Anthocyanin metabolites in human urine and serum

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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* 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 (oecin chloride), peonidin 3-glucoside chloride, and pelargonidin 3-glucoside chloride (callistephen) 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 was acquired from K&K Rare & Fine Chemicals (Costa Mesa, CA, USA).

Abbreviations: DAD, diode array detector; Emax, maximum absorbance intensity; m/z, mass:charge ratio.

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Anthocyanin extraction

Anthocyanins were extracted from biological fluids using disposable solid-phase extraction C18 cartridges (Supelco, Bellefonte, PA, USA). The Tri-Sil Z (Pierce, Rockford, IL, USA), methanol (MeOH), chloroform (Chromasolv, Scientific, 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/m2 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) in 250 ml water. The extract contained four cyanidin 3-glycosides (mg): cyanidin 3-galactoside, 899; cyanidin 3-arabinoside, 321; cyanidin 3-xylloside, 51; cyanidin 3-glucoside, 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 the 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.

Mass spectrometry analysis

MS identification of individual compounds was conducted post-separation via HPLC (as described above). Individual peaks were collected on a Foxyl 200 X-Y fraction collector (ISCO, Inc., Lincoln, NE, USA), concentrated, and analysed by electrospray ionisation–MS. A Micromass QZ 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 N2 flow rate, 95 litres/h; desolvation N2 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
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 N₂ and re-dissolved in 150 µl mobile phase (as outlined earlier; 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.

Novel cyanidin 3-glycoside metabolites 935.https://doi.org/10.1079/BJN20041126
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 silylated 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-glucoside) 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
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, \( \lambda_{\text{max}} \) vis) and absorbance intensity at 440 nm \( \times \) maximum absorbance intensity (\( E_{440} \times E_{\text{max}} \)) 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 also 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) \( \mu \)mol/l) and 66·0 % (390·6 (range 119·4–661·9) \( \mu \)mol/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 weights for parent (anthocyanin) and daughter (anthocyanidin or aglycone; when obtainable) fragments, along with HPLC–DAD data (retention time, \( \lambda_{\text{max}} \) vis) and absorbance intensity at 440 nm \( \times \) maximum absorbance intensity (\( E_{440} \times E_{\text{max}} \)) 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 also 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) \( \mu \)mol/l) and 66·0 % (390·6 (range 119·4–661·9) \( \mu \)mol/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 weights for parent (anthocyanin) and daughter (anthocyanidin or aglycone; when obtainable) fragments, along with HPLC–DAD data (retention time, \( \lambda_{\text{max}} \) vis) and absorbance intensity at 440 nm \( \times \) maximum absorbance intensity (\( E_{440} \times E_{\text{max}} \)) 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 also 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) \( \mu \)mol/l) and 66·0 % (390·6 (range 119·4–661·9) \( \mu \)mol/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
(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) mmol/l) of the identified metabolites. Cyanidin glucuronide and glucuronide derivatives accounted for 10·6 % (1·9 (range 1·5–2·4) mmol/l), with the remaining 5·0 % or 0·9 (range 0·8–1·0) mmol/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).

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

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rt* (min)</th>
<th>λmax (vis) (nm)</th>
<th>E440:Emax (as %)</th>
<th>Molecular weight (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>24·1</td>
<td>518</td>
<td>31·7</td>
<td>449</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>26·6</td>
<td>517</td>
<td>32·6</td>
<td>449</td>
</tr>
<tr>
<td>Cyanidin-3-arabinoside</td>
<td>29·2</td>
<td>517</td>
<td>30·7</td>
<td>419</td>
</tr>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>32·3</td>
<td>502</td>
<td>44·0</td>
<td>433</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>36·0</td>
<td>518</td>
<td>32·6</td>
<td>463</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>38·5</td>
<td>528</td>
<td>27·5</td>
<td>493</td>
</tr>
<tr>
<td>Cyanidin-3-xyloside†</td>
<td>38·9</td>
<td>517</td>
<td>43·6</td>
<td>419</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>43·0</td>
<td>526</td>
<td>23·5</td>
<td>287</td>
</tr>
</tbody>
</table>

Rt, retention time; E440:Emax, 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).

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![Fig. 3. Proposed pathway for the formation of anthocyanin metabolites in human urine and serum post-consumption of cyanidin 3-glycosides.](https://www.cambridge.org/core/core.png)
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-xylloside, 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 (λmax (vis) in the 500 nm range). Analysis of the total peak areas at 525 nm (all integratable peaks with λmax (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, λmax (vis) and...
E_{440}/E_{\text{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 glucuro-
nides (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 docu-
mented (Werneille 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 deriva-
tives (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_{\text{max}} to the peonidin 3-glucoside standard. Only a few researchers have reported 
methylation of 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; 
Werneille et al. 1983; Hollman & Katan, 1998; Miyazawa et al.
1999; Okushio et al. 1999; Day & Williamson, 2001; Donovan et al. 2001). In addition, oxida-
tive 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 (rep-
resented by m/z 477, peak 9; Fig. 1). This structure (m/z 
493/331) matched the molecular weights for parent and 
daugther fragments of malvidin 3-galactoside, had a simi-
lar retention time to that of the malvidin 3-glucoside stan-
dard 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 con-
centrations 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 anthocya-
nin 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 exclu-
sively in the kidney, accumulates in the kidney, or the con-
centration 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 pre-
viously 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 character-
ised in animal models, where these metabolites were identi-
ified in the urine (Buset & Scheline, 1980; Hollman & 
Katan, 1998). In theory, dimethylation of the B-ring, result-
ing in reduced polarity, could merit further phase I cyto-
chrome 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 derivatisa-
tion of the B-ring, which has been documented as a prob-
able 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 glu-
curonide conjugation in man are unknown. The glucuronida-
tion 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 identi-
fication when sample impurities exist, prevents the undeni-
able 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 deriva-
tives of cyanidin 3-galactoside) accounted for 84.0% 
(15.0 (range 11.5–18.7) μmol/l) of the identified anthocya-
nins. 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). Accord-
ingly, cyanidin galactosides accounted for 89.4% 
(529·3 (range 1600–898·6) nmol/l) of the anthocyanins 
(8.6% or 51·1 (range 30·8–71·4) nmol glucuronides/l; 
Table 1) in the serum, with 660·0 % (390·6 (range 
119·4–661·9) nmol/l) being the parent compound cyanidin
3-galactoside. The high percentage of anthocyanin glyco-
sides 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 CO2 (Rozman, 1986; Hollman & Kathan, 1998). Tsuda et al. (1999) reported that cyanidin 3-glycosides administered to rats were metabolised to protocatecholic acid. In the present investigation, protocatecholic acid was observed in trace amounts in both the urine (μg concentrations) and serum (ng concentrations) samples; however, the source of the protocatecholic 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.

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References


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