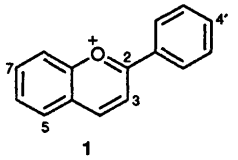
Polyphenol Interactions. Part 5.[1] Anthocyanin Co-pigmentation

Tarankumar V. Mistry, Ya Cai, Terence H. Lilley and Edwin Haslam*
Department of Chemistry, University of Sheffield, Sheffield S3 7HF, UK

In aqueous media, anthocyanins undergo several structural transformations and exist in a series of equilibria between carbinol-base **6a**, **6b**, flavylium cation **3**, quinonoidal anhydro-base **4a**, **4b** and chalcone **7a**, **7b** forms. A detailed interpretation of the proton NMR (400 MHz) spectra of malvin **3** in D_2O is given for the first time in the context of these equilibria. The phenomenon of co-pigmentation is reviewed and the efficacy of various phenolic flavonoids, galloyl and hexahydroxycinnamyl esters as natural co-pigments is determined quantitatively. Association constants at 22 °C in H_2O/CF_3CO_2H (0.02% v/v) of 1686 (±58) and 987 (±37) $dm^3\ mol^{-1}$ were measured by UV-visible spectroscopy for the complexation of **3** with quercetin-3-β-D-galactoside **11** and β-1,2,3,4,6-penta-*O*-galloyl-D-glucose **10**, respectively. In D_2O/CF_3CO_2D (1.1% v/v) at 45 °C an association constant of 508 (±8) $dm^3\ mol^{-1}$ was determined by proton NMR for the interaction of **3** with **10**. DNA, RNA and ATP also act as effective co-pigments for the flavylium ion **3** but both caffeine **16** and theophylline **17** preferentially stabilise the quinonoidal-base forms **4a**, **4b** of the anthocyanin. In media (pH 3.5 to 7.0), they give rise to stable violet through to blue and finally green colours of the anthocyanin **3**. Proton NMR studies of all these intermolecular co-pigmentation reactions are reported and the phenomenon is provisionally interpreted in terms of hydrophobically reinforced 'π-π' stacking of anthocyanin and co-pigment molecules in the aqueous environment.

Floral colours derive from a small group of pigments–principally carotenoids, betacyanins (*Centrospermae*), and anthocyanins and other flavonoids.[2,3] The anthocyanins are pre-eminent and they may vary the colour of flower petal cells from salmon and pink, through scarlet, magenta and violet to purple and blue.[4,5] Anthocyanins are glycosylated polyhydroxy/methoxy derivatives of 2-phenylbenzopyrilium (flavylium) salts **1**. Six aglycones (anthocyanidins) dominate



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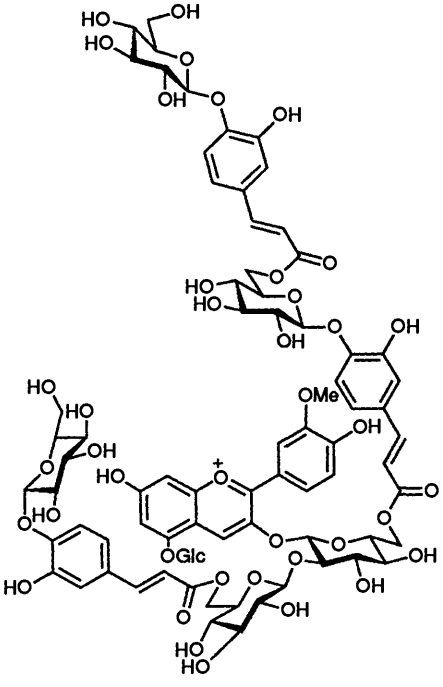
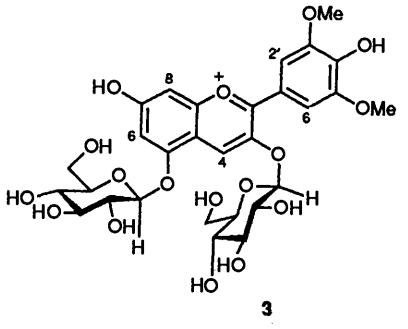
natural anthocyanin structures.[5] Sugars are attached most frequently at O-3 and O-5 and in many cases these sugars are acylated by *p*-coumaric, caffeic, ferulic, sinapic, *p*-hydroxybenzoic, malonic or acetic acids.[3] No anthocyanin has yet been found in Nature in which all the phenolic hydroxy groups are glycosylated or methylated, indeed a free hydroxy group at one or more of the positions 5, 7, 4′ is essential in order to generate *in vivo* all of the colours responsible for fruit and flower pigmentation. An example of the structural complexity present in natural anthocyanins is provided by the 'heavenly blue anthocyanin' **2** (HBA, M_R 1759) from *Ipomoea purpurea* which is a paeonidin-3-sophoroside-5-glucoside substituted with three caffeylglucose residues.[6,7]

Although, as Willstatter originally suggested,[8] the pH of the cell vacuole is of utmost importance in floral pigmentation, attention has focussed in recent years not only on pH but on other molecular mechanisms which underly flower colour variation.[5] Thus, under the physico-chemical conditions appertaining to those in typical cell vacuoles (*e.g.*, weakly acidic) and in the absence of other substrates, most anthocyanins exist substantially in stable colourless forms. The question of how, therefore, anthocyanins give rise to such a striking range of flower colours is an important one and Brouillard[9] has suggested that the ability of anthocyanins to



2 (HBA, M_r = 1759)

exist as several stable colourless forms is a prerequisite to a full expression of floral pigmentation. The structural changes which accompany the dissolution of anthocyanins in aqueous media have been extensively studied by Brouillard and his collaborators[5,10–12]. Under very weakly acidic conditions, four anthocyanin structural types exist in equilibrium: the flavylium cation **3**, the quinonoidal anhydro base **4a**, **4b**, the colourless carbinol bases **6a**, **6b** (in principle four diastereoisomeric forms) and the pale yellow 'reversed chalcones' **7a**, **7b** (Fig. 1) (malvin or 3,5-bis-β-D-glucosylmalvidin). Equilibration between the anhydro base **4a**, **4b** and the carbinol bases **6a**, **6b** occurs exclusively *via* the flavylium cation



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3[10–12] and Brouillard and Dubois[10] quote K (carbinol base/anhydrobase) as 1.6×10^2 at 4 °C.

The appearance of the proton NMR spectrum of malvin **3** in D_2O (400 MHz) is critically dependent (Figs. 2, 3, and 8–10) on the position of these equilibria and hence upon operating conditions,[13–14] particularly those of temperature, concentration and pre-equilibration time. At 20 °C, the protonation–deprotonation **3**⇌**4** is fast[11] and these species appear as a single set of line-broadened signals (Fig. 2); precise chemical shift values are dependent on anthocyanin concentration and the position of the equilibrium (4-H, δ 8.50; 2′-H, 6′-H, δ 7.33; 6-H, 8-H, δ 6.73 and 6.60; two D-glucose anomeric protons, δ 5.20 and 5.25, J 8.5 Hz). Two other major species, the carbinol base **6a** (two diastereoisomeric forms at C-2; 2′-H, 6′-H, δ 6.80 and 6.83; 4-H, δ 6.58 and 6.56; 6-H, 8-H, two pairs of *meta* coupled protons, δ 6.40 and 6.22, J 2.5 Hz), and two minor species, the carbinol base **6b** (two diastereoisomeric forms at C-4; 2′-H, 6′-H, δ 6.79 and 7.07; 4-H, δ 6.77 and 6.55; 6-H, 8-H, δ 6.03 and 5.85, J 2.5 Hz and δ 5.88 and 5.70, J 2.5 Hz), are also distinguished (Fig. 2). At 45 °C (Fig. 16, *vide infra*) additional signals have been assigned to the 'reversed chalcones' **7a**, **7b** (2 × 2′-H, 6′-H, δ = 7.32; 2 × 4-H, δ 7.13 and 6.74; 2 × 6-H, 8-H, two pairs of *meta* coupled protons, δ 6.33 and 6.22 and δ 6.25 and 6.16, J 2.5 Hz). Reactions **3**→**6a**, **6b**→**7a**, **7b** are endothermic[11] and these observations are therefore consistent with the view that the rise in temperature favours the formation of the open-chain chalcone forms **7a**, **7b** with respect to other species. When the solution was cooled to 20 °C, the spectrum retained the presence of the chalcone species **7a**, **7b**. The temperature change from 20 to 45 °C also caused the signals from the species **3**⇌**4** to sharpen considerably and to show strong *downfield* shifts (4-H, Δδ 0.27 ppm; 2′-H, 6′-H, Δδ 0.28 ppm and 6-H, 8-H, Δδ 0.17 and 0.28 ppm) until the spectrum due to **3**⇌**4** closely resembles that of the flavylium ion **3** itself (Fig. 16). This observation is consistent with the thermal disruption of the anthocyanin anhydro base 'π–π'/hydrophobic self-association[14] and with the endothermic nature of the transformation **4**→**3** and hence in a shift in the equilibrium towards the flavylium ion **3**. Confirmation of these various assignments came from the titration of the malvin solution (at 20 and 45 °C) with aliquots of NaOD. Proton NMR signals from species containing readily ionisable phenolic protons (**3**, **4a**, **4b**, **7a**, **7b**) showed a progressive line-broadening until the spectrum (Fig. 3, at 20 °C) closely resembled that of the carbinol bases **6a**, **6b**.

The flavylium ion **3** is red and stable in strongly acidic media. As the pH is raised, there are corresponding changes in colour from red to violet, blue and finally green, due to the formation of increasing amounts of the anhydro base **4a**, **4b** and then the anion **5**. These colours are transient and very rapidly fade. One of the few authenticated examples of an anthocyanin in which the anhydro-base form is stabilised is the 'heavenly blue anthocyanin' **2** in which two of the caffeyl ester groups are thought to be stacked intramolecularly with the anthocyanin

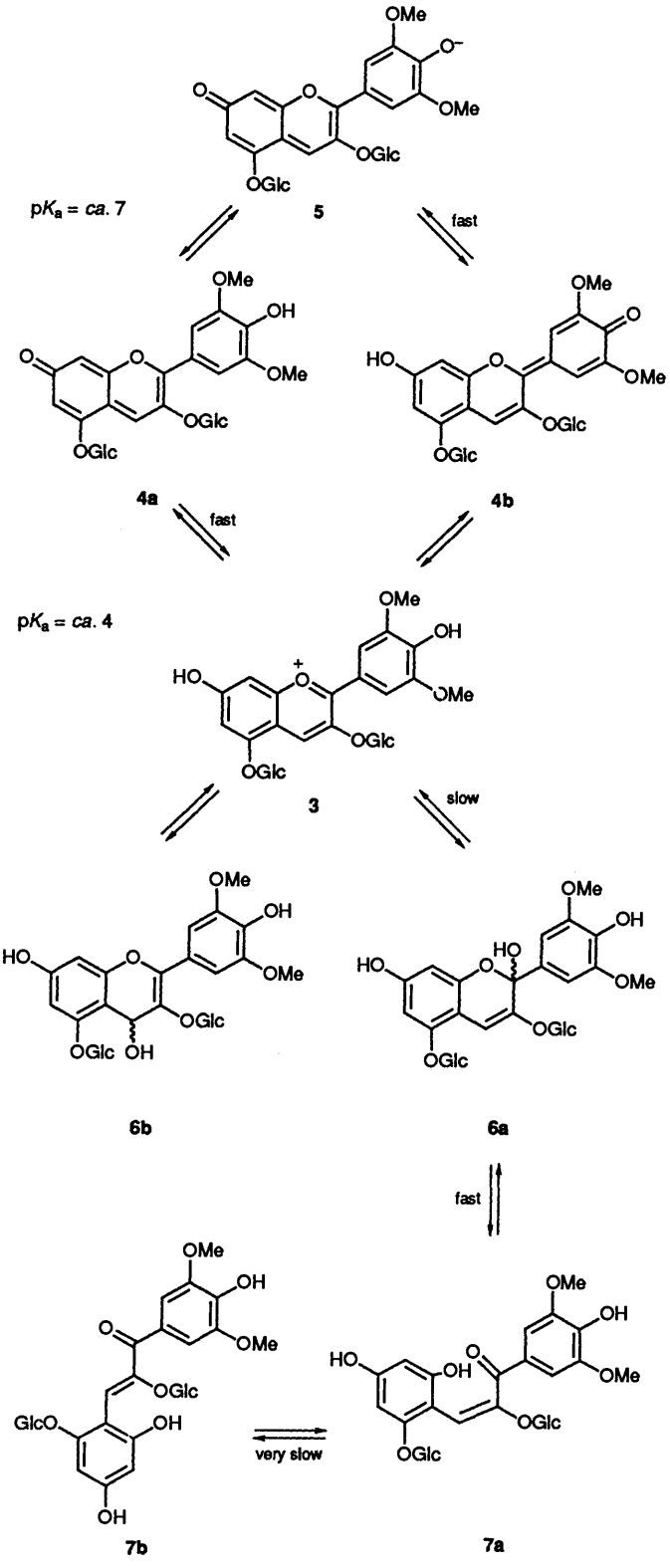


Fig. 1 Anthocyanin equilibria (Brouillard and Lang[12])

nucleus (*e.g.*, **8**).[6,7] Such 'π–π' interactions[14,15] are thought to stabilise the flavylium-cation/anhydro-base forms and this, it has been suggested,[5,7] prevents the usual reaction with water to form the colourless carbinol-base forms (Fig. 1). Saito and his colleagues[16] have reported that the blue ternatins A–F, isolated from the butterfly pea (*Clitoria ternatea*) flower and based upon delphinidin-3,3′,5′-triglucoside are more stable than **2**; a similar rationale has been put forward.

The observation that the colour of isolated anthocyanins

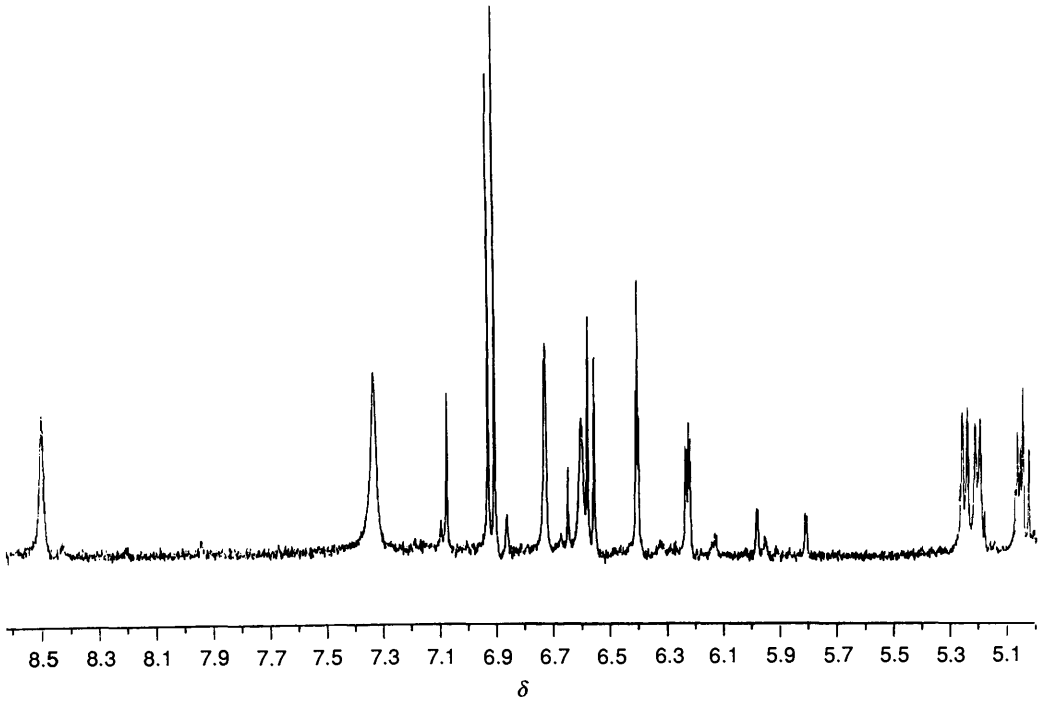


Fig. 2 Proton NMR spectrum (400 MHz) of malvin chloride (4×10^{-3} mol dm^{-3}) in D_2O at 20 °C; δ 5.1 to 8.5, external reference TSP

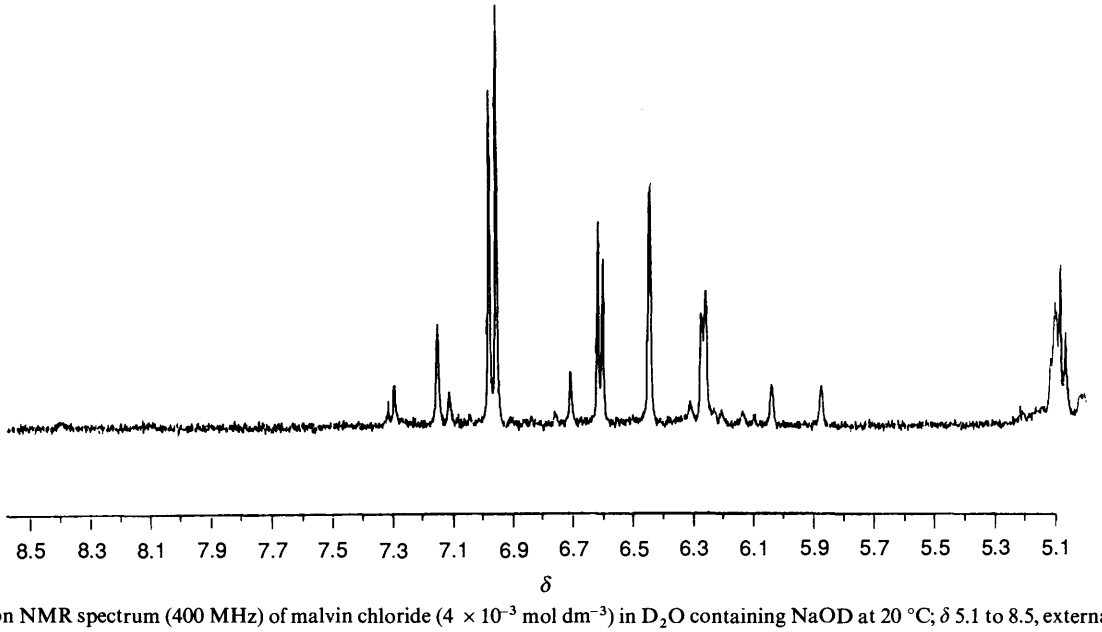
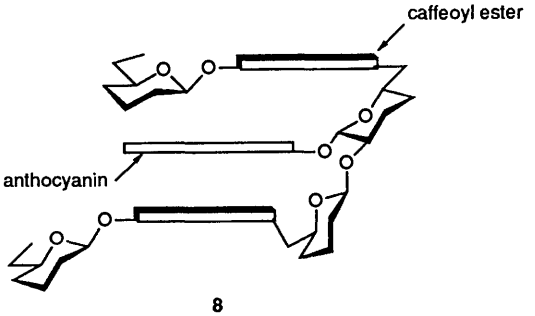


Fig. 3 Proton NMR spectrum (400 MHz) of malvin chloride (4×10^{-3} mol dm^{-3}) in D_2O containing NaOD at 20 °C; δ 5.1 to 8.5, external reference TSP



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could be varied by the presence of other substances (co-pigmentation) was first made by Willstatter and Zollinger[17] and Robinson and Robinson.[18,19] Both groups noted that 'tannin' induced a bathochromic shift in the visible absorption spectrum of anthocyanins. The Robinsons[18,19] observed that co-pigmentation was almost universal in floral colours and they concluded that, 'the phenomenon has little to do with salt formation and occurs in the presence of a large excess of mineral acid; it is entirely the result of the formation of weak additive complexes which are dissociated at elevated temperatures or by the action of solvents'. Co-pigments have no or little visible colour by themselves but when added to an anthocyanin solution greatly enhance the colour of that solution[5–7,20–23] (*e.g.*, Fig. 4, malvin chloride and quercetin-3-galactoside). Co-pigmentation has been shown to be dependent on anthocyanin and co-pigment type, concentration, pH, temperature and metal salts. Although various groups of compounds have been identified as potential co-pigments[20–23]—particularly

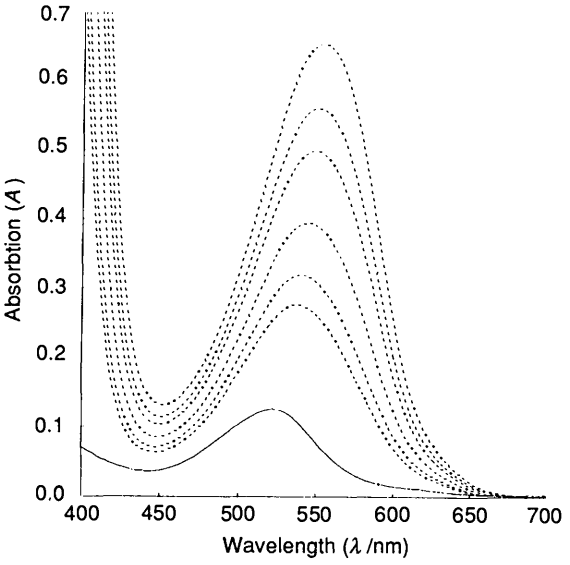


Fig. 4 Malvin chloride (1.0×10^{-4} mol dm^{-3}) in acetate buffer (0.2 mol dm^{-3}, pH 3.65). Changes in anthocyanin visible spectrum (——) produced by the addition of quercetin-3-β-D-galactoside (**11**, – – – –). Final pigment to co-pigment molar ratio (1:30).

Table 1 Malvin chloride–polyphenol co-pigmentation—Flavonoids[a]

Polyphenol	$A - A_o/A_o \times 100\%$	$\lambda - \lambda_o$/nm
(+)-catechin[b]	17	0.8
(+)-gallocatechin[c]	18	0.8
(+)-catechin-3-gallate[d]	48	3.2
(−)-epiafzelechin	13	0.5
(−)-epicatechin[b]	18	0.8
(−)-epigallocatechin[e]	21	1.6
(−)-epigallocatechin-3-gallate[e]	44	2.3
procyanidin B-2-[(−)-epicatechin]$_2$[b]	15	—
procyanidin B-3-[(+)-catechin]$_2$[b]	17	—
procyanidin B-4-[(+)-catechin-(−)-epicatechin][b]	17	—
procyanidin C-1–[(−)-epicatechin]$_3$[b]	19	—
Sorghum polymer–(+)-catechin-[(−)-epicatechin]$_5$[f]	14	—
quercetin-3-β-D-galactoside **11**[g]	173	18.9

[a] Malvin chloride (1.0×10^{-4} mol dm^{-3}), polyphenol (2.0×10^{-4} mol dm^{-3}) in 0.2 mol dm^{-3} acetate buffer pH 3.65. A_o = absorption of malvin chloride at λ_o, A = absorption of malvin chloride plus polyphenol at λ, all at 22 °C. [b] Ref. 34. [c] Ref 45. [d] Ref. 46. [e] Ref. 37. [f] Ref. 35. [g] Ref. 36.

flavonoids and hydroxycinnamyl esters—few have been subject to detailed quantitative investigation. This paper describes work undertaken with a range of co-pigments—natural polyphenols (vegetable tannins), caffeine and theophylline, ATP, DNA and RNA. A preliminary account of some aspects of this work has been published.[24]

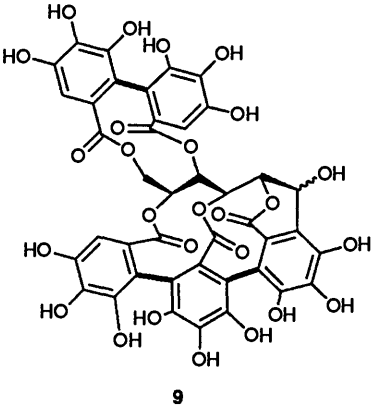
At pH 3.65 (0.2 mol dm^3 acetate buffer) and 22 °C, natural proanthocyanidins[25] (condensed tannins) and related flavan-3-ols display very small co-pigmentation effects with both malvin chloride **3** (Table 1) and cyanin chloride. Natural galloyl esters of D-glucose (hydrolysable tannins)[25] however give rise not only to significant bathochromic shifts in the visible absorption maximum of malvin chloride **3** (Table 2, $\lambda - \lambda_o$) but also to increases in absorptivity ($A - A_o$). These effects are enhanced in the presence of magnesium chloride (0.25 mol dm^3) but are reduced as the temperature is increased (22–60 °C). Galloyl esters of flavan-3-ols similarly show significant co-pigmentation effects compared to the parent flavan-3-ols (Table 1).

Table 2 Malvin chloride–polyphenol co-pigmentation—Phenolic esters[a]

Polyphenol	$A - A_o/A_o \times 100\%$	$\lambda - \lambda_o$/nm
methyl gallate	18	2.4
aesculin **12**	14	0.5
2,6-digalloyl-1,5-anhydro-D-glucitol[b]	10	2.0
β-1,3,6-trigalloyl-D glucose[b]	50	3.2
β-1,2,4,6-tetragalloyl-D glucose[b]	60	4.8
β-1,2,3,4,6-pentagalloyl-D glucose **10**[b]	121	12.0
β-1,2,3,4,6-pentagalloyl-D glucose **10**[b] *plus* $MgCl_2$ (0.25 mol dm^{-2})	488	12.0
tellimagrandin-1[c]	18	0.8
davidiin[d]	78	8.8
vescalagin, castalagin **9**[e]	7	0.5
sanguin H-6[c]	115	8.0
5-O-*p*-coumaryl quinic acid **14**[g,h]	101	1.6
chlorogenic acid **15**[f,h]	143	4.0

[a] Malvin chloride (1.0×10^{-4} mol dm^{-3}), polyphenol (2.0×10^{-4} mol dm^{-3}) in 0.2 mol dm^{-3} acetate buffer pH 3.65. A_o = absorption of malvin chloride at λ_o, A = absorption of malvin chloride plus polyphenol at λ, all at 22 °C. [b] Ref. 39. [c] Ref 41. [d] Ref. 40. [e] Ref. 29, 30. [f] Ref. 42. [g] Ref. 43. [h] Polyphenol (6.0×10^{-4} mol dm^{-3}), malvin chloride (7.7×10^{-4} mol dm^{-3}).

As in related studies of polyphenol complexation,[26–28] the introduction (biosynthetic) of a hexahydroxydiphenoyl group in place of two vicinal galloyl ester groups by dehydrogenation generally gives rise to metabolites with a substantially reduced ability towards co-pigmentation. This is seen most clearly in the case of the diastereoisomers castalagin and vescalagin **9**[29,30], both formally analogues of β-1,2,3,4,6-penta-*O*-galloyl-D-glucose **10** but with six hydrogens less. They **9** are severely constrained in their conformational flexibility and the accessibility of their phenolic nuclei by the intramolecular (C–C) bridging of the galloyl ester groups. These observations are consistent with those of Eugster and Nayeshiro[31] who recently demonstrated that ellagitannins act as very poor



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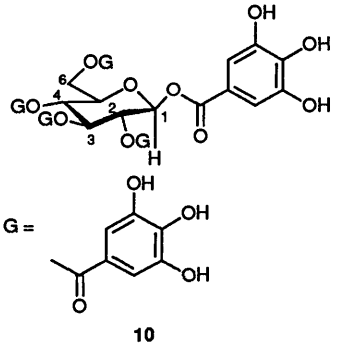

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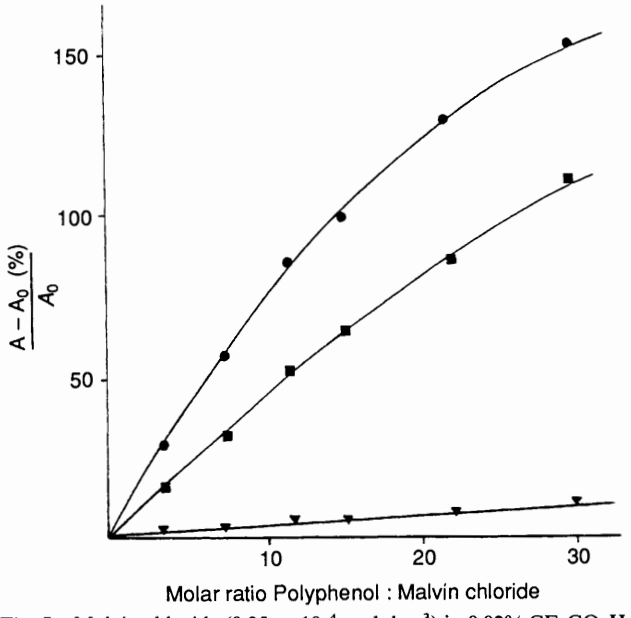


Fig. 5 Malvin chloride (0.25×10^{-4} mol dm^{-3}) in 0.02% CF_3CO_2H–H_2O (v/v). Comparisons of changes in absorbtivity ($A - A_0/A_0$) with different co-pigments: quercetin-3-β-D-galactoside (**11**, ●), β-1,2,3,4,6-pentagalloyl-D-glucose (**10**, ■), aesculin (**12**, ▼).

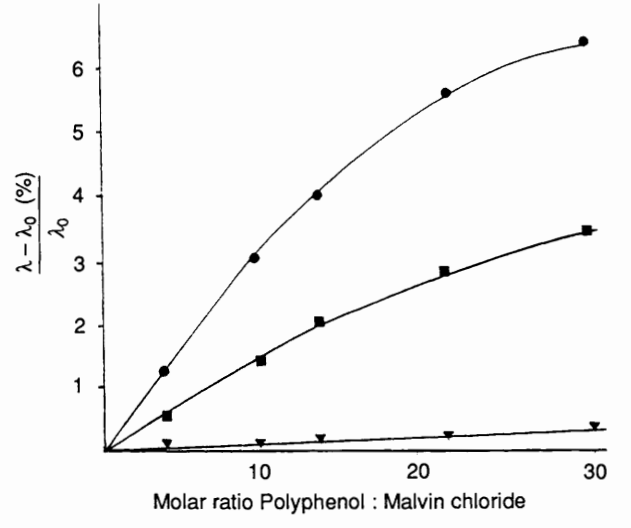
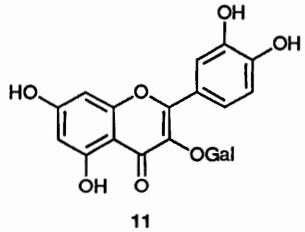


Fig. 6 Malvin chloride (0.25×10^{-4} mol dm^{-3}) in 0.02% CF_3CO_2H–H_2O (v/v). Comparisons of changes in bathochromic shift ($\lambda - \lambda_0/\lambda_0$) with different co-pigments: quercetin-3-β-D-galactoside (**11**, ●), β-1,2,3,4,6-pentagalloyl-D-glucose (**19**, ■), aesculin (**12**, ▼).

anthocyanin co-pigments in rose petals. None of the natural galloyl esters examined, however, was as effective a co-pigment as the flavonol glycosides[5–7,20,21], *e.g.*, quercetin-3-β-D-galactoside **11**. Comparative data on a molar basis is shown in Figs. 5



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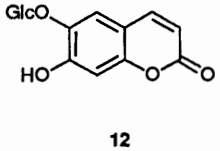

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and 6 for β-1,2,3,4,6-pentagalloyl-D-glucose **10**, quercetin-3-β-D-galactoside **11** and the coumarin aesculin **12**.

Although it has been speculated that co-pigmentation is facilitated by hydrogen bonding, Goto[6,7] proposed that the

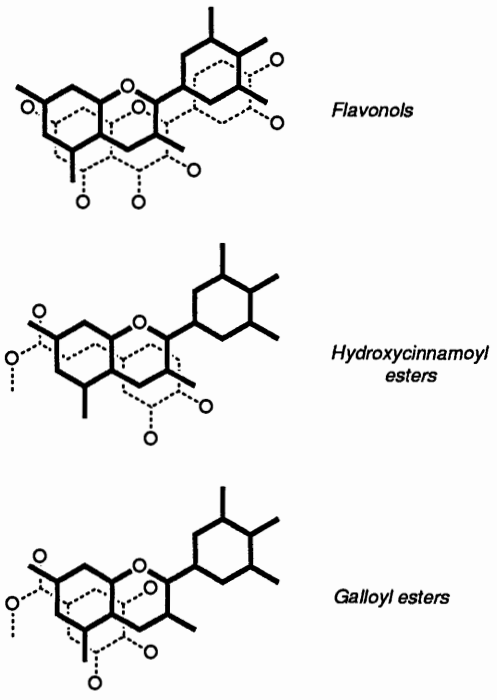


Fig. 7 Anthocyanin co-pigmentation, 'π–π' overlap–some comparisons

phenomenon arises either intramolecularly or intermolecularly by vertical 'hydrophobic stacking' of the aromatic nuclei of the anthocyanin and the co-pigment, *e.g.*, intramolecularly as in the 'heavenly blue anthocyanin' **8**. Brouillard and his colleagues[22] came to a similar conclusion and suggested that its origins were unique to the aqueous environment in which co-pigmentation naturally takes place. They proposed[5,22,23] that the flavylium cation **3** is stabilised by its interaction with the co-pigment, that the flavylium cation–co-pigment complex does not hydrate (Fig. 1) and therefore, at a given pH, more flavylium ions are present. This model broadly rationalises the above observations since, simply on the basis of spatial characteristics, most efficient 'π–π' interactions would occur with the planar flavonol **11** as compared to the galloyl ester group (*e.g.*, **10**), the caffeyl group in chlorogenic acid **15** and the coumarin aesculin **12** (Fig. 7) and the non-planar flavan-3-ols and sterically constrained ellagitannins (*e.g.*, **9**). Using the procedures of Brouillard[22,23] led to plots of ln $A - A_0/A_0$ *versus* ln (polyphenol) with straight lines of slope *ca.* 1.0 for the co-pigmentation of malvin **3** with quercetin-3-β-D-galactoside **11**, β-1,2,3,4,6-pentagalloyl-D-glucose **10** and aesculin **12**, which suggests the formation of a 1:1 complex in each case. Determination of the 1:1 association constant in water (containing 0.02% CF_3CO_2H) for malvin chloride **3** with both **10** and **11** was carried out by following the change in visible absorption at two wavelengths in the co-pigmentation experiment. This gave at 22 °C for quercetin-3-β-D-galactoside **11**, measured at λ_1 520.1 nm and λ_2 564.6 nm, a value of K 1686 (±58) dm^3 mol^{-1}, and for β-1,2,3,4,6-pentagalloyl-D-glucose **10**, measured at λ_1 520.1 nm and λ_2 539.8 nm, a value for K 987 (±37) dm^3 mol^{-1}. Measurements of the chemical shift changes ($\Delta\delta$, 400 MHz) induced by the addition of increasing aliquots of β-1,2,3,4,6-pentagalloyl-D-glucose **10** to malvin chloride in D_2O–CF_3CO_2D (1.1% v/v), (*e.g.* Fig. 8) and application of a modified Benesi–Hildebrand equation to the data[32] gave a value for the association constant for the formation of a 1:1 complex between **3** and **10** of 508 (±8) dm^3 mol^{-1} at 45 °C.

In aqueous trifluoroacetic acid (1.1% v/v), malvin chloride exists exclusively in the flavylium cation form **3**, and the visible absorbtion at λ_{max} 520.1 nm is at a maximum. Addition of

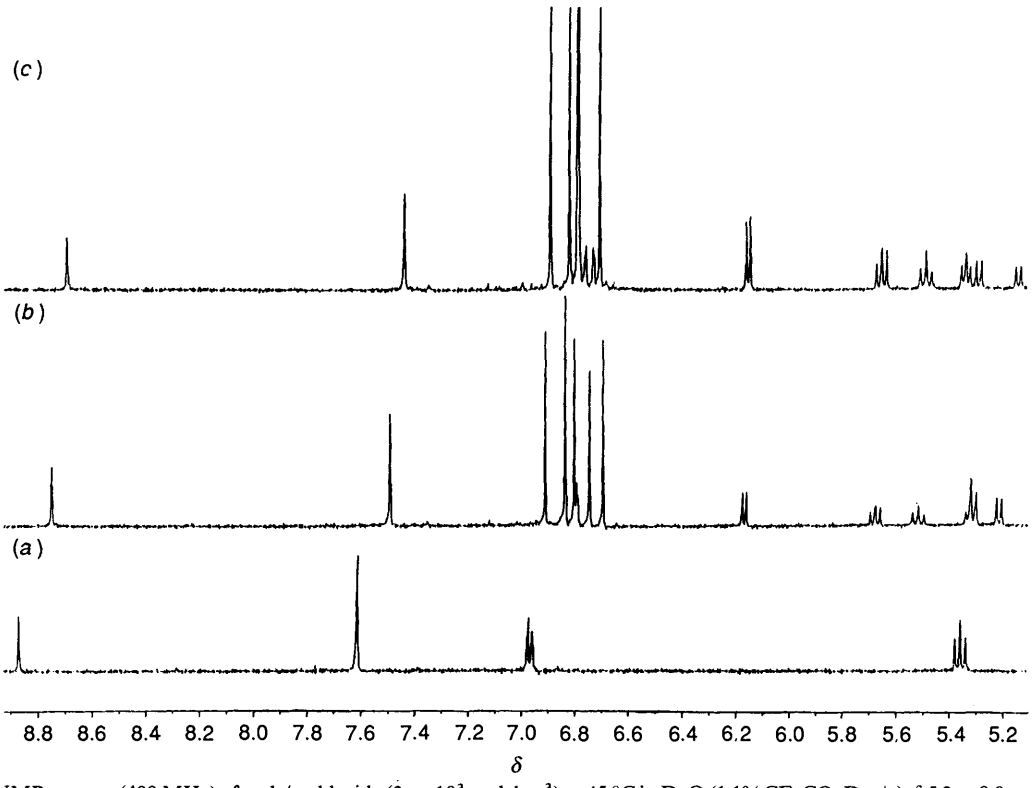


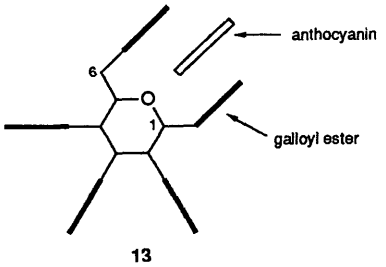
Fig. 8 Proton NMR spectra (400 MHz) of malvin chloride (3×10^3 mol dm^{-3}) at 45 °C in D_2O (1.1% CF_3CO_2D, v/v), δ 5.2 to 9.0, external reference TSP: (*a*) malvin chloride, (*b*) malvin chloride plus β-1,2,3,4,6-pentagalloyl-D-glucose **10**, 1:1, (*c*) malvin chloride plus β-1,2,3,4,6-pentagalloyl-D-glucose **10**, 1:2

polyphenols (*e.g.*, **10**, **11** and **12**) to such solutions produces no increase in absorption (*A*) but only the appropriate shift to longer wavelength in the visible λ_{max} (Tables 1 and 2). This observation strongly supports the view expressed by Brouillard[5,22–23] that, in solutions of intermediate pH, co-pigmentation results primarily from complexation of the polyphenol with the flavylium cation **3** and displacement of the equilibria towards this form of the pigment (Fig. 1).

Earlier work[1] has shown some polyphenols, and in particular flavan-3-ols, to become encapsulated within the hydrophobic cavity provided by β- and γ-cyclodextrins in aqueous media. The cyclodextrins themselves had no effect on the visible absorption spectrum of malvin chloride (0.2 mol dm^3 acetate, pH 3.42) nor on the co-pigmentation of various galloyl esters (*e.g.*, **10**) and this is consistent with the view that the rings A and B of **3** and the solvated galloyl ester group are too large/hydrophilic to enter the cavity of β- or γ-cyclodextrin.[1] However, addition of β- or γ-cyclodextrin effectively reduced the co-pigmentation of malvin chloride **3** by quercetin-3-β-D-galactoside **11** in water containing CF_3CO_2H (0.02% v/v). Using a 1:20 ratio of pigment to co-pigment, we recorded the following *decreases* in absorption maxima and wavelength of maximum absorption (malvin chloride 0.2×10^{-4} mol dm^{-3}): β-cyclodextrin (*A* 13%, λ 12%) and γ-cyclodextrin (*A* 60%, λ 40%). These observations are consistent with the Brouillard model, suggesting that the cyclodextrins reduce the effective concentration of the co-pigments in solution. High resolution proton NMR (400 MHz) studies suggested, on the basis of earlier work,[1] that encapsulation of the quercetin derivative takes place by independent entry of rings A and B into the cyclodextrin cavity.

The enhanced effectiveness of many natural galloyl esters of D-glucose as anthocyanin co-pigments probably also lies in their ability to form a cleft or pocket with which the anthocyanin may intercalate. High resolution proton NMR (400 MHz) studies in which aliquots of **3** were added to β-1,2,3,4,6-pentagalloyl-D-glucose **10**, in D_2O (containing 1.1% CF_3CO_2D) showed that the most significant chemical shift changes ($\Delta\delta$) occurred for the protons associated with the galloyl ester groups at C-1 and C-6, indicating a preference for anthocyanin complexation at or near the galloyl ester groups at C-1 and C-6 on the D-glucopyranose ring. This suggests a model such as **13** for the formation of a 1:1 complex[33] in which the two galloyl ester groups at C-1 and C-6 are maintained in the optimum position and separation to provide a sandwich conformation for hydrophobic stacking with the anthocyanin, by the buttressing effect of the remaining galloyl ester groups on the D-glucopyranose ring. This type of model is similarly valid for the association of **10** with a range of other substrates such as caffeine,[1] methylene blue and the carcinostatic antibiotic daunomycin.[24] An analogous type of model permitting intercalation is probably also operative in the case of the hexahydroxydiphenoyl ester (ellagitannin) davidiin,[33] (Table 2).

Although the effectiveness of a particular co-pigment seems to be related to its planarity and the potential surface area available for hydrophobic/'π–π' stacking (Fig. 7) other factors are of importance. Lady Robinson[19] first noted that chlorogenic acid **15** is an important natural co-pigment and its properties have been extensively studied by Brouillard.[22] Comparison as co-pigments of chlorogenic acid **15** and 5-*O*-*p*-coumarylquinic



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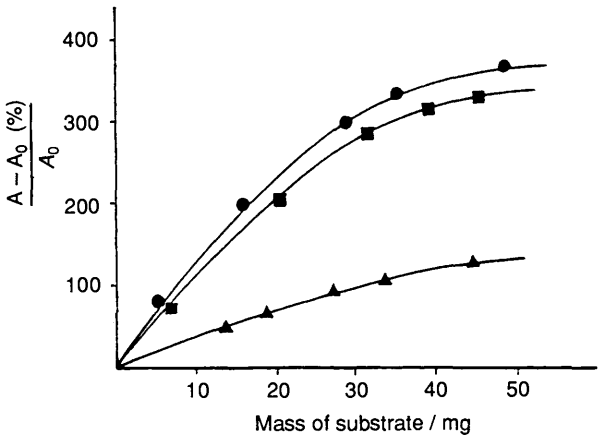


Fig. 9 Malvin chloride in acetate buffer (0.2 mol dm^{-3}, pH 3.42), comparisons of changes in absorbtivity ($A - A_0/A_0$). DNA and RNA (malvin chloride, 3.46 × 10^{-5} mol dm^{-3}), ATP (malvin chloride, 2.2 × 10^{-5} mol dm^{-3}). DNA, ●; RNA, ■; ATP, ▲.

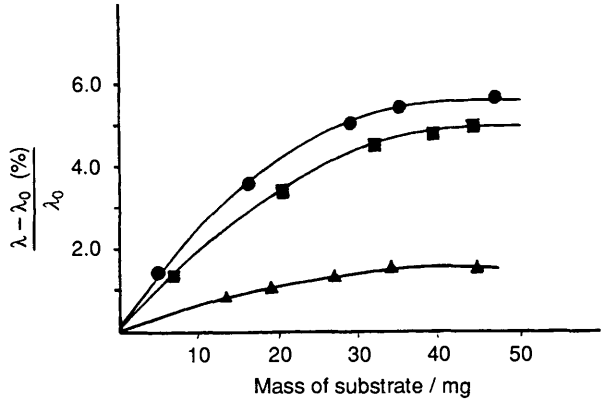
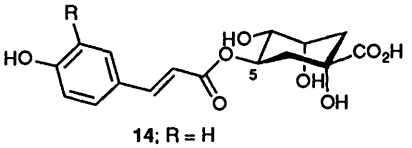


Fig. 10 Malvin chloride in acetate buffer (0.2 mol dm^{-3}, pH 3.42), comparisons of changes in bathochromic shift ($\lambda - \lambda_0/\lambda_0$). DNA and RNA (malvin chloride, 3.46 × 10^{-5} mol dm^{-3}), ATP (malvin chloride, 2.2 × 10^{-5} mol dm^{-3}). DNA, ●; RNA, ■; ATP, ▲.

acid **14** with malvin chloride (0.77 × 10^{-3} mol dm^{-3}, pigment–co-pigment ratio, 1:6) showed that the former phenolic substrate is significantly more effective as a co-pigment: **15** gave a red shift $\Delta\lambda$ 4.0 nm, $\Delta A/A_0$ 143%; **14** gave a red shift $\Delta\lambda$ 1.6 nm, $\Delta A/A_0$ 101%. It thus seems probable that the electron

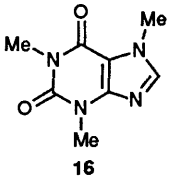


14; R = H
15; R = OH

donating capacity of the phenolic co-substrate is also of importance in the stabilisation of the flavylium cation **3**. Analogous proton NMR (400 MHz) experiments conducted with chlorogenic acid **15** and malvin chloride **3** in D_2O (containing 1.1% CF_3CO_2D) showed typical upfield shifts of the proton resonances of the flavylium cation **3** with increasing molar ratios of the co-pigment (0.5–10.0) (*cf.* Fig. 8) entirely consistent with the earlier interpretation of the co-pigment effect by 'π–π' stacking. Addition of increasing aliquots of chlorogenic acid **15** to a D_2O solution of malvin chloride **3** (3 × 10^{-3} mol dm^{-3}) in contradistinction resulted in increasing *downfield* shifts of the proton signals of the equilibriating species **3**⇌**4** and an enhancement of their intensity relative to those of the various carbinol species **6a**, **6b** (*cf.* Fig. 2). The distinctive downfield shifts of the proton signals of **3**⇌**4** presumably arise from the change of anthocyanin–anthocyanin self-associative 'π–π'/hydrophobic stacking to analogous anthocyanin– chlorogenic acid complexation and to the changing balance of magnetic anisotropic effects. The relative enhancement of intensity of the signals of the coloured forms **3**⇌**4** of the anthocyanin is nevertheless indicative of the general features of co-pigmentation.

Asen, Stewart and Norris[20] originally suggested that anthocyanin co-pigmentation arose by association of the co-pigment with, 'the red flavylium ion and the purple anhydro base'. Whilst several examples of intramolecular stabilisation of anthocyanins to give violet–blue floral colours have recently been put forward[6,7,16] (*e.g.*, **8**), there have been very few convincing demonstrations of intermolecular co-pigmentation with the anhydro-base form **4a**, **4b** recorded.[6,7,9]

The concept of anthocyanin stabilisation occuring *via* the mechanism of 'π–π'/hydrophobic stacking led naturally to the study of purine and pyrimidine bases, nucleotides and nucleic acids as co-pigments. Both RNA (sodium salt *ex.* calf thymus gland) and DNA (sodium salt *ex.* salmon testes) proved to be good co-pigments for the flavylium cation **3** at pH 3.42 (Figs. 9 and 10). The DNA–malvin chloride complex could be readily precipitated from solution by the addition of magnesium chloride (0.1 mol dm^{-3}). Comparisons (on a weight basis) with the disodium salt of ATP suggested that the two nucleic acids probably act in an analogous manner to the galloyl esters (*e.g.*, **13**, *vide supra*) by providing sites for 'π–π'/hydrophobic stacking and intercalation between adjacent strand base pairs. The *N*-methylated xanthines, caffeine **16** and theophylline **17** also



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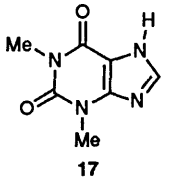

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displayed distinctive co-pigmentation effects (caffeine, Figs. 11–14). However, some comparisons are instructive in respect of the stabilisation of the flavylium cation **3**. In water containing 0.02% v/v CF_3CO_2H with malvin chloride (0.025 × 10^{-3} mol dm^{-3}), the following approximate pigment to co-pigment ratios were required to effect a change of 100% in $\Delta A/A_0$: quercetin-3β-D-galactoside **11**, 15:1; β-1,2,3,4,6-pentagalloyl-D-glucose **10**, 25:1; and caffeine **16**, 240:1. Additionally, in contrast to all other co-pigments examined, both caffeine (and theophylline) gave a distinctive shoulder at λ *ca.* 620 nm in the anthocyanin spectra (*cf.* Fig. 11). At pH 3.42, this shoulder is more pronounced, as is the asymmetry of the principal absorption maximum (Fig. 12). The solutions are visually red–violet and these effects have been ascribed to preferential stabilisation of the anhydro-base form **4a**, **4b** of the anthocyanin by the caffeine (and theophylline). Similar co-pigmentation by caffeine (and theophylline) occurs at pH 4.99, 6.2 and 6.85 (Figs. 13 and 14) and leads to the stabilisation of violet and finally blue forms of the anthocyanin (anhydrobase and possibly the anion **5**). At pH values >7.0 turquoise–green colours are formed. All these complexes are stable at 20 °C and the colours are retained for >21 d. At the higher pH values (*e.g.*, pH 4.99, Fig. 13) the co-pigmentation by caffeine (and theophylline) only leads to enhanced absorption at the principal absorption maxima: significant bathochromic shifts in the position of the absorption maxima do not occur. At these higher pH values, caffeine, and theophylline, are much more effective co-pigments than the hydroxycinnamyl esters **14**, **15**, various galloyl esters, RNA, DNA and ATP.

The co-pigmentation of malvin chloride **3** with caffeine **16** has

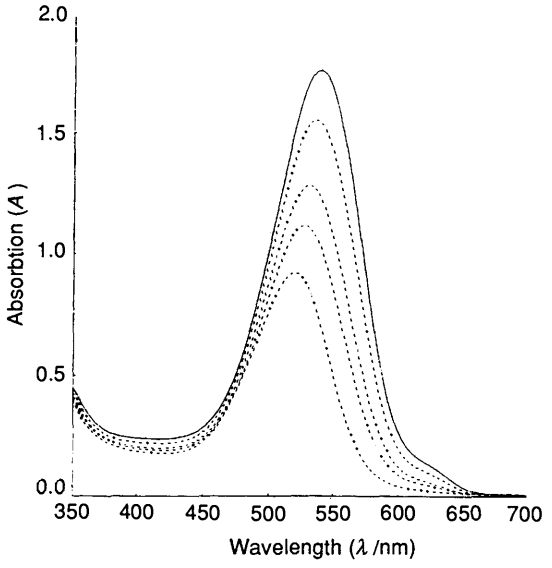

Fig. 11 Malvin chloride (1.56×10^{-4} mol dm^{-3}) in 0.02% CF_3CO_2H–H_2O, v/v. Changes in visible absorption spectrum produced by the addition of caffeine **16**. Pigment to co-pigment molar ratios, 1:11, 1.19, 1:58; final pigment to co-pigment ratio (——, 1:84).

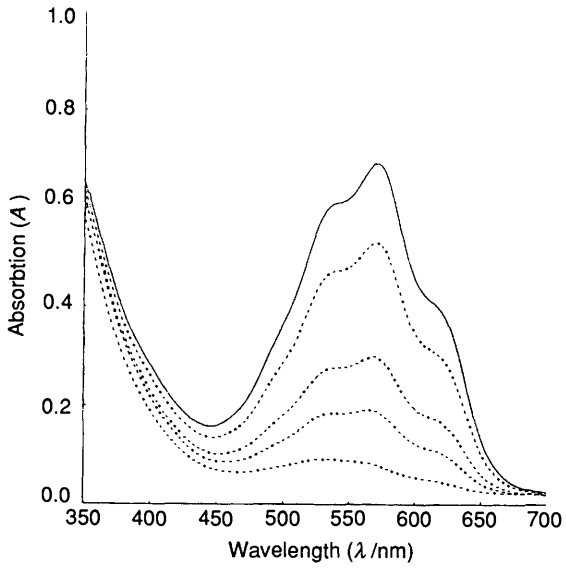

Fig. 13 Malvin chloride (2.3×10^{-4} mol dm^{-3}) in acetate buffer (0.2 mol dm^{-3}, pH 4.99). Changes in visible absorption spectrum produced by the addition of caffeine **16**. Pigment to co-pigment molar ratios, 1:25, 1:50, 1:100; final pigment to co-pigment ratio (——, 1:150).

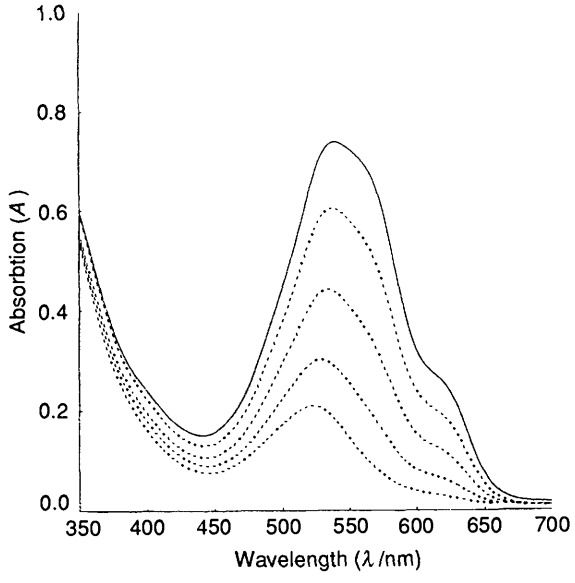

Fig. 12 Malvin chloride (2.6×10^{-4} mol dm^{-3}) in acetate buffer (0.2 mol dm^{-3}, pH 3.42). Changes in visible absorption spectrum produced by the addition of caffeine **16**. Pigment to co-pigment molar ratios, 1:25, 1:50, 1:100; final pigment to co-pigment ratio (——, 1:150).

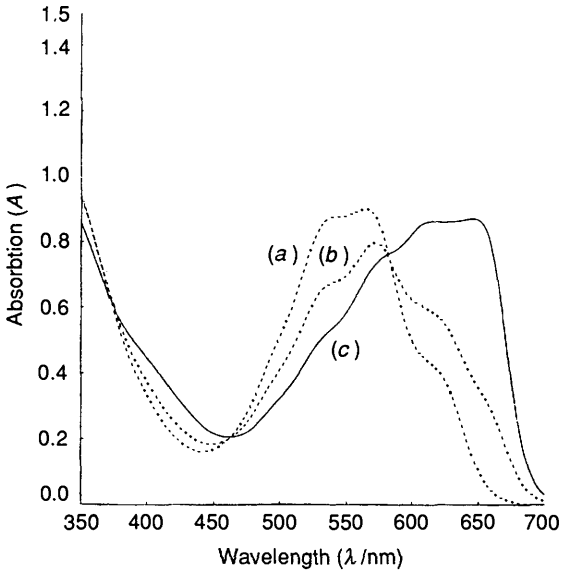

Fig. 14 Malvin chloride (2.35×10^{-4} mol dm^{-3}) in acetate buffer (0.2 mol dm^{-3}, pH 3.75) containing caffeine **16** (pigment to co-pigment molar ratio, 1:150). Changes in visible absorption spectrum produced by the addition of aliquots of NaOH (0.1 mol dm^{-3}): (*a*) pH 3.75, (*b*) pH 6.2, (*c*) pH 6.85.

been studied by proton NMR (400 MHz) in D_2O at 20 and 45 °C (Figs. 15 and 16). At 20 °C, addition of increasing aliquots of caffeine to the anthocyanin caused (Fig. 15) *upfield* shifts of the proton signals of all the species **6a**, **6b** but downfield shifts in those derived from the equilibriating species **3**⇌**4**, entirely analogous to the effects observed with chlorogenic acid **15**, *vide supra*. Presumably the explanation is similar. Support for this view was derived from measurements at 45 °C (Fig. 16). All the proton signals (**3**⇌**4**, **6a**, **6b**, **7a**, **7b**) move upfield upon the addition of caffeine, consistent with the view that, at this temperature, the species **3**⇌**4** is no longer self associated, *vide supra*.

A range of natural phenomena have been rationalised on the basis of the existence of strong attractive interactions between different 'π' systems.[15,47,48] The property of anthocyanin co-pigmentation is most satisfactorily explained in terms of the existence of similar attractive forces between anthocyanin and co-pigment molecules, driven initially in the aqueous medium by hydrophobic effects. In earlier work,[1] we have commented upon the structural features of caffeine **16** and galloyl esters (*e.g.* **10**) which contribute towards the mutual recognition and attraction of these molecules for one another. In the context of anthocyanin complexation natural phenolic esters may be regarded similarly as electron-rich systems able to associate with the electron-deficient flavylium cation **3**. These concepts, and the reasons for the preferential stabilisation of the anhydro-base form **4a**, **4b** of the anthocyanin by caffeine **16** and theophylline **17**, will be elaborated in later work.

Experimental

Natural Polyphenols.—Substrates were isolated as described previously: (−)-epicatechin, (+)-catechin, procyanidins B-2, B-3, B-4 and C-1 (all reference 34); sorghum procyanidin

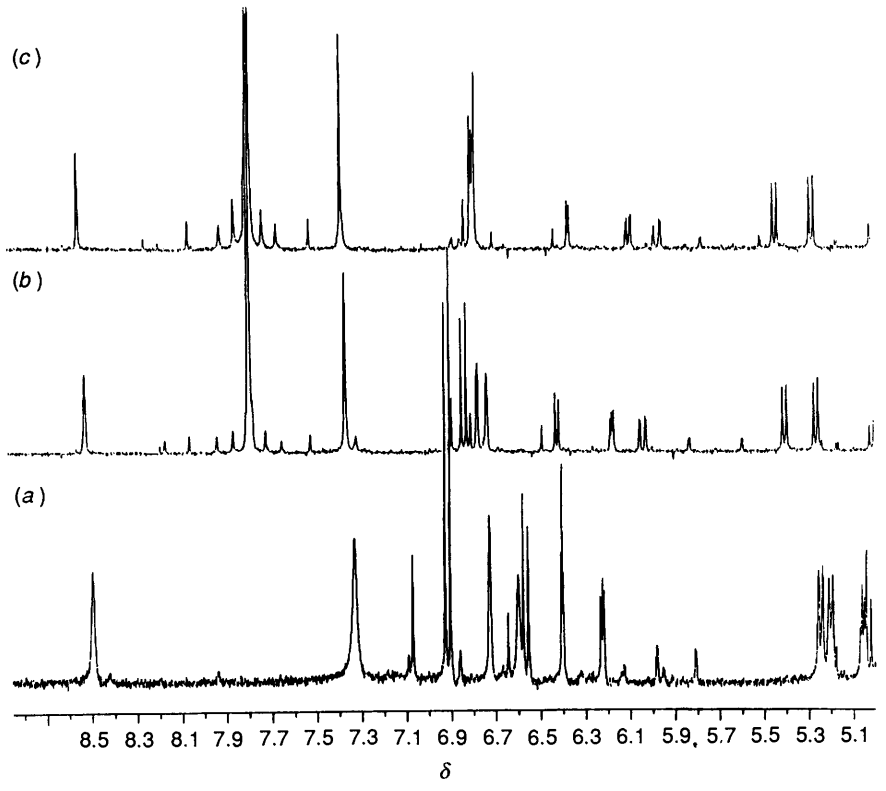

Fig. 15 Malvin chloride (4.0×10^{-3} mol dm^{-3}) proton NMR spectrum (400 MHz) in D_2O at 20 °C; δ 5.1 to 8.5, external reference TSP: (*a*) malvin chloride, (*b*) malvin chloride plus caffeine **16** 1:10, (*c*) malvin chloride plus caffeine **16**, 1:20.

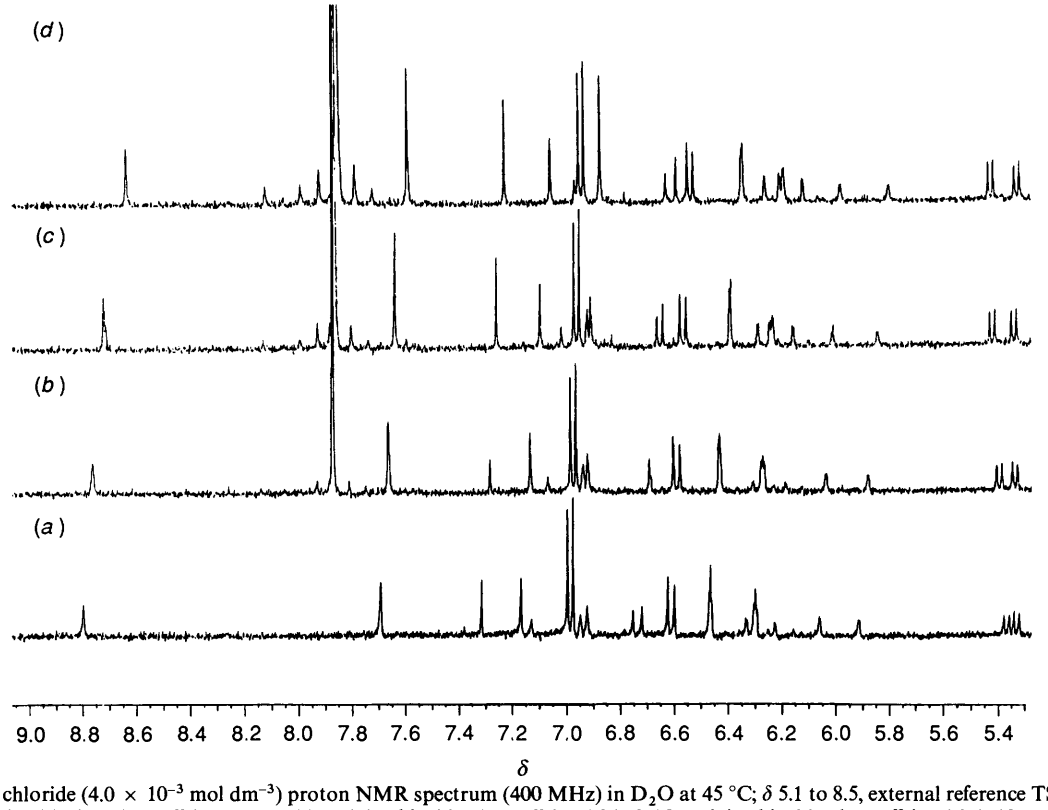

Fig. 16 Malvin chloride (4.0×10^{-3} mol dm^{-3}) proton NMR spectrum (400 MHz) in D_2O at 45 °C; δ 5.1 to 8.5, external reference TSP: (*a*) malvin chloride, (*b*) malvin chloride plus caffeine **16** 1:2, (*c*) malvin chloride plus caffeine **16** 1:6, (*d*) malvin chloride plus caffeine **16**, 1:10.

polymer;[35] quercetin-3-β-D-galactoside **11** from *Calluna vulgaris*;[36] (−)-epigallocatechin and (−)-epigallocatechin-3-gallate,[37] (+)-gallocatechin;[45] (+)-catechin-3-gallate;[46] 2,6-digalloyl-1,5-anhydro-D-glucitol;[38,39] β-1,3,6-trigalloyl-D-glucose, β-1,2,4,6-tetragalloyl-D-glucose and β-1,2,3,4,6-pentagalloyl-D-glucose **10**—all reference 39; vescalagin and castalagin **9**;[29,30] davidiin;[40] sanguin H-6;[41] chlorogenic acid **15**[42] and 5-*O*-*p*-coumaryl quinic acid **14**.[43] Malvin chloride was obtained from black grapes[11,44] and from Aldrich and Extrasynthese, France.

Ultra violet/Visible Spectroscopy.—Measurements were carried out with a PU-8270 UV/VIS scanning spectrophotometer equipped with a thermostatted cell. Observations in strongly acidic media employed 0.02–0.2% CF_3CO_2H/H_2O (v/v) and 0.2 mol dm^{-3} acetate buffer solutions to give media of pH 3.4–6.0. Malvin chloride solutions in the concentration range 10^{-4}–10^{-5} mol dm^{-3} were used for co-pigmentation experiments. The anthocyanin was dissolved in the appropriate medium and mixed with an aliquot of the co-pigment in the same medium to give a pigment concentration in the range specified and a given pigment–co-pigment ratio. Final solutions were equilibrated at 22 °C for 60 min before measurements were made. Typical pigment to co-pigment ratios employed were: (i), quercetin-3-β-D-galactoside **11**, 1:1 to 1:30; (ii), β-1,2,3,4,6-pentagalloyl-D-glucose **10**, 1:1 to 1:30; (iii), aesculin **12**, 1:1 to 1:20; (iv), caffeine **16** and theophylline **17**, 1:10 to 1:250.

Experiments with the sodium salts of RNA (calf thymus, BDH), DNA (salmon testes, Sigma) and ATP (Sigma) were made on a weight basis (0–50 mg) in buffer solution (10 cm^3).

Proton NMR Spectroscopy.—Measurements were carried out using a Bruker WH 400 spectrometer at 20, 30, 45 and 60 °C. Malvin chloride was dried over P_2O_5 at 0.5 mmHg for 48 h before use and solutions in deuterium oxide ($3.0–5.0 \times 10^{-3}$ mol dm^{-3}) were employed in all measurements. TSP were used as an external reference. Solutions were allowed to equilibrate for 30 min prior to examination and measurement of spectra. Studies of the flavylium cation **3** were carried out in deuterium oxide–CF_3CO_2D (1.1% v/v). Association constants were determined by procedures as outlined previously.[1]

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