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Characterisation of anthocyanin–betalain mixtures for food colouring by chromatic and HPLC-DAD-MS analyses

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Abstract

Anthocyanins and betalains that are mutually exclusive in nature were mixed to investigate their potential synergism for food colouring purposes. The blends obtained from four commercial anthocyanic (black carrot, elderberry, sour cherry and strawberry) and one betalainic extract (red beet) were studied with respect to colour evolution over three weeks. While new colour shades were produced by blending anthocyanins with betalains, chroma and hue angle of the mixtures changed over time. The greatest number of new colour shades with acceptable chroma was obtained at pH 4.5 and 5, the stability maxima of red beet, succeeded by pH 3.0. Interestingly, at pH 3.5 only one combination yielded an acceptable chroma, whereas at pH 7 no mixture was stable. To determine individual anthocyanins and betalains in mixtures in a single run, a HPLC method was developed and combined with a mass spectrometer for the identification of specific pseudomolecular and daughter ions.

Keywords: Anthocyanins; Betalains; Colouring foodstuff; Black carrot; Elderberry; Red beet; Sour cherry; Strawberry; Mixtures; CIE$L^*C^*h^\circ$; HPLC-DAD-MS

1. Introduction

In contrast to synthetic dyes with an annual growth of 1% on the European market until 2008, colouring foodstuffs are forecasted to gain 10–15% in the same time range (Anonymous, 2004). Although not clearly legally defined, colouring foodstuffs are produced by an unselective extraction of fruits, vegetables or spices using water- or oil-based solvents. Since their characteristic composition remains unchanged, the resulting extracts are considered as food and consequently do not require declaration with an E-number. Therefore, consumer-friendly labelling "coloured with fruit or vegetable extract" is possible (Stich, Kloos, & Hoeck, 1999; Stintzing & Carle, 2004) giving them the character of an ingredient rather than an additive.

Besides carotenoids and chlorophylls, the wide range of colours of flowers, fruits, vegetables and grains results from the presence of the water-soluble anthocyanins and betalains (Delgado-Vargas, Jiménez, & Paredes-López, 2000; Mazza & Miniati, 1993; Stintzing & Carle, 2004). Due to their divergent biosynthetic pathways on the arogenate level, the precursor of phenylalanine (anthocyanins) and tyrosine (betalains), respectively (Springob, Nakajima, Yamazaki, & Saito, 2003; Strack, Vogt, & Schliemann, 2003), anthocyanins and betalains have never been found jointly in plant tissues. A study by Vogt (2002) provided further evidence that anthocyanin accumulation appeared earlier in evolution than betalains. It has therefore been hypothesized that the two classes substitute each other

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with respect to their biochemical functions such as UV-protection, attraction of pollinators and seed dispersers (Stintzing & Carle, 2004). While the anthocyanins impart orange, red, purple and bluish tints to virtually all members of the Angiospermae, betalain occurrence is restricted to 13 families of the order Caryophyllales (Clement & Mabry, 1996). Anthocyanins and betalains also greatly differ with respect to their pH-stabilities. Whereas the former are generally most intensely coloured in low pH media, the latter are characterized by a stability optimum of pH 5–7 (Francis, 2000). Consequently, food commodities with pH values up to 3 are usually coloured with anthocyanins, those exhibiting lower acidities with betalains, respectively. Although red cabbage, purple sweet potato and black carrot extracts offer an extended stability at low acid pH due to their anthocyanin structures acylated with hydroxycinnamic acid moieties, they are not qualified to fill the betalain gap (Stintzing & Carle, 2004). While both pigment classes have only been investigated separately with respect to their colour qualities in food (e.g., Bassa & Francis, 1987; Cabrita, Fossen, & Andersen, 2000; Cai, Sun, & Corke, 1998; Cevallos-Casals & Cisneros-Cevallos, 2004; Duhard, Garnier, & Mégard, 1997; Giusti & Wrolstad, 2003; Kuusi, Pyysalo, & Pippuri, 1977; Pasch, von Elbe, & Sell, 1975; Pasch & von Elbe, 1977; Sapers, Taffer, & Ross, 1981; Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002; Stintzing, Schieber, & Carle, 2003), to the best of our knowledge, an investigation on intermixtures of anthocyanins with betalains has not yet been scientifically considered.

Therefore, the present study aimed at producing defined blends from various commonly applied anthocyanic fruit and vegetable concentrates with red beet to find out whether the pH-dependent applicability of anthocyanins could be extended by the addition of red beet. Secondly, it was hypothesized that blending might produce tonalities that could not be achieved with either concentrate at a given pH. For these reasons, characterisation of anthocyanin–betalain mixtures was performed assessing the colour qualities over a period of three weeks at different pH conditions. To identify both pigment classes in one run, a HPLC method was developed combined with mass spectrometric detection allowing to monitor their specific pseudomolecular and daughter ion spectra.

2. Materials and methods

2.1. Pigment material

Elderberry, strawberry and sour cherry concentrates were provided by Wild (Heidelberg, Germany). Black carrot and red beet concentrates were from Jahncke (Drochtersen, Germany) and Ernteband (Winnenden, Germany), respectively.

2.2. Solvents and reagents

Reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Deionised water was used throughout.

2.3. Total soluble solids

Total soluble solids were measured at 20 °C with an Abbe refractometer (Carl Zeiss, Oberkochen, Germany).

2.4. Tinctorial strength and quantification of anthocyanins and betalains

Each anthocyanin or betacyanin concentrate was diluted 1 + 6 with deionised water. Further dilutions were performed with McIlvaine buffer to adjust a constant absorbance of 1.00 for each commodity. The resulting total dilution factors ranged from 63 for strawberry up to 1050 for elderberry. From these values, the tinctorial strengths of each concentrate were calculated. The monomeric anthocyanin content was assessed using a pH-differential method (Giusti & Wrolstad, 2001) and expressed as cyanidin 3-glucoside (ε = 26,900 L/mol cm; molecular weight = 449.2 g/mol and λ = 510 nm) for elderberry, sour cherry, and black carrot and pelargonidin 3-glucoside (ε = 15,600 L/mol cm; molecular weight = 433.2 g/mol and λ = 496 nm) for strawberry, respectively. The betalains were quantified according to Stintzing et al. (2003) at pH 6.5 in buffered solution considering the absorption of non-betalainic substances at 600 nm. The values obtained were expressed as betanin (ε = 60,000 L/mol cm; molecular weight = 550.1 g/mol and λ = 538 nm).

2.5. HPLC analyses

The HPLC system was a Merck-Hitachi (Merck, Darmstadt, Germany) with an auto sampler L-7200, an interface module D-7000, an L-7100 pump, an L-7350 column-oven with a Peltier cooling module and an L-7400 UV–Vis detector in series with an L-7450A diode array detector. Optimum separation of anthocyanins and betalains was achieved on an analytical scale (250 × 3 mm i.d.) LUNA $C_{18(2)}$-reversed phase column with a particle size of 5 μm (Phenomenex, Torrance, CA), fitted with a security guard C_{18} ODS (4 × 3.0 mm i.d.) at a flow rate of 1 mL/min and a constant temperature of 25 °C. Eluent A was 5% formic acid and B was MeCN/water (60/40, v/v). Separation was accomplished starting with 3% B in A, followed by a linear gradient to 20% B in A at 30 min and then to 50% B at 40 min.

Absorption maxima of betalains tended to be higher than those of the anthocyanins. Therefore, an intermediate monitoring wavelength of 530 nm was chosen to consider both pigment groups. A 1 + 6 (w/w) aqueous dilution of each concentrate was prepared from which anthocyanin–betalain blends were produced at ratios of 1 + 1, 1 + 2 and 2 + 1 (v/v). Aliquots of unmixed and blended samples of 20 μL were injected for analyses. Duplicate determinations were performed throughout.

2.6. High performance liquid chromatography-tandem mass spectrometric analyses

LC–MS/MS analyses were carried out with an Agilent HPLC series 1100 instrument (Agilent, Waldbronn, Germany) equipped with ChemStation software, a degasser (G1322A), a binary gradient pump (G1312A), a thermoautosampler (G1329/1330A), a column oven (G1316A), and a diode array detector (G1315A) in series with a Bruker Esquire 3000+ ion trap mass spectrometer (Bremen, Germany). Specific mass data were obtained by applying the same solvent system as described above with the ESI source running in the positive ionisation mode (range: *m*/*z* 50–1000). Nitrogen was used as the dry gas at a flow rate of 12.0 L/min and a pressure of 70.0 psi. The nebulizer temperature was set to 365 °C. Using helium as the collision gas (4.1×10^{-6} mbar) with a fragmentation amplitude of 1.2 V (MS^2) collision-induced dissociation was monitored.

2.7. Colour and spectral analyses

For colour and absorbance measurements, a UV–Vis spectrometer (Perkin–Elmer, Überlingen, Germany) equipped with a UV–Vis (UVWinLab V 2.85.04) and a colour (Wincol V 2.05) software (Perkin–Elmer Instruments, Norwalk, CT) was used. Colour evaluation was performed under six pH conditions: pH 1 for highest anthocyanin stability, pH 3 for monitoring loss of flavylium cation stability and the start of the betalain stability range, pH 3.5 as a typical pH for food, pH 4.5 at the absorption sink of anthocyanins, pH 5 for maximum betalain stability and pH 7 for assessing pigment appearance at neutrality (Giusti & Wrolstad, 2001; Stintzing et al., 2002, 2003; Von Elbe, 1975). Clark-Lubbs (HCl-KCl) and McIlvaine (Citrate-phosphate) buffer solutions were applied for pH 1 and at pH 3, 3.5, 4.5, 5, 7, respectively. Each concentrate was first re-diluted with deionised water at a constant ratio of 1 + 6 (w/w). Stock solutions were further thinned down with buffer solutions at the respective pH optima of anthocyanins and betalains, then allowed to equilibrate for 60 min at room temperature, before absorption was normalized to 1.00 ± 0.10. Resulting dilutions of defined colouring strength were applied for blending individual anthocyanin samples with red beet at ratios of 1 + 1, 1 + 2 and 2 + 1 (v/v) in the pH range from 1 to 7 as mentioned above. Spectral reflectance curves (380–780 nm) were subsequently monitored in 1 cm pathlength disposable cuvettes from which a^* and b^* values were determined using illuminant D_{65} and 10° observer angle. Metric chroma C^* and hue angle $h°$ were obtained according to $C^* = (a^{*2} + b^{*2})^{0.5}$ and $h° = (\arctan b^*/a^*)$, respectively. While the former is an index of the brilliance or saturation of a solution, the latter expresses the tonality on a colour wheel counterclockwise from 0/360° (magenta-red), 90° (yellow), 180° (bluish-green), to 270° (blue). All determinations were performed in duplicate and weekly repeated over a period of 22 days. Between measurements cuvettes were covered with parafilm, stored refrigerated at 2 °C in the dark, and allowed to reach room temperature before data acquisition.

3. Results and discussion

3.1. Pigment characterisation and quantification

A concentrate from red beet (*Beta vulgaris* L. ssp. *vulgaris* 'Garden Beet Group'; Lange, Brandenburg, & De Bock, 1999) was chosen as the only commercially available betalain source. Selected anthocyanin-based extracts were sour cherry (*Prunus cerasus* L.), elderberry (*Sambucus nigra* L.) and black carrot (*Daucus carota* L. ssp. *vulgaris* var. *atrorubens* Alef.) for cyanidin, as well as strawberry (*Fragaria* × *ananassa* Duch.) for pelargonidin derivatives, respectively (Glässgen, Seitz, & Metzger, 1992; Mazza & Miniati, 1993). While the major strawberry pigment is known to be extremely prone to colour loss, black carrot extracts provide an interesting amount of acylated structures that have shown to be stable at pH values otherwise detrimental for anthocyanins (Giusti & Wrolstad, 2003; Stintzing et al., 2002). Elderberry and sour cherry are mainly composed of cyanidin-glycosides (Mazza & Miniati, 1993) and were therefore expected to display intermediate stability.

3.1.1. Tinctorial strength and pigment content

The tinctorial strengths expressing the colouring power of the respective concentrates are given in Table 1. Elderberry reached the highest value, followed by black carrot, sour cherry and strawberry at pH 1, while red beet at pH 5 displayed a value similar to black carrot. The pigment contents obtained from spectrophotometric determinations ranged from 13.2 g/kg in elderberry to 1.1 g/kg in strawberry (Table 1).

3.1.2. LC-DAD-MS/MS

To differentiate between betalains and anthocyanins, various tests have been reported (Delgado-Vargas et al.,

Table 1
Characteristic data of anthocyanic (pH 1) and red beet (pH 5) concentrates

Pigment concentrate	TSS[a] (° Bx)	Total dilution factor	A at λ_{max}	Tinctorial strength[b]	Pigment content[c] (mg/kg)	Calculated as[d]
Red beet	69.0	630	1.00	630	4077.0	Betanin
Sour cherry	63.2	105	1.08	113	5919.7	Cyd 3-glc
Elderberry	63.5	1050	1.03	1081	13263.0	Cyd 3-glc
Strawberry	61.6	63	1.02	64	1134.5	Pel 3-glc
Black carrot	58.6	630	1.02	643	4880.0	Cyd 3-glc

[a] TSS, total soluble solids.
[b] Tinctorial strength of concentrate (at optimum pH) = Absorbance (A) at λ_{max} * total dilution factor.
[c] Pigment content in concentrate.
[d] Cyd 3-glc, cyanidin 3-glucoside and Pel 3-glc, pelargonidin 3-glucoside.

2000; Kremer, 1982; Nielson & Harley, 1996). An official HPLC method providing fingerprints of common fruit juices to detect adulterations among anthocyanic or with red beet extracts has also been published (IFU, 1998). However, the separation of red beet betalains was not accomplished and black carrot, now commonly available as commercial concentrate, was not included. Therefore, a new method allowing simultaneous determination of betalains and anthocyanins by HPLC-DAD-MS/MS was established. The chromatograms of the respective 1 + 1 (anthocyanin extract + red beet) combinations are given in Fig. 1. Based on comparison with the literature data, anthocyanins and betalains were readily identified by their retention time order, spectral and mass characteristics including product ion, daughter ion and neutral loss scanning (Table 2). As earlier reported (IFU, 1998), betacyanins were generally more polar than anthocyanins since betanin (**1**, betanidin 5-*O*-glucoside) eluted considerably earlier than cyanidin 3-glucoside (**11**) the most polar anthocyanin monoglucoside in this study (Table 2). The principle chemical structures of the characteristic anthocyanins and betacyanins in the concentrates applied are shown in Fig. 2.

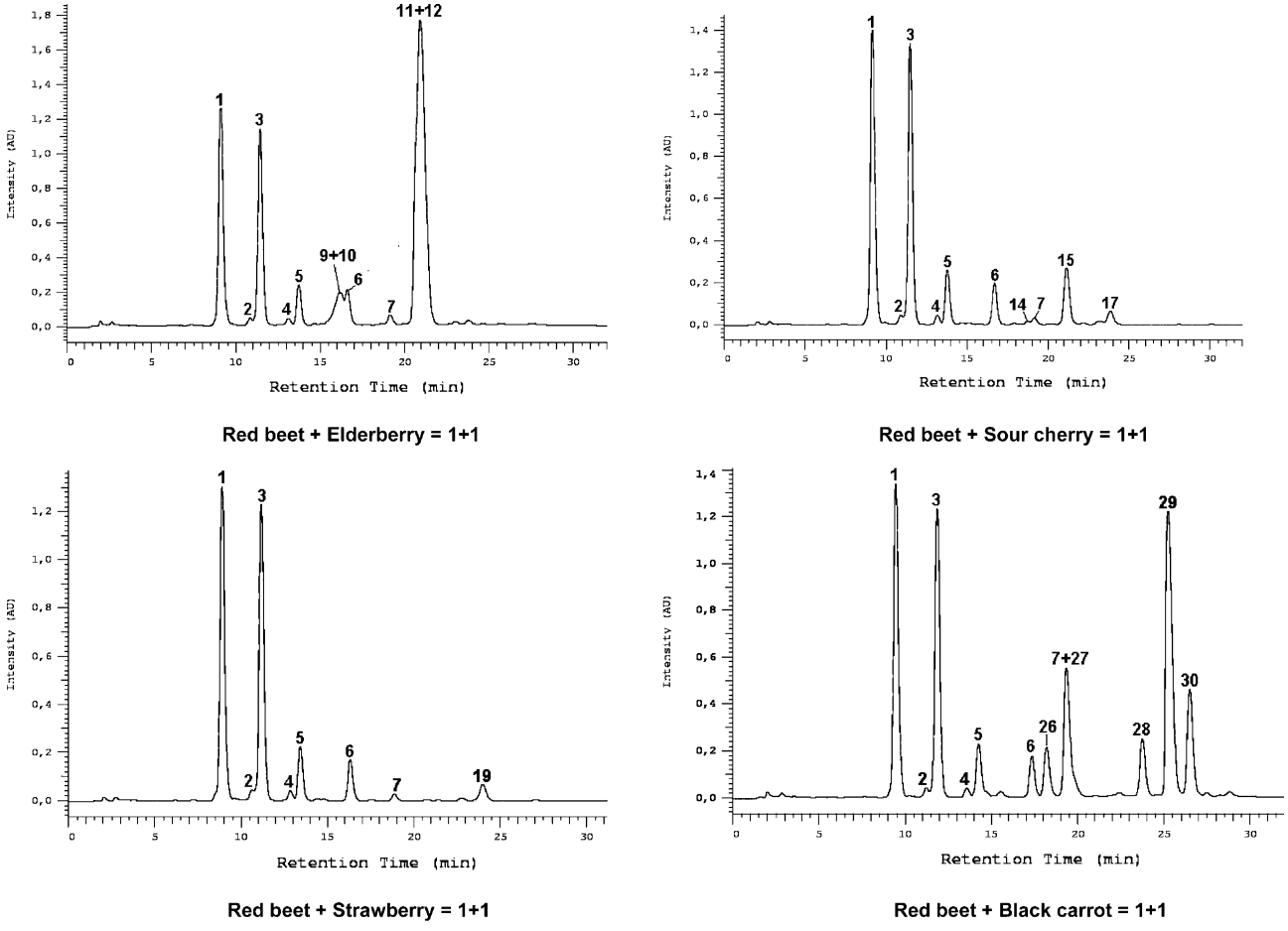

Fig. 1. HPLC separation of red beet-anthocyanin blends (λ_{max} = 530 nm; Peak assignment is given in Table 2).

Table 2
Peak assignment for betacyanins and anthocyanins of five commercial concentrates

	Compound name[a]	Rt (min)	UV–Vis$_{max}$ (nm)	*m/z* $[M + H]^+$	*m/z* MS^2 of $[M + H]^+$	Area at 530 nm (%)
Red beet						
1	Betd 5-glc	9.2	271.9/293.6/538.9	551.1	389.0	40.6
2	17-Decarboxy-betd 5-glc	10.9	266.4/505.4	507.1	345.1	1.2
3	Isobetd 5-glc	11.5	271.9/293.6/538.9	551.1	389.0	40.2
4	17-Decarboxy-isobetd 5-glc	13.2	268.0/505.4	507.1	345.1	1.3
5	Betd	13.8	273.6/544.2	389.0	n.d.	8.4
6	Isobetd	16.7	273.6/544.2	389.0	n.d.	3.9
7	Neobetd 5-glc	19.2	268.0/505.4	549.1	387.0	3.9
8	–[b]	22.9	–[c]	–[c]	–[c]	0.5
	Compound name[a]	Rt (min)	UV–Vis$_{max}$ (nm)	*m/z* $[M]^+$	*m/z* MS^2 of $[M]^+$	Area at 530 nm (%)
Elderberry						
9	Cyd 3-sam-5-glc	16.2	280.4/514.3	743.1	581.1/449.0/287.0	–[d]
10	Cyd 3, 5-di-glc	16.2	280.4/514.3	611.1	287.0	10.1[d]
11	Cyd 3-glc	20.8	283.7/514.3	449.0	287.0	–[d]
12	Cyd 3-sam	20.8	283.7/514.3	581.1	449.0/287.0	89.9[d]
Sour cherry						
13	–[b]	9.2	–[c]	–[c]	–[c]	0.5
14	Cyd 3-glc-rut	18.8	283.7/514.3	757.1	611.0/287.0	4.0
15	Cyd 3-rut	21.2	283.0/514.3	595.1	449.0/287.0	73.2
16	–[b]	22.2	278.0/505.4	–[c]	–[c]	1.4
17	Peo 3-rut	23.9	283.7/514.3	609.1	463.0/301.0	19.8
18	–[b]	30.1	283.7/514.3	–[c]	–[c]	1.1
Strawberry						
11	Cyd 3-glc	21.0	281.0/514.3	449.0	287.0	7.5
19	Pel 3-glc	24.3	277.9/501.0	433.0	271.0	82.1
20	Pel 3-rut	27.3	283.0/496.9	579.1	433.0/271.0	4.9
21	–[b]	29.0	–[c]	–[c]	–[c]	1.7
22	–[b]	33.1	262.2/509.8	–[c]	–[c]	1.6
23	–[b]	34.6	277.9/501.1	–[c]	–[c]	2.2
Black carrot						
24	–[b]	14.2	–[c]	–[c]	–[c]	0.6
25	–[b]	15.0	–[c]	–[c]	–[c]	0.7
26	Cyd 3-xyl-glc-gal	18.3	283.7/514.3	743.1	287.0	7.6
27	Cyd 3-xyl-gal	19.4	283.7/514.3	581.1	287.0	21.0
28	Cyd 3-xyl-glc-gal-sin	23.7	287.8/335.5/523.8	949.2	287.0	7.9
29	Cyd 3-xyl-glc-gal-fer	25.2	287.8/334.3/519.0	919.2	581.1/287.0	45.3
30	Cyd 3-xyl-glc-gal-coum	26.4	287.8/319.0/519.0	889.2	287.0	16.9

[a] Aglycons: Betd, betanidin; Cyd, cyanidin; Peo, peonidin; Pel, pelargonidin. Sugar substituents: Gal, galactose; Glc, glucose; Rut, rutinose; Sam, sambubiose; Xyl, xylose. Acyl substituents: Coum, *p*-coumaric acid; Fer, ferulic acid; Sin, sinapic acid.
[b] Not identified.
[c] No or no unambiguous signal available.
[d] Sum of two coeluting compounds.

3.1.3. Red beet

In red beet, betanin (**1**) and its C_{15}-epimer isobetanin (**2**) amounted to 80.8% altogether, while the corresponding aglycons betanidin (**5**) and isobetanidin (**6**) yielded 12.3%, respectively. Compounds **2** and **4** have been very recently found in *Boerhavia erecta* L. (Stintzing et al., 2004) and in heated red beet preparations (Herbach, Stintzing, & Carle, 2004) and were assigned as 17-decarboxy-betanin and 17-decarboxy-isobetanin, respectively. Neobetanin (14,15-dehydrobetanin) (**7**) has been reported as a genuine compound of red beet (Alard, Wray, Grotjahn, Reznik, & Strack, 1985; Kujala, Loponen, & Pihlaja, 2001) and has only recently been reported to result from heating of red beet juice (Herbach et al., 2004). Although its visible absorption maximum (λ_{max} = 484.9 nm) is in the range of the yellow betaxanthins, neobetanin is generally grouped with the betacyanins (Fig. 2). An additional betacyanin-structure (**8**) was detected at minute amounts, however, lacking signals did not allow closer identification.

3.1.4. Elderberry

Concordant with previous work (Chandra, Rana, & Li, 2001; Inami, Tamura, Kikuzaki, & Nakatani, 1996;

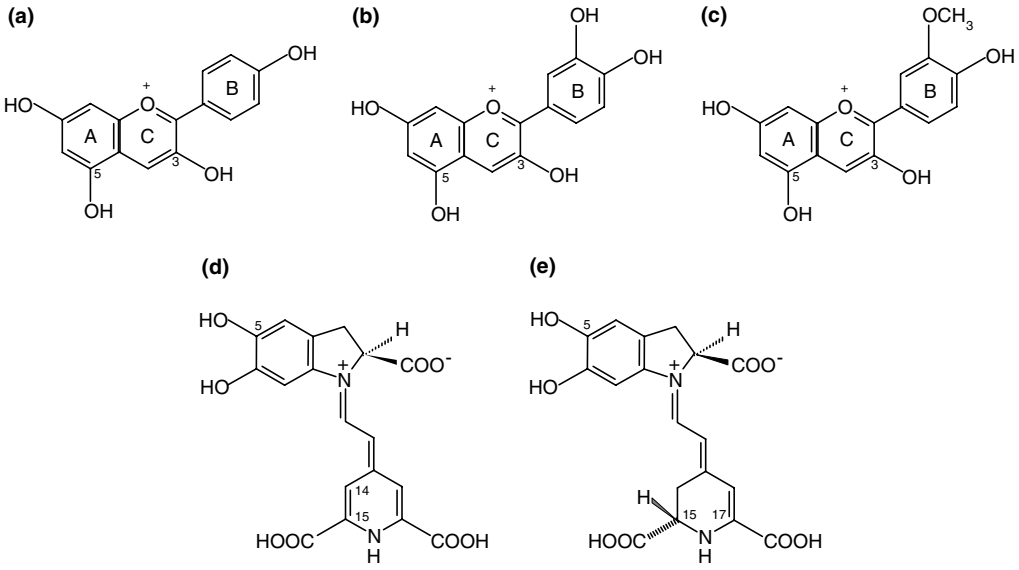

Fig. 2. Anthocyanidin [pelargonidin (a), cyanidin (b), peonidin (c)], and betacyanidin [neobetanidin (d), and betanidin (e)] structures of pigment concentrates.

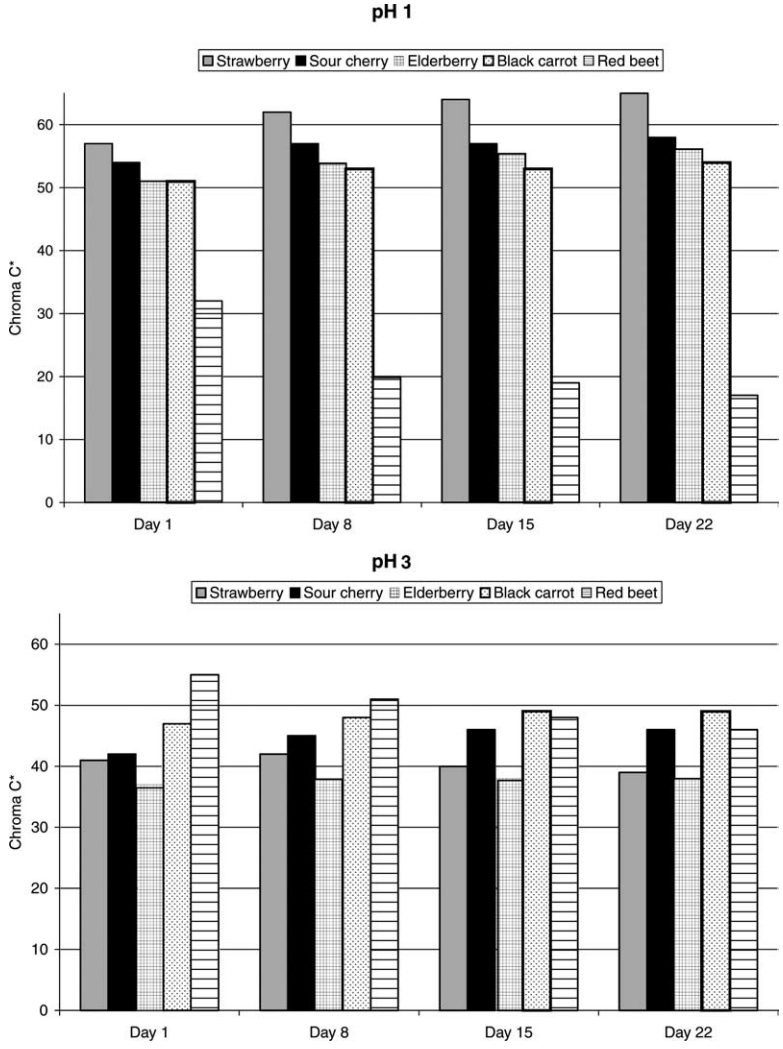

Fig. 3. Chroma development of anthocyanin and red beet solutions at varying pH over 22 days.

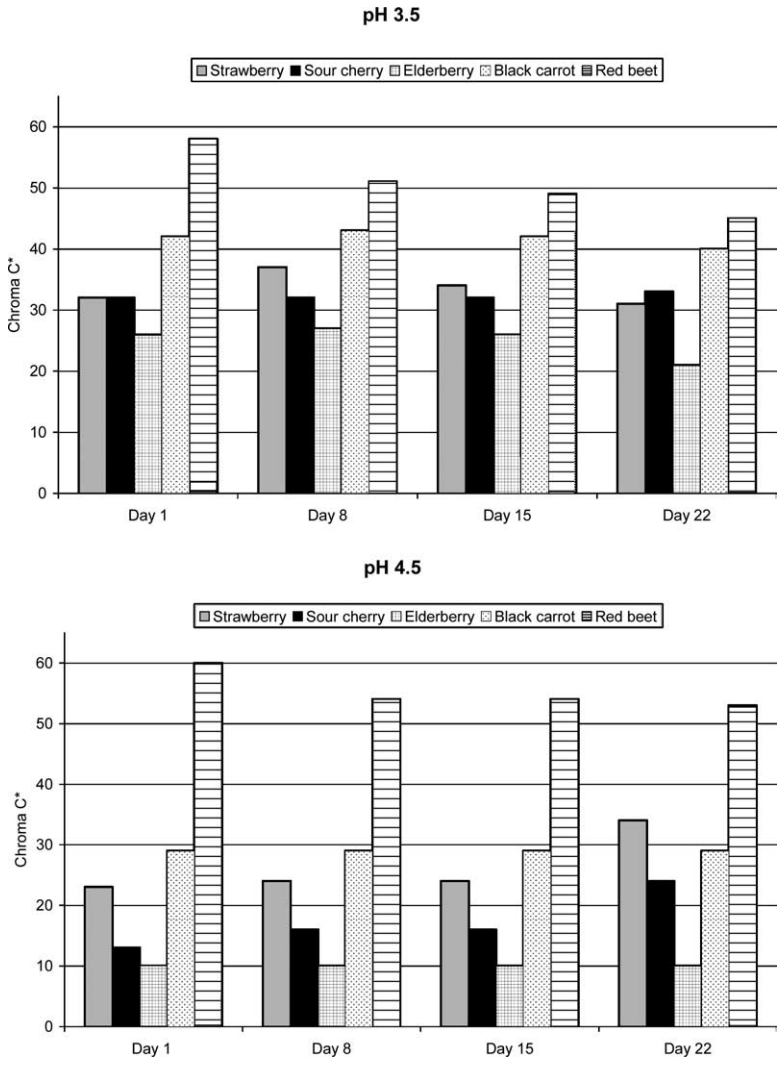

Fig. 3 (*continued*)

Stintzing et al., 2002), the pigment pattern of *Sambucus nigra* L. is characterised by the absence of acylated anthocyanins. Although different stationary phases and various eluent systems were evaluated (data not shown) neither cyanidin 3-sambubioside 5-glucoside (9) and cyanidin 3, 5-diglucoside (10) nor cyanidin 3-glucoside (**11**) and cyanidin 3-sambubioside (**12**) could be separated, when red beet and elderberry pigments were simultaneously assessed (Fig. 2).

3.1.5. Sour cherry

Cyanidin 3-rutinoside (**15**) was the main compound in sour cherry (73.2%), followed by peonidin 3-rutinoside (19.8%) (**17**) and cyanidin 3-glucosylrutinoside (4.0%) (**14**). Further minor compounds were detected at 9.2 min (**13**), at 22.2 min (**16**), and 30.1 min (**18**) amounting to a relative peak area of 3.0% altogether (Table 2). Although the relative peak areas differed, qualitative data corresponded well with studies of Goiffon, Mouly, and Gaydou (1999) and Chandra et al. (2001).

3.1.6. Strawberry

In accordance with previous studies (i.e., García-Viguera, Zafrilla, & Tomás-Barberan, 1997; Hong & Wrolstad, 1990; Lopez-da-Silva, de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002), pelargonidin 3-glucoside (**19**) was the predominant anthocyanin (82.1%) in strawberry together with further compounds of which cyanidin 3-glucoside (7.5%) (**11**) and pelargonidin 3-rutinoside (4.9%) (**20**) were the second major ones.

3.1.7. Black carrot

Black carrot showed the typical cyanidin derivatives (**26**–**29**) together with two minor more polar compounds (**24**, **25**) which were not studied further. When compared to an earlier investigation (Stintzing et al., 2002), the present extract had higher levels of acylated anthocyanins (70.1%) and additionally contained the coumaroyl-derivative (**30**) as described by Glässgen et al. (1992). Again, the hydroxybenzoyl-ester could not be

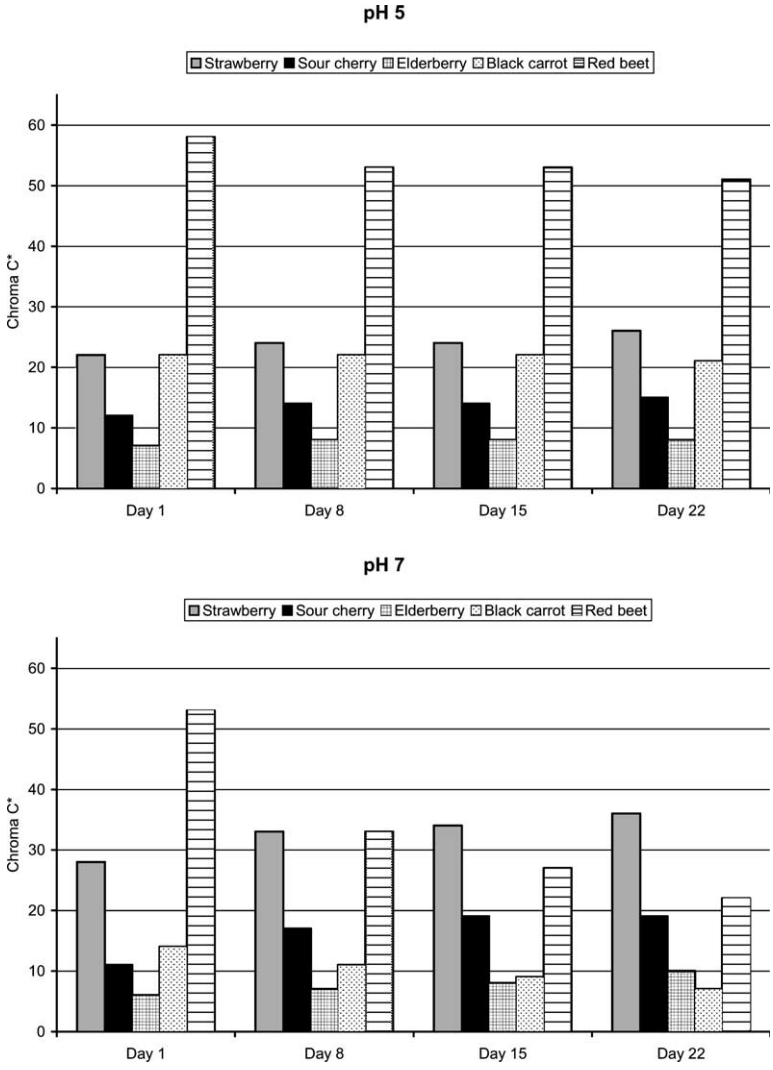

Fig. 3 (*continued*)

detected confirming recent findings by Kammerer, Schieber, and Carle (2003). Therefore, anthocyanin patterns are not only found to be typical of a respective plant tissue, but may also result from differing preharvest and postharvest conditions.

3.2. Chromatic characterisation of commercial concentrates and mixtures

3.2.1. Colour characteristics of commercial concentrates at optimum pH

3.2.1.1. Lightness. Normalization of maximum absorbance resulted in comparable L^*-values (68–72) for anthocyanin solutions at pH 1 and red beet at pH 5 (data not shown).

3.2.1.2. Chroma. Maximum C^*-values were obtained for all anthocyanin dilutions at pH 1 and for the red beet sample at pH 4.5, respectively (Fig. 3). Red beet (60) and strawberry (57) yielded the highest chroma, followed by sour cherry (54), elderberry (51) and black carrot (51). Interestingly, during 22 days, a slight increase of 3–8 chroma units could be observed for the anthocyanin samples in part due to possible flavylium regeneration from hemiketal and chalcone structures, while a lower chroma of 53 was found for red beet (Fig. 3).

3.2.1.3. Hue angle. The hue angle at optimum pH remained virtually constant for each anthocyanin extract with 50° for strawberry, about 20° for sour cherry and elderberry and 10° for black carrot with a slight tendency to higher values (redshift). In the same time range, red beet showed a colour angle of about −10° (Fig. 4). The pelargonidin-based strawberry sample could thus be easily distinguished from the cyanidin-containing sour cherry, elderberry and black carrot extracts. This corroborates common conception that increasing hydroxylation on the B-ring (Fig. 2) brings about a

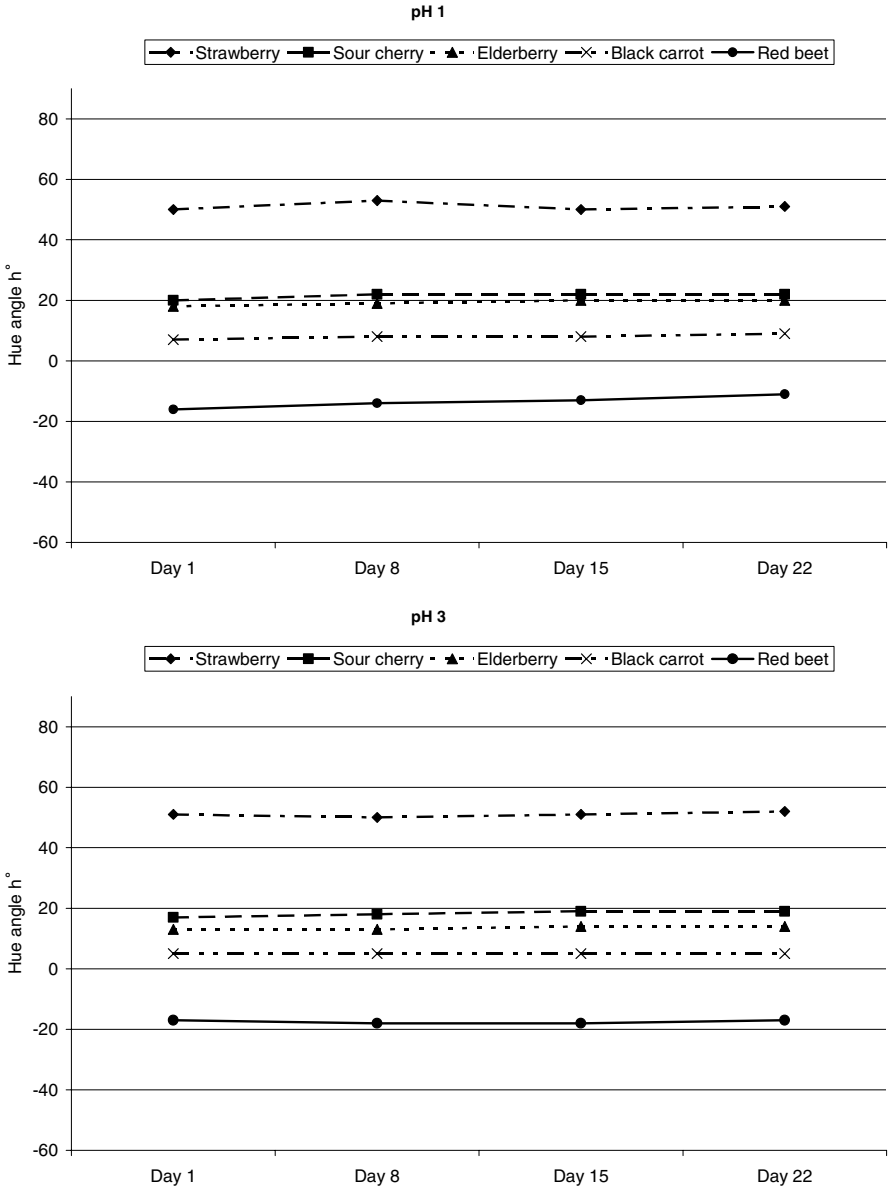

Fig. 4. Hue angle development of anthocyanin and red beet solutions at varying pH over 22 days.

bathochromic shift (Stintzing & Carle, 2004). Consistent with the literature data, acylation of anthocyanins in black carrot reduced the hue angle (Cevallos-Casals & Cisneros-Cevallos, 2004; Giusti & Wrolstad, 2003; Malien-Aubert, Dangles, & Amiot, 2001; Stintzing et al., 2002).

3.2.2. Chromatic characterisation of commercial concentrates at crucial pH

3.2.2.1. Chroma. For all anthocyanic extracts, the chroma values expressing colour brilliance or purity increased over the period of 22 days at pH 3 (Fig. 3). From pH 4.5 and up, the extracts showed diverging behaviour which may be ascribed to individual pigment compositions with differing pH sensitivities towards hemiketal and chalcone formation (Malien-Aubert et al., 2001; Nielsen, Haren, Magnussen, Dragsted, & Rasmussen, 2003; Stintzing et al., 2002). Black carrot at pH 3, 3.5 and 4.5 exhibited highest C^*-values of all anthocyanin extracts confirming the improved stability of acylated pigments towards fading. Elderberry was the least brilliant extract, while sour cherry and strawberry were more stable and at similar levels at pH 3 and 3.5 on day 1 diverging upon storage. Surprisingly, strawberry showed similar (pH 5) or higher (pH 7) chroma compared to black carrot and even red beet (pH 7) after two weeks. C^*-values for red beet declined over time at all pH values except at 4.5 and 5, where they remained virtually unchanged

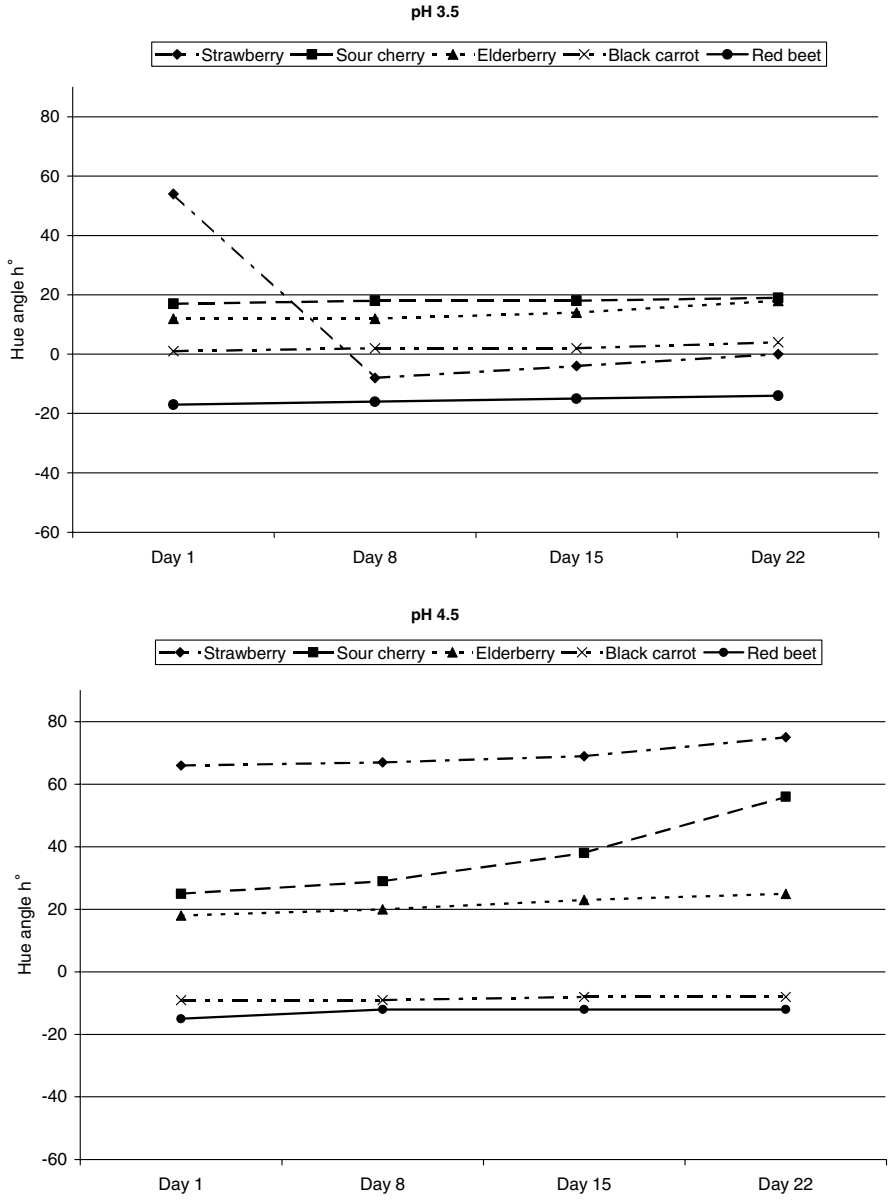

Fig. 4 (*continued*)

after 8 days (Fig. 3). Since decreasing chroma reflects the loss of genuine pigments through degradation or transformation (Cai et al., 1998; Sapers et al., 1981; Stintzing et al., 2002, 2003), chroma development can be applied as an indicator for anthocyanin and betalain stability.

3.2.2.2. Hue angle. Hue angle also changed to a small extent over time in a pH-dependent manner. Although differing among commodities (Fig. 4), the anthocyanic extracts strawberry, elderberry and sour cherry showed a general trend to increasing hue angles (redshift) over time at pH 4.5 and pH 7, while for red beet solutions a change was only registered at pH 7. Absolute $h°$-values for each anthocyanin extract remained unchanged or only slightly declined from pH 1 to 3.5 while at pH 4.5 an abrupt change to hue angles surmounting those at acidic pH was monitored further increasing with each pH increment until reaching a similar yellow-orange tint at pH 7. In contrast, black carrot containing a fair amount of acylated anthocyanins exhibited a general downward shift towards blue with a switch from red-purple to purplish-blue between pH 3.5 and pH 4.5. It is worth mentioning that red beet produced a more reddish hue than black carrot at 5 and 7, while the inverse was registered for all other pH regimes where red beet exhibited the most intense blue colour shade of all extracts.

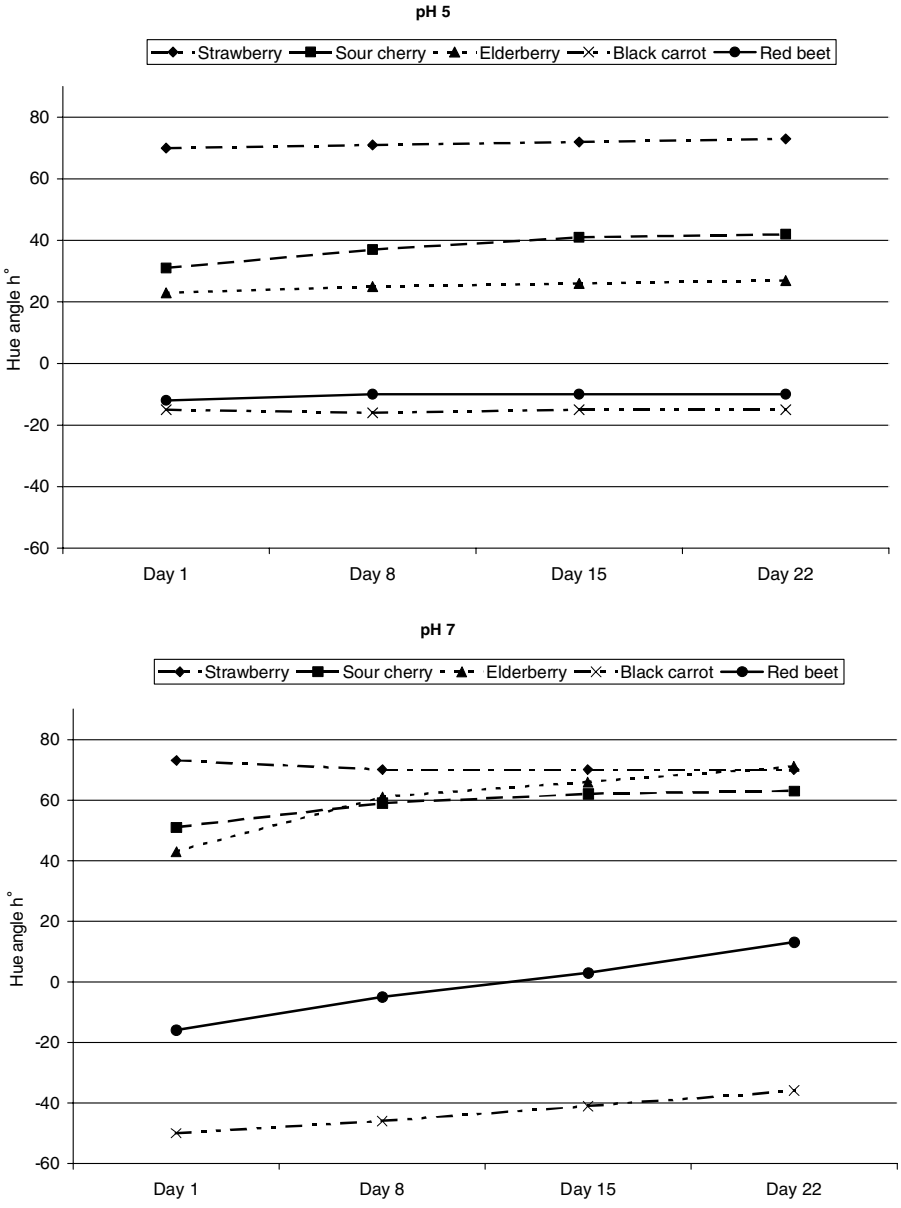

Fig. 4 (*continued*)

At all pH values except pH 3.5 and pH 7, strawberry could be readily distinguished from sour cherry and elderberry and the latter two from the black carrot and red beet preparations, respectively. Possibly being ascribed to the instability of pelargonidin 3-glucoside of strawberry in contrast to the primarily cyanidin-based sour cherry and elderberry, remarkable changes were observed at pH 3.5 for strawberry when values dropped below the 0°/360°-line while sour cherry and elderberry did not. Despite peonidin 3-rutinoside amounting to about 20% of the anthocyanin contents in sour cherry, the latter behaved very similar to elderberry samples exclusively showing cyanidin glucoside derivatives.

3.2.3. Chromatic characterisation of anthocyanin–betalain blends

Fig. 4 shows that $h°$-values of the unmixed solutions were at different levels depending on the pH and type of commodity. However, the following tonality ranges were not covered by the original anthocyanic and red beet samples over the whole period: $-15 < h° < 5$ and $25 < h° < 50$ at pH 1, $-15 < h° < 5$ and $20 < h° < 50$ at pH 3, $-15 < h° < 0$ and $h° > 20$ at pH 3.5, $-5 < h° < 20$ as well as $30 < h° < 65$ at pH 4.5, $-10 < h° < 20$ as well as $45 < h° < 70$ at pH 5 and finally $h° < 40$ at pH 7. It was therefore suspected, filling one of the missing tonalities would be possible by blending anthocyanic commodities with red beet. Minimal

chroma should reach 30 units after 22 days to effectuate an acceptable colour purity. This value was chosen since it is equivalent to half the value attained at the optimum pH for the original anthocyanin (pH 1) or red beet (pH 5) solutions. Because food is usually between pH 3 and 7, pH 1 was not included in this evaluation. Based on an absorption value of 1 at their respective pH optima, the following blends were included in the present investigation: 1 + 1 for equal colouring strength levels of betalains and anthocyanins, as well as 1 + 2 and 2 + 1 for greater colouring power of betalains and anthocyanins, respectively.

Inspection of the spectral curves at different pH-values confirmed the typical pH-optima for anthocyanins at pH 1 and betalains at pH 4.5 to 5 (Delgado-Vargas et al., 2000; Giusti & Wrolstad, 2001; Nielsen et al., 2003; Stintzing et al., 2002; Von Elbe, 1975). Through blending anthocyanins with betalains, absorbance dropped with increasing proportions of red beet at pH 1. Thus, any mixture was disadvantageous yielding lower values than either red beet or the respective anthocyanin solution alone. The same effect was true for pH 3.5. At pH 3.0, 4.5, 5 and 7, increasing amounts of betalains reflected in higher absorptivity of the blends, decreasing with higher anthocyanin proportions (data not shown). Since neither monitoring the wavelengths at maximum absorption nor absorption readings can express the appearance of coloured solutions properly (Fiorini, Barbirolli, & Pifferi, 1999; Gonnet, 1998; Stintzing et al., 2002, 2003), CIE$L^*C^*h^\circ$ measurements were performed for further evaluation.

All combinations from anthocyanic samples with red beet exhibited a shift towards increasing lightness during storage (data not shown). Anthocyanin samples displayed higher L^*-values with increasing pH, which apart from pH 1 was antagonized by rising betalain proportions. Consequently, hue angle and chroma changes were more closely inspected.

3.2.3.1. pH 3. At pH 3, all black carrot-red beet blends yielded h°-values that were not achieved with either of the concentrates (Table 3). However, these mixtures changed over time coming along with a redshift (h° increase) and a concomitant loss in C^*. Furthermore, colour purity generally decreased with rising red beet proportions during the 3-week period. The same effects were registered for strawberry-red beet and elderberry-red beet blends to various extents.

3.2.3.2. pH 3.5. Possibly due to the instability of anthocyanins and moderate stability of betalains at pH 3.5, one single mixture produced a new shade with an acceptable C^*-value. Reaching a value of 59, the hue angle of the elderberry-red beet blend was not found intermediate of the two original solutions after 22 days as was expected, but reached an orange-yellow colour.

Table 3
Colour development for anthocyanin–betalain blends yielding hue angles not achievable with either an anthocyanin or a red beet concentrate alone

Sample	Ratio	Day			
		h°		C^*	
		1	22	1	22
pH 3.0					
Red beet		−17	−17	55	46
Black carrot		5	5	47	49
Black carrot + red beet	2 + 1	−4	3	50	40
	1 + 1	−7	2	51	38
	1 + 2	−11	−2	52	35
Elderberry		13	14	37	38
Elderberry + red beet	1 + 1	−6	9	45	30
Strawberry		51	52	41	39
Strawberry + red beet	2 + 1	24	48	39	31
pH 3.5					
Red beet		−17	−14	58	45
Elderberry		12	18	26	21
Elderberry + red beet	2 + 1	−6	59	37	32
pH 4.5					
Red beet		−15	−12	60	53
Elderberry		18	25	10	10
Elderberry + red beet	1 + 2	−15	−10	48	36
Sour cherry		25	56	13	24
Sour cherry + red beet	1 + 1	−12	−3	40	32
Strawberry		66	75	23	34
Strawberry + red beet	1 + 2	−9	−1	45	30
pH 5.0					
Red beet		−12	−10	58	51
Elderberry		23	27	7	8
Elderberry + red beet	1 + 1	−12	−7	38	31
	1 + 2	−13	−9	45	38
Sour cherry		31	42	12	15
Sour cherry + red beet	1 + 1	−9	−1	37	30
	1 + 2	−11	−7	45	37
Strawberry		70	73	22	26
Strawberry + red beet	1 + 2	−7	−2	44	35

Chroma decreased marginally within 3 weeks ending up with a value between elderberry and red beet solutions (Table 3). The reason for this exceptional behaviour could not be disclosed. Although chroma for either red beet or black carrot exceeded 30 units (Fig. 3), their combinations produced lower values indicating that chroma was substractive (data not shown).

3.2.3.3. pH 4.5. Even though the C^*-values of the original solutions were lower compared to pH 3.5 (Fig. 3), a number of blends filling the hue angle gap at pH 4.5 were found (Table 3). Closer inspection of the values obtained on day 22 revealed that chroma increased with rising red beet concentrations from 33 to 41 exceeding those of black carrot extracts with 29 chroma units (Fig. 3), but new hue angles were not obtained (−11 and −10) and thus were not considered in Table 3. These findings are in contrast to those at pH 3 where inverse

effects were found (Table 3). All these blends were not stable over time and showed a decrease in both hue angle (blueshift) and chroma. Similar developments were registered in mixtures of red beet with elderberry, sour cherry and strawberry, respectively. It is worth to be mentioned that highest betalain proportions were necessary in elderberry or strawberry blends to reach chroma values of 30 and above. Finally, sour cherry-red beet (1 + 1) and strawberry-red beet (1 + 2) mixtures afforded similar colour values (Table 3) with the latter blend being less brilliant.

3.2.3.4. pH 5. At pH 5, again a couple of blends bridging the hue angle gaps of anthocyanic and red beet solutions were identified, with C^*-levels comparable to those at pH 4.5 (Table 3). Acylated anthocyanins from black carrot brought about a similar tonality compared to red beet but lower chroma (Fig. 4). Therefore, in accordance with the literature data, red beet is best suited to be applied at pH 4.5–5 (Stintzing et al., 2003; Von Elbe, 1975) and new colour shades can be best obtained by blending red beet with sources merely carrying anthocyanin glycosides (Table 3).

No blend afforded new stable tints at pH 7.

4. Conclusion

Altogether, these findings may open new opportunities for creating defined colours from foodstuffs. Red beet addition increased chroma at regions of anthocyanin instability. Additionally, tonalities not achievable with either anthocyanin or red beet concentrate could be produced through blending. Since greatest colour changes occurred during the first week, blends should be left for 8 days to allow for assessment of the prospective hue angle and chroma values. Whereas colour stability was best at pH 4.5, 5 and 3, inferior stability was observed at 3.5 and 7. These data confirm the moderate stability of anthocyanins at pH 3, the optimum betacyanin stability at pH 4.5 and 5 and high lability at pH 7 for both pigment classes. At pH 3.5, no beneficial effect could be achieved through combinations and an even counterproductive effect was registered with black carrot-red beet blends. To get an insight into the respective pigment patterns responsible for the overall appearance, a liquid chromatographic method compatible with mass spectrometric detection was developed allowing simultaneous resolution and characterization of a great number of betalains and anthocyanins. Since coloured concentrates differ in their phenolic and anthocyanin or betalain compositions, the data presented can only provide a general trend for the behaviour of anthocyanin–betalain blends. Future studies will be necessary to investigate whether the present findings can be effectively applied for producing tailor-made food colorants. Finally, the proposed HPLC method may be applied for authenticity evaluation of such mixtures.

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References

Alard, D., Wray, V., Grotjahn, L., Reznik, H., & Strack, D. (1985). Neobetanin: isolation and identification from *Beta vulgaris*. *Phytochemistry, 24*, 2383–2385.

Anonymous (2004). Spiel mit den Farben. *Lebensmitteltechnik, 36*, 43–44.

Bassa, I. A., & Francis, F. J. (1987). Stability of anthocyanins from sweet potatoes in a model beverage. *Journal of Food Science, 52*, 1753–1754.

Cabrita, L., Fossen, T., & Andersen, O. M. (2000). Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry, 68*, 101–107.

Cai, Y., Sun, M., & Corke, H. (1998). Colorant properties and stability of *Amaranthus* pigments. *Journal of Agricultural and Food Chemistry, 46*, 4491–4495.

Chandra, A., Rana, J., & Li, Y. (2001). Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *Journal of Agricultural and Food Chemistry, 49*, 3515–3521.

Cevallos-Casals, B. A., & Cisneros-Cevallos, L. (2004). Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. *Food Chemistry, 86*, 69–77.

Clement, J. S., & Mabry, T. J. (1996). Pigment evolution in the Caryophyllales: a systematic overview. *Botanica Acta, 109*, 360–367.

Delgado-Vargas, F., Jiménez, A. R., & Paredes-López, O. (2000). Natural pigments: carotenoids, anthocyanins and betalains. Characteristics, biosynthesis, processing, and stability. *Critical Reviews in Food Science and Nutrition, 40*, 173–289.

Duhard, V., Garnier, J.-C., & Mégard, D. (1997). Comparison of the stability of selected anthocyanin colorants in drink model systems. *Agro-Food Industry Hi-Tech, 8*, 28–34.

Fiorini, M., Barbirolli, G., & Pifferi, P. G. (1999). Effect of pH and temperature changes on the colour of anthocyanins. *Journal of Commodity Science, 38*, 15–30.

Francis, F. J. (2000). Anthocyanins and betalains: composition and applications. *Cereal Foods World, 45*, 208–213.

García-Viguera, C., Zafrilla, P., & Tomás-Barberan, F. A. (1997). Determination of authenticity of fruit jams by HPLC analysis of anthocyanins. *Journal of the Science of Food and Agriculture, 73*, 207–213.

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV–visible spectroscopy. In R.

E. Wrolstad (Ed.), *Current protocols in food analytical chemistry* (pp. 1–13). New York: Wiley, Unit F1.3.: Pigments and Colorants.

Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*, 217–225.

Glässgen, W. E., Seitz, H. U., & Metzger, J. W. (1992). High-performance liquid chromatography/electrospray mass spectrometry and tandem mass spectrometry of anthocyanins from plant tissues and cell cultures of *Daucus carota* L. *Biological Mass Spectrometry, 21*, 271–277.

Goiffon, J.-P., Mouly, P. P., & Gaydou, E. M. (1999). Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography. *Analytica Chimica Acta, 382*, 39–50.

Gonnet, J.-F. (1998). Colour effects of co-pigmentation of anthocyanins revisited–1. A colorimetric definition using the CIELAB scale. *Food Chemistry, 63*, 409–415.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2004). Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. *Journal of Food Science, 69*, C491–C498.

Hong, V., & Wrolstad, R. E. (1990). Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. *Journal of Agricultural and Food Chemistry, 38*, 698–708.

Inami, O., Tamura, I., Kikuzaki, H., & Nakatani, N. (1996). Stability of anthocyanins of *Sambucus canadensis* and *Sambucus nigra*. *Journal of Agricultural and Food Chemistry, 44*, 3090–3096.

International Federation of Fruit Juice Producers (1998). IFU method 71: Anthocyanins by HPLC. IFU, Zug, Switzerland.

Kammerer, D., Schieber, A., & Carle, R. (2003). Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 17*, 2407–2412.

Kremer, B. P. (1982). The anthocyanin–betalain alternative: an experimental approach. *Biochemical Education, 10*, 137–140.

Kujala, T., Loponen, J., & Pihlaja, K. (2001). Betalains and phenolics in red beetroot (*Beta vulgaris*) peel extracts: extraction and characterisation. *Zeitschrift für Naturforschung/Biosciences, 56c*, 343–348.

Kuusi, T., Pyysalo, H., & Pippuri, A. (1977). The effect of iron, tin aluminium, and chromium on fading, discoloration, and precipitation in berry and red beet juices. *Zeitschrift für Lebensmittel – Untersuchung und –Forschung, 163*, 196–202.

Lange, W., Brandenburg, W. A., & De Bock, T. S. M. (1999). Taxonomy and cultonomy of beet (*Beta vulgaris* L.). *Botanical Journal of the Linnean Society, 130*, 81–96.

Lopez-da-Silva, F., de Pascual-Teresa, S., Rivas-Gonzalo, J., & Santos-Buelga, C. (2002). Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC using DAD and ESI-MS detection. *European Food Research Technology, 214*, 248–253.

Malien-Aubert, C., Dangles, O., & Amiot, M. J. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 49*, 170–176.

Mazza, G., & Miniati, E. (1993). *Anthocyanins in fruits, vegetables, and grains*. Boca Raton, Ann Arbor, London, Tokyo: CRC Press.

Nielsen, I. L. F., Haren, G. R., Magnussen, E. L., Dragsted, L. O., & Rasmussen, S. E. (2003). Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. *Journal of Agricultural and Food Chemistry, 51*, 5861–5866.

Nielson, L. R., & Harley, S. M. (1996). Chemotaxonomy: simple test for distinguishing between anthocyanins and betacyanins. *Journal of Biological Education, 30*, 88–90.

Pasch, J. H., von Elbe, J. H., & Sell, R. J. (1975). Betalains as colorants in dairy products. *Journal of Milk and Food Technology, 38*, 25–28.

Pasch, J. H., & von Elbe, J. H. (1977). Red and yellow pigments from betalaines hold promise as substitutes for colors banned by the FDA. *Candy & Snack Industry, 142*, 32–35.

Sapers, G. M., Taffer, I., & Ross, L. R. (1981). Functional properties of a food colorant prepared from red cabbage. *Journal of Food Science, 46*, 105–109.

Springob, K., Nakajima, J.-I., Yamazaki, M., & Saito, K. (2003). Recent advances in the biosynthesis and accumulation of anthocyanins. *Natural Product Reports, 20*, 288–303.

Stich, E., Kloos, K., & Hoeck, H. (1999). Natur, Technik und Recht: miteinander, nicht gegeneinander. *Zucker- und Süßwarenwirtschaft, 52*, 10–14.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science and Technology, 15*, 19–38.

Stintzing, F. C., Kammerer, D., Schieber, A., Adama, H., Nacoulma, O. G., & Carle, R. (2004). Betacyanins and phenolic compounds from *Amaranthus spinosus* L. and *Boerhavia erecta* L. *Zeitschrift für Naturforschung/Biosciences, 59c*, 1–8.

Stintzing, F. C., Stintzing, A. S., Carle, R., Frei, B., & Wrolstad, R. E. (2002). Color and antioxidant properties of cyanidin-based anthocyanin pigments. *Journal of Agricultural and Food Chemistry, 50*, 6172–6181.

Stintzing, F. C., Schieber, A., & Carle, R. (2003). Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology, 216*, 303–311.

Strack, D., Vogt, T., & Schliemann, W. (2003). Recent advances in betalain research. *Phytochemistry, 62*, 247–269.

Vogt, T. (2002). Substrate specificity and sequence analysis define a polyphyletic origin of betanidin 5- and 6-*O*-glucosyltransferase from *Dorotheanthus bellidiformis*. *Planta, 214*, 492–495.

Von Elbe, J. H. (1975). Stability of betalaines as food colors. *Food Technology, 29*, 42–46.