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Stabilisation of the colour of anthocyanins in solutions by admixture with phytocomponents from apple

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SUMMARY
The colour of anthocyanins can be enhanced by planar chemical complex formation, or stabilised by the formation of adducts. We investigated these properties using fruit extracts. Delphinidine and its glucoside, myrtilline, at 0.1 mM were mixed with 10 mM caffeoylquinic acid (CQA; chlorogenic acid) or 6 mM ferulic acid (FeA). In a second experiment CQA, ferulic acid, and a CQA-rich apple extract were added in variable concentrations to a blackcurrant pigment preparation and incubated at 37°C in 0.02 M citrate buffer at pH 1.5, pH 3.0, or pH 4.5. The colour change was followed by measuring the absorbance at 540 nm and the composition of the solutions was assayed by HPLC using diode array detection. Deglycosylation of anthocyanins leads to a loss of colour and chemical stability. It is therefore of major importance that the enzyme preparations used during juice extraction be devoid of glycosidases. Addition of phenolic acids to an anthocyanin solution increases and stabilises the colour, which is accompanied by an acceleration of the loss of the native anthocyanins and the formation of coloured adducts, presumably based on the pyranoanthocyanin structure. The glycosyl residue in anthocyanins is necessary for colour stabilisation, which appears to be due to the formation of new pigments. Caffeoylquinic acid appeared to be more efficient than ferulic acid, and a CQA-rich extract from apple also resulted in stabilisation of the colour of a blackcurrant extract.

An attractive red colour is one of the main sensory characteristics of berry products, notably berry juice. However, the colour of red berry juices is linked to the presence of anthocyanins and is highly susceptible to change, mainly browning.

The colours of anthocyanins vary with pH. The general scheme (Brouillard, 1988) is as follows: at pHs up to 3.0, the flavylium cation dominates, leading to bright orange to purple colours. As the pH rises, the carbon in position 2 may become hydrated, leading to colourless carbinol pseudo-bases, which can open to form yellow retro-chalcones, while proton transfer reactions with the carboxylic acid groups lead to more violet-coloured quinonoidal bases. Further deprotonation at pH 6.0 –7.0 results in the formation of more bluish-coloured, resonance-stabilised quinonoid cations. At pH values typical for fruit products, the anthocyanins can thus exist under a variety of more or less coloured and reactive forms.

Two distinct phenomena occur when anthocyanins are mixed with phenolics in solution. In the short term, the colour of anthocyanins can be enhanced by co-pigmentation, a phenomenon in which complexing compounds such as flavonoids or phenolics bind through weak hydrophobic bonds to the anthocyanins. This is a relatively rapid phenomenon, leading to hyperchromic effects (i.e., an increase in absorbance) and to bathochromic shifts (i.e., a shift toward higher wavelengths, hence bluer colours; Boulton, 2001). In the long term, it has been reported that their colour may be retained, notably in the presence of phenolic acids, by the formation of more stable adducts, known as pyranoanthocyanins (Rentzsch *et al*., 2007b). These pyranoanthocyanins differ from anthocyanins in their colour, usually with a hypsochromic shift towards shorter wavelengths, and therefore a more orange colour. Due to the formation of an additional pyran ring, their colour is also more stable with pH. Many forms of pyranoanthocyanins have been described, mostly in red wine (e.g., hydroxyphenol-pyranoanthocyanins, also known as vinylphenol-pyranoanthocyanins, vitisins, vinylflavonol-pyranoanthocyains, or portisins) depending on the nature of the added molecule. Vinylphenol-pyranoanthocyanins have been detected in blood orange juice (Hillebrand *et al*., 2004), or in strawberry and raspberry juices after the addition of ferulic acid (FeA; Rein *et al*., 2005), or in cherry beverages (Rentzsch *et al*., 2007a). Model systems have been used to study the factors responsible for their formation (Gris *et al*., 2007; Yawado and Morita, 2007; Rein and Heinonen, 2004). These adducts have less intense and yellower colours, but they are also considerably more stable than anthocyanins.

Within the EU Project "ISAFRUIT", we tested the possibility of colour stabilisation through adduct formation for blackcurrant nectars. We started by evaluating the impact of the addition of phenolic acids on the colour of anthocyanin and anthocyanidin solutions, then used apple extracts rich in phenolic acids on a blackcurrant extract.

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MATERIALS AND METHODS

We used acetonitrile of HPLC-grade (Biosolve, Valkenswaard, The Netherlands), and acetic acid (Biosolve) and formic acid (Merck, Darmstadt, Germany) of analytical grade. All the water used was purified using a Millipore system (MilliQ, Millipore SA, Molsheim, France).

Caffeoylquinic acid (CQA) and ferulic acid (FeA) were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Delphinidine and its glucoside, myrtilline, were from Extrasynthèse (Lyon, France). The CQA-rich extract (PA 039) was obtained by Val-de-Vire (Condé sur Vire, France) from a water-extract of cider apple pomace, using a proprietary process. The blackcurrant pigment preparation was a kind gift from CHR Hansen (St Germain Les Arpajon, France).

Incubation of pure compounds

Stabilisation of the colour of anthocyanidins and anthocyanins was tested using delphinidine and its glucoside, myrtilline, at approx 0.1 mM, in contact with 10 mM CQA or 6 mM FeA. The latter was used due to solubility problems at higher concentrations of FeA. The mixtures were incubated at 37°C in 0.02 M citrate buffers at pH 1.5, 3.0, or 4.5 for up to 38 d. The colour change was followed by measuring the absorbance at 540 nm with a UV-visible spectrophotometer (Molecular Devices, St Grégoire, France).

Incubation of extracts

CQA, FeA, and the apple extract were added to a blackcurrant pigment preparation. The total anthocyanin concentration in the blackcurrant extract was 25 g l^{-1}. It was diluted 500-fold to obtain a molarity of approx. 0.125 mM for all experiments. CQA and FeA were added at three-, ten-, 30- and 100-times the molarity of the anthocyanins in the blackcurrant extract. The apple extract was used only at a molarity of 1.5-, three-, ten-, and 16-times that of the anthocyanins. All samples were incubated at 37°C in 0.02 M citrate buffer at pH 3.0 for up to 38 d. Solubilisation of FeA was facilitated by the use of dimethylsulphoxide (DMSO)

Analytical methods

A Waters HPLC apparatus (Milford, MA, USA) System 717, with an autosampler equipped with a cooling module set at 4°C, a 600 E multisolvent system, a 996 photodiode array detector (DAD), and a Millenium 2010 Manager system were used. A Purospher RP18 endcapped column (4 mm × 250 mm; 5 µm; Merck) was used. The solvent system was a gradient of solvent A (890 ml water, 100 ml formic acid, and 10 ml acetonitrile,) and solvent B (acetonitrile). The first 0.5 min was 1% (v/v) B, which increased linearly to 7% B from 0.5 to 1 min, followed by a plateau at 7% B from 1 to 4 min, then increased linearly from 4 to 9 min to 14% B, then increased linearly from 9 to 15 min to 100% B, followed by a plateau from 15 to 35 min at 100% B, followed by washing and reconditioning of the column.

Liquid chromatography with mass spectroscopy detection (LC-MS) was carried out on a composite HPLC system including an SCM1000 vacuum membrane degasser (ThermoQuest, San Jose, CA, USA), a Surveyor autosampler (ThermoFinnigan, San Jose, CA, USA), an 1100 Series binary pump, and a 1100 Series variable wavelength detector (Agilent Technologies, Palo Alto, CA, USA). Samples (3 µl) were injected onto a Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm; 3.5 µm; Agilent Technologies). The oven temperature was 30°C. The eluant was a gradient of solvent A (10 ml l^{-1} formic acid in water) and solvent B (10 ml l^{-1} formic acid in acetonitrile). The following gradient was applied at a flow rate of 0.2 ml min^{-1}: the column was condition at 3% B, which was increased linearly from 0 to 5 min to 9%, then linearly from 5 to 15 min to 16% B, then (from 15 to 45 min) to 50% B, followed by washing and reconditioning of the column.

The ion trap mass spectrometer was an LCQ Deca (ThermoFinnigan) equipped with an electropsray ionisation source. The whole column effluent at 0.2 ml min-1 was injected into the mass spectrometer source, without splitting. Experiments were carried out in the negative ion mode. The source parameters were: spray voltage (3.7 kV), capillary voltage (70.0 V), sheath gas (65 arbitrary units), auxiliary gas (20 arbitrary units), and capillary temperature (250°C). Nitrogen was used as the nebulising gas. Helium was used as the damping gas. The auto gain control mode was used to optimise injection time. Full scan spectra were acquired from *m/z* values of 100–1,000. Data were collected and processed using Version 1.2 Xcalibur software (ThermoFinnigan).

RESULTS AND DISCUSSION

Model solutions

Bright red solutions were obtained at pH 1.5 (initial

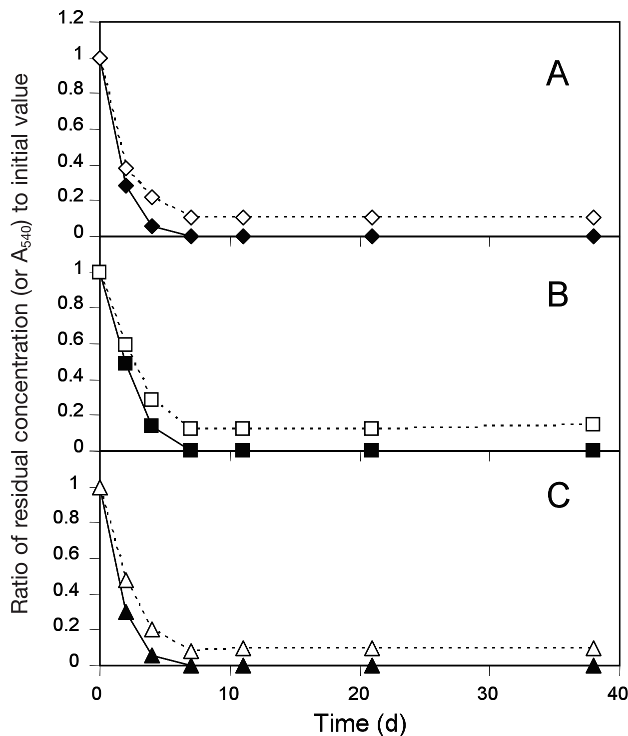

FIG. 1
Relative molecular degradation (loss of concentration) and the loss of absorbance at 540 nm of delphinidine alone (Panel A), with ferulic acid (Panel B), or with caffeoylquinic acid (Panel C) at pH 1.5 and 37°C over 38 d of incubation.All data are presented relative to an initial value at t = 0 d. Concentrations: ♦, alone; ■, with ferulic acid; ▲, with caffeoylquinic acid. Absorbance at 540 nm: ◇, alone; □, with ferulic acid; or Δ, with caffeoylquinic acid.

absorbances values at 540 nm of 0.5 with delphinidine and 0.7 for myrtilline) and for myrtilline at pH 3.0 (initial A_{540} of 0.3). At pH 4.5, the solutions were colourless or slightly yellow, in accordance with the known colour properties of anthocyanins and anthocyanidins. At all pHs, delphinidine disappeared rapidly (< 10 d) from the solution and this was accompanied by the loss of the red colour at pH 1.5 (Figure 1). Ferulic acid had a slight stabilisation effect, but CQA did not. This underlines the need to avoid the presence of glycosidases in the enzyme cocktails used to extract blackcurrant juice.

Myrtilline had much greater stability (Figure 2), particularly at pH 1.5 and at pH 4.5, with losses of approx. 20% in 20 d. The absorbance of myrtilline solutions at 540 nm was better maintained at pH 3.0 in the presence of CQA, while, at pH 1.5, FeA caused an accelerated loss of colour. We used a molar ratio of anthocyanin:CQA of 1:100, identical to that used by Eiro and Heinonen (2002), and an anthocyanin:FeA molar ratio of 1:60, due to the lower solubility of FeA. At pH 3.37, Eiro and Heinonen (2002) observed little or no effect of chlorogenic acid on the colour loss of pelargonidin, cyanidin, and malvidin glycosides, while FeA enhanced the A_{540} of pelargonidin-3-O-glucoside and cyanidin-3-O-glucoside, and stabilised the A_{540} of malvidin-3-O-glucoside, but not cyanidin-3-[2”-xylosyl-6”(coumaroyl-glucosyl)]-galactoside, an acylated anthocyanin.

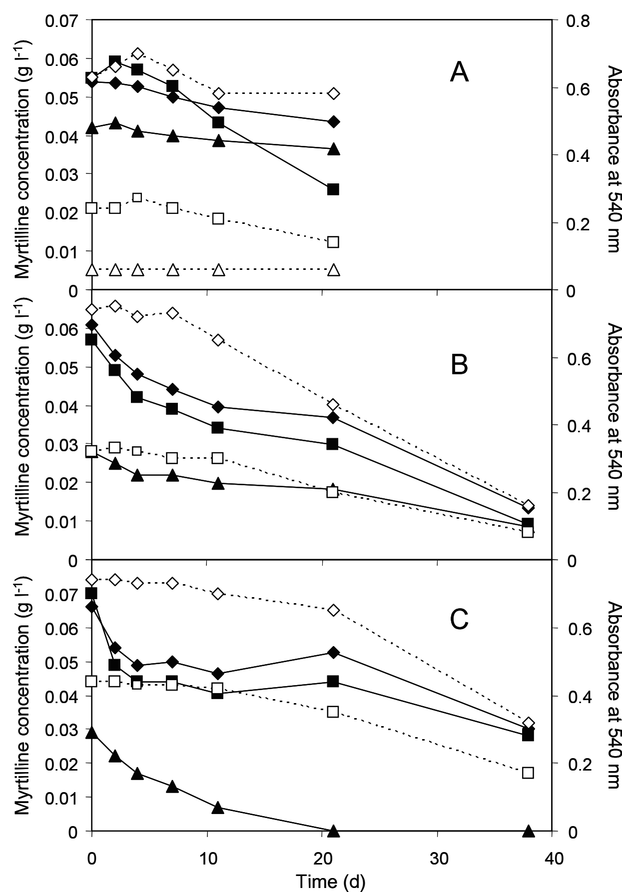

FIG. 2
Absolute molecular degradation (loss of concentration) and loss of absorbance at 540 nm of myrtilline alone (Panel A), with ferulic acid (Panel B), or with caffeoylquinic acid (Panel C) at pH 1.5, 3.0, or 4.5, and 37°C over 38 d incubation. Concentrations: ◆, pH 1.5; ■, pH 3.0; ▲, pH 4.5. Absorbance at 540 nm: ◇, pH 1.5; □, pH 3.0; or Δ, pH 4.5.

In our mixtures, myrtilline disappeared more rapidly in the presence of phenolic acids. The disappearance of the native anthocyanins was more rapid with CQA, and this was particularly marked at pH 4.5. Adducts that absorbed at 540 nm appeared in the chromatograms. The LC-MS chromatograms of the adducts indicated molecular ions at *m/z* 611 with CQA, and at 597 with FeA, corresponding to vinylphenol adducts formed with the loss of the carboxylic acid portion of these molecules, as described by Rein *et al.* (2005) among others.

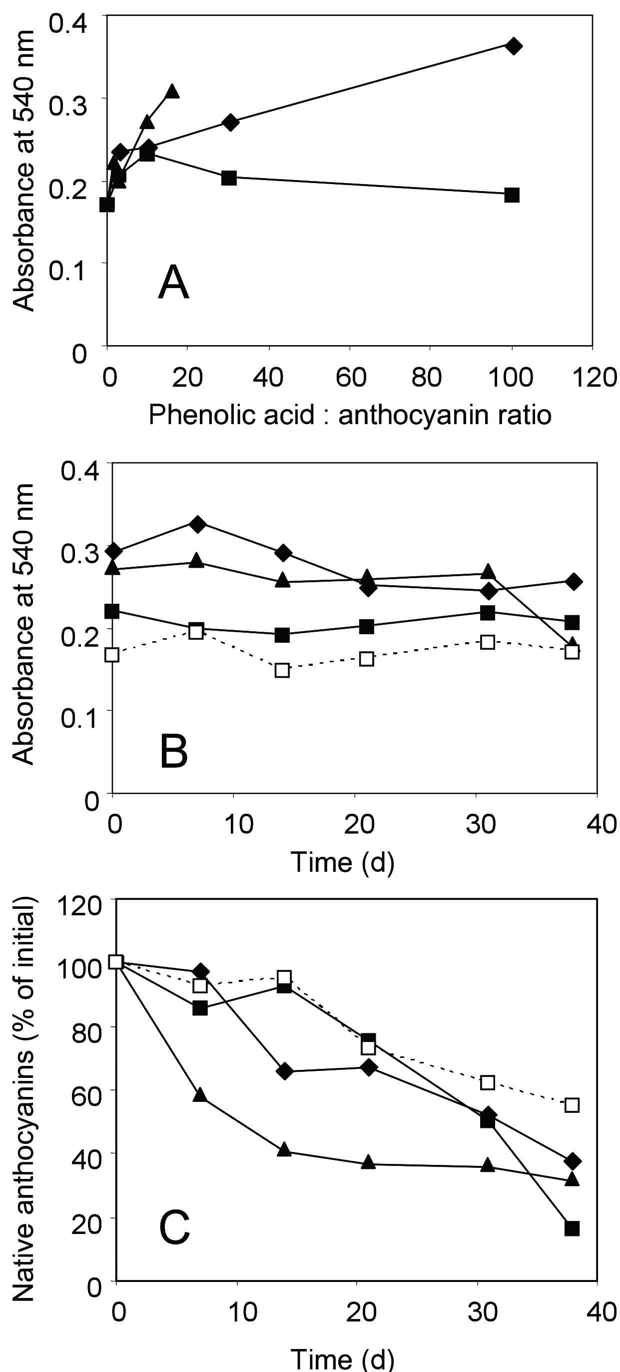

FIG. 3
Impact of the addition of phenolic acids on the colour of a blackcurrant extract at pH 3.0 and 37°C. The effect of the phenolic acid:anthocyanin molar ratio (Panel A), or of the duration of incubation at a molar ratio of 10:1 on the absorbance at 540 nm (Panel B), or on the disappearance of native anthocyanins at a molar ratio of 10:1 (Panel C). ■, with ferulic acid; ▲, with caffeoylquinic acid; ◆, with a CQA-rich apple extract; □, alone.

Blackcurrant pigment preparation

The blackcurrant pigment from CHR Hansen was a viscous liquid with approx. 30% (w/v) dry matter (DM), which contained 100 mg anthocyanins g^{-1} DM, essentially cyanidin-3-O-galactoside and rutinoside, plus some myrtilline. The apple extract (a dry powder) contained 114.6 g kg^{-1} CQA, 8.8 g kg^{-1} *p*-coumaroylquinic acid, 1.0 g kg^{-1} caffeic acid, and 29.8 g kg^{-1} flavan-3-ols. The remainder consisted primarily of carbohydrates and oxidised polyphenols.

As shown above, the colour of the blackcurrant pigment solution was quite stable at pH 3.0 for durations up to 38 d (Figure 3A). The addition of phenolic acids led to an increase in A_{540} (Figure 3B). For CQA, and the CQA-rich apple extract, the A_{540} increased with the molar ratio of phenolic acids to anthocyanins. However the concentration of the apple extract could not be increased beyond a phenolic acid:anthocyanin ratio of 20:1, due to the low solubility of some components of this extract. In addition, the apple extract, which was yellowish-to-brown when dissolved, imparted a brownish tint to the solution. For FeA, the A_{540} was maximum at a FeA:anthocyanin ratio of 10:1, then decreased. Over prolonged periods of storage (< 38 d at 37°C), at a molar ratio of 10:1, the effect of CQA decreased; while, with FeA or the apple extract, the A_{540} appeared stable. HPLC chromatograms indicated a loss of native anthocyanin structures faster than the loss of colour (Figure 3C) and the formation of adducts.

CONCLUSIONS

We have confirmed that deglycosylation of anthocyanins leads to a loss of colour and molecular stability. It is therefore of major importance that the enzyme preparations used for fruit juice extraction are devoid of glycosidases. The addition of phenolic acids, free or esterified, to an anthocyanin solution, can increase and stabilise the colour. This was accompanied by an acceleration in the rate of disappearance of native anthocyanin molecules and the formation of coloured adducts of the vinylphenol-pyranoanthocyanin structure. Caffeoylquinic acid appeared to be more efficient than FeA at stabilising colour at pH 3.0, with a more rapid loss of the native molecules. Esterification might have increased the reactivity of the vinylphenol moiety. The use of extracts from plants, rather than pure compounds, has the drawback that phenolic extracts are usually already coloured, and so can alter the original colour; moreover, they may contain insoluble material. Stabilisation of the colour of red fruit juices does not therefore appear to be achievable by the straightforward application of phenolic-rich extracts.

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