

## Formation of Anthocyanin Ion-pairs. A Co-Pigmentation Effect

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The influence of the addition of ionic salts (NaCl, NaBr, NaI, NaClO<sub>4</sub>) on anthocyanin equilibria is studied in this work. The effect of their addition is a colour enhancement through the formation of an ion-pair between the charged flavylium cation and the anion, which displaces the equilibria towards the coloured flavylium form. The ion-pair formation is an alternative model to that which suggests a preferable hydration of salt anions instead of the flavylium cation. Throughout this work two natural anthocyanins are studied (malvidin 3,5-diglucoside and malvidin 3-glucoside) as well as one anthocyanidin (malvidin). The thermal and photochemical stability of the ion-pairs formed is also examined.

Experiments with a synthetic anthocyanin analogue (4',7-dihydroxyflavylium chloride) which has photochromic properties are also described for comparison purposes.

Anthocyanins are very interesting natural colourants, responsible for most of the red, blue and purple colours of flowers and fruits.<sup>1,2</sup> Several factors are known to affect their colourant properties: for example, the chemical structure and concentration of the anthocyanin, the pH of the medium and the presence of other molecules which may act as co-pigments. In aqueous solutions, at acidic pH values (pH < 6) the anthocyanins can exist as an equilibrium mixture of four different structural forms: the coloured flavylium cation (AH<sup>+</sup>) and the quinoidal bases (A), the colourless hemiacetal (B) and chalcones (C), as depicted in Scheme 1. The potential use of anthocyanins as food colourants, is thus hampered by the fact that at the physicochemical conditions required by the food industry and in the absence of other substrates they exist mainly in colourless forms.<sup>3</sup> Moreover, under such conditions anthocyanins also undergo thermal and photochemical degradation.<sup>4</sup>

Anthocyanin co-pigmentation<sup>5</sup> by other molecules (generally other polyphenols or flavonoids), which by themselves are colourless, seems to be one of the most promising ways to circumvent the loss of colour. However, to our knowledge, with two exceptions,<sup>6,7</sup> no work is known about the influence of the addition of salts on the anthocyanin equilibria depicted in Scheme 1.

In both papers strong hyperchromic effects on the visible absorption band due to the flavylium cation were observed, and the authors interpreted the phenomenon as a decrease in water activity due to the anion solvation which will hinder the hydration of the flavylium form, thus displacing the equilibrium toward this form, with the consequent gain of colour.

In this work, we propose a complementary model to explain the colour enhancement of anthocyanins by salt addition, through the formation of ion-pairs between the charged flavylium cation and the anions. The thermal and photochemical stability of the ion-pairs formed by several anthocyanins [malvin (malvidin 3,5-diglucoside), oenin (malvidin 3-glucoside), malvidin and 4',7-dihydroxyflavylium] is also studied.

### Experimental

The natural anthocyanins (malvin and oenin) and the anthocyanidin malvidin were purchased from Extrasynthèse and used as received after purity verification by HPLC. The synthetic anthocyanin 4',7-dihydroxyflavylium chloride was

kindly supplied by the group of Prof. R. Brouillard in Strasbourg. All other chemicals used were of analytical grade.

The pH was measured with a Metrohm 713 pH meter. Adjustments to the desired pH value were accomplished by addition of a few mm<sup>3</sup> of NaOH, HCl or HClO<sub>4</sub>, except for the NMR experiments where it was adjusted with NaOD or DCl.

Absorption spectra were recorded on a Perkin-Elmer Lambda 6 spectrophotometer. A constant temperature of 24 °C was achieved using a Haake thermostatted bath.

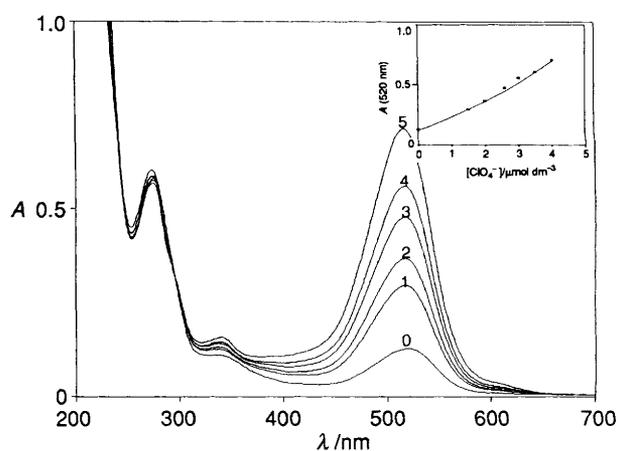
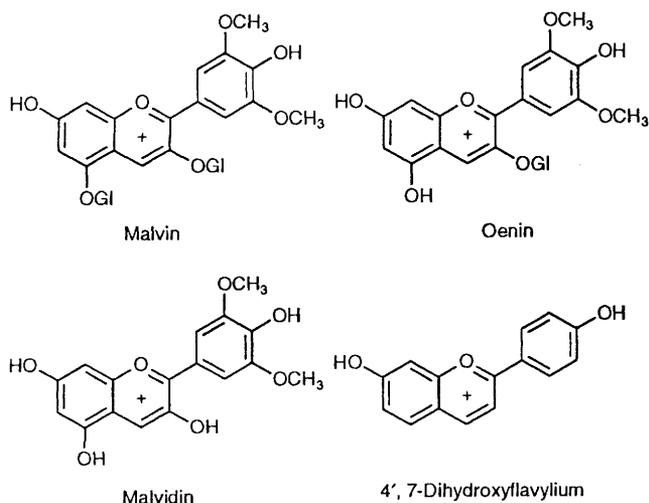
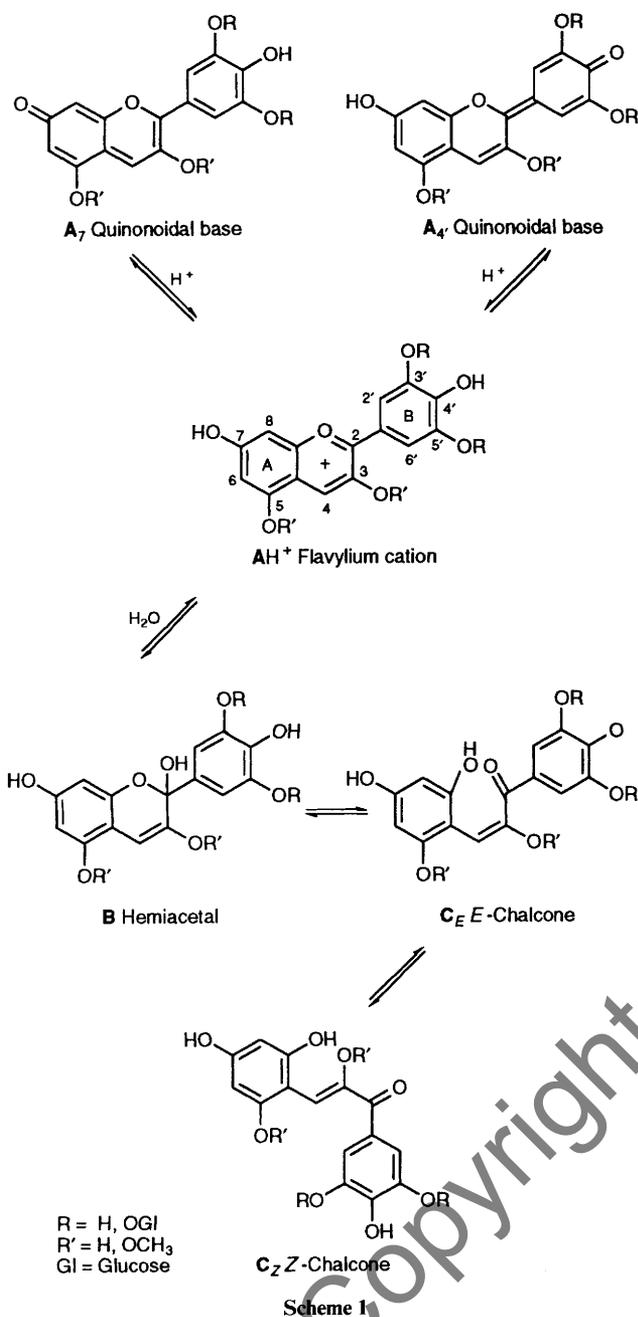
<sup>1</sup>H NMR spectra were performed on a Bruker AMX-300 spectrometer.

Light excitation at 313 nm was carried out by a medium pressure Hg lamp as previously described.<sup>8</sup> The incident light intensity was measured by ferric oxalate actinometry.<sup>9</sup>

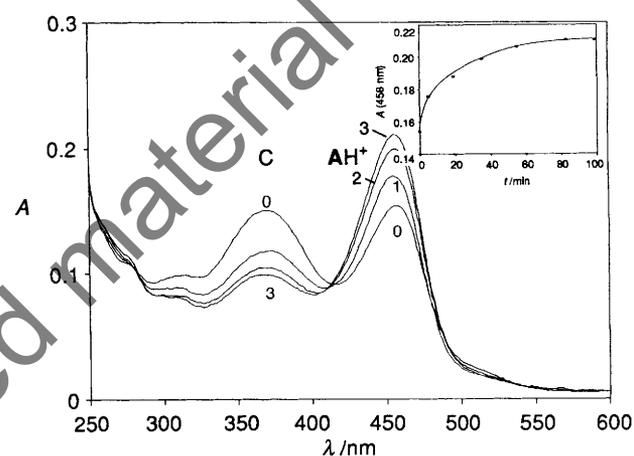
### Results and Discussion

The addition of NaClO<sub>4</sub>, NaCl, NaI or NaBr to previously equilibrated solutions of malvin to a final pH of 2.2–3.3 results in a great hyperchromic shift of the flavylium cation visible band ( $\lambda_{\max} = 520$  nm), as exemplified in Fig. 1 for the case of perchlorate addition. The effect is noticeable only upon addition of the salts in concentrations greater than 1 mol dm<sup>-3</sup>, *i.e.* tens of thousands times the concentration of malvin. Moreover, such hyperchromism increases almost linearly with the concentration of anion added (inset of Fig. 1).

In order to confirm that the colour enhancement is due to a displacement of the equilibria depicted in Scheme 1, we compared the <sup>1</sup>H NMR spectrum of a solution of malvin at pH 2.25 with one of malvin and NaClO<sub>4</sub> at the same pH. The spectrum of the latter showed a great increase in the peaks attributed to the flavylium cation and a decrease in those due to the hemiacetal and chalcone forms, compared with the spectrum of malvin alone. This result clearly indicates a displacement in the equilibria towards the flavylium form. Furthermore, by comparing the two spectra, an upfield shift of the peak attributed to the 2' and 6' protons of the flavylium cation is observed when the anion is added, as opposed to the downfield shifts observed for the remaining flavylium protons. Such an upfield shift may be due to a charge effect due to the proximity of the anion to the charged oxygen atom of the pyrylium ring which increases the shielding of the protons of the neighbouring B ring, thus displacing upfield the respective NMR peaks.<sup>10</sup> These data also support the



**Fig. 1** Ion-pair association of malvin with perchlorate. [malvin] =  $4.8 \times 10^{-5}$  mol dm<sup>-3</sup>; pH 2.7;  $T$  24 °C; [ClO<sub>4</sub><sup>-</sup>] = 0 mol dm<sup>-3</sup> (0); 1.51 mol dm<sup>-3</sup> (1); 2.01 mol dm<sup>-3</sup> (2); 2.6 mol dm<sup>-3</sup> (3); 3.01 mol dm<sup>-3</sup> (4); 4.02 mol dm<sup>-3</sup> (5). *Inset* variation of the absorption at 520 nm (AH<sup>+</sup> maximum) with [ClO<sub>4</sub><sup>-</sup>] added.



**Fig. 2** Effect of the addition of NaClO<sub>4</sub> to an equilibrated solution of 4',7-dihydroxyflavylium, pH 3.5;  $T$  24 °C; [4',7] =  $10^{-5}$  mol dm<sup>-3</sup>; NaClO<sub>4</sub> is in large excess. Solution equilibrated in the dark (0); 2 min after NaClO<sub>4</sub> addition (1); 35 min (2); 83 min (3). *Inset* the increase of AH<sup>+</sup> absorbance ( $\lambda_{\max}$  485 nm) vs. time after addition of perchlorate.

model of the ion-pair, by confirming the proximity of the two charged molecules.

This behaviour is also observed in the synthetic anthocyanin analogue 4',7-dihydroxyflavylium chloride. This synthetic compound is particularly interesting owing to the great degree of *Z*-chalcone formation at mildly acidic pH values (*ca.* 3.5), see curve 0 of Fig. 2. The *Z*-isomer co-exists in equilibrium with the flavylium cation form, but the equilibrium can be shifted in the direction of AH<sup>+</sup> formation through acidification or photochemical irradiation.<sup>11</sup> Moreover, this isomerization is slow enough to be followed by <sup>1</sup>H NMR spectroscopy.

Taking advantage of these properties, an experiment of adding NaClO<sub>4</sub> to a previously equilibrated solution of 4',7-dihydroxyflavylium at pH 3.3 (buffered solution) was performed. A recovery of the absorption due to the flavylium form ( $\lambda_{\max}$  458 nm) can be observed (Fig. 2). This result also points towards an interaction between the flavylium cation of the synthetic anthocyanin and the anion which displaces the equilibria toward the flavylium.

Such ion-ion interactions can be interpreted on the basis of the ion-pair model,<sup>12</sup> which is an alternative and/or complementary model to that advanced in refs. 6 and 7.

This model is based on the assumption that two charged

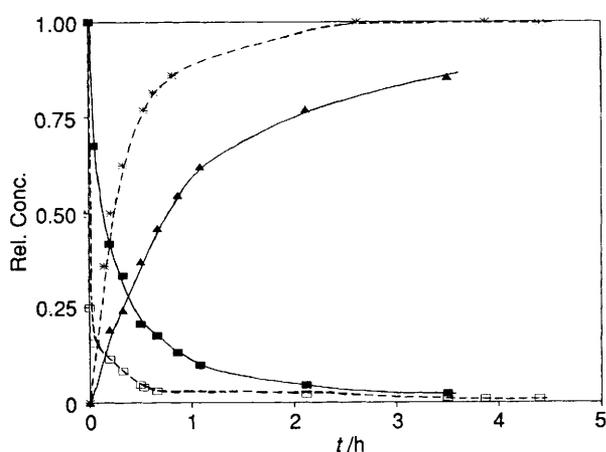


Fig. 3 Comparison of the kinetics for thermal chalcone formation, between malvidin and the ion-pair formed by malvidin and perchlorate. pH 3.2;  $T$  24 °C.  $AH^+$  (ion-pair) (■); C (ion-pair) (△);  $AH^+$  (malvidin) (□); C (malvidin) (\*).

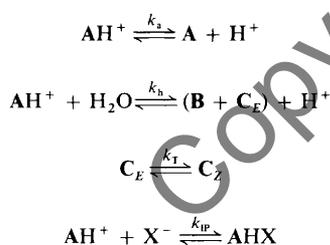
Table 1 Thermal degradation constants<sup>a</sup> for malvidin and for the malvidin-perchlorate ion-pair; pH 3.2;  $T$  24 °C

Compound	$k_1/s^{-1}$
Malvidin <sup>18</sup>	$8.3 \times 10^{-4}$
Malvidin + $ClO_4^-$	$1.2 \times 10^{-5}$

<sup>a</sup> Within an error of 10%.

molecules when at a critical distance from each other can interact, producing an ion-pair association. Such interactions may produce only slight changes or even no changes at all on the absorption spectra of the compound in the presence of the salt, although those changes can be followed by NMR spectroscopy.<sup>13</sup>

The new equilibria for the ion-pair association can be described by the following kinetic scheme, valid for mildly acidic pH values (Scheme 2), where  $X^-$  is the anion and AHX



Scheme 2

represents the ion-pair. The quinonoidal bases, hemiacetal and *E*-chalcone, do not form ion-pairs since they possess no charge. The hydrated B and  $C_E$  forms are extremely quick to equilibrate<sup>14</sup> and, thus, have been written together in an overall hydration equilibrium. In the case of the natural pigments the very minor Z-chalcone can be neglected.

From the NMR spectroscopic data it is possible to measure an overall hydration constant ( $k'_h$ ) for the new anthocyanin

$$k'_h = \frac{([B] + [C_E])[H^+]}{[AH^+] + [AHX]} \quad (1)$$

equilibria in the presence of the anion. This value is  $7.33 \times 10^{-4}$  (for the ion-pair of malvin with perchlorate) while the value for  $k_h$  (malvin alone) measured by NMR spectroscopy<sup>15</sup> is  $9.68 \times 10^{-3}$ .

**Thermal Stability Studies.**—Anthocyanins are known to undergo thermal degradation at acidic pH values, the extent of the reaction being dependent on the chemical structure of the molecule.<sup>16</sup> For example, in the malvidin family [malvidin, malvidin 3-glucoside (oenin) and malvidin 3,5-diglucoside (malvin)] the stability increases in the order malvidin < oenin < malvin.<sup>17</sup> The two glucosylated structures are much more stable than the anthocyanidin. The anthocyanidin flavylium cation is thermally converted into chalcone, which suffers a cleavage yielding 2,4,6-trihydroxybenzaldehyde originated from ring A and 4-hydroxy-3,5-dimethoxybenzoic acid from ring B.<sup>18</sup> The formation of ion-pairs by the glucosylated pigments with perchlorate does not notably increase its stability compared with the pigments by themselves, since the pigments are already stable. The exception is the ion-pair formed by malvidin which is clearly more stable than the anthocyanidin alone.

Thermal degradation of the ion-pair formed by malvidin and perchlorate is followed by absorption spectroscopy, according to the method described in ref. 18. The degradation follows the simplified kinetic scheme:



The kinetic curves for the thermal formation of chalcone, for the ion-pair formed by malvidin and perchlorate, and for malvidin alone are represented in Fig. 3. Comparison of the values obtained for  $k_1$  (Table 1) denote smaller values for the ion-pair. The values for  $k_2$  are of the same magnitude in both cases. These results lead to the conclusion that the formation of ion-pairs by the highly unstable anthocyanidins contribute in a considerable way to its stabilization. A possible model to account for such stabilization may have two causes: (a) an electrostatic interaction between the anion and the charged flavylium cation, displacing the equilibria toward this form, which implies a smaller rate of chalcone formation; (b) a preferable hydration of the anion, thus protecting the anthocyanin molecule against the nucleophilic water attack and consequent cleavage.

**Photochemical Stability Studies.**—The ion-pairs formed by malvin, oenin and malvidin with  $NaClO_4$  were irradiated with light of 313 nm, which is known to promote the degradation of anthocyanins and anthocyanidins with the formation of the same final products described for the case of thermal degradation, although through a different kinetic pattern.<sup>18,19</sup> The result of such irradiation is the photodegradation of the oenin-perchlorate ion-pair. Table 2 lists the ratios ( $\phi$ ) of the quantum yields for the photochemical degradation of the ion-pairs,  $\Phi_{ip}^c$  vs. those for the pigments,  $\Phi^c$  (corrected for the light absorbed by the flavylium cation, which increases when the ion-pair is considered) as defined in eqn. (4) where  $\Delta A$  is the

$$\phi = \frac{\Phi^c}{\Phi_{ip}^c} = \frac{\Delta A_{520}(1 - 10^{-A_{313}^{IP}})A_{313}^{IP}A_{520}^{IP}\Delta t^{IP}}{\Delta A_{520}^{IP}(1 - 10^{-A_{313}^A})A_{313}^A A_{520}^A \Delta t} \quad (4)$$

absorption intensity interval for the considered wavelength and  $\Delta t$  the interval of irradiation time.

The results reported in Table 2 indicate a protective effect against photodegradation on the ion-pairs compared with the parent anthocyanins, which in addition to the great colour enhancement they produce, makes those simple anions a good source of stabilization for that important group of natural pigments.

The data in Table 2 indicate that for malvin, the anion  $Br^-$

**Table 2** Ratio<sup>a</sup> of the corrected photodegradation quantum yields at 313 nm for several anthocyanin ion-pairs. pH 2.7;  $I_0$   $6.98 \times 10^{-7}$  Einstein  $\text{min}^{-1}$ ; air equilibrated solutions

Compound	Anion	$\phi^b$
Malvin	$\text{ClO}_4^-$	1.10
Malvin	$\text{Br}^-$	1.35
Oenin	$\text{ClO}_4^-$	1.55

<sup>a</sup> As defined in eqn. (4). <sup>b</sup> Estimated error  $\pm 10\%$ .

exhibits a higher protective effect to light exposure compared with perchlorate.

**A Photochromic Ion-pair.**—It has already been stated that the synthetic anthocyanin analogue 4',7-dihydroxyflavylium chloride also forms an ion-pair with perchlorate. Since this anthocyanin analogue has photochromic properties,<sup>11</sup> it is interesting to study the influence of the ion-pair on such properties. The photochromic cycles of flavylium recovery upon excitation at 313 nm have a smaller amplitude than those for the synthetic compound alone, since the equilibria in the ion-pair are already displaced to  $\text{AH}^+$ , with less Z-chalcone in solution to be photochemically converted into  $\text{AH}^+$ .

**Conclusions.**—The addition of ionic salts to anthocyanins and the consequent formation of ion-pairs with the charged flavylium cation is an alternative to the use of natural co-pigments to enhance the colour properties of anthocyanins. However, the formation of ion-pairs does not prevent the thermal and photochemical degradations of the pigments, although it does reduce their efficiency. This reduction is possibly due to the displacement of the equilibria toward the flavylium form, which diminishes the rate of chalcone formation and also to the protection against water attack on the anthocyanin, by preferable hydration of the anion.

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#### References

- R. Brouillard, in *The Flavonoids, Advances in Research* (ed. J. B. Harborne), Chapman and Hall, London, 1988, p. 525.
- T. Goto and T. Kondo, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 17.
- G. Mazza and R. Brouillard, *Food Chemistry*, 1987, **25**, 207.
- E. Maccarone, V. Ferrigno, M. L. Longo and P. Rapisarda, *Annali di Chimica*, 1987, **77**, 499.
- S. Asen, *Phytochemistry*, 1984, **23**, 2523.
- T. Goto, T. Hoshino and M. Ohba, *Agr. Biol. Chem.*, 1976, **40**, 1593.
- O. Dangles and R. Brouillard, *Can. J. Chem.*, 1992, **70**, 2174.
- M. T. Gandolfi, M. F. Manfrin, A. Juris, L. Moggi and V. Balzani, *Inorg. Chem.*, 1974, **13**, 1342.
- C. G. Hatchard and C. A. Parker, *Proc. R. Soc. London Ser. A*, 1956, **235**, 518.
- R. J. Abraham, J. Fisher, P. Loftus, in *Introduction to NMR Spectroscopy*, Wiley, Chichester, 1988, p. 19.
- P. Figueiredo, J. C. Lima, H. Santos, M.-C. Wigand, R. Brouillard, A. L. Maçanita and F. Pina 'Photochromism of the Synthetic 4',7-dihydroxyflavylium Chloride' *J. Am. Chem. Soc.*, in press.
- H. Yokoyama and H. Yamatera, *Bull. Chem. Soc. Jpn.*, 1975, **48**, 1770, and references therein.
- J. Sotomayor, H. Santos and F. Pina, *Can. J. Chem.*, 1991, **69**, 567.
- R. Brouillard and J. Lang, *Can. J. Chem.*, 1990, **68**, 755.
- H. Santos, D. L. Turner, J. C. Lima, P. Figueiredo, F. Pina and A. L. Maçanita, *Phytochemistry*, 1993, **33**, 1227.
- J. B. Harborne in *Biochemistry of Plant Phenolics* (eds. T. Swain, J. B. Harborne and C. F. Van Sumere), Plenum, New York, 1979, p. 457.
- G. Hrazdina, A. J. Borzell and W. B. Robinson, *Am. J. Enol. Vitic.*, 1970, **21**, 201.
- P. Furtado, P. Figueiredo, F. Pina and H. C. Neves, 'Photochemical and Thermal Degradation of Anthocyanidins', *J. Photochem. Photobiology A: Chemistry*, 1993, **75**, 113.
- P. Figueiredo, M. G. Silva, H. C. Neves and F. Pina, 'Photodegradation of Malvidin 3,5-diglucoside Followed by RP-HPLC', I-APS Fourth Winter Conference, Clearwater Beach, FL, USA, Jan. 1-5, 1992.

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