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Thermal stability of 3-deoxyanthocyanidin pigments

Liyi Yang [a,*], Linda Dykes [a], Joseph M. Awika [a,b]

[a] Department of Soil & Crop Sciences, Texas A&M University, 2474 TAMU, College Station, TX 77843-2474, USA
[b] Department of Nutrition & Food Science, Texas A&M University, 2253 TAMU, College Station, TX 77843-2253, USA

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ABSTRACT

3-Deoxyanthocyanidins are promising natural colourants due to their unique properties compared to anthocyanins. However, thermal stability of 3-deoxyanthocyanidins is largely unknown. Thermal stability of crude and pure 3-deoxyanthocyanidins was determined at 95 °C/2 h and 121 °C/30 min, at pH 1–7 using HCl, formic or citric acid as acidulants. The colour retention of crude and pure 3-deoxyanthocyanidins (79–89% after 95 °C/2 h and 39–118% after 121 °C/30 min) was high compared to literature reports for anthocyanins under similar treatments. pH significantly affected the thermal stability of 3-deoxyanthocyanidins: Colour retention was better at pH 1–2 (70.2–118%) than at pH 3–7 (39.0–86.8%). Chalcones were identified as the major heat degradation products at pH 3–7. Slow rate of chalcone formation and resistance to C-ring fission were identified as the major contributors to thermal stability of 3-deoxyanthocyanidins. Overall, the heat stability of 3-deoxyanthocyanidins indicates good potential for food use.

1. Introduction

Natural colourants have gained increased attention due to growing consumer demand for natural wholesome foods. Among the natural colourants, anthocyanins are the most widely used water soluble pigments. However, the utilization of anthocyanins in food products is limited due to poor stability to various food processing and handling conditions (Stintzing & Carle, 2004). Heat treatment of anthocyanins often results in colour change (browning) and colour loss. For example, blackcurrant anthocyanins lost about 22% after pasteurization (103 °C 30–45 s) in juice making process (Woodward, McCarthy, Pham-Thanh, & Kay, 2011). Strawberry anthocyanins decreased 9–14% after pasteurization (85 °C 5 s) during puree processing (Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008). The common heat induced reactions of anthocyanins include deglycosylation (Adams, 1973), opening of the pyrylium ring and formation of chalcone (Hrazdina, 1971), and generation of C6–C3–C6 structure fragments (e.g., protocatechuic acid) (Sadilova, Carle, & Stintzing, 2007). Anthocyanins can also polymerize during thermal processing, which can improve their colour stability (Cardona, Lee, & Talcott, 2009; Weinert, Solms, & Escher, 1990).

3-Deoxyanthocyanidins are a group of water-soluble pigments which are structurally related to anthocyanins but do not have the substitution at C-3 position (Fig. 1). Sorghum is the known major edible source of these pigments. Sorghum grains and leaf sheaths of red/purple pericarp/plant colour accumulate high amounts of 3-deoxyanthocyanidins (Awika, Rooney, & Waniska, 2004; Dykes, Seitz, Rooney, & Rooney, 2009; Kayodé et al., 2011). 3-Deoxyanthocyanidins are more stable to pH changes (Mazza & Brouillard, 1987) and bleaching effects of common food additives, such as ascorbic acid and sulphites (Ojwang & Awika, 2008) than anthocyanin analogues. Besides advantages of colour stability, 3-deoxyanthocyanidins also possess chemopreventive properties which make them likely suitable as bioactive food ingredients. For example, the 3-deoxyanthocyanidins induce the activity of quinone reductase, a phase II protective enzyme, and inhibit the growth of human oesophageal and colon cancer cells (Yang, Browning, & Awika, 2009). The cytotoxic effect of 3-deoxyanthocyanidins against human colon cancer cells is stronger than their anthocyanidin counterparts (Shih et al., 2007). These properties indicate good food application potential of 3-deoxyanthocyanidin pigments.

The pH of food matrix has a big influence on the colour and stability of anthocyanins and 3-deoxyanthocyanidins. When in solution, anthocyanins and 3-deoxyanthocyanidins establish a complex equilibrium between coloured flavylium cation, quinoidal base, colourless chalcone and carbinol pseudobase. The acidity (ideally pH 1–2) of the matrix favours the ionization reaction for the formation and stability of flavylium cation. Increase in pH shifts the equilibrium towards hydration of flavylium cation into colourless species, which results in loss of characteristic colour (Brouillard & Dubois, 1977; Mazza & Brouillard, 1987). The pH of foods and food products varies from ~2.0 (lime juice) to ~7.9

* Corresponding author. Tel.: +1 979 845 2925; fax: +1 979 845 0456.
E-mail address: lyang@ag.tamu.edu (L. Yang).

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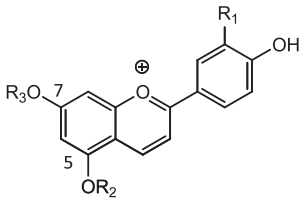

3-Deoxyanthocyanidins	R_1	R_2	R_3
Apigeninidin (APG)	H	H	H
7-*O*-Methyl-apigeninidin (7-OMe-APG)	H	H	CH_3
5-*O*-Methyl-apigeninidin (5-OMe-APG)	H	CH_3	H
5,7-*O*-Methyl-apigeninidin (5,7-OMe-APG)	H	CH_3	CH_3
Luteolinidin (LUT)	OH	H	H
5,7-*O*-Methyl-luteolinidin (5,7-OMe-LUT)	OH	CH_3	CH_3

Fig. 1. 3-Deoxyanthocyanidins used in thermal stability study.

(Graham crackers), the majority fall in between 3.0 and 6.0 (FDA., 2009). Most anthocyanins have low stability in this pH range, whereas the 3-deoxyanthocyanidins remain relatively stable (Awika et al., 2004; Mazza & Brouillard, 1987). However, how thermal processing affects stability of 3-deoxyanthocyanidins is not known. Determining thermal stability of 3-deoxyanthocyanidins will thus add valuable information to the potential of these pigments as bioactive food ingredients. In addition, knowing the degradation mechanisms is valuable in looking for strategies to stabilize the 3-deoxyanthocyanidins during processing.

The goal of this study was to evaluate thermal stability of 3-deoxyanthocyanidins at various pH (1–7) in presence of different acidulants (HCl, formic acid and citric acid). The 3 selected acidulants were aimed to compare the effect of mineral acid (HCl) with common organic acids (formic acid and citric acid). We hypothesize that type of acidulant and not pH alone, has an impact on thermal stability of 3-deoxyanthocyanidins.

2. Materials and methods

2.1. Materials

2.1.1. Crude 3-deoxyanthocyanidin pigments

Crude 3-deoxyanthocyanidin extract was obtained from dried red sorghum leaf sheaths (Health Forever Products, Lagos, Nigeria). This material was previously described by Geera, Ojwang, and Awika (2012). Acidified aqueous methanol (formic acid:water:methanol = 1:49:50, v:v:v) was used to extract the sample, with 2 h shaking at room temperature. Solvent was removed to complete dryness under vacuum at 40 °C using a Multivapor system (Büchi, Flawil, Switzland).

2.1.2. 3-Deoxyanthocyanidin compounds

In order to evaluate the effect of 3-deoxyanthocyanidin structure on their thermal stability, several pure 3-deoxyanthocyanidin compounds (Fig. 1) were used in this study. Apigeninidin (APG), 5-*O*-methyl-apigeninidin (5-OMe-APG), 7-*O*-methyl-apigeninidin (7-OMe-APG), 5,7-*O*-methyl-apigeninidin (5,7-OMe-APG), luteolinidin (LUT), and 5,7-*O*-methyl-luteolinidin (5,7-OMe-LUT) were purchased from AlsaChim (Strasbourg, France). All pure compounds were synthetic and of at least 95% purity.

2.2. Methods

2.2.1. Sample solution preparation

Crude 3-deoxyanthocyanidin extract (10 mg/mL) and pure 3-deoxyanthocyanidins (2 mg/mL, 5.94–6.84 mM) were reconstituted with HPLC grade water (pH = 6.79) or acidified water of pH 1–6 prepared with different acidulants (HCl, formic acid, and citric acid) in Pyrex test tubes with plastic stoppers. Only acids were used in all solutions in order to eliminate the stabilizing effect from constituents of buffer systems. The pH of heated sample was also measured to ensure no change occurred after heat treatment. Samples were sonicated at 40% output energy for 30 s using a tip probe (6 mm diameter) sonicator (VibraCell 40, Sonics & Materials Inc., Danbury, CT) to improve solubility. Immediately after sonication, 0.5 mL of each solution was transferred to a 2.0 mL microcentrifuge tube for thermal treatment.

2.2.2. Heat treatment

The thermal stability of 3-deoxyanthocyanidins was initially evaluated at 95 °C over different time periods (30 min, 1, 2 h) in a water bath (VWR, Radnor, PA). Since heat treatment at 95 °C for up to 2 h did not result in meaningful changes, the thermal stability of 3-deoxyanthocyanidins was further evaluated at 121 °C for 30 min at pH 1–6 and in water, using an autoclave (SM-300, YAMATO Scientific Co., Ltd., Tokyo, Japan). At the end of heat treatment, the samples were put in ice to stop any further degradation reaction. An aliquot of each sample after 30 min equilibration in ice bath was taken to analyse colour intensity change (UV–*vis* spectroscopy) and degradation profiles (HPLC and UPLC–MS). The experiments were performed in duplicates.

2.2.3. Change in colour intensity

For crude 3-deoxyanthocyanidin extract, 150 μL heated sample was diluted 20-fold with 1% HCl in methanol (pH = 1.0). For pure 3-deoxyanthocyanidin compounds, 50 μL of heated sample was diluted 60-fold using the same solvent for analysis. The absorbance at their respective λ_{max} (A) was measured 5 min after dilution by a spectrophotometer (Shimadzu UV2450, Shimadzu Scientific Instruments North America, Columbia, MD). A non-heat treated sample was diluted the same way and served as control (A_0). Colour retention after heat treatment was calculated by comparing A with its respective control A_0.

2.2.4. HPLC analysis

To profile changes of 3-deoxyanthocyanidins due to thermal treatment, 200 μL of treated crude 3-deoxyanthocyanidin extract and 100 μL of treated pure 3-deoxyanthocyanidins were diluted 5-fold and 10-fold, respectively, with 4% formic acid in methanol (pH = 1.0), then filtered through a 0.2 μm nylon membrane syringe filter. A non-heat treated sample was diluted and filtered the same way and served as control. An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) was used for this analysis. It included a quaternary pump (with degasser), an autosampler, a column compartment, and a diode array detector (DAD). A Luna C-18 column (150 mm × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA) was used to carry out the separation with a two solvent gradient: Solvent A 1% formic acid in water and solvent B 1% formic acid in acetonitrile. The gradient based on solvent B was as follows: 0–3 min, 10%; 5 min, 18%; 10 min, 20%; 23 min, 26%; 25 min, 28%; 28 min, 40%; 30 min, 60%; 30–32 min, 60%; 34–40 min 10%. The column was kept at 40 °C during analysis and the flow rate was 1.0 mL/min. The injection volume was 20 μL for crude 3-deoxyanthocyanidin extract and 5 μL for all pure 3-deoxyanthocyanidin compounds.

2.2.5. UPLC-PDA-ESI-TQD-MS analysis

To determine the structures of thermal degradation products, the 3-deoxyanthocyanidin samples used in this experiment were treated the same as for HPLC quantitative analysis. A Waters-ACQUITY UPLC-PDA-ESI-TQD-MS system (Waters Corp., Milford, MA) was used which consisted of a binary solvent manager,

autosampler (sample manager), column heater, and photodiode array eλ detector (PDA) and interfaced with a tandem quadrupole (TQD) mass spectrometer equipped with an ESI source. The separation was performed on a Kinetex C18 column (150 mm × 2.10 mm, 2.6 μm, Phenomenex, Torrance, CA) at 40 °C with following gradient at 0.4 mL/min: Solvent A (0.05% formic acid in water), solvent B (acetonitrile), and the percentage of solvent B was 12–41% from 0 to 23.5 min, 41–75% from 23.5 to 25.5 min, 75% isocratic from 25.5 to 28.5 min, then 75–12% from 28.5 to 29.5 min, and 12% isocratic for 5 min to equilibrate the column. The injection volume was 1 μL. The monitoring wavelength for 3-deoxyanthocyanidin pigments was at 485 nm; while thermal degradation products were monitored at 280 nm and 340 nm. Mass spectrometric data were acquired in positive mode for 3-deoxyanthocyanidins, and in negative mode for degradation compounds. Empower 2 software (Waters Corp.) was used to acquire and analyse data. The MS scan was recorded in the range of 100–1000 Da. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/h for desolvation and at the cone, respectively. The source block temperature and desolvation temperature were set at 150 and 400 °C, respectively. Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and performed for each individual peak/compound detected. Mass parameters were optimized as follows: Capillary voltage, 3.5/3.0 kV; and cone voltage, 60/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage of 45/(30–40) V and collision energy of 35/(15–30) V. Compound identification was based on matching UPLC retention profile, UV–*vis* spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in the literature.

2.2.6. Statistical analysis

Data were analysed with one way analysis of variance (ANOVA) and treatment means were compared by Tukey's HSD test. Differences between two groups of samples were compared by Student's t-test. All statistical analysis was performed by SAS 9.2 (Cary, NC).

3. Results and discussion

3.1. Thermal stability of crude 3-deoxyanthocyanidin extract

The dried leaf sheath from red sorghum has very intense red colour. It is commonly used in West Africa as a source of dye for various food and non-food applications (Kayodé et al., 2011). The 3-deoxyanthocyanidins in the crude extract comprised mostly apigeninidin (7.5 mg/g), 7-*O*-methyl-apigeninidin (0.51 mg/g), and luteolinidin (0.27 mg/g), which was similar to what was reported previously (Geera et al., 2012). It also contained dimeric 3-deoxyanthocyanidin pigments (Geera et al., 2012). Sorghum with red secondary plant colour tends to accumulate mainly apigeninidin type pigments (Dykes et al., 2009). Besides 3-deoxyanthocyanidins, the crude 3-deoxyanthocyanidin extract also had a fair amount of apigenin (0.84 mg/g). Other phenolic compounds usually found in red sorghum, such as phenolic acids and flavanones, were detected in trace amounts.

The crude 3-deoxyanthocyanidin extract retained 89% of colour in distilled water treated at 95 °C for 2 h (data not shown). The colour loss was generally small compared with literature reports of loss of anthocyanins extracted from various resources after similar thermal treatment. For example, purple-flesh potato anthocyanin extract lost approximately 42% of colour after heating at 98 °C for 2 h (Reyes & Cisneros-Zevallos, 2007); colour intensity of red cabbage anthocyanin extract lost about 36% after 2 h heat treatment at 90 °C (Fernández-López, Angosto, Giménez, & León, 2013).

Table 1
Colour retention (%) measured at λ_{max} of crude 3-deoxyanthocyanidin extract after 121 °C/30 min heat treatment, at pH 1–6 and neutral aqueous solutions.

pH	HCl	Formic acid	Citric acid
1	102 ± 1.2^{ab}	61.3 ± 2.2^{def}	118 ± 0.90^{a}
2	93.4 ± 7.5^{bc}	47.6 ± 0.0^{fg}	70.7 ± 0.90^{de}
3	76.1 ± 1.7^{cd}	65.2 ± 0.78^{def}	44.8 ± 0.90^{fg}
4	68.2 ± 2.6^{de}	56.0 ± 0.12^{defg}	56.3 ± 1.1^{defg}
5	46.9 ± 0.30^{fg}	60.5 ± 0.95^{def}	53.2 ± 1.1^{efg}
6	39.0 ± 0.40^{g}	52.9 ± 2.3^{efg}	44.8 ± 1.8^{fg}
Water (6.8)		65.0 ± 11^{def}	

Data are expressed as percentage colour retention (mean ± SD) of duplicate runs. Treatments with the same letter do not significantly differ (Tukey's HSD, $p < 0.05$). Colour retention was defined as absorbance of heat treated sample compared with corresponding non-heated control.

After 121 °C/30 min treatment in HCl, colour retained best in low pH conditions, i.e., pH 1 and 2, with less than 7% loss of absorbance (Table 1). As expected, the colour retention decreased with increase in pH between pH 3 and 6 (76–39%). Colour retention in distilled water was 65%. In citric acid, the trend was similar to HCl; an 18% increase in colour intensity was observed after 121 °C/30 min treatment pH 1 (Table 1). The λ_{max} of crude 3-deoxyanthocyanidin extract in HCl and citric acid solutions, before and after heat treatment, was 481 nm. The hyperchromic shift suggests that copigmentation or formation of coloured products of higher molar absorption coefficients than the original 3-deoxyanthocyanidin pigments may have contributed to the improved colour intensity after heat treatment in pH 1 citric acid solution. In pH 2 and 3 citric acid solutions, a drastic decrease of colour intensity was observed after heat treatment, with 71% and 45% colour retained, respectively (Table 1). Colour retention in citric acid solutions at pH 4–6 was generally similar and ranged from 45% to 56%.

Interestingly, formic acid showed a different trend in low pH conditions. Colour retention in pH 1 and 2 formic acid solutions was 61% and 48%, respectively (Table 1), which were much lower compared with those in HCl and citric acid ($p < 0.05$). Colour retention at pH 3–6 formic acid solutions was comparable to the other two acids (53–65%).

The abundance of H^+ in pH 1 solutions stabilizes the flavylium cation, and contributes to thermal stability of 3-deoxyanthocyanidins. However the stability of 3-deoxyanthocyanidin pigments in formic acid solutions was lower than the other two acids at pH 1 and 2. This may be because of the reducing properties of formic acid, particularly at high temperatures (Gibson, 1969). Due to the presence of an aldehyde group, at high concentrations and temperatures, formic acid may get involved with redox reactions of the pigments, negatively affecting the thermal stability of 3-deoxyanthocyanidins. This indicates that the chemical properties of the acidulant need to be taken into consideration when selecting ingredients for different processing conditions. The three acidulants did not have significant effect on colour retention in the pH range of 4–6. This indicates that in a mildly acidic environment, the chemical characteristics of acidulant may not be critical to thermal stability of 3-deoxyanthocyanidins.

The crude 3-deoxyanthocyanidin extract showed higher overall colour retention (39–118%) than anthocyanins after 121 °C/30 min treatment. For example, the purified anthocyanin fraction from purple potatoes retained approximately 9.6% of total anthocyanins after heating at 120 °C for 30 min (Nayak, Berrios, Powers, & Tang, 2011). These evidence suggest better thermal stability of 3-deoxyanthocyanidins. In addition, no significant browning, or bathochromic/hypsochromic shift was observed for the crude extract after heat treatment. However, the crude 3-deoxyanthocyanidin extract

tended to precipitate easily after heat treatment, especially at higher pH, which indicates that solubility of the 3-deoxyanthocyanidins may be a disadvantage in aqueous applications. Additional work is needed to improve the solubility of sorghum 3-deoxyanthocyanidins in aqueous system over time.

3.2. Thermal stability of 3-deoxyanthocyanidins

After 95 °C/2 h heat treatment, the pure 3-deoxyanthocyanidins retained 79–88% of colour intensity at neutral conditions (data not shown), which were generally comparable to the crude 3-deoxyanthocyanidin extract (colour retained 89%) after similar heat treatment. Anthocyanins in crude pigment extracts are generally more stable compared with purified pigment fractions, due to, among other factors, the stabilizing effect from copigmentation with other phenolic constituents in the crude extract. However, the stability of pure 3-deoxyanthocyanidins was comparable to the crude pigment extract, which suggests that the key to thermal stability of the 3-deoxyanthocyanidins lies in their structure. The half-life values of various pelargonidin (anthocyanidin analogue of apigeninidin) and cyanidin (anthocyanidin analogue of luteolinidin) glycosides in purified fruit anthocyanin fractions were determined as between 1.6 and 2.2 h at 95 °C at pH 3.5 (Sadilova et al., 2007).

The pure 3-deoxyanthocyanidin compounds were remarkably stable under the severe heat treatment of 121 °C/30 min. HCl and citric acid treatments showed a similar trend of highest thermal stability at pH 1 and 2, with >80% colour retention (Table 2). At pH 3–6, the colour retention remained high and was relatively similar among all treatments with values of 68–78%. As observed for the crude 3-deoxyanthocyanidin extract, formic acid treatments showed the opposite trend: Colour was least retained at pH 1 (7.8–46%), followed by pH 2 (26–60%, Table 2). Treatments at the other pH values had similar colour retention as HCl and citric acids (64–79%, Table 2). The reducing properties of formic acid as previously mentioned (Gibson, 1969) likely negatively affected thermal stability in pH 1 and 2 formic acid solutions. Samples treated in neutral aqueous solutions showed similar stability as the pH 3–6 treatments, with 68–84% colour retained (Table 2).

The thermal stability of 3-deoxyanthocyanidins was also significantly affected by molecular structure. This effect was most apparent in formic acid treatments at low pH values (1 and 2). The methoxylated 3-deoxyanthocyanidins were less stable (7.8–39% colour retention) compared with non-methoxylated ones (45–60% colour retention) in pH 1 and 2 formic acid treatments. Additionally, the position of *O*-methyl substitution seemed to affect thermal stability: 5-OMe-APG had the lowest stability among all 3-deoxyanthocyanidin compounds (7.8% and 26% in pH 1 and pH 2 formic acid solutions, respectively). Compared with the C-7 *O*-methyl substituted 7-OMe-APG (31% and 39% in pH 1 and pH 2 formic acid solutions, respectively), the lower stability of C-5 *O*-methyl substituted 5-OMe-APG may be due to higher reactivity of C-5 than C-7 position (de Freitas & Mateus, 2006). The 5,7-*O*-methyl substituted compounds had intermediate thermal stability compared to the C-7 and C-5 mono-*O*-methyl substituted 3-deoxyanthocyanidins (19–35% in pH 1 and 2 formic acid solutions).

In general, the 3-deoxyanthocyanidins showed greater colour retention after 121 °C/30 min heat treatment compared with the crude 3-deoxyanthocyanidin extract (Tables 1 and 2). This was somewhat unexpected. The presence of other phenolic compounds, e.g., phenolic acids, flavones, flavonols, or flavanones, in a crude extract could serve as copigments of anthocyanins, hence improve stability of the crude extract. However, under severe heat treatment, degradation of these non-pigment phenolic compounds may produce reactive fragments that can trigger degradation reactions of the pigments, e.g., oxidation, hence reducing the relative colour stability of the crude 3-deoxyanthocyanidin extract. It will be important to study the thermal stability of additional natural 3-deoxyanthocyanidin extracts with different pigment and copigment compositions, in order to determine the relationship between composition of 3-deoxyanthocyanidins and their thermal stability.

3.3. Heat induced structural changes to 3-deoxyanthocyanidins

The heat induced structural changes of 3-deoxyanthocyanidins were elucidated by HPLC and UPLC–MS analysis. For all three acids, structural changes at pH 3–6 and neutral conditions were very

Table 2
Colour retention (%) measured at λ_{max} of 3-deoxyanthocyanidins after 121 °C/30 min heat treatment, in pH 1–6 and neutral aqueous solutions.

Acid/pH values	APG	7-OMe-APG	5-OMe-APG	5,7-OMe-APG	LUT	5,7-OMe-LUT
HCl						
1	101 ± 1.4^{a}	99.5 ± 2.5^{a}	93.7 ± 2.6^{a}	101.7 ± 8.7^{a}	98.7 ± 0.54^{a}	98.7 ± 0.0^{a}
2	93.4 ± 1.2^{ab}	86.5 ± 4.0^{bc}	80.2 ± 1.1^{cd}	86.5 ± 0.48^{abcd}	83.2 ± 2.1^{bc}	70.2 ± 2.3^{cd}
3	80.5 ± 1.1^{bcde}	65.7 ± 3.8^{efg}	76.7 ± 1.6^{cde}	78.8 ± 3.0^{bcdef}	79.1 ± 0.44^{bcd}	73.5 ± 0.17^{cd}
4	69.0 ± 1.2^{ef}	61.7 ± 0.69^{g}	72.9 ± 3.3^{de}	72.1 ± 1.6^{def}	76.1 ± 2.0^{bcd}	67.6 ± 0.46^{d}
5	70.1 ± 3.4^{ef}	72.1 ± 9.0^{defg}	74.4 ± 0.85^{de}	77.0 ± 0.11^{bcdef}	78.1 ± 1.5^{bcd}	75.6 ± 0.40^{bcd}
6	75.3 ± 0.71^{de}	66.3 ± 0.29^{efg}	69.8 ± 0.14^{e}	70.5 ± 1.3^{def}	71.6 ± 0.29^{cde}	67.8 ± 1.6^{d}
Formic acid						
1	45.4 ± 1.0^{g}	31.3 ± 0.44^{h}	7.80 ± 0.11^{g}	19.1 ± 0.06^{g}	45.6 ± 0.0^{f}	21.5 ± 0.10^{e}
2	58.8 ± 1.1^{fg}	38.9 ± 0.28^{h}	26.4 ± 0.21^{f}	34.5 ± 0.45^{g}	60.6 ± 1.8^{e}	31.0 ± 0.17^{e}
3	74.9 ± 2.6^{de}	63.9 ± 0.68^{fg}	77.9 ± 0.73^{cd}	74.1 ± 5.1^{cdef}	72.4 ± 0.34^{cde}	65.2 ± 0.69^{d}
4	77.9 ± 0.76^{cde}	74.7 ± 1.3^{cedf}	75.0 ± 1.3^{de}	77.3 ± 2.8^{bcdef}	72.1 ± 1.8^{cde}	63.8 ± 2.9^{d}
5	75.3 ± 4.6^{de}	76.3 ± 7.0^{cde}	73.0 ± 1.6^{de}	67.9 ± 5.1^{ef}	75.2 ± 0.65^{cd}	65.8 ± 2.5^{d}
6	79.0 ± 6.0^{cde}	61.4 ± 1.7^{g}	74.6 ± 2.3^{de}	63.0 ± 9.2^{f}	67.1 ± 0.62^{de}	68.2 ± 0.56^{d}
Citric acid						
1	80.0 ± 8.8^{bcde}	95.8 ± 0.30^{ab}	91.3 ± 2.8^{ab}	91.6 ± 2.1^{ab}	88.1 ± 5.1^{ab}	90.3 ± 1.8^{ab}
2	89.7 ± 4.3^{abc}	84.2 ± 1.4^{a}	84.7 ± 1.2^{bc}	89.5 ± 0.60^{abc}	84.1 ± 0.065^{bc}	89.4 ± 15^{ab}
3	79.0 ± 0.94^{cde}	80.7 ± 1.2^{cd}	75.8 ± 2.0^{de}	74.5 ± 0.58^{cdef}	73.8 ± 2.7^{cd}	68.4 ± 0.11^{d}
4	76.9 ± 0.73^{cde}	75.7 ± 0.20^{cdef}	73.9 ± 0.87^{de}	77.9 ± 1.8^{bcdef}	74.3 ± 0.24^{cd}	72.2 ± 0.18^{cd}
5	73.9 ± 2.3^{de}	69.8 ± 0.11^{defg}	75.2 ± 2.4^{de}	78.0 ± 0.85^{bcdef}	73.1 ± 1.5^{cde}	71.1 ± 1.0^{cd}
6	86.8 ± 4.4^{bcd}	74.9 ± 1.3^{cdef}	76.5 ± 0.42^{de}	73.3 ± 0.71^{cdef}	74.8 ± 0.50^{cd}	73.7 ± 0.41^{cd}
Water (pH = 6.8)	78.0 ± 4.6^{cde}	68.6 ± 0.51^{efg}	79.2 ± 4.7^{cd}	82.9 ± 2.3^{bcde}	77.6 ± 11^{bcd}	84.4 ± 5.6^{abc}

Data are expressed as percentage colour retention (mean ± SD) of duplicate runs. Treatments within the same column with the same letter do not significantly differ (Tukey's HSD, $p < 0.05$). Colour retention was defined as absorbance of heat treated sample compared with corresponding non-heated control.

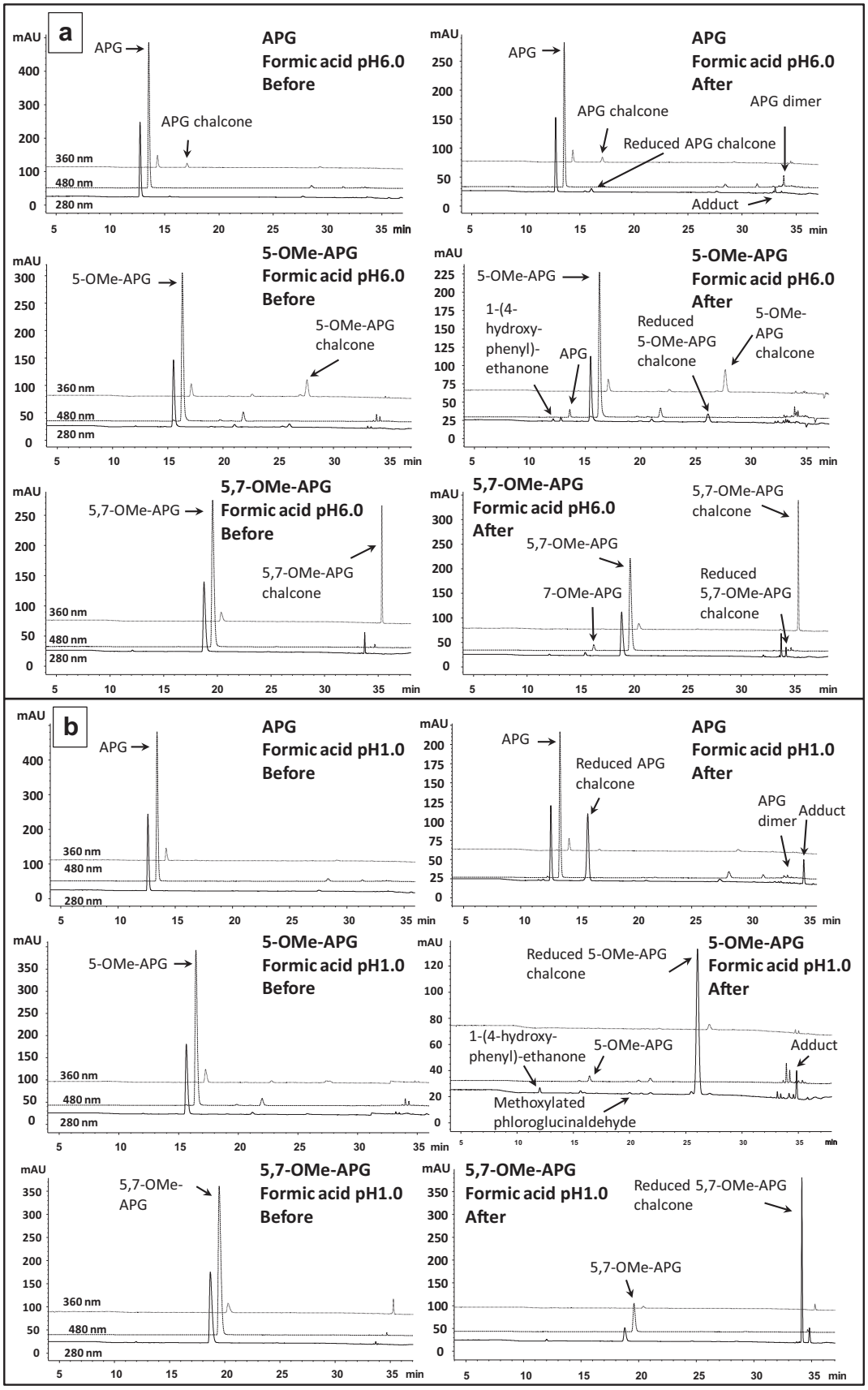

Fig. 2. HPLC chromatograms of apigeninidin (APG), 5-*O*-methyl-apigeninidin (5-OMe-APG), and 5,7-*O*-methyl-apigeninidin (5,7-OMe-APG) in pH 6 (a) and pH 1 (b) formic acid aqueous solutions before and after heat treatment. Chromatograms at 280, 480, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance.

similar. For treatments at low pH conditions (pH 1 and 2), structural changes were minimal except for formic acid treatments. Fig. 2 shows chromatograms of APG, 5-OMe-APG, and 5,7-OMe-APG heated in pH 6 and pH 1 formic acid solutions as examples.

Demethylation only occurred for C-5 $-OCH_3$ substituted 3-deoxyanthocyanidins to form their C-5 –OH substituted compounds (Table 3 and Fig. 2). On the other hand, condensation only occurred for C-5 –OH substituted 3-deoxyanthocyanidins to form their corresponding dimers and trimers (Table 3 and Fig. 2). These two reactions were the only ones which generated coloured 3-deoxyanthocyanidin derivatives with intact flavylium cation (λ_{max} 470–490 nm) (Table 3). This indicates 3-deoxyanthocyanidin structure affects reactions that may influence thermal stability. The fact that C-7 *O*-methyl substituted compound (7-OMe-APG) was not demethylated, may be due to the higher stability of the C-7 than C-5 as reported for anthocyanins (de Freitas & Mateus, 2006). The 5-OCH_3 structure prevented condensation reaction of 3-deoxyanthocyanidins between C-4 and C-8, probably due to reduced electrophilic properties of C-4 position in 5-OCH_3 substituted pigments compared with 5-OH substituted ones. The 5-OCH_3 substitution also prevented the cyclic condensation reactions between 3-deoxyanthocyanidins and pyruvic acid (Ojwang & Awika, 2008).

Anthocyanins from various sources, such as grape pomace (Cardona et al., 2009), blackberry juice (Hager, Howard, & Prior, 2008), wine (Salas, Guerneve, Fulcrand, Poncet-Legrand, & Cheynier, 2004), black olives (Caro, Azara, Delogu, Pinna, & Piga, 2006), condense during processing and storage. Due to the presence of other phenolic compounds, flavanol–anthocyanin and pyrano-anthocyanin type dimeric or oligomeric products are commonly detected. These condensed products are considered to contribute to stability of colour during long term storage. The formation of condensed dimers and trimers from 5-OH substituted 3-deoxyanthocyanidins would possibly contribute to colour stability after heat treatment.

Formation of chalcones was identified as a common and major degradation pattern in all samples. Moreover, formation of reduced chalcones was identified as a minor degradation product in neutral conditions and the most significant product in pH 1 and 2 formic acid treatments. Minor peaks of C-ring fragmentation products and chalcone adducts were also detected (Fig. 2). Fig. 3 summarizes a general degradation scheme of 3-deoxyanthocyanidins due to heat treatment.

Chalcone is an opened C-ring product of 3-deoxyanthocyanidin as a result of hydration at C-2 and formation of carbinol pseudobase in mildly acidic aqueous solutions (Brouillard, Iacobucci, & Sweeny, 1982). Chalcones were detected as the major degradation product in samples heated in neutral and pH 3–6 solutions (Fig. 2a and Table 3). The type of acidulant did not have a significant effect on the extent of chalcone formation.

Structure of 3-deoxyanthocyanidins had an effect on the formation of the chalcones. Non-methoxylated 3-deoxyanthocyanidins, APG and LUT, formed very small quantities of chalcones, while methoxylation increased chalcone formation during heat treatment (Fig. 2a). The C-5 and C-7 *O*-methyl substitution eliminates the electron-donating capacity of –OH at these positions compared to APG and LUT, thus strongly favours the cation hydration reaction towards more carbinol pseudobase and chalcone formation (Brouillard, 1982). In fact, at pH 4–6 and neutral conditions, chalcones were detected before heat treatment as a result of spontaneous hydration reaction at C-2 position. Methoxylated anthocyanidins (such as malvidin, peonidin, petunidin) have been

Table 3
Identification of compounds detected after 121 °C/30 min heat treatment of 3-deoxyanthocyanidins in water and formic acid pH 2 aqueous solutions.

		3-Deoxythocyanidin compounds					
		APG	7-OMe-APG	5-OMe-APG	5,7-OMe-APG	LUT	5,7-OMe-LUT
Parent compound	*m/z* ($[M+H]^+$)	255	269	269	283	271	299
	λ_{max} (nm)	471	469	474	472	487	487
Opened ring products	Chalcone *m/z* ($[M-H]^-$)	271	285	285	299	287	315
	λ_{max} (nm)	375	373	375	372	379	377
	Reduced chalcone *m/z* ($[M-H]^-$)	273	287	287	301	289	317
	λ_{max} (nm)	277	275	277	276	278/306	276/309/377
Condensed products	Dimer/trimer *m/z* ($[M+H]^+$)	509	537/805	–	–	–	–
	λ_{max} (nm)	471	486				
	Chalcone/reduced chalcone adducts *m/z* ($[M-H]^-$)	525/527	555	–	–	–	–
	λ_{max} (nm)	365/264	273				
De-methylated products	*m/z* ($[M+H]^+$)	–	–	255	269	–	285
	λ_{max} (nm)			471	469		487
	Proposed identification			APG	7-OMe-APG		7-OMe-LUT[a]
C-ring fission products	B-ring fragment *m/z* ($[M-H]^-$)	135	–	135	137	–	–
	λ_{max} (nm)	282		276	255		
	Proposed identification	1-(4-Hydroxyphenyl)ethanone		1-(4-Hydroxyphenyl)ethanone	*p*-Hydroxy-benozic acid		
	A-ring fragment *m/z* ($[M-H]^-$)	–	–	167	–	–	153
	λ_{max} (nm)			294			218/258/293
	Proposed identification			Methoxylated phloroglucin-aldehyde			Phloroglucin-aldehyde

Identification of the products was based on UPLC retention time (t_R), UV–*vis* spectra, and MS spectroscopic pattern. Ionization was performed in the positive mode for 3-deoxyanthocyanidins and in the negative mode for the other compounds.
– Product within the category was not identified.
[a] Based on the demethylation pattern of 5,7-OMe-APG.

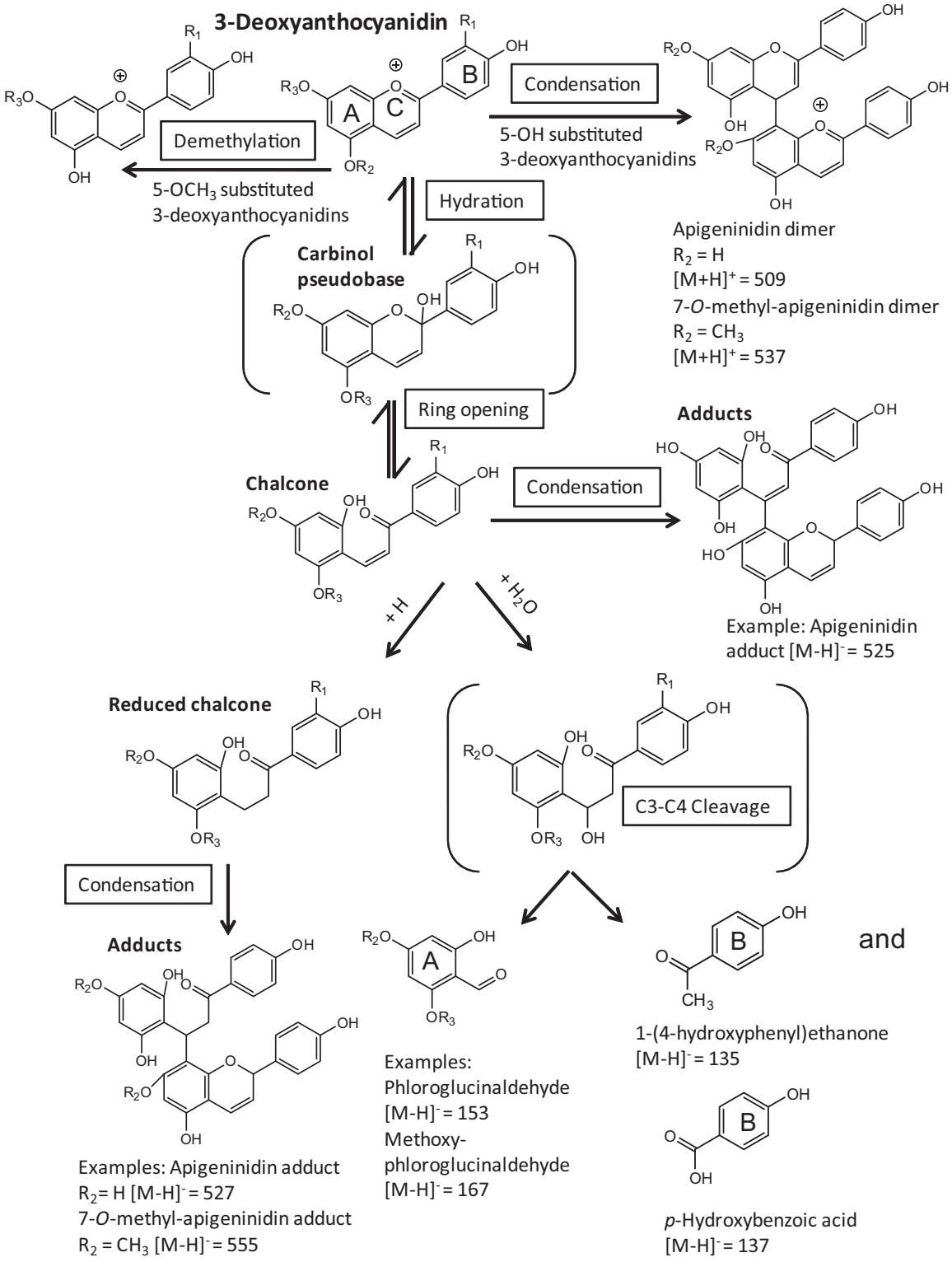

Fig. 3. A schematic illustration of thermal degradation mechanism of 3-deoxyanthocyanidins. Intermediates [in brackets] were not detected.

reported to form more chalcones than non-methoxylated ones (such as delphinidin and cyanidin) in pH 4–7 aqueous solutions (Hrazdina, Borzell, & Robinson, 1970).

In samples treated in low pH formic acid solutions (pH 1 and 2), reduced chalcones were the major product formed after heat treatment (Table 3 and Fig. 2b). This effect was unique to formic acid, and only at pH 1 and 2. This clearly confirms that formic acid acted as a reducing agent under 121 °C/30 min conditions in this study at the high concentrations needed to achieve pH 1 and 2. Based on this reaction pattern, we thus conclude that the lower thermal stability of 3-deoxyanthocyanidins in pH 1 and 2 formic acid solutions was driven by formation of reduced chalcones, which in turn favoured increased chalcone formation from flavylium cation (Fig. 2b). The relative quantities of reduced chalcones followed the same trend as for formation of chalcones: Methoxylation increased the level of reduced chalcones formed (Fig. 2b).

Several peaks were identified as fragments from C-ring fission based on MS pattern, elution pattern, and the spectrophotometric characteristics. In 7-OMe-APG neutral and formic acid pH 2 solutions, as well as 5-OMe-APG formic acid pH 2 solution after heat treatment (Table 3), 1-(4-hydroxyphenyl)ethanone, a B-ring fragment, was identified based on degradation pattern of anthocyanidins (Sadilova et al., 2007). Similarly, *p*-hydroxybenzoic acid was identified as another B-ring fragment in 5,7-OMe-APG formic acid pH 2 solution after heat treatment (Table 3). In 5-OMe-APG formic acid pH 2 solution after heat treatment, 2-methoxy-phloroglucinaldehyde, an A-ring fragment, was identified based on patterns of anthocyanidin degradation (Sadilova et al., 2007). Similarly, phloroglucinaldehyde was also detected in 5,7-OMe-LUT formic acid pH 2 solution after heat treatment (Table 3).

Formation of C-ring cleavage products is a common degradation scheme of anthocyanidins. Products such as phloroglucinaldehyde, *p*-hydroxybenzoic acid and its hydroxylated or methoxylated derivatives have been reported in different studies (Sadilova et al., 2007). Formation of chalcone is considered as the initial step for anthocyanidins to yield C-ring cleavage products. All C-ring cleavage products were detected as minor peaks in pH 1 and 2 formic acid solutions, mostly in the methoxylated 3-deoxyanthocyanidins, in this study. Thus without the reducing effect of formic acid in these reactions, the evidence actually suggests that 3-deoxyanthocyanidins resist fragmentation during thermal treatment. 3-Deoxyanthocyanidins have greater deprotonation rate constant (k_a) than hydration rate constant (k_h) (Brouillard et al., 1982), hence convert less to the colourless carbinol pseudobases in aqueous solutions, which would lead to less formation of chalcones at high pH conditions and after heat treatment compared to anthocyanins. Formation of carbinol pseudobase due to hydrophilic attack at the C-2 position, and corresponding formation of chalcones is the first step of anthocyanins degradation due to thermal treatment (Hrazdina, 1971; Lopes et al., 2007). Thus, the higher resistance of 3-deoxyanthocyanidins to chalcone formation may help explain their better thermal stability relative to anthocyanins. Recently Sousa et al. demonstrated increased chalcone formation at higher pH for 3-deoxyanthocyanidins (Sousa et al., 2013), which may account for the lower stability of the 3-deoxyanthocyanidins at higher pH conditions (Tables 1 and 2).

Minor dimeric adducts were detected in most samples after heat treatment except the two dimethoxylated 3-deoxyanthocyanidins, but only a few were identified (Tables 3). Most of these were chalcone or reduced chalcone adducts based on UV–*vis* characteristics, MS pattern and MS/MS fragmentation pattern. Some examples of proposed structures are shown in Fig. 3. Sadilova et al. (2007) observed several adducts after heat treatment of purified anthocyanin fractions from different fruits/vegetables. The adducts were proposed to be derived from fragments of either the A-ring or the B-ring of anthocyanidins. Interestingly, the adducts detected in our study were mostly condensed with intact C-rings. This further indicates higher resistance of 3-deoxyanthocyanidins to C-ring cleavage than anthocyanidins during heat treatment, which may additionally contribute to their thermal stability.

4. Conclusion

Both crude 3-deoxyanthocyanidin extract and pure 3-deoxyanthocyanidins showed good thermal stability after heat treatments at 95 °C/2 h and 121 °C/30 min. The pure 3-deoxyanthocyanidins were particularly stable over a broad range of pH, which suggests good potential as natural colourants. The crude 3-deoxyanthocyanidin extract tend to be less stable than the pure 3-deoxyanthocyanidins at 121 °C/30 min. This suggests that other phenolic constituents in the crude extract could affect thermal stability of the pigments, especially at temperatures which degradation of polyphenols could occur. More studies are needed to establish the effect of 3-deoxyanthocyanidin and copigment composition of crude pigment extract on thermal stability of sorghum 3-deoxyanthocyanidins. The type of acid affected thermal stability of 3-deoxyanthocyanidins, and this indicates characteristics of acidulants should be taken into considerations when processing products containing 3-deoxyanthocyanidin pigments. Molecular structure could also affect thermal stability of 3-deoxyanthocyanidins under certain conditions. For example, compared with non-methoxylated 3-deoxyanthocyanidins, methoxylated ones had reduced thermal stability when treated in pH 1 and 2 formic acid solutions. This suggests the composition of 3-deoxyanthocyanidin pigments should also be considered when utilizing these pigments in products. Overall, this study showed that 3-deoxyanthocyanidins possess good thermal stability, even at neutral and low acidic conditions (pH 4–6). This property makes 3-deoxyanthocyanidins suitable to be utilized as natural food colourants in various food and beverage products of low acidity.

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