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Caffeine interaction with synthetic flavylium salts. A flash photolysis study for the adduct involving 4′,7-dihydroxyflavylium

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Abstract

The adducts formed between caffeine and three hydroxyflavylium salts (4-methyl-7-hydroxyflavylium, 4′-hydroxyflavylium, and 4′,7-dihydroxyflavylium) are described and the last studied by flash photolysis. A consistent evidence of the caffeine effect on the rate constants of this system is given for the first time. A net hyperchromic effect was only observed for the adduct involving 4′-hydroxyflavylium. The results confirm previous observations that co-pigmentation is essentially a phenomenon that changes the molar fraction distribution of the several species in equilibrium, and not a result of the formation of highly colored charge transfer bands. A simple mathematical model is used to account for the experimental results.

Keywords: Adducts; Caffeine; 4′,7-Dihydroxyflavylium

1. Introduction

Synthetic flavylium salts have been used as model compounds for anthocyanins [1–3] which are one of the most important sources of color in flowers and fruits [4,5]. Both families of compounds, in neutral and acidic aqueous solutions, undergo structural transformations that are dependent on pH, according to a common general kinetical scheme, here represented for 4′,7-dihydroxyflavylium [1–7] (Scheme 1).

One of the characteristics of synthetic flavylium salts is their versatility, because depending on the different substituents, the kinetical and thermodynamical properties can change dramatically [2–5]. In addition, and in contrast with anthocyanins, some of these compounds are easy to synthesize and purify.

It has been firmly established that anthocyanins by themselves can not account for the color observed in plants [4,5]. In natural systems, anthocyanins are located inside of the vacuoles (pH ca. 3.5–6) [8]. However, in aqueous solutions at these pH values, the colorless form, hemiacetal, is the dominant species. The red colored form, flavylium cation, is only stable in very acidic solutions, while the blue colored species, quinoidal base, is present at moderate acidic solutions, but in very low molar fraction. One of the interpretations for the existence of color in natural systems is the formation of adducts [4,5] with other natural products (generally not colored), the so-called co-pigments [9–18] [19,20].

Recently, we have shown that synthetic flavylium salts present very interesting photochromic properties and constitute an attractive model to a new class of nano-memory devices for computers [21–26]. In this work, we present the interactions of caffeine with three synthetic flavylium salts, 4-methyl-7-hydroxyflavylium, 4′-hydroxyflavylium, and 4′,7-dihydroxyflavylium, the last adduct being studied by flash photolysis following a procedure recently described [25,26] (Scheme 2).

2. Experimental

2.1. Materials

The synthetic flavylium salts were prepared according to the published procedure [27,28]. All other chemicals used were of analytical grade.

2.2. pH measurements

The pH was measured with a Metrohm 713 pH meter. The pH of the solutions was adjusted by addition of $HClO_4$ ($pH < 2$) or buffer solutions for higher pH values. In this case, the concentration of the buffer was maintained lower

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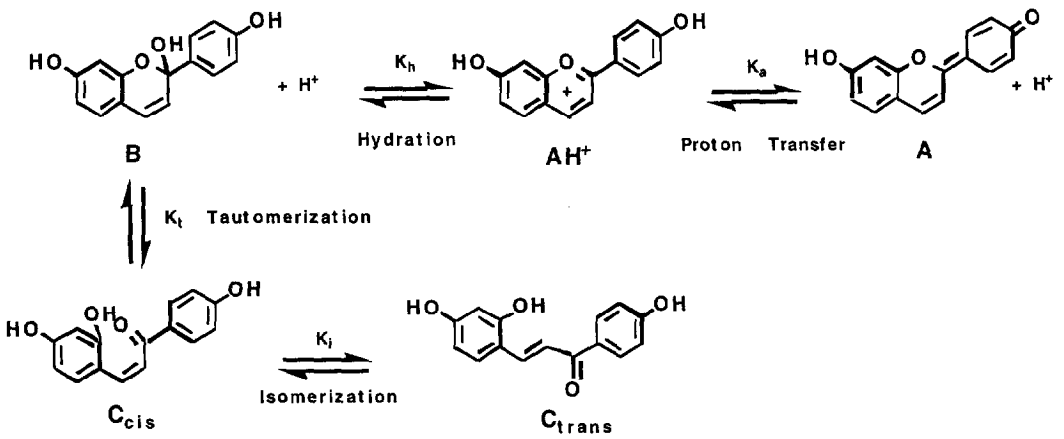
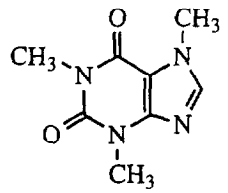


Scheme 1.



Caffeine

Scheme 2.

than 0.01 M in order to avoid buffer effects. Where pH values lower than 1 were used, the negative logarithm of the analytical concentration of the added acid was used.

2.3. Absorption spectroscopy

Spectra were recorded on a Perkin-Elmer lambda 6 spectrophotometer. A constant temperature of 25°C in the quartz cell ($d = 1$ cm) was obtained by use of a Haake thermostated water bath.

2.4. Flash photolysis and photochemical experiments

The flash photolysis experiments were performed as described elsewhere [25,26]. Light excitation was carried out using a medium pressure mercury arc lamp; the irradiation wavelengths were isolated with interference filters (Oriel). The incident light intensity was measured by ferrioxalate actinometry [29].

3. Results and discussion

3.1. Thermal reactions

3.1.1. 4-Methyl, 7-hydroxy flavylium. Exclusive proton transfer

The rather complex system of equilibria depicted in Scheme 1 is very simplified in the case of the compound 4-methyl, 7-hydroxyflavylium, because as reported previously in literature [3], and recently confirmed by ^{1}H NMR studies [30], only two species are present in solution, the flavylium cation (AH^+ absorption maximum at 417 nm) and the quinoidal base (A absorption maximum at 465 nm) (see Fig. 1).

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \qquad pK_a = 4.4 \qquad (1)$$

In Fig. 2, the effect of the addition of Caffeine to this compound is shown.

According to this figure, both forms of 4-methyl, 7-hydroxyflavylium interact with caffeine, but the spectral variations induced by the presence of this co-pigment are not only very small, but also reduce slightly the coloring capacity of the compound. The stoichiometry of the adducts are difficult to determine due to the small spectral variations involved in the co-pigmentation effect. However, it seems that for concentrations of caffeine lower than 0.01 M, a set of isosbestic points are formed, while for greater concentrations of Caffeine a second set of isosbestic points appears. This fact can

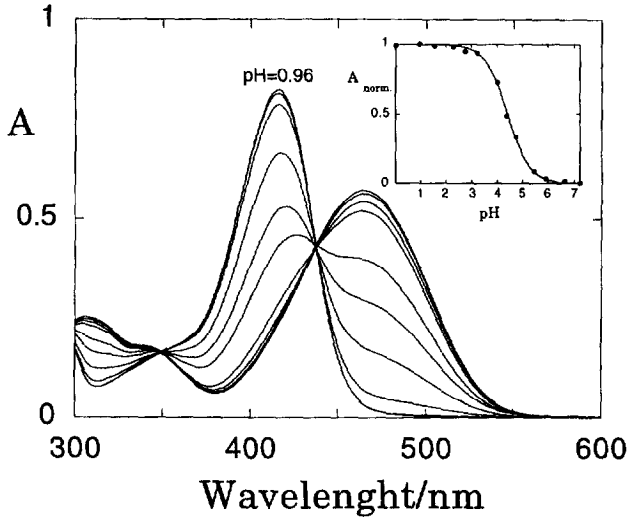


Fig. 1. Spectral variations of the compound 4-methyl-7-hydroxyflavylium as a function of pH: 0.96; 2.28; 3.22; 4.0; 4.36; 4.73; 5.46; 5.92; 6.65; 7.23. Inset—Determination of the pK_a by a fitting of Eq. (3) to Eq. (2), at 417 nm.

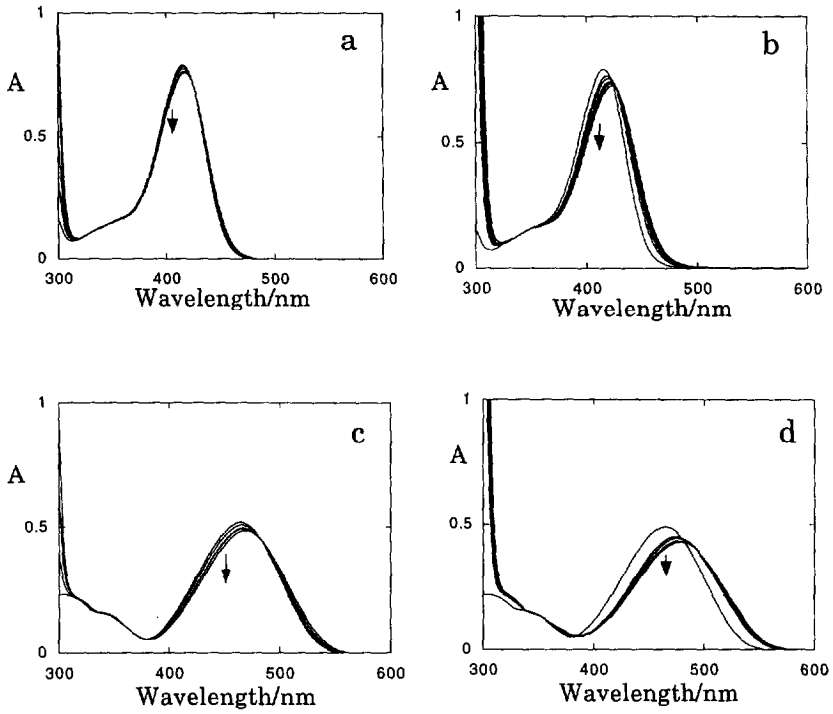


Fig. 2. Spectral variations of the compound 4-methyl-7-hydroxyflavylium as a function of the concentration of added Caffeine. (a) pH = 1.5, 0; 0.0013; 0.0032; 0.0057; 0.0091 M. Isosbestic point at 424 nm. (b) pH = 1.5, 0; 0.02; 0.03; 0.04; 0.05; 0.06; 0.08 M. Isosbestic point at 427 nm. (c) pH = 6.0, 0; 0.0013; 0.0032; 0.0057; 0.0091 M. Isosbestic point at 483 nm. (d) pH = 6.0. 0; 0.02; 0.03; 0.04; 0.06 coincident with 0.08 M. Isosbestic point at 492 nm.

lead to the hypothesis that caffeine can form two different adducts with flavylium cation (and quinoidal base) probably with 1:1 and 1:2 stoichiometry.

In order to quantify the experimental results, we are considering, for simplicity, the situation in which the concentration of caffeine is lower than 0.01 M, and without loss of generality the stoichiometry equal to 1:1 (Eqs. (2) and (3)).

$$AH^+ + X \overset{K_{cp}^{I}}{\rightleftharpoons} AXH^+ \quad (2)$$

$$A + X \overset{K_{cp}^{II}}{\rightleftharpoons} AX \quad (3)$$

As is shown in Appendix A, in the presence of large and constant excess of co-pigment, the system still behaves as a single acid–base equilibrium in which the acidity constant is substituted by K_{ap} Eq. (4), which can be determined by a fitting procedure [24,30].

$$K_{ap} = \frac{K_a + K_a K_{cp}^{II}[X]}{1 + K_{cp}^{I}[X]} \quad (4)$$

Calculation of pK_{ap} for a constant concentration of caffeine 0.01 M, gives a value of 4.15 ± 0.05, that compares with 4.40 ± 0.05 in the absence of co-pigment. This shift immediately indicates that the association constant of the co-pigment with quinoidal base is larger than with flavylium cation; otherwise, we would not observe an increase on the apparent acidity constant (see Eq. (4)). The lower limit of the association constant of the caffeine–quinoidal base adduct can be obtained from Eq. (4) (considering $K_{cp} = 0$), $K_{cp}' = 78\ M^{-1}$, but actually will be larger. Attempts to calculate the association constants of the caffeine adducts with flavylium (pH = 1.0) and quinoidal base (pH = 6.0), using the Benesi–Hildebrand equation [31] were not conclusive due to the excess of error, motivated by the very small spectral variations involved.

In conclusion, the ground state interaction of caffeine with 4-methyl, 7-hydroxyflavylium is characterized by changes on the molar fraction distribution of the species, together with small variations on the molar absorption coefficient of the adducts. The most stable of these two adducts is the one obtained with quinoidal base. No co-pigmentation effect characterized by a hyperchromic effect was observed. These results are compatible with formation of weak interactions probably resulting from van der Waals type forces explaining not only the weak constants but also the lack of large modification in the absorption spectra.

3.1.2. 4′-Hydroxyflavylium

In the case of this compound, all the five forms AH^+, A, B, C_{cis} and C_{trans} can be observed in solution. However due to the particularities of its kinetics it is possible to work in a time scale where C_{trans} does not interfere, or in alternative, wait for the final equilibrium and carry out the experiments in the presence of almost exclusively C_{trans}. In effect, the structural changes observed in this compound upon a pH jump from 1 to the neutral and moderately acidic region are constituted by three steps. In a very rapid step, quinoidal base (A) is formed at expenses of flavylium cation, (AH^+). In a more slow step depending on the final pH an equilibrium between the forms AH^+, A, B and C_{cis} is reached. In this step

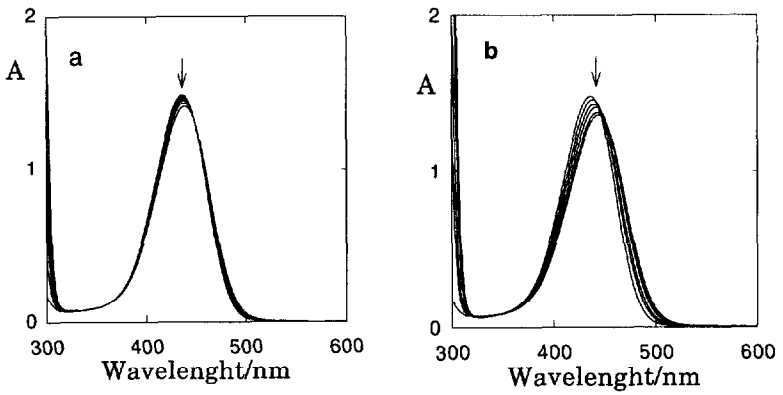


Fig. 3. Spectral variations of the compound 4-hydroxyflavylium as a function of the concentration of added caffeine, pH = 1. (a) 0; 0.0016; 0.0032; 0.0049; 0.0066; 0.098; 0.016 M. Isosbestic point at 440 nm. (b) 0.01; 0.02; 0.04; 0.06; 0.08 M. Isosbestic point at 450 nm.

at pH ca. 6–8, practically no flavylium is present in solution and approximately 40% A, 20% B and 20% C_{cis} are in equilibrium [1,31]. In a much more slow process, these species are transformed into the thermodynamically stable form, the *trans*-chalcone, in a conversion ratio more than 99%. Due to the large interval of time needed to form *trans*-chalcone, it is possible to study the interaction of caffeine with the species A, B and C_{cis}, without interference of this species.

3.1.3. Interaction with Caffeine during the intermediate state

As we mentioned above, at pH = 1, AH^+ is the sole species present in solution and at pH = 6–8 the species A, B, C_{cis} are in equilibrium. In Figs. 3 and 4, the interaction of Caffeine at pH = 1 and 7.4, respectively, obtained in a time scale where the interference of the species C_{trans} can be neglected, are shown. Similar to what was observed for the previous compound, in this case there is also a small interaction between the AH^+ species of 4′-hydroxyflavylium and caffeine (Fig. 3).

As in the previous example, the isosbestic points are not maintained, as long as the concentration of Caffeine increases. The most interesting behavior is however at pH = 7.4. At this pH value, the interaction with quinoidal base and caffeine gives rise to a hyperchromic effect, and a slight red shift of the absorption band maximum, a typical co-pigmentation effect as observed for natural anthocyanins (Fig. 4a).

In principle, this increasing of absorbance can be due to an increase in the molar absorption coefficient and/or to a shift of the equilibrium towards quinoidal base at the expense of B and C_{cis}. In general, the increase in the molar absorption coefficient is obtained when a charge transfer occurs. This type of interaction generally gives rise to a completely new absorption band, which is not the present case. Our results are more compatible with an increase in the molar fraction distribution of the total quinoidal base (free plus complexed), and as in the previous interactions, the adduct probably does not change appreciably its molar absorption coefficient. According to Appendix B, it is straightforwardly demonstrated that whenever the molar absorption coefficient of the adduct and free species are identical,[1] Eq. (5) is observed.

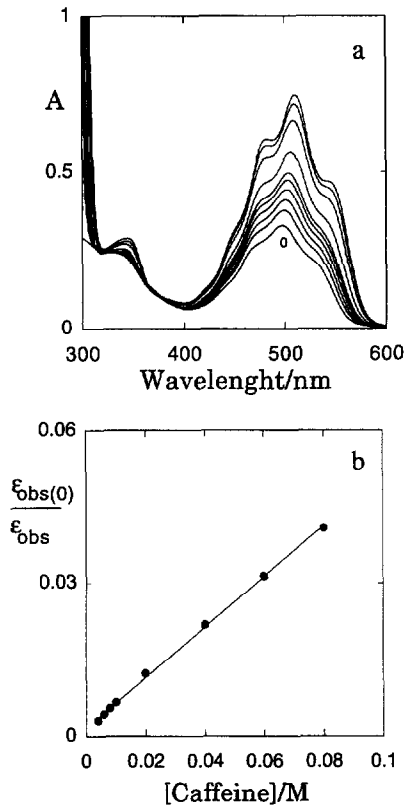


Fig. 4. (a) Spectral variations of the compound 4-hydroxyflavylium 4.3×10^{-5} M at pH = 7.4 in the presence of several concentrations of caffeine: 0; 0.001; 0.002; 0.004; 0.006; 0.008; 0.02; 0.04; 0.06; 0.08 M. (b) Eq. (a21) followed at 498 nm. The best fitting was achieved for $K_{cp}' = 550\ M^{-1}$ and $(K_{cp}')_{ap} = 1.2\times10^{-2}$.

$$\frac{\varepsilon_{obs}(0)}{\varepsilon_{obs}}[X] = \frac{1}{(1+K_{cp}')} + \frac{\frac{(K_{cp}')_{ap}}{K_a'}}{(1+K_{cp}')}[X] \quad (5)$$

This equation was used at 498 nm with a correlation factor

[1] This equation is valid at the isosbestic point and is a good approximation when the molar absorption coefficients of the free species and its adduct are similar.

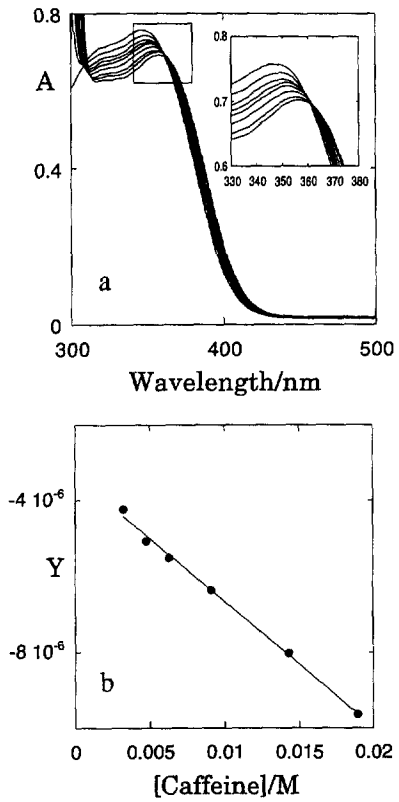


Fig. 5. (a) Spectral variations of the compound 4-hydroxyflavylium 4.3×10^{-5} M at pH = 7.4 (3 days in the dark in order to obtain C_{trans}), upon addition of caffeine: 0; 0.0016; 0.0032; 0.0047; 0.063; 0.0091; 0.014; 0.019; 0.023 M. Isosbestic point at 360 nm. (b) Benesi–Hildebrand representation followed at 330 nm.

of 0.999 (Fig. 4b) and $(K_{ap}')_{cp}=1.2\times10^{-2}$ and $K_{cp}'=550$ M^{-1} were calculated using the value of $K_a'=4.47\times10^{-5}$ from experiences in the absence of co-pigment.

3.1.4. Interaction with caffeine in final state pH = 6–7

As mentioned above, the final state of the compound 4′-Hydroxyflavylium at pH 6–7 is the *trans*-chalcone species [1,31].

In Fig. 5, the interaction of this species with Caffeine is presented. Inspection of this figure shows that, as in the case of flavylium cation, the adduct of *trans*-chalcone with caffeine decreases the color capacity of this form, and the isosbestic point is lost as long as the concentration of caffeine increases. Due to the fact that the spectral variations are larger than in the precedent examples, the association constant can be measured by the Benesi–Hildebrand equation (for a caffeine concentration where the isosbestic points are still maintained) giving a value of $K_{cp}^{IV}=99\ M^{-1}$.

3.1.5. 4′,7-Dihydroxyflavylium

This compound shows an equilibrium between the flavylium cation and its 'conjugate base' constituted by ca. 90% of *trans*-chalcone and 10% of quinoidal base. The final equilibrium is reached in a few hours (depending on pH) and thus the experiments here described concern the final state. Following the examples of 4-methyl,7-hydroxyflavylium and 4′-hydroxyflavylium, we verified an interaction between caffeine and flavylium cation, *trans*-chalcone as well as quinoidal base (Fig. 6).

The spectral variations resulting from addition of caffeine are very small, and the apparent pK_{ap}' for a concentration of caffeine 0.04 M is 2.4 in comparison with 3.05 in the absence of caffeine, leading to the immediate conclusion that the association constants with the 'conjugate base' are larger than with flavylium cation. Inspection of Fig. 6 also shows that while the characteristic absorption of quinoidal base ca. 500 nm (pH > 4.5) is lower in the presence of caffeine, the characteristic absorption ca. 360–370 nm of *trans*-chalcone is more or less the same. The molar fraction of quinoidal base at the equilibrium can be measured from the ratio of the absorbance at 500 nm immediately after a pH jump from 1 to 5, and at the end of this kinetic process [32]. In absence of caffeine is ca. 10%, while in the presence of caffeine 0.05 M is ca. 6%. The net effect of the interaction between caffeine and the 'conjugate base' corresponds to an increase in the concentration of the *trans*-chalcone adduct at the expense of the quinoidal base. This conclusion contradicts one of the proposed explanations for the co-pigmentation effect involving anthocyanins, which states that the co-pigment prevents the hydration reaction [10]. Such interpretation is not com-

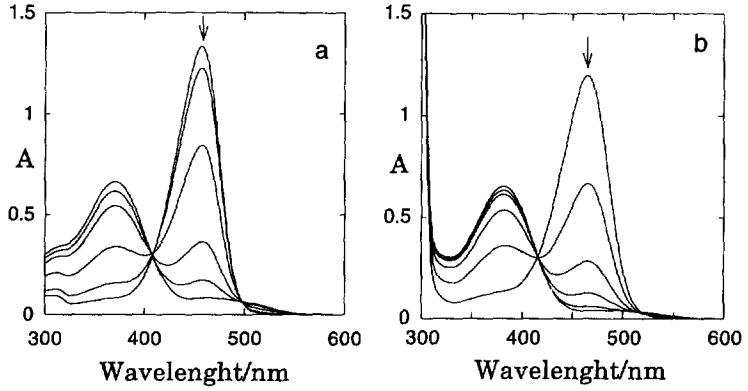


Fig. 6. (a) Spectral variations of the compound 4′,7-dihydroxyflavylium as a function of pH, in the absence of caffeine: pH = 1.0; 2.3; 3.03; 3.74; 4.26; 4.94; (b) The same as in (a) but in the presence of caffeine 0.04 M: pH = 1.0; 2.3; 2.91; 3.43; 3.99; 4.61.

patible with an increase in the molar fraction distribution of *trans*-chalcone in comparison with the quinoidal base as we observed.

3.1.6. Flash photolysis and pH jumps experiments

The photoisomerization of the carbon–carbon double bond is a common process with very important biological consequences [33]. Recently, we used this effect to study the kinetic processes involving synthetic flavylium salts [25,26]. The procedure uses a pulse of light that gives rise to the formation of *cis*-chalcone at the expense of the *trans*-chalcone. Depending on the pH and substituents of the flavylium unit, this last species can form B, AH^+ or A, in competition with the back reaction to recover *trans*-chalcone (see Scheme 1). In the case of the compound 4′-methoxyflavylium [21,22] or 4′-hydroxyflavylium [32], there is a large kinetic barrier from *cis* to *trans*-chalcone, and immediately after the pulse of light, the system proceeds into the direction of the colored species AH^+ and A, and no back reaction is observed. This photochemical effect is more or less permanent at room temperature and constitutes the bases of the proposed memories devices [21,22]. In contrast, in the case of 4′,7-dihydroxyflavylium, a significant part of the *cis*-chalcone formed through the photochemical reaction can be recovered a few seconds after the pulse, in competition with the formation of AH^+ and A. Finally, the excess of AH^+ and A species that are the net result of the photoreaction disappear in a second and slower process and thus the system reaches the equilibrium before irradiation. The observed rate constant of the process immediately after the pulse of light is accounted by Eq. (6) [25]

$$k_{obs(photo)} = k_i + k_{-h}[H^+] \tag{6}$$

where the rate constants are defined by Eq. (7):

$$C_t \underset{k_{-i}}{\overset{k_i}{\leftrightharpoons}} C_c + B \underset{k_{-h}[H^+]}{\overset{k_h}{\leftrightharpoons}} AH^+/A \tag{7}$$

The flash photolysis experiments can be complemented with pH jumps. Usually the system is placed in equilibrium at pH = 1, a certain amount of base is added and the kinetic process followed by spectrophotometry. As described elsewhere [25], in the case of the compound 4′,7-dihydroxyflavylium, Eq. (8) accounts for the global process.

$$k_{obs(pH\ jump)} = \frac{[H^+]}{[H^+]+K_a} \cdot \frac{k_i k_k}{k_i + k_{-k}[H^+]} + \frac{k_{-i} k_{-k}[H^+]}{k_i + k_{-k}[H^+]} \tag{8}$$

The rate constants of Eq. (7) can be obtained by a simultaneous fitting of Eqs. (6) and (8).

In Fig. 7, the observed first order rate constant upon a set of pH jumps carried out in the compound 4′7-dihydroxyflavylium in the presence of Caffeine 0.05 M, as well as a representative decay trace of the process immediately after the pulse of light, are represented. From these data, the rate constants were obtained and are compared with those in the absence of caffeine, Table 1.

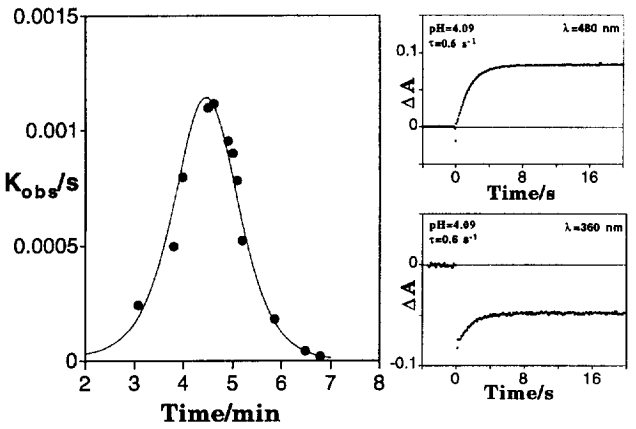


Fig. 7. Left: Variation of the observed pseudo-first order rate constant resulting from the relaxation of the system containing 4′,7-dihydroxyflavylium 5×10^{-5} M and caffeine 0.05 M upon a pH jump from 1. Right: Decay trace of the flash photolysis of the same system at pH = 4.09 followed at 360 nm and 480 nm. The rate constants were calculated by a simultaneous fitting to Eqs. (6) and (8).

Table 1
Thermodynamic and kinetic constants measured by means of pH jump and flash photolysis

	4′,7	4′,7 + Caffeine[a]
K_a	10^{-4}	$10^{-3.4}$
K_h	1.4×10^{-6}	8.8×10^{-7}
K_i	1.4×10^{3}	1.1×10^{4}
k_h	$1.8 \times 10^{-2}\ s^{-1}$	$5.3 \times 10^{-3}\ s^{-1}$
k_{-h}	$1.3 \times 10^{4}\ s^{-1}$	$6 \times 10^{3}\ s^{-1}$
k_i	$0.26\ s^{-1}$	$0.18\ s^{-1}$
k_{-i}	$1.8 \times 10^{-4}\ s^{-1}$	$1.7 \times 10^{-5}\ s^{-1}$

[a]Caffeine 0.05 M.

Inspection of these data shows that all the rate constants are decreased by the complexation. However, from a thermodynamic point of view the equilibrium constant of the *trans–cis* isomerization is more affected than the equilibrium constant of the hydration reaction. The net effect of the interaction is thus to increase the amount of *trans*-chalcone, as mentioned previously in this work.

4. Conclusions

Through this work, we proved that interaction with caffeine is a general phenomenon involving the several species of synthetic flavylium salts, and not only flavylium cation and/or quinoidal base as reported in literature for the case of co-pigmentation involving anthocyanins. Our results confirm the previous observation [10] that co-pigmentation is essentially a phenomenon that changes the molar fraction distribution of the species, and does not appreciably change the molar absorption coefficient of the adducts. The co-pigmen-

tation phenomenon can be viewed as a competition between the several forms of the compound for the co-pigment and in principle there is no reason to neglect association with any form. Through the mathematical approach proposed in this work, a complex system as 4′-hydroxyflavylium or 4′,7-dihydroxyflavylium can be treated as the simplest case of 4-methyl, 7-hydroxyflavylium, assuming that in all systems, we are confronted with an equilibrium between an acid and its conjugate base. The net effect of co-pigmentation can be viewed as a consequence of the balance between the several association constants. The higher the association constant, the lower the energy level of the adduct and the larger its molar fraction distribution for a determinate pH value.

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Appendix A

5. Single equilibrium without complexation

When considering a single acid–base equilibrium (Eq. (a1)):

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \quad \text{(a1)}$$

The pH dependent absorbance, A, taken at any wavelength, divided by the absorbance obtained at a pH value, sufficiently acidic to assure that the sole species in solution is the form AH^+, is given by Eq. (a2):

$$\alpha = \frac{\frac{A}{A_0} - Ct}{1 - Ct} \quad \text{(a2)}$$

where α is the molar fraction of the acidic species defined by Eq. (a3):

$$\alpha = \frac{[AH^+]}{C_0} = \frac{[H^+]}{[H^+] + K_a} \quad \text{(a3)}$$

and Ct is the ratio $\varepsilon_{AH^+}/\varepsilon_A$ obtained as A/A_0 at a pH value where the only absorbing species is the basic form.

6. Single equilibrium with complexation

Whenever complexation occurs with both forms as in the case of 4-methyl, 7-hydroxyflavylium, Eqs. (a4) and (a5) must be added:

$$AH^+ + X \overset{K_{cp}}{\rightleftharpoons} AXH^+ \quad \text{(a4)}$$

$$A + X \overset{K_{cp'}}{\rightleftharpoons} AX \quad \text{(a5)}$$

If the subtract is used in large excess respecting to the flavylium salt, Eq. (a2) is still valid with α substituted by α_{ap} (Eq. (a6))

$$\alpha_{ap} = \frac{[H^+]}{[H^+] + K_{ap}} = 1 - \beta_{ap} \quad \text{(a6)}$$

and K_{ap} given by Eq. (a8)

$$K_{ap} = \frac{K_a + K_a K_{cp}'[X]}{1 + K_{cp}[X]} \quad \text{(a7)}$$

The molar fraction distribution of the four species is now given by Eqs. (a8), (a9), (a10) and (a11):

$$\frac{[AH^+]}{C_0} = \frac{1}{1 + K_{cp}[X]} \cdot \alpha_{ap} \quad \text{(a8)}$$

$$\frac{[AHX^+]}{C_0} = \frac{K_{cp}[X]}{1 + K_{cp}[X]} \cdot \alpha_{ap} \quad \text{(a9)}$$

$$\frac{[A]}{C_0} = \frac{K_a}{K_{ap}(1 + K_{cp}[X])} \cdot \beta_{ap} \quad \text{(a10)}$$

$$\frac{[AX]}{C_0} = \frac{K_a K_{cp}[X]}{K_{ap}(1 + K_{cp}[X])} \cdot \beta_{ap} \quad \text{(a11)}$$

7. Multiple equilibrium of anthocyanins and flavylium salts

In the case of anthocyanins and flavylium salts, Eqs. (a12), (a13), (a14) and (a15) are observed

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \quad \text{(a12)}$$

$$AH^+ \overset{K_h}{\rightleftharpoons} B + H^+ \quad \text{(a13)}$$

$$B \overset{K_t}{\rightleftharpoons} C_{cis} \quad \text{(a14)}$$

$$C_{cis} \overset{K_i}{\rightleftharpoons} C_{trans} \quad \text{(a15)}$$

introducing the 'conjugate base'

$$[CB] = [A] + [B] + [C_{cis}] + [C_{trans}] \quad \text{(a16)}$$

the equilibrium can be once more treated as a single acid–base equilibrium

$$AH^+ \overset{K_a'}{\rightleftharpoons} CB + H^+ \quad \text{(a17)}$$

with $K_a' = K_a + K_h + K_h K_t + K_h K_t K_i$ and

$$\alpha' = \frac{[H^+]}{[H^+] + K_a'} = 1 - \beta' \quad \text{(a18)}$$

and Eq. (a2) is once more observed with α substituted by α' and the molar fraction distribution of the five species are given by Eqs. (a19), (a20), (a21), (a22) and (a23):

$$\frac{[AH^+]}{C_0}=\alpha'=\frac{[H^+]}{[H^+]+K_a'} \quad (a19)$$

$$\frac{[A]}{C_0}=\frac{K_a}{[H^+]}\alpha'=\frac{K_a}{K_a'}\frac{K_a'}{[H^+]+K_a'}=\frac{K_a}{K_a'}\beta' \quad (a20)$$

$$\frac{[B]}{C_0}=\frac{K_b}{K_a'}\beta'; \quad (a21)$$

$$\frac{[C_{cis}]}{C_0}=\frac{K_tK_b}{K_a'}\beta' \quad (a22)$$

$$\frac{[C_{trans}]}{C_0}=\frac{K_iK_tK_b}{K_a}\beta' \quad (a23)$$

8. Multiple equilibrium of anthocyanins and flavylium salts with complexation

This is the most general case in which co-pigmentation can occur with all the species in solution. Considering by simplicity the 1:1 equilibrium and without loss of generality

$$AH^+ + X \overset{K_{cp}}{\rightleftharpoons} AHX^+ \quad (a24)$$

$$A + X \overset{K_{cp}^{I}}{\rightleftharpoons} AX \quad (a25)$$

$$B + X \overset{K_{cp}^{II}}{\rightleftharpoons} BX \quad (a26)$$

$$C_{cis} \overset{K_{cp}^{III}}{\rightleftharpoons} C_{cis}X \quad (a27)$$

$$C_{trans} \overset{K_{cp}^{IV}}{\rightleftharpoons} C_{trans}X \quad (a28)$$

From the total concentration given by Eq. (a29)

$$C_0=[AH^+]+[A]+[B]+[C_{cis}]+[C_{trans}]+[AHX^+]+[AX]+[BX]+[C_{cis}X]+[C_{trans}X] \quad (a29)$$

As in the previous examples, Eq. (a2) is still valid with α substituted by α_{ap}' Eq. (a30)

$$\alpha_{ap}'=\frac{[H^+]}{[H^+]+K_{ap}'} \quad (a30)$$

and K_{ap}' given by Eq. (a31)

$$K_{ap}'=\frac{K_a'+(K_aK_{cp}^{I}+K_hK_{cp}^{II}+K_{cp}^{III}K_hK_t+K_hK_tK_iK_{cp}^{VI})[X]}{(1+K_{cp}[X])} \quad (a31)$$

the molar fraction distribution of the several species can be written

$$\frac{[AH^+]}{C_0}=\frac{1}{1+K_{cp}[X]}\cdot\alpha_{ap}' \quad (a32)$$

$$\frac{[AHX^+]}{C_0}=\frac{K_{cp}[X]}{1+K_{cp}[X]}\cdot\alpha_{ap}' \quad (a33)$$

$$\frac{[A]}{C_0}=\frac{K_a}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a34)$$

$$\frac{[AX]}{C_0}=\frac{K_aK_{cp}^{I}[X]}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a35)$$

$$\frac{[B]}{C_0}=\frac{K_h}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a36)$$

$$\frac{[BX]}{C_0}=\frac{K_hK_{cp}^{II}[X]}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a37)$$

$$\frac{[C_{cis}]}{C_0}=\frac{K_hK_t}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a38)$$

$$\frac{[C_{cis}X]}{C_0}=\frac{K_{cp}^{III}K_hK_t[X]}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a39)$$

$$\frac{[C_{trans}]}{C_0}=\frac{K_hK_tK_i}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a40)$$

$$\frac{[C_{trans}]}{C_0}=\frac{K_hK_tK_iK_{cp}^{IV}[X]}{K_{ap}'(1+K_{cp}[X])}\cdot\beta_{ap}' \quad (a41)$$

Appendix B

One of the most useful methods to calculate association constants is the Benesi–Hildebrand equation [27]:

$$\frac{[X]}{\varepsilon_{obs}-\varepsilon_0}=\frac{1}{\Delta\varepsilon K_{cp}}-\frac{[X]}{\Delta\varepsilon} \quad (b1)$$

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). This equation can be used whenever one sole species is present in solution. It is very useful for example to calculate the association constants with flavylium cation (at very acidic pH values). However, it can not be used when several species are in equilibrium.

9. Calculation of the association constants

Considering the pH sufficiently basic to have exclusively the 'conjugate base' in solution and using a wavelength for which only the quinoidal base and its adduct absorb. Working

at the isosbestic point or at any wavelength for which the molar absorption coefficients do not change appreciably:

$$A=\varepsilon_A[A]+\varepsilon_{AX}[AX]\approx\varepsilon_A\{[A]+[AX]\} \quad (b2)$$

using Eqs. (a37) and (a38) and Eq. (a46)

$$A=\varepsilon_A\frac{K_a+K_aK_{cp}'[X]}{K_a'+(K_{cp}')_{ap}[X]}C_0 \quad (b3)$$

In the absence of co-pigment, A_0 is given by:

$$A_0=\varepsilon_A\frac{K_a}{K_a'}C_0 \quad (b4)$$

leading to

$$\frac{\varepsilon_{obs}}{\varepsilon_{obs}(0)}=\frac{(1+K_{cp}')[X]}{1+\frac{(K_{cp}')_{ap}}{K_a'}[X]} \quad (b5)$$

inverting this last equation, Eq. (b6) is obtained:

$$\frac{\varepsilon_{obs}(0)}{\varepsilon_{obs}}[X]=\frac{1}{(1+K_{cp}')}+\frac{\frac{(K_{cp}')_{ap}}{K_a'}}{(1+K_{cp}')}[X] \quad (b6)$$

from which $(K'_{cp})_{ap}$ and K_{cp} can be obtained.

References

[1] R.A. McClelland, S. Gedge, J. Am. Chem. Soc. 102 (1980) 5838.
[2] R.A. McClelland, G.H. McGall, J. Org. Chem. 47 (1982) 3730.
[3] G. Mazza, R. Brouillard, J. Agric. Food Chem. 35 (1987) 422.
[4] R. Brouillard, in: J.B. Harborne (Ed.), The Flavonoids, Advances in Research, Chapman & Hall, London, 1988, p. 525.
[5] R. Brouillard, in: P. Markakis (Ed.), Anthocyanins as Food Colors, Academic Press, New York, 1982, p. 1.
[6] R. Brouillard, J.E. Dubois, J. Am. Chem. Soc. 99 (1977) 1359.
[7] R. Brouillard, B. Delaporte, J. Am. Chem. Soc. 99 (1977) 8461.
[8] R.N. Stewart, K.H. Norris, S. Asen, Phytochemistry 14 (1975) 937.
[9] G.M. Robinson, R. Robinson, Biochem. J. 25 (1931) 1687.
[10] R. Brouillard, G. Mazza, Z. Saad, A.M. Albrecht-Gary, A. Cheminat, J. Am. Chem. Soc. 111 (1989) 2604.
[11] T. GoTo, T. Kondo, Angew. Chem., Int. Ed. Engl. 30 (1991) 17.
[12] Y. Cai, T.H. Lilley, E. Haslam, J. Chem. Soc. Chem. Commun. (1990) p. 380.
[13] G. Mazza, R. Brouillard, Phytochemistry 29 (1990) 1097.
[14] T.V. Mistry, Y. Cai, T.H. Lilley, E. Haslam, J. Chem. Soc., Perkin Trans. 2 (1991) 1287.
[15] S. Asen, R.N. Stewart, K.H. Norris, Phytochemistry 11 (1972) 1139.
[16] O. Dangles, N. Saito, R. Brouillard, J. Am. Chem. Soc. 115 (1993) 3125.
[17] T. GoTo, T. Hoshino, M. Ohba, Agric. Biol. Chem. 40 (1976) 1593.
[18] G.M. Robinson, C.R. Robinson, Biochem. J. 25 (1931) 1687.
[19] O. Dangles, R. Brouillard, Can. J. Chem. 70 (1992) 2174.
[20] H. Liao, Y. Cai, E. Haslam, J. Sci. Food Agric. 59 (1992) 230–299.
[21] F. Pina, M.J. Melo, M. Maestri, R. Ballardini, V. Balzani, J. Am. Chem. Soc. 119 (1997) 5556.
[22] C. Crabb, New Scientist 2091 (1997) 12.
[23] P. Figueiredo, J.C. Lima, H. Santos, M.C. Wigand, R. Brouillard, A.L. Maçanita, F. Pina, J. Am. Chem. Soc. 116 (1994) 1249.
[24] F. Pina, L. Benedito, M.J. Melo, A.J. Parola, M.A. Bernardo, J. Chem. Soc., Faraday Trans. 92 (1996) 1693.
[25] F. Pina, M.J. Melo, R. Ballardini, L. Flamigni, M. Maestri, New J. Chem. 21 (1997) 969.
[26] M. Maestri, R. Balardini, F. Pina, M.J. Melo, J. Chem. Educ. 74 (1997) 1314.
[27] C. Michaelis, R. Wizinger, Helv. Chim. Acta 34 (1951) 1761.
[28] C. Bullöw, H. Wagner, Ber. Dtsch. Chem. Gess. 34 (1901) 1782.
[29] C.G. Hatchard, C.A. Parker, Proc. R. Soc. London, Ser. A. 235 (1956) 518–536.
[30] F. Pina, M.J. Melo, I. Abreu, J.C. Lima, M.H. Santos, New J. Chem., accepted for publication.
[31] H.A. Benesi, J.H. Hildebrand, J. Am. Chem. Soc. 71 (1949) 2703.
[32] F. Pina, A. Roque, M.J. Melo, M. Maestri, L. Belladelli, V. Balzani, Chem. Eur. J., in press.
[33] J. Saltiel, Y.-P. Sun, in: H. Dürr, H. Bouas-Laurent (Eds.), Photochromism Molecules and Systems, Chap. 3, Elsevier, Oxford, 1990.