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New insights into the bioavailability of red raspberry anthocyanins and ellagitannins

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Abbreviations:

ADME, absorption, disposition, metabolism and excretion; $C_{max,}$ peak plasma concentration;

CID, collision-induced dissociation; ESI, electrospray interface; GIT, gastrointestinal tract; PDA, photodiode array; T_{max}, time to reach peak plasma concentration; UHPLC-MS; ultra high performance liquid chromatography-mass spectrometry

ABSTRACT

Red raspberries, containing ellagitannins and cyanidin-based anthocyanins, were fed to volunteers and metabolites appearing in plasma and urine were analysed by UHPLC-MS. Anthocyanins were not absorbed to any extent with sub nmol/L concentrations of cyanidin-3-glucoside and a cyanidin-*O*-glucuronide appearing transiently in plasma. Anthocyanins excreted in urine corresponded to 0.007% of intake. More substantial amounts of phase II metabolites of ferulic acid and isoferulic acid, along with 4'-hydroxyhippuric acid, potentially originating from pH-mediated degradation of cyanidin in the proximal gastrointestinal tract, appeared in urine and also plasma where peak concentrations were attained 1-1.5 h after raspberry intake. Excretion of 18 anthocyanin-derived metabolites corresponded to 15.0% of intake, a figure substantially higher than obtained in other anthocyanin feeding studies. Ellagitannins pass from the small to the large intestine where the colonic microbiota mediate their conversion to urolithins A and B which appeared in plasma and were excreted almost exclusively as sulfate and glucuronide metabolites. The urolithin metabolites persisted in the circulatory system and were excreted in urine for much longer periods of time than the anthocyanin metabolites although their overall urinary recovery was lower at 7.0% of intake. It is events originating in the proximal and distal gastrointestinal tract, and subsequent phase II metabolism, that play an important role in the bioavailability of both anthocyanins and ellagitannins and it is their metabolites which appear in the circulatory system, that are key to elucidating the mode of action(s) underlying the protective effects of these compounds on human health.

Keywords Raspberries; Anthocyanins; Ellagitannins; Bioavailability; In vivo metabolites; Plasma; Urine

Introduction

Red raspberries (*Rubus idaeus* L.) are a rich source of polyphenolic compounds, the main components being anthocyanins and ellagitannins [1]. There is growing evidence linking consumption of these compounds with beneficial effects on human health: anthocyanins, such as cyanidin-3-*O*-glucoside, have cardioprotective effects [2], while ellagitannins exhibit anti-inflammatory and anti-cancer properties [3]. However, the mechanisms involved remain poorly understood principally because of a limited understanding of the absorption, disposition, metabolism and excretion (ADME), synonymous with bioavailability in nutrition research, of ellagitannins and anthocyanins.

Ellagitannins, which occur in raspberries as well as other berries and fruits [4], are not absorbed per se but are subjected to the action of the colonic microbiota releasing ellagic acid that is converted to urolithins which are absorbed into the circulatory system mainly as sulphate and glucuronide phase II metabolites [5, 6]. Anthocyanins are widely reported to have low bioavailability, with the majority of studies recording peak plasma concentrations (C_{max}) ranging from 1 to 120 nmol/L [7] and urinary recoveries <2% of intake [8,9]. Much lower levels of excretion were evident after feeding Concord grape juice [10] while Wu et al. [11] reported a urinary recovery of blueberry anthocyanins fed to elderly women corresponding to 0.004% of intake. However, a recent study has shown extensive colonic microbiota-mediated degradation of $^{13}C_5$-labeled cyanidin-3-*O*-glucoside which resulted in the production of a wide range of phenolic metabolites over a 0-48 h period. Relative bioavailability assessed by recovery of the ^{13}C-label was 6.9% in breath as CO_2, with 5.4% in urine and 32% in feces in the form of ^{13}C-labeled phenolic and aromatic compounds [12,13]. Unlike other (poly)phenols, anthocyanins are subject to pH-dependent transformations before reaching the large bowel [14]. The early presence of anthocyanin breakdown products in the bloodstream could be associated with chemical decomposition at neutral pH [15] as well as first pass metabolism in the intestinal wall and/or the liver [16].

Potential in vivo health effects of anthocyanins are almost certainly the consequence of the presence of their metabolites in the circulatory system rather than the parent compounds that are present in the ingested foods, and the same applies to ellagitannins. An understanding of the ADME of anthocyanins and ellagitannins is, therefore, crucial for the elucidation of the mechanisms responsible for the health benefits of raspberry polyphenols. This study used UHPLC-MS to further explore the identity and amounts of the phenolic metabolites of anthocyanins and ellagitannins in human plasma and urine following acute ingestion of raspberries, building on earlier studies by González-Barrio et al. [5,6].

Materials and methods

Chemicals and reagents

Raspberries cv. Glen Magna were obtained from a commercial grower in Angus, U.K. Cyanidin-3-*O*-glucoside, ellagic acid, ferulic acid, 3'-methoxy-4'-hydroxyphenylacetic acid (aka homovanillic acid), 3',4'-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxybenzoic acid (aka vanillic acid), 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (aka protocatechuic acid), hippuric acid, acetic acid and formic acid were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). 3',4'-Dihydrocaffeic acid-3'-sulfate (aka 4'-hydroxyphenylpropionic acid-3'-sulfate), ferulic acid-4'-*O*-sulfate and isoferulic acid-3'-*O*-D-glucuronide, were obtained from Toronto Research Chemical, (Toronto, Canada). Urolithin A, urolithin B and urolithin B-3-*O*-glucuronide were provided by Prof. O. Dangles (INRA, Avignon, France). HPLC grade solvents were obtained from Fisher Scientific (Loughborough, UK).

Study protocol

The study and protocol were approved by the West of Scotland Research Ethics Service (ref: 12/WS/0192) and registered at ClinicalTrials.gov (NCT02520596). The criteria for volunteers to participate in the study were to be in good health, a non-smoker, not taking any medication or nutritional supplement, not being pregnant or breast feeding. Ten volunteers (5 female, 5 male) were recruited and gave their written consent to participate in this study. One male volunteer subsequently withdrew from the study. The remaining 9 volunteers were aged between 22 and 44 years, with an average height of 1.72 ± 0.10 m (mean $\pm$ SD), an average weight of 73.3 ± 17.0 kg, and a mean body mass index of 24.6 ± 5.0 kg/m. Subjects were required to follow a diet low in (poly)phenolic compounds by avoiding fruits and vegetables, nuts, high-fibre products, and beverages such as tea, coffee and fruit juices, as well as to abstain from consuming alcohol, for 48 h prior to and during the study. Following an overnight fast, the volunteers consumed 300 g of homogenized raspberries and, except for water intake to maintain adequate levels of hydration, but no other food or drink was allowed for the next 3 h. Lunch was provided and consisted of a selection of white rolls, ham, margarine spread, cheese and ready salted crisps. Volunteers continued the low (poly)phenolic diet for a further 48 h. All urine excreted for 12 h prior to supplementation was collected in acidified methanol as well as that excreted over 48 h after ingestion of raspberries which was collected over the following periods: 0-4 h, 4-8 h, 8-24 h, 24-32 h and 32-48 h. Blood samples were collected in heparin tubes before (0 h) and after raspberry consumption at 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, and 24 h. Plasma obtained by centrifugation of whole blood at 2000 g for 15 min at 5°C, was separated into 1mL aliquots which were acidified to pH 3 with 15 μL of 50% formic acid, and stored at -80°C. The volumes of urine excreted were measured, and aliquots were stored at -80°C.

Extraction of raspberries

Five grams of raspberries were homogenised in 15 mL of methanol/water/formic acid (75:25:1, v/v/v) and ultrasonicated for 1 h at 5°C. Samples were then centrifuged at 5°C for 10 min at 4000 g and

10 µL of the supernatant analysed by HPLC with photodiode array (PDA) and mass spectrometric (MS) detection.

Processing of urine and plasma

Urine samples were defrosted, adjusted to pH 2.5, vortexed, centrifuged at 16110 g for 10 min at 5°C, and passed through 0.45 µm filter discs prior to the analysis of 5 µL aliquots by UHPLC-MS.

Plasma samples were defrosted, vortexed and 400 µL aliquots were mixed with 1 mL of 2% formic acid in acetonitrile. The samples were then vortexed and ultrasonicated for 10 min. After centrifugation at 16110 g for 10 min, supernatants were reduced to dryness in vacuo using a Speedvac concentrator (Thermo Scientific Inc., San Jose, CA) and resuspended in 100 µL of methanol:water:formic acid (50:50:0.1, v/v/v) which was centrifuged at 16100 g for 10 min and 5 µL aliquots of the supernatant analysed by UHPLC-MS^n and UHPLC- MS^2.

Qualitative and quantitative analysis of anthocyanins, ellagic acid and ellagitannins in raspberries by HPLC-PDA-MS^2

Quantitative analysis of (poly)phenols in raspberries was carried out using a Surveyor HPLC (Thermo Scientific Inc.) with an autosampler cooled at 6°C and a PDA detector scanning from 200-600 nm. The injection volume was 10 µL. Chromatography was carried out at 40°C using a Synergy 4 µm Polar-RP 250 x 4.6 mm, reversed phase column (Phenomenex, Macclesfield, UK). The mobile phase, pumped at a flow rate of 1 mL/min, was (A) water/trifluoroacetic acid (99:1, v/v) and (B) methanol/ trifluoroacetic acid (99:1, v/v). The gradient started with 10% B at time 0 min and reached 45% B after 70 min. Chromatograms were recorded at 280, 365 and 520 nm.

Identifications were confirmed using the same instrument but after passing through the PDA detector flow cell, the eluate was split and 0.3 mL/min directed to a LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (ESI) (Thermo Scientific Inc.). The mobile phase, pumped at a flow rate of 1mL/min, was water/ formic acid (99:1, v/v) (A) and methanol/formic acid (99:1, v/v) (B). The gradient started with 10% B at time 0 min and reached 45% B at 70 min. The mass spectrometer was operated in positive ionization mode for anthocyanin analysis and in negative ionization for ellagitannin and ellagic acid identifications. Capillary temperature was 300°C, sheath gas flow was 40 units, while the auxiliary gas was set to 10 units. In negative ionization mode the source voltage was 3 kV. The capillary and tube lens voltage were 4 and 25 V, respectively. When used in positive ionization mode the source voltage was 3.5 kV, while capillary and tube lens voltage were -30 and -60 V, respectively. Analysis was initially carried out in full-scan, data-depending scanning from *m/z* 150 to 2000 and identification was confirmed by selected reaction monitoring (SRM). Data processing was performed using Xcalibur software (Thermo Scientific).

Anthocyanins were quantified by PDA at 520 nm, and expressed as cyanidin-3-*O*-glucoside equivalents, whereas ellagitannins and ellagic acid conjugates were quantified at 280 nm and 365 nm, respectively, and expressed as ellagic acid equivalents.

Qualitative and quantitative analysis of ellagic acid, urolithins and phenolic acid derivatives in urine and plasma samples by UHPLC-MS[2]

Ellagic acid, urolithins and phenolic derivatives were analysed using a DIONEX Ultimate 3000 UHPLC equipped with a TSQ Vantage triple quadrupole mass spectrometer fitted with a heated-ESI (Thermo Scientific Inc.). For UHPLC, mobile phase A was 0.5% formic acid in water and mobile phase B was acetonitrile containing 0.5% formic acid. Separations were performed with a Kinetex PFP (50 × 2.1 mm), 2.6 μm particle size, column (Phenomenex). The gradient started with 1% B, isocratic conditions

were maintained for 1 min, and reached 40% B after 15 min, followed by 2 min at 80% B and then 4 min at 1% B to re-equilibrate the column. The flow rate was 0.3 mL/min, the injection volume was 5 µL, and the column temperature was at 35°C.

The applied MS method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in SRM mode, with negative ionization. The capillary temperature was 270 °C, and the source was operated at 200 °C. The sheath gas flow was 50 units, and auxiliary gas pressure was set to 5 units. The source voltage was 3 kV. Ultra high-purity argon gas was used for collision-induced dissociation (CID). Data processing was performed using Xcalibur software (Thermo Scientific Inc.).

Quantification was performed with calibration curves of standards, when available. Dimethylellagic acid glucuronide, (iso)urolithin A-*O*-glucuronides and sulfoglucuronide were quantified in urolithin-B-3-*O*-glucuronide equivalents. Urolithin-A sulfate was expressed as urolithin A equivalents, while isoferulic acid-3'-*O*-glucuronide and 3'-sulfate were quantified respectively as ferulic acid-4'-*O*-glucuronide and 3'-sulfate equivalents. Hydroxybenzoic acid-sulfates and caffeic acid-3'-sulfate were quantified as dihydrocaffeic acid-3'-sulfate equivalents and 4'-hydroxyhippuric acid by reference to a hippuric acid standard curve.

Qualitative and quantitative analysis of anthocyanins in urine and plasma samples by UHPLC-MS

Anthocyanins were analysed using an Accela UHPLC 1250 with a LTQ XL linear ion trap-mass spectrometer fitted with a heated-ESI probe (Thermo Scientific Inc.). Separation was performed with a Kinetex PFP (50 × 2.1 mm), 2.6 µm particle size column (Phenomenex, Macclesfield, UK). The volume injected was 5 µL and column oven was set to 30°C. Elution was carried out at a flow rate of 0.2 mL/min. The gradient started with 10% acetonitrile in 0.1% aqueous formic acid, and after 1 min a 9-min linear gradient of 10% to 60% acetonitrile was applied. From 10 to 11 min the acidified acetonitrile increased to

80%, and after 2 min was reduced to 10% acetonitrile to re-equilibrate the column. The MS was

operated in positive ionization mode with a capillary temperature of 275°C, with the source temperature

of 300°C. The sheath gas flow was 40 units and the auxiliary gas set to 5 units. The source voltage was

4.5 kV. The capillary and tube lens voltage were 20 and 95 V, respectively. Analyses were carried out

using full scan, data-dependent MS^3 scanning from *m/z* 100 to 600, with CID of 35 (arbitrary units).

Helium gas was used for CID. Data processing was performed using Xcalibur software (Thermo Scientific).

Anthocyanins were quantified using a cyanidin-3-*O*-glucoside calibration curve.

Statistical analysis

Results are presented as mean values ± standard error. For all metabolites found in urine prior to

supplementation, paired comparisons between baseline values and post-supplementation values on an

excretion per hour basis were performed using the Wilcoxon matched-pairs signed rank test. Differences

were considered significant at $p \leq 0.05$. All statistical analyses were performed using the STATA v.12.0

software package.

Results

(Poly)phenol content of raspberries

A total of 8 anthocyanins, 2 ellagic acid conjugates, 3 ellagitannins, 1 hydroxycinnamate and 1

phenolic acid were identified and quantified in the raspberry supplement (Table 1). HPLC retention times

and mass spectra of the compounds detected are presented in Table S1 of the Supplementary

Information. The main anthocyanins were cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2''-*O*-

glucosyl)rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. Sanguiin H-6 was the principal

ellagitannin followed by lambertianin C and a much lower amount of sanguiin H-10. Small quantities of

an ellagic acid-*O*-pentoside and an ellagic acid-*O*-hexoside were also detected along with trace amounts

of ferulic acid and 4-hydroxybenzoic acid. The berries did not contain detectable quantities of either caffeoylquinic, feruloylquinic or *p*-coumaroylquinic acids. The 300 g of blended raspberries consumed by the volunteers contained, in total, 553 µmol of (poly)phenols of which anthocyanins comprising 53% and ellagitannins 45%, with ellagic acid conjugates, ferulic acid and 4-hydroxybenzoic acid making up the remaining 2% (Table 1). For structures see Fig. 1.

Excretion of (poly)phenol metabolites in urine

A total of 27 compounds were detected in urine over the 48 h collection period after ingestion of raspberries and typical UHPLC-MS-SRM traces illustrated in Fig. 2. The HPLC retention times and mass spectra of 23 of these compounds and their occurrence urine and plasma are presented in Table S2 of the Supplementary Information.

Table 2 summarizes quantitative aspects of the urinary excretion of anthocyanins and their phenolic metabolites. None of the parent anthocyanins were detected except for low nmol levels of cyanidin-3-*O*-glucoside excreted during the first 24 h. In addition, trace levels of peonidin-3-*O*-glucoside were found in the 0-4 h and 4-8 h urine samples. This 3'-*O*-methyl analogue of cyanidin-3-*O*-glucoside was not present in raspberries and its appearance in urine points towards in vivo methylation as a minor step in anthocyanin metabolism.

A further 16 phenolic metabolites derived from anthocyanins were detected in urine, namely 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, 4-hydroxybenzoic acid-3-sulfate, 3-hydroxybenzoic acid-4-sulfate, 3',4'-dihydroxyphenylacetic acid, 3'-methoxy-4'-hydroxyphenylacetic acid, 4'-hydroxyhippuric acid, hippuric acid, caffeic acid-3'-sulfate, dihydrocaffeic acid-3'-sulfate, ferulic acid, and sulfated and glucuronidated metabolites of ferulic acid and isoferulic acid (Table 2). These metabolites were excreted in much higher amounts than the anthocyanins themselves. Low levels of certain phenolic acids were detected in baseline urine collected over 12 h before raspberry

consumption and the amounts of these compounds detected over the 0-48 h of the study were used to

correct for baseline excretion. Benzoic acid and phenylacetic acid derivatives were detected in the urine

of some but not all volunteers, with 4-hydroxybenzoic acid, 2 hydroxybenzoic acid sulfates, 3'4'-

dihydroxyphenylacetic acid, and 3'-methoxy-4'-hydroxyphenylacetic acid present in quantifiable

amounts. Total 0-48 h excretion of benzoic acid and phenylacetic acid derivatives was respectively 6.9 ±

5.0 and 3.1 ± 1.7 µmol,.

Caffeic acid-3'-sulfate and dihydrocaffeic acid-3'-sulfate were identified and quantified in all

volunteers with a total urinary excretion of 1.0 ± 0.1 µmol and 1.1 ± 0.3 µmol over 48 h post-

consumption. Other urinary phenylpropanoid derivatives were detected in urines from all volunteers

with a total 0-48 h excretion of 17.8 ± 5.3 µmol (Table 2). 4'-Hydroxyhippuric and hippuric acid were also

present in urine collected from all the volunteers after raspberry intake with total 0-48 h excretion

values of 16.1 ± 1.9 µmol, and 239 ± 55 µmol, respectively. Although hippuric acid, formed by hepatic

glycination of benzoic acid, has been described as a common metabolite of many flavonoids [17], it was

not included in the calculation of the percentage recovery because its high background levels, originating

from other dietary and endogenous sources [18-20], make it difficult to determine the amounts derived

from the metabolism of anthocyanins. Thus, the overall recovery of anthocyanins and their metabolites

observed in this study was 43.9 ± 8.0 µmol which corresponds to 15.0% of anthocyanin intake (Table 2).

In contrast to anthocyanins and their metabolites, no ellagitannin metabolites were detected in

the 0-4 h and 4-8 h urine samples. Low levels of urolithins A and B were detected in 8-48 h urine while

there were much larger amounts of their phase II metabolites, namely a urolithin A-*O*-glucuronide, a

urolithin A-sulfate and urolithin B-3-*O*-glucuronide with total 8-48 h excretion of 3.4 ± 1.2 µmol, 6.7 ±

3.2 µmol, and 5.2 ± 2.6 µmol, respectively (Table 3). Substantial inter-individual differences were

observed. Urolithin A-*O*-glucuronide and urolithin-B-3-*O*-glucuronide were found in urine from all

volunteers but the amounts varied considerably, with 0-48 h excretion ranging from 0.4 to 11 µmol and

0.3 and 24 µmol, respectively. Urolithin A-sulfate was produced by 8 volunteers and in amounts ranging

from 0.9 to 30 µmol. Five out of 9 volunteers produced urolithin A-sulfate-*O*-glucuronide with excretion values ranging between 1.0 and 1.6 µmol while isourolithin A-*O*-glucuronide was produced only by 4 volunteers with total excretion between 0.4 and 3.6 µmol.

From the 257 µmol of ingested ellagitannins and ellagic acid conjugates, 17.9 ± 4.0 µmol were recovered in 0-48 h urine as ellagic acid and urolithin derivatives which represents 7.0 % of intake.

Analysis of plasma

A total of 15 compounds were identified and quantified in plasma, and pharmacokinetic parameters of each compound, except for hippuric acid, are summarized in Tables 4 and 5. Typical UHPLC-MS traces of plasma metabolites are illustrated in Fig. S1 of the Supplementary Information. Two anthocyanins were detected in sub-nmol/L levels. Their pharmacokinetic profiles are illustrated in Fig. 3. Cyanidin-3-*O*-glucoside was present in the samples of 7 volunteers and reached a peak plasma concentration (C_{max}) of 0.2 ± 0.1 nmol/L 1.0 h after ingestion of raspberries. The individual values ranged between 0.1 and 0.7 nmol/L. A cyanidin-*O*-glucuronide, which was not detected in urine, was found in the plasma of 3 volunteers at 2 h, 4 h, and 6 h, at concentrations that ranged from 0.1 to 0.3 nmol/L. Peonidin-3-*O*-glucoside which occurred in low amounts in urine (Table 2) was not detected in plasma. The raspberries contained low levels of two pelargonidin-3-O-glycosides (Table 1) but, unsurprisingly, no pelargonidin-based anthocyanins were detected in either plasma or urine.

Three ferulic acid metabolites were identified and quantified in plasma samples from all 9 volunteers (Table 4). Pharmacokinetic profiles of these compounds are presented in Fig. 4. The C_{max} of ferulic-4'-*O*-sulfate was 47 ± 14 nmol/L and the time to reach C_{max} (T_{max}) was 1 h after the consumption of raspberries. The 2 glucuronide derivatives, ferulic acid-4'-*O*-glucuronide and isoferulic-3'-*O*-glucuronide, had a T_{max} of 1.5 h with C_{max} values of 18 ± 2 nmol/L and 14 ± 2 nmol/L, respectively. At 24 h

these hydroxycinnamate metabolites were still present in plasma albeit at substantially reduced concentrations (Fig. 4).

Hippuric acid and 4'-hydroxyhipurric acid were found in all plasma samples. While pharmacokinetic profile of the predominant component, hippuric acid, showed a more or less steady 1-2 μM concentration throughout the study, 4'-hydroxyhippuric acid had a C_{max} of 78 ± 12 nmol/L 1 h after raspberry intake (Table 4, Fig. 5). 3',4'-Dihydroxyphenylacetic acid was found in plasma samples of 7 volunteers and attained a mean C_{max} of 180 ± 89 nmol/L after 6 h (Fig. 4, Table 4) and at 24 h was still present in the plasma of 3 volunteers at concentrations of 174-387 nmol/L.

Six metabolites derived from the ingested ellagitannins and ellagic acids were quantified in plasma, namely, a dimethylellagic acid-*O*-glucuronide, 2 urolithin A-*O*-glucuronide isomers (urolithin A-*O*-glucuronide and isourolithin A-*O*-glucuronide), urolithin A-sulfate, (iso)urolithin A-sulfate-*O*-glucuronide and urolithin B-3-*O*-glucuronide (Table 5). Their pharmacokinetic profiles are illustrated in Fig. 6. Dimethylellagic acid-*O*-glucuronide was found in plasma samples from all volunteers and appeared at low concentrations after 2 h with more substantial amounts present from 3 h onwards. The C_{max} of this ellagic acid metabolite, 4.1 ± 1.0 nmol/L, was reached 8 h after raspberry consumption (Fig. 6, Table 5). However, levels ranging from 1.5 to 15 nmol/L, were detected in the plasma of 3 volunteers 24 h after raspberry intake. All 5 urolithin derivatives detected in plasma showed highest concentrations at 24 h (Fig. 6) and, as with their urinary excretion, there was considerable inter-individual differences. For instance, the major metabolite urolithin A-sulfate, which was present in the plasma of 8 volunteers, with individual C_{max} values ranging from 121 to 1591 nmol/L and a mean of 450 ± 161 nmol/L (Fig. 6, Table 5). There were similar person-to-person variations in the plasma profiles of the other 4 urolithin metabolites.

Discussion

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In this study the ADME of anthocyanins and ellagitannins, was investigated by using UHPLC-MS to analyse plasma and urine collected after the acute ingestion of 300 g of blended raspberries. From the total of 25 compounds identified in urine, 17 were anthocyanin-derived compounds and 8 were metabolites of ellagitannins. Analysis of plasma samples led to the identification of 9 anthocyanin metabolites and 6 ellagitannin metabolites.

Except for low amounts of cyanidin-3-O-glucoside, no parent raspberry anthocyanins were detected in plasma or urine arguably because they were almost all di-and trisaccharides (see Table 1 and Fig. 1) and as such, without cleavage of the sugar moieties, are unlikely to be absorbed in even trace quantities [7]. Cyanidin-3-O-glucoside exhibited extremely low plasma concentrations with a C_{max} of 0.2 nmol/L reached 1 h post-consumption (Fig. 3, Table 4) and the highest amount, 9.3 ± 5.4 nmol, was excreted in urine 0-4 h after raspberry intake (Table 2). These results are in keeping with other reports on low amounts of anthocyanins in their native form appearing in plasma and urine following absorption from the proximal gastrointestinal tract (GIT) [10,21]. In the current study, very small amounts of two phase II metabolites of cyanidin-3-O-glucoside were detected in plasma and urine. Peonidin-3-O-glucoside, the methylated analogue of cyanidin-3-O-glucoside, was found in urine at 0-4 h and 4-8 h. A cyanidin-O-glucuronide appeared in the plasma of 3 subjects peaking 2-4 h post-ingestion (Fig. 3, Table 4). However, the low amounts of these phase II metabolites indicate they are not major metabolites of anthocyanins.

The levels of anthocyanins in plasma (Fig. 3) were substantially lower than those observed by de Ferrars et al. after the ingestion of a 1,114 μmol bolus of [$^{13}C_5$]cyanidin-3-O-glucoside [13]. This could be due, in part, to a matrix effect but is probably a reflection of the relatively low 37 μmol cyanidin-3-O-glucoside content of the raspberry supplement (see Table 1). From a quantitative perspective, of the 292 μmol of anthocyanins ingested only 21.3 nmol were excreted in 0-48 h urine which corresponds to a recovery of 0.007%. Even when related to the 37 μmol cyanidin-3-O-glucoside intake (Table 1), anthocyanin excretion (Table 2) was equivalent to only 0.06% of intake.

Compared with anthocyanins, much more substantial amounts of phenolic metabolites were detected in plasma and urine. Anthocyanins undergo pH-mediated degradation in the distal GIT [14] forming A-ring derived phloroglucinaldehyde, and hydroxylated and methylated benzoic acid derivatives depending upon the substituent group(s) on the B-ring [22,23]. In the case of cyanidin and pelargonidin, putative B-ring-derived degradants are, respectively, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid [6,15]. However, these phenolic acids were not detected in plasma and only small quantities were excreted in urine (Table 2). Similarly, only small amounts of two sulfated hydroxybenzoic acids were found only in urine while the corresponding glucuronides were not detected, in contrast to the results obtained after ingestion of [^{13}C$_5$]cyanidin-3-O-glucoside, albeit at a markedly higher intake [13].

The main plasma phenolic acid was 3'4'-dihydroxyphenylacetic acid (Fig. 5, Table 4). Although variable amounts were also found at earlier time points indicating that the phenylacetic acid might be formed and absorbed to some extent in the small intestine, its C_{max} of 180 ± 89 nM was reached at 6 h pointing towards absorption in the distal GIT after transformation of cyanidin-based anthocyanins by colonic microbiota.

Ferulic and isoferulic acid metabolites were detected in plasma with a C_{max} at 1-1.5 h (Table 4) and an apparent secondary, minor increase in plasma concentration at 6-8 h, with trace amounts still present after 24 h (Fig. 4). The amounts excreted, 15.7 µmol, were well in excess of the 1.2 µmol of ferulic acid present in the 300 g raspberry supplement so the majority of these phenylpropenoic acid metabolites must have been originated from a different source. Ferulic acid and isoferulic acid metabolites form after ingestion of coffee containing caffeoylquinic acids [24] but the raspberry supplement did not contain detectable quantities of either caffeoylquinic, feruloylquinic or p-coumarylquinic acids (Table 1), so this route is implausible. Although hydroxycinnamates have been proposed to be produced from anthocyanins as a result of bacterial-mediated cleavage of the B-ring in the colon [25], the pharmacokinetic profiles in Fig. 4 indicate that most absorption of ferulic acid derivatives takes place in the proximal rather than the distal GIT. This is supported by the rapid appearance of B-ring-derived

[$^{13}C_2$]ferulic acid in the circulatory system after the ingestion of a [$^{13}C_5$]cyanidin-3-*O*-glucoside [13]. In the current raspberry study, pH-mediated breakdown of cyanidin in the small intestine could result in the formation of caffeic acid which, the findings of Stalmach et al. [24] indicate, is converted to ferulic acid and isoferulic acid and their phase II metabolites via enterocyte-based methylation and sulfation/glucuronidation steps. Although urinary excretion of caffeic acid-3'-sulfate increased after raspberry consumption (Table 2), free caffeic acid was not detected in either plasma or urine, arguably as consequence of a rapid rate of turnover.

Two glycine conjugates of benzoic acids were found in plasma and urine of all volunteers, namely 4'-hydroxyhippuric and hippuric acid. 4'-Hydroxyhippuric acid reached C_{max} 1 h post-ingestion (Fig. 6, Table 4) pointing towards upper GIT absorption and rapid hepatic glycination of 4'-hydroxybenzoic acid. Low amounts of 4-hydroxybenzoic acid (2.3 µmol/300 g) were detected in the raspberry supplement (Table 1) and might have contributed to the pool of the glycinated benzoic acid derivative. However, the relatively high level of urinary excretion of 4'-hydroxyhippuric acid, 16.1 µmol, compared to the traces amounts of 4-hydroxybenzoic acid in raspberries suggest that the hydroxylated hippuric acid is formed in the body by an alternative route. One possibly is via 3-dehydroxylation of cyanidin-derived 3,4-dihydroxybenzoic acid in the proximal GIT, followed by side chain glycination. In keeping with its plasma pharmacokinetic profile (Fig. 6), almost 65% of the excretion of 4'-hydroxyhippuric acid occurred 0-8 h post-ingestion (Table 2) reflecting a rapid clearance from the body.

Potential pathways for the in vivo metabolism of the cyanidin-based anthocyanins are summarised in Fig. 7.

Stable isotope-labelled hippuric acid has been reported as a metabolite of [$^{13}C_5$]cyanidin-3-*O*-glucoside in humans [12]. However, as noted earlier, hippuric acid is also produced from other dietary and endogenous sources, which results in high background levels that can vary substantially from one volunteer to another. As a consequence, the absence of an isotopic label in the present study made it

impossible to determine to what extent degradation of cyanidin-based anthocyanins contributed to the quantity of hippuric acid excreted after raspberry supplementation. Hippuric acid excretion in urine was, therefore, not taken into account when calculating the 15.0% urinary recovery of anthocyanin-derived products (Table 2). None-the-less, this is well in excess of the 5.4% urinary recovery of [$^{13}C_5$]cyanidin-3-*O*-glucoside-derived metabolites reported by Czank et al. [12]. Arguably, this is a consequence of the raspberry matrix coupled with an anthocyanin intake of 292 μmol in the current study compared with the 1114 μmol in the Czank et al. study.

A previous raspberry feeding study with ileostomist revealed a 23% recovery of the ellagitannin, sanguiin H-6, in ileal fluid. In volunteers with a functioning colon this would pass from the small to the large intestine where it would be subject to the action of the microbiota releasing ellagic acid that is further converted to urolithins [5,6] which are absorbed and subjected to phase II sulfation or glucuronidation, seemingly in the liver [26,27]. In the current investigation, after the consumption of raspberries containing 257 μmol of ellagitannins and ellagic acids, no parent compounds were detected in either plasma or urine which is in agreement with previous bioavailability studies [5, 28, 29]. The first ellagitannin-derived metabolite detected in plasma was a dimethylellagic acid-*O*-glucuronide, which appeared in trace amounts 2 h post-ingestion, with more substantial amounts occurring from 3 h onwards and reaching T_{max} at 8 h (Fig. 6, Table 3).

In addition to the dimethylellagic acid-*O*-glucuronide, 5 urolithin metabolites were detected in plasma with their concentrations increasing from 8 h onwards with highest levels being found 24 h post-raspberry intake (Fig. 6, Table 5). As 24 h was the last blood sampling time point, the pharmacokinetic profiles obtained do not allow a determination of the time that C_{max} was reached. Urolithins in urine were first detected in 8-24 h samples, consistent with their formation in the large intestine, and highest amounts were excreted 32-48 h after supplementation indicating that the plasma C_{max} was attained >24 h after raspberry intake. The urinary and plasma pharmacokinetics of the urolithins demonstrate their persistence in the body after consumption of ellagitannin-rich foods. Similar results were obtained in

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previous bioavailability studies with ellagitannins [29,30]. Espin et al. [31] conducted a study in which pigs were fed acorns, a rich source of ellagitannins, and it was found that large amounts of urolithin metabolites accumulated in the gall bladder. These results are indicative of enterohepatic recirculation and could explain the prolonged presence of urolithin metabolites in the circulatory system observed in this (Fig. 6) and other human studies.

While methylated anthocyanin metabolites, arguably formed mainly in the proximal GIT, were produced in sizable amounts (Table 2, Fig. 3), colon-derived dimethylellagic acid-*O*-glucuronide was the sole methylated ellagic acid/ellagitannin metabolite. This raises the possibility that methylation is less prevalent in the large intestine than the upper GIT and, likewise, that there is minimal hepatic methylation of metabolites.

Urolithins are formed in the distal GIT as the result of microbial degradation of ellagitannins and person-to person variations in the composition of the colonic microflora would appear to have a considerable impact on ellagitannin metabolism and bioavailability [5,6]. It has been reported that two human intestinal *Gordonibacter* species, *G. urolithinfaciens* and *G. pamelaeae* are able to convert ellagitannins and ellagic acid-derived compounds to urolithins [32]. Thus, biological activities ascribed to ellagitannin-rich foods, such as anti-inflammatory and anti-carcinogenic properties [33,34], may differ substantially depending on an individual's gut microbiota. Oral supplementation with urolithins might be considered as a potential strategy to overcome their selective production and make their putative health benefits available to a wider population [35].

In addition to their specific anti-inflammatory activity, urolithins have also been shown to be potential candidate modulators of redox status. They have strong antioxidant properties in defined in vitro conditions, but behave mostly as pro-oxidants in cell-based assays]36]. In agreement with this observation, in a cellular experimental bladder cancer model with T24 cells, urolithins were able to decrease intracellular reactive oxygen species and malonaldehyde levels, and to increase superoxide

dismutase activity when the cells were treated with H_2O_2, acting, therefore, as direct and indirect antioxidants [37].

While the current study has provided more detailed information on the ADME of raspberry anthocyanins and ellagitannins than earlier investigations [5,6], especially with regard to plasma pharmacokinetics of metabolites, it is noteworthy that the profiles of urinary metabolites, while similar, are not identical to those obtained in the earlier investigations. This could, in part, be due to the use of HPLC-MS and GC-MS to analyse samples in the earlier studies compared to the different UHPLC-MS systems with enhanced sensitivity in the current investigation. However, another contributory factor is likely to be variations in the colonic microbiota of the volunteers participating in the different studies. A comparison of the baseline levels of hippuric acid excreted prior to supplementation in different investigations indicates this is a more substantial factor than previously envisaged. For instance, in studies with black tea the reported 0-24 h baseline levels of hippuric acid excretion have been 597 ± 98 μmol [38], 1.89 ± 0.28 mmol [39] and 2.5 ± 0.2 mmol [40]. In bioavailability studies carried out in Glasgow with different supplements, 24 h baseline hippuric acid excretion have been 137 ± 43 μmol [41], 243 ± 38 μmol [42], and 589 ± 95 μmol [6]. In line with these values, in a recent Glasgow-based orange juice bioavailability study, baseline excretion of hippuric acid was 232 ± 21 μmol/24 h [43]. In contrast, an orange juice study using Glasgow protocols that was carried out in Adelaide, in collaboration with Australian colleagues, produced baseline hippuric acid excretion at 1029 ± 188 μmol/24 h [44], a 4.4-fold increase. The reasons for this are undoubtedly complex and diverse, one of which could be country-to-country variations in the gut microbiota of the participating volunteers. A recent study examined a catalog of reference genes in the human gut microbiome of healthy Danish and Chinese individuals and found marked, country-specific differences in microbiota regarding many aspects of nutrient metabolism as well as xenobiotic detoxification [45], which, it was speculated, could have been shaped by diet and environment and other influences including host genetics. These are factors that are going to have to be

increasingly taken into account in studies on the ADME of dietary (poly)phenols and the involvement of the gut microbiome in potential beneficial effects on health.

Summary

The results show an extensive degradation of raspberry anthocyanins and ellagitannins as they pass through the body with a great variety of metabolites, including methyl-, glycine-, glucuronide- and sulfate derivatives of phenolic acids, phenylpropanoids, ellagic acid and urolithins. Anthocyanin and ellagitannin bioactivity is likely to be mediated by their phenolic metabolites as opposed to the parent structures. Unexpectedly, metabolism of anthocyanins appears to start in the upper GIT, possibly pH initiated, with 4'-hydroxyhippuric acid and ferulic acid derivatives circulating in plasma with a $C_{max\ of}$ 1-1.5 h after consumption of raspberries. Previously, the formation of these compounds has been thought to be associated exclusively with the action of the colonic microflora. Compounds originating from the ingested ellagitannins were principally phase II metabolites of the colon-derived urolithins A and B, which had plasma C_{max} times of >24 h and were still being excreted in urine 32-48 h after the ingestion of raspberries indicating their high persistence in the body. Moreover, the amounts and type of urolithin conjugates produced differed considerably among the participants presumably as a consequence of variations in the microbial composition of their colonic microflora.

Authors' Contributions

I.A.L. assisted with the feeds, conducted the HPLC-MS and UHPLC-MS analysis and contributed to the interpretation of data and the drafted the manuscript and its revisions; P.M., L.C., G.P.-C., L.B. assisted with the HPLC-MS and UHPLC-MS analyses and contributed to interpretation of data and to the draft of the manuscript and its revisions; G.B. organised and assisted with the feeds and contributed to the draft of the manuscript and its revisions; D.D.R. supervised the HPLC-MS and UHPLC-MS analyses and

contributed to the draft of the manuscript and its revisions; M.E. J. L. obtained ethical permission for the study and contributed to the revision of the manuscript; A.C. designed the project, supervised the feeds and drafted the manuscript and its revisions.

Conflict of interests

None of the authors have any conflict of interest

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at

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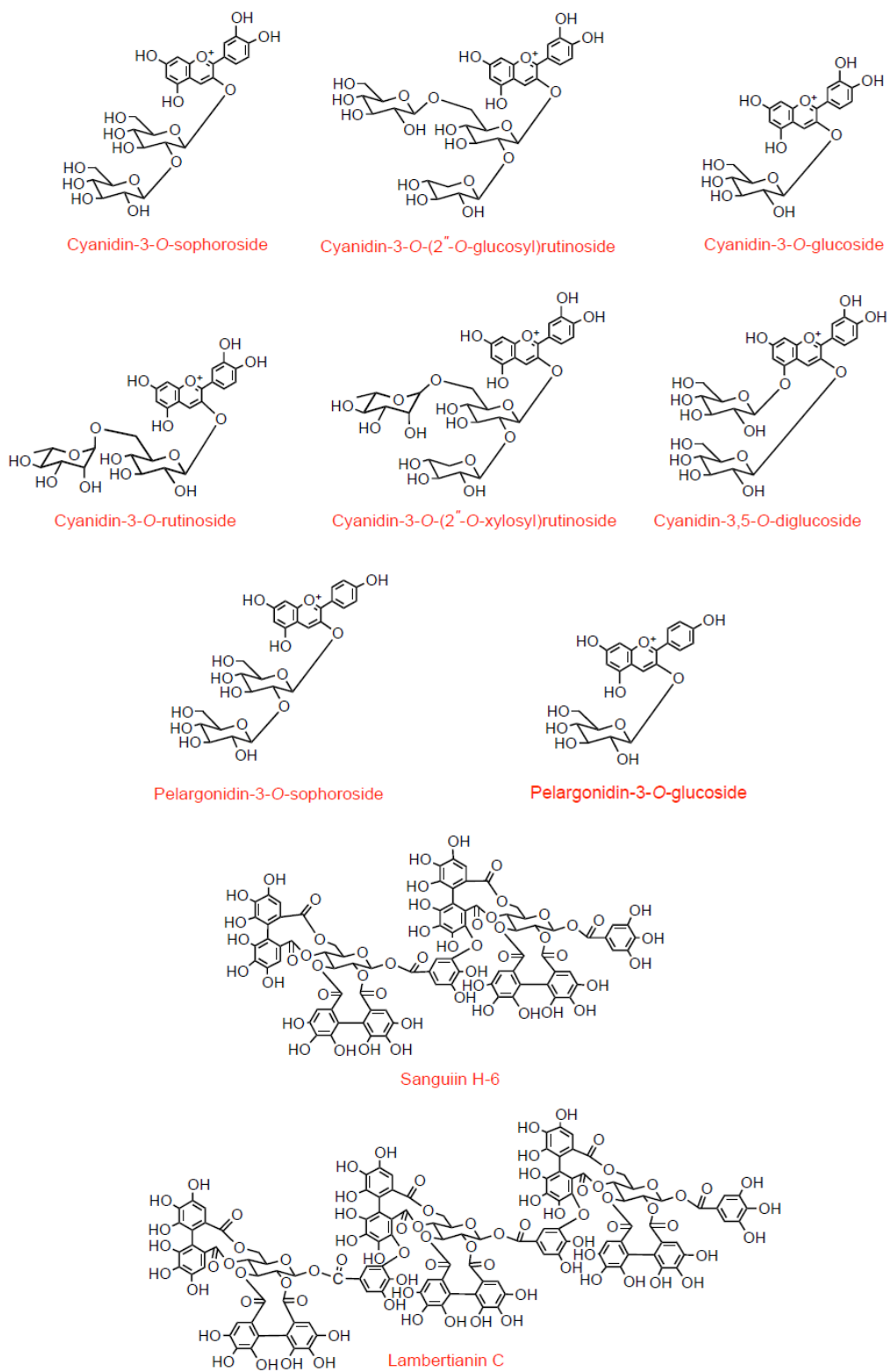
**Fig. 1.** Structures of raspberry anthocyanins and the ellagitannins sanguiin H-6 and lambertianin.
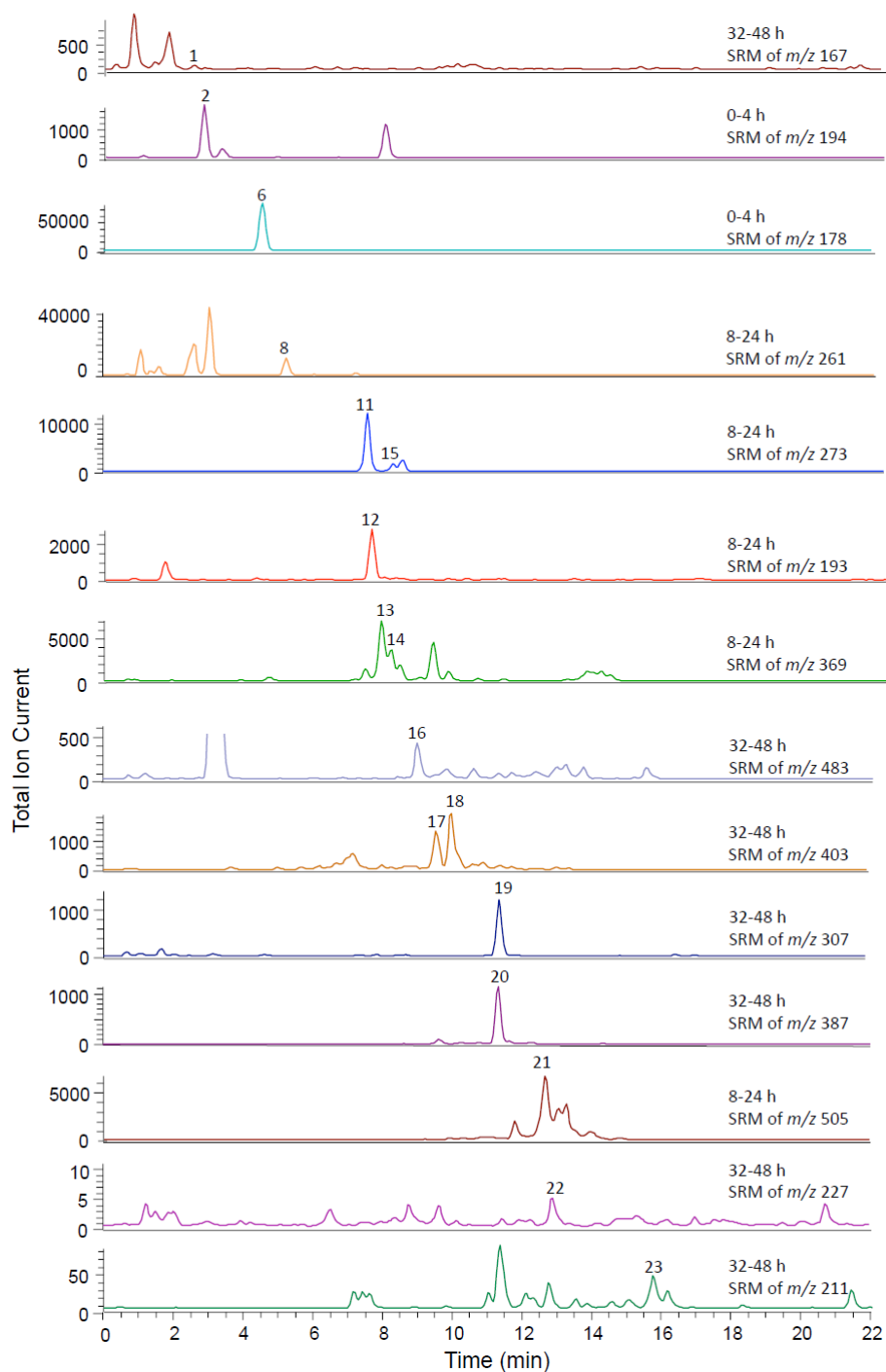
Fig. 2. UHPLC-MS-SRM traces of major phenolic metabolites identified in urine collected at various time points over a 48 h period after ingestion of 300 g of raspberries. Peak identification: **1** - 3',4'-dihydroxyphenyl acetic acid, **2** - 4'-hydroxyhippuric acid, **6** – hippuric acid, **11** - ferulic acid-4'-sulfate, **12** - ferulic acid, **13** - ferulic acid-4'-*O*-glucuronide, **14** – isoferulic acid-3'-*O*-glucuronide, **15** - isoferulic acid-4'-sulfate, **16** - (Iso)urolithin A-sulfate-O-glucuronide, **17** - urolithin A-*O*-glucuronide, **18** - isourolithin A-*O*-glucuronide, **19**- urolithin A-sulfate, **20** - urolthin B-3-*O*-glucuronide, **21** - demethylellagic acid-*O*-glucuronide, **22** - urothin A, and **23** - urolithin B.
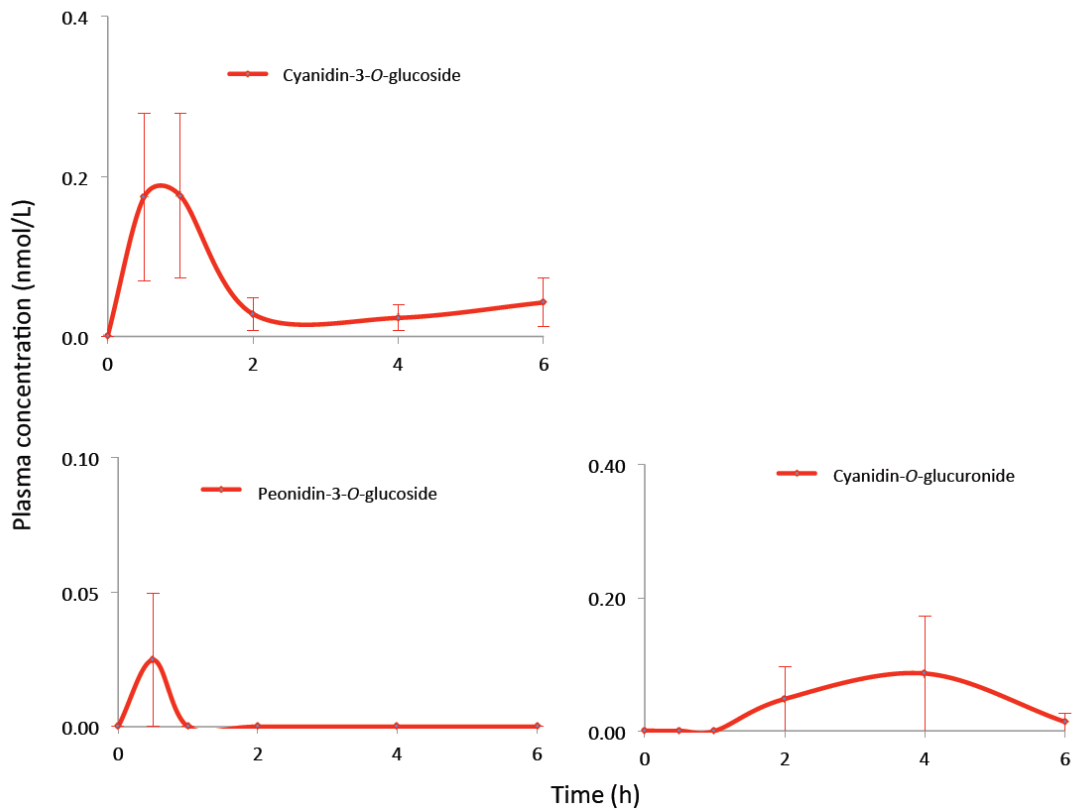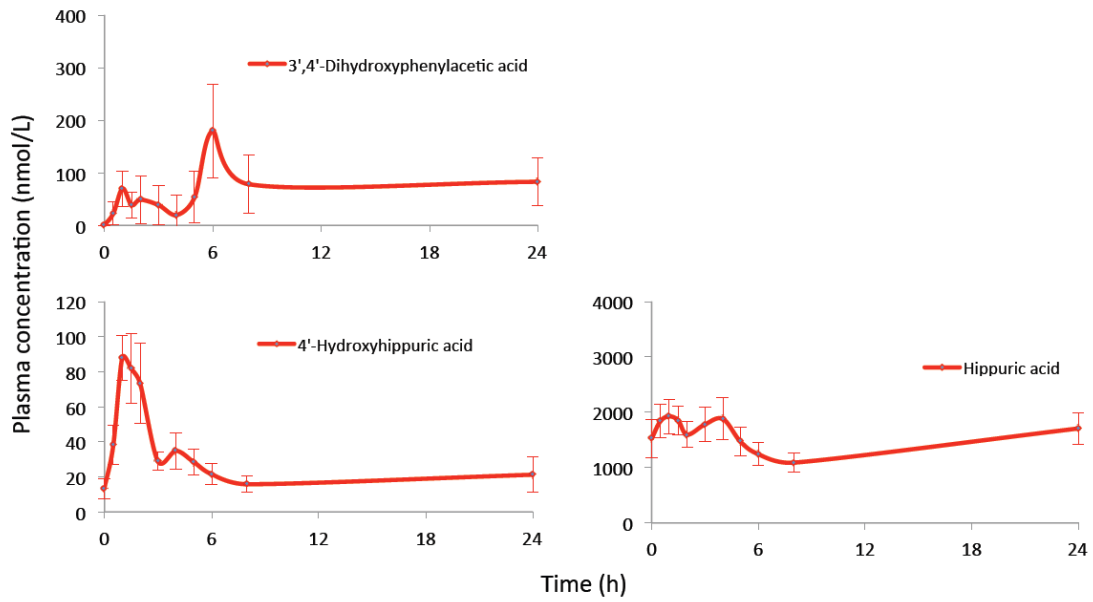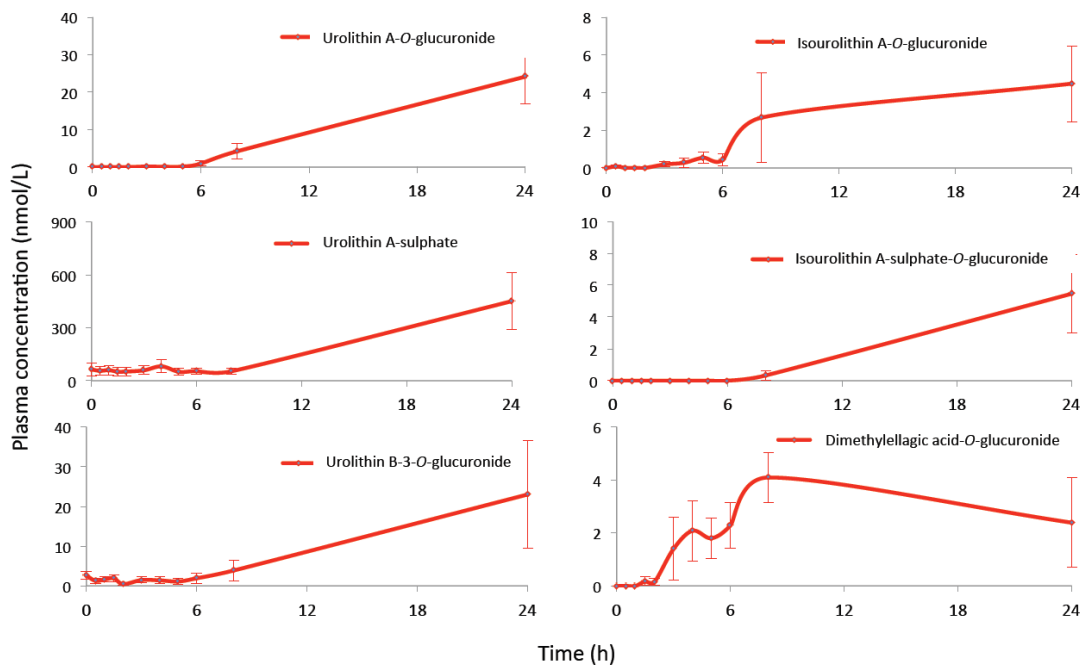
Fig. 3. Pharmacokinetic profiles of anthocyanins in plasma following raspberry consumption. Data expressed as mean values ± SEM (cyandin-3-*O*-glucoside, n = 7; cyanidin-*O*-glucuronide, n =3)
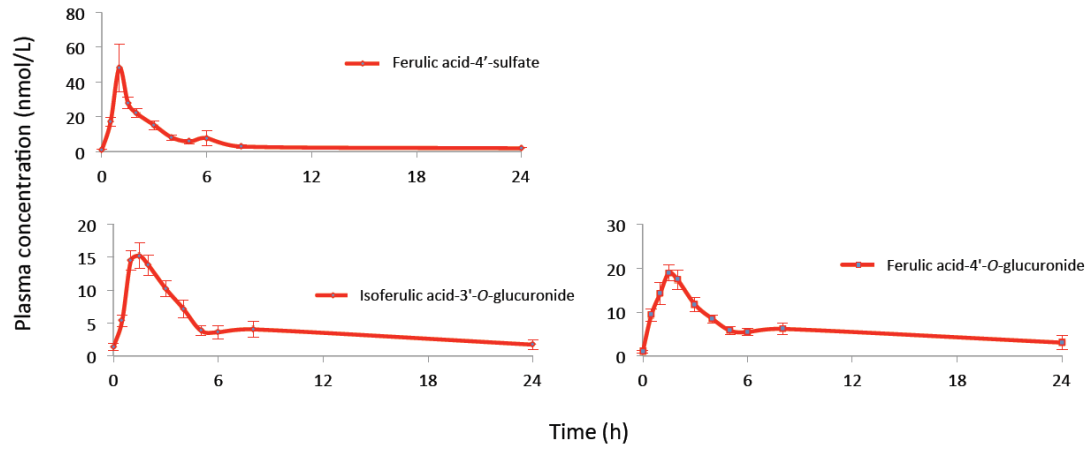
Fig. 4. Pharmacokinetic profiles of ferulic acid metabolites in plasma following raspberry consumption. Data expressed as mean values ± SEM (n = 9)

Fig. 5. Pharmacokinetic profiles of 3',4'-dihydroxyphenylacetic acid , 4'-hippuric acid and hippuric acid in plasma following raspberry consumption. Data expressed as mean values ± SEM (hippuric acid and 4'-hydroxyhippuric acid, n = 9; 3',4'-dihydroxyphenylacetic acid, n = 7)

Fig. 6. Pharmacokinetic profiles of urolithins and ellagic acid derivatives in plasma following raspberry consumption. Data expressed as mean values ± SEM (urolithin A-*O*-glucuronide, n = 9; isourothin A-*O*-glucuronide, n = 4; urolithin A-sulfate, n = 8; isourolithin A-sulfate-*O*-glucuronide, n = 5; urolithin B-3-*O*-glucuronide, n = 7; dimethylellagic acid-*O*-glucuronide, n = 9).
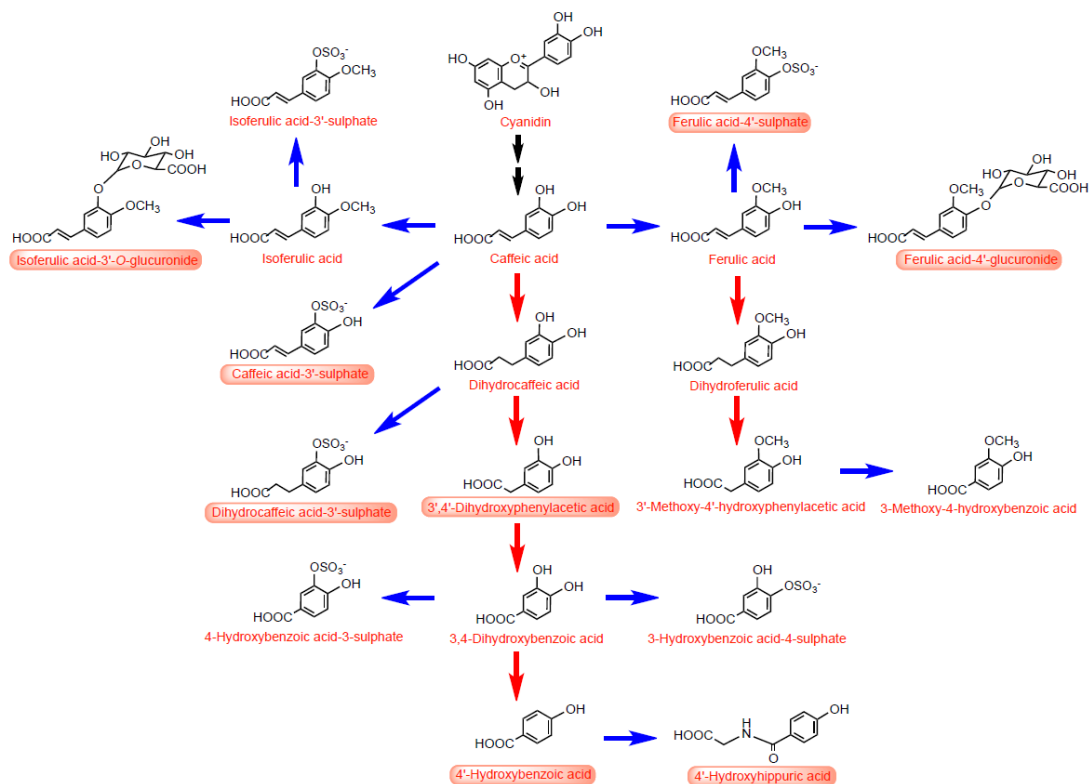
Fig. 7 Potential pathways for the metabolism of cyanidin-based anthocyanins following cleavage of the sugar moiety in the GIT after ingestion of raspberries. It is assumed that the initial degradation step is the pH-mediated breakdown of cyanidin in the proximal GIT leading to the production of caffeic acid (black arrows). Subsequent modification by shortening of the side chain and dehydroxylations, steps likely to be mediated by the colonic microbiota, are indicated with red arrows while blue arrows indicate phase II metabolism. All compounds with the exception of caffeic acid, dihydrocaffeic acid, dihydroferulic acid and isoferulic acid were detected in plasma and/or urine. Compounds with boxed names indicate main components in that 48 h excretion after raspberry consumption increased by at least 1.0 µmol (see Table 2). The proposed metabolism of caffeic acid is in keeping with the findings of Stamach et al. [25] who monitored the conversion caffeoylquinic acids in the proximal GIT to hydroxycinnamate derivatives, including caffeic acid, after the consumption of coffee

Table 1

(Poly)phenolic compounds identified and quantified in raspberries by HPLC-PDA-MS[a]

(Poly)phenolic compounds	µmol per 300 g raspberries
Cyanidin-3-*O*-sophoroside	175 ± 6
Cyanidin-3-*O*-(2''-*O*-glucosyl)rutinoside	56 ± 2
Cyanidin-3-*O*-glucoside	37 ± 1
Cyanidin-3-*O*-rutinoside	20 ± 1
Cyanidin-3-*O*-(2''-*O*-xylosyl)rutinoside	2.7 ± 0.1
Pelargonidin-3-*O*-sophoroside	1.2 ± 0.0
Pelargonidin-3-*O*-glucoside	1.1 ± 0.0
Cyanidin-3,5-*O*-diglucoside	traces
Total anthocyanins	**292 ± 10**
Ellagic acid-*O*-pentoside	3.2 ± 0.2
Ellagic acid-*O*-hexoside	3.1 ± 0.2
Total ellagic acids	**6.3 ± 0.3**
Sanguiin H-6	195 ± 7
Sanguiin H-10	5.7 ± 0.4
Lambertianin C	50 ± 1
Total ellagitannins	**251 ± 3**
Ferulic acid	1.2 ± 0.1
4-Hydroxybenzoic acid	2.3 ± 0.1
Total (poly)phenols	***553 ± 19***

[a] Data expressed as mean value ± S.E. (n=4). The berries did not contain detectable quantities of either caffeoylquinic, feruloylquinic or *p*-coumaroylquinic acids.

Table 2

Urinary excretion of anthocyanins and phenolic compounds 0-48 h after acute intake of 300 g blended raspberries containing 292 µmol of anthocyanins [a,b]

Compounds	Baseline[c]	0-4 h	4-8 h	8-24 h	24-32 h	32-48 h	Total (0-48 h)
Cyanidin-3-*O*-glucoside (nmol)	-	9.3 ± 5.4	6.0 ± 3.1	4.6 ± 1.5	-	-	19.9 ± 9.1*
Peonidin-3-*O*-glucoside (nmol)	-	0.9 ± 0.4	0.5 ± 0.2	-	-	-	1.4 ± 0.4*
Total anthocyanins (nmol)	-	*10.2 ± 5.5*	*6.5 ± 3.2*	*4.6 ± 1.5*			*21.3 ± 9.28*
4-Hydroxybenzoic acid[b]	-	-	0.9 ± 0.9	-	1.6 ± 1.5	3.9 ± 3.7	6.4 ± 4.8*
3,4-Dihydroxybenzoic acid	-	-	-	traces	traces	traces	
3-Methoxy-4-hydroxybenzoic acid	-	-	-	-	-	traces	
4-Hydroxybenzoic acid-3-sulfate	0.1 ± 0.0	-	-	0.2 ± 0.1	traces	0.1 ± 0.0	0.3 ± 0.1*
3-Hydroxybenzoic acid-4-sulfate	0.1 ± 0.0	-	-	0.1 ± 0.0	traces	0.1 ± 0.0	0.2 ± 0.1*
Total benzoic acid derivatives	*0.2 ± 0.0*	-	*0.9 ± 0.9*	*0.3 ± 0.1*	*1.6 ± 1.5*	*4.1 ± 3.7*	*6.9 ± 5.0**
3',4'-Dihydroxyphenylacetic acid	0.4 ± 0.2	0.2 ± 0.1	0.5 ± 0.3	0.7 ± 0.4	0.5 ± 0.3	1.0 ± 0.6	2.9 ± 1.3*
3'-Methoxy-4'-hydroxyphenylacetic acid	-	-	-	traces	0.2 ± 0.2	-	0.2 ± 0.2
Total phenylacetic acids	*0.4 ± 0.2*	*0.2 ± 0.1*	*0.5 ± 0.3*	*0.7 ± 0.4*	*0.7 ± 0.5*	*1.0 ± 0.6*	*3.1 ± 1.7**
Caffeic acid-3'-sulfate	traces	0.6 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	traces	traces	1.0 ± 0.1*
Dihydrocaffeic acid-3'-sulfate	traces	0.4 ± 0.1	0.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	traces	1.1 ± 0.3*
Ferulic acid	1.8 ± 0.3	0.9 ± 0.4	0.7 ± 0.2	1.9 ± 0.6	0.8 ± 0.4	1.6 ± 1.0	5.9 ± 2.1*
Ferulic acid-4'-*O*-sulfate	1.6 ± 0.2	1.6 ± 0.5	0.8 ± 0.2	1.3 ± 0.6	0.8 ± 0.3	2.0 ± 1.3	6.5 ± 2.6*
Isoferulic acid-3'-sulfate	traces	traces	traces	0.2 ± 0.1	traces	0.3 ± 0.1	0.5 ± 0.2*
Ferulic acid-4'-*O*-glucuronide	0.7 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	1.8 ± 0.5*
Isoferulic acid-3'-*O*-glucuronide	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.4*
Total phenylpropanoid derivatives	*4.4 ± 0.6*	*3.8 ± 0.9*	*2.1 ± 0.4*	*4.3 ± 1.2*	*2.2 ± 0.9*	*4.4 ± 2.5*	*17.8 ± 5.3**
4'-Hydroxyhippuric acid	7.0 ± 2.1	7.1 ± 1.0	3.3 ± 0.5	3.3 ± 1.3	1.6 ± 0.8	0.8 ± 0.5	16.1 ± 1.9*
Hippuric acid	294 ± 47	100 ± 21	58 ± 19	26 ± 13	35 ± 18	20 ± 10	239 ± 55*
Total anthocyanin and phenolic acid derivatives (excluding hippuric acid)	*12.0 ± 2.3*	*11.1 ± 1.8*	*6.8 ± 1.3*	*8.5 ± 2.6*	*6.1 ± 1.9*	*10.3 ± 4.3*	*43.9 ± 8.0** *(15.0%)[d]*

[a] Except for anthocyanins, data expressed as in µmol as mean values ± S.E. (n = 9) after subtraction of baseline excretion from each volunteer. Anthocyanins expressed in nmol.

[b] Detected only in the urine of 2 of 9 volunteers

[c] Content of urine collected for 12 h prior to supplementation and on an excretion per hour basis used to subtract from excretion values obtained after raspberry consumption to obtain the values cited in the Table.

[d] The amount excreted as a percentage of the dose of anthocyanins ingested

- not detected

* Significantly higher excretion on per hour basis from that at baseline (p ≤ 0.05).

Table 3

Urinary excretion of urolithins and ellagic acid derivatives 0-48 h after acute intake of 300 g of blended raspberries containing a total of 257 μmol of ellagitannins and ellagic acid derivatives[a]

		Urinary excretion after intake of 300 g of raspberries (μmol)					
Compound	Baseline	0-4 h	4-8 h	8-24 h	24-32 h	32-48 h	Total (48 h)
Urolithin A	-	-	-	-	-	traces	traces
Urolithin B	-	-	-	-	-	traces	traces
Urolithin A-*O*-glucuronide	-	-	-	1.4 ± 0.6	1.0 ± 0.3	1.1 ±0.3	3.4 ± 1.2
Isourolithin A-*O*-glucuronide	-	-	-	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.8 ± 0.4
Urolithin A-sulfate	-	-	-	2.4 ± 1.0	2.0 ± 0.9	2.3 ± 1.2	6.7 ± 3.2
(Iso)urolithin A-sulfate-*O*-glucuronide	-	-	-	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.8 ± 0.2
Urolithin B-3-*O*-glucuronide	-	-	-	1.5 ± 0.9	1.5 ± 0.7	2.2 ± 0.9	5.2 ± 2.6
Dimethylellagic acid-*O*-glucuronide	-	-	-	0.4 ± 0.1	0.3 ± 01	0.3 ± 0.1	1.0 ± 0.2
Total urolithins and ellagic acid derivatives				*6.1 ± 1.5 (2.4%)[b]*	*5.4 ± 1.3 (2.1%)[b]*	*6.4 ± 1.9 (2.5%)[b]*	*17.9 ± 4.0 (7.0%)[b]*

[a] Data expressed as mean value ± S.E. (n=9).

[b] numbers in parentheses represent the amount excreted as a percentage of the dose of ellagitannins ingested

- not detected

Table 4

Anthocyanins and their phenolic degradents identified and quantified in plasma after ingestion of 300 g of raspberries containing 292 µmol of anthocyanins [a]

Compound	n[b]	C_{max} (nmol/L)	T_{max} (h)[c]
Cyanidin-3-*O*-glucoside	7	0.2 ± 0.1	1
Cyanidin-*O*-glucuronide	3	0.1 ± 0.1	4
3',4'-Dihydroxyphenylacetic acid	7	180 ± 89	6
Ferulic acid-4′-sulfate	9	47 ± 14	1
Ferulic acid-4′-*O*-glucuronide	9	18 ± 2	1.5
Isoferulic acid-3'-*O*-glucuronide	9	14 ± 2	1.5
4'-Hydroxyhippuric acid	9	78 ± 12	1

[a] Data expressed as mean value ± S.E. (n=9).
[b] Metabolites detected in n = number of participants
[c] Time to reach C_{max}

Table 5

Urolithins and ellagic acid metabolites identified and quantified in plasma after ingestion of 300 g of raspberries[a]

Compound	n[b]	C_{max} (nmol/L)	T_{max} (h)[c]
Urolithin A-*O*-glucuronide	9	24 ± 7	24
Isourolithin A-*O*-glucuronide	4	4.5 ± 2.0	24
Urolithin A-sulfate	8	450 ± 161	24
(Iso)urolithin A-sulfate-*O*-glucuronide	5	5.5 ± 2.5	24
Urolithin B-3-*O*-glucuronide	7	23 ± 14	24
Dimethylellagic acid-*O*-glucuronide	8	3.5 ± 1.0	8

[a] Data expressed as mean value ± S.E. (n=9).
[b] Metabolites detected in n = number of participants
[c] Time to reach C_{max}