British Journal of Nutrition (2004), **91**, 933–942
DOI: 10.1079/BJN20041126

Anthocyanin metabolites in human urine and serum

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(Received 26 September 2003 – Revised 2 February 2004 – Accepted 11 February 2004)

In the present study we investigated the metabolic conversion of cyanidin glycosides in human subjects using solid-phase extraction, HPLC–diode array detector, MS, GC, and enzymic techniques. Volunteers consumed approximately 20 g chokeberry extract containing 1·3 g cyanidin 3-glycosides (899 mg cyanidin 3-galactoside, 321 mg cyanidin 3-arabinoside, 51 mg cyanidin 3-xyloside and 50 mg cyanidin 3-glucoside). Blood samples were drawn at 0, 0·5, 1, and 2 h post-consumption of the extract. Urine samples were also collected at 0, 4–5, and 22–24 h. We have confirmed that human subjects have the capacity to metabolise cyanidin 3-glycosides, as we observed at least ten individual anthocyanin metabolites in the urine and serum. Average concentrations of anthocyanins and anthocyanin metabolites in the urine reached levels of 17·9 (range 14·9–20·9) μmol/l within 5 h post-consumption and persisted in 24 h urine samples at levels of 12·1 (range 11·1–13·0) nmol/l. In addition, average total levels of anthocyanins and anthocyanin metabolites detected in the serum were observed at 591·7 (range 197·3–986·1) nmol/l within 2 h post-consumption. Cyanidin 3-galactoside accounted for 55·4 % (9·9 (range 7·2–12·6) μmol/l) and 66·0 % (390·6 (range 119·4–661·9) nmol/l) of the detected anthocyanins in the urine and serum samples, respectively. The metabolites were identified as glucuronide conjugates, as well as methylated and oxidised derivatives of cyanidin 3-galactoside and cyanidin glucuronide. Conjugation probably affects the biological activity of anthocyanins and these metabolic products are likely in part responsible for the reported health benefits associated with the consumption of anthocyanins.

Flavonoids: Cyanidin 3-glycosides: Glucuronide: Methylated anthocyanins

In recent years, numerous studies have suggested that anthocyanins, present in fruit and vegetable products, are protective against many chronic degenerative diseases (Kamei *et al.* 1995; Laplaud *et al.* 1997; Andriambeloson *et al.* 1998; Trevithick & Mitton, 1999; Mazza, 2000; Parthasarathy *et al.* 2001). However, there is little reliable information on their absorption and metabolism in human subjects. Several investigators report that anthocyanins are transported in biological fluids exclusively as intact glycosides (Miyazawa *et al.* 1999; Murkovic *et al.* 2000; Mazza *et al.* 2002; Mülleder *et al.* 2002; Suda *et al.* 2002), while few have identified glucuronide or sulfide derivatives (Wu *et al.* 2002; Felgines *et al.* 2003). An adequate identification of metabolised anthocyanins must be established before the elicitation of their health effects. Since conjugation and derivatisation probably alter the bioactive properties of anthocyanins, future *in vitro* studies should be conducted using anthocyanins in their metabolised forms, as they appear in the human body.

The aim of the present study was to investigate the metabolic fate of cyanidin 3-glycosides through the identification of intact or conjugated structures in human urine and serum.

Experimental methods

Material and reagents. The chokeberry extract (no. 74190000, lot L18010) was purchased from Artemis International, Inc. (Fort Wayne, IN, USA). The β-glucuronidase (type-3), β-galactosidase (Aspergillus), β-glucosidase (from almonds) and sulfatase (aryl sulfatase) were purchased from Sigma (Oakville, ON, Canada). The anthocyanin standards, cyanidin 3-glucoside chloride, cyanidin 3-galactoside chloride (ideain chloride), malvidin 3-glucoside chloride (oenin chloride), peonidin 3-glucoside chloride, and pelargonidin 3-glucoside chloride (callistephin) were purchased from Extrasynthese (ZI Lyon Nord, Genay, France). The phenolic acid standards, syringic acid, vanillic acid, p-hydroxybenzoic acid, protocatechuic acid, caffeic acid, and gallic acid were purchased from Sigma (Oakville, ON, Canada). Ferulic acid was acquired from K&K Rare & Fine Chemicals (Costa Mesa, CA, USA) and *p*-hydroxycinnamic acid

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Abbreviations: DAD, diode array detector; E_{440}:E_{max}, absorbance intensity at 440 nm *v.* maximum absorbance intensity; m+, increased mass; MeOH, methanol; *m/z*, mass:charge ratio.

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(or *trans*-*p*-hydroxycinnamic acid) was obtained from Baker Chemical Co. (Phillipsburg, NJ, USA). The Tri-Sil Z (Pierce, Rockford, IL, USA), methanol (MeOH), chloroform (Caledon, Toronto, ON, Canada), formic acid (Fisher Scientific, Springfield, NJ, USA), oxalic acid (Baker Chemical Co., Phillipsburg, NJ, USA), amyl alcohol, HCl, and trifluoroacetic acid (DH Ltd, Toronto, ON, Canada) were all reagent grade. All solvents used for HPLC analysis were HPLC grade.

Clinical procedures

Two healthy, male volunteers, aged 27 and 54 years, participated in the chokeberry consumption trial. The subjects had an average BMI of 27·5 kg/m^2 and were absent of clinical disease as determined using a medical history questionnaire. The subjects were instructed to consume an essentially anthocyanin-free diet for 2 d before the study and to avoid taking aspirin or anti-inflammatory medications, and antioxidant or herbal supplements for 2 weeks before the investigation. The major constituents of the anthocyanin-free wash-out diet were milk, tuna, white bread, chicken, and white rice. No energy restrictions were imposed. The 2 d wash-out diet was performed before baseline sampling. After the overnight fast (12–14 h), the volunteers consumed approximately 20 g chokeberry extract (containing 1·3 g cyanidin glycosides) dissolved in 250 ml water. The extract contained four cyanidin 3-glycosides (mg): cyanidin 3-galactoside, 899; cyanidin 3-arabinoside, 321; cyanidin 3-xyloside, 51; cyanidin 3-glucuoside, 50 (as determined by HPLC–diode array detector (DAD)). The chromatogram of the chokeberry extract is given in Fig. 1 (trace B). The extract was consumed directly following a baseline blood sample (time zero). Subsequent blood samples were taken at 0·5, 1, and 2 h post-consumption of the extract. Blood samples were drawn by venepuncture from a brachial vein into 10 ml evacuated glass tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). The blood samples were allowed to clot at room temperature for 25 min. Samples were then immediately centrifuged (2500 rpm, 1000 ***g***) for 15 min at 5°C to recover the serum. Urine voids were collected in the morning of the study date (first void, time zero) along with 4–5 and 22–24 h samples. The serum and urine were stored at − 70°C upon removal or collection.

Anthocyanin extraction

Anthocyanins were extracted from biological fluids using disposable solid-phase extraction C$_{18}$ cartridges (Supelclean ENVI-18 6 ml 500 mg; Sigma, Oakville, ON, Canada). Cartridges were pre-conditioned using 7 ml acidified MeOH (0·1 % trifluoroacetic acid, pH 2·1), followed by 7 ml acidified water (10 mM-oxalic acid, pH 2·2). Unfiltered blood serum (1·5 ml) or unfiltered urine (1 ml) was acidified (6 M-HCl; 40 μl), diluted with 10 mM-oxalic acid (equal volume), vortexed, and loaded directly onto the solid-phase extraction cartridge. The sample was drained under gravity (about 1 drop/s) and washed with 2 volumes of acidified water (10 mM-oxalic acid, approximately 12 ml). The remaining anthocyanin extract was eluted with 6 ml acidified MeOH (0·1 % trifluoroacetic acid). The extract was evaporated at ambient temperature in a SpeedVac Plus-SC110A condenser (Savant Instruments Inc., Farmingdale, NY, USA) to 0·1–0·5 ml. The residual elute was brought to dryness under N$_2$ at room temperature and the residue was re-dissolved in 200 μl HPLC mobile-phase solution and filtered through a 13 mm 0·45 μm GHB Acrodisc syringe filter (lot no. A10310523; Pall-Gelman, Ann Arbor, MI, USA). Anthocyanins were quantified by comparison with a standard curve obtained using known concentrations of cyanidin 3-galactoside.

High-pressure liquid chromatography analysis

HPLC analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) using a Zorbax SB C$_{18}$ (5 μm, 4·6 × 250 mm; Agilent Technologies, Palo Alto, CA, USA) reverse-phase column with an Inertsil Spherisorb guard column (C$_{18}$ 5 μm, 4·6 × 30 mm; Phenomenex, Torrance, CA, USA). The column temperature was set at 35°C and the injector temperature at 15°C, with an injection volume of 50 μl. The mobile phase consisted of 4·5 % (v/v) formic acid in water (solvent A) and 100 % MeOH (solvent B). The flow rate was 1·0 ml/min and the solvent gradient program used as follows: 10 % B at 0 min, 25 % B at 30 min, 45 % B at 50 min, 100 % B at 55 min, maintaining 100 % for 10 min. A DAD (G1315B; Agilent Technologies, Palo Alto, CA, USA) monitored absorbance at 280, 360, and 525 nm. Peaks on the chromatogram corresponding to anthocyanins as identified by spectral analysis (peaks detected at 525 nm with λ_{max} 250–300, 500–550) were collected from the analytical column (Foxy 200 X-Y fraction collector; ISCO, Inc., Lincoln, NE, USA) and concentrated at ambient temperature in a vacuum condenser (SpeedVac; Savant, Farmingdale, NY, USA) for further analysis. All water used for HPLC was 18 MΩ × cm Milli-Q water and degassed using an inline Agilent 1100 micro vacuum degasser (Agilent Technologies, Palo Alto, CA, USA).

Mass spectrometry analysis

MS identification of individual compounds was conducted post-separation via HPLC (as described above). Individual peaks were collected on a Foxy 200 X-Y fraction collector (ISCO, Inc., Lincoln, NE, USA), concentrated, and analysed by electrospray ionisation–MS. A Micromass ZQ single quadrupole mass spectrometer with electrospray interface and MassLynx 3.5 software (Micromass UK Ltd, Manchester, UK) was used for data acquisition. The mass spectrometer parameters settings were: ionisation mode, electrospray positive ion mode; capillary voltage, 3·25 kV; source temperature, 130°C; desolvation temperature, 280°C; nebuliser N$_2$ flow rate, 95 litres/h; desolvation N$_2$ gas flow rate, 610 litres/h; LM resolution, 15; HM resolution, 15; ion energy, 0·8 V; multiplier voltage, 650 V; cone voltage, 20 V; RF lens, 0·5 V; extractor, 6 V. The flow rate of a built-in syringe pump was set at 20 μl/min. For the flow injection, samples (HPLC fractions) and chokeberry extracts (2·4 mg) were

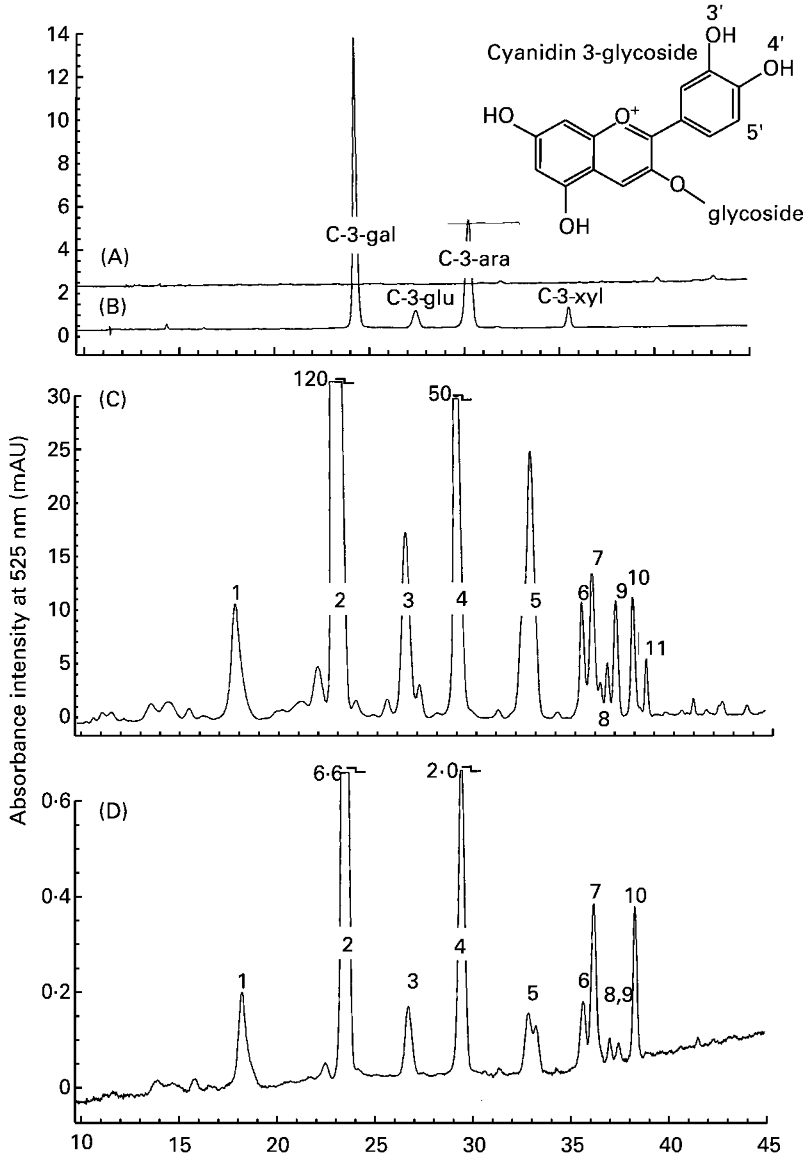

Fig. 1. Anthocyanins in chokeberry extract, and human urine and serum post-consumption of extract. (A), Chromatogram of baseline urine sample; (B), chromatogram of chokeberry extract; (C), chromatogram of 5 h urine sample; (D), chromatogram of 2 h serum sample; C-3-gal, cyanidin 3-galactoside; C-3-glu, cyanidin 3-glucoside; C-3-ara, cyanidin 3-arabinoside; C-3-xyl, cyanidin 3-xyloside. HPLC analysis was as outlined on p. 934. Identification of chokeberry anthocyanins was based on that of known standards. When standards were not available identification was based on retention times and spectral data from the literature (Chandra *et al.* 2001). Urine and serum concentrations of each compound represented by peaks 1 to 11 are given in Tables 1 and 2, respectively. Urine and serum concentrations of anthocyanins were determined using UV-visible HPLC and quantified in relation to known concentrations of a C-3-gal standard.

re-dissolved in 2 ml acetonitrile–water (50:50) containing 0·1 % formic acid. Spectra were recorded by scanning a mass range from mass:charge ratio (*m*/*z*) 100 to 1000 with a scan time of 1 s, an inter-scan time of 0·02 s, and a run duration of 0·5 min.

Enzymic hydrolysis

The dried anthocyanin extract obtained using the solid-phase extraction procedure outlined earlier (p. 934) was subjected to enzymic hydrolysis using β-galactosidase, β-glucuronidase, β-glucosidase, and sulfatase. The dried extract was mixed with 0·1 M-sodium acetate buffer (1 ml) containing activated enzyme. The four individual enzyme buffer solutions (700 U β-galactosidase, 500 U β-glucuronidase, 500 U β-glucosidase, 75 U sulfatase) were created by adding 1 ml pre-incubated (37°C) sodium acetate buffer (pH 3·8) to the pre-weighed enzymes. The enzyme buffer solutions were then vortexed and added to the dried anthocyanin extract. The mixtures were further vortexed and incubated at 37°C for 1 h. The anthocyanin enzyme extracts were then diluted with 1 ml acidified MeOH (4·5 % formic acid in MeOH, pH 2·1) and centrifuged for 10 min at 14 000 rpm. The supernatant fractions were removed, evaporated under N_2 and re-dissolved in 150 μl mobile phase (as outlined earlier;

p. 934). If precipitate persisted, the sample was re-centrifuged (10 min at 14 000 rpm) before filtration. The enzymically hydrolysed aglycone-rich extracts were injected into the HPLC column and analysed using the HPLC methodology outlined earlier (p. 934).

Gas chromatography analysis, acid hydrolysis and derivatisation of sugars

Chemical characterisation of the glycosylating compounds was conducted using capillary GLC post-acid hydrolysis. This was accomplished using methods as published by Gao & Mazza (1994), with slight modifications. Samples were silyated after concentration by adding 50 μl Tri-Sil Z, and incubated at 65°C for 1 h. GC analysis of the derivatised sugars was conducted on a Hewlett Packard 5890A gas chromatograph with a flame ionisation detector (Hewlett Packard (now Agilent Technologies), Palo Alto, CA, USA) using a fused silica capillary column (J&W DB-1701, 30 m × 0·32 mm × 1 μm; J&W Scientific Inc., Folsom, CA, USA). The injector and detector temperatures were 250°C. The carrier gas was He at a head pressure of 80 kPa, flow of 1·8 ml/min, and a linear velocity of 350 mm/s. The sample (1 μl) was injected into the column via a split/splitless injector in split mode using a split ratio of 11:1. The initial column temperature was increased from 120 to 180°C at a rate of 20°C/min, then increased from 180 to 200°C at a rate of 5°C/min and held for 8 min.

Results

The consumption of chokeberry extract containing four cyanidin glycosides (899 mg cyanidin 3-galactoside, 321 mg cyanidin 3-arabinoside, 51 mg cyanidin 3-xyloside, and 50 mg cyanidin 3-glucuoside) resulted in the appearance of at least ten anthocyanin metabolites (as separated by reverse-phase HPLC–DAD) in the human serum and urine (Figs. 1 and 2). Concentrations of individual anthocyanins in the urine and serum extracts are given in Tables 1 and 2 respectively. HPLC–DAD analysis of anthocyanin standards together with their molecular

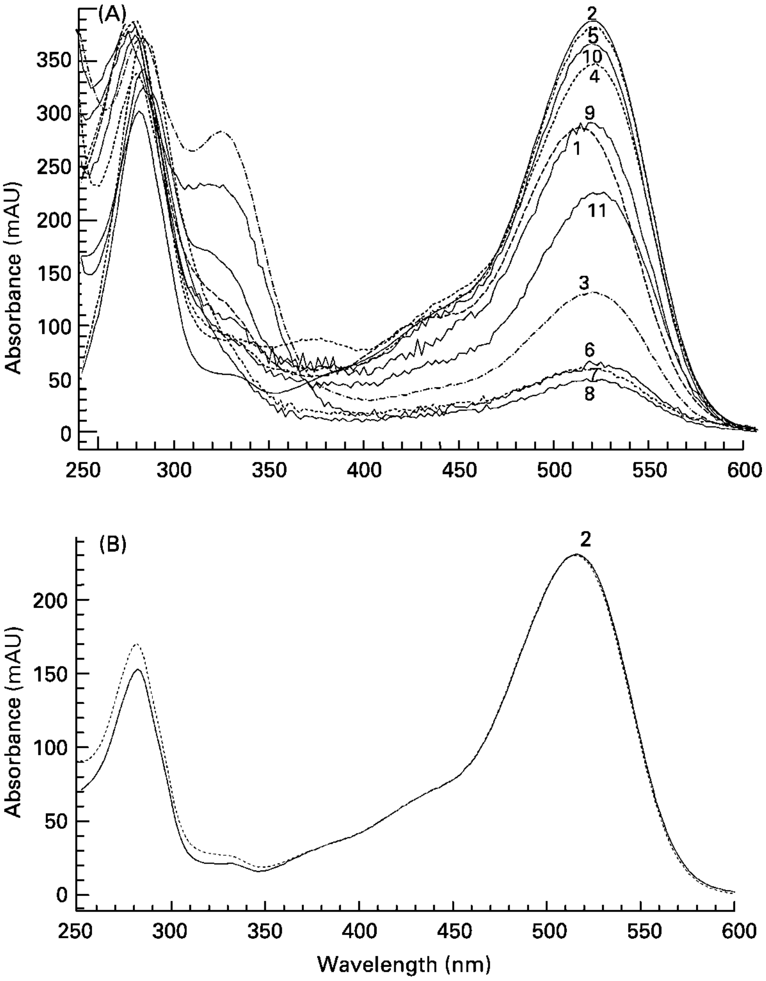

Fig. 2. UV-visible absorption spectra of anthocyanins in human urine and serum samples post-consumption of chokeberry extract monitored at 525 nm by HPLC–diode array detector (DAD). (A), Typical UV-visible absorption spectra of individual peaks in human urine and serum samples post-consumption of the extract; (B), UV-visible absorption spectra of peak 2 (—) and cyanidin 3-galactoside standard (- - -). HPLC chromatogram of urine and serum samples are given in Fig. 1. Urine and serum concentrations of each compound represented by peaks 1 to 11 are given in Tables 1 and 2, respectively. Urine and serum concentrations of anthocyanins were determined using HPLC–DAD and quantified in relation to known concentrations of a cyanidin 3-galactoside standard.

Table 1. Identification and concentration of anthocyanins and anthocyanin metabolites in human urine

Peak	Rt* (min)	λ_{max}* (nm)	E_{440}:E_{max}* (as %)	*m/z*† (total/aglycone)	Conjugation and derivatization‡	Concentration (μmol/l)§ Subject 1	Subject 2	Average
1	18·1	510	39	449	Unknown	0·5	0·7	0·6
2	23·4	515	31	449	Intact C-3-gal	12·6	7·2	9·9
3	26·5	516	24	463	Gluc	0·7	0·9	0·8
4	29·3	517	37	463/301	CH_3	3·3	2·2	2·7
5	32·9	515	36	463/301	CH_3	2·1	1·6	1·8
6	35·5	516	33	447	Gluc + CH_3	0·2	0·5	0·3
7	36·0	514	40	447	Gluc + CH_3	0·1	0·7	0·4
8	36·8	516	38	Unknown	Unknown	0·3	0·3	0·3
9	37·7	517	30	447	2 × CH_3	0·6	0·4	0·5
10	38·5	520	30	491	Gluc + 2 × CH_3	0·5	0·4	0·4
11	39·0	518	31	493/331	2 × CH_3 + OH	0·1	0·1	0·1
Total						20·9	14·9	17·9

Rt, retention time; E_{440}:E_{max}, absorbance intensity at 440 nm *v.* maximum absorbance intensity; *m/z*, mass:charge; C-3-gal, cyanidin 3-galactoside; Gluc, glucuronide acid.

* Rt and spectral data were obtained by HPLC as outlined on p. 934. For an HPLC chromatographic plot of individual peaks, see Fig. 1.

† Data were obtained by MS. Anthocyanins were ionized in their molecular cation forms under MS positive ion mode (as outlined on p. 934).

‡ Identification was based on relative polarity (Rt), MS, and spectral data (as outlined on p. 934) using standards of known composition when available. For spectral characteristics of anthocyanin standards, see Table 3.

§ Concentrations (μmol/l) 5 h following consumption of two subjects determined using UV-visible HPLC and quantified in relation to known concentrations of a C-3-gal standard. No anthocyanins were detected in baseline samples.

Table 2. Concentrations of identifiable anthocyanins in human serum*

		Subject						Total/peak		
		1	2	1	2	1	2	1	2	
Peak†	Rt‡ (min)	t = 0·5 h	t = 0·5 h	t = 1 h	t = 1 h	t = 2 h	t = 2 h	t = 0·5–2 h	t = 0·5–2 h	Average total (*n* 2) t = 0·5–2 h
1	18·1	0·0	0·0	0·0	4·0	5·5	9·3	5·5	13·3	9·4
2	23·4	6·5	66·4	14·9	228·5	98·0	367·0	119·4	661·9	390·6
3	26·5	1·4	5·3	3·0	13·4	7·1	18·6	11·5	37·3	24·4
4	29·3	2·2	23·4	4·7	72·9	29·3	108·5	36·2	204·8	120·5
5	32·9	0·0	0·0	0·9	9·7	2·4	19·6	3·3	29·3	16·3
6	35·5	0·9	0·0	1·8	0·0	2·0	0·0	4·6	0·0	2·3
7	36·0	1·5	0·0	4·2	0·0	3·2	2·3	8·9	2·3	5·6
8	36·8	0·0	0·0	0·0	0·0	1·0	2·8	1·0	2·8	1·9
9	37·7	0·0	0·0	0·0	0·0	1·1	2·6	1·1	2·6	1·8
10	38·5	0·0	4·0	1·1	11·1	4·7	16·6	5·8	31·8	18·8
Total		12·5	99·2	30·6	339·6	154·3	547·3	197·3	986·1	591·7
Average/h		55·8		185·1		350·8				

* Concentrations (nmol/l) of two subjects were determined using UV-visible HPLC and quantified in relation to known concentrations of a cyanidin 3-galactoside standard. For an HPLC chromatographic plot of individual peaks, see Fig. 1; for MS and HPLC identification of individual peaks, see Table 1. No anthocyanins were detected in baseline samples.

† Individual peaks were identified as anthocyanins by analysis of UV-visible spectra having maxima in the 250–300 and 500–550 nm range.

‡ Retention times were obtained by HPLC as outlined on p. 934.

weights are given in Table 3. The UV-visible spectral analysis of all potential metabolites is given in Fig. 2. Initial attempts to fully characterise anthocyanin metabolites in the urine by enzymic and chromatographic methods were unsuccessful and results of both enzymic hydrolysis (detection of aglycone via HPLC–DAD) and acid hydrolysis (detection of derivatised sugars and glucuronic acid via capillary GC) experiments were deemed inconclusive. MS was necessary for the adequate identification of urinary metabolites. Post-MS analysis, the identification of anthocyanins (Table 1) was based on the matching of molecular weights for parent (anthocyanin) and daughter (anthocyanidin or aglycone; when obtainable) fragments, along with HPLC–DAD data (retention time, $\lambda_{max\ (vis)}$ and absorbance intensity at 440 nm *v.* maximum absorbance intensity (E_{440}:E_{max})) with that of available standards (Table 3). Identification was based on metabolites isolated from pooled urine samples. The concentration of unidentified metabolites is based on cyanidin 3-galactoside molar equivalents. The results indicated that cyanidin 3-galactoside was the primary anthocyanin in the chokeberry extract accounting for 68·0 % of its total anthocyanins. Cyanidin 3-galactoside was also the primary anthocyanin identified in the urine and serum samples accounting for 55·3 % (9·9 (range 7·2–12·6) μmol/l) and 66·0 % (390·6 (range 119·4–661·9) μmol/l) of the identified anthocyanins, respectively. No anthocyanins were detected in the baseline serum or urine samples. The results indicated the presence of both cyanidin 3-galactoside (*m/z* 449; Fig. 1, peak 2) and cyanidin glucuronide (*m/z* 463; Fig. 1, peak 3). The results also indicated the presence of mono- and dimethylated cyanidin galactosides (increased mass

Table 3. Ultraviolet-visible high-pressure liquid chromatography characteristics of anthocyanin standards

Peak	Rt* (min)	Absorption spectra		Molecular weight (*m/z*)	
		$\lambda_{max\ (vis)}$ (nm)	E_{440}:E_{max} (as %)	Glycoside	Aglycone
Cyanidin-3-galactoside	24·1	518	31·7	449	287
Cyanidin-3-glucoside	26·6	517	32·5	449	287
Cyanidin-3-arabinoside†	29·2	517	30·7	419	287
Pelargonidin-3-glucoside	32·3	502	44·0	433	271
Peonidin-3-glucoside	36·0	518	32·6	463	301
Malvidin-3-glucoside	38·5	528	27·5	493	331
Cyanidin-3-xyloside†	38·9	517	43·6	419	287
Cyanidin	43·0	526	23·5		287

Rt, retention time; E_{440}:E_{max}, absorbance intensity at 440 nm *v.* maximum absorbance intensity; *m/z*, mass:charge ratio.

* Rt and spectral data were obtained by HPLC (as outlined on p. 934) using standards of known composition when available.

† Identification of cyanidin 3-arabinoside and cyanidin 3-xyloside in the chokeberry extract was based on retention times and spectral data (as standards were not available) and was confirmed in the literature (Chandra *et al.* 2001).

(m+)14 and m + 28; Fig. 1, peaks 4 and 5 respectively) and glucuronides (m + 14; Fig. 1, peaks 6 and 7 and m + 28; Fig. 1, peak 10). Oxidative modification was also indicated in one peak (*m/z* 493/331; Fig. 1, peak 11) by an increased mass (m + 16) above the di-methyl derivative of cyanidin 3-galactoside (represented by *m/z* 477, Fig. 1, peak 9). A proposed pathway for these compounds is given in Fig. 3.

The HPLC quantitative analysis of the individual anthocyanin peaks in the urine revealed that cyanidin 3-galactoside and derivatives of cyanidin 3-galactoside accounted for an average of 84·0 % (15·0 (range 11·5–18·7) μmol/l) of the identified metabolites. Cyanidin glucuronide and glucuronide derivatives accounted for 10·6 % (1·9 (range 1·5–2·4) μmol/l), with the remaining 5·0 % or 0·9 (range 0·8–1·0) μmol/l uncharacterised (Fig. 1, traces C and D, peaks 1 and 8). In addition to the urinary analysis of metabolites, comparisons were made between urinary and serum metabolites using HPLC–DAD data (Figs. 1 and 2, and Tables 1 and 2).

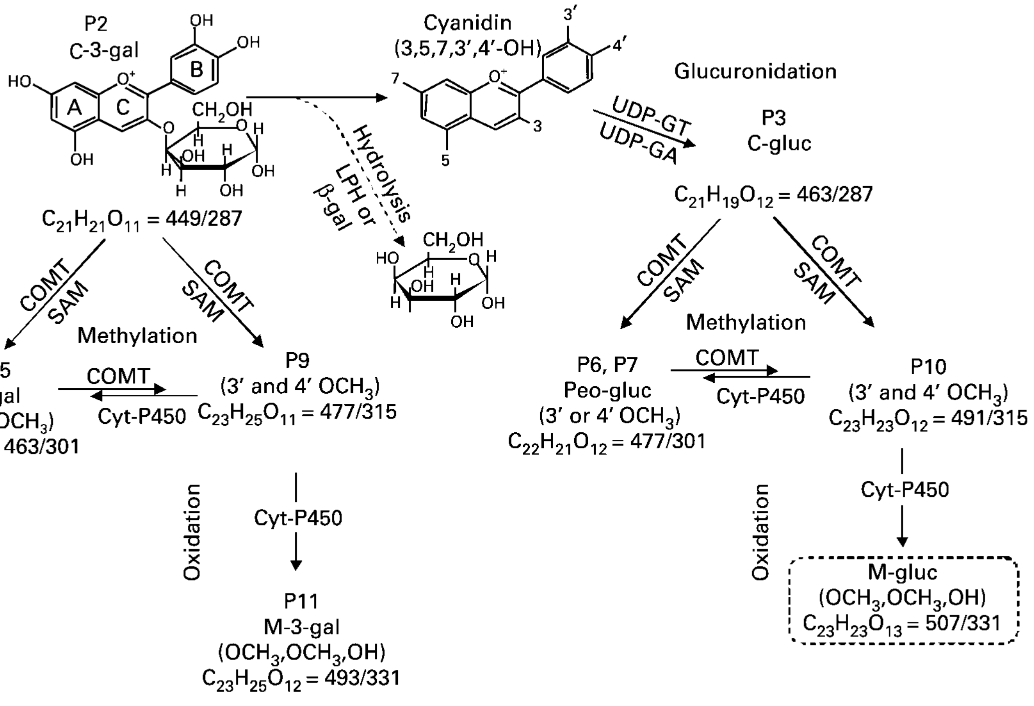

Fig. 3. Proposed pathway for the formation of anthocyanin metabolites in human urine and serum post-consumption of cyanidin 3-glycosides. P, peak; C-3-gal, cyanidin 3-galactoside; C-gluc, cyanidin glucuronide; M-3-gal, malvidin 3-galactoside; M-gluc, malvidin glucuronide; Peo-3-gal, peonidin 3-galactoside; Peo-gluc, peonidin glucuronide; COMT, catechol-*O*-methyltransferase; SAM, S-adenosyl methionine; LPH, lactase–phlorizin hydrolase; β-gal, β-galactosidase; UDP-GT, uridine diphosphate glucuronosyltransferase; UDP-GA, UDP-glucuronic acid; Cyt-P450, cyotchrome P450. Molecular weights were obtained by MS (as outlined on p. 934). Identification was based on relative polarity (retention time), MS, and UV-visible data (as outlined on p. 934 and Table 1) and compared with those of known standards (Table 3). For an HPLC chromatographic plot of individual peaks, see Fig. 1. The circled metabolite is proposed but not identified. P1 and P8 are not illustrated as there were insufficient data available to elicit a potential structure. P9 and P10 have not been named as no anthocyanins of comparable structure have been reported to date.

The results indicated that the serum peaks matched (both retention times and UV-visible spectra) the peaks identified in the urine with the exception of one compound (peak 11) that was observed in the urine but did not appear in the serum (Fig. 1, traces C and D). Cyanidin 3-galactoside and derivatives of cyanidin galactoside identified in the serum accounted for 89·4 % (529·3 (range 160·0–898·6) nmol/l) of the metabolites. Glucuronide and glucuronide derivatives accounted for 8·6 % (51·1 (range 30·8–71·4) nmol/l), with the remaining 1·9 % or 11·3 (range 6·5–16·1) nmol/l uncharacterised (Fig. 1, traces C and D, peaks 1 and 8).

Discussion

The purpose of the present study was to identify potential metabolites of cyanidin 3-glycosides in human urine and serum. To date there is little reliable information on the absorption and metabolism of anthocyanins in human subjects, and the studies available have reported contradictory results. The results of the present investigation indicate that a large proportion of anthocyanins consumed are metabolised before entry into the circulation and their metabolites will probably be responsible for many of the reported health effects associated with anthocyanin consumption.

The chokeberry extract utilised in the present trial was chosen because it contained only one anthocyanidin species, cyanidin. Most other fruit extracts contain two or more anthocyanidins making it difficult to identify the origin of any one metabolic by-product. Unfortunately, the highly concentrated chokeberry extract contained a high percentage of phenolics and polyphenolics. As a result, it was very astringent and its palatability (when dispersed in water) was low. Consequently, incomplete subject compliance made it difficult to establish the exact concentration of the supplement consumed by the two subjects. For this reason, no attempt has been made to establish the percentage of anthocyanins appearing in the urine and serum relative to the initial dose (bioavailability). Future studies will establish bioavailability using an encapsulated extract or a product more similar in composition to the intact fruit. The aim of the present study was simply to consume a high-enough concentration of cyanidin glycosides to identify their possible metabolites in biological fluids and to elicit a potential metabolic pathway for anthocyanin biotransformation in human subjects. It is important to note that the structural derivatives of anthocyanins proposed in the present study result from the consumption of a high dose of anthocyanins (1·3 g). The metabolic route under these circumstances may differ from the route following the ingestion of a more ‘typical’ dose of anthocyanins, as would be encountered with high fruit and vegetable consumption or with moderate to high wine consumption.

The consumption of four cyanidin glycosides (cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-xyloside, and cyanidin 3-glucoside) resulted in the appearance of at least ten individual anthocyanin metabolites in the human urine and serum (Figs. 1 and 2). To our knowledge, no other study has identified this number of anthocyanin metabolites in the urine or serum to date. Findings from studies using similar concentrations of total anthocyanins but reporting fewer metabolites are probably the result of individual metabolite concentrations being below the detection limit of the methodologies used. In the present study, only cyanidin was consumed, with the majority (68 %) attributed to one cyanidin glycoside (cyanidin 3-galactoside), therefore, resulting in fewer numbers and higher concentrations of individual metabolites. In the present investigation, the total concentration of identifiable anthocyanins in the urine reached an average of 17·9 (range 14·9–20·9) μmol/l within 5 h post-consumption of the chokeberry extract (Table 1). Urine samples (22–24 h) showed cyanidin 3-galactoside and metabolised derivatives of cyanidin 3-galactoside to persist in the urine at levels of 0·011–0·013 nmol/l. The identification of anthocyanin metabolites in 24 h urine samples has also recently been noted by Felgines *et al.* (2003), and may signify the potential for minor tissue accumulation. Additionally, the concentrations of identifiable anthocyanins and anthocyanin metabolites in the serum (2 h sample) were observed at a level of 350·8 (range 154·3–547·3) nmol/l within 2 h post-consumption, with a cumulative total (0–2 h) serum concentration reaching 591·7 (range 197·3–986·1) nmol/l over the 2 h sampling period (Table 2).

Magnification of the chromatograms revealed the appearance of many small peaks that were at concentrations too low to adequately identify but were well above any baseline noise. Adequate structural identification was not possible at this concentration but many peaks had spectral characteristics representative of anthocyanins ($\lambda_{max\ (vis)}$ in the 500 nm range). Analysis of the total peak areas at 525 nm (all integratable peaks with $\lambda_{max\ (vis)} > 500$ nm not observed in baseline samples) revealed that the urine total concentration of anthocyanins and/or ‘anthocyanin-like’ compounds reached levels as high as 22·7 μmol/l within 5 h post-consumption. The total (0–2 h) serum levels reached 997·5 nmol/l within 2 h post-consumption of the extract. (The concentration of unknown compounds was based on cyanidin 3-galactoside molar equivalents.) These results indicate that the body may have the capacity to transform anthocyanins into numerous metabolites, many of which probably go undetected as a result of their substantial numbers and subsequently low concentrations.

Efforts to identify the main anthocyanin metabolites through enzymic hydrolysis experiments were unsuccessful, as the enzymes β-galactosidase, β-glucuronidase, and β-glucosidase had affinities for all anthocyanin metabolite peaks to varying extents. This led us to suspect the presence of both glycoside and glucuronide derivatives of cyanidin in our samples. GC analysis, post-acid hydrolysis and derivatisation of sugars were also utilised to identify the possible glycosylating structures (galactose, glucose, arabinose, xylose, glucuronic acid). These results suggested that both glycosides and glucuronides were present. However, as a result of the low concentrations and large numbers of derivatised sugars produced, adequate chromatographic identification was not possible. Further analysis using electrospray ionisation–MS was necessary for sufficient identification of the potential metabolites.

Identification of urinary metabolites (Table 1) was based on the matching of molecular weights for parent and daughter fragments, along with the retention time, $\lambda_{max\ (vis)}$ and

E_{440}:E_{max}, to that of available standards (Table 3 and Fig. 2). The data obtained indicated the presence of both cyanidin 3-galactosides (*m/z* 449; Fig. 1, peak 2) and cyanidin glucuronides (*m/z* 463; Fig. 1, peak 3). There are little data regarding the identification of anthocyanin glucuronides to date (Wu *et al.* 2002; Felgines *et al.* 2003). However, the urinary excretion of other flavonoid and isoflavonoid glucuronides such as catechin, quercetin, and genisten has been well documented (Wermeille *et al.* 1983; Hollman & Katan, 1998; Piskula & Terao, 1998; Holder *et al.* 1999; Okushio *et al.* 1999; Walle *et al.* 2000; Williamson *et al.* 2000; Oliveira *et al.* 2002).

Mono- and dimethylated cyanidin 3-galactoside derivatives (m + 14, peaks 4 and 5, and m + 28, peak 9; Fig. 1) and glucuronide derivatives (m + 14, peaks 6 and 7, and m + 28, peak 10; Fig. 1) were also indicated in the present investigation (Table 1). Monomethylated derivatives had similar retention times and $E_{440}:E_{max}$ to the peonidin 3-glucoside standard. Only a few researchers have reported methylated anthocyanins in the urine and blood of human subjects and animals (Miyazawa *et al.* 1999; Tsuda *et al.* 1999). However, methylated derivatives of quercetin and catechin have been documented extensively (Harborne, 1958; Wermeille *et al.* 1983; Hollman & Katan, 1998; Miyazawa *et al.* 1999; Okushio *et al.* 1999; Day & Williamson, 2001; Donovan *et al.* 2001). In addition, oxidative modification was also indicated in one metabolite (peak 11; *m/z* 493) by an increased mass (m + 16) above the di-methyl derivative of cyanidin 3-galactoside (represented by *m/z* 477, peak 9; Fig. 1). This structure (*m/z* 493/331) matched the molecular weights for parent and daughter fragments of malvidin 3-galactoside, had a similar retention time to that of the malvidin 3-glucoside standard but differed in spectral characteristics (Tables 1 and 3). No other oxidised derivatives of anthocyanins have been previously identified to our knowledge. Comparisons were made between anthocyanin metabolites in the serum and those in the urine; unfortunately, as a result of low concentrations of individual anthocyanins in the serum, the collection and concentration of a sufficient quantity of sample was not possible. Consequently, MS analysis using the above methods (p. 934) could not be utilised. However, retention times and UV-visible spectra were obtainable and the data indicated that the serum peaks matched the peaks identified in the urine (Table 2 and Fig. 1). When comparisons were made between urinary and serum metabolites, it was apparent that one anthocyanin metabolite (peak 11) occurred in the urine but not in the serum (Fig. 1, traces C and D). The identification of an anthocyanin metabolite exclusively in the urine may indicate that this metabolic product is either formed exclusively in the kidney, accumulates in the kidney, or the concentration of this metabolite in the serum may have been below the detection limit of our methodology. MS analysis (of the urinary metabolite) revealed this compound (peak 11) to be a highly metabolised cyanidin 3-galactoside (indicated by two methylations and one hydroxylation) having the same mass and daughter fragment as malvidin 3-galactoside (*m/z* 449/331). Tsuda *et al.* (1999) have previously reported the methylation of cyanidin 3-glucoside at both the 3′ and 4′ positions in the liver of rats. Also, the addition of hydroxyl groups to flavones has been characterised in animal models, where these metabolites were identified in the urine (Buset & Scheline, 1980; Hollman & Katan, 1998). In theory, dimethylation of the B-ring, resulting in reduced polarity, could merit further phase I cytochrome P450 oxidation, as observed with other flavonoids (Griffiths, 1982), in an attempt to increase the water solubility of the structure for elimination in the urine. This may explain the appearance of this compound in the urine while not in the serum. The parent aglycone cyanidin was not identified in any urine or serum samples. This result is consistent with other studies on anthocyanin metabolism as anthocyanin aglycones are generally regarded as unstable at physiological pH (Tsuda *et al.* 1999; Wu *et al.* 2002; Felgines *et al.* 2003).

Compounds having identical molecular weights but different retention times and absorption spectra were observed and are probably the result of methylation of the ortho hydroxyls of the cyanidin B-ring (i.e. 3′ *v.* 4′ methylation, peaks 4 and 5, 6 and 7; Fig. 1). Methylation at different sites of the B-ring may cause a slight shift in retention time, producing two distinct peaks on an HPLC chromatogram. It should be noted that the pathway we have proposed, in Fig. 3, only accounts for the derivatisation of the B-ring, which has been documented as a probable site for methylation and oxidation of flavonoids (Griffiths, 1982; Hollman & Katan, 1998; Tsuda *et al.* 1999; Doostdar *et al.* 2000). However, methylation and hydroxylation at other sites of cyanidin may be possible and cannot be excluded using the methodologies used in the present study. In addition, the sites of anthocyanin glucuronide conjugation in man are unknown. The glucuronidation of quercetin, a flavonoid of similar structure to anthocyanins, has been documented at the 4′, 3′, 7, and 3 positions of the polyphenol ring (Day *et al.* 2000). The absence of available standards for anthocyanin metabolites, along with the problems associated with compound identification when sample impurities exist, prevents the undeniable identification of the metabolites. NMR is required to elicit the actual positioning of the glucuronide, hydroxyl, or methyl subgroups.

Total urinary analysis of identified peaks revealed that cyanidin galactosides (cyanidin 3-galactoside and derivatives of cyanidin 3-galactoside) accounted for 84·0 % (15·0 (range 11·5–18·7) μmol/l) of the identified anthocyanins. Of these, 55·3 % (9·9 (range 7·2–12·6) μmol/l) was the parent compound cyanidin 3-galactoside (10·6 % glucuronides; 1·9 (range 1·5–2·4) μmol/l; Table 1). Accordingly, cyanidin galactosides accounted for 89·4 % (529·3 (range 160·0–898·6) nmol/l) of the anthocyanins (8·6 % or 51·1 (range 30·8–71·4) nmol glucuronides/l; Table 1) in the serum, with 66·0 % (390·6 (range 119·4–661·9) nmol/l) being the parent compound cyanidin 3-galactoside. The high percentage of anthocyanin glycosides in relation to glucuronides as observed in the present trial has also been reported by Wu *et al.* (2002) (90 % anthocyanin glycosides) but is not consistent with other flavonoid studies found in the literature. Most studies of flavonoid glycoside consumption indicate that the major metabolites in the urine are the glucuronide derivatives of the parent compound (Piskula & Terao, 1998; Holder *et al.* 1999; Kuhnle *et al.* 2000; Donovan *et al.* 2001; Oliveira *et al.* 2002; Felgines *et al.* 2003). Felgines *et al.* (2003) recently reported that approximately 80 % of excreted anthocyanins were monoglucuronides. The high percentage of glycoside observed in the present trial may be the result of the consumption of a high dose of anthocyanins and may not occur under more normal physiological concentrations.

Total analysis of identified peaks revealed that methyl derivatives of cyanidin galactosides and glucuronides accounted for 37 and 33 % of the identified metabolites in the urine and serum, respectively. Although a high percentage of metabolites were methylated in the present investigation, the level of methylation, as well as oxidation, observed may have been, as suggested earlier, the result of the high dose of anthocyanins consumed.

We found no evidence of sulfation in the present study; however, studies indicate this pathway can be easily saturated (Dutton, 1980; Laitinen & Watkins, 1986; Williamson *et al.* 2000; Oliveira *et al.* 2002). As the sulfation pathway is substrate limited, it is therefore possible that this pathway was overwhelmed by the high concentration of anthocyanin glycosides in the present investigation, and any sulfate derivatives appearing in the urine or blood may have been at a concentration below the detection limit of our methodology. It is also possible that metabolites of lower concentration had similar retention times (overlapping peaks on the chromatogram) to those of more concentrated metabolites and were not identified and collected. This illustration could also be used to explain why no arabinosides, glucosides, or xylosides were identified in the urine and serum. Alternatively, the route of absorption for anthocyanin glycosides may have specificity towards galactosides over, arabinosides or xylosides (Williamson *et al.* 2000).

Researchers have reported that many flavonoid glycosides are cleaved before entry into intestinal cells where they are either transported to the liver as aglycones, or metabolised to glucuronide and/or sulfide conjugates before transport (Hollman & Katan, 1998; Spencer *et al.* 1999; Kuhnle *et al.* 2000; Williamson *et al.* 2000; Donovan *et al.* 2001; Oliveira *et al.* 2002). In the present investigation, both glycoside and glucuronides were identified in the urine and serum. This evidence leads us to believe that there may be more than one route of absorption. The combination of the present results and others in the literature seems to indicate that a dose-dependent or saturatable metabolic pathway may predominate (Mizuma *et al.* 1994; Hollman *et al.* 1995; Wolffram *et al.* 1995). This could explain the inconsistency in findings reported between studies using varying doses of anthocyanins and flavonoids.

The metabolism of anthocyanins (particularly by colonic microflora) may also result in the formation of phenolic acids, phenolic acid residues, H, or CO_2 (Rozman, 1986; Hollman & Katan, 1998). Tsuda *et al.* (1999) reported that cyanidin 3-glycosides administered to rats were metabolised to protocatechuic acid. In the present investigation, protocatechuic acid was observed in trace amounts in both the urine (μg concentrations) and serum (ng concentrations) samples; however, the source of the protocatechuic acid could not be determined and results have therefore been excluded from the present report. In addition, colonic microflora have significant potential to transform flavonoids into lower-molecular-weight compounds, which may also have protective biological activities. Future research in this area is essential to establish a more complete understanding of flavonoid metabolism.

To conclude, the present results indicate that orally administered cyanidin 3-glycosides are absorbed and are transported in human serum and urine as glycosides and glucuronides, both of which appear to undergo further methylation and oxidation. The contribution of each individual structure to the reported bioactivity (health effects) of anthocyanins should be the focus of future research.

Acknowledgements

The present study (Pacific Agri-Food Research Center contribution no. 2225) was supported by the National Sciences and Engineering Research Council of Canada, and the Ontario Ministry of Agriculture, Food and Rural Affairs.

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