Mol. Nutr. Food Res. 2007, *51*, 1461 – 1471 DOI 10.1002/mnfr.200700179

Research Article

Thermal degradation of anthocyanins and its impact on color and *in vitro* antioxidant capacity

Eva Sadilova, Reinhold Carle and Florian C. Stintzing

Institute of Food Science and Biotechnology, Hohenheim University, Stuttgart, Germany

The aim of the current study was to thoroughly investigate the structural changes of anthocyanins at pH 3.5 in purified fractions from black carrot, elderberry and strawberry heated over 6 h at 95°C. Degradation products were monitored by HPLC-DAD-MS^3 to elucidate the prevailing degradation pathways. In addition, alterations of color and antioxidant properties observed upon heating were scrutinized. Most interestingly, the degradation pathways at pH 3.5 were found to differ from those at pH 1. Among others, chalcone glycosides were detected at 320 nm in heat-treated elderberry and strawberry pigment isolates, and opening of the pyrylium ring initiated anthocyanin degradation. In the case of acylated anthocyanins, acyl-glycoside moieties were split off from the flavylium backbone, first. Finally, for all pigment isolates, phenolic acids and phloroglucinaldehyde were the terminal degradation products as remainders of the B- and A-ring, respectively. Maximum and minimum antioxidant stabilizing capacities were found in black carrot and strawberry, respectively, which was explained by the high degree of acylation in the former. After heating, decline of trolox equivalent antioxidant capacity (TEAC) was observed in all samples, which was attributed to both anthocyanins and their colorless degradation products following thermal exposure. As deduced from the ratio of TEAC value and anthocyanin content, the loss of anthocyanin bioactivity could not be compensated by the antioxidant capacity of newly formed colorless phenolics upon heating.

Keywords: Anthocyanin stability / Antioxidant capacity / Color / Half-life / Thermal degradation

Received: May 17, 2007; revised: June 12, 2007; accepted: June 13, 2007

1 Introduction

Stable and attractive colors are a highly valued attribute on a most competitive food and beverage market and recent trends ask for replacement of synthetic dyes with natural alternatives [1]. However, the latter are suffering from poorer stability with a higher lability towards temperature, pH, oxygen and light both during processing and storage. Thus, the conditions governing pigment degradation need to be understood to establish adequate measures securing sufficient stability. Orange, red, violet sometimes even blue color shades may be obtained from anthocyanin-based preparations derived from diverse fruits and vegetables such as elderberry, strawberry and black carrot. Anthocyanins most obviously change their color depending on pH. While in strongly acidic aqueous media (at pH 2 and below) the red-colored flavylium cation is predominant, a colorless hemiacetal is mainly observed between pH 2 and 4, which itself is converted to *cis*- and *trans*-chalcones. A further pH shift to 6 leads to uncharged quinoidal bases with purple and finally anionic structures with a bluish tint [1–3].

Whereas these pH-dependent structural changes are well documented, the impact of temperature as the second most crucial parameter on anthocyanin stability is not sufficiently understood.

Most studies have simply reported the kinetics of anthocyanin fading disrespecting the underlying chemical transformations [4–6]. Notably, the latter are not only important from a chemical point of view, but will also affect color and the biological activity of anthocyanin-containing food. Therefore, deeper knowledge is required to evaluate the impact of heating on both the visual and nutritional properties of anthocyanins. This is even more important, as anthocyanins witness a growing interest in the prevention of age-related diseases, including cardiovascular diseases and cancer, mainly rooting in their antioxidant activity [7, 8]. The latter feature is made plausible by the electron deficiency of anthocyanins and their ability to quench free radicals at the site of oxidative events. This can be monitored applying

---

Correspondence: Dr. Florian C. Stintzing, Wala Heilmittel GmbH – Quality Control, Dorfstrasse 3, 73087 Bad Boll/Eckwälden, Germany
E-mail: florian.stintzing@wala.de
Fax: +49-7164-930-228

Abbreviation: TEAC, trolox equivalent antioxidant capacity

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

various *in vitro* tests. While numerous studies reported the antioxidant capacities of extracts from fruits, vegetables, cereals, nuts and sweets [9, 10], little is known about purified anthocyanins, and their changes induced by thermal treatment common to food preparation practices [11, 12].

Whereas Markakis (1957) [13] and Hrazdina (1971) [14] postulated opening of the pyrylium ring and chalcone formation as a first degradation step, Adams (1973) [15] proposed hydrolysis of the glycosidic moiety and aglycon formation as the initial reaction. Tanchev and Ioncheva (1976) [16] detected several thermal degradation products, among them quercetin, phloroglucinaldehyde and protocatechuic acid, which were identified by paper chromatography. In a recent study [17] pigment breakdown was investigated at pH 1 to scrutinize the effect of heat at the pH optimum of anthocyanin stability. In contrast, the present work focused on the formation of heat degradation products and their impact on color and antioxidant potential at pH 3.5, representing the typical pH of fruit juices.

For this purpose, the most commonly used anthocyanin concentrates derived from elderberry, strawberry and black carrot were selected. To obtain purified anthocyanin fractions colorless phenolics, sugars and other matrix compounds were removed. This allowed both to compare pelargonidin (strawberry) and cyanidin-derivatives (elderberry, black carrot) as well as the impact of number and type of acylation (elderberry, black carrot). In addition, copigmentation of anthocyanins with colorless phenolics was thus excluded. Color alterations and half-life values ($T_{1/2}$) of purified pigment preparations and individual anthocyanins as well as the degradation products formed were monitored by spectrophotometry and LC-MS. The antioxidant capacity of the heated extracts was assessed by the trolox equivalent antioxidant capacity (TEAC)-assay because its pH comes close to the physiological conditions, thus mimicking the *in vivo* condition.

2 Materials and methods

2.1 Solvents and reagents

Reference standards such as *p*-coumaric acid and phloroglucinaldehyde were from Fluka (Buchs, Switzerland) while protocatechuic, 4-hydroxybenzoic and ferulic acids were from Roth (Karlsruhe, Germany), respectively. All other reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Sephadex LH-20 and XAD-16-HP were from Amersham (Uppsala, Sweden) and from Rohm & Haas (Darmstadt, Germany), respectively. Purified water was used throughout.

2.2 Plant material

Commercial concentrates from elderberry (*Sambucus nigra* L.), strawberry (*Fragaria x ananassa* Duch.) and black carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) were provided by Ernteband (Winnenden, Germany) and stored at –30°C. Prior to sample work-up, concentrates were diluted (1:7, w/w) with purified water.

2.3 Anthocyanin purification

To remove sugars and salts, samples were pre-purified on a column (800 × 43 mm id) filled with XAD-16 HP. Prior to sample application, the resin was conditioned and equilibrated by successive rinsing with 500 mL methanol and 1000 mL pH 2 water (acidified with TFA), respectively. Subsequently, 5 mL elderberry, 5 mL black carrot and 10 mL strawberry sample, respectively, were applied and washed with 1000 mL pH 2 water (acidified with TFA) for removal of salts and sugars. For elution of the pigment fraction, at least 500 mL methanol acidified with pH 2 water (acidified with TFA) (95:5, v/v) were applied until the column was colorless. The so obtained samples were concentrated *in vacuo* and re-diluted in 2 mL purified water. For cleaning purposes, the column was rinsed with 250–500 mL pure methanol and re-equilibrated to starting conditions with 1000 mL pH 2 water (acidified with TFA).

To separate anthocyanins from colorless phenolics, further purification was performed on a Sephadex LH-20 column (660 × 26 mm id) pre-equilibrated by applying 500 mL methanol followed by 500 mL pH 2 water (acidified with TFA). Two to four milliliters of sample was put on the column and rinsed with pH 2 water (acidified with TFA). From the strawberry, elderberry and black carrot samples two, three and three fractions, respectively, were obtained. After elution of the fractions, the column was rinsed with 500 mL methanol to remove colorless phenolics and subsequently re-equilibrated with pH 2 water (acidified with TFA) (95:5, v/v). Individual anthocyanin fractions were checked for purity by HPLC-DAD at a monitoring wavelength of 280 nm. Fraction 1 of strawberry, fraction 1 and 2 of elderberry and fractions 1 and 2 of black carrot, respectively, were sufficiently pure to be used for heating experiments, *i.e.* anthocyanin content was at least 93% of the chromatogram area at 280 nm.

The purified anthocyanin fractions were gently evaporated to dryness at 30°C, re-dissolved with deionized water and adjusted to about 180 mg anthocyanin/L for each sample and pH 3.5 using a few drops of 2N NaOH. NaOH was chosen to exclude stabilizing effects from typical buffer anions such as citrate or phosphate.

2.4 Heating experiments

Heating experiments were performed in duplicate at 95°C in Pyrex tubes (100 × 14 mm id, Bibby Sterilin, Stone Staffs, UK) equipped with a magnetic stirrer and a plastic stopper. After 0.5, 1, 2, 4 and 6 h, the tubes were immedi-

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

ately cooled in an ice-bath for 1 min to stop thermal degradation. An aliquot of each sample was passed through a PTFE filter (0.45 µm, polypropylene, VWR International, West Chester, PA, USA) and analyzed by HPLC-DAD. The remainder was taken for antioxidant assays, anthocyanin quantification and colorimetric measurements after 30 min of equilibration. An unheated control was treated identically.

2.5 Quantification and color measurements

All samples were analyzed with a UV-Vis spectrophotometer (Perkin Elmer, Überlingen, Germany) equipped with UV-Vis (UV-Winlab version 2.85.04) and color (Wincol version 2.05) softwares (Perkin Elmer, Norwalk, CT, USA) based on equivalent tinctorial strengths of 1.00 ± 0.05 and individual absorption maxima at pH 1 before pH adjustment to pH 3.5.

Determination of total anthocyanin content was based on a pH-differential method and expressed as cyanidin-3-glucoside (cyd-3-glc) equivalents for elderberry and black carrot and pelargonidin-3-glucoside (pel-3-glc) equivalents for strawberry, respectively [18, 19] according to the following formula:

$$c\,[\mathrm{mg/L}] = A \cdot MW \cdot DF \cdot 1000/\varepsilon_M \cdot d$$

A = absorption value, MW = molecular weight of cyd-3-glc [449 g/mol], ε_M = molar extinction coefficient of cyd-3-glc at pH 1 [26,900 L/mol · cm] for elderberry and black carrot, MW = molecular weight of pel-3-glc [433 g/mol], ε_M = molar extinction coefficient of pel-3-glc at pH 1 (15 600 L/mol · cm) for strawberry, DF = dilution factor and d = path length of the cuvette (1 cm).

Half-life values $T_{1/2}$ were determined by calculating the ratio of the anthocyanin content in the unheated juice sample (A_0) and in the juice samples heated at 95°C for 0.5 to 6 h (A_t). The natural logarithm of ratio A_0/A_t was plotted against the heating period. The slope of the graph through the origin obtained by connecting the data points was equated with *k*, from which the half-life values ($T_{1/2} = \ln 2/k$) were deduced. For individual half-life values of anthocyanins the relative peak area ratios of the particular pigment at 520 nm were registered after their identification by HPLC-DAD-MS^3 measurements (see below). Based on absorption measurements covering the range from 380 to 780 nm, objective color (CIEL*a*b*) was assessed after 30 min of equilibration in McIlvaine buffer at pH 3.5. Chromaticity C* [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle h° [$h° = \arctan(b^*/a^*)$] were calculated from a*- and b*-values at D_{65} and an observer angle of 10°. The hue angles were expressed on a 360°-color wheel where 0°/360° presents purplish-red, 90° yellow, 180° bluish-green, 270° blue. Also color differences $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ [20] were calculated between samples heated for 0.5, 1, 2, 4 and 6 h. For both quantification and color measurements, two separate samples were analyzed in duplicate.

2.6 Antioxidant capacity measurements

The TEAC assay was applied [21] for antioxidant capacity measurements of samples reflecting the reducing capacity at neutral conditions (pH 7.4) by scavenging the ABTS$^+$ radical cation and monitoring at 734 nm. The resulting values were expressed as vitamin C equivalents based on a 5-point calibration curve ($R^2 \geq 0.99$). Blank samples of strawberry, elderberry and black carrot were treated the same way.

2.7 HPLC-DAD and LC-MS^3 analyses

Using a Merck LaChrom Elite HPLC System (Merck-Hitachi, Darmstadt, Germany) equipped with an autosampler L-2200, a pump L-2130, a diode array detector L-2450, and a JetStream column oven, anthocyanins were separated on an analytical C18 Sunfire column (250 × 4.6 mm id, 5 µm; Waters, Wexford, Ireland) with a C 18 pre-column (4 × 3.0 mm id, Phenomenex, Torrance, CA, USA) at a constant temperature of 25°C and a flow rate of 1 mL/min. Eluent A was 5% aqueous formic acid, 100% ACN was used as B. Starting isocratically with 100% A for 5 min, linear gradients were ramped to 10% B at 20 min, 13% at 40 min, 20% at 44 min, 25% at 50 min, 100% at 55 min before re-equilibration to initial conditions. Simultaneous monitoring was performed at 520, 320 and 280 nm.

Using the same method, MS detection was carried out on an Agilent Series 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with G1322A degasser, a G1312A binary gradient pump, a G1329/1330A autosampler, a G1316A column oven, and a G1315A DAD interfaced with a Bruker Model Esquire 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany) fitted with an ESI source operating in the positive ionization mode. Data acquisition and processing were performed with Esquire control software. Nitrogen was used at a flow rate of 12 L/min and a pressure of 70 psi both as drying and nebulizing gas. The nebulizer temperature was set at 365°C and a potential of –4000 V was used on the capillary. CID was performed with a fragmentation amplitude of 1.0 V using helium as a collision gas at a pressure of 1.17×10^{-8} bar.

3 Results and discussion

3.1 Color alterations

The absorption maxima of strawberry, elderberry and black carrot anthocyanins at pH 3.5 were 498, 514 and 524 nm, respectively, showing a bathochromic shift compared with an earlier study performed at pH 1 [17]. Color properties of all purified anthocyanins before and after thermal treatment at 95°C, were determined by CIEL*a*b* measurements as

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Color properties of purified elderberry, strawberry and black carrot anthocyanins at pH 3.5 upon heating at 95°C on an equivalent tinctorial strength basis of 1.00 ± 0.05 (mean ± SD, *n* = 4)

Heating period (h)	Strawberry	Elderberry	Black carrot
	Lightness L*		
0	85.03 ± 0.80	81.60 ± 0.28	69.60 ± 0.40
0.5	86.29 ± 0.32	82.87 ± 0.41	n.d.
1	86.71 ± 1.08	85.17 ± 0.53	73.15 ± 0.13
2	89.33 ± 0.13	86.44 ± 0.36	75.37 ± 0.38
4	90.91 ± 0.58	90.52 ± 0.33	80.09 ± 0.24
6	n.d.	n.d.	83.30 ± 0.64
	Hue angle h°		
0	35.14 ± 0.19	7.68 ± 1.31	3.48 ± 0.74
0.5	35.25 ± 0.28	8.60 ± 1.43	n.d.
1	35.42 ± 0.24	9.30 ± 1.57	1.74 ± 0.56
2	36.23 ± 0.77	13.49 ± 2.13	1.05 ± 0.95
4	41.19 ± 1.24	25.59 ± 4.03	358.88 ± 0.13
6	n.d.	n.d.	358.17 ± 0.49
	Chroma C*		
0	23.46 ± 0.54	28.60 ± 0.41	49.77 ± 0.79
0.5	21.51 ± 0.83	25.23 ± 0.36	n.d.
1	18.64 ± 0.46	21.65 ± 0.42	43.61 ± 0.59
2	13.97 ± 0.28	15.96 ± 0.10	37.39 ± 0.40
4	7.57 ± 0.58	9.05 ± 0.34	27.61 ± 0.37
6	n.d.	n.d.	19.94 ± 0.35
	ΔE*		
0	0.00	0.00	0.00
0.5	2.32	3.62	n.d.
1	5.10	7.84	7.25
2	10.42	13.71	13.78
4	17.00	22.06	24.56
6	n.d.	n.d.	32.84

n.d., Not determined.

shown in Table 1. In accordance with a previous study [17], lightness and hue angle values increased, except for the hue angle in black carrot, while chroma showed a considerable decrease for all samples corresponding to a color fading (Table 1), being less important for black carrot.

A pH increment comes along with hemiacetal, chalcone and finally quinoidal base formation [1]. This change appeared to proceed as far as the quinoidal structures in strawberry and elderberry both being devoid of acylated anthocyanins. The latter exhibiting higher pH-values. The total color difference (ΔE*) was highest in black carrot and lowest in isolated strawberry anthocyanins. Since a threshold value of ΔE = 1 is noticeable by the human eye, heating at 95°C for 30 min was sufficient to produce a visible color change (Table 1).

Unexpectedly, color difference values observed after heating at pH 1 [17] were higher than at pH 3.5. This may be explained by the fact that chroma values in unheated samples were already lower at pH 3.5 through pH dependence of color and after heating no major changes were triggered.

3.2 Anthocyanin degradation and half-life value

For a closer investigation of color degradation, purified anthocyanins fractions were diluted with pH 3.5 water to adjust anthocyanin concentration to 170–194 mg/L in all samples. The anthocyanin content was calculated before and after 0.5, 1, 2, 4 h of heating at 95°C for strawberry and elderberry and after 1, 2, 4, 6 h of heating for black carrot, respectively. The results obtained are shown in Table 2 together with the respective half-life values for the purified anthocyanin fractions.

As expected, the total anthocyanin content of all samples considerably decreased during heating at 95°C. Elderberry exhibited the highest initial anthocyanin content (194 mg/L). After 4 h of heating only minor residual amounts of anthocyanin (46 mg/L) were found. Almost

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Anthocyanin contents (mg/L) and half-life ($T_{1/2}$) values (h) for pigment isolates from strawberry, elderberry and black carrot concentrates at pH 3.5 during heating at 95°C based on spectrophotometric measurements (mean ± SD, *n* = 4)

Heating period (h)	Strawberry	Elderberry	Black carrot
0	171.35 ± 1.86	194.09 ± 5.52	185.66 ± 2.39
0.5	148.82 ± 3.74	166.43 ± 5.19	n.d.
1	129.43 ± 4.88	136.85 ± 6.18	147.08 ± 8.07
2	84.55 ± 5.70	101.49 ± 3.64	99.36 ± 6.31
4	40.39 ± 2.86	45.62 ± 1.39	71.66 ± 1.99
6	n.d.	n.d.	42.77 ± 2.31
$T_{1/2}$	1.95 ± 0.11	1.96 ± 0.06	2.81 ± 0.05

n.d., Not determined.

identical contents were obtained for black carrot (43 mg/L) after 6 h of thermal exposure, reflecting its higher heat resistance. A similar drop of the anthocyanin decrease content was observed for strawberry, with a half-life value almost identical with elderberry ($T_{1/2}$ ~ 1.9 h). Maximum half-life value was obtained for the anthocyanin fraction of black carrot isolate ($T_{1/2}$ ~ 2.8 h). Consequently, acylation of anthocyanins in black carrot considerably prolong their half-life compared with nonacylated derivatives from strawberry and elderberry isolates.

The present findings show that degradation kinetics of the total pigment fraction from strawberry, elderberry and black carrot followed a first-order reaction with an identical correlation coefficient of R^2 = 0.99. While in a previous study on anthocyanin isolates (pH 1), much higher half-life values of 4.1 and 3.2 h were registered for black carrot and strawberry under comparable temperature conditions [17], the present study was performed at pH 3.5, the typical pH of fruit juices. Thereby, improved thermal anthocyanin stability at higher acidity of the medium could be confirmed [22].

3.3 Assessment of anthocyanins and their degradation products by HPLC-DAD-MS^3

To monitor anthocyanins and their thermal degradation products HPLC-DAD-MS^3 analyses were applied and peaks were identified by comparing their characteristic MS data and retention times with commercial standards and published data [12, 17, 23–25].

3.3.1 Strawberry

The predominant anthocyanin in the unheated strawberry isolate was pelargonidin-3-glucoside (pel-3-glc, 18) showing a molecular ion at *m/z* 433, followed by traces of cyanidin-3-glucoside (cyd-3-glc, 9) with shorter retention and a molecular ion at *m/z* 449. A novel peak was monitored at 320 nm with a retention time of 41.1 min and an absorption maximum of 342 nm. Its molecular ion *m/z* 473 was assigned to pelargonidin chalcone glucoside, generating a typical fragment at *m/z* 289 (= 473-22(Na^+)-162(glc-H_2O), 22). Its formation is plausible due to the structural transformation by the pH increase to 3.5. Since reversible occurrence of chalcones occur at higher pH is well known, samples were acidified with HCl to provide evidence of the assumed chalcone structure by its disappearance after acidification (data not shown).

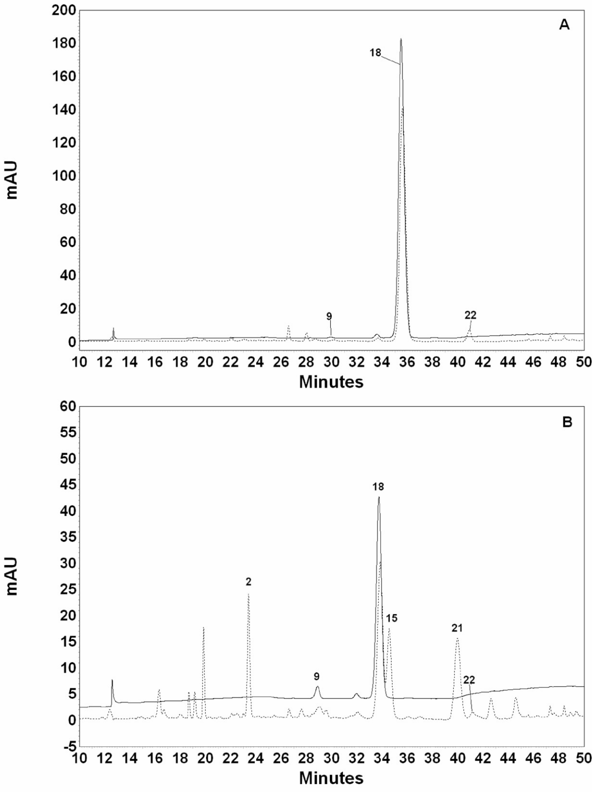

Figure 1. HPLC-DAD profile of purified strawberry anthocyanins (A: unheated, B: heated for 4 h at 95°C) monitored at 520 nm (solid line) and 280 nm (dotted line), respectively. Peak assignments are given in Table 4.

After 4 h of heating, a second degradation product was detected displaying an absorption maximum at 253 nm and a retention time of 23.5 min (2) (Fig. 1). Its molecular ion *m/z* 139 pointed to 4-hydroxybenzoic acid as the cleavage

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

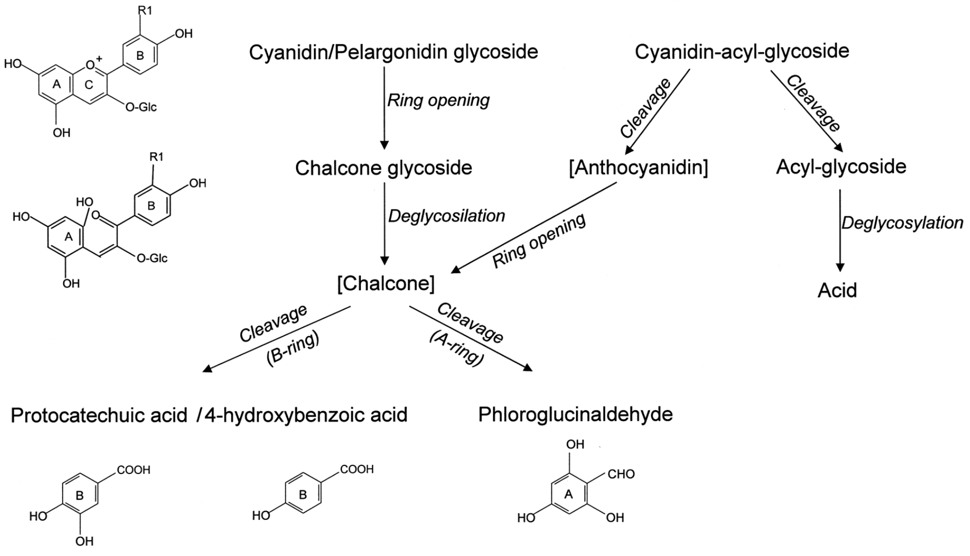

Figure 2. Scheme of anthocyanin degradation at pH 3.5. Compounds in brackets could not be detected.

Table 3. Quantification (µg/mL) of phenolic degradation products in purified anthocyanins from strawberry, elderberry and black carrot during heating at 95°C and pH 3.5 (mean ± SD, *n* = 4)

	4-hydroxy-benzoic acid	**Protocatechuic acid**		**Phloroglucinaldehyde**		
Time (h)	**Strawberry**	**Elderberry**	**Black carrot**	**Strawberry**	**Elderberry**	**Black carrot**
0	–a)	–a)	–a)	–a)	–a)	–a)
0.5	3.01 ± 0.12	2.98 ± 0.02	n.d.	1.38 ± 0.02	2.28 ± 0.23	tr
1	5.50 ± 0.34	6.39 ± 0.01	6.34 ± 1.56	2.04 ± 0.35	4.00 ± 0.13	tr
2	7.92 ± 0.62	12.64 ± 0.28	16.31 ± 0.20	3.84 ± 0.03	5.77 ± 0.00	tr
4	11.89 ± 0.01	25.52 ± 1.11	26.13 ± 0.82	4.89 ± 0.03	8.69 ± 0.22	tr
6	n.d.b)	n.d.	33.24 ± 0.49	n.d.	n.d.	tr

a) not detectable.
n.d., Not determined; tr, traces.

product of the pelargonidin B-ring (Fig. 2). Quantification of 4-hydroxybenzoic acid was based on a 5-point calibration ($R^2 = 0.99$), showing time-dependent content ranging from 3.0 to 11.9 µg/mL after heating for 0.5 and 4 h, respectively (Table 3).

In accordance with previous findings [17], the A-ring of the anthocyanin was degraded to phloroglucinaldehyde (15) with a molecular ion at *m/z* 155. To improve sensitivity of detection of the colorless artifacts the target mass was reduced from *m/z* 400 to *m/z* 200. Phloroglucinaldehyde content was quantified based on a 5-point calibration ($R^2 = 0.99$) and increased from 1.4 to 4.9 µg/mL after 0.5 and 4 h, respectively. Monitoring at 320 nm revealed a loss of the chalcone glucoside coinciding with the formation of a novel peak. Since further characterization was hindered by coelution of compounds chromatographic conditions were optimized to achieve their separation: 10% B at 20 min, 10% at 35 min, 20% at 44 min, 25% at 50 min and 100% at 55 min. In addition, temperature was lowered from 25 to 20°C. The novel compound, (21), showed an absorption maximum at 293 nm and a molecular ion at *m/z* 275. It was tentatively assigned to a dimeric compound formed from phloroglucinaldehyde and 4-hydroxybenzoic acid (=275-18(H_2O)-154(phloroglucinaldehyde) =139). However, a heating of a mixture of 4-hydroxybenzoic acid and phloroglucinaldehyde over 4 h at 95°C did not prove this assumption.

3.3.2 Elderberry

As major compounds of the genuine elderberry pigment isolate cyanidin-3-glucoside-xyloside (cyd-3-glc-xyl, 8) with a molecular ion at *m/z* 581 and a typical fragmentation 581→287, together with cyanidin-3-glucoside (cyd-3-glc, 9) displaying an *m/z* of 449 and 287 for the molecular ion

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

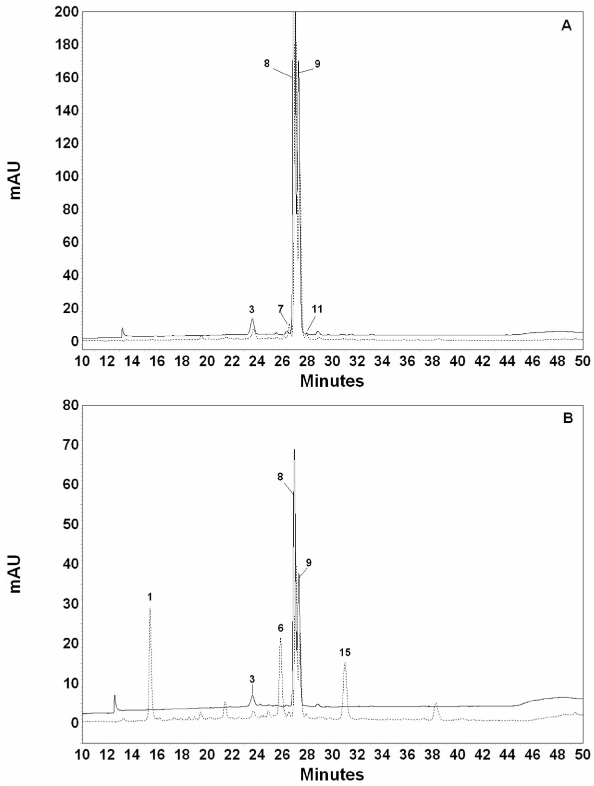

Figure 3. HPLC-DAD profile of purified elderberry anthocyanins (A: unheated, B: heated for 4 h at 95°C) monitored at 520 nm (solid line) and 280 nm (dotted line), respectively. Peak assignments are given in Table 4.

and aglycon, respectively, were found. To improve their separation, the injection volume was reduced to 10 µL, yielding cyd-3-glc-xyl at a retention time 27.2 min and cyd-3-glc at 27.6 min. Besides traces of cyanidin-3,5-diglucoside (cyd-3,5-diglc, 3) eluting at 23.9 min with a prominent ion at *m/z* 611 the cyanidin aglycon was identified at *m/z* 287, demonstrating a loss of two hexose moieties equivalent to *m/z* 324. These findings are in agreement with previous studies [26–28].

Consistent with the typical anthocyan strawberry pattern, the chalcone glycoside of cyanidin-glc-xyl anthocyanin was detected at 320 nm at a retention time of 26.7 min. This compound (7) produced a signal at *m/z* 621 and an absorption maximum at 326 nm. Fragmentation in the MS/MS experiment yielded an *m/z* 305 ion corresponding to the chalcone [=621-22(Na^+)-162(glc-H_2O)-132(xyl-H_2O)]. The base peak (11) at 28.2 min with *m/z* 489 and an absorption maximum of 342 nm exhibited an identical fragment of *m/z* 305 [=489-22(Na^+)-162(glc-H_2O)].

After heating for 4 h, further degradation products were identified at 280 nm (Fig. 3). Compound 1 was characterized as protocatechuic acid by comparison of retention time and mass characteristics with a commercial standard. The formation of protocatechuic acid during the heating experiment was quantified by HPLC based on a 5-point calibration (R^2 = 0.99) and was found to increase from 3.0 to 25.5 µg/mL after 0.5 and 4 h, respectively (Table 3). Compound 15 was identified as phloroglucinaldehyde by comparison of characteristic data with those of a commercial reference. As mentioned in a previous study [17], protocatechuic acid is the remainder of the B-ring while phloroglucinaldehyde derives from the A-ring of cyanidin (Fig. 2). The amount of the latter ranged from 2.3 to 8.7 µg/mL after 0.5 to 4 h, respectively (Table 3). Moreover, an unknown peak (6) was detected with an absorption maximum of 293 nm and a mass signal at *m/z* 291. Its correspondence to a dimer formed from protocatechuic acid and protocatechuic acid and phloroglucinaldehyde, respectively, was assumed. Its content increased with prolonged thermal exposure. It is noteworthy, that neither the anthocyanin aglycon nor the chalcone were detectable as intermediate degradation compounds (Fig. 3).

3.3.3 Black carrot

As reported in previous studies, the typical profile of unheated black carrot anthocyanins consisting of five peaks was confirmed [12, 29]. The major compounds were cyanidin-3-galactoside-xyloside-glucoside-ferulic acid (cyd-3-gal-xyl-glc-fer, 13), followed by cyanidin-3-galactoside-xyloside (cyd-3-gal-xyl, 5), cyanidin-3-galactoside-xyloside-glucoside-coumaric acid (cyd-3-gal-xyl-glc-coum, 16), cyanidin-3-galactoside-xyloside-glucoside-sinapic acid (cyd-3-gal-xyl-glc-sin, 12) and cyanidin-3-galactoside-xyloside-glucoside (cyd-3-gal-xyl-glc, 4) exhibiting molecular ions at *m/z* 919, 581, 889, 949 and 743, respectively. In contrast to unheated strawberry and elderberry anthocyanin fractions, chalcone glycosides could not be detected in black carrot. This may be explained by the smaller amounts of cyd-3-gal-xyl-glc (6.1 mg/L) and cyd-3-gal-xyl (37.7 mg/L) compared with cyd-3-glc-xyl (126.2 mg/L) and cyd-3-glc (67.9 mg/L) from elderberry or pel-3-glc (171.4 mg/L) from strawberry.

Most interestingly, eight novel peaks were detected after 6 h of heating (Fig. 4). Compound 1 with the shortest retention time at 15.7 min and a typical molecular ion at *m/z* 155 was assigned to protocatechuic acid (B-ring). This corroborates the findings for elderberry, as protocatechuic acid resulting from B-ring is identical in both elderberry and black carrot (Fig. 2). Quantification revealed that protocatechuic acid content increased from 6.3 to 33.2 µg/mL after 1 and 6 h, respectively (Table 3).

A second compound (6) yielded *m/z* 291 with a retention time 26 min, exhibited the same fragmentation (291→273→137) as compound 6 in elderberry and was therefore tentatively assigned to a dimer formed from protocatechuic acid or protocatechuic acid and phloroglucinaldehyde, respectively. However, phloroglucinaldehyde (15) was only detected in traces after heating. Compounds 10, 14 and 17 exhibited molecular ions at *m/z* 643, 673 and 703

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

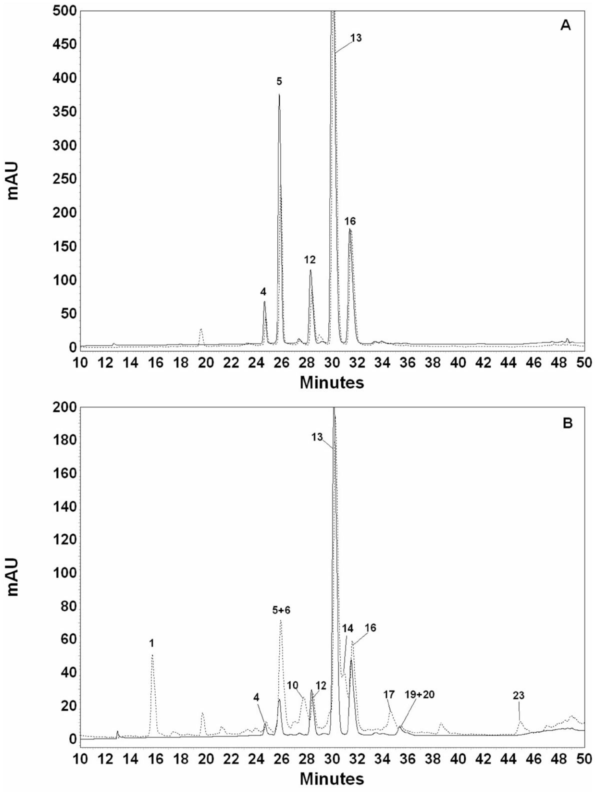

Figure 4. HPLC-DAD profile of purified black carrot anthocyanins (A: unheated, B: heated for 6 h at 95°C) monitored at 520 nm (solid line) and 280 nm (dotted line), respectively. Peak assignments are given in Table 4.

with absorption maxima of 308, 323 and 317 nm typical for coumaric, ferulic and sinapic acid, respectively. These phenolics with retention times of 27.8, 31.1 and 34.7 min were supposed to be the Na-adducts of coumaric (= 643-xyl(132-H_2O)-glc(162-H_2O)-gal(162-H_2O)-Na^+(22)), ferulic (= 673-xyl(132-H_2O)-glc(162-H_2O)-gal(162-H_2O)-Na^+ (22)) and sinapic (= 703-xyl(132-H_2O)-glc(162-H_2O)-gal(162-H_2O)-Na^+(22)) acids conjugated with glucose, galactose and xylose, respectively. In contrast to a previous study [17], only traces of cyd-3-gal-glc-fer (19) and cyd-3-gal-glc-coum (20) were found at 520 nm with their characteristic molecular ions at *m/z* 787 and 757 (Table 4, Fig. 4), while the sinapic acid adduct was not detectable. These findings imply that xylose cleavage is a negligible event at pH 3.5. Consequently, in contrast to pH 1 no colored intermediates were formed. Compound 23 with an *m/z* 177 and a retention time of 45 min was readily confirmed by coinjection of a commercial standard of anhydrous ferulic acid. Based on this observation, it is assumed that acylated anthocyanins are cleaved into the corresponding acyl-glycosides and the aglycones the latter being readily transformed into the intermediate chalcone which itself is instantly cleaved into protocatechuic acid and phloroglucinaldehyde due to its high thermolability and thus not detectable by HPLC-DAD (Fig. 2).

3.4 Anthocyanin degradation and antioxidant capacity

TEAC measurements were performed to evaluate the thermally induced changes of the antioxidant capacity as compared to the unheated samples of equal anthocyanin content.

Heated samples at pH 3.5 and 95°C were measured at pH 7.4 to mimic the conditions prevailing in the small intestine and blood [30]. At this pH, the flavylium cations are deprotonized to form quinoidal bases. Depending on the respective pK values of the individual pigments, the molar ratio of flavylium cation and quinoidal bases is determined, which is higher for acylated compared to the non-acylated anthocyanin glycosides [12, 31, 32]. This was earlier explained by intramolecular stacking of acylated anthocyanins protecting against hydration, *i.e.* decolorization [33] and is reflected by the half-life values of the individual anthocyanins (Table 5): Maximum half-life values were found for the anthocyanins acylated with ferulic, sinapic and coumaric acids, with the feruloyl-derivative exhibiting the best heat stability ($T_{1/2}$ ~ 3.4 h). It was thus demonstrated that the acyl moiety enhances anthocyanin resistance toward thermal degradation. Unexpectedly, the half–life value of pel-3-glc from strawberry ($T_{1/2}$ ~ 2.1 h) came close to that of cyd-3-gal-xyl-glc from black carrot ($T_{1/2}$ ~ 2.2 h). No differences in half-life values were also found for the galactose and glucose derivatives (cyd-3-glc-xyl versus cyd-3-gal-xyl).

These data help explaining the TEAC data: For the unheated anthocyanin samples, the highest antioxidant capacity value was assessed in black carrot (835.7 mg vitamin C equivalents/L) and the lowest in strawberry (185.4 mg vitamin C equivalents/L). Antioxidant capacities of di-glycosylated cyanidin from elderberry (476.8 mg vitamin C equivalents/L) and monoglycosylated pelargonidin (185.4 mg vitamin C equivalents/L), as the major compound from strawberry, differed considerably (Table 6). Its divergence was suspected to the higher number of hydroxyl group of the cyanidin structure. By adjusting all test solutions to the same anthocyanin concentration, the measured differences could be related to the individual anthocyanin structures after heating: Antioxidant capacities of all black carrot, elderberry and strawberry samples decreased. It also became evident that acylated anthocyanins contribute stronger to antioxidant capacity than their glycosylated counterparts, which is consistent with earlier studies [12].

Increasing TEAC/anthocyanin ratios (Table 6) could be ascribed to a progression of degradation products upon thermal exposure (Table 3). For elderberry and strawberry, 3.6- and 3.2-fold increases of the TEAC/anthocyanin ratios were observed after 4 h of heating, while it took 6 h for black carrot to reach 3.6-fold ratio. This pointed to the fact

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 4. HPLC-DAD-MS^3 data of anthocyanins and their degradation products from pigment isolates from strawberry (S), elderberry (E) and black carrot (BC) concentrates at pH 3.5 heated at 95°C

Peak no.	Compound	R_t	λ_{max}	*m/z* $[M]^+$	*m/z* $[M+H]^+$	*m/z*		S	E	BC
						MS^2	MS^3			
1[a]	Protocatechuic acid	15.6	259		155	111	93		x	x
2[a]	4-hydroxybenzoic acid	23.5	253		139	95	95	x		
3[b]	Cyd-3,5-di-glc	23.9	518	611		449;287	287		x	
4[b]	Cyd-3-gal-xyl-glc	24.8	523	743		287				x
5[b]	Cyd-3-gal-xyl	26.1	518	581		287				x
6[a,c]	Adduct 1	26.0	293		291	273	137		x	x
7[c]	Cyd-chalcone-glc-xyl	26.7	326		621[e,f]	305	305		x	
8[b]	Cyd-3-glc-xyl	27.2	516	581		287	287		x	
9[b]	Cyd-3-glc	27.6	517	449		287	287	x	x	
10[a,c]	*p*-Coumaric acid derivative	27.8	308		643[e]	451	391			x
11	Cyd-chalcone glucoside	28.2	342		489[e]	471	305		x	
12[b]	Cyd-3-gal-xyl-glc-sin	28.5	532	949		581;287				x
13[b]	Cyd-3-gal-xyl-glc-fer	30.3	528	919		581;287	287			x
14[a,c]	Ferulic acid derivative	31.1	323		673[e]	481	421			x
15[a]	Phloroglucinaldehyde	31.1	292		155	127	81	x	x	x
16[b]	Cyd-3-gal-xyl-glc-coum	31.6	527	889		581;287	287			x
17[a,c]	Sinapic acid derivative	34.7	317		703[e]	511	451			x
18[b]	Pel-3-glc	35.5	501	433		271	271	x		
19[a]	Cyd-3-gal-glc-fer	36.1	–[d]	787		287	287			x
20[a]	Cyd-3-gal-glc-coum	36.9	–[d]	757		287				x
21[a,c]	Adduct 2	40.1	293		275	257	161	x		
22	Pel-chalcone glucoside	41.1	342		473[e]	455	289	x		
23[a]	Ferulic acid	45.0	323		177[g]	145	118			x

a) Degradation products.
b) Genuine anthocyanins prior to heating. Cyd: cyanidin, Pel: pelargonidin, gal: galactoside, glc: glucoside, xyl: xyloside, coum: coumaric acid, fer: ferulic acid, sin: sinapic acid.
c) Tentatively assigned.
d) Not detectable.
e) The *m/z* value represents the respective pseudomolecular ion + Na^+.
f) Weak signal.
g) The *m/z* value represents the respective pseudomolecular ion –H_2O.

that anthocyanin degradation products of elderberry and strawberry exhibit a higher antioxidant capacity than those from black carrot.

Closer inspection revealed that on the same level of anthocyanin retention (24%), TEAC retention was 85 and 74% in elderberry and strawberry, respectively after 4 h of heating. According to Table 3, the major contribution of phenolics to TEAC is provided by phloroglucinaldehyde and protocatechuic acid in elderberry and phloroglucinaldehyde and 4-hydroxybenzoic acid in strawberry with higher total amounts in elderberry after 4 h of heating. Anthocyanin retention in black carrot reached 39% with a corresponding TEAC retention of 87%. In addition to protocatechuic acid the major phenolic compounds responsible for TEAC-activity were ferulic, coumaric and sinapic acids conjugated with xylose, galactose and glucose, respectively. The antioxidant capacity of diluted anthocyanin samples adjusted to those of the heated samples was assessed in a separate experiment and a linear relationship between anthocyanin concentration and vitamin C equivalent was found (data not shown). In comparison with the respective data for the phenolics in the TEAC assay, it could be concluded that the phenolics generated exhibited a higher antioxidant capacity than the parent anthocyanins on the same concentration basis (data not shown). Though two new HPLC methods were applied on the heated black carrot sample, only traces of phloroglucinaldehyde were detectable. Hence, it will be interesting to complete elucidation of its formation mechanism.

Table 5. Half-life values of individual anthocyanins in purified elderberry, black carrot and strawberry pigment isolates at pH 3.5 upon heating at 95°C (mean ± SD, *n* = 2)

Pigment	**Source**	**$T_{1/2}$ (h)**
Pel-3-glc	strawberry	2.12 ± 0.00
Cyd-3-glc	elderberry	1.82 ± 0.03
Cyd-3-glc-xyl	elderberry	1.68 ± 0.01
Cyd-3-gal-xyl-glc	black carrot	2.18 ± 0.28
Cyd-3-gal-xyl	black carrot	1.59 ± 0.04
Cyd-3-gal-xyl-glc-sin	black carrot	2.94 ± 0.11
Cyd-3-gal-xyl-glc-fer	black carrot	3.43 ± 0.08
Cyd-3-gal-xyl-glc-coum	black carrot	3.10 ± 0.11

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 6. Antioxidant capacity expressed as vitamin C equivalents (mg/L), retention of anthocyanin content and TEAC values in% and calculated TEAC/anthocyanin ratio for elderberry, black carrot and strawberry pigment isolates during heating at 95°C (means ± SD, *n* = 4)

Time (h)	TEAC ± SD	Retention TEAC (%)	Retention anthocyanin[a)] (%)	Ratio (TEAC/ACN)
	Elderberry			
0	476.81 ± 1.31	100.0	100.0	2.46
0.5	470.00 ± 1.25	98.6	85.7	2.82
1	441.88 ± 6.37	92.7	70.5	3.22
2	424.06 ± 1.81	88.9	52.3	4.18
4	403.56 ± 4.31	84.6	23.5	8.85
	Black carrot			
0	835.71 ± 13.40	100.0	100.0	4.50
1	790.54 ± 75.04	94.6	79.2	5.37
2	751.43 ± 48.64	89.9	53.5	7.56
4	723.14 ± 44.97	86.5	38.6	10.08
6	698.33 ± 38.61	83.6	23.0	16.32
	Strawberry			
0	185.38 ± 2.78	100.0	100.0	1.08
0.5	178.43 ± 1.13	96.3	86.8	1.19
1	174.95 ± 1.39	94.4	75.5	1.35
2	158.65 ± 3.22	85.6	49.4	1.88
4	138.00 ± 1.37	74.4	23.6	3.41

a) Based on the data from Table 2.

4 Concluding remarks

Since the experimental set-up was the same in a previous study conducted at pH 1 [17], the reasons for other compounds generated are suspected to be divergent degradation pathways and different stabilities of the compounds at the particular pH-values. In comparison with an earlier investigation [17], it was found that at pH 3.5 half-life values of anthocyanins glycosylated with xylose increased. For cyd-3-gal-xyl-glc, half-life values doubled at pH 3.5 as compared to pH 1. It is thus assumed that xylose possesses a stabilizing effect on the individual anthocyanin towards thermal degradation at pH 3.5.

The presence of chalcone glycosides and the absence of aglycons at pH 3.5 demonstrate pH-dependent degradation pathways. Supposedly, the first step of thermal degradation at pH 3.5 is not anthocyanin deglycosylation, but opening of the pyrylium ring and chalcone glycoside formation. The latter would then be cleaved to yield the chalcone, which due to its thermolability instantly degrades into a phenolic acid and aldehyde, respectively. These data appear to be most useful, because few data are available on anthocyanin chalcones [34–37], especially on their determination by HPLC [26, 38].

In contrast to a previous study [17], formation rates of phenolic degradation products (4-hydroxybenzoic acid, protocatechuic acid, phloroglucinaldehyde) increased when shifting the pH from 1 to 3.5, which underlines the lower thermal stability of anthocyanins at pH 3.5.

Most importantly, the loss of bioactivity through anthocyanin degradation could not be compensated by the respective colorless phenolics generated upon heating. In summary, these findings are considered viable not only from a technological point of view but may also be instructive to trace anthocyanins during their passage in the gastrointestinal tract thereby obtaining reliable data on their metabolic fate.

5 References

[1] Stintzing, F. C., Carle, R., *Trends Food Sci. Technol.* 2004, *15*, 19–38.

[2] Cabrita, L., Fossen, T., Andersen, O. M., *Food Chem.* 2000, *68*, 101–107.

[3] Cevallos-Casals, B. A., Cisneros-Zevallos, L., *Food Chem.* 2004, *86*, 69–77.

[4] Adams, J. B., Ongley, M. H., *J. Food Technol.* 1973, *8*, 139–145.

[5] Drdak, M., Daucik, P., *Acta Alimentaria* 1990, *19*, 3–7.

[6] Kirca, A., Özkan, M., Cemeroglu, B., *Food Chem.* 2006, *97*, 598–605.

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[7] Beattie, J., Crozier, A., Duthie, G. G., *Curr. Nutr. Food Sci*. 2005, *1*, 71–86.

[8] Ghosh, D., *Curr. Topics Nutraceut. Res.* 2005, *3*, 113–124.

[9] Pellegrini, N., Serafini, M., Salvatore, S., Del Rio, D. *et al*., *Mol. Nutr. Food Res*. 2006, *50*, 1030–1038.

[10] Nilsson, J., Pillai, D., Önning, G., Persson, Ch. *et al*., *Mol. Nutr. Food Res.* 2005, *49*, 239–246.

[11] Wang, H., Cao, G., Prior, R. L., *J. Agric. Food Chem.* 1997, *45*, 304–309.

[12] Stintzing, F. C., Stintzing, A. S., Carle, R., Frei, B., Wrolstad, R. E., *J. Agric. Food Chem.* 2002, *50*, 6172–6181.

[13] Markakis, P., Livingstone, G. E., Fillers, G. R., *Food Res.* 1957, *22*, 117–30.

[14] Hrazdina, G., *Phytochemistry* 1971, *10*, 1125–1130.

[15] Adams, J. B., *J. Sci. Food Agric.* 1973, *24*, 747–762.

[16] Tanchev, S., Ioncheva, N., *Nahrung* 1976, *20*, 889–893.

[17] Sadilova, E., Stintzing, F. C., Carle, R., *J. Food Sci.* 2006, *71*, 504–512.

[18] Giusti, M. M., Wrolstad, R. E., in: Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H. *et al*. (Eds.) *Handbook of Food Analytical Chemistry. Pigments, colorants, flavors, texture, and bioactive food components.* Wiley & Sons: Hoboken, NJ/USA 2005, pp. 19–31.

[19] Lee, J., Durst, R. W., Wrolstad, R. E., *J. AOAC Int.* 2005, *88*, 1269–1278.

[20] Gonnet, J.-F., *Food Chem.* 1999, *66*, 387–394.

[21] Van den Berg, R., Haenen, G. R. M. M., Van den Berg, H., Bast, A., *Food Chem.* 1999, *66*, 511–517.

[22] Tanchev, S., Iontcheva, N., *Nahrung* 1975, *19*, 629–633.

[23] Giusti, M., Rodriguez-Saona, L. E., Griffin, D., Wrolstad, R. E., *J. Agric. Food Chem.* 1999, *47*, 4657–4664.

[24] Stintzing, F. C., Trichterborn, J., Carle, R., *Food Chem.* 2006, *94*, 296–309.

[25] Aaby, K., Skrede, G., Wrolstad, R. E., *J. Agric. Food Chem.* 2005, *53*, 4032–4040.

[26] Brønnum-Hansen, K., *J. Chromatogr.* 1983, *262*, 385–392.

[27] Inami, O., Tamura, I., Kikuzaki, H., Nakatani, N., *J. Agric. Food Chem.* 1996, *44*, 3090–3096.

[28] Wu, X., Gu, L., Prior, R. L., McKay, S., *J. Agric. Food Chem.* 2004, *52*, 7846–7856.

[29] Kammerer, D., Carle, R., Schieber, A., *Eur. Food Res. Technol.* 2004, *219*, 479–486.

[30] Clifford, M. N., *J. Sci. Food Agric.* 2000, *80*, 1063–1072.

[31] Hoshino, T., Tamura, H., in: Ikan, R. (Ed.), *Naturally Occurring Glycosides*, John Wiley & Sons, New York Weinheim 1999, Chapter 2, pp. 43–82.

[32] Redus, M., Bakers, D. C., Dougall, D. K., *J. Agric. Food Chem.* 1999, *47*, 3449–3459.

[33] Dangles, O., Saito, N., Brouillard, R., *Phytochemistry* 1993, *34*, 119–124.

[34] Jordheim, M., Fossen, T., Andersen, Ø. M., *J. Agric. Food Chem.* 2006, *54*, 9340–9346.

[35] Furtado, P., Figueiredo, P., Neves, H. Ch., Pina, F., *J. Photochem. Photobiol. A. Chem*. 1993, *75*, 113–118.

[36] Lopes, P., Richard, T., Saucier, C., Teissedre, P. L. *et al*., *J. Agric. Food Chem*. 2007, *55*, 2698–2704.

[37] Santos, H., Turner, D. L., Lima, J. C., Figueiredo, P. *et al*., *Phytochemistry* 1993, *33*, 1227–1232.

[38] Preston, N. W., Timberlake, C. F., *J. Chromatogr.* 1981, *214*, 222–228.