Colonic Catabolism of Ellagitannins, Ellagic acid and Raspberry Anthocyanins: In Vivo and In Vitro Studies

Rocío González-Barrio, Christine A. Edwards, and Alan Crozier

Joseph Black Building, Centre for Population and Health Sciences, School of Medicine, College of Medical, Veterinary and Life Sciences. University of Glasgow, Glasgow G12 8QQ, United Kingdom (R.G.-B., A.C.); Centre for Population and Health Sciences, School of Medicine, College of Medical, Veterinary and Life Sciences. University of Glasgow, Yorkhill Hospital, Glasgow G3 8SJ, United Kingdom (C.A.E)
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ABBREVIATIONS: HPLC-PDA-MS, high-performance liquid chromatography-photodiode array-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; BSTFA, N,O-Bis(Timethylsilyl)trifluoroacetamide; OFN, oxygen-free nitrogen; TMCS, trimethylchlorosilane; SPE, solid phase extraction; SDB-L, styrene-divinylbenzene polymer.
ABSTRACT:

Red raspberries contain principally anthocyanins and ellagitannins. Following ingestion of raspberries by humans trace levels of anthocyanins, absorbed in the upper gastrointestinal tract, are excreted in urine in amounts corresponding to <0.1% of intake. Urine also contains urolithin-O-glucuronides derived from colonic metabolism of the ellagitannins. Raspberry feeds with ileostomists show that substantial amounts of the anthocyanin and ellagitannin intake are excreted in ileal fluid. In subjects with an intact functioning colon, these compounds would pass to the large intestine. The aim of this study was to identify raspberry-derived phenolic acid catabolites that form in the colon and those that are subsequently excreted in urine. *In vitro* anaerobic incubation of ellagitannins with fecal suspensions demonstrated conversion to ellagic acid and several urolithins. Fecal suspensions converted 80% of added ellagic acid to urolithins. *In vivo*, urolithins are excreted in urine as O-glucuronides not aglycones indicating that the colonic microflora convert ellagitannins to urolithins while glucuronidation occurs in the wall of the large intestine and/or post-absorption in the liver. Unlike ellagitannins, raspberry anthocyanins were converted *in vitro* to phenolic acids by anaerobic fecal suspensions. Urinary excretion of phenolic acids following ingestion of raspberries indicates that after formation in the colon some phenolic acids undergo phase II metabolism resulting in the formation of products that do not accumulate when anthocyanins are degraded in fecal suspensions. There is a growing realisation that colonic catabolites such as phenolic acids and urolithins may have important roles in the protective effects of a fruit and vegetable-rich diet.
INTRODUCTION

There is evidence from a diversity of sources that a berry-rich diet offers potential protective effects on health (Beattie et al., 2005; Del Rio et al., 2010) with daily consumption of red raspberry juice for 12 weeks by hamsters on an atherogenic diet reducing aortic lipid deposition by ~90% (Rouanet et al., 2010). Red raspberries contain the ellagitannins sanguin H-6 and lambertianin C as well as a characteristic spectrum of anthocyanins, the major constituents being cyanidin-3-O-sophoroside, cyanidin-3-O-(2″-O-glucosyl)rutinoside and cyanidin-3-O-glucoside with smaller quantities of cyanidin-3-O-rutinoside, cyanidin-3-O-(2″-O-xylosyl)rutinoside pelargonidin-3-O-sophoroside, pelargonidin-3-(2″-O-glucosyl)rutinoside, pelargonidin-3-O-glucoside and pelargonidin-3-O-rutinoside (Mullen et al., 2002; Borges et al., 2010). There is evidence linking intake of ellagitannins with health benefits. This based principally on research with pomegranate juice which contains ellagitannins in the form of punicalins and punicalagin (Aviram, et al., 2000; Pantuck et al., 2006; Rosenblant et al., 2006; Belal et al., 2009; Larrosa et al., 2010; Verzelloni et al., 2011).

In our earlier study, after oral ingestion of 300 g of raspberries by human subjects, no anthocyanins were detected in plasma and <0.1% of intake was excreted in urine 0-7 h after ingestion, indicating low level absorption in the proximal gastrointestinal tract (González-Barrio et al., 2010). No ellagitannins were detected in either plasma or urine collected after raspberry ingestion. However, there was evidence of colonic catabolism of ellagitannins with the appearance of urolithin A-O-glucuronide, two of its isomers, and urolithin B-O-glucuronide in urine collected 7-48 h after raspberry consumption. A parallel feeding study, in which 300 g of raspberries were ingested by ileostomists, also detected trace amounts of urinary anthocyanins, but in keeping with their formation in the colon, urolithins were not excreted in urine. Ileal fluid collected after raspberry
consumption contained 40% of the anthocyanin intake and 23% of the ingested sanguin H-6. In healthy subjects with a functioning colon, these compounds would pass from the small to the large intestine where they would be subject to the action of the microbiota (González-Barrio et al., 2010).

This paper reports on further studies into the fate of ellagitannins and anthocyanins in the colon in which, after raspberry consumption by healthy humans, urinary excretion of phenolic acid catabolites was monitored. In addition, products formed during anaerobic in vitro fecal fermentation of i) raspberry anthocyanins, ii) the ellagitannin punicalagin and iii) ellagic acid were investigated.

**Materials and Methods**

**Raspberries and Chemicals.** Raspberries cv. Glen Ample obtained from a commercial grower in Angus, UK were used in the feeding study with humans. Raspberry juice, rich in anthocyanins but containing only traces of ellagitannins and other minor compounds including hydroxycinnamic acid derivatives and flavonols, supplied by Ella Drinks Ltd (Alloa, Clackmannanshire, Scotland), was freeze-dried and the resultant powder was used in in vitro incubations with fecal slurries. Cyanidin-3-*O*-glucoside was purchased from Apin Chemicals (Abingdon, Oxford, UK), cyanidin-3-*O*-sambubioside-5-*O*-glucoside from Polyphenols (Sandnes, Norway) and phenolic acids from Sigma-Aldrich Co Ltd. (Poole, Dorset, UK), Fisher Scientific (Leicestershire, UK), Extrasynthese (Genay, France) and AASC Ltd (Southampton, Hampshire, UK). Punicalagin was supplied by Chromadex (Irvine, CA, USA) and urolithins A and B were a generous gift from Dr Navindra Seeram (University of Rhode Island, Kingston, RI, USA). HPLC grade solvents were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, UK). Formic acid, sodium diethylthiocarbamate, trytone and resazurin...
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were purchased from Sigma-Aldrich. All chemicals and reagents used in the preparation of buffers, macromineral, micromineral and reducing solutions were obtained from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK), Fisher Scientific and BDH Laboratory Supplies (Poole, UK).

**Human Feeding Studies.** The Glasgow Royal Infirmary Research Ethics Committee and the Glasgow University Ethical Committee approved the study protocol. Nine healthy humans (four female and five male) were recruited and gave their written consent to participate in the study. They were non-smokers, not on medication, aged between 25 and 31 years. Subjects were required to follow a diet low in (poly)phenolic compounds by avoiding fruits and vegetables, nuts, high fiber products and beverages such as tea, coffee, fruit juices, as well as to abstain from consuming alcohol 60 h prior to the beginning of the study. After an overnight fast, the volunteers consumed 300 g of raspberries and after 3 h followed the low phenolic diet until the final urine sample was collected. All urine excreted 24 h before and over five time periods, 0-4, 4-7, 7-24, 24-32 and 32-48 h, after raspberry consumption was collected. After recording the volumes of urine, aliquots were stored at –80 ºC until analysis.

**Human Fecal Samples.** For the *in vitro* fecal incubation experiments, healthy subject who participated in the study had a normal diet, were non-smokers, aged from 22-30, had no digestive disease and had not received antibiotics for at least 6 months prior to the study. The donors followed the special diet low in (poly)phenolic compounds for 60 h prior to feces collection. Fecal samples, collected by the donors in a special plastic tub containing an AnaeroGen sacket (Oxoid Ltd., UK) to generate anaerobic conditions, were processed within 1 h of passage.

**Fermentation Medium.** The fermentation media was prepared as described by Jaganath et al. (2009). Briefly, 2 g of tryptone was mixed in 400 ml of distilled water
and 100 μL of micromineral solutions (consisting of 13.2 g of CaCl\_2·2H\_2O, 10 g of MnCl\_2·4H\_2O, 1 g of CoCl\_2·6H\_2O, 8 g of FeCl\_3·6H\_2O, and distilled water up to 100 ml), 200 ml of buffer (2 g of NH\_4HCO\_3, 17.5 g NaHCO\_3, and distilled water up to 500 ml), 200 ml of macromineral solution (2.85 g of NA\_2HPO\_4, 3.1 g KH\_2PO\_4, 0.3 g MgSO\_4·7H\_2O, and distilled water up to 500 ml), and 1 ml of 1% (w/v) of resazurin solution (a redox indicator). The medium was adjusted to pH 7 using HCl, boiled and allowed to cool under oxygen-free nitrogen (OFN) to remove oxygen. Reducing solution (312 mg of cysteine hydrochloride, 2 ml of 1 M NaOH, 312 mg Na\_2S·9H\_2O, and distilled water up to 50 ml) was added at 0.5 ml per 10 ml of medium after which the solution was purged with OFN until anaerobic conditions.

**In Vitro Incubations.** Samples of freshly voided human feces were homogenized with phosphate buffer to obtain 32% fecal suspensions. Five ml of suspension was added to 44 ml of the pre-reduced fermentation medium and 1 ml of substrate in a 100 ml McCartney bottle. Raspberry anthocyanins, punicalagin and ellagic acid, in presence of 0.5 g of glucose, were added to fecal suspensions. Blank fecal suspensions without the addition of substrate were incubated simultaneously. To distinguish between enzymatic and chemical degradation, substrates were also incubated in the presence of heat-inactivated microflora (100 °C, 1 h) obtained from one of the donors.

After the substrates were added, the fermentation bottles were purged with OFN, sealed airtight and placed horizontally in a shaking water bath shaking at 60 stokes/min and protected from the light. The fermentation bottles were incubated at 37 °C over 48 h (raspberry anthocyanins) or 72 h (punicalagin and ellagic acid), aiming to simulate the conditions in the colonic lumen (Edwards et al., 1996). Aliquots of fecal suspensions (6 ml) collected after 0, 2, 4, 6, 24 and 48 h (raspberry anthocyanin incubations), and after 0, 2, 5, 24, 48, and 72 h of (punicalagin and ellagic acid incubations) were stored at –80
ºC prior to analysis. The average initial pH of the cultures was 7.2 and at the end of the incubation period this had dropped to pH 5.8, pH 6.5 and pH 6.3 after raspberry anthocyanins, punicalagin and ellagic acid incubations, respectively.

**Purification of Fecal Suspension Incubations.** Anthocyanins, punicalagin, ellagic acid and urolithins in fecal suspensions were purified using a SDB-L cartridge (500 mg/6 ml, Strata, Phenomenex, Macclesfield, Cheshire, UK) prior to analysis by HPLC-PDA-MS². Aliquots of fecal suspensions (1 ml) were added to 5 ml of 1% aqueous formic acid, vortexed and centrifuged at 4000g for 5 min at 4 ºC. The supernatant obtained was loaded on to a preconditioned SDB-L cartridge, which was washed with 5 ml of 1% aqueous formic acid before eluting anthocyanins, punicalagin, ellagic acid and urolithins with 3.5 ml of MeOH acidified with 1% formic. The methanolic phase was reduced to dryness under nitrogen, dissolved in 100 μl of 1% formic acid in MeOH and 200 μl of 1% aqueous formic acid, vortex-mixed, centrifuge at 16110g for 10 min at 4 ºC and analyzed by HPLC-PDA-MS². Blank fecal suspensions were spiked with known amounts of cyanidin-3-O-sambubioside-5-O-glucoside, ellagic acid, punicalagin and urolithin A in order to assess recovery efficiencies. The solid phase extraction gave a recovery rate of 75–80% for anthocyanins, 98–102% for punicalagin, 60–62% for ellagic acid, and 85–87% for urolithin A.

**Purification of Phenolic Acids in Urine and Fecal Suspensions.** Phenolic acid purification, derivatization and analysis of urine were adapted from Roowi et al. (2010) with some modifications. A 30 μg internal standard of 2,4,5-trimethoxycinnamic acid was added to 1 ml of urine. Samples were acidified by adding 65 μl of 1 M HCl and partitioned three times against 1.5 ml volumes of ethyl acetate. The upper organic phases were combined, transferred to an amber glass vial and dried at 37 ºC under a stream of nitrogen. A 200 μl volume of dichloromethane added to rinse the wall of the
vials was dried under nitrogen at 37 °C. Derivatization reagent (50 µl of BSTFA + 10% TMCS) was added to the vials, which were flushed with a gentle flow of nitrogen prior to sealing. Samples were heated at 70 °C for 4 h with vortexing every 30 min to achieve complete silylation. Anhydrous hexane (350 µl) was added to each sample, prior to analysis by GC-MS.

Purification and analysis of phenolic acids in fecal suspensions was carried out as follows. Briefly, 1 ml of fecal suspension was added to 4 ml of aqueous 0.2 M HCl and the mixture centrifuged at 4000g for 5 min at 4 °C. 2,4,5-Trimethoxycinnamic acid was added to the supernatant which was loaded on to a preconditioned 500 mg SDB-L cartridge which was then washed with 0.1 M HCl before eluting the phenolic acids with ethyl acetate. Residual aqueous phase was separated from the organic phase by centrifugation at 4000g for 10 min at 4 °C. The ethyl acetate extract was then transferred to an amber glass and dried at 37 °C under a slight stream of nitrogen. Samples were further dried by the addition and evaporation of dichloromethane and the dried extract was derivatized as described for urine samples.

**Qualitative and Quantitative Analysis by GC-MS.** Derivatized phenolic acids in urine and fecal suspensions were analyzed on a Trace GC interfaced to a DSQ mass spectrometer equipped with a split/splitless injector and an AI3000 autosampler (Thermo Fisher, Hemel Hampsted, UK). Samples (1 µL) were injected in split mode with a 25:1 ratio. The inlet temperature was maintained at 220 °C. The oven was programmed from 40 °C to 160 °C at 20 °C/min, to 200 °C at 1.5 °C/min, to 250 °C at 10 °C/min to a final temperature of 300 °C at 40 °C/min, held for 5 min. The transfer line was maintained at 310 °C. The helium carrier gas flow rate was 1.2 ml/min. Data acquisition was performed in the positive ionization mode in full scan (m/z 50-470) with an ionization energy of 70 eV, from 5 to 35 min. Acquisition and analysis of GC-MS
data were performed on Xcalibur version 2. Phenolic acids were identified by comparison with the retention times and mass spectra of authentic standards. Quantification was based on 2.5–50 ng calibration curves constructed for a set of phenolic acid standards that were derivatized and analyzed ($R^2>0.96$).

**Qualitative and Quantitative Analysis by HPLC-PDA-MS**

Urine and purified fecal samples were analyzed on a Surveyor HPLC system comprised of an HPLC pump, PDA detector, scanning from 200 to 700 nm, and an autosampler cooled to 4° C (Thermo Finnigan, San Jose, USA). Separations of punicalagin, ellagic acid, urolithins and anthocyanins were performed using a Synergi 4μm RP-POLAR 80Å 250 x 4.6 mm i.d. reverse phase column (Phenomenex, Macclesfield, UK), and maintained at 40° C. The mobile phase, pumped at a flow rate of 1 ml/min, was 0.1 % formic acid and 100% MeOH, establishing a linear gradient from 10 to 40% MeOH over 60 min to analyse anthocyanins, and from 10 to 65% MeOH over 50 min to analyse punicalagin, ellagic acid and urolithins. After passing through the flow cell of the PDA detector the column elute was split and 0.3 ml/min was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface (ESI) in positive mode for anthocyanins and in negative mode for ellagitannins, ellagic acid and urolithins. Analyses were carried out using full scan, data dependant MS$^2$ scanning from $m/z$ 150-1500. With the ESI in positive ionization mode, capillary temperature was 300 °C, sheath gas 50 units, auxiliary gas 35 units, and the source voltage 2 kV. For negative ionization, capillary temperature was 200 °C, sheath gas 60 units, auxiliary gas 20 units, and the source voltage was 6 kV. Anthocyanins were quantified by reference to a 1–1000 ng cyanidin-3-O-glucoside calibration curve ($R^2=0.999$) at 520 nm. Punicalagin and ellagic acid were quantified against standard curves for reference compounds at 280 nm (10–1500 ng, $R^2=0.997$) and 365 nm (1–1000 ng $R^2=0.999$), respectively. Urolithin
A and urolithin B were quantified against standards at 305 nm (0.4–800 ng, $R^2=0.998$) while isourolithin A and urolithin C were quantified at 305 nm in urolithin A equivalents. The identification of these compounds was according to their absorbance and MS spectra based on data previously reported (Borges et al., 2010; Espín et al., 2007; González-Barrio et al., 2011).

**Statistical Analyses.** All samples were analyzed in triplicate and the quantitative estimates represent mean values ± standard error. Data were subjected to statistical analysis of variance (ANOVA). Normality of variances was tested by Shapiro-Wilk test, before determining the ANOVA. Statistical were performed using SPSS V.14 for Windows.

**Results**

**Analysis of Raspberries.** The analysis of the raspberries used in the feeding study has been described previously (González-Barrio et al., 2010). Each 300 g serving contained a total of 204 μmol of anthocyanins and 123 μmol of ellagitannins of which 82% was sanguin H-6. The total ellagic acid (free and conjugated) content was 17 μmol.

**Urinary Excretion of Phenolic acids After the Consumption of Raspberries.** Data on the mean quantities of 10 phenolic acids in urine collected over 24 h periods before and after the ingestion of raspberries by eight subjects are summarized in Table 1. These phenolic acids were identified based on GC-MS analysis by comparison with the retention times and mass spectra of authentic standards (Supplemental Table S-1). All the phenolic acids were present in urine collected both before and after raspberry intake. Small but significant increases in 4'-hydroxymandelic acid, 3',4'-dihydroxyphenylacetic acid, 3-(4'-hydroxyphenyl)lactic acid, 4'-hydroxyhippuric acid
and hippuric acid were observed after raspberry consumption. Excretion of some of the other phenolic acids may well have increased after raspberry intake but because of their presence in control samples, the increases were not statistically significant. This is compounded further by substantial differences in the phenolic acid excretion profile of the individual volunteers, which reflects variations in their colonic microflora. This can be assessed by consulting the information in Table 1 on instances where significant increases in the excretion of specific phenolic acids were observed with some but not all eight volunteers. More detailed information on the phenolic acid excretion pattern of the individual volunteers can be found in supplemental data (Supplemental Table S-2).

In preliminary studies some urine samples were treated with β-glucuronidase and sulfatase enzymes at different concentrations (20, 50, 100 and 200 U of sulfatase H-2 from *Helix pomatia*, 2150 U, which contains residual β-glucuronidase activity) prior to analysis by GC-MS. This did not significantly increase the quantities of phenolic acids detected nor did it change the phenolic acid profiles. This indicates that the phenolic acids monitored in this study are excreted principally as free acids and not as glucuronide or sulfate conjugates.

**Anaerobic Degradation of Raspberry Anthocyanins by Fecal Suspensions.**

The two main anthocyanins identified by HPLC-PDA-MS² in the raspberry juice extract were cyanidin-3-O-sophoroside ($m/z$ 611 → 287) and cyanidin-3-O-(2′′-O-glucosyl)rutinoside ($m/z$ 757 → 611, 287) representing 50% and 30% of the total anthocyanins, respectively. In addition to these compounds, other minor anthocyanins were identified including cyanidin-3-O-glucoside ($m/z$ 449 → 287), pelargonidin-3-O-sophoroside ($m/z$ 595 → 271), cyanidin-3-O-rutinoside ($m/z$ 595 → 287) and pelargonidin-3-O-(2′′-O-glucosyl)rutinoside ($m/z$ 741 → 595, 271). HPLC-PDA-MS² analysis of fecal suspensions incubated with the raspberry anthocyanin extract at a
concentration of 100 μM showed a time-dependent loss of the anthocyanins. None-the-less >20% still remained intact after 48 h (Figure 1). The individual anthocyanins were degraded at a similar rate (data not shown). In order to distinguish between enzymatic and chemical degradation, the raspberry anthocyanin extract was also incubated with fecal suspensions previously deactivated by treating at 100 °C, for 1 h. The heat-treated fecal suspension did not degrade the anthocyanins (Figure 1) demonstrating the losses observed with the activated suspensions were a consequence of the enzymatic activity of the colonic microbiota.

In addition to analysing the anthocyanin content of the fecal incubations, phenolic acid levels were determined by GC–MS. The data obtained are presented in Table 2. Typical GC traces are illustrated in Figure 2 while retention time data and mass spectra of the trimethylsilyl derivatives can be found in Supplemental Table S-1. Although the concentration of 3-phenyllactic acid and 3-(4’-hydroxyphenyl)propionic acid rose, there was a high background and the increases were not statistically significant, in contrast to the increases in catechol, resorcinol, pyrogallol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, tryrosol, 3-(3’-hydroxyphenyl)propionic acid and 3-(3’,4’-dihydroxyphenyl)propionic acid. The concentration of 3-(4’-hydroxyphenyl)lactic acid also increased but because of the high background this was significant only at the 24 h time point (Table 2). Incubations with heat–treated fecal suspensions, in keeping with their inability to degrade the raspberry anthocyanins did not accumulate phenolic acid demonstrating the role of the colonic microbiota in the formation of these catabolites (data not shown).

**Anaerobic Degradation of Ellagitannins Incubated with Fecal Suspensions.**

The raspberry ellagitannins sanguin H-6 and lambertianin C were not readily available in quantity so fecal incubations from five volunteers were carried out with punicalagin,
a structurally similar ellagitannin. Punicalagin is found in pomegranates and, like sanguin H-6 and lambertianin C, can be hydrolyzed to yield ellagic acid which is converted to urolithins (Clifford and Scalbert, 2000; Cerdá et al., 2005; Crozier et al., 2009). Although punicalagin was added to the incubations at a concentration of 100 µM only ~50 µM was detected at the beginning of the incubation period. Ellagic acid was also detected at time 0 h time point, suggesting that the reduction in intitial punicalagin concentration may, in part, be attributed to hydrolysis of the ellagitannin releasing ellagic acid (Figure 3). Ellagic acid declined between the 0 h and 5 h time points and thereafter was present albeit in low concentrations. The concentration of total urolithins increased over the incubation period reaching a plateau after 48-72 h. The concentration of punicalagin declined for the first five hours of incubation after which it remained stable at ~ 20 µM (Figure 3).

As with the excretion of urinary phenolic acids, there was marked variation in both the rate of decline of punicalagin and the appearance of ellagic acid and urolithins in fecal suspensions obtained from different volunteers (Table 3). Residual punicalagin concentrations at the end of the 72 h incubation period were 26, 21, 13 15 and 1.3 µM while final total urolithin concentrations were 27.4, 2.9, 1.3, 0.3 and 0 µM. The type of urolithin to accumulate also varied substantially with low amounts of urolithins A and C being most prevalent while a high concentration of isourolithin A was produced exclusively by donor IV (Table 3). Marked person-to-person variations were previously observed in the urinary excretion of urolithins following consumption of raspberry and other ellagitannin-containing food (Cerdá et al., 2005; González-Barrio et al., 2010).

In contrast to the production of urolithins, incubation of punicalagin with fecal suspensions did not result in the synthesis of significant amounts of phenolic acids.
Anaerobic Incubation of Ellagic acid Fecal Suspensions. In view of its role as an intermediate in the conversion of ellagitannins to urolithins (Larrosa et al., 2006), ellagic acid was incubated with fecal suspensions at a concentration of 50 µM for periods of up to 72 h. At the 0 h time point the ellagic acid concentration had declined by 50% possibly because of its limited solubility (Figure 4). During the initial phase of the incubation ellagic acid declined further and after 5 h the mean concentration was <1.0 µM. Urolithins began to appear in the incubates after 5 h and thereafter rose rapidly and the average concentration after 72 h was 22 µM compared to the initial mean 0 h ellagic acid concentration of 28 µM. This indicates a ~80% conversion of ellagic acid to urolithins. As with the punicalagin incubations there was much variation in the profile of urolithins that were formed from ellagic acid with urolithin A, iso-urolithin A, and urolithin C being detected in most incubations while the fecal suspension from one volunteers also produced urolithin B (Table 4). These data are based on HPLC-PDA-MS² analysis with typical traces illustrated in supplemental data (Figure S-1). More detailed information on the identification of urolithins based on absorbance and mass spectra can be found in Supplemental Figure S-2.

Discussion

In our earlier human feeding study in which subjects ingested 300 g of raspberries anthocyanins were detected in urine in amounts corresponding to 0.1% of intake. Urolithin-\(O\)-glucuronides, derived from colonic breakdown of the ellagitannins sanguiin H-6 and lambertianin C, were also excreted with much person-to-person variation in the amounts (0–8.5% of ellagitannin intake) and the spectrum of urinary urolithins which comprised \(O\)-glucuronides of urolithin A, two isomers of urolithin A and urolithin B. Following an intake of 300 g of raspberry by volunteers with an ileostomy 23% of the
ellagitannin intake was detected in ileal fluid. In subjects with an intact functioning colon the ellagitannins would pass from the small to the large intestine where they would be converted to urolithins by the colonic microflora (González-Barrio et al., 2010). These events were investigated in more detail in the current investigation.

The in vitro anaerobic metabolism of ellagitannins and ellagic acid by fecal suspensions was investigated. The fecal suspensions converted punicalagin to ellagic acid, urolithin A, isourolithin A and urolithin C with substantial variation in the profile obtained with the individual fecal samples (Table 3, Figure 3), reflecting the person to person differences in the colonic microflora. Ellagic acid was readily converted to urolithins by the fecal suspensions with yields of ~80% (Table 4, Figure 4). Although formed in greater amounts than from punicalagin, the type of urolithins that were produced was very similar except for the additional presence of small amounts of urolithin B. In these in vitro incubations only urolithin aglycones were produced while in vivo urolithin-O-glucuronides are excreted in urine (González-Barrio et al., 2010). This indicates that the colonic microflora are responsible for the conversion of ellagitannins to urolithins, probably by the pathway illustrated in Figure 5, while glucuronidation is a feature of phase II metabolism either in the wall of the large intestine and/or post-absorption in the liver. It is also of interest to note that incubation of punicalagin and ellagic acid with the fecal suspensions did not result in any significant increases in phenolic acid so both substrates appear to be resistant to bacterial-mediated ring fission.

In our earlier raspberry feeds with ileostomists ~40% of the anthocyanin intake was detected in ileal fluid (González-Barrio et al., 2010). The fate of these anthocyanins was investigated in the current study i) by monitoring the in vitro catabolism of raspberry anthocyanins when incubated with fecal suspensions and ii) by analysing
phenolic acids excreted in urine collected from healthy subjects, with a functioning colon, after raspberry consumption.

Incubation of the predominantly cyanidin-based raspberry anthocyanins with fecal suspensions for 48 h resulted in their partial degradation by the colonic microflora. Surprisingly, as the incubations were not carried out under acidic conditions, ~20% of the anthocyanins remained intact at the end of the incubation period (Figure 1). The in vitro colonic microflora-mediated breakdown of the anthocyanins resulted in the accumulation of significant quantities of catechol, resorcinol, pyrogallol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, tryrosol, 3-(3’-hydroxyphenyl)propionic acid, 3-(3’,4’-dihydroxyphenyl)propionic acid and 3-(4’-hydroxyphenyl)lactic acid (Table 2). Analysis of urine after the consumption of 300 g of raspberries by healthy volunteers enabled the phase II metabolism of these phenolic acids to be evaluated during their passage through the wall of the colon and transport through the circulatory system prior to excretion. As noted previously ellagitannins and ellagic acid were not converted in significant amounts to phenolic acids in by fecal suspensions so the urinary phenolics are in all probability derived principally from the raspberry anthocyanins. After raspberry consumption significant increases were observed in urinary excretion of 4’-hydroxymandelic acid, 3’,4’-dihydroxyphenylacetic acid, 3-(4’-hydroxyphenyl)lactic acid, 4’-hydroxyhippuric acid and hippuric acid (Table 1). Possible routes for the production of these phenolic acids, from ring A and ring B during the C-ring fission of cyanidin skeleton, which are in keeping with proposals of Aura (2008), are illustrated in Figure 6.

It is likely that the colonic breakdown of cyanidin-based anthocyanins is more complex that the routes illustrated in Figure 6. Excretion of some of the other phenolic acids listed in Table 1 may well have increased after raspberry intake but because of
their high background levels, the increases were not statistically significant. This is further compounded by both the substantial differences in the phenolic acid excretion of the individual volunteers, which reflects variations in their colonic microflora, and the fact that some of the phenolic acids are produced by additional routes from sources unrelated to the ingested cyanidin-based anthocyanins. It is, for instance, well known that there are pathways to hippuric acid from benzoic acid, quinic acids (Clifford et al., 2000), tryptophan, tyrosine and phenylalanine (Self et al., 1960; Grümmer, 1961). None-the-less, the elevated urinary excretion of hippuric acid occurring after raspberry consumption is likely to be partially derived from anthocyanin degradation.

Monitoring the in vitro and in vivo fate of ellagitannins in the colon is more straightforward than that of anthocyanins and other flavonoids because they appear to be converted predominantly to urolithins, which unlike phenolic acids, are otherwise not produced by the body. While the type of study we have undertaken provides useful evidence of the degradation of dietary flavonoids and related compounds to phenolic acids, a full elucidation of the pathways involved awaits studies using stable isotope labelled dietary flavonoids. Finally, there is a growing awareness that colon-derived phenolic acid may play a role in the potential protective effects of dietary flavonoids and related compounds. Colonic catabolites from (poly)phenolic compounds present in several fruits and beverages have been shown to have antiglycative and neuroprotective effects (Verzelloni et al., 2011) and phenolic catabolites are also reported to have anti-inflammatory effects (Larrosa et al., 2009).
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Authorship Contributions

Participated in research design: González-Barrio, Crozier and Edwards.

Performed data analysis: González-Barrio.

Wrote or contributed to the writing of the manuscript: González-Barrio and Crozier wrote the manuscript which was edited by Edwards.


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Address correspondence to: Professor Alan Crozier, North Laboratories, Joseph Black Building, Centre for Population and Health Sciences, School of Medicine, College of Medical, Veterinary and Life Sciences. University of Glasgow, Glasgow G12 8QQ, United Kingdom. E-mail: alan.crozier@glasgow.ac.uk
Figure Legends

FIG. 1. Anaerobic degradation of raspberry anthocyanins in the presence of heat-inactivated (○) and active microflora (●) in fecal suspensions. Bars represent standard errors of mean values (n = 6). The initial concentration of anthocyanins glycosides was 100 μM.

FIG. 2. GC-MS traces of phenolic acids in fecal suspensions of healthy donors incubated with raspberry anthocyanins, collected at the times indicated. (1) catechol, (2) resorcinol, (3) pyrogallol, (4) tyrosol, (5) 3-phenyllactic acid, (6) 4-hydroxybenzoic acid, (7) 4'-hydroxyphenylacetic acid, (8) 3-(3'-hydroxyphenyl)propionic acid, (9) 3-(4'-hydroxyphenyl)propionic acid, (10) 3-(4'-hydroxyphenyl)lactic acid.

FIG. 3. Anaerobic metabolism of punicalagin to ellagic acid and urolithins in fecal suspensions. Bars represent standard errors of mean values (n = 5). The initial concentration of punicalagin was 100 μM.

FIG. 4. Anaerobic metabolism of ellagic acid to urolithins in fecal suspensions. Bars represent standard errors of mean values (n = 4). The initial concentration of ellagic acid was 50 μM.

FIG. 5. Proposed pathways for the conversion of punicalagin to ellagic acid and urolithins in anaerobic fecal suspensions.

FIG. 6. Proposed pathways for the conversion of cyanidin-based red raspberry anthocyanins to phenolic acids. Following consumption of 300 g of raspberries ~40% of the ingested anthocyanins, principally cyanidin-O-glycosides, pass into the large intestine. When raspberry anthocyanins are incubated with fecal suspensions under
anerobic conditions the cyanidin aglycone is released and catabolized by the colonic microflora undergoing C-ring fission releasing phenolic acids, originating from both the A- and B-rings, which are metabolised via the pathways illustrated. Analysis of urine collected after raspberry consumption indicates that some of the colonic catabolites enter the circulatory and undergo further metabolism before being excreted in urine. These catabolites are thus detected in urine but not fecal suspensions. F – catabolites detected in fecal suspensions; U – catabolites detected in urine (also highlighted in grey); * – potential intermediates that did not accumulate in detectable quantities.
TABLE 1

Quantities of phenolic acids excreted in urine by humans 24 h before and after the consumption of 300 g of raspberries

<table>
<thead>
<tr>
<th>Urinary phenolic acids</th>
<th>Control</th>
<th>0-24 h</th>
<th>Subjects with significant increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>4'-Hydroxybenzoic acid</td>
<td>6.4 ± 1.1</td>
<td>7.4 ± 1.5</td>
<td>1/8</td>
</tr>
<tr>
<td>4'-Hydroxymandelic acid</td>
<td>10 ± 2</td>
<td>15 ± 1*</td>
<td>4/8</td>
</tr>
<tr>
<td>3'-Methoxy-4'-hydroxymandelic acid</td>
<td>13 ± 2</td>
<td>15 ± 1</td>
<td>3/8</td>
</tr>
<tr>
<td>3',4'-Dihydroxyphenylacetic acid</td>
<td>2.0 ± 0.6</td>
<td>3.4 ± 0.3*</td>
<td>4/8</td>
</tr>
<tr>
<td>4'-Hydroxyphenylacetic acid</td>
<td>65 ± 12</td>
<td>87 ± 7</td>
<td>3/8</td>
</tr>
<tr>
<td>3-(3'-Hydroxyphenyl)hydracrylic acid</td>
<td>16 ± 6</td>
<td>14 ± 4</td>
<td>2/8</td>
</tr>
<tr>
<td>3-(4'-Hydroxyphenyl)lactic acid</td>
<td>3.8 ± 0.7</td>
<td>6.5 ± 0.8*</td>
<td>2/8</td>
</tr>
<tr>
<td>3'-Methoxy-4'-hydroxyphenylacetic acid</td>
<td>18 ± 2</td>
<td>17 ± 2</td>
<td>1/8</td>
</tr>
<tr>
<td>4'-Hydroxyhippuric acid</td>
<td>28 ± 5</td>
<td>50 ± 9*</td>
<td>4/8</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>589 ± 95</td>
<td>1390 ± 134*</td>
<td>6/8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>751 ± 108</strong></td>
<td><strong>1606 ± 137</strong>*</td>
<td><strong>6/8</strong></td>
</tr>
<tr>
<td><strong>Total without hippuric acid</strong></td>
<td><strong>163 ± 24</strong></td>
<td><strong>216 ± 17</strong></td>
<td><strong>2/8</strong></td>
</tr>
</tbody>
</table>

aData expressed as mean values in μmol ± standard error (n=8). * denotes mean values increased significantly after raspberry consumption (p < 0.05).
### TABLE 2

Mean concentration of phenolic acids detected in fecal suspensions from six donors incubated for 0, 6, 24 and 48 h with raspberry anthocyanins at a concentration 100 μM

<table>
<thead>
<tr>
<th>Phenolic acids</th>
<th>Incubations</th>
<th>0 h</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 2.7 ± 0.7*</td>
<td>2.7 ± 1.0*</td>
<td>2.9 ± 1.3*</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 1.2 ± 0.4*</td>
<td>1.4 ± 0.4*</td>
<td>1.4 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>Resorcinol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 3.0 ± 1.6*</td>
<td>2.3 ± 1.3*</td>
<td>2.3 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>Pyrogallol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 3.0 ± 1.6*</td>
<td>2.3 ± 1.3*</td>
<td>2.3 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 5.1 ± 0.4*</td>
<td>13 ± 1.4*</td>
<td>15 ± 1.5*</td>
<td>14 ± 1.6*</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 7.4 ± 0.4*</td>
<td>8.3 ± 1.0*</td>
<td>8.7 ± 1.2*</td>
<td>11 ± 1.8*</td>
</tr>
<tr>
<td>Tyrosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 2.6 ± 0.2*</td>
<td>15 ± 2.2*</td>
<td>17 ± 2.1*</td>
<td>15 ± 2*</td>
</tr>
<tr>
<td>3-Phenyllactic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>1.7 ± 1.0</td>
<td>52 ± 18</td>
<td>50 ± 22</td>
<td>41 ± 19</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>3.8 ± 1.2</td>
<td>40 ± 7.6</td>
<td>82 ± 15</td>
<td>91 ± 25</td>
</tr>
<tr>
<td>3-(4'-Hydroxyphenyl)lactic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>0.2 ± 0.2</td>
<td>10 ± 3.1</td>
<td>14 ± 4.6</td>
<td>14 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>1.0 ± 0.5</td>
<td>15 ± 1.5</td>
<td>29 ± 3.2*</td>
<td>31 ± 7.0</td>
</tr>
<tr>
<td>3-(3'-Hydroxyphenyl)propionic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>1.1 ± 1.1</td>
<td>1.0 ± 1.0</td>
<td>1.1 ± 0.8</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>2.0 ± 1.0</td>
<td>13 ± 1.9*</td>
<td>13 ± 2.3*</td>
<td>12 ± 2.6*</td>
</tr>
<tr>
<td>3-(4'-Hydroxyphenyl)propionic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>5.3 ± 4</td>
<td>7.9 ± 6.2</td>
<td>10 ± 7.1</td>
<td>11 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>6.8 ± 3.7</td>
<td>13 ± 5.9</td>
<td>16 ± 5.3</td>
<td>15 ± 5.2</td>
</tr>
<tr>
<td>3-(3',4'-Dihydroxyphenyl)propionic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>n.d. 2.3 ± 2.3</td>
<td>2.7 ± 2.7</td>
<td>2.1 ± 2.1</td>
<td></td>
</tr>
</tbody>
</table>

* Data expressed as mean values in μM ± standard error (n=6). * indicates values significantly higher than blank incubations values at p<0.05. n.d., not detected. *Treated and blank incubations, with or without the addition of raspberry anthocyanins, respectively.
**TABLE 3**

In vitro anaerobic metabolism of punicalagin to ellagic acid and urolithins in fecal suspensions from five donors. Quantities of substrate and products detected in fecal samples taken 0, 6, 24 and 48 h after incubation of punicalagin at a concentration 100 μM.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Compounds</th>
<th>0 h</th>
<th>2 h</th>
<th>5 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Punicalagin</td>
<td>60 ± 2</td>
<td>67 ± 2</td>
<td>33 ± 1</td>
<td>27 ± 1</td>
<td>19 ± 8</td>
<td>13 ± 1</td>
</tr>
<tr>
<td></td>
<td>Ellagic Acid</td>
<td>2.7 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.3 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.3 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>II</td>
<td>Punicalagin</td>
<td>44 ± 0</td>
<td>52 ± 2</td>
<td>20 ± 0</td>
<td>24 ± 0</td>
<td>25 ± 2</td>
<td>21 ± 9</td>
</tr>
<tr>
<td></td>
<td>Ellagic Acid</td>
<td>3.1 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>0.4 ± 0.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.4 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.4 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>III</td>
<td>Punicalagin</td>
<td>65 ± 2</td>
<td>64 ± 2</td>
<td>33 ± 1</td>
<td>33 ± 0</td>
<td>33 ± 0</td>
<td>26 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Ellagic Acid</td>
<td>2.8 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>IV</td>
<td>Punicalagin</td>
<td>46 ± 6</td>
<td>22 ± 0</td>
<td>5.8 ± 0.3</td>
<td>1.9 ± 0.1</td>
<td>1.1 ± 0.5</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Ellagic Acid</td>
<td>2.6 ± 0.9</td>
<td>0.9 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4 ± 0.0</td>
<td>2.0 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Isourolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.2 ± 0.0</td>
<td>23 ± 0.6</td>
<td>24 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.2 ± 0.0</td>
<td>2.9 ± 0.1</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4 ± 0.0</td>
<td>4.4 ± 0.1</td>
<td>26 ± 0.7</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>V</td>
<td>Punicalagin</td>
<td>60 ± 4</td>
<td>21 ± 0.3</td>
<td>13 ± 0.7</td>
<td>16 ± 2</td>
<td>14 ± 3</td>
<td>15 ± 3</td>
</tr>
<tr>
<td></td>
<td>Ellagic Acid</td>
<td>2.7 ± 0.5</td>
<td>0.7 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.2 ± 0.0</td>
<td>1.8 ± 0.0</td>
<td>2.0 ± 0.1</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.8 ± 0.0</td>
<td>1.8 ± 0.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.2 ± 0.0</td>
<td>1.8 ± 0.0</td>
<td>2.8 ± 0.0</td>
<td>2.9 ± 0.0</td>
</tr>
</tbody>
</table>

*aData expressed as mean values of individual donor in μM ± standard error (n=3).
TABLE 4

*In vitro anaerobic metabolism of ellagic acid to urolithins in fecal suspensions from four donors. Quantities of substate and products detected in fecal samples taken 0, 6, 24 and 48 h after incubation of ellagic acid at a concentration 50 μM*<sup>a</sup>

<table>
<thead>
<tr>
<th>Donor</th>
<th>Compounds</th>
<th>0 h</th>
<th>2 h</th>
<th>5 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ellagic Acid</td>
<td>18 ± 0</td>
<td>12 ± 0</td>
<td>6.5 ± 0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.5 ± 0</td>
<td>0.4 ± 0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Isourolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8.5 ± 0</td>
<td>10 ± 0</td>
<td>12 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.9 ± 0</td>
<td>4.9 ± 0</td>
<td>5.6 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.5 ± 0</td>
<td>13 ± 0</td>
<td>15 ± 0</td>
<td>18 ± 0</td>
</tr>
<tr>
<td>II</td>
<td>Ellagic Acid</td>
<td>23 ± 2</td>
<td>11 ± 0</td>
<td>10 ± 0</td>
<td>1.6 ± 0</td>
<td>1.3 ± 0</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.7 ± 0</td>
<td>16 ± 0</td>
<td>11 ± 0</td>
<td>1.1 ± 0</td>
</tr>
<tr>
<td></td>
<td>Isourolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.9 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.6 ± 0</td>
<td>12 ± 0</td>
<td>23 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin B</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.9 ± 0</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.7 ± 0</td>
<td>17 ± 0</td>
<td>23 ± 0</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>III</td>
<td>Ellagic Acid</td>
<td>32 ± 6</td>
<td>21 ± 0</td>
<td>9.2 ± 0</td>
<td>1.0 ± 0</td>
<td>0.6 ± 0</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.9 ± 0</td>
<td>14 ± 0</td>
<td>9.0 ± 0</td>
<td>6.7 ± 0</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.9 ± 0</td>
<td>14 ± 0</td>
<td>15 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.9 ± 0</td>
<td>17 ± 0</td>
<td>23 ± 0</td>
<td>22 ± 0</td>
</tr>
<tr>
<td>IV</td>
<td>Ellagic Acid</td>
<td>40 ± 1</td>
<td>30 ± 0</td>
<td>13 ± 0</td>
<td>0.9 ± 0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin C</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.3 ± 0</td>
<td>8.3 ± 0</td>
<td>0.1 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Isourolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6.6 ± 0</td>
<td>15 ± 0</td>
<td>13 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4.1 ± 0</td>
<td>7.8 ± 0</td>
<td>8.2 ± 0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urolithin B</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.8 ± 0</td>
<td>1.7 ± 0</td>
<td>1.1 ± 0</td>
</tr>
<tr>
<td></td>
<td>Total Urolithins</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.3 ± 0</td>
<td>19 ± 0</td>
<td>23 ± 0</td>
<td>23 ± 0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed as mean values of individual donor in μM ± standard error (n=3)
Figure 2.
Figure 3.
Figure 4.