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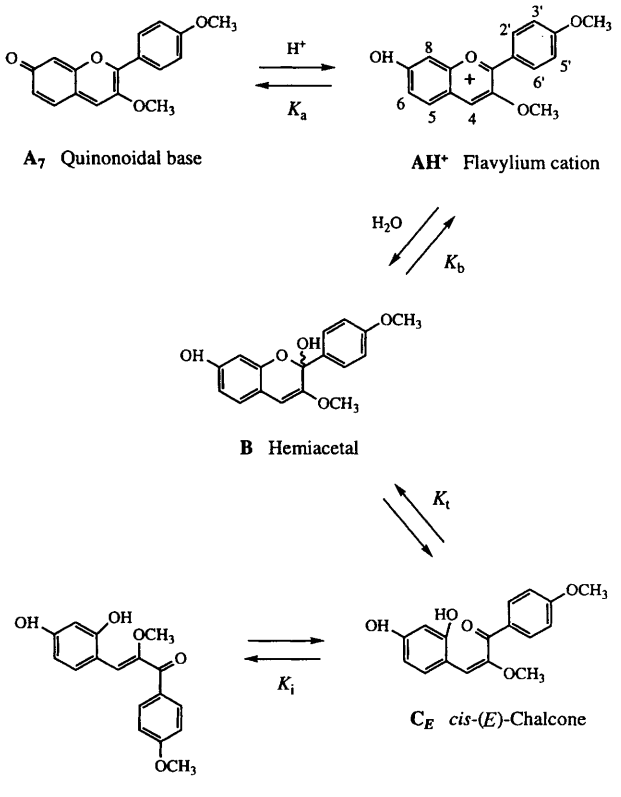
Photochemistry of 3,4′-dimethoxy-7-hydroxyflavylium chloride

Photochromism and excited-state proton transfer

Fernando Pina,* Lina Benedito, Maria João Melo, A. Jorge Parola and Alexandra Bernardo
Centro de Química Fina e Biotecnologia, Departamento de Química, FCT/UNL, Quinta da Torre, 2825 Monte de Caparica, Portugal

The synthetic compound 3,4′-dimethoxy-7-hydroxyflavylium chloride gives rise, in aqueous solution at moderately acidic pH, to a pH-dependent equilibrium between the flavylium cation, hemiacetal, (*Z*)-chalcone and a small amount of quinonoidal base. The distribution, as a function of pH, of the molar fractions of the several species present in solution have been calculated on the basis of ^{1}H NMR and pH jump experiments monitored by stopped-flow and conventional UV–VIS spectrophotometry, and high-performance liquid chromatography (HPLC). The compound shows interesting photochemical properties: (i) at pH 4.0 it presents a photochromic effect that converts (*Z*)-chalcone into hemiacetal, the reaction being reversible in the dark and (ii) excited-state proton transfer is observed between the flavylium cation and quinonoidal base. An appropriate formalism to quantify the experimental results has been developed. The formalism allows determination of the pH-dependent molar fraction distribution of the several anthocyanin forms present at equilibrium, as well as predicting the distribution of the molar fractions prior to equilibrium.

Synthetic flavylium salts are excellent model compounds of natural anthocyanins, the most important plant pigments,[1] with potential applications in the food industry as colourants. Like natural anthocyanins these compounds, in slightly acidic aqueous solutions, undergo structural transformation as depicted in Scheme 1,[2,3] for the synthetic 3,4′-dimethoxy-7-hydroxyflavylium chloride.



Scheme 1

Recently, we described the photochromic effect that occurs upon light absorption of the (*Z*)-chalcone form of the synthetic flavylium salt 4′,7-dihydroxyflavylium.[4] This photochromic reaction converts the *trans*-chalcone into the flavylium cation, and is based on a *trans–cis* isomerization of a C=C bond, a process which is also used in nature by the Schiff base of 11-(*Z*)-retinal.[5]

In spite of the importance and the challenges that emerge from the study of the photochemistry of anthocyanins and their synthetic analogues, there is a lack of studies on this subject. In the present paper, we report the photochromic effect observed in the 3,4′-dimethoxy-7-hydroxyflavylium chloride, together with another interesting photochemical phenomenon, excited-state proton transfer between the flavylium cation and quinonoidal base.

Experimental

Materials

The flavylium salt was prepared according to the published procedure.[6] All other chemicals used were of analytical grade.

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, except for NMR experiments, where a different procedure was used (see below). Where pH values lower than 1 were used, namely in the fluorescence emission experiments, the negative logarithm of the analytical concentration of the added acid was used.

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.

Photochemical experiments

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

Table 1 Conditions for HPLC measurements

time/min	A(%)	B(%)
0	80	20
10	70	30
20	60	40
25	40	60
30	0	100

measured by iron(III) oxalate actinometry.[7] Estimated errors for quantum yields are 10%.

HPLC

The HPLC equipment consisted of an L-6200A Merck–Hitachi intelligent pump, with a Rheodyne 7125 injection valve, fixed volume 20 μl. The effluent was monitored by means of a diode array UV–VIS detector Merck–Hitachi L-4500. Quantitative measurements were made with a Merck–Hitachi D-6000 interface connected to a computing integrator. The temperature was held constant with an L-5025 Merck–Hitachi column thermostat.

The chromatographic assays were performed with a Nucleosil 100 $5C_{18}$ column (Macherey-Nagel, Düren), particle size 5 μm, 250 × 8 × 4 mm^3. The mobile phase comprised A (aqueous solution of $HClO_4$, pH = 1.5) and B (methanol) in a gradient mode according to Table 1. The flow was 0.8 ml min^{-1} and the temperature 40 °C. A Nucleosil 100-$5C_{18}$, 11 × 8 × 4 mm^3, particle size 5 μm was used as guard column.

NMR

3,4′-Dimethoxy-7-hydroxyflavylium chloride was dissolved in DCl (*ca.* 1 mol l^{-1} in D_2O) to a final concentration of *ca.* 0.1 mmol l^{-1}; the pH of the starting solution was typically 0.5 and was increased by the addition of NaOD. The pH measurements were made in the NMR tube using an Ingold glass electrode, and quoted values are direct meter readings without correction for the isotopic effect[8] and are denoted with an asterisk.

^1H NMR spectra were obtained on a Bruker ARX-400 spectrometer operating at 400.13 MHz. The following conditions were used: presaturation of the residual HDO resonance for 2 s, 70° flip angle, 4.3 s total recycle time and 32 K acquisition data points. Spectra were processed with 1 Hz line-broadening prior to Fourier transformation. Nuclear Overhauser effect spectroscopy (NOESY) was carried out according to ref. 4. Spectra were acquired over a 10 ppm bandwidth, collecting 2048(t_2) × 512(t_1) data points. Spectra were recorded in the phase-sensitive mode by the time-proportional incrementation method and transformed to produce real matrices consisting of 1024 × 1024 data points. Mixing times of 1.0 s were used.

Fluorescence emission

The fluorescence emission spectra were measured using a SPEX F111 Fluorolog spectrofluorimeter. Emission and excitation spectra are corrected.

Stopped-flow apparatus

Stopped-flow experiments were performed with Hi-Tech Scientific equipment incorporating an SU-40 spectrophotometer unit and a Preparative Quench and Stopped-Flow SHU PQ/SF-53. Experiments were carried out using acquisition times of 20 ms, 0.1 s and 10 s at a constant temperature of 23 °C. For the two last acquisition times a filter of 33 ms was used.

Results

Structural transformation in the ground state

***(a)* Equilibrated solutions.** Aqueous solutions of 3,4′-dimethoxy-7-hydroxyflavylium chloride, upon reaching equilibrium in the dark, exhibit pH-dependent absorption spectra, which qualitatively resemble those previously published for similar compounds[1] (Fig. 1). The main feature that emerges from these spectra is the progressive decrease, with increasing pH, of the absorption band centred at 489 nm (inset of Fig. 1) which can be unequivocally attributed to the flavylium cation.

This attribution was confirmed by ^1H NMR spectroscopy which allowed the assignment of the resonances due to the flavylium cation (see Table 2). A spectrum was recorded at a pH* *ca.* 0.5, where the only detectable species in solution is flavylium cation (Fig. 2, A). Resonances due to protons 2′ + 6′ and 3′ + 5′ at 8.23 and 6.84 ppm are immediately recognized from the expected relative intensity of the two protons. The final assignment of these protons (2′ + 6′ at 8.23 ppm) was made based on a NOESY spectrum (see later). The peak centred at 7.15 ppm can be assigned to proton 6 owing to its interaction with proton 5 ($^3J_{6,5}$ = 8.8 Hz) and 8 ($^4J_{6,8}$ = 2.2 Hz); the observation of a 2.2 Hz splitting in the signal at 7.11 ppm allows its assignment to proton 8. The remaining doublet at 7.74 and the singlet at 8.49 ppm, can only be attributed respectively to proton 5 ($^3J_{5,6}$ = 8.8 Hz) and proton 4. Finally

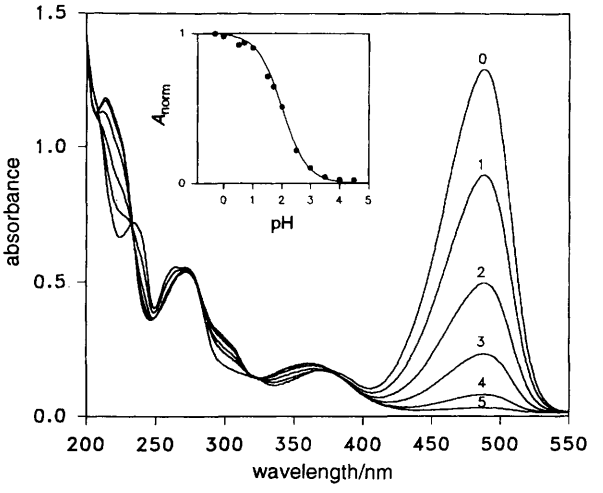


Fig. 1 Absorption spectra of 3,4′-dimethoxy-7-hydroxyflavylium chloride (4.5 × 10^{-5} mol l^{-1}) upon equilibration in the dark, as a function of pH: 0, 1.44; 1, 1.97; 2, 2.46; 3, 2.96; 4, 3.51; 5, 4.03. Inset: normalized absorption of flavylium cation followed at 489 nm.

Table 2 Chemical shifts (δ) and scalar couplings (J/Hz) of the three forms of 3,4′-dimethoxy-7-hydroxyflavylium chloride by ^1H NMR in D_2O at 27 °C

	flavylium[a]		hemiacetal[b]		(*Z*)-chalcone[b]	
	δ	J	δ	J	δ	J
H(2′) + H(6′)	8.23	8.9	7.38	8.8	7.68	8.8
H(3′) + H(5′)	6.84	8.9	6.86	8.8	6.94	8.8
H_4	8.49	—	5.88	—	6.86	—
H_5	7.74	8.8	6.94	8.7	7.81	8.3
H_6	7.15	8.8	6.41	8.7	6.38	8.3
		2.2		2.4		2.3
H_8	7.11	2.2	6.30	2.4	6.27	2.3
3-CH_3	3.94	—	3.46	—	3.56	—
4′-CH_3	3.69	—	3.68	—	3.74	—

[a] pH* 0.5. [b] pH* 4.0.

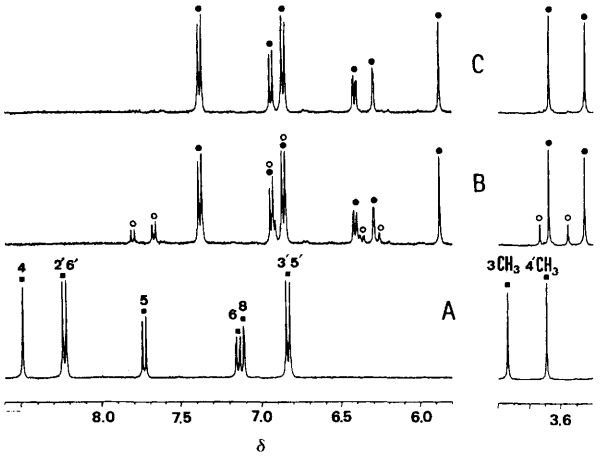


Fig. 2 400 MHz ^{1}H NMR spectra of 3,4′-dimethoxy-7-hydroxyflavylium chloride, 1 mmol l^{-1} in D_2O at 27 °C: A, pH* 0.5; B, pH* 4.0; C, upon 20 min of irradiation. (■) flavylium cation, (●) hemiacetal, (○) (*Z*)-chalcone.

the absorption peaks of the methoxy groups appear at 3.94 and 3.69 ppm. Assignments are summarized in Table 2.

The ^{1}H NMR spectrum of solutions of 3,4′-dimethoxy-7-hydroxyflavylium equilibrated at pH* 4.0 (Fig. 2, B) was also recorded. It is compatible with the existence of two main, different compounds with vestiges of a third. This is particularly clear on the basis of the existence of two sets of peaks relative to the methoxy substituents: one set at 3.74 and 3.56 ppm, the other at 3.68 and 3.46 ppm, with relative areas of 18% and 80% (the remaining 2% may be attributed to quinonoidal base, see below). These relative areas facilitate the assignments in the low-field part of the spectrum, the predominant species having signals at 5.88, 6.30, 6.41 and 7.38 ppm and the minor species at 6.27, 6.38, 7.68 and 7.81 ppm. The signals in the region 6.8–7.0 ppm have contributions from both species. Irradiation of the sample at 366 nm gives rise to an NMR spectrum (Fig. 2, C) containing only the major species and this allows clear identification of the signals in the 6.8–7.0 ppm region. Table 2 also lists these assignments.

At pH* 4.0 it might be expected that the hemiacetal, B, the *trans*-(*Z*)-chalcone, C_Z and the *cis*-(*Z*)-chalcone, C_E, all exist. In order to identify the two species in the spectrum, NOE measurements were run on the equilibrated solution at pH* = 4.0. The minor species was assigned to (*Z*)-chalcone, on the basis of observed NOE connections between H(4) and H(2′) + H(6′), and between the protons of the methyl group at the 3 position and H(5). The major species shows an NOE connection between H(4) and the protons of the methyl group at the 3 position. This is compatible with the presence of B or C_E as the major species. However the chemical shift of H(4) at a rather high field, seems to belong to a poorly conjugated species as is the case with the hemiacetal B, where the presence of the hydroxy groups at the 2 position interrupts resonance between the two benzene moieties.

In order to assign the resonances of the methyl groups, as well as those of the doublets 2′ + 6′ and 3′ + 5′, a NOESY experiment was run on a sample equilibrated at pH* 3.5. The spectrum (not shown) reveals the existence of three species, AH^+, B and C_Z, as well as positive (chemical exchange) and negative (NOE connections) cross-peaks. The first two species, AH^+ and B, are in equilibrium on the NMR timescale and, as expected, equilibrium between B and C_Z is too slow to be observed. The assignments of resonances due to methyl groups in the hemiacetal were made based on the observed NOE connection between H(4) and the methyl group at the 3 position. The other methyl group, at the 4′ position, shows an NOE connection with H(3′) + H(5′), which permits assignment of resonances due to H(2′) + H(6′) and H(3′) + H(5′) in the hemiacetal. Positive cross-peaks due to chemical exchange between AH^+ and B led to total assignment of peaks in both the hemiacetal and the flavylium cation. In the (*Z*)-chalcone species similar assignments were made beginning with an observed NOE connection between H(3′) + H(5′) and the methyl group at the 4′ position.

(*b*) Non-equilibrated solutions. Stopped-flow experiments were carried out by monitoring the absorbance at 489 nm, following a pH jump from 1 to 3.6. The results are depicted in Fig. 3A, and show that the absorbance decreases monoexponentially with a decay time of 0.475 ± 0.002 s^{-1}. No other processes were detected for faster acquisition times (0.1 s or 20 ms). However, this decay is followed by a much slower process, well observed at *ca.* 362 nm (see later). The UV–VIS spectrum obtained at the end of the fast decay is shown in Fig. 4 (curve 0) and presents a small absorption band at 489 nm attributed to the flavylium cation and a strong absorption band at 275 nm. The shape of this last band and its relative position, are identical to the hemiacetal absorption bands detected for other analogous compounds, *i.e.* anthocyanins.[1] Moreover, similar lifetimes for the kinetics of the covalent hydration of the pyrilium nucleus to produce hemiacetal[1] were observed. All these facts lead to the conclusion that upon a pH jump from 1 to 3.6, flavylium cation is transformed into hemiacetal in a few seconds.

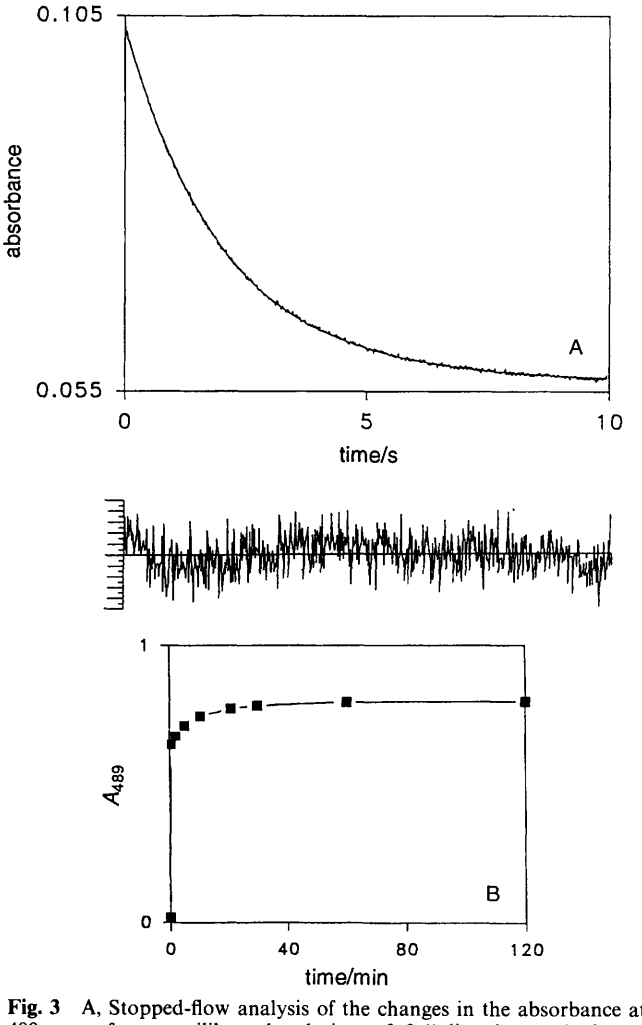


Fig. 3 A, Stopped-flow analysis of the changes in the absorbance at 489 nm of an equilibrated solution of 3,4′-dimethoxy-7-hydroxyflavylium chloride at pH 1.0, upon a pH jump to pH 3.6. B, Changes in the absorbance measured at 489 nm, by means of a conventional spectrophotometer, of a solution of 3,4′-dimethoxy-7-hydroxyflavylium chloride previously equilibrated at pH 4.0, upon a pH jump to pH 0.5.

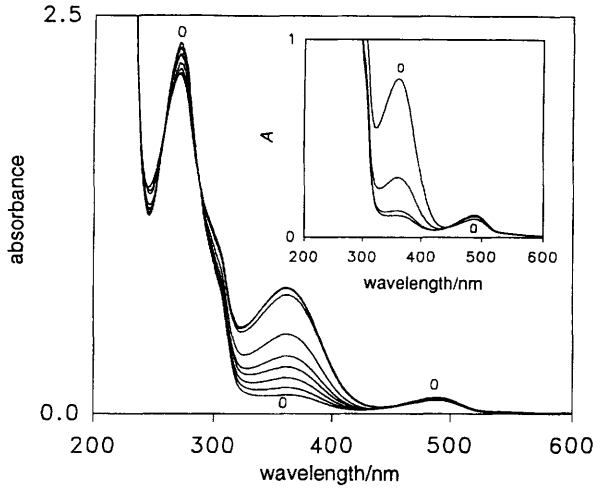


Fig. 4 Thermal evolution in the dark of 3,4′-dimethoxy-7-hydroxyflavylium chloride (1.5×10^{-4} mol l^{-1}) at pH 4.0. 0, initial time and 1, 12; 2, 22; 3, 35; 4, 60; 5, 150; 6, 1000 and 7, 1450–2450 min. Inset: spectral variations upon irradiation at 366 nm of previously thermal equilibrated solutions of 3,4′-dimethoxy-7-hydroxyflavylium chloride (6×10^{-5} mol l^{-1}) at pH 4.0. Light intensity = 5.4×10^{-7} Einstein min^{-1}; 0, initial; and after 1, 10; 2, 20 and 3, 30–40 min.

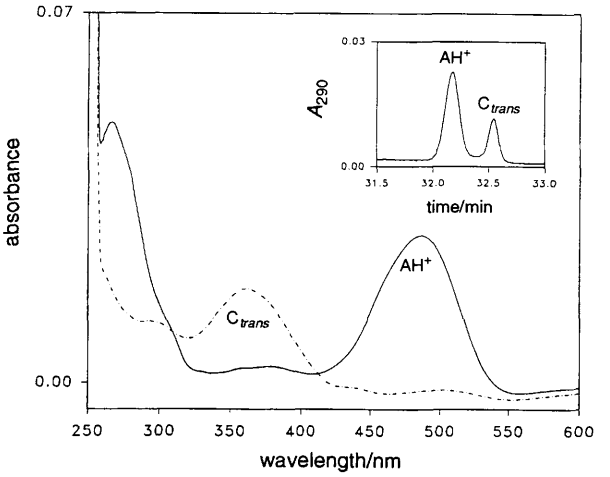


Fig. 5 Absorption spectra of the two species of 3,4′-dimethoxy-7-hydroxyflavylium chloride, separated by HPLC, from a solution previously equilibrated at pH 4.0. The obtained chromatogram is included in the inset.

In addition to this fast process, a second one was observed, (Fig. 4) giving rise to spectral variations that denote a slow formation of a band centred at *ca.* 362 nm which is also the same band observed in the equilibrated solutions at the same pH value (Fig. 1). The shape and relative position of this final band are in accordance with a chalcone absorption. Moreover, its growth corresponds to a decrease in the hemiacetal absorption band at 275 nm. This reaction is very slow, being completed on a timescale of hours, and (on the basis of the above results obtained for the system at equilibrium) can be unequivocally attributed to partial conversion of hemiacetal into (*Z*)-chalcone.

HPLC of solutions previously equilibrated at pH < 1.0 and pH 4.0 was also performed. Following the approach described by Preston and Timberlake,[9] the water component of the eluent gradient was acidified to pH 1.5. This is a very useful way of separating all species that are not involved in a fast equilibrium with the flavylium cation. As expected, HPLC of the solution of the compound previously equilibrated at pH < 1 only shows a single peak with a retention time of 32.2 min and a recorded absorption spectrum characteristic of the flavylium cation. The HPLC of the solution previously equilibrated at pH 4.0, exhibits a second peak with retention time 32.6 min and an absorption spectrum (Fig. 5) characteristic of a chalcone species, in addition to the characteristic peak of flavylium cation. This last HPLC experiment can thus be interpreted as: (i) the first peak is due to the pH jump occurring just upon column injection, which converts into flavylium all the species that are in a fast equilibrium with it and (ii) the second peak, in accordance with previous measurements for other parent compounds,[9] may be attributed to a chalcone species.

The HPLC results were confirmed by monitoring the spectral UV–VIS changes at 489 nm (flavylium absorption maximum) in a conventional spectrophotometer. After a pH jump to 0.5, of a solution previously equilibrated (in the dark) at pH 4, an 'instantaneous' recovery of the flavylium absorption was followed by a subsequent recovery that is complete in less than 1 h (Fig. 3 B). This result is compatible with the existence of a species in fast equilibrium with the flavylium cation, which may be calculated to be 82% of the total, the remaining 18% resulting from the recovery of the form that is in slow equilibrium with AH^+.

In conclusion, from UV–VIS absorption, NMR, stopped-flow and HPLC experimental results, the reaction of the flavylium cation to give its equilibrium products at moderately acidic pH values seems to be composed of (i) very rapid conversion of flavylium cation into hemiacetal followed by (ii) a slower partial conversion of hemiacetal into (*Z*)-chalcone.

Structural transformations involving the excited state

***(a)* Photochromic effect.** Irradiation of equilibrated solutions in the dark at pH 4.0, were carried out at 313 or 366 nm. The reaction was followed by ^1H NMR (Fig. 2C) which clearly shows that irradiation converts the minor (*Z*)-chalcone species into the major hemiacetal species. The photochemical reaction was also followed by UV–VIS spectroscopy (inset of Fig. 4) and the spectral variations are once more consistent with the proposed photochemical reaction. Furthermore, by standing the irradiated solution in the dark, the hemiacetal reconverts to the (*Z*)-chalcone and the previous equilibrium situation prior to irradiation is regained. This corresponds to a photochromic effect in which light converts the minor species into the major species, the reaction reverting thermally in the dark. The quantum yield for the photochemical reaction, measured during the early part of the irradiation was calculated to be 0.006 at 366 nm.

***(b)* Adiabatic proton transfer in the excited state.** Adiabatic excited-state proton transfer (ESPT) is an interesting phenomenon that consists of the transfer of a proton between two excited species.[10–12] In accordance with what has been described for other phenols, namely the classic examples of naphth-1-ol and -2-ol,[11,12] we detected ESPT in the compound 3,4′-dimethoxy-7-hydroxyflavylium.† The results are summarized in Fig. 6. Excitation of a very acidic solution (0.3 mol l^{-1} in $HClO_4$) of the compound at 489 nm gives rise to a fluorescence emission band, with a maximum centred at 534 nm, as depicted in curve 0, Fig. 6A, the respective excitation spectra collected at the emission wavelength of 534 nm, being coincident with the absorption spectrum of the flavylium cation. As the pH is increased up to 4, the fluorescence emission intensity (λ_{exc} = 489 nm) decreases, as would be expected, because at this excitation wavelength the absorption is exclusively due to the flavylium cation. However, the shapes of the fluorescence emission spectra of the very acidic solution when

† A similar effect was also detected in 4′,7-dihydroxyflavylium.[13]

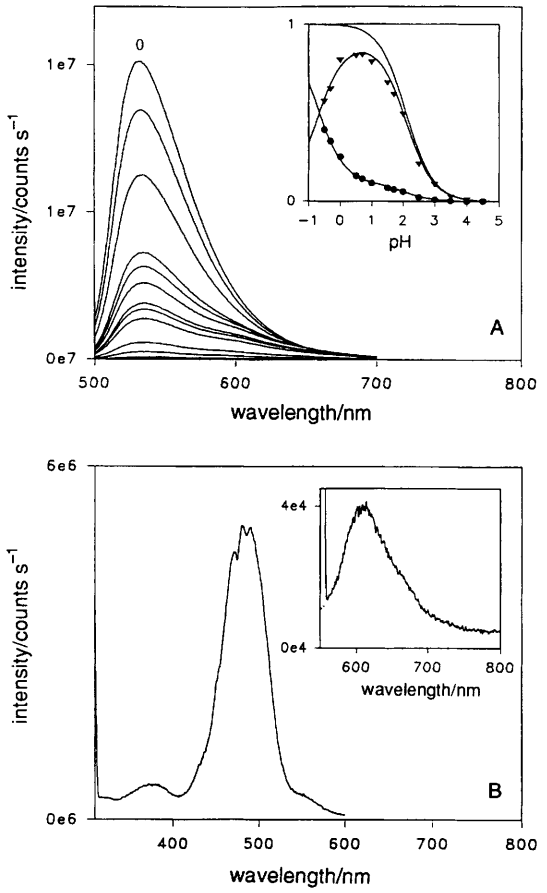


Fig. 6 A, Fluorescence emission spectra (λ_{exc} = 489 nm) of 3,4′-dimethoxy-7-hydroxyflavylium chloride (5×10^{-6} mol l^{-1}) as a function of pH. The global intensity of the spectra decreases with increasing pH, but the shape of the curves are different, showing the influence of the emission from the basic species occurring at less acidic pH values. Inset: qualitative trend of the normalized fluorescence emission of the flavylium cation (●) and quinonoidal base (▼), followed at their respective emission maxima, upon separation of both emissions as described in the literature.[11,12] B, Excitation spectra (λ_{em} = 610 nm) of 3,4′-dimethoxy-7-hydroxyflavylium chloride (5×10^{-5} mol l^{-1}) at pH 4.0. Inset: emission spectrum obtained by selective excitation of the quinonoidal base, λ_{exc} = 550 nm, pH 4.0.

compared with those of less acidic solutions are not coincident. The ratio of the emission intensities at 610 and 534 nm increases with increasing pH. An obvious candidate to explain this new emission at 610 nm is the quinonoidal base formed *via* ESPT.

The emission from the quinonoidal base was confirmed by careful analysis at pH 4.0. At this pH the total amount of quinonoidal base was calculated from ^{1}H NMR to be *ca.* 2%, which is of the same order of magnitude as the flavylium cation. For that reason, in a more concentrated solution than that of the previous experiment, the emission spectrum was measured at an excitation wavelength of 550 nm. This wavelength was chosen because the absorption spectrum of the quinonoidal base is expected to be red-shifted in comparison with that of the flavylium cation. The result is depicted in the inset of Fig. 6B and may be considered as the 'pure' emission from the quinonoidal base.

The presence of ESPT was confirmed by the excitation spectrum carried out at the emission wavelength of 620 nm, (Fig. 6B), where the quinonoidal base is the emissive species. This spectrum shows an intense band analogous to the characteristic absorption of the flavylium cation. In addition a small band at the position expected for the quinonoidal base was observed, confirming the possibility of selective excitation of the quinonoidal base yielding its 'pure' emission spectrum.

Discussion

In the previous section we accounted for the chemical nature of the several pH-dependent species that are formed upon dissolution (or pH jumps) of 3,4′-dimethoxy-7-hydroxyflavylium in aqueous solutions. Here, we discuss and quantify the experimental results through a simple formalism that accounts for both, the molar fraction distribution and the equilibrium constants of the several anthocyanin species. In addition, the formalism can be applied to simulate situations out of the equilibrium, without introducing more parameters than those used in the equilibrium situation.

The formalism is based on two simple functions denoted α and β, defined as follows for a single acid–base equilibrium, characterized by its acidity constant K_a, as described by eqn. (I)

$$RH^+ \overset{K_a}{\rightleftharpoons} R + H^+ \qquad \text{(I)}$$

The activities of the species RH^+ and R may be substituted by the corresponding concentrations, as long as the ionic strength is maintained constant, or alternatively by calculation of the relevant activity coefficients. If, under our working conditions, the autoprotolysis equilibrium of water can be neglected, the molar fraction of the acidic and basic forms will be given by eqn. (1) and (2).

$$\alpha = \frac{[RH^+]}{[RH^+] + [R]} = \frac{[H^+]}{[H^+] + K_a} \qquad (1)$$

$$\beta = \frac{[R]}{[RH^+] + [R]} = \frac{K_a}{[H^+] + K_a} \qquad (2)$$

The shape of the α (and $\beta = 1 - \alpha$) functions *vs.* pH, is the well known sigmoid, an example of this shape being depicted in the inset of Fig. 1.

α or β can be determined experimentally by *e.g.* ^{1}H NMR[15] or, more easily using UV–VIS spectrophotometry. In the latter case the following expression is straightforwardly deduced:

$$\alpha = \frac{A/A^0 - C_t}{1 - C_t} \qquad (3)$$

where A is the absorbance, monitored at a fixed wavelength, for different pH values, and $A^0 = \varepsilon_{RH^+} C_0$, the normalizing factor obtained at a sufficiently acidic pH value to yield exclusively the acidic form, (ε_{RH^+} being the molar absorption coefficient of the acidic species and C_0 the total concentration). α is thus accessible experimentally through eqn. (3) because the constant, C_t, is also obtained from experimental data, being equal to the ratio A/A^0 at extremely basic pH. In effect, the two limiting situations can be observed: (i) at sufficiently low pH in order to assure that the acidic form is the sole species present in solution, $A = A^0 = \varepsilon_{RH^+} C_0$ and by consequence the ratio A/A^0 is equal to unity, and $\alpha = 1$; (ii) for sufficiently high pH values, the basic form is the exclusive species in solution, $A = \varepsilon_R C_0$ ($\alpha = 0$) and the ratio A/A^0 is equal to $C_t = \varepsilon_R/\varepsilon_{RH^+}$.

The experimental α obtained through eqn. (3), can be adjusted by fitting to a theoretical α as defined by eqn. (1), leading to the value of K_a. Alternatively eqn. (1) can be converted to a linear relation, as shown in eqn. (4), and K_a can be obtained from its slope.

$$\frac{1}{\alpha} = 1 + K_a \frac{1}{[H^+]} \qquad (4)$$

The simple picture described above is complicated in the case of anthocyanins and related compounds. These species are involved in pH-dependent multi-equilibria as was previously shown in Scheme 1. However, in spite of its more complicated form, the molar fractions of all these species can be

written in terms of the equilibrium constants, as shown in Appendix 1. The main feature that emerges from these expressions, according to previous results from other authors,[1] is that the flavylium cation behaves like the acidic form of a single acid–base equilibrium, an α function in which the acidity constant, K_a, is substituted by K'_a

$$\frac{[AH^+]}{C_0} = \frac{[H^+]}{[H^+] + K'_a} = \alpha' \quad (5)$$

$$K'_a = K_a + K_b + K_t K_b + K_i K_t K_b \quad (6)$$

In contrast with the flavylium cation that decreases in concentration as the pH increases, all the remaining species increase their molar fraction with increasing pH, presenting a shape which is a product of a factor (lower than 1) by the complementary β function. Moreover, the sum of all the molar fractions of the species other than flavylium cation, is coincident with the $\beta' = 1 - \alpha'$ function.

In order to determine α' experimentally, exactly the same procedure used to calculate K_a can be employed to calculate K'_a. Finally, this method simplifies the determination of the molar fractions of the several species in equilibria, using the following procedure: (i) from the spectrophotometric measurements the molar fraction of flavylium cation is obtained and consequently K'_a, as well as α' and β'; (ii) from a single ^{1}H NMR spectrum taken at a pH value of the basic plateau, the equilibrium molar fractions of the remaining species can be determined. These data are sufficient to determine all the molar fractions of the equilibrium species as well as the equilibrium constants, see Appendix 1. Moreover, this procedure avoids the very time-consuming ^{1}H NMR titration, a method which has been previously used for the same purposes.[14]

Application of this general formalism to the compound 3,4′-dimethoxy-7-hydroxyflavylium chloride, gives rise to a simplification, because in the equilibria only hemiacetal and (*Z*)-chalcone, as well as vestiges of quinonoidal base, were detected.

The experimental value of α' was calculated by means of eqn. (3), monitoring at the absorption maximum of flavylium cation at 489 nm. Fitting was achieved for $pK'_a = 2.1$ ($K'_a = 7.94 \times 10^{-3}$), see inset of Fig. 1.

As mentioned above, one great advantage of this formalism is that determination of the molar fraction of the species other than flavylium cation only needs the calculation of the pre-factors by which β must be multiplied, eqn. (A5)–(A8) of the appendix. These pre-factors determine the respective plateaux, which are reached at sufficiently basic pH values, (*i.e.* when β' becomes equal to 1). These plateau concentrations were previously calculated from ^{1}H NMR and pH jump experiments described in the Results section. On the basis of these data, the values for the molar fractions at the plateau of hemiacetal, (*Z*)-chalcone and quinonoidal base are, respectively, 0.80, 0.18 and 0.02, leading to $K_b = 6.2 \times 10^{-3}$, the product $K_i K_t = 0.26$ and $K_a = 1.6 \times 10^{-4}$. In Fig. 7 the molar fractions of the several species in equilibrium with 3,4′-dimethoxy-7-hydroxyflavylium chloride are represented as a function of pH (full lines).

Non-equilibrium systems

Let us assume that the flavylium cation is dissolved at a particular pH value, or that the system suffers a pH jump from the acidic region where flavylium is the dominant species to a less acidic pH value. According to the results described above, the system will reach, in less than 10 s, what may be considered a pre-equilibrium state involving all the species except the (*Z*)-chalcone. This occurs because the isomerization is the rate-controlling step, and is much slower than all the other processes. The expressions for the pre-equilibrium molar fractions are readily defined, by neglecting the *cis–trans* isomerization equilibrium ($K_i = 0$), as shown in Appendix 1. This is especially useful in predicting the nature of the anthocyanin species that are formed in a timescale of a few seconds, just upon dissolution of flavylium cation in moderate acidic aqueous solutions (or upon a pH jump from very acidic to less acidic values).

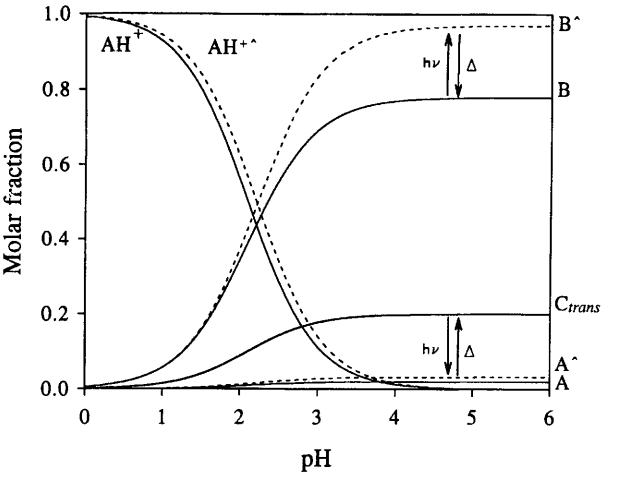


Fig. 7 Representation of the molar fraction distribution of the several species present in solution of 3,4′-dimethoxy-7-hydroxyflavylium chloride as a function of pH. Full lines correspond to the equilibrium situation; dotted lines correspond to the predictions for a pre-equilibrium situation, obtained a few seconds after a pH jump from a very acidic medium to a pH value of the basic plateau (*ca.* pH 4 to pH 6). Calculations were carried out through the mathematical approach described in the Discussion.

The pre-equilibrium molar fractions of 3,4′-dimethoxy-7-hydroxyflavylium chloride (Fig. 7, dotted lines) can be calculated using eqn. (A4)–(A6) of the appendix and $K_a^\wedge = 6.36 \times 10^{-3}$. Inspection of this figure explains why: (i) at pH = 2.52 (not shown) the system presents a pre-equilibrium of flavylium cation and hemiacetal as well as a small amount of quinonoidal base, and the final equilibrium consists of flavylium cation, hemiacetal and (*Z*)-chalcone; (ii) at pH 4 the pre-equilibrium is mainly hemiacetal and a small amount of quinonoidal base to give a final equilibrium of hemiacetal and (*Z*)-chalcone.

***(a)* Photochromic effect.** The effect of light on 3,4′-dimethoxy-7-hydroxyflavylium, at pH 4, can be explained as a *trans–cis* photoisomerization which is reversible in the dark. Fig. 7 is also useful to visualize the photochromic effect. Light places the system in the position of the pre-equilibrium. For example, at pH 4 the hemiacetal is formed at the expense of (*Z*)-chalcone. In the dark the hemiacetal is restored and again coexists in equilibrium with (*Z*)-chalcone.

***(b)* Adiabatic proton transfer.** The two emissions observed at the excitation wavelength of 498 nm, were separated and normalized following procedures described in the literature,† and are shown in the inset of Fig. 6A. The picture must be viewed as a qualitative trend because the emission maximum of the flavylium cation is not accessible experimentally, probably lying at pH values lower than −1. Moreover, normalization of the emission from the basic species is subject to large errors.[12,15] However, in spite of its qualitative aspect, the picture clearly shows the ESPT phenomenon. Considering that the lifetimes of the quinonoidal base and flavylium cation

† The normalization of the emission from the basic form was carried out considering that the sum of the normalized emissions from the basic and acidic species is equal to 1. (See ref. 11, 12). However, this is only valid if quenching effects of proton and buffer are not present.

are of the same order of magnitude,[11,12] a rough value of the excited state $pK^* = -1$ can be assumed.‡ From the Förster cycle,[10,14] based on both the emission and absorption maxima of the acidic and basic species, the difference between the ground and excited pKs, $\Delta pK = pK_a - pK_a^*$ was calculated to be *ca.* 4.7. This value allows one to estimate the excited state $pK_a^* = -0.86$ (using the above calculated value of $K_a = 1.58 \times 10^{-4}$), which qualitatively justifies the previous assumptions.

Conclusions

The existence of *trans*-chalcones in anthocyanins, as well as in their synthetic analogues is definitively proved.[1–4,16] In spite of this, the role played by these species in the interconversion kinetics of several anthocyanins has not been sufficiently emphasized. In effect, the *cis–trans* isomerization seems to be the determining step in the thermal processes of ring-opening and closure. In addition, we have shown that the same reaction is the key step in the photochromic effect observed in synthetic flavylium salts. The existence of this slower process (*cis–trans* isomerization) allows definition of a pre-equilibrium state upon a pH jump or UV–VIS irradiation. The nature of the anthocyanin species involved in this pre-equilibrium state is easily predicted by application of the model developed here.

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Appendix 1

Let us consider the overall pigment concentration characterized by the equilibrium constants, K_a, K_b, K_t, K_i according to Scheme 1:

$$C_0 = [AH^+] + [A] + [B] + [C_Z] + [C_Z] \quad \text{(A1)}$$

$$C_0 = [AH^+]\left(1 + \frac{K_a}{[H^+]} + \frac{K_b}{[H^+]} + \frac{K_t K_b}{[H^+]} + \frac{K_i K_t K_b}{[H^+]}\right) \quad \text{(A2)}$$

Introducing

$$K'_a = K_a + K_b + K_t K_b + K_i K_t K_b \quad \text{(A3)}$$

and rearranging eqn. (A2), the molar fraction of the acidic form can be obtained through eqn. (A4)

$$\frac{[AH^+]}{C_0} = \alpha' = \frac{[H^+]}{[H^+] + K'_a} \quad \text{(A4)}$$

The other remaining species are calculated using the same strategy

$$\frac{[A]}{C_0} = \frac{K_a}{[H^+]}\alpha' = \frac{K_a}{K'_a}\frac{K'_a}{[H^+] + K'_a} = \frac{K_a}{K'_a}\beta' \quad \text{(A5)}$$

$$\frac{[B]}{C_0} = \frac{K_b}{K'_a}\beta' \quad \text{(A6)}$$

$$\frac{[C_E]}{C_0} = \frac{K_t K_b}{K'_a}\beta' \quad \text{(A7)}$$

$$\frac{[C_Z]}{C_0} = \frac{K_i K_t K_b}{K'_a}\beta' \quad \text{(A8)}$$

with β' defined by eqn. (A9)

$$\beta' = \frac{K'_a}{[H^+] + K'_a} = 1 - \alpha' \quad \text{(A9)}$$

In the cases where the (*Z*)-chalcone is not observed (as in the case of 3,4′-dimethoxy-7-hydroxyflavylium chloride), the same equations are once more valid if K'_a is substituted by K''_a.

$$K''_a = K_a + K_b + K_i K_t K_b \quad \text{(A10)}$$

Eqn. (A1) to (A9) can also be used to account for the molar fraction distribution in the case of the pre-equilibrium. Assuming a pH jump from previously equilibrated solutions at pH 1.0 to pH 4.0, eqn. (A1) to (A9) are also applicable if K'_a is substituted by $K_a^\wedge$ defined according to eqn. (A11).

$$K_a^\wedge = K_a + K_b + K_t K_b \quad \text{(A11)}$$

In the case of a pH jump from a previously equilibrated solution at pH 4.0 to pH 1.0 the pre-equilibrium is once more accounted for by eqn. (A1) to (A9) and $K_a^\wedge$ defined according to eqn. (A11), but C_0 must be substituted by $C_0^\wedge$.

The pre-equilibrium equations applied to the compound 3,4′-dimethoxy-7-hydroxyflavylium chloride are simpler because of the lack of (*E*)-chalcone and $K_a^\wedge$ is given by the sum of $K_a + K_b$.

‡ With $K_{ap}^* = (\tau/\tau')\,(k_a^*/k_{-a}^*)$ the symbols representing, respectively, lifetime of the acid and basic excited species and rate constants for excited state deprotonation and protonation.

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