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Thermodynamics and Kinetics of Cyanidin 3-Glucoside and Caffeine Copigments

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ABSTRACT: The multiequilibrium system of reactions of cyanidin 3-glucoside at acidic and mildly acidic pH values was studied in the presence of caffeine as a copigment. The thermodynamic and kinetic constants were determined using the so-called direct and reverse pH jump experiments that were followed by conventional UV−vis spectroscopy or stopped flow coupled to a UV−vis detector, depending on the rate of the monitored process. Compared with that of free anthocyanin, the copigmentation with caffeine extends the domain of the flavylium cation up to less acidic pH values, while in a moderately acidic medium, the quinoidal base becomes more stabilized. As a consequence, the hydration to give the colorless hemiketal is difficult over the entire range of pH values. At pH 1, two adducts were found for the flavylium cation−caffeine interaction, with stoichiometries of 1:1 and 1:2 and association constants of 161 M^{-1} (K_1) and 21 M^{-1} (K_2), respectively.

KEYWORDS: *anthocyanin, cyanidin 3-glucoside, caffeine, copigmentation, color stabilization, hydration*

INTRODUCTION

Anthocyanins are the ubiquitous compounds responsible for most of the red and blue colors found in flowers and fruits.[1] Noncovalent interactions involving the colored species related to anthocyanins (namely, flavylium cation and quinoidal base) and other natural compounds are a matter of great importance with regard to color expression in plants. Noncovalent interactions have been extensively studied under the category of copigmentation.[2−5]

Anthocyanins in acidic to moderately acidic solutions are involved in a pH-dependent network of reversible reactions (Figure 1).[6−9] The flavylium cation (usually red colored) is the only stable species under very acidic conditions, and at higher pH values, the colorless hemiketal is the dominant species together with quinoidal base (blue color), with chalcones as minor components. A convenient way to study the network of chemical reactions involving anthocyanins and related compounds is to conduct pH jumps:[10] a direct pH jump is here defined as a change in pH from equilibrated solutions at very low pH values (where flavylium cation is stable) to higher pH values. Conversely, reverse pH jumps[11] are defined as the addition of acid to equilibrated solutions at higher pH values (typically pH 4−6) to reach a very acidic pH where flavylium is stable.

When a direct pH jump is performed, the flavylium cation (AH^+) transfers a proton to water, leading to a zwitterionic species that rearranges to the quinoidal base A [Figure 1, deprotonation (eq 1)].

The flavylium cation is also involved in a parallel reaction that is much slower than the previous one because of the addition of water at position 2, giving hemiketal species B2 (which will hereafter be termed B) [Figure 1, hydration (eq 2)].[a]

On the other hand, the hemiketal (chromene) reacts via a tautomeric process that leads to a ring opening with formation of a *cis*-chalcone species (Cc). This reaction usually occurs on a subsecond to second time scale [Figure 1, tautomerization (eq 3)].

The hydration reaction is strongly dependent on the proton concentration, while the tautomerization is catalyzed at very acidic and basic pH values.[12] In Figure 2, a simulation of the rate constant of these two kinetic processes is given. Below pH 2, hydration is much faster than tautomerization and occurs on a millisecond to subsecond time scale. However, at pH >2.5, hydration is usually much slower than tautomerization.

In conclusion, after a pH jump from a very acidic solution containing the flavylium cation to a moderately acidic solution, quinoidal base is formed immediately and constitutes a kinetic product (because it is usually less stable thermodynamically) that slowly disappears to reach a pseudoequilibrium involving all the species except the *trans*-chalcone.

Finally, the thermodynamic equilibrium is attained from the *cis*-chalcone isomerization to give the *trans*-chalcone (Ct), in a process that in anthocyanins usually takes a few hours [Figure 1, isomerization (eq 4)].

The three kinetic processes are simulated in Figure 3 for the case of anthocyanins, and because of their clear separation in time, they can be studied separately, which simplifies the kinetic analysis of the system. Moreover, the change of regime in the case of the second kinetic process is also illustrated in the figure. Only at lower pH values is the tautomerization reaction slower than the hydration.

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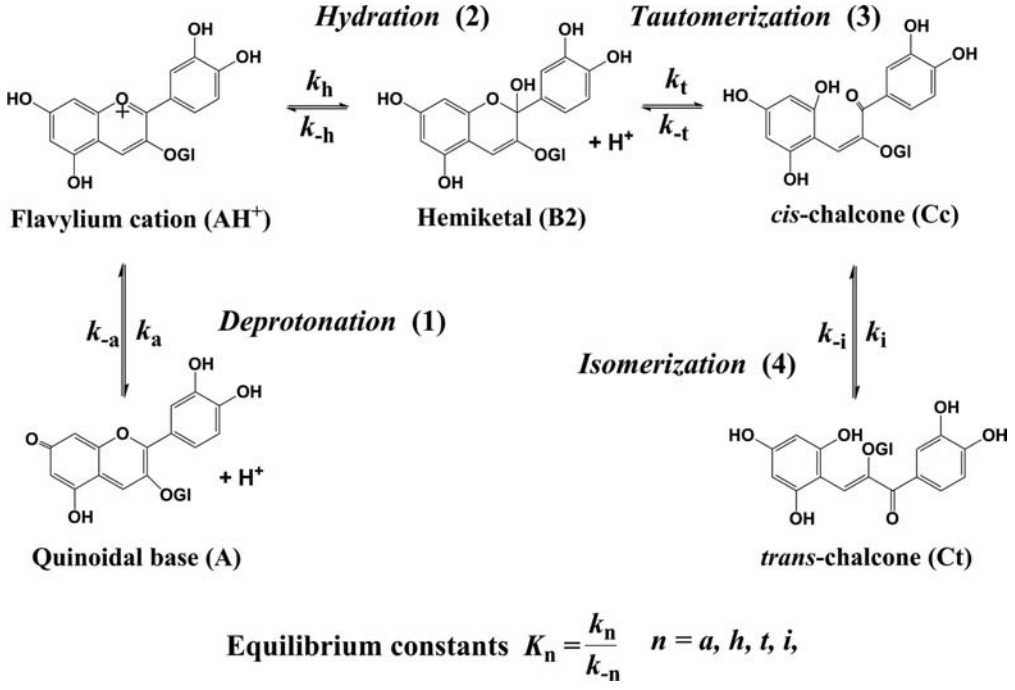

Figure 1. Network of chemical reactions of cyanidin 3-glucoside in acidic to moderately acidic solutions.

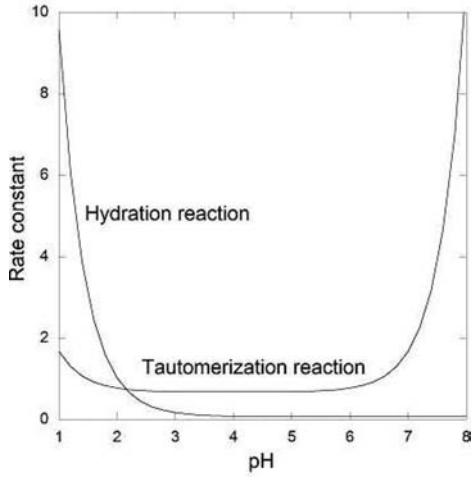

Figure 2. Simulation of the pH-dependent rate constants of hydration and tautomerization using the values reported in this paper: k_h = 0.07 s^{-1}; k_{-h} = 95 s^{-1} M^{-1}; k_t = 0.07 s^{-1}; and k_{-t} = 0.6 s^{-1}. Reliable acid and basic catalysis constants for the tautomerization reaction were estimated to be k^H = 10 and k^{OH} = 10^7 s^{-1} M^{-1}, respectively.

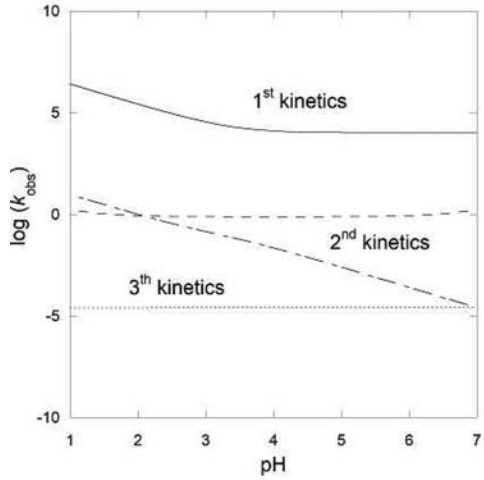

Figure 3. Simulation of the rate constants of the three well-separated kinetic processes taking place in anthocyanins, first kinetics (—), second kinetics (– – – and —·—), and third kinetics (···): k_a = 10^4 s^{-1}; k_{-a} = $10^{7.4}$ s^{-1} M^{-1}; k_h = 0.07 s^{-1}; k_{-h} = 95 s^{-1} M^{-1}; K_a = $10^{-3.36}$; K_t = 0.09; K_h = 7.3 × 10^{-4} M^{-1}; k_i = 7.4 × 10^{-6} s^{-1}; k_{-i} = 2.5 × 10^{-5} s^{-1}; k_t + k_{-t} = 0.7 s^{-1}; k^H = 10 s^{-1} M^{-1}; and k^{OH} = 10^7 s^{-1} M^{-1}.

In spite of numerous and well-documented works regarding the copigmentation with anthocyanins, the effect of copigmentation on the rate and equilibrium constants of the network described above has been less studied.[13,14] For this purpose, the copigmentation involving cyanidin 3-glucoside and caffeine was selected (Figure 4). The copigmentation effect of caffeine has been studied for malvin[13,15] and several synthetic flavylium compounds.[16−18] The main driving force for the association seems to be $\pi-\pi$ stacking between the chromophore and the copigment.[15]

Caffeine and derivatives of cyanidin 3-glucoside are found together in seeds of cacao[19] and in the leaves of a line of red tea recently developed by natural crossing.[20] Additionally, caffeine is a common food additive, and from this point of view, it is

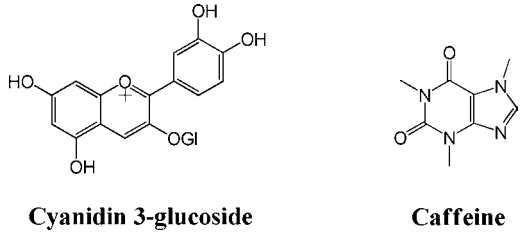

Figure 4. Chemical structures of cyanidin 3-glucoside and caffeine.

interesting to know how its presence can affect the reactivity of anthocyanins, usually employed as food colorants. Recently, we reported on the effect of self-aggregation on the thermody-

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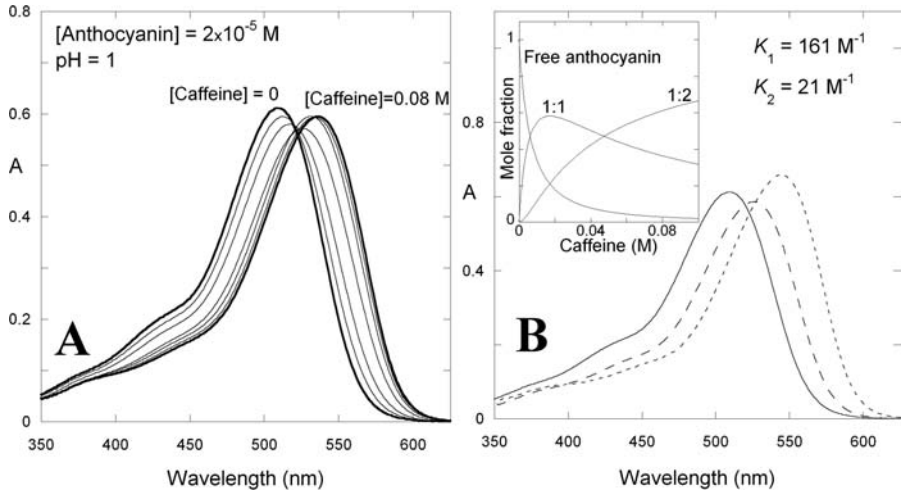

Figure 5. (A) Spectral variations upon addition of caffeine to cyanidin 3-glucoside (2.3×10^{-5} M) at pH 1.0. The spectra correspond to 0, 0.002, 0.006, 0.02, 0.04, 0.06, and 0.08 M caffeine. The spectra for 0 and 0.08 M caffeine are shown as bold lines. (B) Mathematical treatment of the data in panel A allowed us to obtain the pure spectra corresponding to the 1:1 (−−−) and 1:2 (···) flavylium cation−caffeine adducts. The spectrum of free anthocyanin is plotted as a solid line. The inset shows the molar fractions of the adducts and free anthocyanin as a function of caffeine concentration.

namics and kinetics of the six most common anthocyanidin 3-glucosides, including cyanidin 3-glucoside.[11] In this work, we report for the first time an extensive study of the effect of caffeine copigmentation on the thermodynamics and kinetics of the network of chemical reactions of cyanidin 3-glucoside in diluted solutions, where self-aggregation effects can be neglected.

MATERIALS AND METHODS

Cyanidin 3-glucoside chloride (Kuromanin chloride) was purchased from Extrasynthese; caffeine was purchased from Merck. All the reagents (≥96%) were used without any further purification. The solutions were prepared in Millipore water. The pH of the solutions was adjusted by the addition of HCl, NaOH, and 8.3×10^{-3} M citrate buffer and was measured on a CRISON pH-Meter BASIC 20^{+} instrument. The ionic strength of the solutions was kept constant at 0.1 M by the addition of NaCl. UV−vis absorption spectra were recorded on Varian Cary 100 Bio and Varian Cary 5000 spectrophotometers. The stopped flow experiments were conducted in an Applied Photophysics SX20 stopped flow spectrometer provided with a PDA.1/UV photodiode array detector with a minimum scan time of 0.65 ms and a wavelength range of 200−735 nm.

RESULTS AND DISCUSSION

In Figure 5A, the spectral variations observed upon addition of increasing concentrations of caffeine (up to 0.08 M) to a diluted solution of cyanidin 3-glucoside (2.3×10^{-5} M) at pH 1 are shown. For lower caffeine concentrations, the absorption red shifts and slightly decreases and an isosbestic point at 521 nm is observed; for higher caffeine concentrations, the absorption shifts to higher wavelengths and gives rise to a small absorption increase and the isosbestic point at 521 nm disappears, with a new one appearing at 534 nm. At pH 1, the flavylium cation is the only species present in solution in the case of the anthocyanin; thus, these spectral changes suggest the formation of two adducts involving the flavylium cation of cyanidin 3-glucoside and caffeine, probably with flavylium cation:caffeine stoichiometries of 1:1 and 1:2. Mathematical treatment of the system[21,22] assuming these stoichiometries allowed us to obtain the absorption spectra of the two adducts, as well as the respective association constants, as shown in Figure 5B.

The 1:1 stoichiometry has been proposed for a number of flavylium cation−copigment complexes, usually resulting, like here, in a bathochromic shift and a small decrease in the molar extinction coefficient.[18,23−25] The formation of a second complex with a 1:2 stoichiometry has been less reported. In this case, we have to take into account the possibility that the formation of this complex will vary, depending on the chemical structure of anthocyanin and copigment as well as the anthocyanin:copigment molar ratio, among other factors. Several years ago,[18] the formation of the 1:2 complex was proposed for the 4-methyl-7-hydroxyflavylium−caffeine and 4′-hydroxyflavylium−caffeine systems at high copigment concentrations, but in both cases, the complexation to additional caffeine induced a greater decrease in the molar extinction coefficient. Here the effect is different; i.e., the formation of the second adduct provokes the increase in the molar extinction coefficient. For some reason (outside the scope of this work), the interaction with more caffeine increases the value of the oscillator strength related to the electronic transition (or transitions) responsible for the absorption band.

Inspection of the inset in Figure 5B shows that concentrations of caffeine higher than the respective solubility limit would be necessary to obtain the 1:2 adduct as the only species. However, for 0.08 M caffeine, practically all the anthocyanin interacts with the copigment through the 1:1 (36%) and 1:2 (61%) adducts, and we selected this ratio of concentrations, i.e., 0.08 M caffeine versus 2×10^{-5} M cyanidin 3-glucoside, to analyze the effect of copigmentation on the thermodynamics and kinetics related to the network of chemical reactions of the anthocyanin (Table 1).

The pH dependence of the absorption spectra of thermally equilibrated solutions of cyanidin 3-glucoside 1.7×10^{-5} M in the presence of caffeine 0.08 M is shown in Figure 6A. Here, K'_a is the global acid−base equilibrium constant that relates the flavylium cation with the other species in the network (eq 5):[26]

$$\begin{aligned} AH^{+} + H_2O \rightleftharpoons A + B \quad K_a' = K_a + K_h + K_hK_t \\ + Cc + Ct + H_3O^{+} \qquad + K_hK_tK_i \end{aligned} \tag{5}$$

When these spectra are compared with the absorption spectra of cyanidin 3-glucoside in the absence of caffeine (Figure 6B),[11]

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Table 1. Thermodynamic Constants of 1.7×10^{-5} M Cyanidin 3-Glucoside in the Presence and Absence of 0.08 M Caffeine

caffeine	pK_a	K_h (M^{-1})	K_t	K_i[b]	pK_a'
0.08 M[a]	3.3(6)	7.4×10^{-4}	0.09	0.3	2.9
–[c]	3.8(0)	3.1×10^{-3}	0.12	–	2.5

[a]Estimated error for all constants except K_i, 15%. [b]The percentage of Ct is ≈2%, and the reported value is a rough estimate. [c]See ref 11. There the reported concentration of anthocyanin was 2×10^{-5} M.

it is clear that the copigment stabilizes the flavylium cation, because its adduct becomes less acidic (pK_a' higher), as well as the quinoidal base, because a significant amount of this species is presented for less acidic solutions.

In Figure 7A, the kinetic process with a direct pH jump from 1 to 5.8 for 1.7×10^{-5} M cyanidin 3-glucoside and 0.08 M caffeine is shown. Immediately after the pH jump, the characteristic absorption spectrum of the quinoidal base appears. This process corresponds to the proton transfer reaction, and its rate that occurs on the microsecond time scale is too fast to be followed by the common techniques, including stopped flow[6] (eq 6).

$$k_{1st} = k_a + k_{-a}[H^+] \quad (6)$$

The observed decrease in the quinoidal base absorption is biexponential, the faster process corresponding to the hydration control (eq 7).[9] In other words, because we are in a range of pH values above 2.5, the hemiketal B equilibrates faster with *cis*-chalcone than flavylium cation and hemiketal (faster tautomerization) and the term $1/(1 + K_t)$ corresponds to the mole fraction of B in equilibrium wtih Cc. Moreover, as reported by Brouillard and Dubois,[6] the quinoidal base does not give directly the hemiketal at moderately acidic and neutral pH values; in fact, only the fraction of flavylium cation available from the initial (faster) equilibrium with quinoidal base leads to hemiketal. This observation is included in eq 7 by the term $[H^+]/([H^+] + K_a)$, corresponding to the mole fraction of the flavylium cation.

$$k_{2nd(hydration\ control)} = \frac{[H^+]}{[H^+] + K_a} k_h + \frac{1}{1 + K_t} k_{-h}[H^+] \quad (7)$$

The slower process in Figure 6A corresponds to the isomerization to give Ct, and its rate depends on the mole fraction of Cc in the pseudoequilibium involving A, AH^+, B, and Cc (eq 8).[9]

$$k_{3rd} = \frac{K_h K_t}{[H^+] + K_a + K_h(1 + K_t)} \times k_i + k_{-i} \quad (8)$$

Because the mole fraction of Ct is very small in anthocyanins (<5%), the latest process is difficult to observe and quantify precisely.

In Figure 7B, the absorption spectra of the quinoidal base (taken immediately after the direct pH jump to pH ≫ pK_a) is compared with that corresponding to cyanidin 3-glucoside in the absence of caffeine.[11] As in the case of the flavylium cation, the copigmentation with caffeine gives rise to a red shift in the absorption spectra, indicating that the excited state of the anthocyanin is more stabilized than the ground state.

The data reported in the inset of Figure 7A allow us to estimate the equilibrium constant K_a from eq 9,[27] where the initial absorbance is due to the complete conversion of the total concentration of the anthocyanin (previously in the form of flavylium cation) to quinoidal base and the final absorbance is caused by the quinoidal base remaining at the equilibrium; from the inset of Figure 7A, 35% of quinoidal base remains at equilibrium (1.7×10^{-5} M cyanidin 3-glucoside and 0.08 M caffeine). From eq 9 and using a K_a' value of $10^{-2.9}$, a K_a value of $10^{-3.36}$ is obtained.

$$\frac{A_f}{A_i} = \frac{K_a}{K_a'} \quad (9)$$

Several pH jumps like that shown in Figure 7A were performed at different final pH values. The faster rate constant of the biexponential process (hydration) is plotted versus pH in Figure 8 (●) and was fit using eq 7 (———) (see below).

The reverse pH jumps give complementary information about the system. In Figure 9, acid was added to equilibrated solutions at pH 5.7 to yield a final pH of 1.3, and the spectral variations were followed by stopped flow (Figure 9A). In this

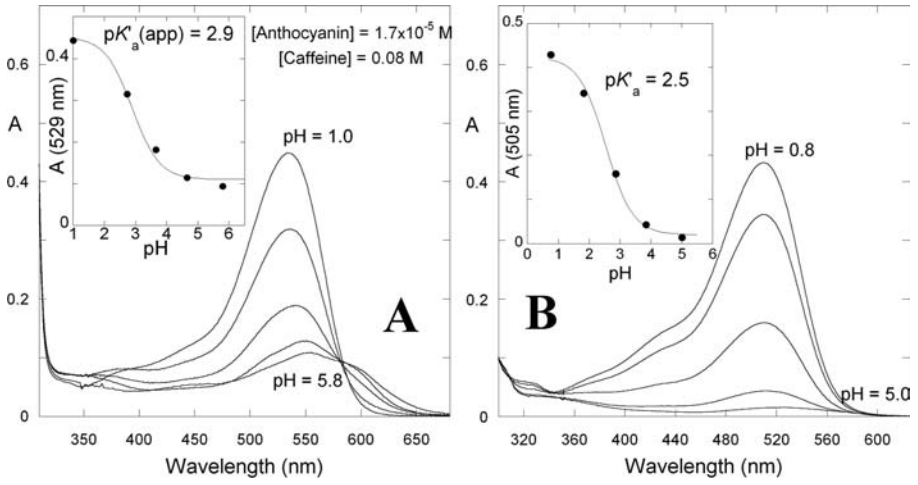

Figure 6. (A) pH-dependent absorption spectra of thermally equilibrated solutions of 1.7×10^{-5} M cyanidin 3-glucoside and 0.08 M caffeine. (B) Same in the absence of caffeine [2×10^{-5} M (see ref 11)].

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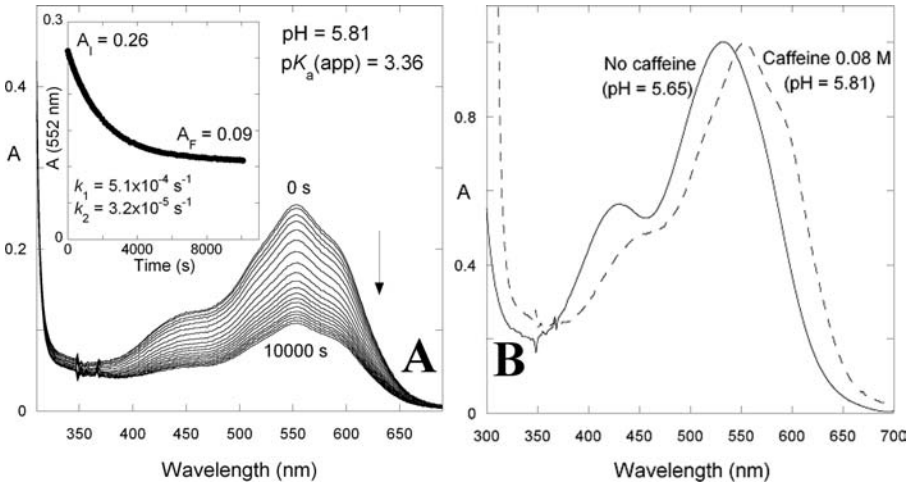

Figure 7. (A) Spectral variations upon a pH jump from 1.0 to 5.8, with 1.7×10^{-5} M cyanidin 3-glucoside and 0.08 M caffeine. (B) Normalized spectra of the cyanidin 3-glucoside quinoidal base in the presence of caffeine obtained immediately after the pH jump (– – –) and analogous spectra of cyanidin 3-glucoside in the absence of caffeine for comparison (see ref 11).

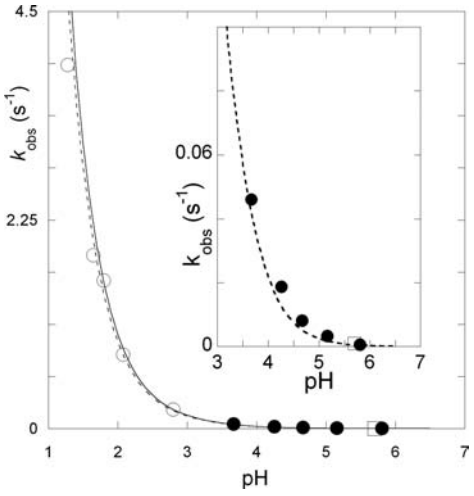

Figure 8. pH dependence of the direct (●) and reverse (○) pH jumps of 1.7×10^{-5} M cyanidin 3-glucoside, in the presence of 0.08 M caffeine. Fitting was achieved by eq 7 (– – –) and eq 10 (—); a point regarding the kinetics of the complexation of cyanidin 3-glucoside at pH 5.7 is included (□).

case (low pH value), hydration becomes faster than tautomerization. The quinoidal base present at pH 5.7 was immediately converted into flavylium cation, a process that took place during the mixing time of the stopped flow. This reaction was followed by the formation of more flavylium cation through a biexponential process: the first corresponding to the formation of flavylium cation from the hemiketal present in the equilibrium at pH 5.7 (eq 10) and the last to the conversion of Cc into flavylium cation via hemiketal (eq 11). In this kind of experiment, no reversibility is expected from B to give Cc.[28]

$$k_{\mathrm{obs1(SF)}} = \frac{[\mathrm{H}^+]}{K_a + [\mathrm{H}^+]} k_h + k_{-h}[\mathrm{H}^+] \tag{10}$$

$$k_{\mathrm{obs2(SF)}} = k_{-t} \tag{11}$$

In eq 11, neither acidic nor basic catalysis was considered for the reverse tautomerization reaction,[12] because at this pH the proton concentration was low for the observation of the first and the hydroxyl concentration was very low for detection of the second.

The amplitudes of the three processes are reported in Figure 9B and give

$$K_t = \frac{\%\mathrm{Cc}}{\%\mathrm{B}} \tag{12}$$

and

$$\frac{K_a}{K_h} = \frac{\%\mathrm{A}}{\%\mathrm{B}} \tag{13}$$

From the percentages shown in Figure 9B, $K_t = 0.09$ and $K_h = 6.3 \times 10^{-4}$ M^{-1}. The kinetic constants of the reverse pH jumps fit with eq 10 are shown in Figure 8 (○).

Knowing K_t and K_a, we can conduct a global fitting of the kinetic data reported in Figure 8 with eqs 7 and 10, leading to a k_h of 0.07 s^{-1} and a k_{-h} of 95 M^{-1} s^{-1}. The ratio between the hydration and dehydration rate constants gives a K_h of 7.4×10^{-4} M^{-1}, in good agreement with the value calculated from the reverse pH jumps (see above). It is worth noting that the similarity of eq 7 with eq 10 results from the low value of K_t in this system. Finally, the slower kinetic process in Figure 9B corresponds to a rate constant k_{-t} of 0.7 s^{-1}, permitting us to calculate a k_t of 0.06 s^{-1} from the respective equilibrium constant.

To corroborate the interpretation of the copigmentation phenomenon, an equilibrated solution of cyanidin 3-glucoside at pH 5.7 was mixed with caffeine at the same pH value (final concentrations of 1.7×10^{-5} and 0.08 M, respectively) and the spectral variations were monitored (Figure 10). An increase in the level of the caffeine–quinoidal base adduct is clearly observed. It is worth noting the rate of the kinetic process shown in Figure 10, which fits with the hydration–dehydration kinetic process presented in the inset of Figure 8 (□).

In summary, the spectral changes upon addition of caffeine to cyanidin 3-glucoside show clearly the interaction of the copigment with the flavylium cation as well as with quinoidal base. The mole fraction distribution of the species of the flavylium network in the absence[11] and presence of caffeine 0.08 M is represented in Figure 11. The domain of the flavylium cation is extended by addition of caffeine as a result of the stabilization of this species, while at moderately acidic pH

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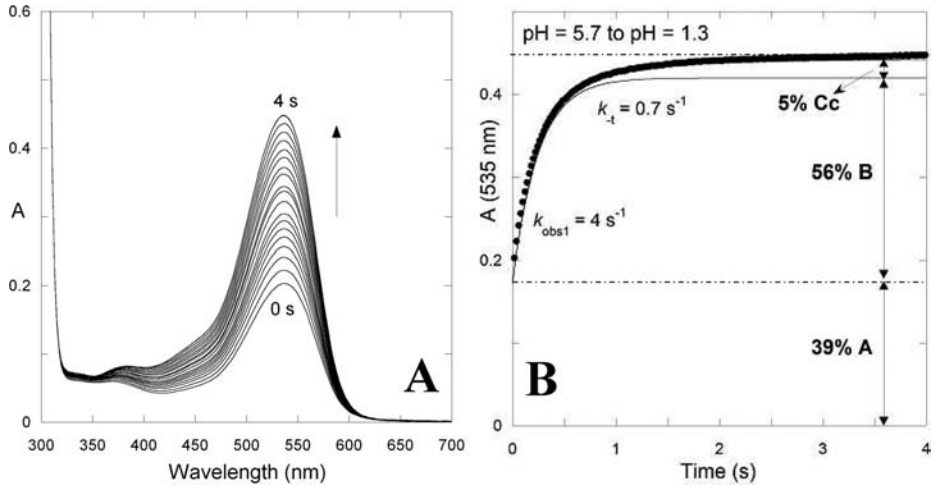

Figure 9. (A) Reverse pH jump from an equilibrated solution of 1.7×10^{-5} M cyanidin 3-glucoside at pH 5.7 in the presence of 0.08 M caffeine to a final pH of 1.3, followed by stopped flow. (B) Trace of the kinetics monitored at 535 nm.

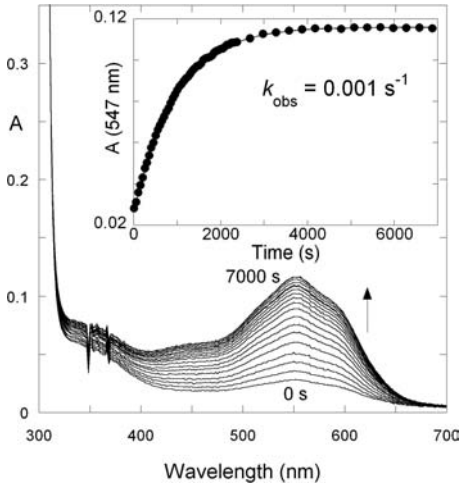

Figure 10. Spectral variations upon addition of caffeine to an equilibrated solution of cyanidin 3-glucoside at pH 5.7. Final concentrations of 0.08 M caffeine and 1.7×10^{-5} M cyanidin 3-glucoside.

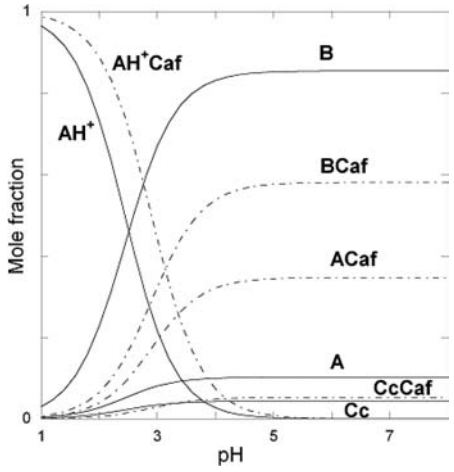

Figure 11. Comparison between the mole fraction distribution of cyanidin 3-glucoside in the absence[11] (—) and presence of 0.08 M caffeine (– – –). In this figure, the terms AH$^+$Caf, BCaf, ACaf, and CcCaf refer to the molar fractions of AH$^+$, B, A, and Cc, respectively, in the presence of caffeine.

values, the level of the quinoidal base−caffeine adduct increases at the expense of the hemiketal.

With regard to the kinetic constants, the most interesting result is the increase in the dehydration rate constant (k_{-h}) for the anthocyanin−caffeine system (Table 2). Somehow, the presence of the copigment facilitates the reaction of the hemiketal to give the flavylium cation. Analogous behavior was previously observed in the case of the self-aggregation of anthocyanins (i.e., dehydration is facilitated in more concentrated and/or aggregated solutions).[11]

In conclusion, copigmentation results in the stabilization of the colored flavylium cation and quinoidal base species, making the hydration to give the colorless hemiketal more difficult. The interaction of the anthocyanin with caffeine mimics in some way what happens in Nature. The flavylium cation is only stable at very acidic pH values, and as a consequence, to achieve the red color inside the vacuoles of the plants (roughly in the pH interval of 3−6), it is necessary to increase the pH domain of the flavylium cation. On the other hand, at higher pH values, the quinoidal base is a very minor species, and thus, interactions with suitable species are needed to stabilize the quinoidal base and obtain blue colors.

Table 2. Kinetic Constants of 1.7×10^{-5} M Cyanidin 3-Glucoside in the Presence and Absence of 0.08 M Caffeine

caffeine	k_h (s^{-1})	k_{-h} (M^{-1} s^{-1})	k_t (s^{-1})	k_{-t} (s^{-1})	k_i (s^{-1})[b]	k_{-i} (s^{-1})[b]
0.08 M[a]	0.07	95	0.06	0.7	7.4×10^{-6}	2.5×10^{-5}
–[c]	0.11	35	0.07	0.6	–	–

[a]Estimated error for all constants except the isomerization constants, 15%. [b]The percentage of Ct is ≈2%, and the reported value is a rough estimate. [c]See ref 11. There the reported concentration of anthocyanin was 2×10^{-5} M.

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Notes

The authors declare no competing financial interest.

■ ADDITIONAL NOTE

[a]This nomenclature is due to the fact that in several systems it is possible to observe the formation of a second hemiketal, from the addition of water to position 4, which is termed B4. However, this reaction is negligible for the system being studied here.

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