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Anthocyanins from red cabbage – stability to simulated gastrointestinal digestion

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Abstract

Anthocyanins were the main polyphenol components in extracts of fresh and pickled red cabbage. The composition of anthocyanins in red cabbage was studied using liquid chromatography mass–spectrometry. Eleven major peaks absorbing at 520 nm were discerned, which represented 18 different anthocyanin structures. Another five minor anthocyanin components could be identified by searching at their respective *m*/*z* values but only in anthocyanin-enriched concentrates produced by sorption to solid phase extraction matrices. The predominant anthocyanins were constructed of cyanidin-3-diglucoside-5-glucoside "cores" which were non-acylated, mono-acylated or di-acylated with *p*-coumaric, caffeic, ferulic and sinapic acids. Pelargonidin-3-glucoside and novel forms of cyanidin-3-*O*-triglucoside-5-*O*-glucoside di-acylated with hydroxycinnamic acids were also detected in extracts of raw red cabbage, commercially pickled red cabbage and anthocyanin-enriched concentrates.

The stability of the anthocyanins to simulated gastrointestinal digestion was assessed. The anthocyanins were effectively stable in the acidic gastric digestion conditions but the total recovery after simulated pancreatic digestion was around 25% compared to around 100% recovery of phenol content. As anthocyanins make up the majority of red cabbage polyphenols, this suggested that anthocyanins broke down to form new phenolic components. The recovery of the individual anthocyanins was monitored by LC–MS^n. All of the anthocyanins were reduced in content after pancreatic digestion but acylated forms were notably more stable than non-acylated forms. There was also a relationship between the type of acylated hydroxycinnamic acid and stability to pancreatic digestion.

Keywords: Red cabbage; Anthocyanins; Polyphenols; Stability; Health benefits; *In vitro* digestion; Hydroxycinnamic acids; Acylation

1. Introduction

Increased anthocyanin content has been a breeding target for the genetic improvement of blackcurrant (Brennan, 1996) and raspberry (Graham et al., 2004) germplasm for both cosmetic reasons and potential health benefits. Anthocyanins are responsible for the red, purple and blue hues of plant fruits, flowers and leaves (Strack and Wray, 1993). Dietary consumption, mainly from red fruits, certain vegetables (such as red cabbage) and red wine (Wu et al., 2006) can reach 200 mg/day. Anthocyanins have a range of biological activities that may produce health benefits; examples range from inhibition of DNA damage in cancer cells *in vitro* (Hou, 2003), inhibition of digestive enzymes (McDougall and Stewart, 2006), induction of insulin production in isolated pancreatic cells (Jayaprakasam et al., 2005), reduction in inflammatory responses (Tall et al., 2004) to protection against age-related decline in brain function (Lau et al., 2006).

Eating anthocyanin-rich fruits, extracts or pure anthocyanins may prevent or suppress disease states *in vivo* (Ramirez-Tortosa et al., 2001; Mazza et al., 2002). Oral intake of anthocyanins increased antioxidant status of the serum (Serafini et al., 1998; Ramirez-Tortosa et al., 2001;

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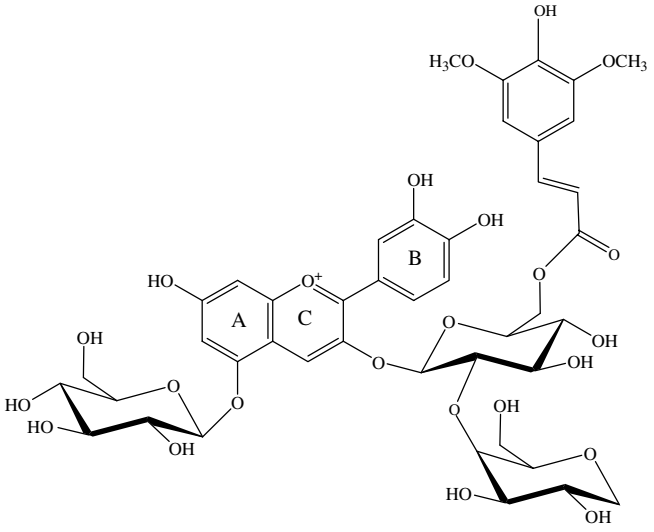
Mazza et al., 2002; Talavera et al., 2006) but this was usually accompanied by very low uptake of anthocyanins into the serum (≪1% of dose) (Lapidot et al., 1998; Bub et al., 2001; Frank et al., 2003; Talavera et al., 2006). The apparent low bioavailability of anthocyanins casts doubt on their ability to cause their proposed beneficial effects throughout the body.

It has become clear from studies on simulated human gastrointestinal digestion that anthocyanins, whilst stable in the acidic conditions of the stomach, are less stable at the elevated pH of the small intestine (Perez-Vicente et al., 2002; Gil-Izquierdo et al., 2002; McDougall et al., 2005a,b). Red cabbage (*Brassica oleracea* L.) is a useful food colouring (Giusti and Wrolstad, 2003; Stintzing and Carle, 2004) because red cabbage anthocyanins are stable over a broader pH range than anthocyanins from (say) blackcurrants, which only retain colour at pH < 4.0 (Markakis, 1982). Therefore, red cabbage anthocyanins are used as colours for foods with neutral pH and are natural alternatives to synthetic blue colourings (Bridle and Timberlake, 1997).

The major anthocyanins of red cabbage are based on a core of cyanidin-3-*O*-diglucoside-5-*O*-glucoside (Fig. 1), which can be non-acylated, mono-acylated or di-acylated with *p*-coumaric, caffeic, ferulic and sinapic acids (Tanchev and Timberlake, 1969; Idaka et al., 1987a,b; Giusti et al., 1999; Wu and Prior, 2005). Anthocyanins exist in equilibrium of four molecular species; the coloured basic flavylium cation and three secondary structures; the quinoidal bases, the carbinol pseudobase and the chalcone pseudobase forms. At pH 2 or below, the flavylium cation form predominates but as the pH is raised towards 7 the colourless chalcone pseudobase begins to dominate. Chalcone formation is also favoured by elevated temperatures and prolonged exposure may enhance degradation between the B and C rings (Fig. 1) resulting in the destruction of the anthocyanin chromophore (Strack and Wray, 1993; Clifford, 2000). The unusual pH stability of the colour of red cabbage anthocyanins is thought to be due to the presence of these acyl groups which "hinder the hydrolysis of the red flavylium cationic form to the colourless carbinol base, allowing preferential formation of the blue quinoidal bases" (Bridle and Timberlake, 1997). Glycosylation at positions 3 and 5 shifts the colour towards the blue and the stability of colour may also be influenced by intramolecular co-pigmentation (Maulien-Aubert et al., 2001).



Cyanidin-3-(sinapoyl)diglucoside-5-glucoside

Fig. 1. Structure of a red cabbage anthocyanin.

In this study, we assess if the known pH stability of red cabbage anthocyanins influences their stability under simulated gastrointestinal (GIT) digestion. The relative stability of the anthocyanins under GIT conditions will determine the pool size for whatever active mechanisms are present in the stomach (Passamonti et al., 2003) or the small intestine (Gee et al., 1998) to transport anthocyanins into the blood stream. Information on the relationship between the structure and gastrointestinal stability of anthocyanins will be fed-back into traditional or marker-assisted breeding programs to facilitate the generation of fruit with enhanced health benefits.

2. Materials and methods

2.1. Extraction procedures

Fresh red cabbage and pickled red cabbage was purchased from a local supermarket. The raw cabbage (500 g) was chopped into small pieces and added to 1 l of ice-cold 0.5% (v/v) acetic acid in water. The material was homogenised in a Waring Blender then filtered through coarse then fine glass sinters. The pickled red cabbage was drained then extracted in the same manner.

Portions of the red cabbage extracts were adjusted to 0.5% (v/v) formic acid prior to application to C18 solid phase extraction columns (C18E units, 1000 mg capacity, Phenomenex Ltd.). The columns were pre-treated with acetonitrile containing 0.5% (v/v) formic acid and pre-equilibrated in 0.5% (v/v) formic acid in water. After a wash with 2 volumes of 0.5% (v/v) formic acid in water, the bound anthocyanin-rich material was eluted with acetonitrile containing 0.5% (v/v) formic acid. Recovery of cyanidin-3-*O*-glucoside from the SPE procedure was around 90%. For the *in vitro* digestion procedure, portions of the bound material was evaporated to remove acetonitrile and formic acid then resuspended in same volume of distilled water.

2.2. Simulated gastrointestinal digestion

The procedure was adapted from the method outlined by Gil-Izquierdo et al. (2002) which itself was adapted from the work of Miller et al. (1981). This work showed significant correlation between *in vitro* and *in vivo* measure-

ments of iron bioavailability. The method has been described previously (McDougall et al., 2005a) and consists of two sequential steps; an initial pepsin/HCl digestion for 2 h at 37 °C to simulate gastric conditions followed by a digestion with bile salts/pancreatin for 2 h at 37 °C to simulate small intestine conditions.

2.3. Anthocyanin and phenol assays

The total anthocyanin concentration was estimated by a pH differential method (Ribereau-Gayon and Stonestreet, 1965). The absorbance value was related to anthocyanin content using the molar extinction coefficient calculated in-house for cyanidin-3-*O*-glucoside. Phenol content was measured using a modified Folin-Ciocalteau method (Singleton and Rossi, 1965). Phenol contents were estimated from a standard curve of gallic acid. All results have been corrected for the presence of phenols in the pancreatin/bile salts mixture.

Prior to LC–MS, samples were acidified to 0.5% (v/v) formic acid by slow addition of 10% formic acid and mixed well. After centrifugation, the phenol and anthocyanin contents of the supernatant and the "insoluble" material, which was soluble in methanol, were assayed. Insoluble material accounted for less than 2% of the total anthocyanin and phenol contents. Samples were dried in a speed-vac (Thermo-Finnegan Ltd.) to suitable phenol concentrations for LC–MS.

2.4. Liquid chromatography–mass spectroscopy (LC–MS)

Samples (containing 20 μg gallic acid equivalents by Folin assay) were analyzed on a LCQ-DECA system, comprising Surveyor autosampler, pump and photo diode array detector (PDAD) and a ThermoFinnigan mass spectrometer iontrap by the method described previously (McDougall et al., 2005a). The MS was calibrated against standard molecules and tuned against cyanidin-3, 5-*O*-diglucoside to maximize response. Standard anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3, 5-*O*-diglucoside and pelargonidin-3-*O*-glucoside) were obtained from ExtraSynthese (Genay, France). For direct infusion studies, the extracts were infused into the MS source at 5 μl/min in 50% acetonitrile. MS and MS^2 spectra were accumulated over 5 min.

The amount of each anthocyanin was estimated using the peak area calculated under the specific m/z value for each molecular species as defined by the software associated with the mass spectrometer (Xcalibur™, ThermoFinnigan). This method gives a reasonable estimate of content even when anthocyanin peaks were not completely separated. The % recovery of the anthocyanins in the IN and OUT samples was calculated as the percentage of the amount of that particular anthocyanin in the post-gastric sample. This comparison overcomes potential problems in differential ionization of individual anthocyanins. All values are averages values obtained from three replicate injections of samples obtained from three replicate experiments ± standard error. A factor was calculated to correct for the degree of concentration of the sample required to give 40 μg phenols/injection on LC–MS.

3. Results and discussion

The red cabbage extract contained a high content of anthocyanins (137.5 ± 2.9 mg/100 g) which after concentration by sorption to C18 solid phase extraction (SPE) units gave an anthocyanin/total phenol ratio of ~1.00. HPLC analysis of the red cabbage extract yielded a number of peaks that absorbed at 520 nm (Fig. 2a). The HPLC profiles at 280 nm and 520 nm (Fig. 2a and b) are very similar which confirms that anthocyanins make up the majority of red cabbage polyphenols. The pickled red cabbage gave a very similar profile with only small variations in the relative abundances of the peaks (results not shown). The first major peak (Fig. 2a, peak 1) gave a PDA spectrum with maxima at 510 nm and 280 nm. This peak gave one major ion $[M]^+$ $m/z = 773$ with $MS^2 = 611$ $[M-Glc]^+$, 449 $[M-2Glc]^+$ and 287 $[M-3Glc]^+$ which is consistent with cyanidin-3-*O*-diglucoside-5-*O*-glucoside (Table 1). Small amounts of cyanidin-3, 5-*O*-diglucoside (T_R = 23.15) and cyanidin-3-*O*-sophoroside ($T_R = 28.23$) could be identified by searching at their shared $m/z = 611$ value. However, these could only be differentiated from in-source fragmentation of the m/z 773 peak in spectra of the concentrated anthocyanin sample produced by solid phase extraction. The identity of the cyanidin-3, 5-*O*-diglucoside peak was also confirmed by co-chromatography with an authentic standard (results not shown).

All of the other main anthocyanin peaks gave PDA spectra with maxima around 320 nm which is characteristic of acylation with hydroxycinnamic acids (Hong and Wrolstad, 1990). The general pattern of anthocyanin peaks in red cabbage was largely similar to that described by Wu and Prior (2005), and they were mainly composed of a core unit of cyanidin-3-*O*-diglucoside-5-*O*-glucoside (Fig. 1) acylated with various hydroxycinnamic acids.

Peak 2 gave $[M]^+$ $m/z = 979$ with $MS^2 = 817$ $[M-Glc]^+$, 449 $[M-2Glc\text{-sinapoyl}]^+$ and 287 $[M-3Glc\text{-sinapoyl}]^+$, which can be assigned to cyanidin-3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside. This structure is shown in Fig. 1. Peak 3 gave $[M]^+$ $m/z = 949$ with $MS^2 = 787$ $[M-Glc]^+$, 449 $[M-2Glc\text{-feruloyl}]^+$ and 287 $[M-3Glc\text{-feruloyl}]^+$, which can be assigned to cyanidin-3-*O*-(feruloyl)diglucoside-5-*O*-glucoside (Wu and Prior, 2005). The difference of 30 a.m.u is characteristic of methoxy groups.

Peak 4 had a PDA spectra with maxima at 495, 430(sh) and 280 nm and gave $[M]^+$ $m/z = 433$ and 271 and $MS^2 = 271$ $[M-Glc]^+$. These are characteristic of pelargonidin-3-*O*-glucoside and this was confirmed by co-chromatography of this peak with an authentic standard (results not shown). Pelargonidin-3-*O*-glucoside has not previously been reported in red cabbage but it was also present in concentrates produced by C18 solid phase

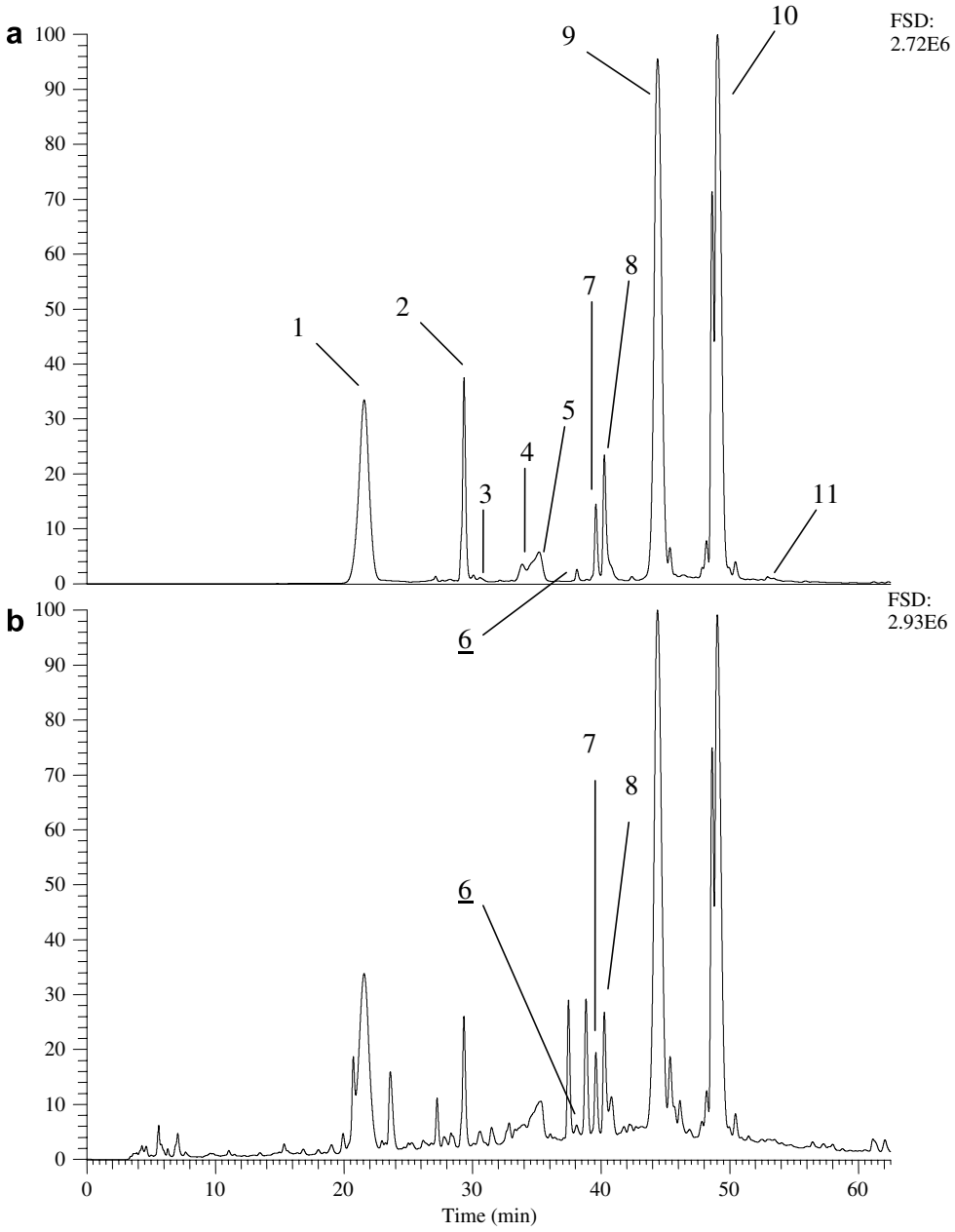


Fig. 2. HPLC traces for red cabbage extract The trace in (a) shows the absorbance at 520 nm and the trace in (b) the absorbance at 280 nm for the red cabbage extract. Arrows in (a) denote peaks discussed in the text. Not all peaks are labeled. The full scale deflection (FSD) is given for each trace.

extraction and in the extracts of commercially pickled red cabbage.

Peak 5 yielded MS data that suggested that this peak was made up of three co-eluting molecular species; peak 5a gave $[M]^+$ $m/z = 1081$ with poor quality $MS^2 = 919$ $[M-Glc]^+$; peak 5b gave $[M]^+$ $m/z = 1111$ with no discernible MS^2 ions and peak 5c gave $[M]^+$ $m/z = 1141$ also with no discernible MS^2 ions. However, direct infusion of the SPE concentrate produced MS^2 spectra for these molecular species (results not shown) that were identical to those previously ascribed to cyanidin-3-*O*-(caffeoyl)(*p*-comaroyl)diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(glucopyranosyl-feruloyl)diglucoside-5-*O*-glucoside and cyanidin-3-*O*-(glucopyranosyl-sinapoyl)diglucoside-5-*O*-glucoside, respectively (Wu and Prior, 2005).

Peak 6 gave the same PDA and MS data as peak 2 and may be an isomer of cyanidin-3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside with a different attachment of the sinapoyl group which gives enhanced retention. This has been noted previously (Wu and Prior, 2005).

Peak 7 gave $[M]^+$ $m/z = 1287$ with $MS^2 = 1125$ $[M-Glc]^+$, 963 $[M-2Glc]^+$ and 449 $[M-3Glc\text{-feruloyl-feruloyl}]^+$. This molecular species $[M]^+$ $m/z = 1287$ has not been identified in red cabbage previously but it has the same mass as cyanidin-3-*O*- (feruloyl)(feruloyl)diglucoside-5-*O*-glucoside with an extra glucose. The MS^2 pattern

Table 1
Peak assignments, retention times and mass spectral data of anthocyanins in red cabbage

Peak	T_R	PDA	$[M^+]$ m/z	MS^2	Putative identity
1	21.57	510, 280	773.0	610.9 449.0 287.2	Cy-3-diGlc-5-Glc
2	29.34	525, 330 280	979.1	817.0 449.0 287.2	Cy-3-(sin)-diGlc-5-Glc
3	30.07	525, 325sh 280	949.1	787.0 449.0 287.2	Cy-3-(fer)-diGlc-5-Glc
4	33.87	495, 430 280	433.0 271.2	271.2	Pg-Glc
5a	34.57	525, 325sh 280	1081.1	919.1[a] 449.0	Cy-3-(caff-*p*C)-diGlc-5-Glc
5b	35.09	525, NR	1111.1	949.1[a] 703.0 449.0	Cy-3-(glucofer)-diGlc-5-Glc
5c	35.27	525, NR	1141.1	979.0[a] 817.0 449.0	Cy-3-(glucosin)-diGlc-5-Glc
6	38.12	525, 325sh	979.1 280	817.0 449.0 287.2	Cy-3-(sin)-diGlc-5-Glc
7	39.59	535, 320sh	1287.2 280	1125.0 963.1 449.0	Cy-3-(fer)(fer)-triGlc-5-Glc
8a	40.26	535, 325	1347.2 280	1185.1 1023.0 449.0	Cy-3-(sin)(sin)-triGlc-5-Glc
8b	40.30	535, NR	1317.2	1155.1 993.0 449.0	Cy-3-(sin)(fer)-triGlc-5-Glc
9a	44.25	525, 325 280	919.1	757.0 449.0 287.2	Cy-3-(*p*C)-diGlc-5-Glc
9b	44.43	525, NR	979.1	817.0 449.0 287.2	Cy-3-(sin)-diGlc-5-Glc
9c	44.66	525, NR	949.1	787.1 449.0 287.2	Cy-3-(fer)-diGlc-5-Glc
10a	48.67	535, 320 285	1125.1	963.1 449.0	Cy-3-(fer)(fer)-diGlc-5-Glc
10b	49.04	535, NR	1155.1	993.1 449.0	Cy-3-(sin)(fer)-diGlc-5-Glc
10c	49.29	535, NR	1185.1	1023.1 449.0	Cy-3-(sin)(sin)-diGlc-5-Glc
11	52.94	525, 325 280	1125.1 1155.1 1185.1	as 10a as 10b as 10c	Cy-3-(sin)(sin)-diGlc-5-Glc Cy-3-(sin)(fer)-diGlc-5-Glc Cy-3-(fer)(fer)-diGlc-5-Glc

Cy = cyanidin, Pg = pelargonidin, Glc = glucoside, glucofer = glucopyranosyl-feruloyl, glucosin = glucopyransoyl-sinapoyl, caff = caffeoyl, *p*C = *p*-coumaroyl, fer = feruloyl, sin = sinapoyl. 325sh refers to a shoulder at 325 nm, NR means PDA spectra not resolved due to co-eluting compounds and in these cases the first PDA values represent the entire peak.

[a] MS^2spectra obtained from direct infusion MS.

also suggests that is similar to that of cyanidin-3-*O*-diglucoside-5-*O*-glucoside, especially in the characteristic ultimate breakdown to 449, so it would appear that the "extra" glucose is attached to the 3-position and the structure could be cyanidin-3-*O*- (feruloyl)(feruloyl)triglucoside-5-*O*-glucoside (Wu and Prior, 2005). Giusti et al. (1999) recorded the presence of mono-acylated derivatives of cyanidin-3-*O*-triglucoside-5-*O*-glucoside but did not provide complete MS or MS^2 data for these compounds.

Peak 8 gave MS data that suggested that it was made up of two co-eluting species; peak 8a gave $[M]^+$ $m/z = 1347$ with $MS^2 = 1185$ $[M-Glc]^+$, 1023 $[M-2Glc]^+$ and 449 [M−3Glc-sinapoyl-sinapoyl]$^+$. Peak 8b gave $[M]^+$ $m/z = 1317$ with $MS^2 = 1155$ $[M-Glc]^+$, 993 $[M-2Glc]^+$ and 449 [M−3Glc-sinapoyl-feruloyl]$^+$. By analogy to peak 7, these compounds may be cyanidin-3-*O*-(sinapoyl)(sinapoyl)triglucoside-5-*O*-glucoside and cyanidin-3-*O*-(sinapoyl)(feruloyl)triglucoside-5-*O*-glucoside, respectively.

Peak 9 was a major anthocyanin component and gave MS data that suggested that it was made up of three co-eluting species; peak 9a gave $[M]^+$ $m/z = 919$ with $MS^2 = 757$ $[M-Glc]^+$, 449 [M−2Glc-*p*-coumaroyl]$^+$ and 287 [M−3Glc-*p*-coumaroyl]$^+$; peak 9b gave $[M]^+$ $m/z = 979$ with $MS^2 = 817$ $[M-Glc]^+$, 449 [M−2Glc-sinapoyl]$^+$ and 287 [M−3Glc-sinapoyl]$^+$ and peak 9c gave $[M]^+$ $m/z = 949$ with $MS^2 = 787$ $[M-Glc]^+$, 449 [M−2Glc-feruloyl]$^+$ and 287 [M−3Glc-*p*-feruloyl]$^+$. These can be putatively identified as cyanidin-3-*O*-(*p*-coumaroyl)diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside and cyanidin-3-*O*-(feruloyl)diglucoside-5-*O*-glucoside, respectively.

Peak 10 was the major anthocyanin peak and gave MS data that suggested that it was made up of three co-eluting species. Peak 10a gave $[M]^+$ $m/z = 1125$ with $MS^2 = 963$ $[M-Glc]^+$ and 449 [M−2Glc-2feruloyl]$^+$; peak 10b gave $[M]^+$ $m/z = 1155$ with $MS^2 = 993$ $[M-Glc]^+$ and 449 [M−2Glc-feruloyl-sinapoyl]$^+$ and peak 10c gave $[M]^+$ $m/z = 1185$ with $MS^2 = 1023$ $[M-Glc]^+$ and 449 [M−2Glc-2sinapoyl]$^+$. These major components can be identified as cyanidin-3-*O*-(feruloyl)(feruloyl)diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(feruloyl)(sinapoyl)diglucoside-5-*O*-glucoside and cyanidin-3-*O*-(sinapoyl)(sinapoyl)-diglucoside-5-*O*-glucoside respectively by comparison of their m/z values and their comparative retention times with previous reports (Giusti et al., 1999; Wu and Prior, 2005).

Peak 11 was a minor component but contained the same m/z signals as peaks 10a–c which suggests that they are isomers of these components that differ in the attachment of the acyl groups.

The ratio of the heights of the peak of the acylated component (around 320 nm) over the visible maxima (around 525 nm) in the PDA spectra can be used to gauge whether the anthocyanins are mono- or di-acylated with hydoxycinnamic acids (Hong and Wrolstad, 1990) with values greater than unity suggesting di-acylation. When calculated, these values support the putative identities given above (results not shown).

Other putative anthocyanins could be identified by searching the MS data at relevant m/z values. These included $[M]^+$ $m/z = 965$ ($T_R = 26.48$) with MS^2 of 803, 449 and 287 which was putatively identified as cyanidin-3-*O*-(*p*-hydroxybenzoyl)(oxaloyl)diglucoside-5-*O*- glucoside; $[M]^+$ $m/z = 935$ ($T_R = 39.54$) with MS^2 of 773, 449 and 287 which was putatively identified as cyanidin-3-*O*-(caffeoyl)diglucoside-5-*O*-glucoside; $[M]^+$ $m/z = 817$ ($T_R = 46.46$) with MS^2 of 655, 449 and 287 which was putatively identified as cyanidin-3-*O*-(sinapoyl)glucoside-5-*O*-glucoside; $[M]^+$ $m/z = 743$ ($T_R = 26.28$) with MS^2 of 611, 419 and 287 which was putatively identified as cyanidin-3-*O*-diglucoside-5-*O*-xyloside. No evidence for the presence of cyanidin-3-*O*-(sinapoyl)diglucoside-5-*O*-xyloside ($[M]^+$ $m/z = 949$) as detected by Wu and Prior (2005) could be obtained.

The total anthocyanin content (as measured by the colorimetric assay) was stable to gastric digestion (Table 2) but was poorly recovered following pancreatic digestion compared to bulk phenols i.e. the combined (IN and OUT) recovery of phenols approached 100% of the amount applied compared to only ~27% of anthocyanins. As anthocyanins make up the majority of the original red cabbage polyphenols, it is likely that phenols detected after gastrointestinal digestion include breakdown products of anthocyanins.

Low recovery of anthocyanins in the IN or "serum-available" fraction from the *in vitro* digestion procedure has been noted before (Perez-Vicente et al., 2002; Gil-Izquierdo et al., 2002; McDougall et al., 2005a,b) and although it approaches the low serum bioavailability of anthocyanins in animal and human feeding trials (e.g. Talavera et al., 2006), partition across dialysis membrane in this *in vitro* method cannot mimic the active transport processes reported to occur in the stomach (Passamonti et al., 2003), in the small intestine (Gee et al., 1998) or the proposed structural changes that may accompany anthocyanin transport from the small intestine (Kuhnle et al., 2000; Day et al., 2000; Nemeth et al., 2003; Walton et al., 2006). Nevertheless, stability under gastrointestinal conditions will severely limit the pool of anthocyanins available to any effective transport mechanism.

The *in vitro* digestion method can indicate which compounds survive GIT conditions and are likely to reach the colon where they can act as antioxidants or be bio-transformed into phenolic antioxidants that can be absorbed from

Table 2
Effect of gastrointestinal digestion on anthocyanin and phenol content

Sample	% Recovery[a]	
	Phenols	Anthocyanins
Post-gastric	107.8 ± 2.1	95.1 ± 2.6
IN	1.9 ± 0.2	20.6 ± 1.7
OUT	24.9 ± 0.9	79.3 ± 2.3

[a] Values are averages of three replicate experiments ± standard errors and are compared to values of the original extract.

the large intestine/colon (Gonthier et al., 2003; Aura et al., 2005). The FSD of the IN and the OUT samples (Fig. 3b and c respectively) was ~10-fold lower than the original extract and all peaks were therefore reduced in abundance compared to the original sample. Even taking this into account, it is apparent that Peak 1 (cyanidin-3-*O*-diglucoside-5-*O*-glucoside) has been greatly reduced relative to other peaks. Peaks 2 (cyanidin-3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside) and peak 9 (a mixture of cyanidin-3-*O*-(*p*-coumaroyl)diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(sinapoyl) diglucoside-5-*O*-glucoside and cyanidin-3-*O*-(feruloyl)diglucoside-5-*O*-glucoside) were noticeably reduced compared to peak 10, which remains the major peak after pancreatic digestion. Pelargonidin-3-*O*-glucoside, cyanidin-3, 5-*O*-diglucoside, cyanidin-3-*O*-sophoroside and other minor components (that could only be discerned in the original extract by searching for specific masses) could not be detected in the IN and OUT samples (results not shown). A few non-anthocyanin peaks (detectable at 280 nm) increased in relative abundance after pancreatic digestion but these candidate anthocyanin breakdown products could not be unambiguously identified (results not shown).

The combined recoveries of the individual anthocyanins after pancreatic digestion are shown in Fig. 4. There is a

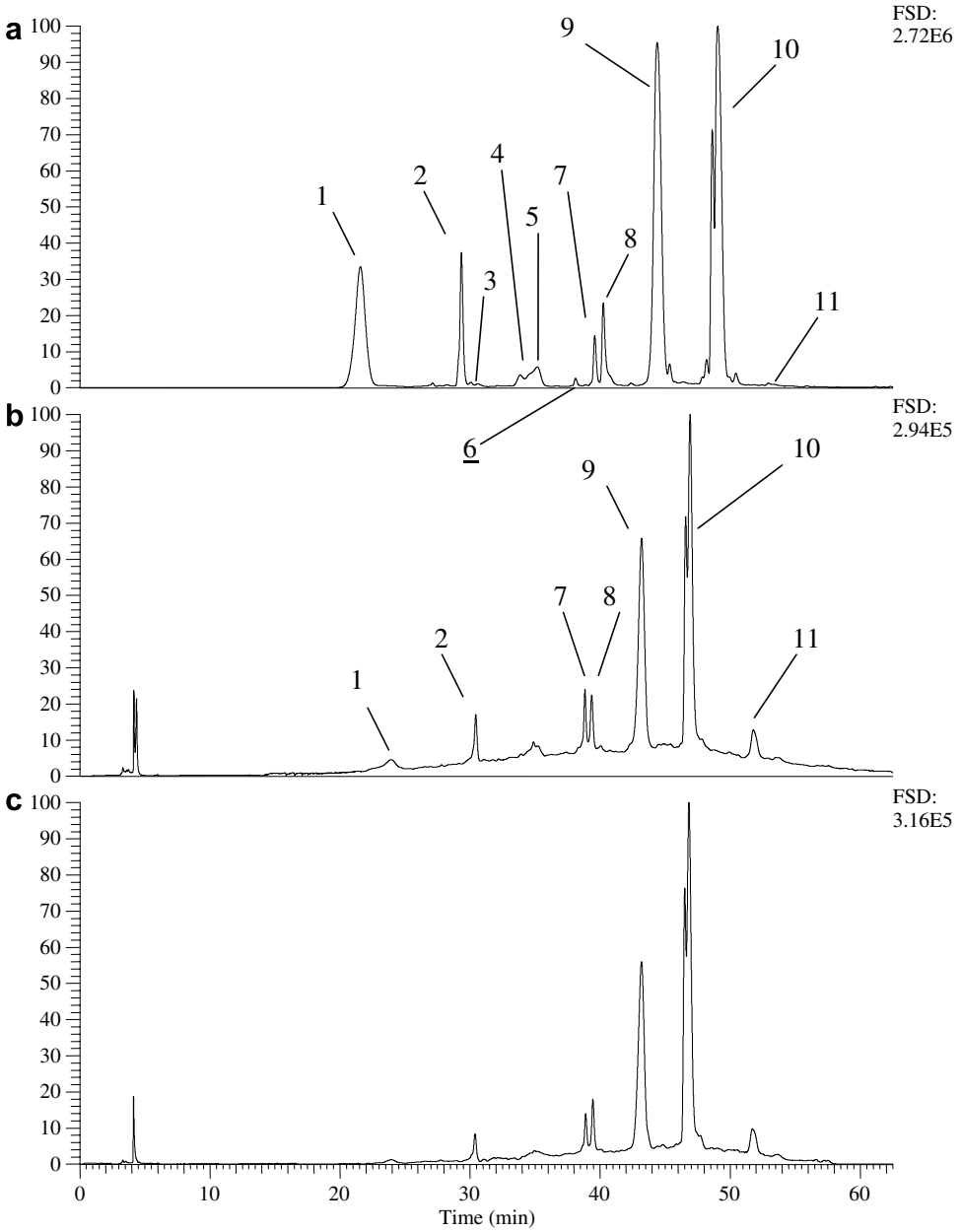


Fig. 3. HPLC traces of samples after *in vitro* digestion of red cabbage The trace in (a) shows the absorbance of peaks at 520 nm for the post-gastric sample, (b) the IN sample and (c) the OUT sample. Arrows in (a) and (b) denote peaks discussed in the text. Not all peaks are labeled. The full scale deflection (FSD) is given for each trace.

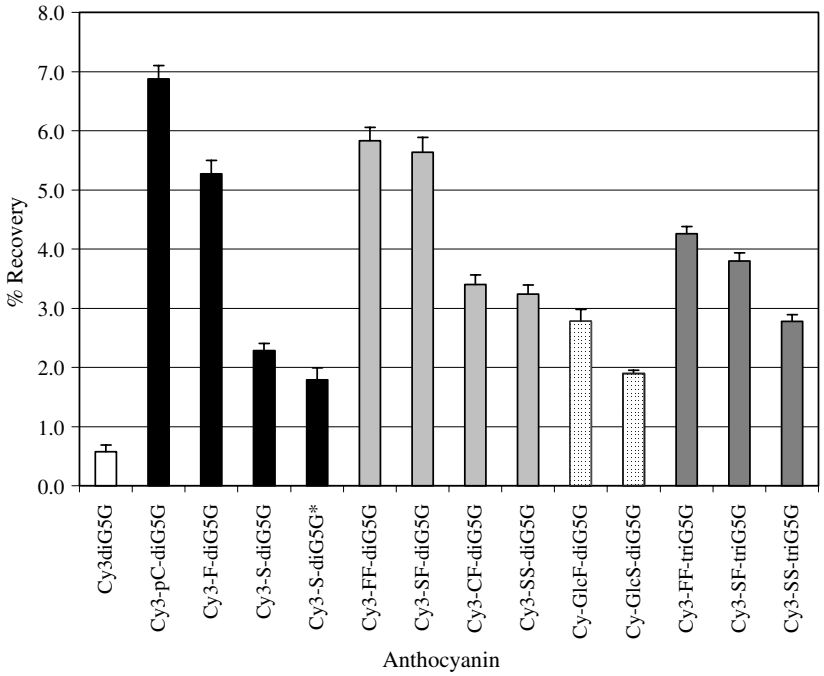


Fig. 4. Recovery of anthocyanins after simulated pancreatic digestion. The recovery of anthocyanins is calculated compared to the amount of each anthocyanin present in the post-gastric sample. The amount present in the IN and OUT samples was combined to give the pancreatic recovery. The values shown are averages values obtained from three replicate injections of samples obtained from three replicate experiments ± standard error.

notable difference between the low recovery of the non-acylated anthocyanin (e.g. cyanidin-3-*O*-diglucoside-5-*O*-glucoside) and the survival of the anthocyanins acylated with hydroxycinnamic acids. This confirms previous reports of the enhanced pH stability of acylated forms (Maulien-Aubert et al., 2001; Stintzing et al., 2002). The type of acylation may also influence pancreatic stability. Within the mono-acylated forms, cyanidin-3-*O*-diglucoside-5-*O*-glucoside (Cy3diG5G) mono-acylated with *p*-coumaric acid (Cy3-*p*C-diG5G) was more stable than Cy3diG5G mono-acylated with ferulic or sinapic acids (i.e. Cy3-*p*C-diG5G > Cy3-F-diG5G > Cy3-S-diG5G or the isomeric form Cy3-S-diG5G*). Within the di-acylated forms of Cy3diG5G, the order of stability was Cy3-FF-diG5G ⩾ Cy3-SF-diG5G > Cy3-CF-diG5G ⩾ Cy3-SS-diG5G. Within the di-acylated forms of Cy3triG5G, the order was Cy3-FF-triG5G > Cy3-SF-triG5G > Cy3-SS-triG5G. Also, cyanidin-3-*O*-(glucopyranosyl-feruloyl)-diglucoside-5-*O*-glucoside was more stable than cyanidin-3-*O*-(glucopyranosyl-sinapoyl)-diglucoside-5-*O*-glucoside. Therefore, acylation with sinapic acid generally reduced stability compared to acylation with other hydroxycinnamic acids. Di-acylation did not reduce stability compared to the equivalent mono-acylated anthocyanin (e.g. Cy3-FF-diG5G = Cy3-F-diG5G) and in certain combinations may increase stability (e.g. Cy3-SS-diG5G ⩾ Cy3-S-diG5G and Cy3-SF-diG5G > Cy3-S-diG5G).

The main stresses in the pancreatic digestion are elevated pH, temperature and the presence of oxygen. It is possible that the sinapoyl derivatives are simply less stable to these stresses. Previous work on raspberry anthocyanins suggested that the low comparative stability of cyanidin-3-*O*-glucoside may have been caused by preferential oxidation due to its higher antioxidant activity (McDougall et al., 2005a). To this end, it is interesting that sinapic acid has the highest antioxidant activity amongst the hydroxycinnamic acids (order sinapic > caffeic ≫ ferulic > *p*-coumaric acids) (Natella et al., 1999).

Red cabbage anthocyanins have been reported to have a range of biological effects; they protect neuron-like PC12 cells against amyloid beta-protein-induced toxicity (Heo and Lee, 2006), *N*-methyl-D-aspartate-induced oxidative damage in rat brain (Lee et al., 2002) and paraquat-induced oxidative stress in rats (Igarashi et al., 2000). These largely antioxidant-related bioactivities require systemic bioavailability and perhaps even access across the blood-brain barrier (Lee et al., 2002). However, our results suggest that the bulk of red cabbage anthocyanins are unstable under small intestine conditions and are unlikely to reach the serum or survive long under serum conditions. This strongly suggests that the biological activities attributed to anthocyanins may be carried out by, as yet, unidentified breakdown products.

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