{-1} cm^{-1}) relative to that of the free flavylium ion ($\epsilon_{497\,nm} = 2.2 \times 10^4$ M^{-1} cm^{-1}).

Absorption spectra of PLG in the presence of the polyphenols in Scheme 2 exhibit similar changes. Spectroscopic data of the corresponding complexes are summarized in Table 1.

2. Electronic Spectra of Anthocyanin−Ferulic Acid Complexes. Absorption spectra of aqueous solutions of the anthocyanins malvidin 3-glucoside (oenin), peonidin 3-glucoside, cyanidin 3,5-diglucoside (cyanin), and malvidin 3,5-diglucoside (malvin), in the presence of FRA at pH 0.45 (Figure 2) and 2.58 (not shown), all gave results similar to those obtained with PLG: the spectra of the flavylium−FRA complexes are red shifted and show lower molar extinction coefficients than the respective free flavylium ions (Figure 2). This trend also holds for synthetic flavylium ions, including 4-methyl-7-methoxyflavylium chloride (MMF), that, unlike natural anthocyanins,

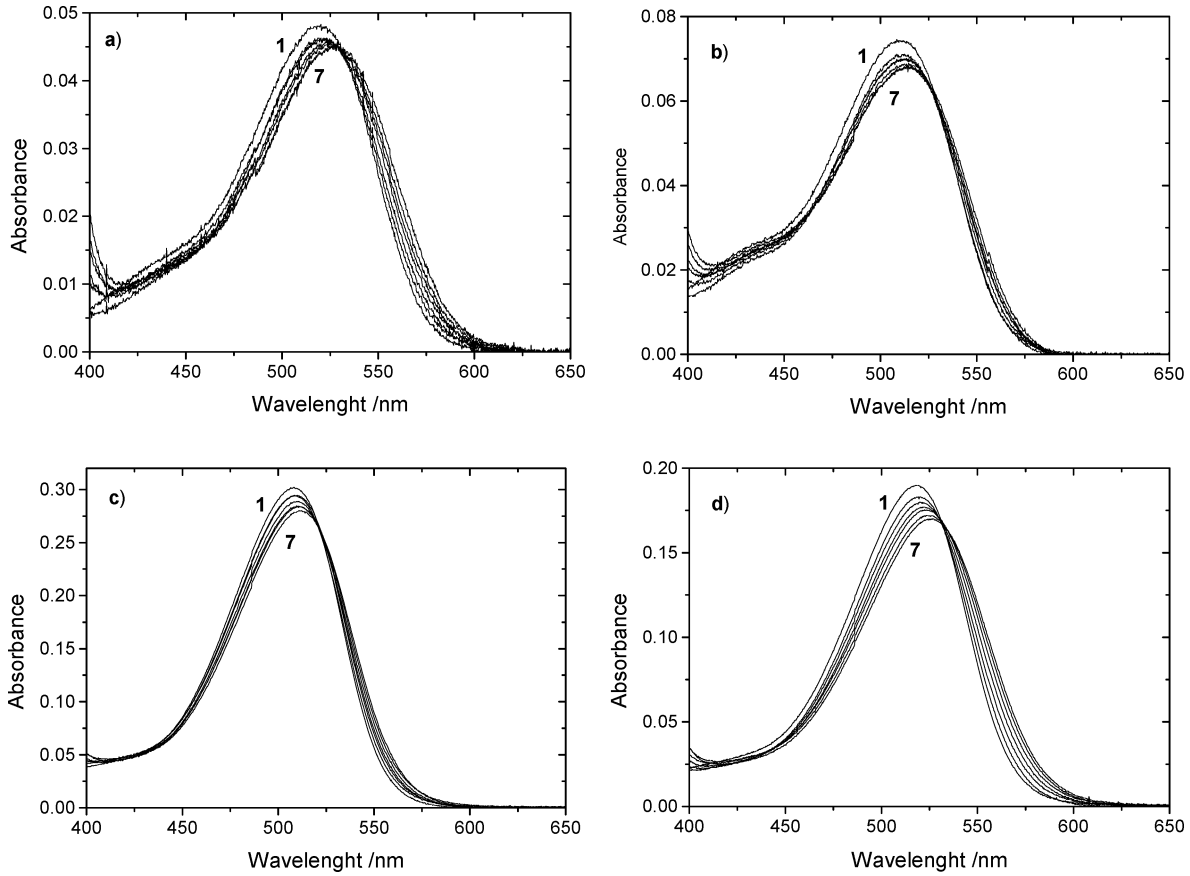


Figure 2. Absorption spectra in aqueous solution at pH 0.45 of (a) oenin (1) no copigment, (2) 3.7 mM (3) 7.4 mM, (4) 11.0 mM, (5) 14.7 mM, (6) 18.4 mM, (7) 22.1 mM; (b) peonidin 3-glucoside (1) no copigment, (2) 3.8 mM (3) 7.6 mM, (4) 11.5 mM, (5) 15.3 mM, (6) 19.1 mM, (7) 22.9 mM; (c) cyanin (1) no copigment, (2) 3.6 mM (3) 7.1 mM, (4) 10.7 mM, (5) 14.3 mM, (6) 17.8 mM, (7) 21.4 mM; and (d) malvin (1) no copigment, (2) 4.0 mM (3) 8.0 mM, (4) 11.9 mM, (5) 15.9 mM, (6) 19.9 mM, (7) 23.9 mM.

TABLE 1: Maximum Wavelengths (λ_{max}) and Molar Extinction Coefficients (ϵ_{max}) of the UV−Vis Absorption of Pelargonin−Polyphenol Complexes[a]

polyphenol	λ_{max} (nm)	$\epsilon_{max} \times 10^{-4}$ (M^{-1} cm^{-1})	IP (eV)	K_{Cop}[b] (M^{-1})	ΔG_{Cop}[c] (eV)
protocatechuic acid			8.75	68	−0.107
gallic acid	510	1.9	8.61	87	−0.113
caffeic acid	504	2.1	8.31	237	−0.138
ferulic acid	513	1.7	8.20	256	−0.140
quercitrin			7.63	1740	−0.188

[a] Calculated ionization potential of the copigment (IP), copigmentation constant (K_{Cop}), and standard Gibbs free energy (ΔG_{Cop}) of complexation. [b] Reference 24. [c] $\Delta G_{Cop} = -RT \ln K_{Cop}$.

TABLE 2: Theoretical Gas Phase Electron Affinities of Anthocyanins (EA) and Polarizabilities (α), Cyclic Voltametry Peak Potential (E_{red}), in MeCN vs SCE, for the Reduction of Anthocyanins in Acetonitrile, Equilibrium Constants (K_{Cop}), and Standard Gibbs Free Energy (ΔG_{Cop}) of Copigmentation of Anthocyanins with Ferulic Acid (FRA)

anthocyanin	EA (eV)	E_{red} (V)	α (Å^3)	K_{Cop}[a] (pH = 2.58)	ΔG_{Cop}[d] (eV)
MMF	6.82	−0.29(3)	31.3	214[b]	−0.136
peonidin	6.82	−0.36(7)		155	−0.127
oenin	6.86	−0.40(0)		243	−0.139
pelargonin	6.90	−0.31(9)	54.5	263	−0.143
cyanin	6.90	−0.29(8)	57.0	210	−0.135
malvin	6.91	−0.32(8)	61.3	265[c]	−0.140

[a] From eq 4. [b] From ref 24, from eq 5, pH = 0.45. [c] pH 2.63. [d] $\Delta G_{Cop} = -RT \ln K_{Cop}$.

cannot deprotonate and does not hydrate in aqueous solution. Table 2 shows the equilibrium constants (K_{Cop}) and standard Gibbs free energy ($\Delta G_{Cop} = -RT \ln K_{Cop}$) of copigmentation of all of these anthocyanins with FRA at pH = 2.58.

Figure 3 presents the calculated energy level diagrams for the singlet states of the MMF cation (Figure 3a), FRA (Figure 3c), and the MMF−FRA complex at the equilibrium MMF−FRA distance of 3.5 Å (Figure 3b) in water (see the Experimental Section). The first singlet excited state of the complex (2.11 eV) is a charge transfer CT state, resulting from an electronic transition from the highest occupied molecular orbital (HOMO) predominantly localized on FRA to the lowest unoccupied molecular orbital (LUMO) localized on MMF (Scheme 3). The $S_1(CT) \leftarrow S_0$ transition is predicted at 594 nm, with a very small oscillator strength value ($f_1 = 0.002$).

The two close-lying second and third singlet excited states of the complex are the symmetric and antisymmetric combinations of the two first local excited LE states of MMF, which in turn result from the HOMO → LUMO and HOMO-1 → LUMO transitions of MMF. The predicted oscillator strengths of the $S_2(LE) \leftarrow S_0$ and $S_3(LE) \leftarrow S_0$ transitions ($f_2 = 0.11$ and $f_3 = 0.31$), at 402 and 390 nm, respectively, are lower than the calculated oscillator strength of the first transition of the isolated MMF cation ($f = 0.67$), at 389 nm. The results are in qualitative agreement with those of the experiment in predicting a small red shift and a decrease of the molar extinction coefficient of the first absorption band of MMF upon complexation with FRA. Additionally, the calculations reveal that the red shift results not only from the lowering in energy of the first electronic transition of MMF but also from the fact (see below) that the first observable absorption band of the complex is due to two transitions (the $S_2 \leftarrow S_0$ and $S_3 \leftarrow S_0$ transitions), one of which

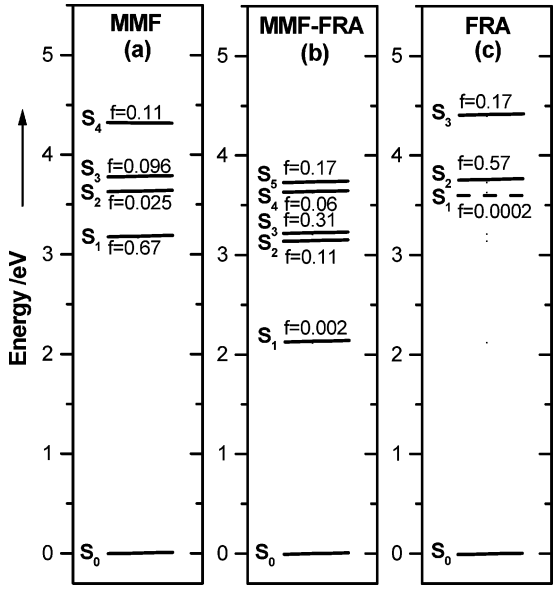
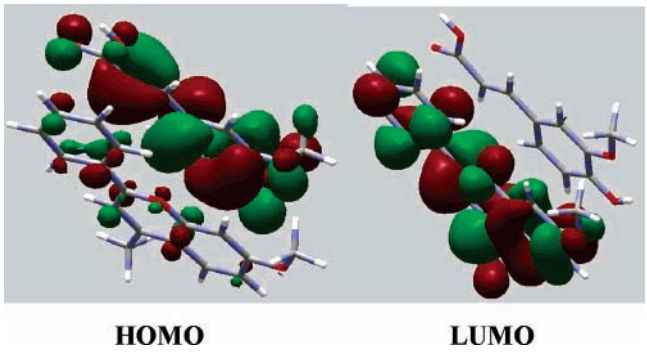


Figure 3. Energy level diagrams of the singlet states of (a) the MMF cation, (b) the MMF−FRA complex at the equilibrium distance, and (c) of FRA, calculated with DFT.

SCHEME 3



is clearly lower in energy than the first transition of MMF. This also explains the broadening of the first absorption band upon complex formation.

The charge-transfer band, predicted at ca. 594 nm, could not be detected, even using 5 cm optical path cells. This suggests that the molar extinction coefficient of the CT transition is very small, which is in fact consistent with the ϵ_{CT} value predicted by the calculations (ca. 300 M^{-1} cm^{-1} for an oscillator strength $f_{CT} = 0.002$).

3. Reduction Potentials and Electron Affinities of Anthocyanins and Ionization Potentials of Polyphenols. The energy of CT states depends on the electron affinity of the electron-accepting moiety and the ionization potential of the donor. The values of the adiabatic gas-phase ionization potentials (IP) of the copigments shown in Scheme 2 and of those listed in Tables 3 and 4 were calculated, as described previously,[24] from the difference between the enthalpies of formation of the copigment radical cation ($\Delta_f H_{Cop\bullet+}$) and the copigment ($\Delta_f H_{Cop}$), i.e., IP = $\Delta_f H_{Cop\bullet+} - \Delta_f H_{Cop}$. The IP values confirm that the hydroxyflavones are excellent electron donors, whereas the hydroxybenzoic acids are relatively poor electron donors.

Table 3 also includes literature[13] values for the fractional increase in absorbance, $(A\text{-}A_0)/A_0$ (see the Discussion), of cyanidin 3,5-diglucoside in the presence of 26 copigments at pH 3.32, whereas Table 4 includes literature values of equilibrium constants, K_{Cop}, for copigmentation of malvidin 3,5-diglucoside at pH 3.65 by a series of other copigments.[18−22]

TABLE 3: Calculated Ionization Potentials (IP) and Polarizabilities (α), of Natural Copigments and Fractional Increase of Absorbance, $(A - A_0)/A_0$, of Cyanidin 3,5-Diglucoside (2×10^{-3} M) in the Presence of the Copigment (6×10^{-3} M) in Aqueous Solution at pH 3.32[13]

copigment	IP (eV)	α (Å^3)	$(A - A_0)/A_0$
quercetin 3-rutinoside (rutin)	7.28	54.8	2.28
kaempferol 3-glucoside	7.53	41.3	2.39
quercitrin	7.63	41.3	2.17
quercetin-3-glucoside	7.70	42.0	1.88
quercetin-7- glucoside	7.82	43.8	1.73
hesperidin	7.97	56.1	1.19
sinapic acid	8.01	22.1	1.17
(+)-catechin	8.09	28.7	0.78
chlorogenic acid	8.14	32.5	0.75
esculin	8.18	30.4	0.66
ferulic acid	8.20	19.6	0.6
phloridzin	8.29	41.7	1.01
naringin	8.29	53.7	0.97
apigenin-7-glucoside	8.30	42.5	0.68
caffeic acid	8.31	17.7	0.56
p-hydroxycinnamic acid	8.35	17.1	0.32
proline	8.54	11.3	0.25
m-hydroxycinnamic acid	8.63	17.1	0.44
o-hydroxybenzoic acid	8.73	13.6	0.09
protocatechuic acid	8.75	14.3	0.23
glutamic acid	8.77	12.8	0.06
p-hydroxybenzoic acid	8.87	13.6	0.19
alanine	8.97	8.4	0.05
aspartic acid	9.01	10.9	0.03
glycine	9.09	6.5	0.09
benzoic acid	9.40	13.0	0.18

TABLE 4: Calculated Ionization Potentials, IP, of Natural Copigments and Equilibrium Constants, K_{Cop},[18−22] for Copigmentation of Malvidin 3,5-Diglucoside at pH 3.65 and Standard Gibbs Free Energy (ΔG_{Cop}) of Complexation

copigment	IP (eV)	K_{Cop} (M^{-1})	ΔG_{Cop} (eV)
rutin	7.28	3300	−0.205
morin	7.29	2300	−0.195
quercetin	7.84	650	−0.164
chlorogenic acid	8.14	280	−0.142
tannic acid	8.16	277	−0.142
caffein	8.19	225	−0.137
apigenin 7-glucoside	8.30	137	−0.124
protocatechuic acid	8.75	80[a]	−0.111

[a] This work.

Theoretical gas-phase electron affinities (EA) of the flavylium ion AH^+ of the anthocyanins peonidin 3-glucoside, oenin, cyanin, and malvin were also calculated from the enthalpies of formation of AH^+ and $AH^\bullet$ (EA = $\Delta_f H_{AH\bullet} - \Delta_f H_{AH+}$)[24] and are shown in Table 2. The calculated electron affinity values are extraordinary high, as found with PLG and MMF,[24] and relatively insensitive to variations in the substituents present on the anthocyanin chromophore.

Cyclic voltammograms of the AH^+ form of these anthocyanins, in acidified MeCN, all show irreversible reduction waves at slightly negative potentials, as earlier observed with PLG and MMF.[24] The values observed by us (Table 2) are quite large, in agreement with published polarographic data[28,29] for other flavylium cations, and also weakly dependent on the substitution pattern of the flavylium. Both electron affinity and reduction potential values agree in predicting that the AH^+ form of anthocyanins is easily reduced, independent of the substitution pattern.

Discussion

1. Copigmentation vs Equilibrium Constants of the PLG−FRA Complex. In mildly acidic aqueous solutions (pH ≈ 3),

the major portion of the anthocyanin is in the hemiacetal form B in equilibrium with the *E*- and *Z*-chalcones C (eq 1, where K_h' represents the apparent hydration constant).[8−11]

$$B/C + H^+ \overset{K_h'}{\rightleftharpoons} AH^+ \quad (1)$$

Addition of a copigment (Cop) that is able to form a complex (CP) with the AH^+ cation (equilibrium constant K_{Cop}) displaces the hydration equilibrium toward AH^+ plus CP (eq 2) and, when the extent of CP hydration is smaller than that of AH^+ (it is in fact negligible, see below), causes an increase in the total concentration of the colored species ([AH^+] plus [CP])

$$B/C + H^+ \overset{K_h'}{\rightleftharpoons} AH^+ + Cop \overset{K_{cop}}{\rightleftharpoons} CP \quad (2)$$

Because both the absorption wavelength and molar extinction coefficient of AH^+ and CP are similar and the hydration reaction (formation of the hemiacetal) of CP is thermodynamically disfavored,[30−32] the peak absorbance in the visible region ($A = \epsilon_{AH+}[AH^+] + \epsilon_{CP}[CP]$) increases with respect to that of AH^+ in the absence of copigment (A_0).

The fractional increase in absorbance at a given wavelength λ_{abs}, $(A - A_0)/A_0$, is proportional to (1) the ratio of the molar extinction coefficients of the complexed (CP) and the free flavylium cation (AH^+) at that wavelength λ_{abs} ($r_\epsilon = \epsilon_{CP}/\epsilon_{AH}+$), (2) the equilibrium constant K_{Cop}, (3) the concentration of copigment [Cop], and (4) to a more or less complex function of the pH and the hydration equilibrium K_h constant.[17,31] When complexation of species other than the flavylium form is neglected, $(A\text{-}A_0)/A_o$ is related to the equilibrium constants of eq 2 by eq 3

$$\frac{A - A_0}{A_0} = r_\epsilon K_{Cop}[CP] \times \frac{1 + \frac{[H^+]}{K_h}\frac{r_\epsilon - 1}{r_\epsilon}}{1 + \frac{[H^+]}{K_h}(1 + K_{Cop}[CP])} \quad (3)$$

Equation 3 can be simplified, under the condition that $[H^+] \ll K_h$, to eq 4, which predicts that the fractional increase $(A - A_0)/A_0$, is a linear function of copigment concentration, [Cop][17,31]

$$(A - A_0)/A_0 = K_{Cop} r_\epsilon [Cop] \quad (4)$$

Determination of copigmentation constants via eq 3 requires evaluation of the ratio of the molar extinction coefficients of the complexed and free flavylium cation at the analysis wavelength ($r_\epsilon(\lambda) = \epsilon_{CP}/\epsilon_{AH}^+$), besides satisfaction of the condition $[H^+] \ll K_h$. On the other hand, at the isosbestic point, $r_\epsilon(\lambda_{iso})$ is necessarily exactly equal to one, making it the optimal wavelength for application of eq 4. The isosbestic wavelength was determined for all complexes at pH 0.25.

The fractional increase of the absorbance $(A - A_0)/A_0$ at 510 nm (isosbestic wavelength), of the PLG−FRA aqueous solutions at pH 2.58 and 3.65, shown in Figure 1, panels a and b, is plotted as function of the concentration of ferulic acid, [FRA], in Figure 4. The plots are linear, as predicted from eq 4; however, the value of the slope at pH 3.65 (695 M^{-1}) is more than 2-fold larger than the value at pH 2.58 (263 M^{-1}). Recall that the slope value should equal the value of K_{Cop} because $r_\epsilon = 1$ at the isosbestic wavelength. The use of eq 3, which does not neglect the K_h terms, corrects the latter value of K_{Cop} to 305 M^{-1}, but does not account for this difference.

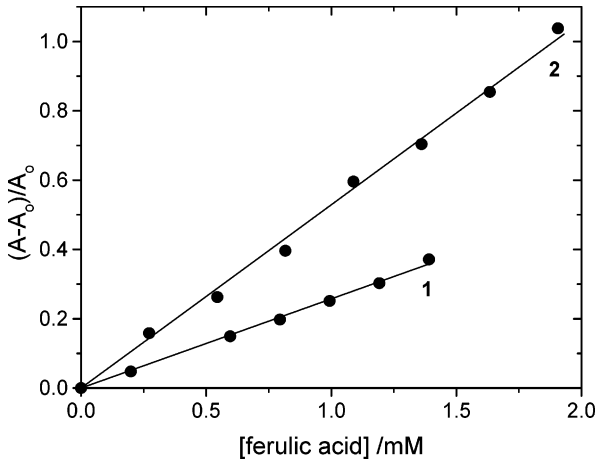


Figure 4. Plot of the fractional increase of absorbance $(A - A_0)/A_0$, at the isosbestic wavelength (510 nm) of PLG aqueous solutions, as a function of [FRA] at (1) pH 2.58 and (2) pH 3.65.

The value of K_{Cop} at pH 2.58 (either 263 or 305 M^{-1}) is slightly larger than the value for the equilibrium constant of the PLG−FRA complex at pH 0.25, K_C, previously obtained from fluorescence quenching of PLG by FRA (256 M^{-1}), i.e., from a plot of the fluorescence intensity ratio vs [FRA] (eq 5) [We distinguish K_C, the true flavylium complexation constant, from K_{Cop}, measured in a pH range where other equilibria are present].[24]

$$\frac{I_0}{I} = 1 + K_C[FRA] \quad (5)$$

The important implication of this observation that K_{Cop} is equal to or larger than K_C, is the validation of the fundamental assumption made in the quantitative analysis of copigmentation, i.e., that hydration of the complexed flavylium ion CP is negligible. In fact, partial hydration of CP would result in $K_C > K_{Cop}$.

The equilibrium constant, K_C, for PLG-FRA complex formation at the strongly acidic pH of 0.25 can alternatively be evaluated from the absorption spectra. At pH 0.25 (Figure 1c), only the free AH^+ and complexed CP forms of pelargonin exist ($[H^+] \gg K_a, K_h$). Under this condition, spectral decomposition (Figure 1d) yields the mole fractions of the two species (α_{AH+} and α_{CP}), and consequently, the complexation constant is straightforwardly obtained ($\alpha_{CP}/\alpha_{AH+} = K_C[Cop]$), bypassing the approximations needed at pH ≈ 3. The spectral decomposition can be further checked using fluorescence data.[24] The value of K_C, at pH 0.25, from absorption spectra (257 M^{-1}) is equal to that (256 M^{-1}) obtained from fluorescence quenching (see below).

Finally, we also attempted to determine K_C at pH 0.25 directly from the absorption spectra (without spectral decomposition). In fact, at sufficiently low pH values, when $[H^+] \gg K_h$, eq 2 simplifies to eq 6 (with no further assumptions)

$$\frac{A_0}{(A - A_0)} = \frac{1}{r_\epsilon - 1} + \frac{1}{(r_\epsilon - 1)K_C}\frac{1}{[Cop]} \quad (6)$$

Plots of $A_0/(A - A_0)$ vs the reciprocal copigment concentration (1/[Cop]) at a number of wavelengths (excluding the isosbestic wavelength) were constructed from the data in Figure 1c. Although the copigmentation constant could, in theory, be obtained from the *y*-intercept/slope ratio of each of these plots, the values of K_C thus obtained showed large scatter, reflecting

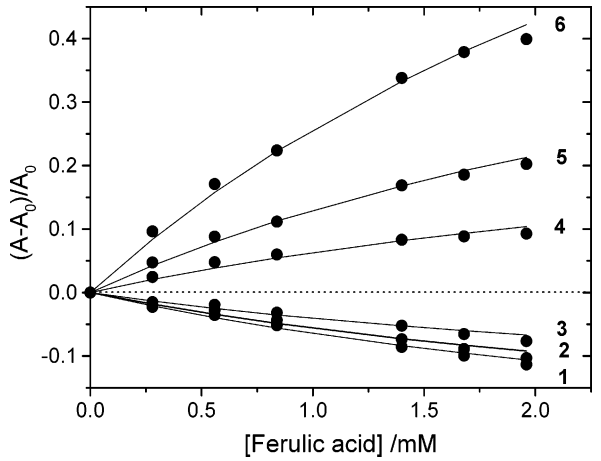


Figure 5. Plot of the fractional increase of absorbance $(A\text{-}A_0)/A_0$, at wavelengths (1) 480 nm, (2) 486.5 nm, (3) 487 nm, (4) 518 nm, (5) 524 nm, and (6) 532 nm, of PLG aqueous solutions at pH 0.25, as a function of [FRA]: ●, experimental data; —, calculated with eq 3.

the inaccuracy of the intercept values (r_ϵ −1 too close to zero). However, global analysis of all data points with eq 3 provided acceptable results, with a value of K_C equal to ca. 258 M^{-1} (Figure 5).

2. pH Dependence of the Equilibrium Constant for Formation of the PLG−FRA Complex. The values of the constants K_C for complexation/copigmentation of PLG by FRA, obtained by the absorption and fluorescence methods, are plotted against the pH in Figure 6a. The values are approximately constant from pH 0.25 up to ca. 2.5, but sharply increase at higher pH values.

An increase of K_{Cop} with pH has been reported for a number of anthocyanin−copigment pairs and generally attributed to the complexation of the neutral base form of the anthocyanin with the copigment.[17,32,33] However, comparison of the normalized spectra of PLG alone with that in the presence of 19.1 mM FRA at pH 3.65 (spectra 1 and 8 in Figure 1a) shows a slight decrease in the absorbance at ca. 570 nm, the wavelength where the base form of PLG predominantly absorbs, relative to the absorption at 498 nm. This is opposite to the expected increase in absorbance if the base were stabilized.

On the other hand, the pK_a value of FRA is 4.56,[34] implying that at pH 3.65 more than 10% of FRA is deprotonated and in the ferulate form, which does not necessarily have the same equilibrium constant for complex formation with PLG as the acid form of FRA. We therefore analyzed the possibility that the observed pH dependence of K_C resulted instead from the existence of two complexing species (the acid and base forms of FRA). The observed K_C (or K_{Cop}) would them be an apparent equilibrium constant, K_C^{app}, consisting of a linear combination of the true equilibrium constants ($K_C^{ferulic}$ and $K_C^{ferulate}$), with coefficients equal to the mole fractions of the base (α) and acid (1 − α) forms of FRA (eq 7), with $\alpha = K_a/(K_a + [H^+])$

$$K_C^{app} = \alpha K_C^{ferulate} + (1 - \alpha) K_C^{ferulic} \qquad (7)$$

In support of this assumption, the plot of K_C^{app} vs α is linear (Figure 6b) as predicted from eq 7, yielding from the intercept the value of $K_C^{ferulic} = 256\ M^{-1}$, and, from the sum of the slope and intercept, $K_C^{ferulate} = 4160\ M^{-1}$. The curve in Figure 6a was calculated with these values and the value of acidity constant of ferulic acid, $K_a = 10^{-4.56}$.

This result has two important implications. First, the value of the copigmentation constants measured in mildly acidic media

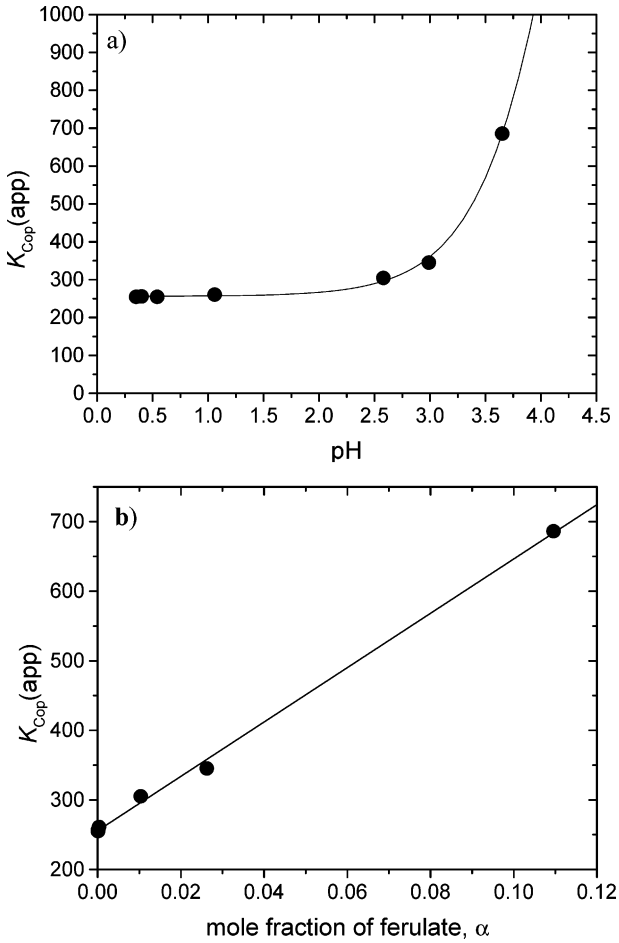


Figure 6. Plot of the apparent equilibrium constant K_C^{app} of the PLG−FRA complex in aqueous solutions as a function of (a) pH and (b) the mole fraction of the base form of FRA (α).

may significantly change when the copigment pK_a is close to the measurement pH. Second, and more important, the fact that the copigmentation constant of the more water-soluble anion of FRA is much larger than that of the neutral acid form implies that the hydrophobic effect alone does not explain the formation of PLG complexes, i.e., PLG copigmentation. The large value of $K_C^{ferulate} = 4160\ M^{-1}$ is, on the other hand, compatible with the much lower value of the gas-phase ionization potential of the ferulate anion (calculated IP = 3.40 eV) plus additional stabilization by ion pairing. In water, the IP value of the ferulate anion is expected to increase and that of ferulic acid to decrease because the reactant is neutral and the product is an ion for ionization of ferulic acid, while the opposite is true for ferulate. Using the Born formalism (eq 8), these correction terms are estimated as ca. 1.7 eV, taking $r^{ferulic} \approx r^{ferulate} = 4.3$ Å (from mean of van der Waals and QSAR molecular volumes). Thus, the IP value of the ferulate ion would still be expected to be ca. 1.4 eV lower than that of ferulic acid

$$IP_{water}^{ferulic} = IP_{gas\ phase}^{ferulic} - \frac{\epsilon - 1}{2\epsilon}\frac{e^2}{r^{ferulic}} \approx 8.2 - 1.7 = 6.5\ eV$$

$$IP_{water}^{ferulate} = IP_{gas\ phase}^{ferulate} + \frac{\epsilon - 1}{2\epsilon}\frac{e^2}{r^{ferulate}} \approx 3.4 + 1.7 = 5.1\ eV \qquad (8)$$

3. Equilibrium Constants of Anthocyanin−Polyphenol Complexes. Table 1 shows also values of the equilibrium constant K_C of PLG in the presence of five representative

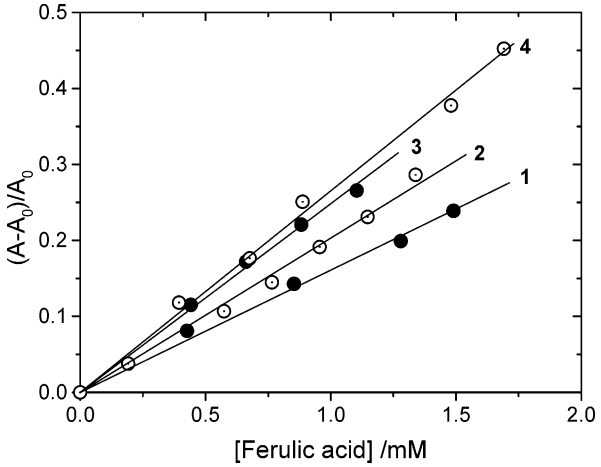


Figure 7. Fractional increase of the absorbance $(A - A_0)/A_0$ at the isosbestic wavelengths of aqueous solutions of (1) peonidin 3-glucoside, (2) oenin, (3) cyanin, and (4) malvin as a function of [FRA] at pH 2.58.

copigments, measured by the fluorescence quenching method. The values of the standard Gibbs free energy for complex formation, $\Delta G°$, are clearly correlated with the IP of the copigment, i.e., the lower the IP, the lower $\Delta G°$.

Figure 7 shows plots of the fractional increase of the absorbance $(A - A_0)/A_0$ at the isosbestic wavelengths for peonidin 3-glucoside, oenin, cyanin and malvin, as a function of the concentration of ferulic acid, [FRA], at pH 2.58. The values of the copigmentation constants (Table 2) do not exhibit large changes, but do tend to increase with the EA of the flavylium ion. Despite the intrinsic errors in predicting electron affinity values[24] it is clear that the EA values of the anthocyanins are all rather similar to each other. Because the hydrophobicity of the anthocyanins changes significantly within this anthocyanin series, the fact that the copigmentation constant does not vary significantly among the five anthocyanins does indicate that copigmentation is not markedly dependent on hydrophobicity. On the other hand, given the small differences in electron affinities and peak reduction potentials of the anthocyanins, the quasi-invariant K_{Cop} values are entirely consistent with a predominant role of charge transfer in copigmentation.

4. Charge-Transfer Nature of Flavylium−Polyphenol Complexes. The nature and properties of ground-state electron-donor−acceptor (EDA) molecular complexes were fully treated by Mulliken[35] in the fifties. Within the context of Mulliken's approach, the simplest description of an EDA complex is in terms of a resonance hybrid of the nonbonded (AD) and charge-transfer (A^-D^+) states (configurations). Thus, the ground state of the complex is characterized by the wave function

$$\psi_g = a\psi_{(AD)} + b\psi_{(A-D+)} \qquad (9)$$

where, generally, $a \gg b$. Within this very simple approach, stabilization of the ground state is due to interaction of the (AD) and (A^-D^+) configurations, besides van der Waals interactions. The degree of charge-transfer interaction, and hence the stability of the EDA complex, is proportional to the magnitude of the coefficient b in eq 9, which increases with decreasing the energy of the (A^-D^+) state, i.e., the energy gap between the (AD) and the (A^-D^+) states (see also Figure 3b). The energy of the (A^-D^+) state is, in turn, dictated by the ionization potential of the electron donor IP, the electron-affinity of the electron acceptor EA and a Coulombic work term associated with bringing the oxidized donor and reduced acceptor together to form A^-D^+ (eq 10):[36] In the present case of a cation acceptor and a neutral donor (charge shift), C is close to zero

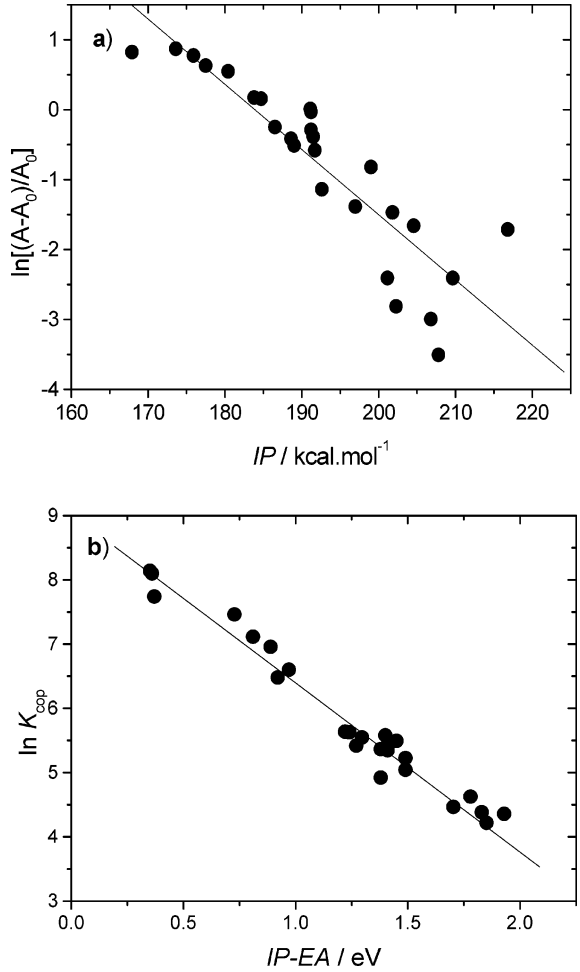


Figure 8. Plots of (a) the natural logarithm of the fractional increase of the absorbance of cyanin, $(A - A_0)/A_{-}$, in aqueous solution at pH 3.32 (Table 3) as a function of the ionization potential IP of 26 copigments ([Cop] = 6 mM) and (b) the natural logarithm of the equilibrium constant, K_{Cop}, of malvin−Cop (Table 4), PLG−Cop (Table 1), MMF−Cop (ref 24), and AH^+−FRA complexes (Table 1), vs the difference between the calculated ionization potential IP of the copigment and anthocyanin electron affinity ***EA***.

$$E(A^-D^+) = IP - EA - C \qquad (10)$$

Equation 10 implies that the lower the copigment IP and the higher the anthocyanin EA, the lower $E(A^-D^+)$ (the energy gap between the CT and ground states), and the more stable the complex with respect to the free flavylium ion. Clearly, then, the existence of a significant contribution of charge-transfer to the stabilization of anthocyanin−copigment complexes should result in systematic correlations of the efficiency of complexation with the ionization potentials and electron affinities of the anthocyanin and copigment.

As shown for the synthetic flavylium cation MMF and the natural anthocyanin PLG with several polyphenols, the complexation constant K_C indeed increases with the decrease in ionization potential of the copigment, pointing to the participation of charge-transfer interactions in the complex. Moreover, the logarithm of K_C, and hence the Gibbs free energy for EDA complex formation, ΔG^0_{Cop}, is a linear function of $IP_{Cop} - EA_{Anthoc}$.[24]

In Figure 8, we show that this trend is apparently quite general. Figure 8a shows that a plot of the logarithm of

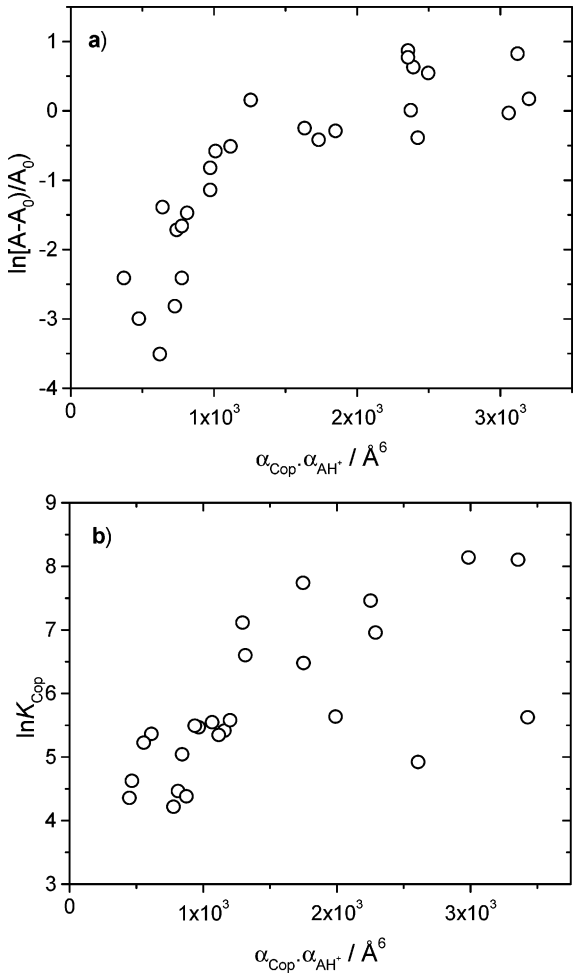


Figure 9. Plots of (a) the natural logarithm of the fractional increase of the absorbance of cyanin, $(A - A_0)/A_0$, in aqueous solution at pH 3.32 (Table 3) vs the product of the polalarizabilities of anthocyanin and copigment (b) the equilibrium constant, K_{Cop}, of malvin−Cop (Table 4), PLG−Cop (Table 1), MMF−Cop (ref 24), and AH^+−FRA complexes (Table 1), vs the product of the polalarizabilities of anthocyanin and copigment.

$(A - A_0)/A_0$ (proportional to K_{Cop}) for cyanin[13] is a linear function of the IE of the copigment, with a slope value similar to that of the MMF and PLG plots. Figure 8b shows that the natural logarithm of K_{Cop} (K_C) for complexation of malvin,[18−22] PLG and MMF by several copigments, as well as the ln K_{Cop} of the anthocyanins in Table 2 with FRA, is a linear function of the difference between the IE of the copigment and the electron affinity EA of the anthocyanin.

Given the large number of potential copigments, any correlation of the copigmentation strength (measured either by K_{Cop} or $(A - A_0)/A_0$) for as wide a range of anthocyanins and copigments as that shown in Figure 8 should also permit estimation of the approximate copigmentation strength for other similar molecules not yet tested. A linear least-squares fit of the data in Figure 8b provides the empirical correlation given by eq 11 (with IP − EA in eV)

$$\ln K_{Cop} = (9.06 \pm 0.11) - (2.68 \pm 0.08)(IE_{Cop} - EA_{Anthoc}) \quad (11)$$

Final Considerations and Conclusion

The model used to interpret the data assumes that charge transfer is the dominant interaction and that it determines the stability of anthocyanin−copigment complexes. Evidently, this may not be true for all mechanisms of copigmentation (e.g., complexation with metals)[37] and does not necessarily exclude the presence of additional contributions from donor−acceptor (e.g., mutual polarizability) interactions, solute−solvent van der Waals' interactions (e.g., hydrophobic interactions)[15] or other electrostatic effects.[38−40] Additional interactions, such as hydrogen bond formation, can also in principle play a role in copigmentation. In the present case, the lack of a correlation between the complexation efficiency (either $(A - A_0)/A_0$ or K_{Cop}) and the mutual polarizability of the anthocyanin and the copigment (Figure 9) rules out the importance of this factor for all but perhaps the poorest electron donor copigments (the hydroxybenzoic acids).

Thus, the central conclusion of the present study is that charge-transfer from the copigment to the anthocyanin indeed makes a significant contribution to the stability of anthocyanin−copigment complexes. Charge-transfer to the anthocyanin has two important consequences for color intensification or copigmentation. First, the positive charge at carbon 2 of the flavylium cation should decrease, resulting in a reduction in the equilibrium constant for hydration. Second, the negative charge at the hydroxyl oxygen(s) should increase, reducing the equilibrium constant(s) for deprotonation.[39,40] The combination of these two effects stabilizes the flavylium cation form of the anthocyanin with respect to both the hemiacetal and the quinonoidal base, respectively. Thus, charge transfer also provides an explanation for the negligible hydration of the flavylium ion in the complexed form CP, as an alternative to the classic explanation based on a reduction in the extent of "exposure of C2 to water".

Although the present results do not rule out the possibility of additional stabilization of the anthocyanin−copigment complex by other factors, they do require that charge transfer be taken into account in any analysis of the copigmentation of anthocyanins by colorless organic molecules.

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