Quantitative Measurement of Proton Dissociation and Tautomeric Constants of Apigeninidin

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Depending on the pH of the medium, the anthocyanidin apigeninidin [5,7-dihydroxy-2-(4'-hydroxyphenyl)benzopyrilium chloride] may deprotonate, leading to the formation of three tautomeric neutral and anionic forms. In order to determine the relative percentages of the various forms which are present in solution at a given pH, the three possible derivatives of apigeninidin in which two hydroxys are replaced by methoxy groups and the three in which only one hydroxy is replaced by a methoxy group were synthesized. From the first three derivatives only one neutral form and from the second three only one anionic form can be generated by proton dissociation; the study of these compounds made possible the measurement of the proton dissociation constants of each tautomer of apigeninidin and allowed us to calculate the tautomeric ratio of its three neutral and anionic forms. Moreover, our results give evidence of the existence of a dianionic form present at pH close to neutrality ($pK'_a = 8.06$). From the results obtained it is possible to calculate the composition of the mixture of the different species which can originate from the cationic form of apigeninidin at any pH by proton dissociation and tautomeric equilibria.

Anthocyanin pigments are glycosylated polyhydroxy derivatives of (2-phenyl)benzopyrilium (flavylium) salts; they are largely responsible for the colour of flowers and fruits, possess pharmacological properties and are also studied as food colouring agents.[1] Depending on the number of hydroxyls present on the flavylium nucleus, the anthocyanins can give rise, by means of proton dissociation, to various neutral or anionic species in prototropic tautomeric equilibrium. These transformations can also be accompanied by a hydration reaction on the flavylium cation, leading to colourless forms, hemiacetals and chalcones.[2]

The formation of the neutral quinoidal bases and the anionic forms of the anthocyanins, with the formation of non-covalent complexes between these coloured forms and other substances, the copigments, not coloured themselves, appear to be one of the main factors responsible for the wide range of colours present in the vegetable world.[3,4]

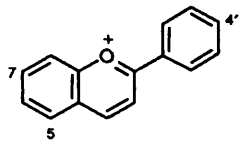
Apigeninidin[5,7-dihydroxy-2-(4'-hydroxyphenyl)benzopyrilium chloride] ($\mathbf{AH^+}$) is one of the most common aglycone of anthocyanins. In slightly acidic media $\mathbf{AH^+}$ may deprotonate, leading to three quinoidal bases (neutral forms) in prototropic tautomeric equilibrium (**A5**, **A7**, **A4'**, Scheme 1); deprotonation may occur at any of its three hydroxy groups (at C-5, C-7 or C-4').[2,5] At increasing pH, another proton may dissociate, giving rise to three anionic forms in prototropic tautomeric equilibrium ($\mathbf{A54'^-}$, $\mathbf{A74'^-}$, $\mathbf{A57^-}$, Scheme 1).[2] Currently, nothing is known about the respective amount of each neutral and anionic tautomer.

Thus, in order to gain an insight into the process by which the colouring of flowers and fruits develops and to establish quantitative relationships between chemical structure and biological activity in the anthocyanin class of compounds, we needed to determine the composition of the mixture of species which, at a given pH, can originate by proton dissociation and tautomeric equilibria.

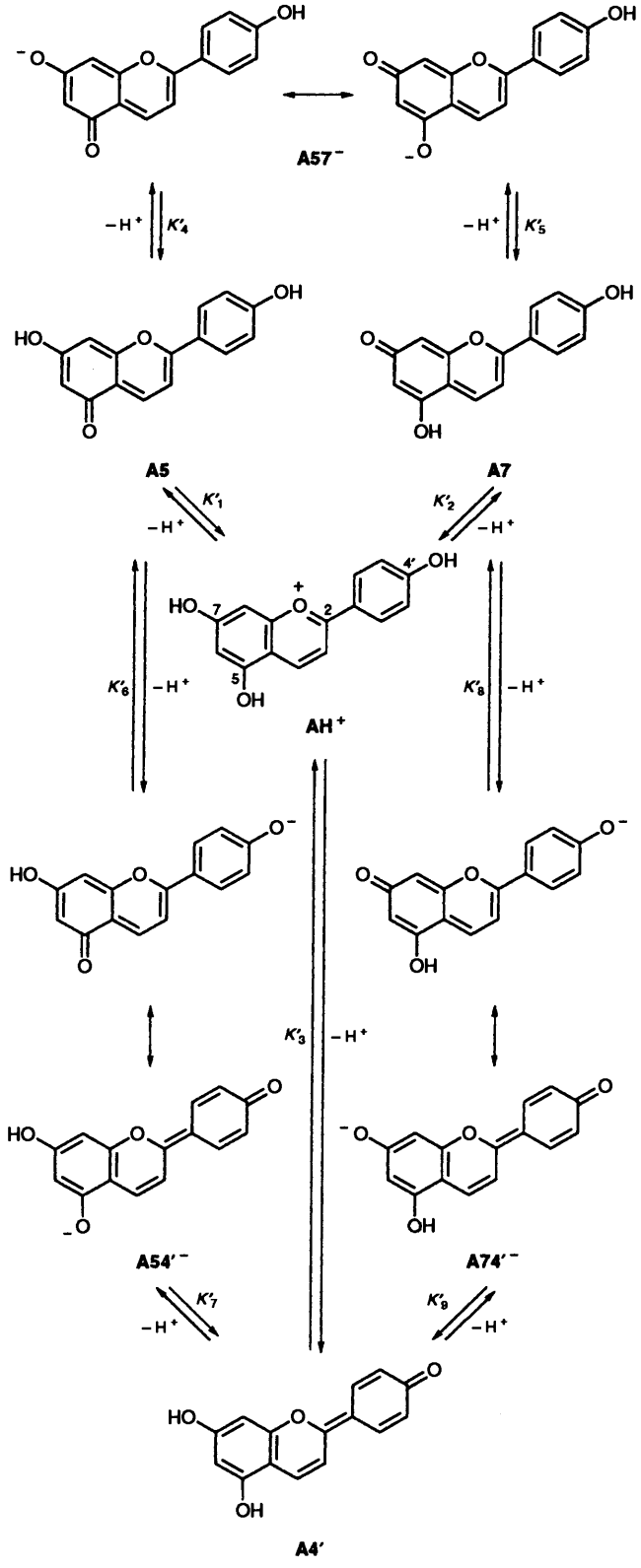
This problem, which we had already examined by means of theoretical molecular orbital calculations,[6] is experimentally solved by means of a simplification of the complex problem of the tautomerism of apigeninidin: to this end we synthesized the three flavylium salts in which two hydroxy groups of apigeninidin are replaced by methoxy groups [5-hydroxy-7-methoxy-2-(4'-methoxyphenyl)- **1**; 7-hydroxy-5-methoxy-2-(4'-methoxyphenyl)- **2**; 2-(4'-hydroxyphenyl)-5,7-dimethoxy-benzopyrilium chloride **3**] and the three in which only one hydroxy group was replaced by one methoxy group [5,7-dihydroxy-2-(4'-methoxyphenyl)- **4**; 5-hydroxy-2-(4'-hydroxyphenyl-7-methoxy- **5**; 7-hydroxy-2-(4'-hydroxyphenyl)-5-methoxy-benzopyrilium chloride **6**]. Proton dissociation, in the case of compounds **1–3**, selectively gives rise to three neutral forms (**A5**, **A7**, **A4'** respectively, Table 1) while in the case of compounds **4–6** it generates three anionic forms ($\mathbf{A57^-}$, $\mathbf{A54'^-}$, $\mathbf{A74'^-}$ respectively, Table 1); all of these forms can potentially be formed by apigeninidin (Scheme 1, Table 1).

By studying these compounds, given that they can only form one tautomer for each molecule and assuming that hydroxy/methoxy substitution does not appreciably affect the acidity of the remaining hydroxys, we determined the proton dissociation constants of each tautomer of apigeninidin. From the measured

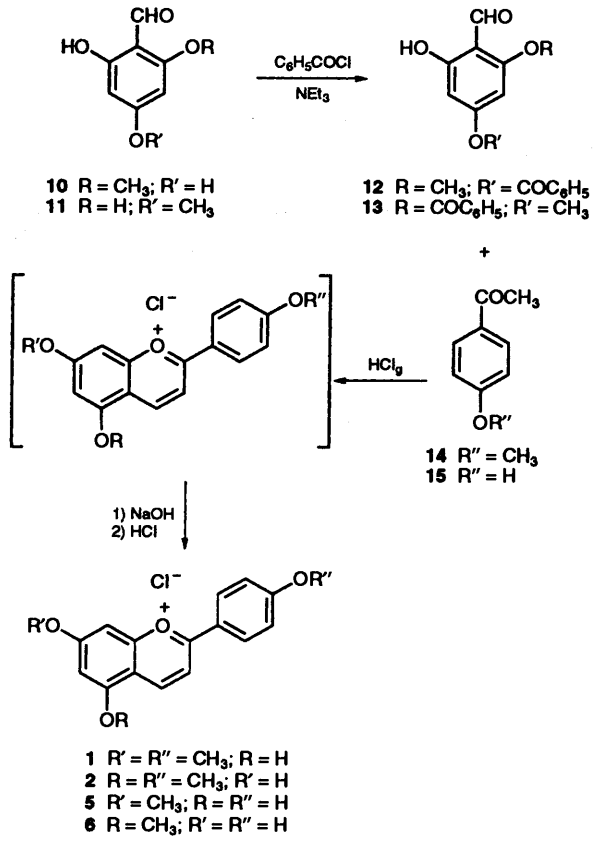
Table 1 Possible tautomeric forms which can originate by proton dissociation from compounds **1–6** and apigeninidin (**7**)



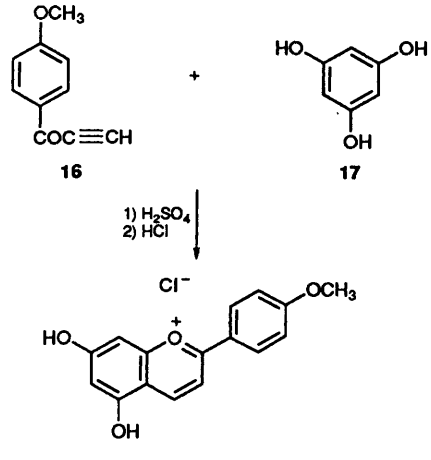
	Compound substitution				
	C-5	C-7	C-4'	Neutral forms	Anionic forms
1	OH	OCH_3	OCH_3	**A5**	
2	OCH_3	OH	OCH_3	**A7**	
3	OCH_3	OCH_3	OH	**A4'**	
4	OH	OH	OCH_3	**A5**, **A7**	$\mathbf{A57^-}$
5	OH	OCH_3	OH	**A5**, **A4'**	$\mathbf{A54'^-}$
6	OCH_3	OH	OH	**A7**, **A4'**	$\mathbf{A74'^-}$
7	OH	OH	OH	**A5**, **A7**, **A4'**	$\mathbf{A57^-}$, $\mathbf{A54'^-}$, $\mathbf{A74'^-}$



Scheme 1 Prototropic equilibria of apigeninidin (AH^+)



Scheme 2



Scheme 3

pK'_a we were then able to determine the abundance percentages of the three neutral and anionic tautomers of apigeninidin. On the basis of UV–VIS spectroscopy, we then evidenced subsequent proton dissociation of the monoanionic forms of apigeninidin (**A57**$^-$, **A54'**$^-$, **A74'**$^-$) with formation of a dianionic species. Considering the existence of this form, it is possible to calculate the composition of the different species of apigeninidin which, at a given pH, proton dissociations and tautomeric equilibria can give rise to.

The new compounds **1**, **2**, **3**, **5** and **6** were synthesized by the reaction between the substituted 2-hydroxybenzaldehyde and the 4-substituted acetophenone. For the synthesis of compounds **1**, **2**, **5** and **6** it was still necessary to protect beforehand the hydroxy group of the substituted benzaldehyde not involved in the formation of the benzopyrilium nucleus, by means of benzoylation; the protecting group was subsequently removed by treatment with sodium hydroxide: **1**, **2**, **5** and **6** are then obtained by reacidification (Scheme 2); compound **4** was synthesized by condensation of the ethynyl *p*-methoxyphenyl ketone with 1, 3, 5-trihydroxybenzene (Scheme 3).

Results

Determination of the Tautomeric Constants of the Neutral Forms of Apigeninidin.—In view of the first dissociation of apigeninidin (AH^+), which leads to formation of the three neutral forms **A5**, **A7** and **A4'** (Scheme 1), we can write:

$$AH^+ \overset{K'_1}{\rightleftharpoons} A5 + H^+$$

$$AH^+ \overset{K'_2}{\rightleftharpoons} A7 + H^+$$

$$AH^+ \overset{K'_3}{\rightleftharpoons} A4' + H^+$$

$$K'_1 = \frac{[A5]\cdot a_{H^+}}{[AH^+]} \quad (1)$$

$$K'_2 = \frac{[A7]\cdot a_{H^+}}{[AH^+]} \quad (2)$$

$$K'_3 = \frac{[A4']\cdot a_{H^+}}{[AH^+]} \quad (3)$$

Assuming that hydroxy/methoxy substitution does not appreciably affect the acidity of the remaining hydroxys of apigeninidin, the values of the equilibrium constants K'_1, K'_2 and K'_3 can be considered to be equal to the proton dissociation constants of the molecules **1**, **2** and **3**, respectively [$pK'_{a(AH^+)}$, Table 2], since each of these molecules can only give rise to one neutral form (Table 1). From K'_1, K'_2 and K'_3 we obtain the tautomeric constants:

$$A7 \rightleftharpoons A5 \quad K_{T_1} = 0.214$$

$$A7 \rightleftharpoons A4' \quad K_{T_2} = 0.009$$

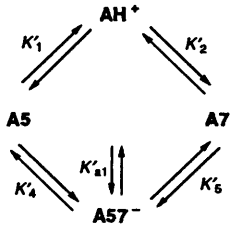
$$A5 \rightleftharpoons A4' \quad K_{T_3} = 0.042$$

The abundance percentages of each neutral tautomer of apigeninidin can be calculated with eqn. (4) (only shown for **A5**)

$$\%A5 = \frac{[A5]}{[A5] + [A7] + [A4']} \cdot 100 \quad (4)$$

where concentrations are related by eqns. (1)–(3). These are: **A7** = 81.8, **A5** = 17.5 and **A4'** = 0.7%.

Determination of the Tautomeric Constants of the Anionic Forms of Apigeninidin.—With regard to the formation of the three anionic forms, $A57^-$, $A54'^-$ and $A74'^-$, of apigeninidin (Scheme 1), the proton dissociation equilibria of compounds **4**–**6** have to be taken into account. In the case of the $A57^-$ form, one can write:



where eqns. (5)–(7) hold with $K'_5/K'_4 = K'_1/K'_2$.

Table 2 Proton dissociation constants (pK'_a) at 20 °C, I = 0.1 mol dm^{-3}

Compound	$pK'_{a(AH^+)}$	λ/nm	$pK'_{a(A)}$	λ/nm
1	4.62 ± 0.03[a]	466		
2	3.95 ± 0.03	470		
3	6.00 ± 0.03	511		
4	3.89 ± 0.05	424	6.71 ± 0.05	424
5	4.54 ± 0.02	466	7.27 ± 0.04	536
6	4.10 ± 0.02	469	7.48 ± 0.01	540
8	4.80 ± 0.02	467		
	4.85 ± 0.04[b]			
9	4.24 ± 0.02	500	7.28 ± 0.03	533
	4.30 ± 0.05[b]			

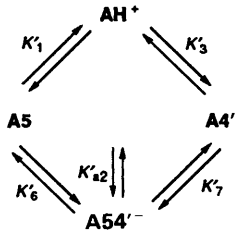
[a] Standard deviation. [b] pK'_a at 25 °C and $I < 10^{-2}$ mol dm^{-3}, obtained by means of pH-jump experiments, from ref. 5

$$K'_4 = \frac{[A57^-]\cdot a_{H^+}}{[A5]} \quad (5)$$

$$K'_5 = \frac{[A57^-]\cdot a_{H^+}}{[A7]} \quad (6)$$

$$K'_{a1} = \frac{[A57^-]\cdot a_{H^+}}{[A5] + [A7]} \quad (7)$$

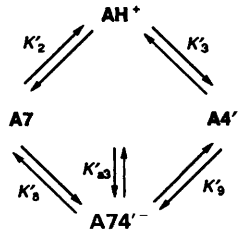
Thus, knowing K'_1 and K'_2, which are the proton dissociation constants of compounds **1** and **2**, respectively, and having determined K'_{a1}, which is the proton dissociation constant of the neutral forms of **4** [$pK'_{a(A)}$, Table 2], eqns. (5)–(7) allow us to calculate K'_5 and K'_4. The other equilibria are dealt with in a similar manner to determine the values of K'_6, K'_7, K'_8 and K'_9 [eqns. (8)–(13)].



$$K'_6 = \frac{[A54'^-]\cdot a_{H^+}}{[A5]} \quad (8)$$

$$K'_7 = \frac{[A54'^-]\cdot a_{H^+}}{[A4']} \quad (9)$$

$$K'_{a2} = \frac{[A54'^-]\cdot a_{H^+}}{[A5] + [A4']} \quad (10)$$



$$K'_8 = \frac{[A74'^-]\cdot a_{H^+}}{[A7]} \quad (11)$$

$$K'_9 = \frac{[A74'^-]\cdot a_{H^+}}{[A4']} \quad (12)$$

$$K'_{a3} = \frac{[A74'^-]\cdot a_{H^+}}{[A7] + [A4']} \quad (13)$$

K'_{a2} and K'_{a3} are the proton dissociation constants of the neutral forms of **5** and **6**, respectively [$pK'_{a(A)}$, Table 2].

The tautomeric constants of the three anionic forms are calculated from the values of K'_4–K'_9:

$$A57^- \rightleftharpoons A54'^- \quad K_{T_4} = 0.051$$

$$A57^- \rightleftharpoons A74'^- \quad K_{T_5} = 0.141$$

$$A54'^- \rightleftharpoons A74'^- \quad K_{T_6} = 2.793$$

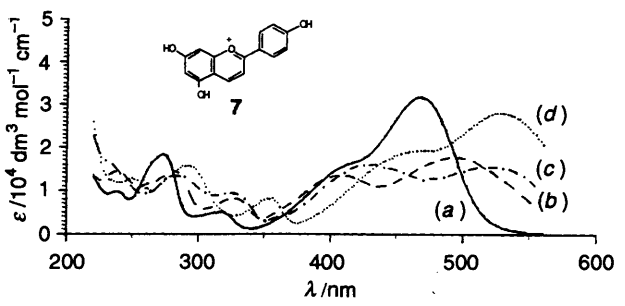


Fig. 1 UV–VIS spectra of apigeninidin (7) in solution at pH of 1.70, 5.59, 6.77 and 11.30 (spectra (*a*)–(*d*) resp.) (I = 0.1 mol dm^{-3}, 20 °C)

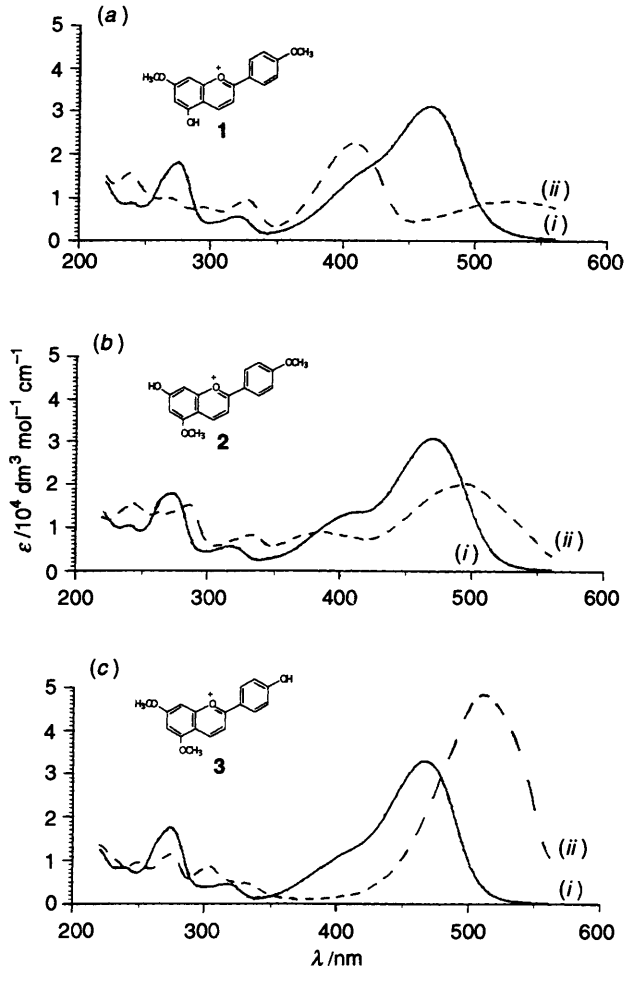


Fig. 2 UV–VIS spectra of the neutral tautomers **A5**, **A7** and **A4′** [spectra (*ii*), Fig. 2 (*a*)–(*c*)] originated from molecules **1**–**3** [cationic forms: spectra (*i*)] respectively (I = 0.1 mol dm^{-3}, 20 °C)

The abundance percentages of each anionic tautomer of apigeninidin were calculated with eqn. (14) (only shown for **A57**$^-$), where the concentrations are related by eqns. (5)–(13):

$$\%\mathbf{A57}^- = \frac{[\mathbf{A57}^-]}{[\mathbf{A57}^-] + [\mathbf{A54'}^-] + [\mathbf{A74'}^-]} \cdot 100 \quad (14)$$

These are: **A57**$^-$ = 83.9, **A54′**$^-$ = 4.2, **A74′**$^-$ = 11.9%.

The Dianionic Form of Apigeninidin.—The behaviour of apigeninidin in solution at different pH levels can be interpreted by comparing its UV–VIS absorption spectra at a given pH (Fig. 1), obtained in the same manner as described in the procedure, relative to the determination of the proton dissociation constants, with the spectra of the neutral tautomers **A5**, **A7** and **A4′**, originating from molecules **1**–**3** [Fig. 2, (*ii*)], and those of the anionic forms, **A57**$^-$, **A54′**$^-$ and **A74′**$^-$, which originate from molecules **4**–**6** [Fig. 3, (*iii*)].

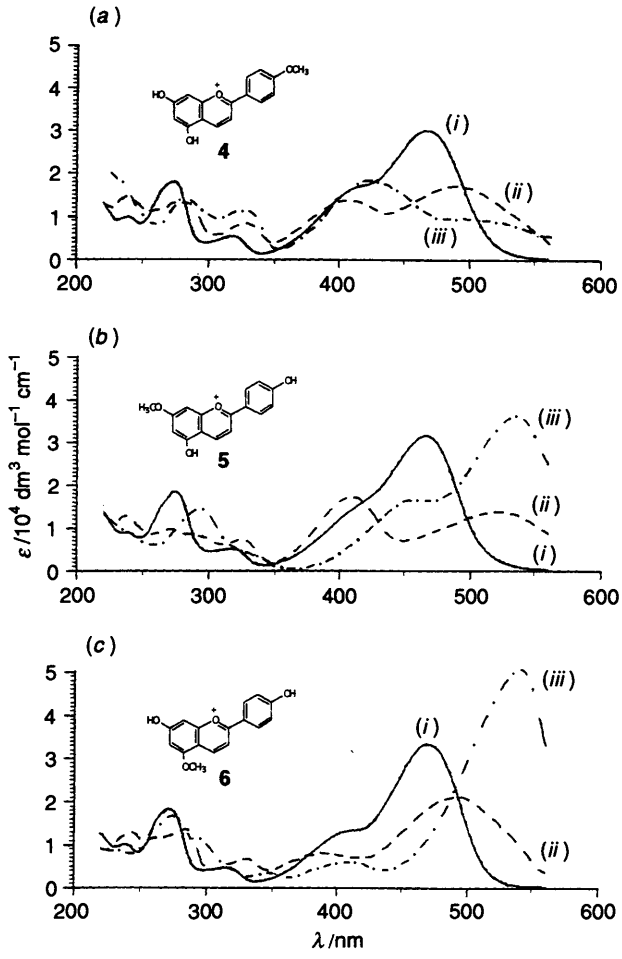


Fig. 3 UV-VIS spectra of the anionic tautomers **A57**$^-$, **A54′**$^-$ and **A74′**$^-$ [spectra (*iii*), Fig. 3 (*a*)–(*c*)] originated from molecules **4**–**6** [cationic forms: spectra (*i*)], respectively. Spectra (*ii*) refer to the respective neutral forms (see Table 1) (I = 0.1 mol dm^{-3}, 20 °C).

The spectra of the neutral forms **A5** and **A7** can be distinguished from that of **AH**$^+$ by a positive shift of the longer wavelength of the absorption maximum (bathochromism) and a hypochromic effect [Figs. 2(*a*) and (*b*), respectively]; also, in the case of **A5**, there exists a more intense band at 410 nm [Fig. 2(*a*)] which shifts to slightly longer wavelengths upon formation of the **A57**$^-$ anion. (424 nm) [Fig. 3(*a*)].

The spectra of the **A4′** tautomer [Fig. 2(*c*)] and of the **A54′**$^-$ and **A74′**$^-$ anionic forms [Fig. 3(*b*) and (*c*)] are characterized by an intense hyperchromic and bathochromic effect with respect to **AH**$^+$.

While the UV–VIS spectra of apigeninidin in solution at pH 5.59 and 6.77 [Fig. 1(*b*) and (*c*)] are clearly attributable to the spectra of the most abundant tautomers, **A7** [Fig. 2(*b*), (*ii*)] and **A57**$^-$ [Fig. 3(*a*), (*iii*)] respectively, at higher pH the spectra markedly differ from the spectra of the most abundant anionic tautomer: a band appears at 528 nm [Fig. 1(*d*) recorded at pH 11.30]; given its position and intensity, this band can be attributed to further ionization of the monoanionic forms with formation of the dianion **A574′**$^=$; this species has a pK'_a (pK'_{10}) value of 8.06 ± 0.05, determined at the isosbestic point between absorption spectra of the neutral and monoanionic forms (521 nm).

Table 3 Solution composition at varied pH

pH	A5	A7	A4′	A54′$^-$	A74′$^-$	A57$^-$	AH$^+$	A574′$^=$
5.59	15.8	73.8	0.7	0.3	1.0	6.8	1.6	—
7.26	3.0	13.9	0.1	3.0	8.5	60.1	—	11.4
7.94	0.5	2.2	<0.1	2.3	6.5	46.4	—	42.0

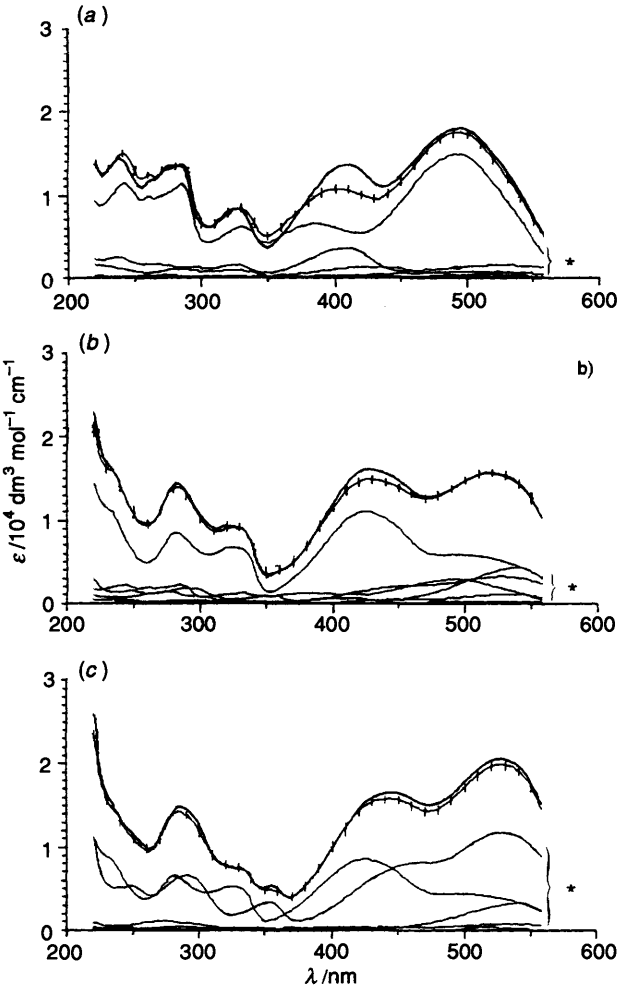


Fig. 4 Comparison between UV–VIS spectra of apigeninidin (**7**) at pH: (*a*) 5.59; (*b*) 7.26 and (*c*) 7.94. (—), I = 0.1 mol dm^{-3}, 20 °C; (+ + +) represents the sum of the spectra of its several possible forms (see Table 1 plus the dianionic form) multiplied by their abundance percentages indicated by *

$$(\mathbf{A57}^- + \mathbf{A54'}^- + \mathbf{A74'}^-) \overset{K'_{10}}{\rightleftharpoons} \mathbf{A574'}^= + H^+$$

$$K'_{10} = \frac{[\mathbf{A574'}^=]\cdot a_{H^+}}{[\mathbf{A57}^-] + [\mathbf{A54'}^-] + [\mathbf{A74'}^-]} \quad (15)$$

Species of Apigeninidin Present in Solution.—Given the proton dissociation constants of each neutral (compounds **1**–**3**) and anionic tautomers (compounds **4**–**6**) of apigeninidin and taking into account the existence of the dianionic species with a pK'_a = 8.06 (Table 2 plus A574′$^=$ form), we can calculate the percentage composition at different pH levels (I = 0.1 mol dm^{-3}, 20 °C), of the various forms of apigeninidin present in solution (Table 3).

According to the calculated percentages, the UV–VIS absorption spectra of apigeninidin at pH 5.59, 7.26 and 7.94, I = 0.1 mol dm^{-3}, 20 °C, are closely reproduced by the spectra representing the sum of the spectra of each form multiplied by its abundance percentage [Fig. 4(*a*)–(*c*), respectively], the maximum difference in the absorption maximum at the longer wavelength being 3%.

Discussion

This is the first report which, through the synthesis and the study of selectively substituted analogs, offers a quantitative clarification of the proton dissociation and the tautomeric equilibria of a complex anthocyanidin like apigeninidin.

As regards the tautomeric equilibria of the neutral forms, **A7** predominates in solution (81.8%); in the case of the anionic forms, the most abundant tautomer is **A57**$^-$ (83.9%), which dominates at pH close to neutrality; moreover, a dianionic form is already present at neutral pH.

The problem of the determination of the tautomeric equilibria of apigeninidin had already been tackled theoretically by us[6] with the use of quantum chemical calculations (AM1);[7] we used the calculated relative stabilities of the different neutral and anionic tautomers generated by the most common anthocyanidins, and in particular by apigeninidin, to predict the dominant tautomeric species present in solution, according to the principle that the most stable forms are those that are most likely to be present.

As regards the neutral forms, there is a qualitative agreement between the relative stabilities of the tautomers calculated *in vacuo* and the experimental data reported here: the **A4′** form was predicted to be less stable than **A5** and **A7** by 4.11 and 2.88 kcal mol^{-1}, respectively, in agreement with the lower presence of **A4′** in solution compared with the other forms. As regards the anionic tautomers, **A54′**$^-$ and **A74′**$^-$ were predicted to be almost equally stable, with **A57**$^-$ about 16 kcal mol^{-1} less stable; however, the present results show that, while **A54′**$^-$ and **A74′**$^-$ are actually present in solution in almost equal amounts, the predominant anionic tautomer in solution is **A57**$^-$. Although solvation model calculations on these anionic forms based on a semiempirical formulation of the virtual charge model[8] indicated no change in the calculated stability trend, the marked preference for **A57**$^-$ must be due to solvent effects.

In fact, since the **A57**$^-$ anionic tautomer has a higher dipole moment than **A54′**$^-$ and **A74′**$^-$ (μ = 21.45, 2.43 and 7.67 D, respectively, calculated according to a constant reference system focused on oxygen O-1) solvation is more likely to favour **A57**$^-$ rather than **A54′**$^-$ and **A74′**$^-$. In order to improve the treatment of solvation, explicit water molecules, instead of a simple virtual representation, proved to be far superior in reproducing the prevalence of **A57**$^-$ in solution. In fact, free energy perturbation[9] calculations (Amber 4.0 program) on the three anionic tautomers solvated by a periodic box of 480 water molecules showed that while **A54′**$^-$ and **A74′**$^-$ have almost the same free energy of solvation, **A57**$^-$ is better solvated by about 20 kcal mol^{-1};[10] as a result, solvation over-compensates for the lower stability of **A57**$^-$ *in vacuo* and correctly accounts for the presence of this form in solution.

In conclusion, the full explanation of the pH-related behaviour of proton dissociation of apigeninidin in aqueous solution, which is important for the determination of the quantitative relationships between chemical structure and biological activity, will likewise be very useful for a better

understanding of phenomena like copigmentation and self-association, in which tautomers may be directly involved.[4,11]

Experimental

Materials.—Reagent-grade chemicals were used without purification. IR spectra were recorded on a Perkin-Elmer 681 spectrophotometer in tetrachloroethylene solution. UV–VIS spectra were obtained with a Perkin-Elmer λ 16 spectrophotometer in MeOH–1% HCl 1 mol dm^{-3}. M.p.s were determined on a Buchi 510 melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Bruker AMX 400 (Centro Interdipartimentale Grandi Strumenti, Modena University) (400 MHz) in CD_3OD containing 5% *v/v* of DCl 20%, unless otherwise stated. Chemical shifts are reported in ppm from tetramethylsilane as internal standard. *J* values are given in Hz. Microanalyses were carried out in the microanalysis laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University. Mass spectra were recorded on a Finnigan MAT SSQ 710. TLC was performed on precoated silica gel F254 plates (Merck). Solvent system: cyclohexane–ethyl acetate–triethylamine (NEt_3) 10:10:1. All the substances were dried to constant weight on P_2O_5 under reduced pressure at room temperature prior to analysis. 7-Hydroxy-4-methyl-2-(4′-methoxyphenyl)benzopyrilium chloride **8** was a generous gift from Coca Cola Co.

4-Benzoyloxy-2-hydroxy-6-methoxybenzaldehyde **12**.—To a solution of 2,4-dihydroxy-6-methoxybenzaldehyde **10**[12] (1.60 g, 9.5 mmol) in anhydrous acetone (50 cm^3), were added at 0 °C, NEt_3 (2.00 cm^3, 14.3 mmol) and, dropwise with stirring, benzoyl chloride (1.10 cm^3, 9.5 mmol). A white precipitate formed almost immediately. The suspension was shaken for 1 h and then left to stand at room temperature for 3 h. The non-soluble material was removed by filtration and the filtrate concentrated under reduced pressure. Crystallization of the residue from ethanol (EtOH) and then from isoamyl alcohol gave the *aldehyde* **12** (2.07 g, 80%), mp 85–86 °C (lit.,[13] 85 °C); R_f 0.80 (Found: C, 66.2; H, 4.4. $C_{15}H_{12}O_5$ requires C, 66.2; H, 4.4%); ν_{max}/cm^{-1} 3061, 3005, 2965, 2937, 2887, 2849, 1744, 1646, 1625, 1588, 1530, 1453, 1424, 1392, 1355, 1306, 1247, 1207, 1176, 1131, 1056 and 1023 (the spectra did not change on dilution); $\delta(CD_3COCD_3)$ 3.98 (3 H, d, *J* 0.28, OMe), 6.51 (1 H, dd, *J* 2.01, 0.60, 3-H), 6.63 (1 H, br d, *J* 2.00, 5-H), 7.61 (2 H, m, 3′-H and 5′-H), 7.75 (1 H, m, 4′-H), 8.17 (2 H, m, 2′-H and 6′-H), 10.27 (1 H, d, *J* 0.60, CHO) and 12.22 (1 H, s, OH). Selective decoupling experiments confirmed the *J* of 0.28 Hz of OMe as a long-range constant with 5-H at δ 6.63, the *J* of 2.01 Hz as *meta* constant between 3-H and 5-H and the *J* of 0.60 Hz between the H of the aldehyde and 3-H. The correct assignment of the structure was confirmed by difference NOE experiments. Irradiation of OMe at δ 3.98 enhanced 5-H at δ 6.63 and the H of the aldehyde at δ 10.27. Irradiation of H at δ 12.22 (OH) enhances the H of the aldehyde at δ 10.27 and 3-H at δ 6.51; *m/z* 272 (M^+, 23.5%) 106 (6.9), 105 (100.0), 78 (2.1), 77 (34.0), 69 (3.1), 51 (7.6) and 50 (2.2).

2-Benzoyloxy-6-hydroxy-4-methoxybenzaldehyde **13**.—Compound **13** was obtained as described for **12**. Starting from 2,6-dihydroxy-4-methoxybenzaldehyde **11**[12] the reaction yielded the *aldehyde* **13** (58%); mp 105–107 °C (from EtOH) (lit.,[14] 109 °C); R_f 0.55 (Found: C, 66.2; H, 4.3. $C_{15}H_{12}O_5$ requires C, 66.2; H, 4.4%); ν_{max}/cm^{-1} 3060, 3006, 2964, 2936, 2877, 2843, 1748, 1644, 1576, 1502, 1432, 1395, 1370, 1325, 1295, 1245, 1194, 1153, 1081, 1037 and 1016 (the spectra did not change on dilution); $\delta(CD_3COCD_3)$ 3.94 (3 H, s, OMe), 6.44 (1 H, dd, *J* 2.32, 0.49, 5-H), 6.59 (1 H, d, *J* 2.33, 3-H), 7.62 (2 H, m, 3′-H and 5′-H), 7.76 (1 H, m, 4′-H), 8.22 (2 H, m, 2′-H and 6′-H), 10.05 (1 H, d, *J* 0.56, CHO) and 12.09 (1 H, s, OH); *m/z* 272 (M^+, 9.9%), 167 (7.9), 106 (6.9), 105 (100.0), 78 (2.0), 77 (32.0), 69 (3.2), 51 (7.1) and 50 (2.2).

5-Hydroxy-7-methoxy-2-(4′-methoxyphenyl)benzopyrilium Chloride **1**.—Gaseous HCl was bubbled through a solution of **13** (1.23 g, 4.5 mmol) and 4-methoxyacetophenone **14** (0.76 g, 5.1 mmol) in EtOH–EtOAc 50:50 (*v/v*) (40 cm^3) for 2 h at 55 °C; the solution turned dark red. It was left to stand at room temp. for 9 h and then at −20 °C overnight. The non-soluble residue was then removed and washed with diethyl ether. The crude 5-benzoyloxy-7-methoxy-2-(4′-methoxyphenyl)benzopyrilium chloride thus obtained was treated with NaOH 6% (13 cm^3 g^{-1} intermediate). The reaction mixture was left to stand for 20 h in the dark, then filtered and the resultant clear red liquid treated with EtOH (5 cm^3 g^{-1} intermediate) and acidified with conc. HCl (5 cm^3 g^{-1} of intermediate). The resultant red solid was finally crystallized from HCl 5 mol dm^{-3}, yielding the title compound **1** (0.11 g, 7%), mp 210–212 °C (decomp.) (Found: C, 60.35; H, 5.4. $C_{17}H_{15}O_4Cl + H_2O$ requires C, 60.6; H, 5.1%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 471(4.50), 325(3.72) and 278(4.26); δ 4.01 (3 H, s, 4′-OMe), 4.11 (3 H, s, 7-OMe), 6.76 (1 H, d, *J* 2.16, 6-H), 7.28 (2 H, m, 3′-H and 5′-H), 7.32 (1 H, dd, *J* 2.16, 0.82, 8-H), 8.26 (1 H, d, *J* 8.76, 3-H), 8.48 (2 H, m, 2′-H and 6′-H) and 9.23 (1 H, dd, *J* 8.74, 0.82, 4-H).

7-Hydroxy-5-methoxy-2-(4′-methoxyphenyl)benzopyrilium Chloride **2**.—A solution of **12** (2.03 g, 7.5 mmol) and 4-methoxyacetophenone **14** (1.16 g, 7.7 mmol) in EtOH–EtOAc 50:50 (*v/v*) (25 cm^3) was subjected to gaseous HCl at 60 °C for 2 h; the solution turned red. It was left to stand at room temp. overnight and the following day diethyl ether (40 cm^3) were added to the reaction mixture; the resultant red precipitate was then collected and washed with diethyl ether. The crude 7-benzoyloxy-5-methoxy-2-(4′-methoxyphenyl)benzopyrilium chloride thus obtained, treated as for compound **1**, yielded compound **2** (0.29 g, 12%) (from HCl 3 mol dm^{-3}), mp 210–213 °C (decomp.) (Found: C, 60.6; H, 4.9. $C_{17}H_{15}O_4Cl + H_2O$ requires C, 60.6; H, 5.1%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 476(4.53), 323(3.75), 275(4.26) and 242(4.02); δ 3.99 (3 H, s, 4′-OMe), 4.12 (3 H, s, 5-OMe), 6.83 (1 H, d, *J* 1.93, 6-H), 7.11 (1 H, dd, *J* 1.94, 0.89, 8-H), 7.26 (2 H, m, 3′-H and 5′-H), 8.19 (1 H, d, *J* 8.74, 3-H), 8.42 (2 H, m, 2′-H and 6′-H) and 9.16 (1 H, dd, *J* 8.71, 0.88, 4-H).

2-(4′-Hydroxyphenyl)-5,7-dimethoxybenzopyrilium Chloride **3**.—A solution of 2-hydroxy-4,6-dimethoxybenzaldehyde (1.00 g, 5.5 mmol) and 4-hydroxyacetophenone **15** (0.80 g, 5.9 mmol) in methanol (30 cm^3) was treated with gaseous HCl at 55 °C for 1 h. The solution turned red and a red precipitate separated out. It was left to stand at room temp. for 4 h, then the non-soluble residue was filtered out, washed with diethyl ether and crystallized from methanol–1% HCl (1 mol dm^{-3}) and HCl (3 mol dm^{-3}), to give the title compound **3** (0.25 g, 13%), mp 170–175 °C (decomp.) (Found: C, 60.8; H, 5.2. $C_{17}H_{15}O_4Cl + H_2O$ requires C, 60.6; H, 5.1%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 476 (4.61), 323(3.59) and 276(4.25); δ 4.12 (3 H, s, 7-OMe), 4.14 (3 H, s, 5-OMe), 6.91 (1 H, d, *J* 2.07, 6-H), 7.11 (2 H, m, 3′-H and 5′-H), 7.39 (1 H, dd, *J* 2.07, 0.76, 8-H), 8.25 (1 H, d, *J* 8.91, 3-H), 8.41 (2 H, m, 2′-H and 6′-H) and 9.16 (1 H, dd, *J* 8.89, 0.78, 4-H).

5,7-Dihydroxy-2-(4′-methoxyphenyl)benzopyrilium Chloride **4**.—A solution of ethynyl *p*-methoxyphenyl ketone **16**[15] (1.23 g, 7.7 mmol) and 1,3,5-trihydroxybenzene + $2H_2O$ **17** (1.66 g, 10.2 mmol) in acetic acid (11 cm^3) at 15 °C was treated with conc. H_2SO_4 (1 cm^3). After 10 h the resultant red precipitate was collected, washed with diethyl ether and crystallized twice from HCl 1 mol dm^{-3} affording the title compound **4** (1.26 g,

54%), mp 300 °C (decomp.) (lit.,[16] 360 °C as hydrate) (Found: C, 63.1; H, 4.2. $C_{16}H_{13}O_4Cl$ requires C, 63.1; H, 4.3%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 475(4.46), 324(3.77), 276(4.26) and 241(3.96); δ 4.00 (3 H, s, 4'-OMe), 6.71 (1 H, d, J 2.01, 6-H), 7.01 (1 H, dd, J 2.02, 0.90, 8-H), 7.26 (2 H, m, 3'-H and 5'-H), 8.13 (1 H, d, J 8.66, 3-H), 8.41 (2 H, m, 2'-H and 6'-H) and 9.16 (1 H, dd, J 8.63, 0.91, 4-H).

5-Hydroxy-2-(4'-hydroxyphenyl)-7-methoxybenzopyrilium Chloride **5**.—Gaseous HCl was bubbled for 2 h through a solution of **13** (1.40 g, 5.1 mmol) and 4-hydroxyacetophenone **15** (0.77 g, 5.7 mmol) in EtOH–EtOAc 50:50 (v/v) (30 cm^3) maintained at 55 °C. The solution turned red and, after 24 h, the precipitate was collected and washed with diethyl ether. Another portion of product was obtained by adding diethyl ether to the reaction liquid. The crude 5-benzoyloxy-2-(4'-hydroxyphenyl)-7-methoxybenzopyrilium chloride thus obtained was treated in the next step as for **1** without purification. A rubbery red precipitate was formed which was recovered and crystallized first from methanol–conc. HCl (3%) and then from HCl (3 mol dm^{-3}), in order to obtain the title compound **5** (0.10 g, 6%), mp 230 °C (decomp.) (lit.,[17] 236–237 °C decomp.) (Found: C, 61.2; H, 4.2. $C_{16}H_{13}O_4Cl + 0.5H_2O$ requires C, 61.25; H, 4.5%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 476(4.56), 325(3.69) and 278(4.28); δ 4.09 (3 H, s, 7-OMe), 6.74 (1 H, d, J 2.14, 6-H), 7.11 (2 H, m, 3'-H and 5'-H), 7.28 (1 H, dd, J 2.13, 0.61, 8-H), 8.19 (1 H, d, J 8.75, 3-H), 8.40 (2 H, m, 2'-H and 6'-H) and 9.17 (1 H, dd, J 8.85, 0.71, 4-H).

7-Hydroxy-2-(4'-hydroxyphenyl)-5-methoxybenzopyrilium Chloride **6**.—A solution of **12** (1.50 g, 5.5 mmol) and 4-hydroxyacetophenone **15** (0.82 g, 6.0 mmol) in EtOH–EtOAc 50:50 (v/v) (20 cm^3) were subjected to gaseous HCl for 1 h at 60 °C. The solution turned red and a red precipitate slowly settled out. The mixture was allowed to stand at room temp. for 6 h, left overnight at −20 °C and then treated as described for **5**. The crude 7-benzoyloxy-2-(4'-hydroxyphenyl)-5-methoxybenzopyrilium chloride thus obtained, treated as for **1**, yielded the title compound **6** (0.15 g, 8%) (from HCl 3 mol dm^{-3}); mp 250–255 °C (decomp.) (Found: C, 61.5; H, 4.4. $C_{16}H_{13}O_4Cl + 0.5H_2O$ requires C, 61.25; H, 4.5%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 481(4.59), 324(3.63), 275(4.25) and 243(4.00); δ 4.11 (3 H, s, 5-OMe), 6.81 (1 H, d, J 1.93, 6-H), 7.08 (1 H, dd, J 1.93, 0.91, 8-H), 7.10 (2 H, m, 3'-H and 5'-H), 8.14 (1 H, d, J 8.85, 3-H), 8.35 (2 H, m, 2'-H and 6'-H) and 9.11 (1 H, dd, J 8.85, 0.91, 4-H).

5,7-Dihydroxy-2-(4'-hydroxyphenyl)benzopyrilium Chloride (Apigeninidin) **7**.—The compound was obtained as described.[18] Mp >300 °C (Found: C, 58.65; H, 4.0. $C_{15}H_{11}O_4Cl + H_2O$ requires C, 58.4; H, 4.2%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 480(4.54), 325(3.69), 277(4.27) and 242(3.98); δ 6.70 (1 H, d, J 2.01, 6-H), 6.98 (1 H, dd, J 2.01, 0.88, 8-H), 7.10 (2 H, m, 3'-H and 5'-H), 8.08 (1 H, d, J 8.71, 3-H), 8.33 (2 H, m, 2'-H and 6'-H) and 9.11 (1 H, dd, J 8.66, 0.82, 4-H).

7-Hydroxy-2-(4'-hydroxyphenyl)benzopyrilium Chloride **9**.—The compound was obtained according to a published procedure.[19] Mp 250–255 °C (decomp.) (Found: C, 61.6; H, 4.3. $C_{15}H_{11}O_3Cl + H_2O$ requires C, 61.55; H, 4.5%); λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 470(4.71), 258(4.08) and 241(4.16). δ data agree with published values.[20]

Determination of the Proton Dissociation Constants (pK'_a).—The compounds being studied were dissolved in methanol–HCl (2%) (95:5 v/v) at 3.4 mmol dm^{-3} concentration. Aliquots of this solution (15 mm^3) were added to buffers (3 cm^3) at constant ionic strength[21] (I = 0.1 mol dm^{-3}) and with pH increasing each time in increments of 0.25 units; UV–VIS spectra were recorded immediately by means of a Perkin-Elmer λ 16 UV–VIS spectrophotometer equipped with a thermostatted cuvette holder (scan rate 960 nm min^{-1}) at 20 ± 0.2 °C. In order to avoid the occurrence of self-association phenomena, anthocyanidin concentrations were kept as low as possible (about 16 μmol dm^{-3}), hence the pigments may be considered to be present just in a monomeric form.[22] After recording each spectrum, the pH of the solution was measured with a combined electrode (Orion SA 520) calibrated with buffer at pH 4.01, 7.00 and 10.01. The numerical values of the proton dissociation constants of the cationic forms AH^+ and of the neutral forms A [as $pK'_{a(AH^+)}$ and $pK'_{a(A)}$] of the compounds under study were evaluated from the change in absorbance[23] at the λ reported (Table 2). The pK'_a values of compound **4**, in which the two dissociations partially overlapped and whose neutral and anionic absorption spectra are very similar, were determined with the aid of a computer program,[24] which determines iteratively the molar extinction coefficient of the neutral form on the basis of the absorbance values of the cationic and anionic forms and the absorbance values at varying pH, thus enabling the pK'_a values to be calculated. These equilibria are not complicated by the hydration reaction, as revealed by the presence of well-defined isosbestic points. Reacidification of the solutions with a few mm^3 of conc. HCl immediately recovers the whole spectrum of the cationic form quantitatively. We also determined the pK'_a values of 7-hydroxy-4-methyl-2-(4'-methoxyphenyl)benzopyrilium chloride **8** and 7-hydroxy-2-(4'-hydroxyphenyl)benzopyrilium chloride **9**; the values found are in good agreement with those determined by the pH-Jump technique[5] (Table 2). The effect of the presence of a small, though constant, percentage of methyl alcohol (< 0.5% v/v) was ignored.

Acknowledgements

We wish to express our gratitude to Professor R. Brouillard (Strasbourg University) for the fruitful discussion and helpful advice. Thanks are due to Dr G. A. Iacobucci from the Coca Cola Co., Atlanta, GA, for the generous gift of 7-hydroxy-4-methyl-2-(4'-methoxyphenyl)benzopyrilium chloride. Financial support from CNR (grant 93.02800.CT03) is gratefully acknowledged.

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Paper 4/05432F
Received 6*th September* 1994
Accepted 13*th October* 1994