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RAPID COMMUNICATION

Colour and stability of pure anthocyanins influenced by pH including the alkaline region

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This study on anthocyanin colour variation (intensity, λ_{max}, ε) over the pH range 1–9 during 60 days of storage, was conducted on petunidin 3-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (petanin) and cyanidin 3-*O*-β-D-glucopyranoside (cy3glc) at 10 and 23°C. Compared to cy3glc, petanin afforded higher colour intensity and higher or similar stability throughout the whole pH range. At pH 4.0, 84% of petanin was intact after 60 days storage at 10°C, while the corresponding solution of cy3glc was totally degraded. At pH 8.1 the colour intensity of petanin was even higher than at the lowest pH values. The visible λ_{max} absorption of petanin after 5 days at pH 8.1 at 10°C was similar or higher than the corresponding absorptions of the fresh solutions of cy3glc at any pH. The use of anthocyanins like petanin as food colorants in slightly alkaline products (bakery, milk, egg, etc.) should therefore be considered—at least in products with limited storage time kept in a refrigerator.

INTRODUCTION

There is considerable interest in the development of food colorants from natural sources to replace synthetic food colorants (Francis, 1989). The role of anthocyanins as food colouring agents becomes very important since they form the reds and the blues of many fruits and vegetables (Mazza and Miniati, 1993), and provide the attractive colour of many fruit juices, wines, jams and preserves. The motive to extend the use of these water-soluble pigments as food colorants is also increasing, along with their potential positive health effects (Bridle and Timberlake, 1997). However, several factors restrict their possibilities. Their colour is easily affected by a number of reactions occurring in food products, and the major problem associated with the storage of anthocyanins is their instability caused by temperature, oxygen, light and some enzymes (Jackman *et al.*, 1987; Francis, 1989). A particular problem is the pH influence on their behaviour. Based on observation of a few relatively simple anthocyanins *in vitro*, the following scheme is generally accepted (Brouillard, 1988): at a pH of approximately 3 or lower, the anthocyanin is orange or red and exists as a flavylium cation. As the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to the acidic hydroxyl groups of the aglycone. While the first reaction gives a colourless carbinol pseudo-base, which can undergo ring opening to a chalcone pseudo-base, the latter reactions give rise to quinonoidal bases. Further deprotonation of the quinonoidal bases can take place at pHs between 6 and 7 with the formation of purplish, resonance-stabilised quinonoid anions.

A lot of work has been done to identify the content of potential anthocyanin sources (Mazza and Miniati, 1993), and the content of the principal commercial available colorant sources covers a variety of different anthocyanins: grape (*Vitis vinifera*) (Wulf and Nagel, 1978), red cabbage (*Brassica oleraceae*) (Idaka *et al.*, 1987*b*), elderberry (*Sambucus nigra, S. canadensis*) (Brønnum-Hansen *et al.*, 1985; Johansen *et al.*, 1991), black carrot (*Daucus carota* spp. *sativa*) (Gläßgen *et al.*, 1992), various *Vaccinium* spp. (Fuleki and Francis, 1967; Baj *et al.*, 1983; Andersen, 1985; Andersen, 1987; Ballington *et al.*, 1987), blackcurrant (*Ribes nigrum*)

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(Chandler and Harper, 1962), roselle (*Hibiscus sabdariffa*) (Du and Francis, 1974), black chokeberry (*Aronia melanocarpa*) (Striegl *et al.*, 1995), purple corn (*Zea mays*) (Harborne and Self, 1987). All these products may be characterised as crude or partially purified extracts containing a mixture of anthocyanins in addition to other components. The information regarding characteristics and the stability of these extracts has increased in recent years. There is, however limited information considering stability of pure anthocyanins during storage. The major reason for this is that most anthocyanins are difficult to purify and have limited commercial accessibility, especially in large quantities. For the pH region 5–7 it has been shown that simple anthocyanins are unstable and are quickly decolourised by hydration at the 2-position of the anthocyanidin skeleton (Brouillard, 1988). Most of the reports treating stability of pure anthocyanins under pH influence, deal with the stability of acylated anthocyanins in weak acid or neutral aqueous solutions (Goto *et al.*, 1983, 1984; Saito *et al.*, 1985, 1995; Idaka *et al.*, 1987a; Yoshida *et al.*, 1991, 1992). It has been shown that these types of anthocyanins (mostly di- or poly-acylated) are more resistant to hydration, and hence possess a higher colour stability in weakly acidic or neutral solutions. These stability studies have never included the alkaline region.

This paper describes how the colour and colour stability of petunidin 3-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (petanin) and cyanidin 3-*O*-β-D-glucopyranoside (cy3glc) vary in the pH range 1–9 during storage. The former pigment is a representative for anthocyanins acylated with aromatic acids existing for instance in the family Solanaceae (Andersen *et al.*, 1991; Opheim and Andersen, 1992; Price and Wrolstad, 1995). We have previously (Nerdal and Andersen, 1991, 1992) described a new mechanism for intermolecular association of anthocyanins based on studies of petanin in acidified methanolic solutions at low temperatures. Cy3glc is a typical representative for the simple type of anthocyanins found in elderberry, blueberry, cowberry, whortleberry, blackcurrant, roselle, black chokeberry, etc. It is generally accepted that anthocyanins exhibit their most intense colour when they are in their flavylium ion form (Francis, 1975). This paper reveals that this is not the case for anthocyanins like petanin, which extends the potential of using anthocyanins as food colorants to these products (bakery, milk, egg, etc.) which are alkaline.

MATERIALS AND METHODS

Sources

Natural rice (*Oriza sativa* L.) a rich source of cy3glc was bought at a local food market, while blue potatoes (*Solanum tuberosum* var. Congo), the source of petanin, were obtained from Norske Potetindustrier.

Pigment isolation

The anthocyanins of blue potatoes were extracted 3 times with 0.2% HCl in MeOH at 4°C. The combined extracts were concentrated under reduced pressure, purified by partition against ethyl acetate and applicated on a XAD-7 Amberlite column. The pigments were further purified by droplet counter-current chromatography using BAW (4:1:5) upper and lower phases as stationary and mobile phases, respectively, and size-exclusion chromatography (Sephadex LH-20 column, 100×1.6 cm, Pharmacia) using H_2O–MeOH–HCl (60:40:0.05, v/v) as eluent. The major pigment, petanin (Fig. 1), was confirmed to be petunidin 3-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside by a combination of chromatographic and spectroscopic parameters (Andersen *et al.*, 1991).

The anthocyanins in the grains of natural rice were extracted 3 times with 0.2% HCl in MeOH. The combined extracts were concentrated under reduced pressure, purified by partition against both ethyl acetate and hexane and applied onto a XAD-7 Amberlite column. The pigments were separated on a Sephadex LH-20 column (100×1.6 cm, Pharmacia) using H_2O–MeOH–

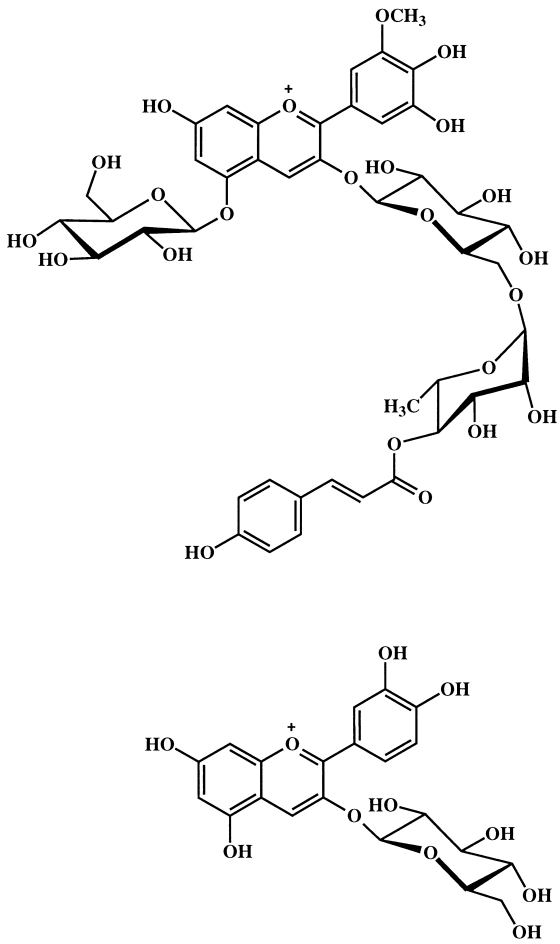

Fig. 1. Structures of petanin (top) and cyanidin 3-glucoside (bottom).

HCl (50:50:0.05, v/v) as eluent. The major pigment was confirmed to be cyanidin 3-*O*-*β*-D-glucopyranoside (cy3glc) (Fig. 1). See Andersen *et al.* (1991) for further experimental details.

Buffer solutions

Buffer solutions of 13 different pH values were prepared in accordance with Table 1. The accurate pH for each buffer solution was measured with a HANNA HI 9224 portable pH Meter equipped with a HI 1230B Combination pH Electrode and a HI 7669/2W Temperature Probe. The spectral behaviour of anthocyanins is dependent on solvent, and substances present in the solutions may influence the colour.

Measurements of colour and stability

The colour stability of petanin and cy3glc was determined at 10 and 23°C, respectively. The chloride salts of the isolated pigments were dissolved in acidified methanol to make stock solutions with concentrations 1 mg/ml and 0.5 mg/ml for petanin and cy3glc, respectively, and aliquots of 2 and 4 ml of these solutions were transferred to sample tubes, evaporated to dryness and dissolved in 20 ml of each of the 13 different buffer solutions (Table 1) to give a final pigment concentration of 0.1 mg/ml. Each anthocyanin buffer solution was divided into two portions which were stored at 10 and 23°C, respectively. The sample tubes were sealed with parafilm and the pigments were stored under air atmosphere. UV/Vis spectra were recorded between 240 and 700 nm on a VARIAN CARY3 UV-Visible Spectrophotometer equipped with a 386 ACER 1120SX computer unit. Pure buffers were used as reference cell solutions. UV/Vis measurements were made after 1 h, 1, 2, 5, 8, 15 and 60 days. The colour intensities were measured as absorbance values at λ_{max} for petanin and cy3glc at each pH value and expressed as molar absorptivity (ε, in $M^{-1}cm^{-1}$).

RESULTS AND DISCUSSION

Colours of petanin and cyanidin 3-glucoside at pH 1–9

The visible absorption maxima for petanin and cy3glc suffered a bathochromic shift on pH increase from pH 1.0 to 8.1 (Fig. 2). From pH 1.0–6.0 the maxima for petanin were in all instances 12–15 nm higher than the corresponding λ_{max} values for cy3glc (Table 2). At pH 6.5–7.3 petanin and cy3glc achieved nearly identical λ_{max} values, but further increase in pH induced an extra bluish shift to reach maxima around pH 8.1, while at pH 8.6–9.0 the λ_{max} values dropped. In aqueous solutions, cy3glc has previously been attributed with absorption maxima around 511 nm at pH 0.8–3.7 (Mazza and Brouillard, 1990; Maccarone *et al.*, 1992). The observed blue-shift of visible λ_{max} of petanin and cy3glc, with respect to pH increase, is in accordance with the work of Mazza and Brouillard (1990); however, their analyses were restricted to non-acylated anthocyanins within the pH range 2.7–5.7.

The pH variation also affected colour intensity of petanin and cy3glc (Fig. 3). A pH increase from 1 to 5 resulted in decreasing colour intensities for both anthocyanins until minima were reached at pH 5. Then, further

Table 1. Solvent proportions (v/v) of the 13 different buffer solutions applied in the pH region 1–9

pH	KCl 0.2 M	KHphthalate 0.1 M	KH_2PO_4 0.1 M	BORAX 0.025 M	HCl 0.2 M	HCl 0.1 M	NaOH 0.1 M
1.0	25				67		
2.4		50				42.2	
3.1		50				18.8	
4.0		50				0.1	
5.0		50					22.6
6.0			50				5.6
6.5			50				13.9
7.0			50				29.1
7.3			50				37.0
7.7			50				43.5
8.1			50				46.1
8.6				50		13.5	
9.0				50		4.6	

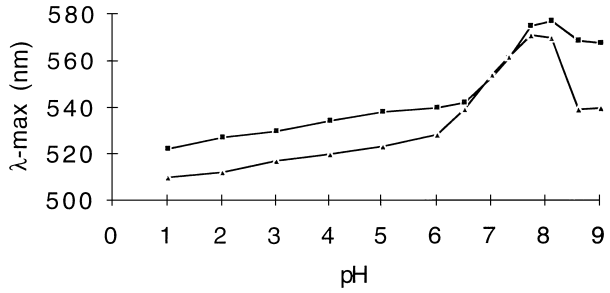

Fig. 2. Visible absorption maxima (λ_{max}, nm) at different pH, for (a) 1.03×10^{-4} M petanin (square) and (b) 2.06×10^{-4} M cyanidin 3-glucoside (triangle) as chloride salts taken 1 h after dissolution in buffered solutions at room temperature.

Table 2. Visible absorption maxima (λ_{max}, nm) and molar absorptivity (ε, $M^{-1}cm^{-1}$) for 1.03×10^{-4} M petanin and 2.06×10^{-4} M cyanidin 3-glucoside chloride salts 1 h after dissolution in buffered solutions (pH 1–9) at room temperature

pH	Petanin λ_{max}	Petanin ε	Cyanidin 3-glucoside λ_{max}	Cyanidin 3-glucoside ε
1.0	522	24 000	510	20 000
2.4	527	18 000	512	18 400
3.1	530	11 600	517	14 600
4.0	534	5200	520	6800
5.0	538	2800	523	1700
6.0	540	5400	528	2000
6.5	542	13 600	539	4200
7.0	553	16 700	554	6900
7.3	561	19 900	562	8400
7.7	575	26 200	571	10 700
8.1	577	31 600	570	12 500
8.6	569	19 400	539	10 100
9.0	568	18 000	540	10 500

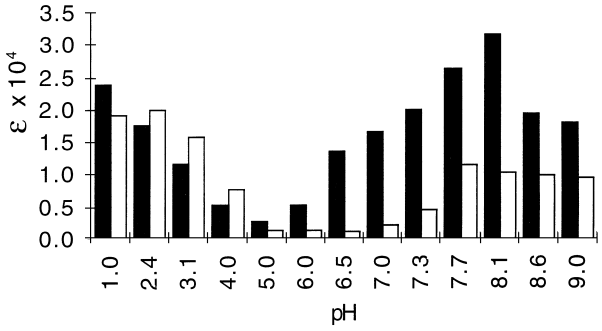

Fig. 3. Absorbance measurements conducted at λ_{max} of 1.03×10^{-4} M petanin (filled boxes) and 2.06×10^{-4} M cyanidin 3-glucoside (open boxes) as chloride salts for each of the pH 1–9 buffer solutions taken 1 h after dissolution.

increase in pH entailed increasing colour intensities until around pH 8.1. Surprisingly, the maximum colour intensity for petanin was reached in the alkaline region at pH 8.1, whereas the corresponding maximum for cy3glc was found as expected at pH 1.0 when this latter pigment occurs in the flavylium cationic state (Figs 4 and 5). Concerning petanin, the shape of the absorption band in the visible spectra at pH 8.1 was in fact narrower than the visible absorption band at pH 1.0 (Fig. 5), thus indicating that petanin occurs mainly as one single or a few similar structures at that pH. Petanin colour intensity was consistently higher than cy3glc throughout the whole pH range, except for the pH interval 2.4–4.0.

Anthocyanin stability on storage at 10 and 23°C, pH 1–9

Petanin and cy3glc were stored for 60 days in buffered solutions at 13 different pH values and two temperatures, and their visible absorption spectra were registered at determined time intervals. Colour stability was described on the basis of absorbance changes measured at the anthocyanins' λ_{max} for each pH value.

During storage at 10°C (Table 3) and at low pH values, both petanin and cy3glc showed rather high

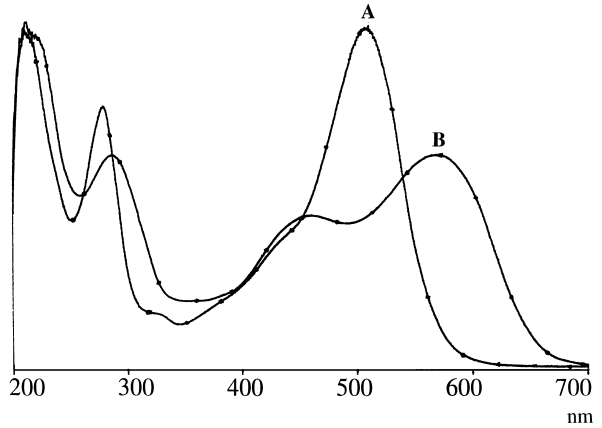

Fig. 4. UV/Visible spectra of 2.06×10^{-4} M cyanidin 3-glucoside as chloride salt taken 1 h after dissolution in buffered solutions at pH 1.0 (A) and 8.1 (B) at room temperature.

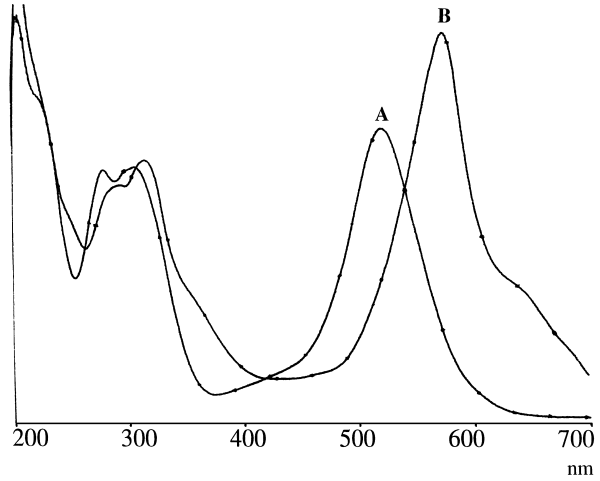

Fig. 5. UV/Visible spectra of 1.03×10^{-4} M petanin as chloride salt taken 1 h after dissolution in buffered solutions at pH 1.0 (A) and 8.1 (B) at room temperature.

visible absorption values. At pH 1.0 to 3.1, where both anthocyanins occur mainly in the flavylium cationic form, at least 90% of the colour was still intact after 60 days. Above pH 3.1, colour stability decreased to reach minimum at pH 6.0 and 7.0 for petanin (totally degraded after 8 days) and cy3glc (totally degraded after 2

Table 3. Absorbance of petanin and cyanidin 3-glucoside measured at pH 1–9 during 60 days of storage at 10°C in the dark using air atmosphere. The absorbances are normalised (conc. 1.03×10^{-4} M)

	Time (days)[a]						
pH	0	1	2	5	8	15	60
1.0	2.47	2.44	2.44	2.44	2.42	2.43	2.39
	2.06	1.91	2.03	2.07	2.08	1.97	2.21
2.4	1.81	1.97	1.91	1.88	1.86	1.86	1.64
	1.90	1.92	1.88	1.77	1.87	1.78	1.67
3.1	1.20	1.44	1.58	1.26	1.28	1.21	1.25
	1.51	1.59	1.57	1.56	1.65	1.53	1.35
4.0	0.54	0.73	0.62	0.54	0.57	0.51	0.45
	0.70	0.78	0.78	0.77	0.86	0.58	0
5.0	0.29	0.35	0.32	0.26	0.27	0.26	0.15
	0.17	0.14	0.13	0.12	0.15	0	0
6.0	0.55	0.27	0.28	0.17	0	0	0
	0.21	0.14	0.17	0	0	0	0
6.5	1.40	0.54	0.42	0.21	0.13	0	0
	0.43	0.12	0.19	0	0	0	0
7.0	1.72	1.12	0.63	0.32	0.22	0.15	0
	0.72	0.23	0	0	0	0	0
7.3	2.05	1.59	1.10	0.54	0.28	0.18	0
	0.87	0.45	0.31	0	0	0	0
7.7	2.71	2.30	2.18	1.48	0.81	0.39	0
	1.10	0.69	0.52	0	0	0	0
8.1	3.27	2.76	2.43	1.72	1.08	0.49	0
	1.29	1.02	0.79	0	0	0	0
8.6	2.01	1.37	0.66	0.29	0.15	0	0
	1.04	0.98	0.87	0.59	0.61	0	0
9.0	1.85	1.20	0.33	0.11	0	0	0
	1.08	0.95	0.92	0.56	0.44	0	0

[a]The upper and lower value in each interval correspond to petanin and cyanidin 3-glucoside, respectively.

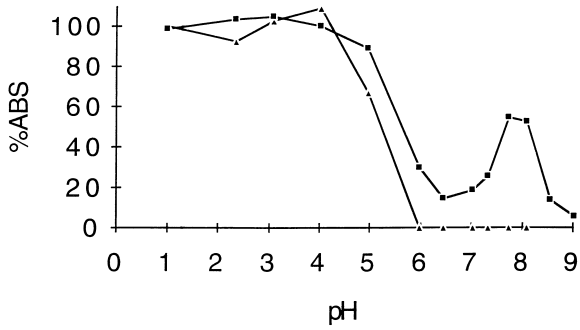

Fig. 6. The percentage of intact petanin (square) and cyanidin 3-glucoside (triangle) after 5 days storage at 10°C measured as absorbance at λ_{max}. The measurements at pH 8.6 and 9.0 for cyanidin 3-glucoside (Table 3) are not included because of the red-orange colour of these solutions.

days), respectively. Then colour stability increased until local maxima were reached around pH 8.1 for petanin and 8.6 for cy3glc, to finally decrease again towards pH 9.0.

In the pH region 1.0–3.1, the colour stabilities of petanin and cy3glc were almost equal upon 60 days of storage, but from pH 5.0–8.1 the colour stability of petanin became much higher than that of cy3glc (Table 3). At pH 4.0 and 5.0, respectively 84% and 52% of petanin were still intact after 60 days storage, while the corresponding solutions of cy3glc were totally degraded within 60 and 15 days, respectively. A comparison between petanin and cy3glc colour stability after 5 days of storage at 10°C, throughout the whole pH range, is provided in Fig. 6. At pH 7.7 and 8.1, more than 50% of petanin was still intact, while all the bluish colour of cy3glc was gone (Fig. 6). In fact, the visible absorption of petanin after 5 days storage at pH 8.1 was similar or higher than the corresponding absorption of the initial solutions of cy3glc at any pH. Around pH 8.6 and 9.0, cy3glc displayed more colour than petanin during storage (Tables 3 and 4). The colour of the solutions for the former pigment were orange-red, and the broad visible absorption bands measured for these solutions suggest the presence of several structures. The initial drops in colour intensity of alkaline anthocyanin solutions have been described by Brouillard (1982) but only on a time scale ranging from minutes to a few hours.

The results suggest that the colour evolution for petanin and cy3glc followed approximately the same pattern at 10 and 23°C over the same pH range, but the anthocyanins were markedly less stable at 23°C. During storage at 23°C, the highest colour stability for these pigments was again achieved at low pH values (Table 4). For instance at pH 1.0 more than 75% of both pigments was intact after 60 days storage. However, their stability decreased dramatically in the region pH 4–9, where colour differences between the two anthocyanins became even more notable than at 10°C (Table 4). While petanin at pH 4.0 was showing 39% absorption after 60 days, cy3glc was totally degraded.

Research on the stability of pure anthocyanins has previously been focused on a limited pH range (5–7). By comparison with these studies we found that the monoacylated pigment petanin has a comparable stability to other anthocyanins with aromatic mono- or di-acylation, as platyconin (Goto *et al.*, 1983; Saito *et al.*, 1985), shisonin and alatanin C (Yoshida *et al.*, 1991), gentiodelphin and the dicaffeylated cyanidin derivatives from *Ipomoea purpurea* (Saito *et al.*, 1985, 1995).

Table 4. Absorbance of petanin and cyanidin 3-glucoside measured at pH 1–9 during 60 days of storage at 23°C in the dark using air atmosphere. The absorbances are normalised (conc. 1.03×10^{-4} M)

	Time (days)[a]						
pH	0	1	2	5	8	15	60
1.0	2.47	2.45	2.44	2.42	2.39	2.36	1.93
	2.06	2.18	2.07	2.01	2.01	2.02	1.90
2.4	1.81	1.73	1.67	1.63	1.64	1.56	1.11
	1.90	1.86	1.87	1.86	1.82	1.82	1.52
3.1	1.20	1.12	1.05	1.04	1.03	1.02	0.74
	1.51	1.48	1.47	1.45	1.42	1.37	0.83
4.0	0.54	0.48	0.44	0.42	0.42	0.38	0.21
	0.70	0.68	0.67	0.60	0.48	0.36	0
5.0	0.29	0.29	0.24	0.22	0.23	0.16	0
	0.17	0.16	0.15	0	0	0	0
6.0	0.55	0.14	0.08	0	0	0	0
	0.21	0.15	0.14	0	0	0	0
6.5	1.40	0.21	0.12	0	0	0	0
	0.43	0.22	0.22	0	0	0	0
7.0	1.72	0.53	0.20	0	0	0	0
	0.72	0.21	0	0	0	0	0
7.3	2.05	1.06	0.33	0.12	0	0	0
	0.87	0.26	0	0	0	0	0
7.7	2.71	1.95	1.08	0.25	0	0	0
	1.10	0.41	0	0	0	0	0
8.1	3.27	2.36	1.22	0.26	0	0	0
	1.29	0.73	0.35	0	0	0	0
8.6	2.01	0.41	0.11	0	0	0	0
	1.04	0.77	0.48	0	0	0	0
9.0	1.85	0.21	0	0	0	0	0
	1.08	0.80	0.49	0	0	0	0

[a]The upper and lower value in each interval correspond to petanin and cyanidin 3-glucoside, respectively.

CONCLUSION

In relatively strong acidic aqueous solutions simple anthocyanins such as cyanidin 3-glucoside, showed high and similar stability to more complex anthocyanins such as petanin, which contains one aromatic acyl group. At least 90% of the two pigments were still intact after 60 days at 10°C. At pH 4.0–8.1 petanin showed both higher colour intensity and higher stability than cy3glc at 10 and 23°C. Around pH 8.1, where petanin has its most bluish colour (λ_{max} at 577 nm), the visible maximum absorption of petanin after 5 days storage at 10°C was similar or higher than the visible absorption of the fresh solutions of cy3glc at any pH. The use of anthocyanins like petanin as food colorants in slightly alkaline products (bakery, milk, egg, etc.) should therefore

be considered—at least in products with limited storage time which are kept in a refrigerator.

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