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Ion-pairing co-pigmentation with 4′,7-dihydroxyflavylium studied by pulse light jumps

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Abstract

Pulse light and pH jumps were used to study the structural transformations of the 4′,7-dihydroxyflavylium cation when involved in ion-pairs with the anions chloride, bromide and perchlorate. The association constants of the ion-pairs were calculated by a new approach based on two spectrophotometric titrations: (i) in the absence and, (ii) in the presence of a constant concentration of co-pigment. This method was also used to calculate the ion-pairing association constants of Malvin and the same anions. On the basis of our results, the co-pigmentation model based on the suppression of the hydration reaction in the flavylium complex is questioned.

Keywords: 4′7-dihydroxyflavylium; Co-pigmentation; Ion-pairing

1. Introduction

Anthocyanins are very important and abundant natural products, responsible for most of the colors of flowers and fruits [1–3]. The structural modifications that appear upon dissolution of anthocyanins in acidic aqueous solution, were elegantly described by Brouillard and Dubois and Brouillard and Delaporte [4,5]. In acidic aqueous solutions, the most common anthocyanins, as for example Malvin, are involved in several equilibria that give rise to the formation of three colored forms, the red flavylium cation AH^+, the blue quinonoidal base A and the pale yellow chalcones C (*cis* and *trans*). These forms are in equilibrium with the two (S and R) colorless hemiacetals B [6], as depicted in Scheme 1.

The role played in vivo by anthocyanins as agents of plant coloring, was subject to much attention. Taking Malvin as an example, in vitro, the red flavylium cation is the dominant form only in extreme acidic conditions, (pH = 1–3). Moreover, as pH becomes less acidic, the prevalent form is the colorless hemiacetal, the other colored forms being present only in small amounts. These facts exclude anthocyanins as the sole responsible for the in vivo color, and point out their participation in some type of supramolecular structure involving other natural compounds.

The increase of the color can be achieved through the simple association of anthocyanins with other polyphenols or flavonoids, which by themselves are colorless. This process is denominated co-pigmentation [7–12], and it has been used with success to stabilize the red flavylium cation or in a few cases, the blue quinonoidal base. This type of complexation is probably dominated by van der Walls forces, but the role played by electrostatic effects is not yet clear [13]. Previous work on the formation of ion-pairs between the flavylium cation of Malvin and simple anions, such as chloride, and perchlorate [14], shows that these effects are necessarily present whenever charges are involved.

In this work we study some ion-pairing co-pigmentation effects with 4′,7-dihydroxyflavylium and Malvidin 3,5-diglucoside (Malvin) at the equilibrium. The use of a simple methodology to calculate the ion-pair association constants is also described. In addition pulse light and pH jumps were used in order to compare the rate constants in the presence and absence of the anions.

2. Experimental

Malvin was purchased from Extrasynthèse, and used as received after purity verification by HPLC. The synthetic 4′,7-dihydroxyflavylium chloride salt was prepared according to the literature [15]. All other chemicals were of analytical grade. The pH was adjusted by the addition of appropriate volumes of acid ($HClO_4$) or base (NaOH), and

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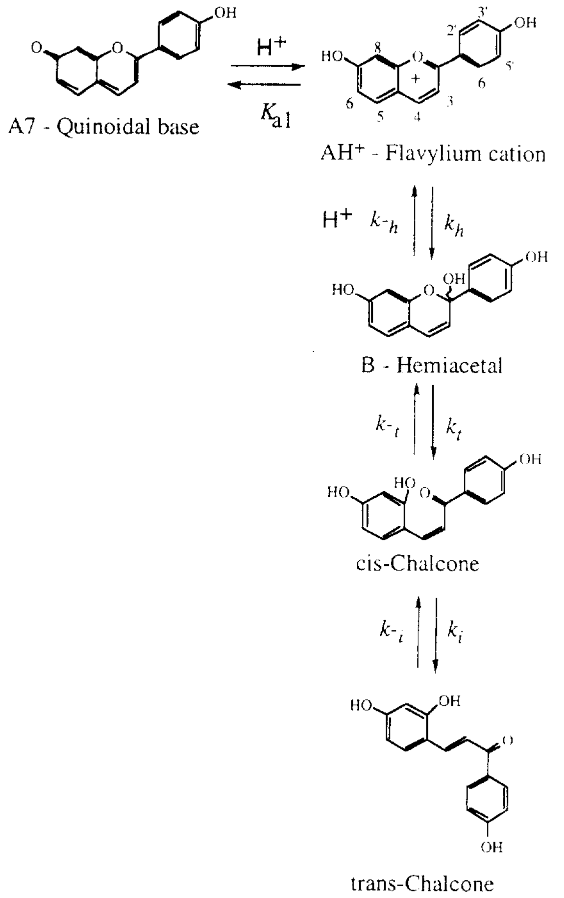

Scheme 1.

measured with a Metrohm 713 pH meter. For pH values lower than pH = 1.0, the analytical concentration of the added acid was used. Absorption spectra were measured on a Perkin-Elmer Lambda 6 spectrophotometer. A constant temperature of 25°C was maintained with a Haake thermostated bath. Pulse light experiments were carried out as described previously [16–18].

3. Results and discussion

3.1. Ion-pairing co-pigmentation with simple anions

In Fig. 1 the pH variation of the normalized[1] absorption of the compound 4′,7-dihydroxyflavylium, ($2\cdot10^{-5}$ M dark equilibrated), measured at 458 nm in the presence of large excess (2 M) of the anions chloride, bromide and perchlorate is represented. The shape of the absorption spectrum of flavylium cation is only very slightly modified by the presence of the anion (not shown), but the apparent pK_a is extended to high pH values.

In the case of Malvin the behavior is identical and very similar curves are obtained.

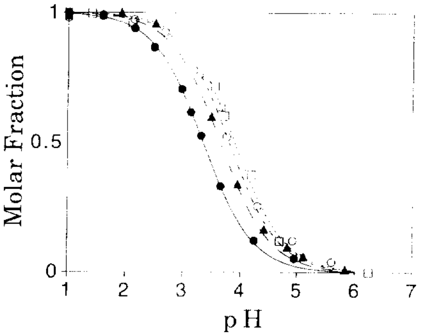

Fig. 1. pH dependence of the absorbance at 458 nm of the salt 4′,7-dihydroxyflavylium, $3\cdot10^{-5}$ M, in the absence and presence of large excess of anions (2 M). The absorbance was normalized according to Eq. (10): (●) in the absence of anion; (▲) chloride, (○) bromide, (□) perchlorate.

In order to rationalize the data of Fig. 1, several equilibria in which anthocyanis and synthetic flavylium salts are involved must be considered [4,5], Eqs. (1)–(4).

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \qquad (1)$$

$$AH^+ \overset{K_h}{\rightleftharpoons} B + H^+ \qquad (2)$$

$$B \overset{K_t}{\rightleftharpoons} C_{cis} \qquad (3)$$

$$C_{cis} \overset{K_i}{\rightleftharpoons} C_{trans} \qquad (4)$$

In this scheme AH^+, A, B and C represent the flavylium cation, quinoidal base, hemiacetal and chalcone forms respectively. The two hemiacetal enantiomers [6] are represented by the symbol B. When co-pigmentation with flavylium cation occurs, Eq. (5) has been added.

$$AH^+ + nX^- \overset{K_{cp}}{\rightleftharpoons} AHX_n^{1-n} \qquad (5)$$

The classical method to calculate association constant of the type of Eq. (5) is the *Benesi–Hildebrand* equation [19]

$$\frac{[X]}{\varepsilon_{obs}-\varepsilon_0} = \frac{1}{\Delta\varepsilon K_{cp}} + \frac{[X]}{\Delta\varepsilon} \qquad (6)$$

where $\varepsilon_{obs} = A/C_0$ is the ratio of the measured absorbance divided by the concentration of the anthocyanin species (which is maintained constant), ε_0 the molar absorption coefficient of the free anthocyanin, $\Delta\varepsilon$ the difference between the molar absorption coefficients of the free anthocyanin and its adduct, K_{cp} the association constant and [X] the variable concentration of the co-pigment (in large excess). The use of this equation assumes that the ionic strength is maintained constant by addition of the so-called 'innocent anions' [20,21] typically of the same sort as those we are reporting in Fig. 1. Therefore we are confronted in this work with the lack of 'innocent ions'. Unless we neglect the control of the ionic strength, the Benesi–Hildebrand equation is of limited usefulness, see below for more details.

Another ingenious mathematical model very useful to calculate association constants involving flavylium cation, was describe by Brouillard et al. [7], Eq. (7).

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[1] According to Eq. (10), see below.

$$\ln\frac{A-A_0}{A_0}=\ln\left(\frac{K_{\mathrm{cp}}\varepsilon_{\mathrm{cp}}}{\varepsilon_{\mathrm{AH^+}}}\right)+n[\mathrm{X}] \qquad (7)$$

In this equation, A is the absorbance upon addition of the co-pigment of concentration $[\mathrm{X}]$, A_0 the absorbance in the absence of co-pigment, $\varepsilon_{\mathrm{cp}}$ and $\varepsilon_{\mathrm{AH^+}}$ are respectively the molar absorption coefficients for the adduct and flavylium cation, and n the number of co-pigment molecules in the flavylium cation adduct. In this equation the stoichiometric number n, can be directly obtained from the slope of the linear relation (Eq. (7)), but calculation of K_{cp} implies previous knowledge of the molar absorption coefficients $\varepsilon_{\mathrm{AH^+}}$ and $\varepsilon_{\mathrm{cp}}$. While the first is easily calculated in the absence of co-pigment, to obtain the second it is necessary to consider that all the anthocyanin is involved on the adduct. When the association constant is very low (as in our case) or the co-pigment is not sufficiently soluble in water, this last assumption is not valid and consequently $\varepsilon_{\mathrm{cp}}$ is not available.

3.1.1. A method to calculate low association constants. Exclusive co-pigmentation with flavylium cation

The limitations of the methods described above led us to develop an alternative approach. In this method we carried out two spectrophotometric titrations as follows: one in the absence of co-pigment, and the other in the presence of an excess and constant concentration of co-pigment.

Considering the first titration, as shown previously [1,2,22] the molar fraction of the flavylium cation species can be obtained from Eqs. (8) and (9)

$$\frac{[\mathrm{AH^+}]}{C_0}=\alpha'=\frac{[\mathrm{H^+}]}{[\mathrm{H^+}]+K_{\mathrm{a}}'} \qquad (8)$$

$$K_{\mathrm{a}}'=K_{\mathrm{a}}+K_{\mathrm{h}}+K_{\mathrm{h}}K_{\mathrm{t}}+K_{\mathrm{h}}K_{\mathrm{t}}K_{\mathrm{i}} \qquad (9)$$

This result is very important because Eq. (8) is formally equivalent to an acid-base equilibrium of a single species. In other words, the molar fraction of the flavylium cation present in the complex equilibria of anthocyanins, can be mathematically treated as one single equilibrium, and its shape as a function of pH is the well known sigmoid curve as depicted in Fig. 1.

The experimental spectrophotometric determination of the function α', measured at any wavelength, can be obtained by a simple expression, Eq. (10)

$$\alpha'=\frac{\dfrac{A'}{A_0'}-Ct}{1-Ct} \qquad (10)$$

where A' is the pH dependent absorbance at the chosen wavelength, A_0' the absorbance at a sufficiently acidic pH value to consider the flavylium cation as the sole species present in solution, and the factor Ct is a constant which is experimentally determined, as the ratio $A':A_0'$, at a pH value sufficiently high to consider $\alpha'=0$.

The spectrophotometric method is thus very useful to calculate K_{a}' by simply fitting Eq. (8) to the experimental α' function. We have used this procedure to calculate the values reported in Table 1, (data in the absence of anions).

Table 1
Ion-pair co-pigmentation parameters of Malvin and the synthetic 4′,7-dihydroxyflavylium

$\mathrm{p}K'_{\mathrm{a}}(K_{\mathrm{cp}})$				
	–	Cl	Br	$\mathrm{ClO_4}$
Malvin	1.5	2.0 (1.1)	2.15 (1.7)	2.0 (1.1)
4′,7-dihydroxyflavylium	3.05	3.6 (1.7)	3.75 (2.0)	3.8 (2.4)

Considering now the second titration, and introducing the parameter K_{ap}, Eq. (11),

$$K_{\mathrm{ap}}=\frac{K_{\mathrm{a}}'}{1+K_{\mathrm{cp}}[\mathrm{X}]^n} \qquad (11)$$

the molar fraction of flavylium cation and its adduct can be written as

$$\frac{[\mathrm{AH^+}]}{C_0}=\frac{1}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}} \qquad (12)$$

$$\frac{[\mathrm{AHX}_n{}^{m}]}{C_0}=\frac{K_{\mathrm{cp}}[\mathrm{X}]^n}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}} \qquad (13)$$

These expressions are constituted by a product of a pre-factor, depending on the association parameters, and once more an α function as defined by Eq. (8), where now K_{a}' is substituted by K_{ap}. Since we are working with a large excess and constant concentration of co-pigment, the pre-factor multiplying the α function, as well as K_{ap}, are constants. It is easy to demonstrate that determination of the apparent α function (α_{ap}) can also be calculated by Eq. (10), see Appendix A. In this case the absorbance of the solution is divided, as before, by the normalizing factor obtained from a solution at sufficiently acidic pH to have exclusively the mixture of both species $\mathrm{AH^+}$ and $\mathrm{AHX}_n{}^+$. The constant Ct is taken as the ratio A/A_0' at high pH values. Fitting of the experimental α_{ap} through Eq. (8) leads to K_{ap}. Our final objective, (calculation of the association constant), only needs the knowledge of the stoichiometric number n, and Brouillard's equation, Eq. (2), can be used for this purpose[2]. However in the present case, ion-pairs of order higher than 1 can be discharged, because the first adduct is already neutral.

We applied this treatment to the data of Fig. 1, and the respective values of the association constants are reported in Table 1.

The values of these constants show that it is not possible to reach a situation where all flavylium cation can be considered associated to the anion (which would require concentrations much higher than the solubility limit of these anions).

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[2] This can be done because in Brouillard's expression, determination of n is directly obtained from the equation, and the knowledge of $\varepsilon_{\mathrm{cp}}$ is not necessary.

This clearly shows that Brouillard's equation, or any other method needing direct calculation of ε_{cp}, cannot be used in the case of ion-pairing. At this point the present method and the Benesi–Hildebrand equation are the alternatives. In both methods the condition $K_{cp}[X] > 1$ must be observed, otherwise the sensibility is very low, as can be proved by inspection of Eq. (11), and reported in Ref. [17] for the Benesi–Hildebrand equation. However, in our method all measurements can be done in the best conditions, at the highest concentration of anion, while in the Benesi–Hildebrand equation the concentration of X must comprise lower values. This implies less accuracy in the first points of the linear relation, resulting in a loss of sensibility. In the present treatment the measurement of K_{ap} is carried out at constant ionic strength, which is maintained by the constant concentration of the anion. This is particularly useful considering that the ionic strength in the other methods is kept constant by addition of the so-called 'innocent ions', precisely of the same sort as those that we are describing in this work.

The weakness of the present method is the calculation of K_a', which was obtained at low ionic strength. In practice we can measure K_a' at very low, but controlled ionic strength, for example 0.05 M of anion. At this ionic strength the ion-pair can be neglected. However we are always comparing K_a' and K_{ap} calculated at different ionic strengths. Corrections can be made but are discussible for values of 2 M or even 1 M. An alternative is to decrease the concentration of the anion down to values for which the activity coefficients can be calculated, but this procedure implies a loss of sensibility. We have chosen in this work to present the constants obtained at a concentration of anion equal to 2 M, with K_a' calculated without any correction for the ionic strength. Unfortunately the problems with the control of the ionic strength using the Benesi–Hildebrand equation are even more drastic. For example to observe an ion pair with chloride, the following strategy could be used [17]: the concentration of chloride changes from 0 to 2 M (or other) and is compensated by addition of the 'innocent' salt to maintain the sum of chloride plus the 'innocent' salt equal to 2 M. The problem is to find an 'innocent' salt without interfering when the concentration of chloride is very low.

As a consequence of the inherent errors associated with the measurements of the ion-pairing constants of Table 1, we did not explore possible explanations for their relative trend.

3.2. Complexation vs. hydration protection effect

One possible interpretation for the existence of co-pigmentation effects with AH^+ is the suppression of the hydration reaction when flavylium cation is involved in the complex [7]. As a consequence of this assumption the kinetical scheme used to account for the co-pigmentation phenomenon contains exclusively Eqs. (1)–(5). In the case of ion-pairing co-pigmentation, the presence of the anions surrounding the carbon 2 of the flavylium structure, could difficult more or less the hydration reaction. However it seems difficult to accept a complete hindrance. On this basis there is no experimental evidence to exclude a mechanism of hydration through the flavylium adduct, as represented by Eq. (14).

$$\mathrm{AHX}_n^{(1-n)+} \overset{K_h'}{\rightleftharpoons} \mathrm{B}(+n\mathrm{X}^-)+\mathrm{H}^+ \tag{14}$$

Eq. (14) accounts for a new channel of hydration in which the process is (in principle) more difficult. Using Eq. (14) the apparent pK_{ap} can be rewritten as shown by Eq. (15)

$$K_{ap}=\frac{K_a'+(K_h'-K_h)\gamma}{1+K_{cp}[X]^n}=\frac{K_a'}{1+K_{cp}[X]^n}+\frac{(K_h'-K_h)\gamma}{\{1+k_{cp}[X]^n\}} \tag{15}$$

In this equation γ is the fraction of flavylium cation that is complexed. The first term of Eq. (15) is due exclusively to the complexation, (as previously reported) and the second accounts for the new channel of hydration. Inspection of Eq. (15) shows that in the case of neglecting the existence of this new channel the calculated association constant is lower than the real value. For this reason the association constants reported in Table 1 must be taken as the lower limit of the ion-pairing co-pigmentation.

3.3. Pulse light and pH jumps

Recently we proved [17] that in the case of the compound 4′,7-dihydroxyflavylium pulse light together with pH jump techniques can give information on the equilibrium constants of the hydration (K_h) and isomerization (K_i) reactions, as well as on their respective rate constants. We carried out these types of experiment in the presence of anions. The results are represented in Fig. 2 for chloride (2 M) and can be interpreted by means of Scheme 2 [17]. From the observed rate constant of the process that occurs upon a pH jump from 1 to ca. $2.5 > \mathrm{pH} > 6.5$ it is possible to calculate [17] the rate constants k_h and k_{-i} and from the pulse light experiments the rate constants k_{-h} and k_i are also available. This permits obtaining both equilibrium constants allowing the construction of an energy level diagram as shown in Scheme 2. Comparing the results in the presence and absence of anions we observed only very small variations on the four rate constants, which are within the limit of our experimental accuracy. In other words we can only exclude variations larger than ca. 20%. However the assumption that co-pigmentation prevents the hydration reaction (and why not the inverse process, the dehydration?) seems not to be supported by our results. A possible explanation for the co-pigmentation effects observed in Fig. 1 can be proposed on the basis of Scheme 2. The ion-pairing co-pigmentation simply decreases the relative energy of the adduct with flavylium, allowing the observation of the red color at more basic pH values. This lowering of the energy level of flavylium cation (in the ion pair) will also have slight influence on the kinetics, because in principle the activation

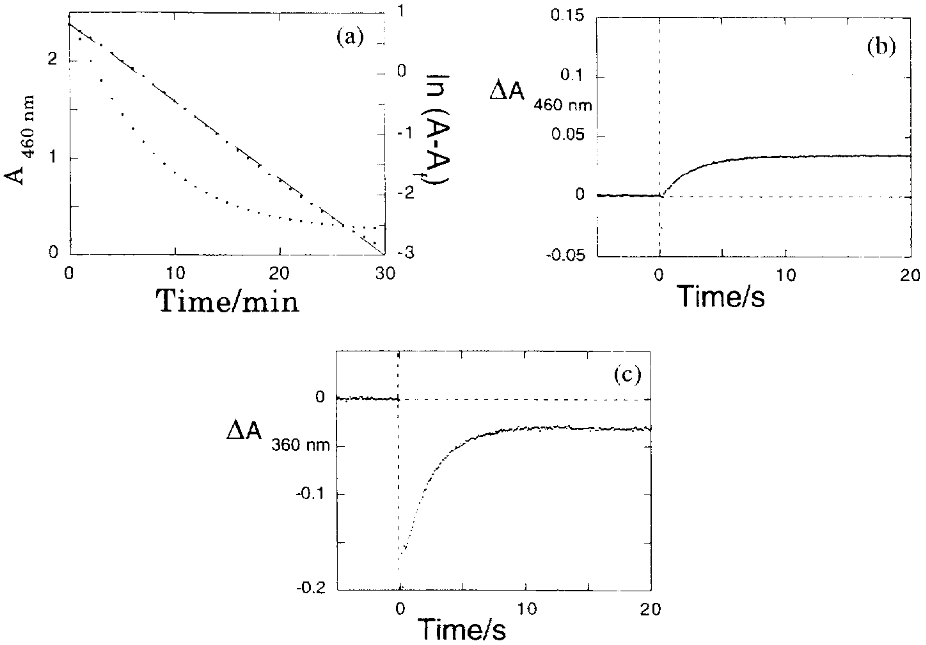
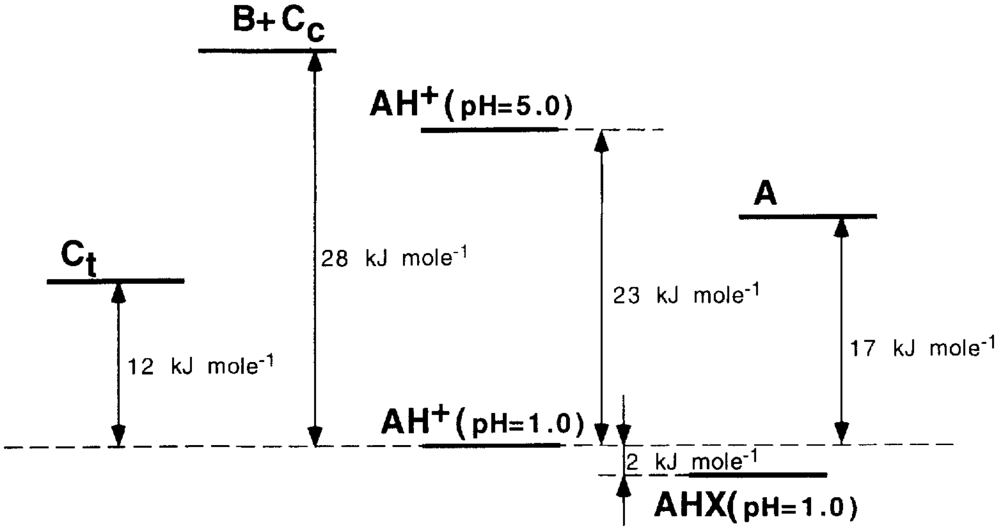


Fig. 2. (a) Variation of the absorbance at 460 nm of the compound 4′,7-dihydroxyflavylium in the presence of NaCl 2 M upon a pH jump from 1 to 4.9. The process follows a first order kinetic law with the constant equal to $2.1 \cdot 10^{-3}$ s^{-1}. (b) Pulse light jump of 4′,7-dihydroxyflavylium in the presence of NaCl 2 M at pH = 5.6 followed at 460 nm. Observed lifetime 0.36 s^{-1}. (c) The same as in (b) followed at 360 nm.



Scheme 2.

energy for the hydration reaction increases and consequently the rate of hydration reaction decreases. In other words the greater the association constant (the lower the energy level of the adduct), more activation energy is expected for the hydration reaction. This fact could also explain why we did not observe any significant modification on the hydration reaction in the presence of anions. The association constants involved are very low and the decreasing of the energy level of flavylium cation in the ion-pair is less than 2 kJ M^{-1}. This value is enough to shift the molar fraction distribution of flavylium cation and its ion-pair, (Fig. 1), but not to influence significantly the activation energy of the hydration reaction.

4. Conclusions

The existence of ion-pairing in the co-pigmentation phenomenon has been generally neglected. However these effects can be important when measuring the association constants at high ionic strengths. In this work we have also questioned the assumption that the flavylium adduct is completely protected from the hydration reaction, which is an hypothesis of the co-pigmentation models used in literature. In effect, the experimental results of the ion-pairing co-pigmentation with 4′,7-dihydroxyflavylium indicate that, in spite of the formation of the ion-pairs, the hydration reaction not only still occurs, but also its rate constant is not significantly affected. Can this behavior be extended to natural anthocyanins and stronger co-pigmentation effects? This is the subject of work in progress.

Acknowledgements

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Appendix A.

From the total concentration given by Eq. (a1)

$$C_0=[\mathrm{AH}^+]+[\mathrm{A}]+[\mathrm{B}]+[\mathrm{C}_{cis}]+[\mathrm{C}_{trans}]+[\mathrm{AHX}_n^+] \qquad (\mathrm{a1})$$

and using Eq. (11), the molar fractions of the several species present in solution in the presence of co-pigment are given by Eq. (a2)Eqs. (a3) and (a4)Eqs. (a5) and (a6)Eqs. (a7) and (a8), as long as co-pigment is used in excess relative to anthocyanin (or synthetic flavylium) concentration.

$$\frac{[\mathrm{AH}^+]}{C_0}=\frac{1}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}} \qquad (\mathrm{a2})$$

$$\frac{[\mathrm{AHX}_n^+]}{C_0}=\frac{K_{\mathrm{cp}}[\mathrm{X}]^n}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}} \qquad (\mathrm{a3})$$

$$\frac{[\mathrm{A}]}{C_0}=\frac{K_{\mathrm{a}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)}\cdot\beta_{\mathrm{ap}} \qquad (\mathrm{a4})$$

$$\frac{[\mathrm{B}]}{C_0}=\frac{K_{\mathrm{h}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)}\cdot\beta_{\mathrm{ap}} \qquad (\mathrm{a5})$$

$$\frac{[\mathrm{C}_{cis}]}{C_0}=\frac{K_{\mathrm{h}}K_{\mathrm{t}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)}\cdot\beta_{\mathrm{ap}} \qquad (\mathrm{a6})$$

$$\frac{[\mathrm{C}_{trans}]}{C_0}=\frac{K_{\mathrm{h}}K_{\mathrm{t}}K_{\mathrm{i}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)}\cdot\beta_{\mathrm{ap}} \qquad (\mathrm{a7})$$

$$\beta_{\mathrm{ap}}=1-\alpha_{\mathrm{ap}} \qquad (\mathrm{a8})$$

The absorbance at any wavelength where only the colored species (AH^+ $\mathrm{AHX}_n{}^+$ and A) absorb, is given by Eq. (a9)

$$A=\{\varepsilon_{\mathrm{AH}^+}[\mathrm{AH}^+]+\varepsilon_{\mathrm{AHX}_n}[\mathrm{AHX}_n^+]+\varepsilon_{\mathrm{A}}[\mathrm{A}]\}C_0 \qquad (\mathrm{a9})$$

which can be rewritten as Eq. (a10)

$$\frac{A}{C_0}=\frac{\varepsilon_{\mathrm{AH}^+}}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}}+\frac{\varepsilon_{\mathrm{AHX}_n}K_{\mathrm{cp}}[\mathrm{X}]^n}{1+K_{\mathrm{cp}}[\mathrm{X}]^n}\cdot\alpha_{\mathrm{ap}}+\frac{\varepsilon_{\mathrm{A}}K_{\mathrm{a}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)}\cdot\beta_{\mathrm{ap}} \qquad (\mathrm{a10})$$

or in a more simple notation as Eq. (a11)

$$A=\{C_1\alpha_{\mathrm{ap}}+C_2\beta_{\mathrm{ap}}\}C_0 \qquad (\mathrm{a11})$$

with

$$C_1=\frac{\varepsilon_{\mathrm{AH}^+}+\varepsilon_{\mathrm{AHX}_n}K_{\mathrm{cp}}[\mathrm{X}]^n}{1+K_{\mathrm{cp}}[\mathrm{X}]^n} \qquad (\mathrm{a12})$$

$$C_2=\frac{\varepsilon_{\mathrm{A}}K_{\mathrm{a}}}{K_{\mathrm{ap}}(1+K_{\mathrm{cp}}[\mathrm{X}]^n)} \qquad (\mathrm{a13})$$

From this point it is immediate that Eq. (a11) can be converted into Eq. (10).

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