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Effect of polysaccharides on the colour of anthocyanins

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The effect of a variety of plant polysaccharides and sugars on anthocyanin colour was investigated. The colour intensity (absorbance), but not the λ_{max}, of solutions of different anthocyanins was found to be diminished in the presence of amylose, amylopectin and α- and β-cyclodextrins whilst glucose, maltose and sucrose caused an increase in colour. This colour change was more apparent at pH 4 than at pH 2.

INTRODUCTION

During our work with coloured potato tubers it was noticed that extracts of anthocyanins often showed decreased colour. This decrease was in contrast to flower (camellia, delphinium, mallow) extracts and it was thought that the high levels of starch present in tubers might be responsible for this effect.

Anthocyanins

Anthocyanins are found in the vacuoles of many plants and their perceived colour depends on several factors such as concentration, solvent, temperature, pH, substitution on the B-ring and presence of copigments (Brouillard, 1982). pH has a large effect on the colour of anthocyanins because the three species of water (i.e. H^+, OH^- and H_2O) are highly reactive towards anthocyanins. Thus, the solvent water plays an important role in influencing both the stability and reactivity, as well as spectral properties of the various structures adopted by anthocyanins in aqueous solutions.

In acidic aqueous solutions, four species of anthocyanin molecule may exist in equilibrium: the quinonoidal base (A), the flavylium cation (AH^+), the pseudobase or carbinol (PB), and the chalcone (C) (Brouillard, 1982; Chen & Hrazdina, 1982), thus

$$AH^+ \Leftrightarrow A + H^+ \text{ acid/base equilibrium} \quad (1)$$

$$AH^+ + H_20 \Leftrightarrow PB + H^+ \text{ hydration equilibrium} \quad (2)$$

$$PB \Leftrightarrow C \text{ ring/chain tautomeric equilibrium} \quad (3)$$

Interconversion between these structures may take place as shown in Fig. 1.

In most higher plants the pH of a mature vacuole is acidic, and a survey of many flowers has shown that the pH of the vacuole ranges from 2·5 to 7·5 (Stewart *et al.*, 1975). For highly acidic cells pigmentation is probably due to the flavylium form (AH^+) alone, whereas in the range pH 3–4, both the flavylium cation and the neutral tautomer (A) may contribute to colour, whilst at pH 4–6 the neutral tautomer dominates due to deprotonation of the hydroxylated flavylium cation (eqn (1), Fig. 1) (Brouillard, 1982). Thus pH is a major factor in anthocyanin colour (Brouillard, 1982; Chen & Hrazdina, 1982).

Protection of the flavylium ring against attack by water is absolutely necessary to maintain intensely coloured solutions. One way of retaining anthocyanin colour is by removal of water and displacement of the hydration/dehydration equilibrium towards the coloured species (i.e. reduce the extent of the hydration reaction) (Brouillard, 1983).

Copigmentation may also protect anthocyanins against hydration thus preserving their red colour. Copigments include flavonoids, polyphenols, alkaloids, amino acids and organic acids. This effect occurs only in aqueous systems and is sensitive to pH, temperature and composition of the solution (Chandra *et al.*, 1993).

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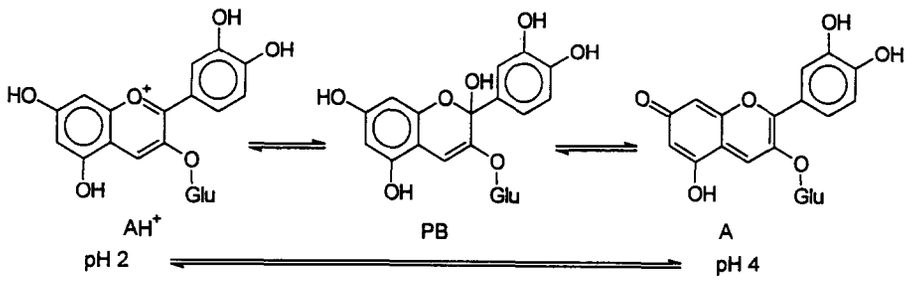

Fig. 1. Effect of pH upon the structure of cyanidin (after Brouillard, 1982). Only reactions at acid pH (i, ii) are considered here.

Plant carbohydrates

Relevant properties of selected plant and food carbohydrates are summarised below.

Most plant starches comprise 20–25% amylose and 75–80% amylopectin. Amylose consists of essentially linear chains of α-(1,4)-linked glucose with occasional α-(1,6) branch points (Morrison & Karkalas, 1990). Because amylose exists as a helical structure, with six successive glucose units per revolution and a 5Å wide cavity in the centre of the helix, it can form inclusion complexes with alcohols, fatty acids and iodine (Noltemeyer & Saenger, 1980). Amylopectin consists of α-(1,4)-glucan chains joined at numerous α-(1,6) branch points which disrupt helix formation and therefore only the short linear chains are capable of forming helices (Kennedy & White, 1979).

Cyclodextrins (CDs) are cyclic oligosaccharides containing α-(1,4)-linked glucose units; the most common are α- and β-cyclodextrin which have six and seven glucose units, respectively (Saenger, 1980; Chandra *et al.*, 1993). Cyclodextrins have hydrophobic cavities with an inner diameter of 6Å for α-CD and 7·5Å for β-CD. Thus they can form reversible inclusion complexes with smaller molecules (often phenolic substances) which fit into the cavity (in both the solid and liquid state), and are known to bind readily to molecules regardless of solvent environment. This is in marked contrast to copigmentation effects which only occur in aqueous systems (Chandra *et al.*, 1993).

Pectins are important components of plant tissues, especially of the parenchyma of fleshy roots (Coultate, 1989); for example, potato tubers contain 0·36% pectin (Robinson, 1987). Pectin is a mainly linear polymer composed of esterified galacturonic acid residues, linked by α-(1,4) glycosidic bonds (Belitz & Grosch, 1987; Robinson, 1987).

Polydextrose is composed almost entirely of randomly cross-linked glucose polymers with the α-(1,6) bond predominating (Leibrand *et al.*, 1985).

Because anthocyanin pigments in plant foods (and derived products) may occur with a variety of saccharides, the latter may affect their structure and stability thus playing a role in the final colour of food products. It was therefore of interest to investigate the effect of selected saccharides on the spectral properties of anthocyanins.

MATERIALS AND METHODS

Preparation of anthocyanins

Anthocyanin extracts were prepared from suitable plant materials:

- pelargonidin-3-glucoside (Pg-3-glu) from freeze-dried strawberries (*Fragaria ananassa*);
- delphinidin-3,5-diglucoside (Dp-3,5-glu) and delphinium-3-glucoside (Dp-3-glu) from *Delphinium* spp. petals;
- malvidin-3,5-diglucoside (Mv-3,5-glu) from mallow petals (*Malva sylvestris*);
- pelargonidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Pg-RF) from tubers of potato (*Solanum tuberosum*) cultivar 'Red Flesh'; and
- malvidin-3-*p*-coumaroyl-rutinoside-5-glucoside (Mv-U) from potato cultivar 'Urenika'.

Tissue was extracted with 15% acetic acid in methanol and the anthocyanins chromatographed on a column of C_{18} silica, eluted with 60% (v/v) methanol, and redissolved in water. Each plant extract also contained other flavonoids and phenolic acids but these did not appear to have any obvious effect on the results since similar patterns were found with the pure anthocyanins. The purity of the two anthocyanin standards, cyanidin-3-rutinoside (Cy-3-rut) and malvidin-3-glucoside (Mv-3-glu) (Plantech), was confirmed by HPLC.

The pH of the anthocyanin solutions was adjusted (with HCl or NaOH) to pH 2 and/or pH 4, giving a final absorbance (at λ_{max}) of 0·6–1·0 at pH 2, or 0·4–0·8 at pH 4. The anthocyanins used are listed in Table 1.

Table 1. Sources of anthocyanins

Anthocyanin	Source	λ_{max} (nm)	
		pH 2	pH 4
Cy-3-rut	Standard (Plantech)	512	518
Dp-3,5-glu	Delphinium flowers	534/6	568
Dp-3-glu	Delphinium flowers	536/8	570
Mv-3-glu	Standard (Plantech)	518	526
Mv-3,5-glu	Mallow flowers	540	546
Pg-3-glu	Strawberry fruit	498	498
Pg-RF	Potato tubers, cv. 'Red Flesh'	504	518
Mv-U	Potato tubers, cv. 'Urenika'	524	538

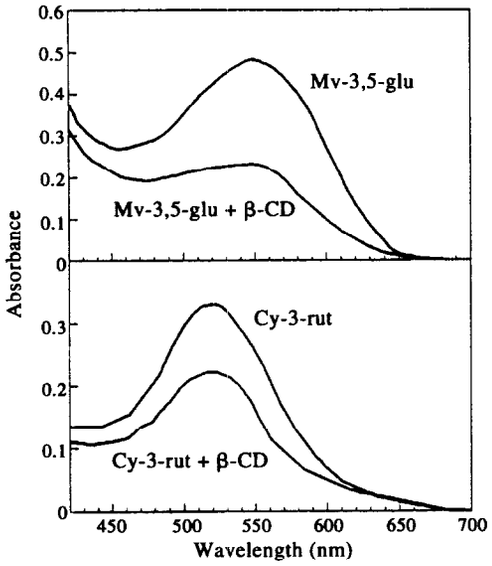

Fig. 2. Spectra of malvidin-3,5-diglucoside and cyanidin-3-rutinoside at pH 4·0 in presence or absence of β-cyclodextrin.

Carbohydrates

Solutions of the following carbohydrates were prepared by dissolving in water followed by adjustment to pH 2 or 4: 1·5% amylopectin (AP), 3% α-cyclodextrin (α-CD) (cyclohexa-amylose), 3% β-cyclodextrin (β-CD) (cyclohepta-amylose), 1·5% pectin, 3% polydextrose (PolyD, Pfizer, USA), 50% sucrose, 50% glucose, 50% fructose, and 50% maltose (all as w/v). Solutions of 1·5% amylose (AM) were dissolved initially in 50% (w/v) KOH and then the pH was adjusted (the final concentration of KOH was 12·5%). All reagents except PolyD, were supplied by Sigma (USA).

Spectrophotometric assay

For the spectrophotometric assays, equal amounts (250 μl) of anthocyanin and carbohydrate solutions were mixed and the spectra (blanked against carbohydrate solution) measured after 60 min. Controls (without added carbohydrate) contained 250 μl anthocyanin plus 250 μl distilled water (at pH 2 or 4) (or KOH/HCl blank for amylose). Results are presented as a percentage of the control absorbtion at its visible λ_{max}. Spectra were recorded on a HP8452A diode-array spectrophotometer.

RESULTS

Representative spectra showing cyanidin-3-rutinoside (standard) and mallow extract (mainly malvidin-3,5-glucoside) both without (control) and with β-cyclodextrin are given in Fig. 2 which shows the decrease in absorbance at 60 min after the addition of β-cyclodextrin. Figures 3(a)–(c) show the effect of added AM, AP,

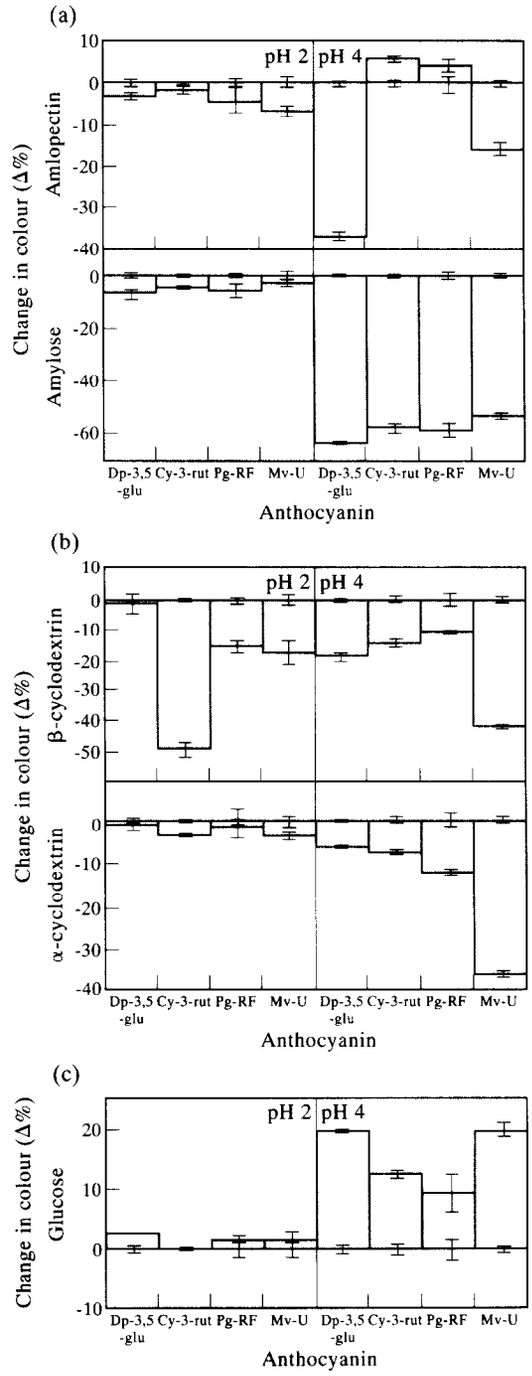

Fig. 3. Effect of added carbohydrate on colour of anthocyanins at pH 2·0 or 4·0. Results are presented as % change in absorbance at λ_{max}.

α-CD, β-CD and glucose on a selection of anthocyanins at pH 2 and 4. In the presence of AM, AP, α-CD and β-CD there was a significant decrease in visible absorbance at pH 4. However, addition of AP and α-CD showed no significant change at pH 2 whereas AM and β-CD also showed a significant decrease in absorbance at pH 2. Typically, the decrease in colour was larger at pH 4 than at pH 2. The addition of glucose resulted in an increase in absorbance, especially at pH 4. The results for sucrose and maltose are similar to the results presented in Fig. 3(c) for glucose.

Table 2. Effect of added carbohydrates on anthocyanin colour (compared with control)

Carbohydrate	ΔA at pH 2	ΔA at pH 4
Amylose[a]	Decreased	Decreased
Amylopectin[a]	No change	Decreased
α-CD[a]	No change	Decreased
β-CD[a]	Decreased	Decreased
PolyD	No change	No change
Pectin	No change	Decreased (Dp), increased (Pg and Mv)
Sucrose	No change	Increased
Glucose[a]	No change	Increased
Fructose	No change	No change
Maltose	No change	Increased
DMSO (33%)	No change	Decreased
KOH/HCl	No change	Increased

[a]See Figs 3(a)–(c) for more detail.

Results with all carbohydrates are summarised in Table 2. Polydextrose and fructose showed no significant effect at either pH, whilst pectin showed a change only at pH 4. Essentially similar results were obtained with anthocyanins from different sources. The degree of colour change appeared to be related primarily to the identity of the anthocyanin aglycone and only secondarily to its sugar substitution and/or acylation pattern.

In all cases there was no change in λ_{max}, only a change in the intensity of absorbance. The fading effect was dependent on both the anthocyanin and carbohydrate concentration. We also confirmed the observation of Yamada *et al.* (1980), that the loss of colour was time-dependent and was stable after 60 min.

DISCUSSION

From Figs 3(a)–(c) it may be seen that the addition of AM, AP, α-CD and β-CD resulted in significant decreases in anthocyanin colour with AM and β-CD showing the largest effect. The α-CD molecule has a smaller inner diameter than β-CD and thus appeared to be less efficient at forming inclusion complexes with anthocyanins. AP is a much more highly branched molecule than AM and can only form helical structures within the unbranched straight chains and therefore caused far less decrease in anthocyanin colour than did AM.

This 'fading' effect has also been reported by Yamada *et al.* (1980) who examined the interaction of only three anthocyanins (pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-(4-(*p*-coumaroyl)-L-rhamnosyl-(1,6)-glucosido)-5-glucoside) with α- and β-cyclodextrin. In their experiments each anthocyanin was adjusted to pH 2 and added to varying concentrations of α- or β-CD. Addition of β-CD resulted in the fading of the two anthocyanin solutions (Pg-3-glu and Cy-3-glu) and this effect was increased with higher concentrations of β-CD. Addition of α-CD resulted in a fading effect only for pelargonidin-3-glucoside and this was less than that measured for β-CD. This effect was found to be reversed at extremely low pH values (pH < 0·5). No change in λ_{max} was observed. Yamada *et al.* (1980) also investigated the effect of amylose (dissolved in 33% DMSO) on these anthocyanins, and found that the addition of AM only caused the fading of pelargonidin-3-glucoside, but the extent of this effect could not be determined because of immediate AM precipitation. The precipitation of AM from DMSO solutions was also observed in our experiments; it was overcome by initially dissolving AM in 50% KOH and then readjusting its pH to 2 or 4. This procedure resulted in the AM remaining in solution and retaining its helical structure, as shown by its characteristic reaction with KI/I_2 solution. Yamada *et al.* (1980) postulated that the fading phenomenon was due to the conversion of a flavylium ion into a pseudobase in two steps: (1), the formation of an inclusion complex of the anthocyanin with the CD, and (2), conversion of the flavylium ion to the pseudobase by catalytic action of the CD.

More recently Chandra *et al.* (1993) reported the stabilising effect of 0·8–1·6% α- and β-cyclodextrin on anthocyanins extracted from tart cherries with β-CD having the largest effect. Juice samples containing these cyclodextrins retained higher levels of anthocyanins after 12 weeks of storage than solutions without added cyclodextrins. This stabilisation was thought to occur because the anthocyanin was protected from attack (by water, etc.) by being in the inclusion complex. If this is so then starch (amylose) might also be expected to exhibit a similar protective effect, which could be useful in the long-term storage of foods containing anthocyanins.

The absorbance of anthocyanins (compared with the control) increased when glucose, sucrose and maltose were added, but this may be due to a decrease in water activity (a_w). Anthocyanin colour is known to increase upon the removal of water by displacement of the hydration/dehydration equilibrium (Fig. 1) towards the coloured species (Brouillard, 1983); it has been found also that sugar molecules are effective at binding water (Coultate, 1989). Copigmentation, where the aromatic residues of the copigments stack with the pyrylium ring of the flavylium cation, also reduces the extent of the hydration reaction and therefore increases the stability of the coloured species (Brouillard, 1983). Typically, copigmentation causes a bathochromic shift in the visible λ_{max} of all anthocyanins, as well as the increase in absorbtion. However, in our experiments no change in λ_{max} was observed.

The presence of sugars (and their breakdown products, furfural and 5-hydroxymethyl-furfural) in anthocyanin solutions has been found by some workers to cause anthocyanin degradation (reviewed in Francis, 1989). When sugars were added to pelagonidin-3-glucoside, fructose led to greater pigment degradation than glucose, sucrose or maltose (Francis, 1989) whilst others (R. E. Wrolstad, pers. comm.) have found an increase in stability of anthocyanins when stored in the presence of added sugars.

The effect of pectin was inconsistent; with malvidin and pelargonidin-glycosides it caused a small increase

in colour, whereas with delphinidin-3,5-glucoside it led to a small decrease in colour. Asen *et al.* (1972) have reported that pectin may act as a copigment.

The effect of carbohydrates on anthocyanin colour was more dramatic at pH 4 than at pH 2, and this was due in part to the greatly reduced absorbance of the anthocyanins at pH 4. Since the pH of plant vacuoles lies between 2·5 and 7·5 (Stewart *et al.*, 1975), it is possible that the presence of other molecules in the vacuole may have an effect on the colour shown by the anthocyanins. Similarly, in fruit juices and homogenates, the colour of anthocyanins could be affected by the levels of starch and sugars, if present, as well as the pH and other copigments. This phenomenon could be of importance for industries which rely on the natural colour of anthocyanins to produce food products with pleasing appearances.

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