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Processing blackcurrants dramatically reduces the content and does not enhance the urinary yield of anthocyanins in human subjects

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Abstract

Blackcurrant (BC) fruits are a rich source of biologically active anthocyanins but little is known of the anthocyanin content of commonly consumed BC products or how processing affects the absorption. We report that whereas fresh and frozen whole BC were a rich source of anthocyanins, containing 897 and 642 mg (100 g FW)$^{-1}$ of total anthocyanins the levels in all other products were substantially lower (0.05–10.3% of the levels in fresh fruit). Further, when the absorption and excretion of BC was assessed in volunteers consuming a portion (100 g) of frozen whole BC (642 mg total anthocyanins) and, 300 g of a BC drink made by diluting concentrated syrup (33.6 mg total anthocyanins), only small quantities of BC anthocyanins were excreted in urine (fruit, 0.053 ± 0.022%; drink, 0.036 ± 0.043%; mean percent urinary yield ± SD) and they were not detected in plasma. These data indicate that fresh and frozen BC, but not processed products, are rich sources of anthocyanins but, regardless of the food source, these anthocyanins are poorly bioavailable.

Keywords: Flavonoids; Phenolics; Polyphenols; Bioavailability; Human metabolism

1. Introduction

Anthocyanins are highly coloured flavonoids that are present in many plants consumed as foods. For example, they are responsible for the rich red, purple, blue, and black colours of berries and currants (e.g. blackcurrants, blueberries, raspberries, redcurrants, strawberries) and other fruits (blood orange, red apples, red and black grapes), but also some vegetables such as purple cabbage and aubergines (Francis, 1989). Anthocyanins have been shown to exhibit a number of biological activities that are in keeping with the protection afforded by fruit and vegetable-rich diets against age-related diseases such as cancer, cardiovascular disease, cataracts and neurological disorders including Alzheimer's (Andriambeloson et al., 1998; Laplaud, Lelubre & Chapman, 1997; Mares-Perlman, 1997; Parthasarathy, Khan-Merchant, Penumetcha, Khan, & Santanam, 2001; Trevithick & Mitton, 1999). *In vitro*, anthocyanins exhibit anti-inflammatory (Moroney, Alcaraz, Forder, Carey, & Hoult, 1988), antioxidant (Ding et al., 2006), vasomodulatory (Bell & Gochenaur, 2006; Fumagalli et al., 2006), and anti-haemostatic (Rechner & Kroner, 2005) activities. There is also considerable evidence, from *in vitro* studies at least, to indicate that anthocyanins possess anti-tumour activities, including inhibition of tumour and tumour cell growth, anti-proliferative and pro-apoptotic activities, and inhibition of pro-inflammatory enzymes (Chen et al., 2005; Cooke et al., 2006; Ding et al., 2006; Munoz-Espada & Watkins, 2006). The anthocyanin content of many fruits

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and vegetables is considerably higher than the content of other classes of flavonoids that are consumed in the diet. For example, the content of anthocyanins in lowbush blueberries, highbush blueberries, raspberries and strawberries have been reported in the range 155–4350 μmol (100 g FW)$^{-1}$ (equivalent to 67–1885 mg (100 g FW)$^{-1}$ (Kalt, Forney, Martin, & Prior, 1999), whereas rich sources of flavonols (e.g. onions), and flavones (e.g. celery) are in the range 1–50 mg (100 g FW)$^{-1}$ (Harnly et al., 2006; Hertog, Hollman, & Katan, 1992).

In order to affect the function of cells and tissues beyond the gut, bioactive components of the diet need to be absorbed and reach the peripheral circulation. There have been a number of studies concerned with the absorption of anthocyanins in human subjects. These indicate that anthocyanins are only partially bioavailable, with nanomolar concentrations observed in plasma and urinary yields commonly less than 0.1% of the oral dose (Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Prior & Wu, 2006). The most bioavailable anthocyanins reported to date are those from red wine (Lapidot, Harel, Granit, & Kanner 1998) and strawberries (Felgines et al., 2003), where urinary yields in excess of 1% have been reported. The relatively high bioavailability of red wine anthocyanins may be due to the alcohol content of the beverage, but recent reports indicating that urinary red wine anthocyanin yields were <1% and no better than dealcoholised red wine or red grape juice are inconsistent with the earlier report (Bub, Watzl, Heeb, Rechkemmer, & Briviba, 2001; Frank, Netzel, Strass, Bitsch, & Bitsch, 2003). Strawberries contain a single major anthocyanin, pelargonidin-3-glucoside, which has been shown to be efficiently transformed during absorption in humans such that the predominant form in urine is a pelargonidin-glucuronide, with other glucuronides, a pelargonidin sulfate and small quantities of pelargonidin glucoside also present (Felgines et al., 2003). The efficient deglucosylation and subsequent phase-2 metabolism of pelargonidin is similar to that observed for flavonols such as quercetin, isoflavones such as genistein and daidzein from soy, citrus flavanones, and flavones such as chrysin and luteolin (Kroon et al., 2004). In contrast, many anthocyanin bioavailability studies report only the original anthocyanins present in plasma and/or urine, and for most of these studies the levels reported are very low (Prior & Wu, 2006).

The blackcurrant (*Ribes nigrum* L.) is a species of currant that is native to central and northern Europe and northern Asia that produces a very dark purple (almost black) berry fruit. The intense colour is due to the high concentrations of anthocyanins, and they are also a rich source of vitamin C. The two main anthocyanidins (aglycones) are delphinidin and cyanidin, and the major anthocyanins are the 3-rutinosides and 3-glucosides. Blackcurrants are a very popular fruit in Europe, and the lower popularity in the United States is almost certainly due to the effects of a ban on currant farming enacted in the early 1900s, which still exists in a few US states today. Although some blackcurrants are consumed as fresh fruit, the majority are processed to produce juices, syrups, cordials, purees, and concentrates and incorporated into jams/conserves, jellies, pie fillings, and various ready-to-drink beverages including smoothies. Blackcurrant extracts are sold as a commodity and are widely used in the food industry, particularly for their colour but also their flavour which can be rather astringent.

The aims of this study were to (1) examine the anthocyanin content of a range of blackcurrant products, and (2) measure the absorption and excretion of blackcurrant anthocyanins in humans, comparing the bioavailability of intact blackcurrant fruits with a blackcurrant drink made from a highly processed blackcurrant syrup.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol and acetonitrile were obtained from Fisher Scientific and Sigma–Aldrich, respectively. Trifluoroacetic acid (TFA), sulfatase (*Helix pomatia* Type H-1), β-glucuronidase (*H. pomatia* Type H-5) and all phenolic acids were purchased from Sigma–Aldrich (UK). All anthocyanin standards were obtained from Extra synthése (Genay, France). All solvents and other chemical used were of HPLC grade and purity was assessed (LC-UV-MS/MS) to be 95% or greater for all phenolic standards.

2.2. Sampling of blackcurrant fruits, juices and other products

Blackcurrant products were purchased locally from a variety of outlets including major supermarkets and smaller retailers. Branded, economy and supermarket own-branded products were purchased. Products included: Fresh fruit, frozen fruit, drinks (economy and high juice squashes/cordials requiring dilution before consumption, ready-to-drink blackcurrant juices and flavoured drinks, fruit smoothies), blackcurrant coulis, various blackcurrant conserves/jams, tinned fruit (blackcurrant fruit filling, blackcurrants in fruit juice).

For the interventions, fresh whole blackcurrants (*Ribes nigrum* var. Ben Alder), were obtained from a local farm (SE & JM Farrow Ltd., Pond Farm, Pound Lane, Toft Monks, Beccles, Suffolk, NR34 0EX, UK). 5 kg were collected and then washed and prepared to give 2.5 kg of clean fresh fruit. Whole blackcurrants were then portioned into 100 g (FW) samples and stored at −20 °C. Prior to consumption, a portion of the blackcurrants were defrosted overnight at 4 °C. The blackcurrant drink was a commercially available syrup concentrate (made with 110 g blackcurrants per 100 g product) that was provided to volunteers diluted (50 g concentrate plus 250 g water).

2.3. Subjects and study design

Ten apparently healthy volunteers (three men, seven women) aged between 20 and 65 yrs were recruited to

participate in this study. All study participants were assessed for eligibility on the basis of a health questionnaire and the results of clinical laboratory tests. The following exclusion criteria applied: smokers; long term medical conditions such as asthma (unless untreated within the past two years), heart disease, gastrointestinal disease, diabetes, cancer; regular prescribed medication (except HRT and oral contraceptive); supplement (unless judged not to affect study outcome) or antibiotic use within 4 weeks prior to the start of the study; pregnancy; blood donation within 4 months prior to the start of the study; BMI <18.5 or >35; clinical results at screening judged by the medical advisor to affect study outcome or be indicative of a health problem. Subject characteristics were (mean ± SD): weight 71.5 ± 13.9 kg (range 50.8–92.9 kg), BMI 24.5 ± 3.2 kg/m^2 (range 20.7–29.7 kg/m^2) and age 49 ± 12 yr (range 25–64 yrs). The study was explained to participants and written informed consent was obtained prior to participation. The study protocol was approved by the Human Research Governance Committee of the Institute of Food Research and the Norwich Research Ethics Committee.

The study was a randomized two-phase crossover design investigating the bioavailability of anthocyanins from fresh and processed blackcurrants. Each test phase comprised a 5 day period of intervention separated by a washout period of at least one week. During each period of intervention subjects followed a low-polyphenol diet and to aid compliance a list of authorized and prohibited foods were given. On day 3 of the intervention, fasted subjects had an intravenous catheter inserted and a baseline blood sample (10 ml) was obtained. Subjects were given a standard breakfast consisting of two slices of white toast (72 g) with spread (10 g) followed by either 100 g whole blackcurrants (containing 642 mg total anthocyanins) or 50 g diluted blackcurrant syrup (33.5 mg anthocyanins). To limit variation in food and drink intakes, subjects refrained from drinking and eating for 1.5 and 4 h, respectively. Blood samples (10 ml) were collected into lithium heparin tubes at 15, 30 and 45 min and 1, 1.5, 2, 3, 4, 6, 8, 24 and 48 h and immediately centrifuged at 1500 g for 10 min. Plasma samples were subsequently acidified with HCl to prevent anthocyanin degradation and frozen at −20 °C prior to analysis. Urine was collected the day before consumption of the test meal and between 0–2, 2–4, 4–6, 6–8, 8–24 and 24–48 h after consumption. The amount of urine in each fraction was measured, and samples were acidified with HCl and analysed immediately for anthocyanins, or frozen at −20 °C for later analysis of phenolic acids.

2.4. Quantification of anthocyanins in blackcurrant fruits, juices and other products

Blackcurrant fruits (fresh and frozen) and derived products were analysed using high-performance liquid chromatography (HPLC). All solvents used were HPLC grade, all water was ultra-pure and the purity of commercial standards was confirmed by HPLC. All analyses were performed at least in triplicate. Fresh and frozen fruits were weighed, freeze dried and subsequently milled to a fine powder. Samples of fruit powder (40 mg) were mixed with 950 μl of 70% aqueous methanol containing 1% HCl and 50 μl 0.5 mg (ml)$^{-1}$ pelargonidin-3-glucoside in methanol (internal standard). All juices, jams, syrups, fruit in juice, tinned fruit and the coulis were analysed directly from a known mass or volume by addition of 7 parts of methanol-HCl (99:1, vol/vol) and 50 μl 0.5 mg ml^{-1} pelargonidin-3-glucoside in methanol. Tinned fruits, fruits in juice, and jams were blended in a food mixer, and then processed in the same way as juices. All samples were centrifuged (13,000*g*, 10 min) and analysed by HPLC as described below, using diode array detection for quantification, and tandem mass spectrometry (positive ion mode) to confirm the identity of the analytes. Recoveries of internal standards for the food/beverage analyses were 95.7 ± 14.6% (mean SD). Intra-day variance values for cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside and delphinidin-3-glucoside in foods and beverages were 7.8%, 9.4%, 8.1% and 9.0%, respectively. Limits of detection (LOD) for cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside and delphinidin-3-glucoside using absorbance at 520 nm were 0.13, 0.14, 0.21 and 0.24 ng injected, respectively.

2.5. Extraction and analysis of anthocyanins from plasma and urine

Prior to extraction of anthocyanins, 50 μl malvidin-3-glucoside (0.1 mg (ml)$^{-1}$) was added to samples of acidified urine (20 ml) or acidified plasma (2.0 ml) as an internal standard. Anthocyanins in plasma and urine were extracted using a solid phase extraction (SPE) cartridge (Varian Bond Elute C_{18}) conditioned with methanol (5 ml) followed by 1% aqueous HCl (10 ml). Following application of urine or plasma, the cartridge was washed with 1% aqueous HCl (10 ml) and anthocyanins eluted directly into vials with 1% HCL in methanol (0.5 ml for urine, 1.0 ml for plasma). The SPE eluate from urine samples was analysed directly by HPLC. Plasma eluates were evaporated to dryness (N_2 stream, 40 °C) and re-dissolved in 1% HCl in methanol (0.2 ml) prior to analysis by HPLC (Agilent HP1100) using a Gemini C_{18} column (150 × 2.00 mm, 5 μm particle size; Phenomenez, Macclesfield, UK) eluted with an increasing gradient of acetonitrile in 0.1% aqueous TFA at a flow rate, 0.3 ml (min)$^{-1}$ over 45 min at 30 °C. Post-column, the eluent passed through a UV-diode array detector monitoring over the range 200–600 nm, and subsequently an electrospray ionisation-mass spectrometer (ESI-MS; Agilent Technologies, Waldbronn, Germany). The mass spectrometer was operated in positive ionisation mode (cone voltage 22 V, source block temp 120 °C, desolvation temperature 300 °C) with multiple reaction monitoring ([*m*/*z*] parent, daughter; delphinidin-3-glucoside – 465.1, 303.05; delphinidin-3-rutinoside – 611.17, 303.05; cyanidin-3-glucoside – 449.11, 287.06; cyanidin-3-rutinoside – 595.17, 287.06; malvidin-3-glucoside – 493.13, 331.08).

Quantification of blackcurrant anthocyanins in plasma, urine and food/beverage samples was based on standard curves (range of 0.1–100 μg $(ml)^{-1}$) for each anthocyanin that were run alongside samples with correction for recovery of the internal standard. All standard curves were linear with regression coefficients >0.999. Recoveries of internal standards for the urine analyses were 94.3 ± 10.9% (mean ± SD). Intra-day variance values for cyanidin-3-rutinoside and delphinidin-3-rutinoside in urine were <9.0 and <11%, respectively. Limits of detection for cyanidin-3-rutinoside and delphinidin-3-rutinoside in urine and plasma were 0.26 and 0.42 ng injected (A_{520} nm), equivalent to 0.66 and 1.05 ng $(ml\ urine)^{-1}$, and 2.6 and 4.2 ng $(ml\ plasma)^{-1}$, respectively (4.4 and 6.9 nmol $(l\ plasma)^{-1}$, respectively).

2.6. Extraction and analysis of phenolic acids in urine

Urine was thawed and samples (1 ml) were mixed with internal standard (*trans*-cinnamic acid; 50 μl of 50 μM aq. solution), phosphate buffer (pH 5, 250 μl), sulfatase (100 μl, 1000 U), β-glucuronidase (100 μl, 1000 U), and 5% HCl (10 μl) and incubated at 37 °C for 16 h. Subsequently, extracts were centrifuged (13,000 rpm × 10 min) and filtered (0.2 μm) and samples (100 μl) were injected directly onto the HPLC column. Samples were injected onto a Luna C_{18} column (250 × 4.6 mm i.d., 5 μm) and eluted at 1.0 ml $(min)^{-1}$ using a gradient from solvent A (1.5% aqueous acetic acid) to solvent B (acetonitrile: water: acetic acid; 80: 18.5: 1.5) starting 0% B, increasing to 1% B at 5 min, 3% B at 10 min, 8% B at 15 min, 16% B at 30 min, 35% B at 40 min and 100% B at 50 min. The eluent was monitored (diode array detector) at 270 and 325 nm and subsequently sprayed into the mass spectrometer interface without splitting. Electrospray ionisation in the negative mode (ESI-) was applied with a mass range of 50–1600, fragmentor setting 100, gas temperature 350 °C, drying gas at 13.0 L $(min)^{-1}$, nebuliser press at 50 psi and capillary voltage at 3000 V. 3-Hydroxy-hippuric acid (3HHA), 4-hydroxy-hippuric acid (4HHA) and hippuric acid (HA) were quantified using external standard curves generated using selected ion monitoring mass spectrometry (*m/z* for $[M-H]^- = 194$, 194 and 178 for 3HHA, 4HHA and HA, respectively) with correction for the internal standard. The limit of detection for phenolic acids in urine was ~50 ng per injection based on signal to noise ratios for several spiked urine samples. Intra-day variance (mean% standard deviation) determined from repeated replicate measurement of a pooled sample (5 replicates over 5 days) was <3% and inter-day variance was <5%.

2.7. Data analysis

Statistical analyses were performed using the R data analysis software (R Development Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.). Repeated Measures models were used to analyse the data and "volunteer" was included as a "random effect". For all models, regression diagnostics were checked to determine if data transformations, outlier omissions, or alternative non-parametric models were required. All results from the models were considered significant if $p < 0.05$. When the metabolite data was split into its two separate components (Delphinidin-3-rutinoside and cyanidin-3-rutinoside) and analysed for differences in fruit versus juice, a Wilcoxon Rank Sum test was used, results were considered significant if $p < 0.05$.

3. Results

3.1. Anthocyanin content and composition of fresh blackcurrants and commercially available blackcurrant products

A range of blackcurrant products were obtained from local supermarkets and smaller outlets and their content of blackcurrant anthocyanins (delphinidin- and cyanidin-3-rutinosides and 3-glucosises) determined (Table 1). Blackcurrant anthocyanins were detected and quantified in all the samples except for one (a blackcurrant and apple squash); in this sample we were not able to detect the blackcurrant anthocyanins, but there were several peaks of absorbance at 520 nm. The composition order of the individual anthocyanins was delphinidin-3-rutinoside > cyanidin-3-rutinoside > delphinidin-3-glucoside > cyanidin-3-glucoside for all the other products. Fresh blackcurrants contained almost 0.9 g anthocyanins per 100 g fresh weight of fruit. The concentration of anthocyanins in frozen fruit was also high (642 mg $(100\ g\ FW)^{-1}$, equivalent to more than 70% of the levels in the fresh fruit sample, but the concentrations in all the other products were substantially lower. Only three products (blackcurrants in syrup, a pie filling, one of the several jams) contained anthocyanin contents >5% of that expected based on the stated blackcurrant content and the mean value for fresh fruit (relative fresh content). The relative fresh contents of the majority of products were less than 1%. The variation within product types was high. For example, blackcurrant anthocyanin concentrations in jams ranged from 4.8 to 60.6 mg $(100\ g\ FW)^{-1}$ and in squash/cordial products ranged from 4.1 to 29.6 mg $(100\ g\ FW)^{-1}$. Estimates of the anthocyanin content of typical portions of each product ranged from 0.5 mg in a branded apple and blackcurrant ready-to-drink carton product to more than 80 mg in a blackcurrant pie filling and a blackcurrants in juice product. However, these portion contents are ~10-fold lower than for fresh and frozen fruit.

3.2. Absorption and urinary excretion of blackcurrant anthocyanins

Following ingestion of blackcurrant fruits or a blackcurrant drink made from concentrate, small quantities of two anthocyanins were detected in urine samples of volunteers.

Table 1
Anthocyanin contents of blackcurrant fruits and commercially available blackcurrant-derived products

Product type	Description	Total anthocyanin content[a]	Stated blackcurrant content (%)	Relative anthocyanin content[b] (%)	Anthocyanins per portion[d]
Fresh fruit	Supermarket, punnet	897	100	100	897
Frozen fruit	Supermarket, bag	642	100	71.6	642
Jams, conserves	BC conserve, supermarket brand	3.9	45	0.44	1.17
	Organic BC conserve, supermarket brand	6.65	40	0.74	2.00
	BC jam, supermarket brand	7.54	35	0.84	2.26
	BC jam, branded	5.59	35	0.62	1.68
	BC jam, branded	24.0	35	2.68	7.20
	Reduced sugar BC jam, branded	9.08	35	1.01	2.72
	Reduced sugar BC jam, supermarket brand	48.58	40	5.42	14.57
Cordial, squash	BC cordial, branded	20.01	4[c]	2.23	10.01
	Apple + BC squash, supermarket brand[e]	–	1[c]	–	–
	High juice BC squash, supermarket brand	32.2	50[c]	3.59	16.1
	Organic BC squash, branded	4.13	12[c]	0.46	2.07
	BC squash, supermarket brand	7.48	10[c]	0.83	3.74
	High juice BC squash, supermarket brand	26.4	35[c]	2.95	13.2
Ready-to-drink	Apple + BC, branded	1.00	5[c]	0.11	0.50
	BC, branded (tetra pack)	3.82	6[c]	0.43	9.55
	BC, light, branded	0.462	6[c]	0.05	1.53
	Apple, pear + BC pressed juice	7.84	5	0.87	15.6
	Probiotic yoghurt smoothie (sup'market brand)	25.97	18	2.90	51.95
	Smoothie, for kids, branded	5.1	4	0.57	10.18
Fruit in tins, jars	BC fruit pie filling, supermarket brand	149.4	45	16.66	149.4
	BC fruit in juice, supermarket brand	103.9	Not specified	11.59	103.9
	BC in syrup, branded	54.4	Not specified	6.07	54.4

[a] Data are the total anthocyanin contents (cyanidin-3-rutinoside + cyanidin-3-glucoside + delphinidin-3-rutinoside + delphinidin-3-glucoside), given as mg (100 g FW)$^{-1}$ for fruit, coulis and jam products and mg (100 ml)$^{-1}$ for squashes, drinks and juices, from a minimum of 3 determinations.
[b] Calculated as a proportion of the anthocyanin content of fresh fruit taking into account the stated blackcurrant content of the product.
[c] Stated content of blackcurrant juice concentrate.
[d] Portion sizes used: Fruit, 100 g; coulis, 28 g; Jam, 30 g; Squash, 50 ml, Juices, 200 ml; Drinks, as sold. Based on the Food Standards Agency (UK) Portion Size booklet, 3rd Edition, 2002.
[e] This product apparently contained anthocyanins (absorbance at 520 nm) but the usual blackcurrant anthocyanins were not present so no data are given.

These appeared as new peaks in HPLC chromatograms monitored at 520 nm and gave anthocyanin-like UV–visible spectra. These two peaks had retention times identical to those for pure standards of delphinidin-3-rutinoside and cyanidin-3-rutinoside, and their identity was confirmed using mass spectrometry which indicated the presence of the parent ions ($m/z = 611.17$ and 595.17) and the anthocyanidin fragment ions ($m/z = 303.05$ and 287.06) for delphinidin-3-rutinoside and cyanidin-3-rutinoside, respectively (data not presented). No other anthocyanins were detected in urine. Although anthocyanins were shown to be stable in acidified plasma for at least 6 days (losses at 4 °C < 5%), they were not stable in the absence of acidification (losses up to 50% in 16 h).

Anthocyanins appeared in urine within 2 h of ingestion of the blackcurrant products. Urinary excretion peaked in the 2–4 h sample and then declined (Fig. 1). The absolute quantities of anthocyanins excreted via urine were low (48 h total urinary excretion 339 ± 139 and 10.2 ± 12.5 μg, from fruit and drink, respectively. The total urinary anthocyanin yields were extremely low (mean ± SD; 0.053 ± 0.022% and 0.036 ± 0.043% for the fruit and drink, respectively. To analyse the data (response = excretion, volunteer treated as fixed effect, all other measured variables treated as explanatory), it was established that a power transformation of 0.25 was appropriate for the explanatory variable. The effect of dose was highly significant ($p < 0.001$), and the difference between delphinidin-3-rutinoside and cyanidin-3-rutinoside excretion was borderline but not significant ($p = 0.078$). To investigate further, fractional excretion data were analysed to take account of the very large differences in dose and, since the data were not normally distributed, a Wilcoxon Rank Sum test was applied. Using a similar approach, the difference in total anthocyanin urinary yield between the fruit and drink matrices was not significant ($p = 0.84$). When the individual metabolites were treated separately, excretion of delphinidin-3-rutinoside was significantly different between the fruit and juice meals (0.058 ± 0.025 versus 0.021 ± 0.017, $p = 0.0015$) but the excretion of cyanidin-3-rutinoside was not ($p = 0.13$). Anthocyanins were not detected in any of the plasma samples, presumably because the levels achieved in plasma were below the limits of detection (4.4 and 6.9 nmol l^{-1} for cyanidin-3-rutinoside and delphinidin-3-rutinoside, respectively).

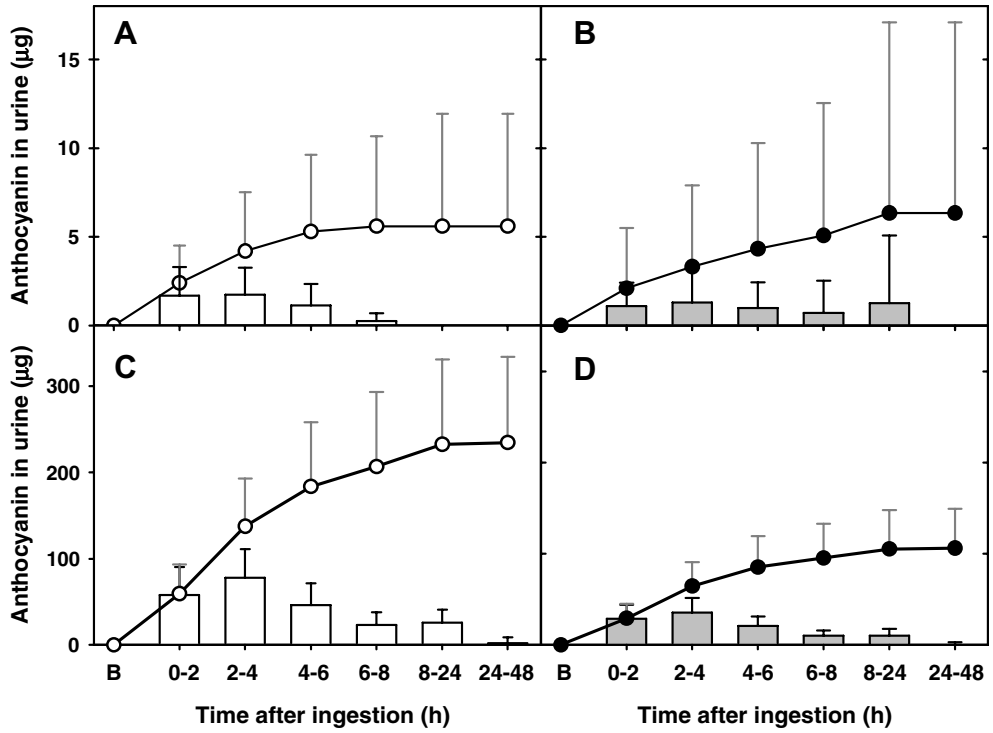

Fig. 1. Urinary excretion of anthocyanins from blackcurrant fruit and juice. Volunteers ($n = 10$) consumed, in random order, whole blackcurrants (panels C and D) or a blackcurrant drink made from concentrate (panels A and B) containing 640 and 33.5 mg total anthocyanins, respectively. Urine was collected at baseline (B) and for the time periods shown and immediately analysed for anthocyanins. Bars; amount of anthocyanin excreted in the time period: circular symbols/lines; cumulative excretion of anthocyanin: open bars and circles; delphinidin-3-rutinoside: filled bars and circles; cyanidin-3-rutinoside.

3.3. Urinary excretion of phenolic acids

The urinary yield of the orally dosed anthocyanins accounted for only a tiny fraction (<0.1%) of the oral dose. Various phenolic acids such as phenylpropionic, phenylacetic, benzoic and cinnamic acids have been reported as major urinary products following ingestion of certain flavonoid-rich foods or supplements (Hodgson et al., 2004; Ito et al., 2005; Olthof, Hollman, Buijsman, van Amelsvoort, & Katan, 2003; Rios et al., 2003). Urine samples were analysed using a method developed for measurement of simple phenolic acids in urine. Comparison of paired baseline and post-intervention UV chromatograms for a subset ($n = 4$) of subject urine samples for the fruit intervention analysed by HPLC indicated that in all four volunteers, three peaks were increasing post intervention (data not presented). These three peaks corresponded to authentic standards of 3-hydroxy-hippuric acid (3HHA), 4-hydroxy-hippuric acid (4HHA) and hippuric acid (HA) in terms of retention time and UV spectra, and their identity was confirmed using selected ion monitoring mass spectrometry. These phenolic acids were quantified and profiles for their excretion determined. Urinary excretion kinetic profiles for the three acids were similar (Fig. 2); the urinary excretion rate peaked in the first 2 h collection, and then declined slowly up to the 6–8 h collection with no significant changes in subsequent samples. Mean baseline daily excretion of the three acids amounted to 24.6 mg, which increased to 41.4 mg on the intervention day and 37.7 mg on the subsequent day (Table 2), which equated to an increase in phenolic acid excretion of 30.0 mg over 48 h.

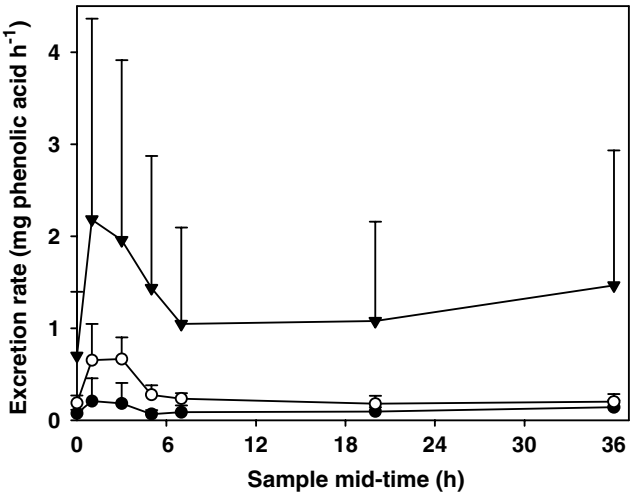

Fig. 2. Kinetics of phenolic acid excretion following blackcurrant consumption. Urine samples from the blackcurrant intervention day of a randomly selected subset ($n = 4$) of volunteers were analysed for phenolic acids. Data are the rates of urinary excretion in mg phenolic acid h^{-1}. ▼, Hippuric acid (HA); ○, 4-hydroxyhippuric acid (4HHA); ●, 3-hydroxyhippuric acid (3HHA).

Table 2
Urinary excretion of phenolic acids following consumption of blackcurrant fruits

	Baseline[a]	0–24 h[a]	24–48 h[a]	Change over baseline[b]
3HHA	1.98 ± 1.13	2.80 ± 1.68	2.95 ± 2.21	1.8 ± 3.8
4HHA	5.35 ± 2.82	8.15 ± 1.86	5.55 ± 1.38	3.0 ± 3.5
Hippuric acid	17.3 ± 0.6	30.5 ± 1.3	29.2 ± 5.0	25.2 ± 7.6
Total	24.6	41.4	37.7	30.0

[a] Data are mg phenolic acid in a 24 h urine collection (mean ± SD).
[b] Data are the differences between the sum of the differences between the baseline and the 0–24 and 24–48 h collections [(0–24) – B + (24–48) – B].

4. Discussion

Blackcurrants are a rich source of highly coloured and potentially health promoting anthocyanins. Since the majority of blackcurrant fruit is processed to products such as syrups, squashes, juices, ready-to-drink beverages and smoothies, pie fillings and sauces, it is important to know the content of anthocyanins in the processed foods and beverages, and how processing might effect the bioavailability. For example, around 95% of the entire UK blackcurrant crop is processed to a juice concentrate and subsequently used to make a range of branded blackcurrant drink products (Ribena™; GlaxoSmithKline Group; http://www.ribena.co.uk/index_flash.html) that are also marketed in Australia and New Zealand. Our data show that the retention of anthocyanins in all the blackcurrant beverages tested appears to be very poor. The blackcurrant beverage products analysed for this report represent those available from major supermarkets in the UK during the period 2005 to early 2007 and include clarified squashes, ready-to-drink products, fresh and long-life juices, fruit smoothies and syrup concentrates for dilution. Hence, we conclude that the amounts of anthocyanins typically ingested by the UK population when consuming blackcurrant drinks, including fresh fruit juices and smoothies made from whole fruit are rather low.

Further, the levels of anthocyanins in a range of other blackcurrant products including those in which the blackcurrant fruit is largely intact (e.g. tinned fruits in syrup or juice, pie fillings) and those in which the whole fruits are macerated (e.g. coulis) were also just a small fraction of those in fresh blackcurrants (Table 1). In contrast, frozen blackcurrant fruits contained high levels of anthocyanins. These data indicate that, with the exception of frozen whole fruit, the levels of intact anthocyanins in all processed blackcurrant products is extremely low, with the highest observed levels (tinned fruit) corresponding to only 9.5% of the concentrations in fresh fruit, and some products containing only trace amounts.

Anthocyanins are unique among the flavonoids in having a positive charge associated with the C-ring in the flavylium ion form (see Fig. 3). In most plant tissues and in some products such as wine, the anthocyanins are in the intensely coloured flavylium form, often due to copigmentation which enhances the anthocyanin colour and stability (Brouillard & Dangles, 1994). However, when the plant cells are ruptured, or the anthocyanins are exposed to higher pH (near neutral), the anthocyanins can form the carbinol pseudo-base, quinoidal-base, or the chalcone; this process is associated with a loss of colour. This process can occur rapidly, but the rate is dependent on the pH and temperature, light conditions, the presence/concentrations of

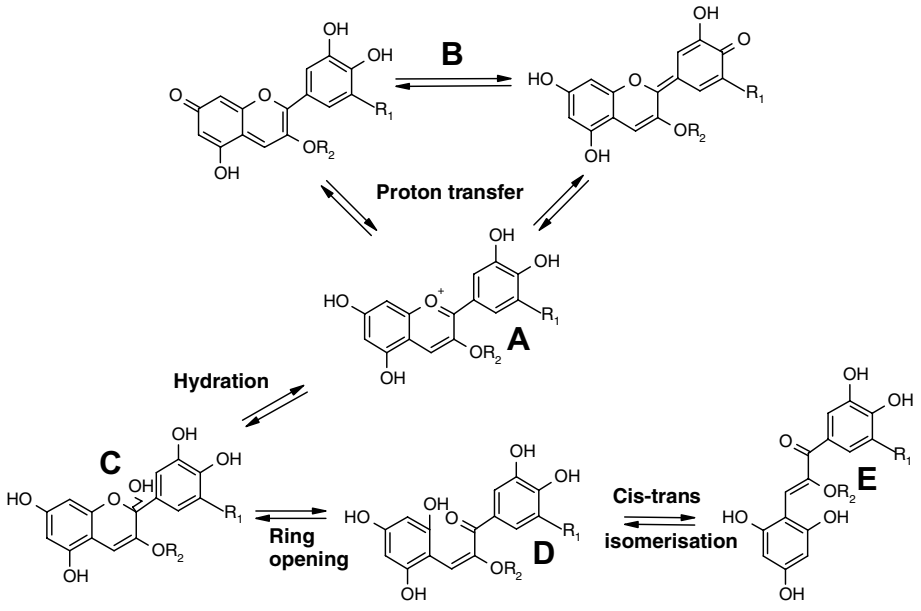

Fig. 3. Structure of blackcurrant anthocyanins and their pH-dependent transformations. R_1 = H, cyanidin; R_1 = OH, delphinidin; R2 = glucose, -3-glucoside; R2 = rhamnose-glucose, -3-rutinoside. The highly coloured flavylium ion (A) is the predominant species in highly acidic solutions. A number of (generally) reversible reactions may occur if the pH is increased, leading to the formation of a quinoidal base (B), which is also highly coloured, or a hemiacetal (C) that can then form the *cis*-chalcone (D) and the *trans*-chalcone (E); C, D and E are lightly coloured or colourless.

metal ions, oxygen, ascorbic acid and enzymes, and may also be affected by the anthocyanin concentration (Mazza & Miniati, 1993). In addition, anthocyanin instability can lead to the formation of polymeric forms (Markakis, 1982) which is associated with a change in the colour to a browner shade (Skrede, Wrolsted, Lea, & Enersen, 1992). Indeed, considerable research effort since the nineteen sixties has focussed on anthocyanin stability, driven principally by the interest in anthocyanins as colours for the food industry and developing products with stable anthocyanin colour (Francis, 1989). More recently, interest in anthocyanins has focussed on their role as biologically active components of fruits and vegetables with considerable antioxidant activity (Ding et al., 2006) and a range of other biological activities that may be responsible in part for the health-promoting effects of diets rich in fruits and vegetables. There is now an extensive literature documenting the biological activities of anthocyanins (recently reviewed by Prior & Wu, 2006). There are also a number of published reports describing the bioavailability of anthocyanins from a variety of plant sources. But, it is important to note that (1) the evidence of anthocyanin biological activities that may be beneficial for human health has been generated, almost exclusively, from *in vitro* studies, and (2) the previously reported bioavailability studies have used isolated anthocyanins or anthocyanin extracts that provide a high dose of generally stabilised anthocyanins. In relation to the first point, there is currently little, if any, evidence that orally delivered anthocyanins can affect established biomarkers of human health, and the data from *in vitro* studies that do not take account of bioavailability and metabolism should be considered with caution (Kroon et al., 2004). Concerning the intakes of anthocyanins, and their bioavailability, we report here that the anthocyanin contents of a broad range of commercial products that represent the majority of blackcurrant foods and beverages consumed in the UK are substantially lower than in the original fruits (Table 1), and that there is no significant difference in bioavailability between whole fruits and a processed syrup. This has important implications when considering the possible impact of blackcurrant anthocyanins on human health since the exposure has been considerably less than might have been previously expected. There is also interest in producing fruits/vegetables with enhanced levels of anthocyanins, for example grapes with enhanced levels of anthocyanins in the skins achieved through chemical treatment (Fumagalli et al., 2006). However, blackcurrants are already very rich sources of anthocyanins, and it may be more productive to focus on retaining more of the anthocyanins in food products.

Our study has shown that only a tiny fraction of the anthocyanins present in an oral dose of blackcurrants is excreted intact in the urine. This observation is in keeping with several other reports concerned with the absorption and excretion of orally dosed anthocyanins (Manach et al., 2005; Prior & Wu, 2006). Flavonoids are degraded by the microflora in the gut, especially the colon. Using human faecal samples as a source of colonic microflora, Deprez et al., (2000) demonstrated that flavonoids (oligomeric flavanols) were efficiently degraded such that the 48 h samples were almost devoid of intact flavanols. Further, the use of radio-labelled compounds facilitated the detection and identification of several small aromatic acids, predominantly of the phenylacetic, phenylpropionic, and phenylvaleric type, which were identified as products of the degradation of these flavonoids. It has been known that phenolic acids are urinary products of flavonoid metabolism for over 50 years (Booth, Jones, & De Eds, 1958; Booth, Murray, Jones, & De Eds, 1956) and of human urine for more than 50 years (Mathieu & Revol, 1968). More recently, a number of studies have provided evidence to show that aromatic acids are significant urinary products in humans following ingestion of certain flavonoids or flavonoid-rich foods and beverages (Hodgson et al., 2004; Ito et al., 2005; Olthof et al., 2003; Rios et al., 2003). For some flavonoids, the urinary molar yield (i.e. the mole fraction of oral dose recovered in urine, assuming a 2:1 molar ratio between flavonoid consumed and potential phenolic acid product) is substantial. For example, it has been reported that, in human subjects, ≈50% of orally ingested chlorogenic acid and 43% of tea polyphenols were excreted as hippuric acid, and ≈50% of ingested quercetin was excreted as a mixture of mono- and di-hydroxylated phenylacetic acids (Olthof et al., 2003). In this report, we show that the urinary excretion of three phenolic acids increased above baseline following ingestion of fresh blackcurrants (Table 2). However, the mean additional quantity excreted amounted to 30.0 mg, which accounts for only 4.68% of the ingested blackcurrant anthocyanins on a mass percent basis. We are not aware of any other reports describing the excretion of phenolic acids from orally ingested anthocyanins. The predominant flavonoids in blackcurrants are anthocyanins. But, there are also (relatively) small quantities of other flavonoids including flavonols (predominantly quercetin glycosides), and it is possible that the observed increased excretion of the benzoic acid and derivatives reported here arise from other components of the fruits. Recently, it was reported that significant concentrations of anthocyanins were present in the faeces of rats that had been fed an anthocyanin-rich diet ((3.85 g anthocyanin (kg diet)$^{-1}$) (He, Magnuson, & Giusti, 2005). Depending on the source of the anthocyanins, it was reported that the rat faeces contained up to 2.0 g kg^{-1} of anthocyanins and that it was highly coloured (purple, black). Although yields were not reported, it is clear that the rats excreted large proportions of the ingested anthocyanins in the faeces as intact or slightly modified (e.g. methylated) anthocyanins.

Currently, the fate of the bulk of ingested anthocyanins in humans is not known. It is possible that a proportion is excreted in the faeces, as has been demonstrated for rats (He et al., 2005). But, it is also likely that as yet unknown metabolites are formed during intestinal transit, colonic

degradation or through human metabolism. Anthocyanins are intrinsically unstable, and during intestinal transit, they will encounter pH conditions that will facilitate their rapid degradation. Future research should focus on the fate of the ingested anthocyanin that cannot be accounted for as intact anthocyanins or phenolic acid degradation products and on identifying the major products of anthocyanin breakdown within the gastrointestinal tract.

In conclusion, this study has shown that processed blackcurrant products retain only a small fraction of the anthocyanins from the original fruit, and that processing has no significant effect on the oral bioavailability in human subjects which is very poor (<0.1% of dose). Future research concerned with the potential impact of anthocyanins on human health should take into account the very low exposures that cells and tissues beyond the gastrointestinal tract will encounter *in vivo*, and efforts to identify and characterise the products of anthocyanin breakdown should also be considered.

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