**Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers**

Rui C. Pimpãoa, Tristan Dewb,c, Maria E. Figueirad, Gordon J. McDougalle, [Derek Stewart](http://link.springer.com/search?facet-author=%22Derek+Stewart%22)e,f, Ricardo B. Ferreiraa,g, Claudia N. Santosa,h, Gary Williamsonb,\*

*aInstituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, EAN, 2781-901 Oeiras, Portugal.*

*bSchool of Food Science and Nutrition*, *University of Leeds, Leeds LS2 9JT, United Kingdom.*

*cCurrent address: Bradford School of Pharmacy, School of Life Sciences, University of Bradford, Bradford, United Kingdom.*

*diMed-UL e Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisboa, Portugal.*

*eThe James Hutton Institute, Dundee DD2 5DA, Scotland, United Kingdom.*

*fBioforsk – Norwegian Institute for Agricultural and Environmental Research, Tromso, Norway.*

*gInstituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal.*

*hInstituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal.*

\* Corresponding author: Prof Gary Williamson, School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK; Phone +44 113 343 8380; Fax: +44 113 343 2982; Email: g.williamson@leeds.ac.uk

List of abbreviations:

BHT, butylated hydroxytoluene; DHC, dihydrocaffeic; DHF, dihydroferulic; glcrnd, glucuronide; MRM, multiple reaction monitoring; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; (poly)phenols, flavonoids, phenolic acids and their phenolic metabolites; UDPGA, uridine 5′-diphosphoglucuronic acid trisodium salt.

Keywords: Berry fruits/ Bioavailability/ Colon/ Conjugates/ Metabolomics

**Abstract**

**Scope:** The colonic metabolism of dietary (poly)phenols is complex and many metabolites and conjugates have not yet been unambiguously identified in humans.

**Methods and results:** Urine samples from 9 healthy human volunteers obtained after the ingestion of a puree of five (poly)phenol-rich berry fruits were analysed using LC-Orbitrap mass spectrometry to provide a preliminary indication of possible metabolites based on exact mass. In most cases, the identity of compounds was confirmed using standards produced either chemically or enzymically followed by analysis using LC-triple quadrupole mass spectrometry. Sulfated, glucuronidated and methylated forms of catechol, pyrogallol and protocatechuic acid mostly appeared in urine after 8 hours, suggesting colonic metabolism. Gallic acid and (-)-epicatechin conjugates appeared mainly before 4 hours, indicative of absorption from the small intestine. Conjugates of ferulic, caffeic, and vanillic acid appeared at intermediate times.

**Conclusion:** We have positively identified metabolites and conjugates, some novel, in the urine of healthy volunteers after intake of multiple phenolics from a mixed puree from berry fruits, with each being excreted at specific and signature times. Some of these compounds could potentially be used as biomarkers of fruit intake. The possible biological activities of these colonic metabolites require further assessment.

**1 Introduction**

The association between consumption of fruits and vegetables and the decrease in risk of suffering from degenerative diseases has been well established. Several studies have provided evidence for the effects of dietary fruit in reducing the risk of cardiovascular/inflammatory diseases [1, 2] as well as of Alzheimer’s disease and other neuropathies [3]. These effects have been at least partially attributed to the presence of (poly)phenols, present in plant foods. Berry fruits are particularly rich in some classes of these compounds, such as phenolic acids, tannins, and flavonoids [4]. However, when trying to correlate the beneficial health effects described for (poly)phenols present in fruits and their bioavailability in humans, it is often observed that some of these compounds are poorly absorbed and may undergo several metabolic modification steps. Additionally, metabolism by the colonic microbiota may result in several catabolites which can be absorbed and contribute to an increased bioavailability [5, 6].

Thus, it is unlikely that the compounds initially present in fruits, or even their aglycones, are responsible solely for the biological effects *in vivo*. Nonetheless, current literature on bioavailability of (poly)phenol metabolites and their degradation pathways is still limited and therefore more studies are necessary to fill this gap. Recently, blueberries, raspberries, blackberries, Portuguese crowberry and strawberry tree fruits were characterized for phenolics [7], and these fruits provide a diverse range of (poly)phenols in the diet, ideal for an intervention study examining the metabolism of a broad mixture of (poly)phenols. In this work, healthy subjects ingested a standardised puree of these fruits and the urinary excretion of phenolic compounds was assessed. The main urinary metabolites in healthy volunteers were initially assessed by using exact mass scanning with a LC-Orbitrap MS, and subsequently confirmed by comparison with chemically and enzymically synthesized standards and then analysed using LC-triple quadrupole MS.

**2 Materials and methods**

**2.1 Preparation of fruit puree**

The consumed fruit puree consisted of 100 g of each of five fruits: blueberries (*Vaccinum* spp. variety Georgia Gem), blackberries (*Rubus* L.subgenus Rubus Watson variety Karaka Black) and raspberries (*Rubus idaeus* L.variety Himbo Top) were harvested at the Fataca experimental field in Odemira, Portugal; strawberry tree fruits (*Arbutus unedo* L.) were harvested in the Alentejo region, Portugal; and Portuguese crowberries (*Corema album* L.) were harvested in the Comporta region, Portugal. Fruits were blended together using a domestic food processor, for 1 min at room temperature. The puree was prepared on the day of the study and was passed through a sieve to remove seeds before being given to the volunteers. A sample of the puree was also freeze-dried and stored at -80 ºC until analysis.

**2.2 Subjects and study design**

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of the Faculty of Pharmacy, University of Lisbon, Portugal (02/CEECFFUL/2012). The protocol was explained to each volunteer and written informed consent was given before the study. Nine individuals (six females and three males) aged between 23 and 54 years with a body mass index of 23.2 ± 3.5 were recruited. Individuals were all considered healthy by a medical questionnaire and standard blood tests where the levels of glucose, urea, creatinine, total cholesterol, HDL cholesterol, triglycerides, C-reactive protein, and the activity of aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, were assessed. Volunteers did not have any history of cardiovascular diseases or any other medical illnesses, were non-smoking, and were not receiving medication or taking vitamins that could interfere with the study.

Volunteers followed a (poly)phenol-free diet for 2 days before the study and throughout the day of the study, and the compliance with this restriction was confirmed through a questionnaire. After an overnight fast, volunteers ingested 500 mL of the above fruit puree with a standard breakfast containing no additional (poly)phenols, consisting of bread, with ham or cheese, yogurt and biscuits.

Urine samples were collected into a container (containing 0.5 g of ascorbic acid) before the ingestion of the puree, and in the periods of 0-2 h, 2-4 h, 4-8 h and 8-24 h, and the volumes recorded. Sodium azide (0.1% w/v) was added to urine samples which were kept at -20 ºC until analysis.

**2.3 Determination of phenolic compounds in the prepared fruit puree**

For determination of (poly)phenols, the dried puree was extracted with ethanol-water (1:1), containing 1 mM butylated hydroxytoluene (BHT) and 0.1 mM daidzein as internal standard. One part of the extract was used directly for HPLC-DAD analysis of total anthocyanins and caffeoylquinic acids. Another part of the extract was incubated with cellulase (EC. 3.2.1.4, Sigma Aldrich, St. Louis, MO, USA) and hesperidinase (EC. 3.2.1.40, Sigma Aldrich, St. Louis, MO, USA) from *Aspergillus niger* and analysed by HPLC-DAD, as described previously [7].

**2.4 Extraction of phenolics from urine**

A 2 mL aliquot of urine was spiked with 10 µL of 1 mg/mL rutin as internal standard and was added to 10 mL of acetonitrile. Samples were centrifuged at 3000 *g* for 15 min. The supernatant was collected, dried under vacuum and reconstituted in 500 µL of 50% (v/v) ethanol with 0.1% (w/v) ascorbic acid and 20 µg/mLof taxifolin as second internal standard for further analysis.

**2.5 Prediction of metabolites in human samples**

A list of metabolites and conjugates potentially present in human urine after ingestion of the fruits was drawn up based on prediction of metabolic pathways. The structures of 205 compounds were drawn and their exact mass calculated by using MarvinSketch software 5.7.0 from Chemaxon.

**2.6 Urine analysis using LC-Orbitrap MS**

A small proportion of each aliquot of urine from each time point from all volunteers were pooled together just for the LC-Orbitrap MS analysis. Phenolics were extracted as described above. Samples were separated on a HPLC Accela 600 HPLC system (Thermo Scientific) using a C18 Synergi Hydro RP18 column (Phenomenex, Macclesfield, UK) 4 µm particle size and dimensions 2 mm ID x 150 mm. The column was fitted with a Security GuardTM guard system containing an Aqua 10 µm C18 Guard Cartridge (2 mm ID x 4 mm) (Phenomenex, Macclesfield, UK) and eluted over a gradient of 100% solvent A (95% H2O, 5% ACN with 0.1% (v/v) formic acid) to reach 15% B (95% ACN, 5% H2O with 0.1% (v/v) formic acid) at 10 min, 25% B at 30 min, 60% B at 60 min, 100% B at 63 min, 100% B at 68 min at a flow rate of 0.26 mL/min. Analysis was done on a LTQ Orbitrap™ XL hybrid mass spectrometer (Thermo Scientific, Bremen, Germany).

MS analysis was performed using data dependent Nth order double play analysis comprising full scan mass range 80-2000 amu , 30000 resolution, Data type Centroid and data dependent MS/MS (60 seconds of exclusion duration) on the top 3 most intense ions detected above threshold automatically in the independent scan event. ESI settings were as follows: source voltage, 3.4 kV; the capillary temperature was 300 ºC with a sheath gas at 40 psi and auxiliary gas at 5 psi.

MS data handling software (Xcalibur QualBrowser software, Thermo Electron Corp.) was used to search for predicted metabolites by their appropriate *m/z* value. All peaks were checked for *m/z* value and fragmentation products.

**2.7 Preparation of liver cytosolic fractions**

For preparation of liver cytosolic fractions, ox and pig liver were rinsed with 250 mM sucrose solution and a sample of 13 g was weighed. Samples were minced and homogenized with 50 mL Tris-HCl buffer 50 mM pH 7.5 using a polytron for 2 min on ice. Samples were centrifuged for 10 min at 17400 *g* at 6 ºC. The supernatant was recovered and centrifuged for 90 min at 3500 *g* and 6 ºC. The supernatant was recovered and stored at -80 ºC until use.

**2.8 Preparation of liver microsomes**

For preparation of microsomes, pig liver was rinsed with 250 mM sucrose solution and a sample of 13 g was weighed. Samples were minced and homogenized with 50 mL of the sucrose solution using a polytron for 2 min on ice. Samples were centrifuged for 10 min at 17400 *g* at 6 ºC, supernatant was recovered and 0.2 mL of 88 mM CaCl2 was added to 20 mL of supernatant. The solute was left to stand on ice for 5 min with occasional swirling. The mixture was centrifuged at 27000 *g* for 15 min at 6 ºC, and after discarding the supernatant the pellet was resuspended in 2.5 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 20% (v/v) glycerol. Microsomal fractions were stored at -80 ºC until use.

**2.9 Sulfation of phenolic substrates**

Individual phenolic compounds used as substrates were incubated at a concentration of 300 µM with 35 µM adenosine 3′-phosphate 5′-phosphosulfate (PAPS) and 5.1 mM *p*-nitrophenyl sulfate (pNPS) as co-factors, 1 mM dithiothreitol, 10 mM sodium sulfite and 100 µM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4. To this solution, 25 L of liver cytosolic fraction was added and the reaction was incubated at 37 ºC for 4 h. The reaction was stopped with addition of 200 L of ice cold, ACN containing 500 mM HCl. Samples were centrifuged twice for 15 min at 17000 *g* and the supernatant was stored at -20 ºC until analysis.

**2.10 Glucuronidation of phenolic substrates**

The protocol is based on the modification of the method followed by Dueñas *et al*. [8]. Individual compounds used as substrates were incubated at a concentration of 800 µM with 25 mM HEPES buffer, pH 7.2, containing 1 mg.mL-1 alamethicin, 2 mM uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), and 1 mM saccharolactone in a final volume of 110 µL. The reaction was started with the addition of 5 µL of pig liver microsomes and incubation was performed at 37 ºC for 4 h. Reaction was stopped by the addition of 200 µL of methanol containing 1.6 mM ascorbic acid. Samples were centrifuged twice for 15 min at 17000 g, and the supernatant was stored at -20 ºC until analysis.

**2.11 Chemical synthesis of hydroxycinnamate metabolites**

The hydroxycinnamate and dihydroxycinnamate-sulfates and glucuronides used as standards were chemically synthesized and characterized as described previously [9] and were kindly provided by Prof. Denis Barron, NIHS, Lausanne, Switzerland.

**2.12 LC-MS/MS analysis**

Individual urine samples were analysed by LC-MS/MS. The HPLC system comprised an Agilent 1200 series micro degasser, SL binary pump, SL autosampler with chilled sample compartment (8 ºC), column oven (30 ºC), and diode array detector (Agilent Technologies, Cheadle, UK). The system was controlled and data processed by Agilent MassHunter software (version B.01.03). An Atlantis T3 Column, 100 Å, 3 µm, 2.1 mm ID x 100 mm HPLC column (Waters, Hertfordshire, UK) was used for chromatographic separations at a flow rate of 0.26 mL/min over a gradient of 100% solvent A (95% H2O, 5% ACN with 0.5% (v/v) formic acid) for 10 min, reaching 15% B (95% ACN, 5% H2O with 0.5% (v/v) formic acid) from 10 to 20 min. Solvent B increased to 25% at 40 min and to 100% B at 43 min where it was maintained for 5 min returning to 0% in 2 min. MS analysis was performed with an Agilent 6410a triple-quadrupole LC-MS-MS with electrospray source at 350 ºC, a source voltage of 4kV , and N2 drying gas flow rate of 11 L/min at a pressure of 30 psi (Peak Scientific, NM30LA, Inchinnan, UK). The analysis was performed in negative Multiple Reaction Monitoring (MRM) mode and optimisation for ion transmission was achieved by repeated injections of individual standards.

Relative amounts of compounds were estimated after normalizing to internal standard (rutin) and corrected by urinary volume.

**2.13 Statistical analysis**

Pharmacokinetic excretion profile of urine metabolites was constructed using GraphPad Prism 5. This package was also used for statistical analysis. Box-and-Whiskers plots for minimum and maximum values were produced. Comparisons in relation to the baseline were performed with two tailed Wilcoxon matched pairs test with a confidence level of 95%.

**3 Results**

**3.1 Identification and quantification of phenolic compounds in fruit puree**

The puree containing blueberries, raspberries, blackberries, Portuguese crowberry and strawberry tree fruits was characterized by HPLC-DAD for the major compounds (Caffeoylquinic acids and anthocyanins, Supplementary Table 1) and the aglycones were also quantified after multi-enzyme hydrolysis of the glycosides (Table 1). The most abundant compounds were anthocyanins, chlorogenic acids and gallic acid. Caffeic acid was abundant after hydrolysis and possibly resulted from esterase activity on caffeoylquinic acids plus glycosidase activity towards glycosides of caffeic acid.

**3.2 Initial determination of metabolites in urine using exact mass data**

Compounds were identified in pooled urine samples for each time point by comparing their exact mass and fragmentation patterns (when available) obtained using an LC- Orbitrap MS with predicted metabolites. By comparing chromatograms of urine samples before ingestion and after ingestion of the fruit puree, it was observed that several metabolites appeared after ingestion. Only metabolites which increased in abundance after ingestion of the fruit puree were selected for confirmation to exclude compounds that could result from endogenous metabolism (Supplementary Tables 2 to 6). Anthocyanins, which were very abundant in the fruit puree, were not evident by LC-Orbitrap MS analysis. After this initial analysis, the most abundant compounds were selected for confirmation by comparison with enzymically or chemically synthesised standards in individual samples using the LC-triple quadrupole MS.

**3.3 Enzymatic synthesis of phenolic compounds**

For production of conjugated compounds, (poly)phenol aglycones were incubated *in vitro* with pig liver microsomes and UDP-GA to generate glucuronide conjugates, and with an ox liver cytosolic fraction in the presence of the co-factors PAPS and *p*-NPS to generate the formation of mono-sulfated compounds. Ox liver cytosolic fraction was used instead of pig liver as it was observed to be more efficient in generating sulfated conjugates. The resulting conjugates were identified by their mass and fragmentation pattern (multiple reaction monitoring - MRM transitions), and are shown in Table 2. When multiple conjugates resulted from the reaction owing to the presence of multiple available hydroxyl groups, it was not possible to distinguish the exact position of the conjugation, with exception for the compounds synthesized chemically.

Gallic acid, although theoretically having only two *different* hydroxyl groups for conjugation, yielded three glucuronidated metabolites (Supplementary Fig. 1 A). Also, vanillic acid, possessing a single hydroxyl group available for conjugation, originated one sulfate as expected, but apparently two glucuronides (Supplementary Fig. 1 B). The one sulfated metabolite of 4-*O*-methylgallic acid was produced as expected; however, protocatechuic acid yielded only one peak (Supplementary Fig. 1 C), even though two hydroxyl groups are available for conjugation. These events suggest a positional specificity of sulfotransferases for these compounds if only one metabolite is being produced, or alternatively overlapping of two metabolites in one peak.

(-)-Epicatechin yielded three sulfates, although five hydroxyl groups are theoretically available for sulfation or glucuronidation. However, glucuronidation of (-)-epicatechin yielded five peaks (Supplementary Fig. 1 D) but with large differences in efficiency of conjugation for the different positions.

Conjugates of catechol, 4-methylcatechol, pyrogallol and its *O*-methylated forms, 1-*O*-methylpyrogallol and 2-*O*-methylpyrogallol, are summarized in Table 2 and occurred as expected. The only exception was 4-methylcatechol which, although having two available hydroxyl groups, yielded only one glucuronide and one sulfate.

Therefore, enzymic synthesis of sulfated compounds is not strictly regio-selective for most compounds, but reveals a preference for certain positions, as revealed by differences in peak areas for synthesized isomers of the same parent compound.

**3.4 Confirmation and kinetics of metabolites in human urine**

Individual urine samples from each volunteer and each time point were analysed using the triple quadrupole LC-MS in MRM mode, using conditions optimized for the standard compounds, to confirm if compounds initially identified by exact mass scanning matched the synthesized standards. The presence of aglycones and conjugates in urine is indicated in Table 2. The aglycones protocatechuic and dihydrocaffeic (DHC) acids, and gallic acid-*O*-glucuronide, were not detected by the LC Orbitrap-MS due to low levels of the compounds combined with the lower sensitivity of this method, but were, however, detected with LC-triple quadrupole MS. On the other hand, some aglycones (such as catechol, 4-methylcatechol, ferulic and isoferulic acids) were tentatively identified using the Orbitrap-MS but were not found by the MRM method. This might result from technical limitations such as misleading identification of the peak due to other compounds with the same exact mass, or due to non-intentional in-source fragmentation of the conjugated compounds resulting in the detection of a “ghost” aglycone.

Therefore, the presence of sulfated and glucuronidated conjugates was confirmed in individual urine samples (Table 2). The levels of compounds which increased in urine after consumption of the fruit puree are shown in Figs. 1to 7.

Gallic acid, found in the free form, appeared in urine at early time points, reaching a maximum between 0 and 4 h post ingestion (Fig. 1A). Phase II metabolites of gallic acid (glucuronide, methyl and methyl-sulphate) reached a maximum excretion in urine between 2 to 4 h, slightly later than gallic acid (Table 2, Fig. 1 B, C and D). *O*-Glucuronidated metabolites of gallic acid were not observed with the initial screening using the LC-Orbitrap MS, but were found using the triple quadrupole LC-MS. One *O*-sulfated conjugate of protocatechuic acid was also found in urine samples (Table 2) and matched the single conjugate synthesized *in vitro*. Protocatechuic acid-*O*-sulfate and phase II metabolites of vanillic acid had an intermediate time of excretion, peaking between 4 and 8 h (Fig. 2). The excretion profiles of phase II metabolites of hydroxycinnamic acids (Table 2) are shown in Figs. 3 and 4.

Several (-)-epicatechin conjugates were enzymically produced *in vitro*, but only two *O*-sulfated and two *O*-glucuronidated metabolites were found in urine (Table 2), thus suggesting a more selective mechanism of conjugation *in vivo* by phase II enzymes. The time course in urine of (-)-epicatechin conjugates is consistent with a rapid excretion (Fig. 5).

Both *O*-glucuronides and *O*-sulfates of catechol and 4-methylcatechol were detected and had very similar profiles of appearance in urine (Fig. 6), starting from low levels at initial time points, and increasing especially after 4 h. The same excretion profile was observed for phase II metabolites of pyrogallol (Fig. 7). Only one sulfated metabolite of 1-*O*-methylpyrogallol was identified, although two metabolites were obtained *in vitro*.

**4 Discussion**

Berries contribute significantly to the dietary intake of polyphenols [10] but many of the metabolites and conjugates produced after consumption are not known. This study focused on the identification of urinary metabolites and their conjugates after ingestion of a berry-rich fruit puree containing a high level of different (poly)phenols. Multi-enzyme hydrolysis was used for identification and quantification of aglycone equivalents in the fruit puree, as previously described for each individual fruit [7]. This hydrolysis also mimics to a certain extent the deglycosylation known to occur *in vivo*, either in small intestine or in colon, prior to absorption [6, 11].

Our results show that, as expected, excretion generally follows phase II conjugation. Gallic acid appears to be an exception, as it was detected in the urine of all volunteers in relatively high amounts in its unconjugated form, although it was also the most abundant aglycone in the fruit puree. This compound was also found in urine after phase II metabolism, i.e. in glucuronidated, methylated and sulfated forms. Gallic acid and 4-*O*-methylgallic acid have been reported in humans after tea intake [12] and, in addition to these compounds, 4-*O*-methylgallic-3-*O*-sulfate was reported in rats after ingestion of gallic acid [13]. In humans, this metabolite was only tentatively identified after ingestion of (poly)phenol-rich juice [14] and red wine [15]. An *O*-glucuronidated conjugate of gallic acid, although not detected with LC-Orbitrap MS, was detected in the urine of volunteers with LC-triple quadrupole MS, due to its very low levels. This compound was confirmed using the standard that we prepared, and, to our knowledge, this is the first report of this metabolite in mammals. Absorption of gallic acid appears to be fast and elimination of this compound and its phase II metabolites in urine peaked 2 to 4 h after ingestion (Fig. 1), even though conjugates are still found in urine between 8 and 24 h. This suggests that gallic acid is still being produced and absorbed several hours after ingestion of the fruit puree, possibly due to degradation by the colonic microbiota of compounds such as esters of gallic acid, although they may also arise as breakdown products of anthocyanins such as malvidin or delphinidin [16-18].

Anthocyanins were very abundant in the puree, mainly glycosides of cyanidin from raspberries and blackberries [19-21], but also glycosides of malvidin, delphinidin, petunidin, cyanidin and peonidin from blueberry [20, 21]. However, their presence in urine was not demonstrated in LC-Orbitrap MS, due to their very low levels. Recent evidence suggests that anthocyanins are found in plasma and urine in very low amounts; nevertheless, the products of their degradation, colonic catabolism and metabolism are much more abundant [16, 22-24]. In a recent study, Czank and co-workers [25] administered isotopically labelled cyanidin-3-*O*-glucoside to human subjects, and revealed that apparent bioavailability of anthocyanin-derived products was far higher than expected; metabolites present in urine and plasma included degradation products of cyanidin such as protocatechuic acid and phloroglucinaldehyde. These reactions are consistent with *in vitro* data [24, 26]. Additionally phenylacetic acids, phenylpropenoic acids, hippuric acids and phase II conjugates of protocatechuic acid as metabolites derived from labelled cyanidin-3-*O*-glucoside were also detected in humans [25].

In our study, phloroglucinaldehyde was not evident in the urine of volunteers by LC-Orbitrap MS analysis. However, protocatechuic acid, at least partly resulting from the degradation of cyanidin, was found in urine essentially as one *O*-sulfated conjugate. Although two hydroxyl groups are available for sulfation in the protocatechuic acid molecule, only one peak was generated both *in vitro* and *in vivo*. However, two isomers have been described in humans for protocatechuic acid-*O*-sulfate [25]. Vanillic acid, possibly resulting from *O*-methylation of protocatechuic acid, was also found in urine, although its isomer isovanillic acid was not. In fact, vanillic acid was found conjugated with sulfate and glucuronic acid, and vanillic acid-*O*-sulfate has been previously identified in humans [25, 27]. Phase II metabolites of protocatechuic acid appeared in urine, particularly between 4 and 8 h (Fig. 2), suggesting a colonic origin. The appearance of one more *O*-glucuronidated metabolites than the number of available hydroxyl groups in the vanillic acid molecule might be due to peak dissociation caused by the chromatography conditions, or possibly glucuronidation of the carboxylic group which has been observed before [27]. The same situation might occur with gallic acid as we detected three glucuronidated metabolites after *in vitro* enzymic synthesis.

Caffeic acid, also found in high amounts in the fruit puree mostly conjugated with quinic acid, was found in urine in sulfated forms. However, other conjugated metabolites were also found, possibly resulting from metabolism of caffeic acid in the digestive tract since caffeic acid generates ferulic and isoferulic acids by methylation, DHC acid by reduction and dihydroferulic (DHF) acid by both events [9]. Although ferulic acid was also initially present in the fruit puree, it is possible that it also results from methylation of caffeic acid, as phase II metabolites of isoferulic acid were found in urine and isoferulic acid was not present in the fruit puree.

Caffeic acid-*O*-sulfates were observed in two temporal phases (Fig. 3 A, B). The first phase (peaking between 2 and 4 h) is consistent with the hydrolysis of 5-*O*-caffeoylquinic acid by esterases in the small intestine [28] but caffeic acid might also be derived from glycosidase action in the small intestine on caffeic acid glycosides. The second phase (peaking between 8 and 24 h) might result from the action of esterases from colonic microbiota on caffeoylquinic acids. Additionally, the late urinary peaks of DHC and DHF acids conjugates (Fig. 4 A to E) could also be explained by the action of the colonic microbiota on caffeic acid, as suggested previously [28]. In fact, studies made with ileostomist patients reveal that a proportion of the ingested caffeoylquinic acids can be recovered in the ileal fluid of humans, and thus reach the colon (between 26 and 78%, depending on the food matrix) [29, 30].

Although not very abundant in fruit puree, the presence of phase II metabolites of (-)-epicatechin in urine of volunteers was observed. Two *O*-sulfates and two *O*-glucuronides of (-)-epicatechin were found in urine, although the position of the conjugation was not confirmed. These metabolites reached a maximum in urine between 2 and 4 h (Fig. 5), indicating early absorption and excretion. Previous work has also indicated that (-)-epicatechin metabolites reached a maximum amount in plasma generally before 4 h after ingestion [14, 31]. Previously, three *O*-glucuronidated conjugates of (-)-epicatechin were found in plasma and urine of human subjects, identified as (-)-epicatechin-3'-*O*-β-D-glucuronide, (-)-epicatechin-4'-*O*-β-D-glucuronide and (-)-epicatechin-7-*O*-β-D-glucuronide [31]. The same study reported the presence of two *O*-sulfated conjugates of (-)-epicatechin characterized as (-)-epicatechin-3'-*O*-sulfate and (-)-epicatechin-4'-*O*-sulfate.

Although not found in fruit puree, phase II metabolites of pyrogallol and catechol were relatively abundant in urine samples. Pyrogallol and catechol in their free forms had been previously found as products of metabolism when Concord grape juice was incubated *in vitro* with faecal slurries [17]. They were also found in the urine of volunteers after ingestion of Concord grape juice especially at later time points, but neither of these compounds were observed in the urine of ileostomist patients [32], which strongly suggests a colonic origin based on degradation of other (poly)phenols. Pyrogallol has also been suggested to be generated from colonic degradation of malvidin-3-*O*-glucoside [16]. In our study, pyrogallol in the free form was detected in low amounts but catechol was not detected. However, methylated, sulfated and glucuronidated metabolites of these compounds increased significantly at later time points in urine (Fig. 6 and 7). Catechol-*O*-sulfate and catechol-*O*-glucuronide were previously found in urine of rats in which catechol had been injected into the renal portal circulation [33] or after administration of a *Glechoma longituba* extract [34]. However, to our knowledge, the presence of catechol-*O*-glucuronide in the urine of humans has never been described, and catechol-*O*-sulfate, although previously detected in human urine, was not related to the consumption of polyphenols [35]. Moreover, this is also the first report of *O*-sulfated and *O*-glucuronidated metabolites of 4-methylcatechol in human urine. Regarding pyrogallol-*O*-glucuronide, it was previously identified in rat urine after ingestion of gallic acid [13]. In the same study, 2-*O*-methylpyrogallol was found as the aglycone and also in a glucuronidated form [13]. Pyrogallol-*O*-glucuronide and pyrogallol-*O*-sulfate were also previously identified in volunteers after green and black tea consumption [27, 36, 37]. Isomers of methylpyrogallol-*O*-sulfate were also found after green and black tea consumption [27]. Phase II metabolites of both pyrogallol and catechol, therefore, appear to be generated from further catabolism of (poly)phenol metabolites by colonic microbiota.

**5 Concluding remarks**

This study focused particularly on detection and identification of phase II metabolites of phenolic acids after ingestion of a (poly)phenol-rich fruit puree. Either directly absorbed or produced via degradation of more complex (poly)phenols, these metabolites showed a significant and substantial increase in urine of volunteers after ingestion of the puree, and represent possible biomarkers for (poly)phenol intake. Several metabolites were for the first time reported and confirmed in human urine after ingestion of (poly)phenols, including 4-*O*-methylgallic acid-3-*O*-sulfate, gallic acid-*O*-glucuronide, catechol-*O*-sulfate and -*O*-glucuronide, and 4-methylcatechol-*O*-sulfate and -*O*-glucuronide. Additionally, when considering the effect of certain dietary (poly)phenols on human health, these metabolites and conjugates should be considered for their possible biological activities.

**6 References**

[1] Basu, A., Rhone, M., Lyons, T. J., Berries: emerging impact on cardiovascular health. *Nutr Rev* 2010, *68*, 168-177.

[2] Gonzalez-Gallego, J., Victoria Garcia-Mediavilla, M., Sanchez-Campos, S., Tunon, M. J., Fruit polyphenols, immunity and inflammation. *Brit J Nutr* 2010, *104*, S15-S27.

[3] Dai, Q., Borenstein, A. R., Wu, Y., Jackson, J. C., Larson, E. B., Fruit and vegetable juices and Alzheimer's disease: The Kame Project. *Am J Med* 2006, *119*, 751-759.

[4] Paredes-Lopez, O., Cervantes-Ceja, M. L., Vigna-Perez, M., Hernandez-Perez, T., Berries: improving human health and healthy aging, and promoting quality life - a review. *Plant Foods Hum Nutr* 2010, *65*, 299-308.

[5] Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M.*, et al.*, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective efects against chronic diseases. *Antiox Redox Sign* 2013, *18*, 1818-1892.

[6] Williamson, G., Clifford, M. N., Colonic metabolites of berry polyphenols: the missing link to biological activity? *Brit J Nutr* 2010, *104*, S48-S66.

[7] Pimpao, R. C., Dew, T., Oliveira, P. B., Williamson, G.*, et al.*, Analysis of phenolic compounds in portuguese wild and commercial berries after multienzyme hydrolysis. *J Agric Food Chem* 2013, *61*, 4053-4062.

[8] Duenas, M., Mingo-Chornet, H., Joaquin Perez-Alonso, J., Di Paola-Naranjo, R.*, et al.*, Preparation of quercetin glucuronides and characterization by HPLC-DAD-ESI/MS. *Eur Food Res Technol* 2008, *227*, 1069-1076.

[9] Fumeaux, R., Menozzi-Smarrito, C., Stalmach, A., Munari, C.*, et al.*, First synthesis, characterization, and evidence for the presence of hydroxycinnamic acid sulfate and glucuronide conjugates in human biological fluids as a result of coffee consumption. *Org Biomol Chem* 2010, *8*, 5199-5211.

[10] Pinto, P., Cardoso, S., Pimpao, R. C., Tavares, L.*, et al.*, Daily polyphenol intake from fresh fruits in Portugal: contribution from berry fruits. *Int J Food Sci Nutr* 2013, *64*, 1022-1029.

[11] Day, A. J., Canada, F. J., Diaz, J. C., Kroon, P. A.*, et al.*, Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters* 2000, *468*, 166-170.

[12] Shahrzad, S., Aoyagi, K., Winter, A., Koyama, A., Bitsch, I., Pharmacokinetics of gallic acid and its relative bioavailability from tea in healthy humans. *J Nutr* 2001, *131*, 1207-1210.

[13] Yasuda, T., Inaba, A., Ohmori, M., Endo, T.*, et al.*, Urinary metabolites of gallic acid in rats and their radical-scavenging effects on 1,1-diphenyl-2-picrylhydrazyl radical. *J Nat Prod* 2000, *63*, 1444-1446.

[14] Borges, G., Mullen, W., Mullan, A., Lean, M. E. J.*, et al.*, Bioavailability of multiple components following acute ingestion of a polyphenol-rich juice drink. *Mol Nutr Food Res* 2010, *54*, S268-S277.

[15] Boto-Ordonez, M., Urpi-Sarda, M., Isabel Queipo-Ortuno, M., Corella, D.*, et al.*, Microbial Metabolomic Fingerprinting in Urine after Regular Dealcoholized Red Wine Consumption in Humans. *J Agric Food Chem* 2013, *61*, 9166-9175.

[16] Hidalgo, M., Oruna-Concha, M. J., Kolida, S., Walton, G. E.*, et al.*, Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J Agric Food Chem* 2012, *60*, 3882-3890.

[17] Stalmach, A., Edwards, C. A., Wightman, J. D., Crozier, A., Colonic catabolism of dietary phenolic and polyphenolic compounds from Concord grape juice. *Food Funct* 2013, *4*, 52-62.

[18] Pawlowska, A. M., De Leo, M., Braca, A., Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: Identification of anthocyanins and gallic acid derivatives. *J Agric Food Chem* 2006, *54*, 10234-10238.

[19] Borges, G., Degeneve, A., Mullen, W., Crozier, A., Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *J Agric Food Chem* 2010, *58*, 3901-3909.

[20] Mullen, W., Larcombe, S., Arnold, K., Welchman, H., Crozier, A., Use of accurate mass full scan mass spectrometry for the analysis of anthocyanins in berries and berry-fed tissues. *J Agric Food Chem* 2010, *58*, 3910-3915.

[21] Cho, M. J., Howard, L. R., Prior, R. L., Clark, J. R., Flavonoid glycosides and antioxidant capacity of varous blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J Sci Food Agric* 2004, *84*, 1771-1782.

[22] Aura, A. M., Martin-Lopez, P., O'Leary, K. A., Williamson, G.*, et al.*, In vitro metabolism of anthocyanins by human gut microflora. *Eur J Nutr* 2005, *44*, 133-142.

[23] Nurmi, T., Mursu, J., Heinonen, M., Nurmi, A.*, et al.*, Metabolism of berry anthocyanins to phenolic acids in humans. *J Agric Food Chem* 2009, *57*, 2274-2281.

[24] Woodward, G. M., Needs, P. W., Kay, C. D., Anthocyanin-derived phenolic acids form glucuronides following simulated gastrointestinal digestion and microsomal glucuronidation. *Mol Nutr Food Res* 2011, *55*, 378-386.

[25] Czank, C., Cassidy, A., Zhang, Q., Morrison, D. J.*, et al.*, Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a C-13-tracer study. *Am J Clin Nutr* 2013, *97*, 995-1003.

[26] Kay, C. D., Kroon, P. A., Cassidy, A., The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. *Mol Nutr Food Res* 2009, *53*, S92-S101.

[27] van der Hooft, J. J. J., de Vos, R. C. H., Mihaleva, V., Bino, R. J.*, et al.*, Structural elucidation and quantification of phenolic conjugates present in human urine after tea intake. *Anal Chem* 2012, *84*, 7263-7271.

[28] Stalmach, A., Mullen, W., Barron, D., Uchida, K.*, et al.*, Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metab Dispos* 2009, *37*, 1749-1758.

[29] Erk, T., Renouf, M., Williamson, G., Melcher, R.*, et al.*, Absorption and isomerization of caffeoylquinic acids from different foods using ileostomist volunteers. *Eur J Nutr* 2013, DOI 10.1007/s00394-00013-00512-z.

[30] Stalmach, A., Steiling, H., Williamson, G., Crozier, A., Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch Biochem Biophys* 2010, *501*, 98-105.

[31] Actis-Goretta, L., Leveques, A., Giuffrida, F., Romanov-Michailidis, F.*, et al.*, Elucidation of (-)-epicatechin metabolites after ingestion of chocolate by healthy humans. *Free Radic Biol Med* 2012, *53*, 787-795.

[32] Stalmach, A., Edwards, C. A., Wightman, J. D., Crozier, A., Gastrointestinal stability and bioavailability of (poly)phenolic compounds following ingestion of Concord grape juice by humans. *Mol Nutr Food Res* 2012, *56*, 497-509.

[33] Rennick, B., Quebbema.A, Site of excretion of catechol and catecholamines - renal metabolism of catechol. *Am J Physiol* 1970, *218*, 1307-1312.

[34] Ni, S., Qian, D., Duan, J.-a., Guo, J.*, et al.*, UPLC-QTOF/MS-based screening and identification of the constituents and their metabolites in rat plasma and urine after oral administration of *Glechoma longituba* extract. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010, *878*, 2741-2750.

[35] Roux, A., Xu, Y., Heilier, J.-F., Olivier, M.-F.*, et al.*, Annotation of the human adult urinary metabolome and metabolite identification using ultra high performance liquid chromatography coupled to a linear quadrupole ion trap-orbitrap mass spectrometer. *Anal Chem* 2012, *84*, 6429-6437.

[36] Daykin, C. A., Van Duynhoven, J. P. M., Groenewegen, A., Dachtler, M.*, et al.*, Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J Agric Food Chem* 2005, *53*, 1428-1434.

[37] Van Dorsten, F. A., Daykin, C. A., Mulder, T. P. J., Van Duynhoven, J. P. M., Metabonomics approach to determine metabolic differences between green tea and black tea consumption. *J Agric Food Chem* 2006, *54*, 6929-6938.

**Funding/acknowledgements:**

We thank to Pedro Oliveira (Instituto Nacional de Investigação Agrária, Oeiras, Portugal) for providing *Vaccinum* spp., *Rubus* spp*,* and *Rubus idaeus*. We also acknowledge all volunteers who participated in the study. This work was supported by Fundação para a Ciência e a Tecnologia under grants PEstOE/EQB/LA0004/2011, SFRH/BD/63615/2009 (RP) and SRFH/BPD/84618/2012 (CNS), The Scottish Government Rural and Environment Science and Analytical Services Division (DS and GJM), Climafruit (Interreg IVB) (DS), EU FP7 EUBerry KBBE-2010-4 265942 (CNS and DS) and the European Research Council (POLYTRUE? 322467 to GW).

**Conflict of Interest:**

The authors have declared no conflicts of interest.

Table 1. Quantification of phenolic compounds in fruit juice, consumed by volunteers, after hydrolysis using hesperidinase and cellulase using HPLC-DAD relative to authentic standards.

|  |  |
| --- | --- |
| **Compound** | **Concentration****(mg/500 mL fruit puree)** |
| **Gallic acid** | 425.9 ± 14.0 |
| **Protocatechuic acid** | 16.7 ± 0.2 |
| **3-Methylgallic acid** | 12.9 ± 0.1 |
| **(+)-Catechin** | 17.6 ± 3.9 |
| **Vanillic acid** | 2.9 ± 0.5 |
| **Caffeic acid** | 140.4 ± 2.2 |
| **Ph**l**oroglucinaldehyde** | 28.8 ± 0.3 |
| **Syringic acid** | 13.2 ± 0.6 |
| **(-)-Epicatechin** | 12.2 ± 1.6 |
| **Ellagic acid** | 24.8 ± 1.3 |
| **Ferulic acid** | 6.9 ± 0.1 |
| **Myricetin** | 15.6 ± 0.2 |
| **Quercetin** | 15.5 ± 0.4 |
| **Kaempferol** | 2.4 ± 0.0 |

Table 2. Summary of HPLC- triple quadrupole MS/MS detection properties for commercial or synthesized standards and indication of their presence in urine. A = aglycone, M = metabolite, RT = retention time.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Metabolite code** | **RT** | **[M-H]- (m/z)** | **Metabolite identity** | **Presence in urine** | **Standards Origin** |
| Precursor | Fragment |
| **A1** | 8.1 | 109.1 |  | Catechol | Χ | C |
| **M1** | 7.12 | 189.1 | 109.1 | Catechol-*O*-sulfate |  | S |
| **M2** | 11.7 | 285.1 | 109.1 | Catechol-*O*-glcrnd |  | S |
| **A2** | 19.3 | 123.1 |  | 4-Methylcatechol | Χ | C |
| **M3** | 18.7 | 203.1 | 123.1 | 4-Methylcatechol-*O*-sulfate \* |  | S |
| **M4** | 20.9 | 299.1 | 123.1 | 4-Methylcatechol-*O*-glcrnd \* |  | S |
| **A3** | 3.1 | 125.1 |  | Pyrogallol |  | C |
| **M5** | 3.5 | 205.1 | 125.1 | Pyrogallol-*O*-sulfate \* |  | S |
| **M6** | 6.2 | 205.1 | 125.1 | Pyrogallol-*O*-sulfate \* |  | S |
| **M7** | 3.7 | 301.1 | 125.1 | Pyrogallol-*O*-glcrnd \* |  | S |
| **M8** | 9. 7 | 301.1 | 125.1 | Pyrogallol-*O*-glcrnd \* |  | S |
| **A4** | 13.1 | 139.1 |  | 1-*O*-Methylpyrogallol | Χ | C |
| **M9** | 10.5 | 219.1 | 139.1 | 1-*O*-Methylpyrogallol-*O*-sulfate \* |  | S |
| **M10** | 11.2 | 219.1 | 139.1 | 1-*O*-Methylpyrogallol-*O*-sulfate \* | Χ | S |
| **A5** | 9.0 | 139.1 |  | 2-*O*-Methylpyrogallol | Χ | C |
| **M11** | 6.0 | 219.1 | 139.1 | 2-*O*-Methylpyrogallol-1-*O*-sulfate |  | S |
| **A6** | 6.5 | 153.1 | 108.1 | Protocatechuic acid |  | C |
| **M12** | 5.9 | 233.1 | 153.1 | Protocatechuic acid-*O*-sulfate \* |  | S |
| **A7** | 16.6 | 167.1 | 151.8 | Vanillic acid |  | C |
| **M13** | 7.8 | 247.1 | 167.1 | Vanillic acid-4-*O-*sulfate |  | S |
| **A8** | 18.5 | 167.1 | 151.8 | Isovanillic acid | Χ | C |
| **M14** | 10.5 | 247.1 | 167.1 | Isovanillic acid-3-*O*-sulfate | Χ | S |
| **M15** | 5.0 | 301.1 | 167.1 | Vanillic acid-*O*- glcrnd \* |  | S |
| **M16** | 8.9 | 301.1 | 167.1 | Vanillic acid-*O*- glcrnd \* |  | S |
| **A9** | 2.9 | 169.1 | 124.9 | Gallic acid |  | C |
| **M17** | 1.3 | 345.1 | 169.1 | Gallic-acid-*O*- glcrnd \* | Χ | S |
| **M18** | 2.4 | 345.1 | 169.1 | Gallic-acid-*O*- glcrnd \* | Χ | S |
| **M19** | 4.6 | 345.1 | 169.1 | Gallic-acid-*O*- glcrnd \* |  | S |
| **A10** | 10.0 | 183.1 | 167.8 | 4-*O*-Methylgallic acid |  | C |
| **M20** | 7.6 | 263.1 | 183.1 | 4-*O*-Methylgallic acid-3-*O*-sulfate |  | S |
| **A11** | 24.1 | 193 | 134, 178 | Ferulic acid | Χ | C |
| **A12** | 25.1 | 193 | 134, 178 | Isoferulic acid | Χ | C |
| **A13** | 18.2 | 174 | 135 | Caffeic acid |  | C |
| **A14** | 15.0 | 181 | 137 | Dihydrocaffeic acid |  | C |
| **A15** | 22.7 | 195 | 136 | Dihydroferulic acid |  | C |
| **M21** | 18.5 | 369 | 193, 113 | Ferulic acid-4-*O*-glcrnd |  | P |
| **M22** | 22.3 | 369 | 193, 113 | Isoferulic acid-3-*O*-glcrnd |  | P |
| **M23** | 17.4 | 357 | 181, 137 | DHC acid-4-*O*-glcrnd | Χ | P |
| **M24** | 18.3 | 357 | 181, 137 | DHC acid-3-*O*-glcrnd |  | P |
| **M25** | 20.3 | 371 | 195, 113 | DHF acid-4-*O*- glcrnd |  | P |
| **M26** | 21.5 | 273 | 193, 178 | Ferulic acid-4-*O*-sulfate |  | P |
| **M27** | 22.7 | 273 | 193, 178 | Isoferulic acid-3-*O*-sulfate |  | P |
| **M28** | 19.2 | 259 | 179, 135 | Caffeic acid-4-*O*-sulfate |  | P |
| **M29** | 20.1 | 259 | 179, 135 | Caffeic acid-3-*O*-sulfate |  |  P |
| **M30** | 17.4 | 261 | 181, 137 | DHC acid-4-*O*-sulfate |  | P |
| **M31** | 17.6 | 261 | 181, 137 | DHC acid-3*-O*-sulfate |  | P |
| **M32** | 20.3 | 275 | 195, 136 | DHF acid-4-*O*-sulfate |  | P |
| **A16** | 21.0 | 289.1 | 244.9 | (-)-Epicatechin | Χ | C |
| **M33** | 17.2 | 465.1 | 289 | Epicatechin-*O*-glcrnd \* |  | S |
| **M34** | 17.8 | 465.1 | 289 | Epicatechin-*O*-glcrnd \* | Χ | S |
| **M35** | 18.3 | 465.1 | 289 | Epicatechin-*O*-glcrnd \* | Χ | S |
| **M36** | 18.9 | 465.1 | 289 | Epicatechin-*O*-glcrnd \* | Χ | S |
| **M37** | 20.8 | 465.1 | 289 | Epicatechin-*O*-glcrnd \* |  | S |
| **M38** | 17.6 | 369.1 | 289 | Epicatechin-*O*-sulfate \* |  | S |
| **M39** | 20.1 | 369.1 | 289 | Epicatechin-*O*-sulfate \* | Χ | S |
| **M40** | 21.9 | 369.1 | 289 | Epicatechin-*O*-sulfate \* |  | S |

\* - Conjugated compounds which were not possible to distinguish between isomers due to more than one available position for conjugation.

Legend: ✓ – present in urine, X – absent in urine, Glcrnd – Glucuronide, P – Commercially available (HPLC grade), S – Enzymically synthesized, D – Provided by Prof. Denis Barron

**Figures**

Figure 1. Box plot of relative quantification based on peak area of conjugated metabolites of gallic acid found in urine at different collection times in human volunteers. (A) Gallic acid (A9), (B) gallic acid-*O*-glucuronide (M19), (C) 4-*O*-methylgallic acid (A10), (D) 4-*O*-methylgallic-3-*O*-sulfate (M20). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

Figure 2. Box plot of relative quantification of conjugated metabolites of protocatechuic acid and vanillic acid found in urine at different collection times in human volunteers. (A) Protocatechuic acid-*O*-sulfate (M12), (B) vanillic acid-4-*O*-sulfate (M13), (C) vanillic acid-*O*-glucuronide (M15), (D) vanillic acid-*O*-glucuronide (M16). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.



Figure 3. Box plot of relative quantification of conjugated metabolites of caffeic acid, ferulic acid and isoferulic acid found in urine at different collection times in human volunteers. (A) Caffeic acid-4-*O*-sulfate (M28), (B) caffeic acid-3-*O*-sulfate (M29), (C) ferulic acid-4-*O*-glucuronide (M21), (D) ferulic acid-4-*O*-sulfate(M26), (E) isoferulic acid-3-*O*-glucuronide (M22), (F) isoferulic acid-3-*O*-sulfate (M27). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

Figure 4. Box plot of relative quantification of conjugated metabolites of DHC acid and DHF acid found in urine at different collection times in human volunteers. (A) DHC acid-4-*O*-sulfate (M30), (B) DHC acid-3-*O*-sulfate (M31), (C) DHC acid-3-*O*-glucuronide (M24), (D) DHF acid-4-*O*-sulfate (M32), (E) DHF acid-4-*O*-glucuronide (M25). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

Figure 5. Box plot of relative quantification of conjugated metabolites of (-)-epicatechin found in urine at different collection times in human volunteers. (A) (-)-Epicatechin-*O*-glucuronide (M33), (B) (-)-epicatechin-*O*-glucuronide (M37), (C) (-)-epicatechin-*O*-sulfate (M38), (D) (-)-epicatechin-*O*-sulfate (M40). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

Figure 6. Box plot of relative quantification of conjugated metabolites from catechol and 4-methylcatechol found in urine at different collection times in human volunteers. (A) Catechol-*O*-sulfate (M1), (B) catechol-*O*-glucuronide (M2), (C) 4-methylcatechol-*O*-sulfate (M3), (D) 4-methylcatechol-*O*-glucuronide (M4). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

Figure 7. Box plot of relative quantification of conjugated metabolites of pyrogallol, 1-*O*-methylpyrogallol and 2-*O*-methylpyrogallol found in urine at different collection times in human volunteers. (A) pyrogallol-*O*-sulfate (M5), (B) pyrogallol-*O*-sulfate (M6), (C) pyrogallol-*O*-glucuronide (M7), (D) pyrogallol-*O*-glucuronide (M8), (E) 1-*O*-methylpyrogallol-*O*-sulfate (M9), (F) 2-*O*-methylpyrogallol-1-*O*-sulfate (M11). No star, not significant; \*, p < 0.05; \*\*, p < 0.01 relative to time zero.

**Supplementary materials**

Table 1. Quantification of total anthocyanins, 5-*O*-caffeoylquinic acid, and 3-*O*-caffeoylquinic acid [mg/500 mL] in fruit puree ingested by volunteers determined by HPLC-DAD (details see materials and methods section)

|  |  |
| --- | --- |
| **Compounds** | **Concentration****(mg/500 mL fruit puree)** |
| **Total anthocyanins1** | 636 ±19 |
| **5-Caffeoylquinic acid** | 130 ± 2 |
| **3-Caffeoylquinic acid** | 5.9 ± 0.1 |

1 Anthocyanin peaks were summed and quantified as cyanidin-3-*O*-glucoside equivalents

Table 2. Structure of metabolites of protocatechuic, vanillic and gallic acids, and presence in human urine using the Orbitrap LC-MS.



|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Metabolite** | **R1** | **R2** | **R3** | **R4** | **Exact mass** | **Detected in urine** |
| Protocatechuic acid | OH | OH | OH | OH | 154.0266 |  Χ |
| Protocatechuic acid-3-*O*-sulfate | OSO3 | OH | H | OH | 233.9834 |  |
| Protocatechuic acid-4-*O*-sulfate | OH | OSO3 | H | OH |
| Vanillic acidIsovanillic acid | OCH3OH | OHOCH3 | HH | OHOH | 168.0423 |  |
| Vanillic acid-4-*O*-sulfate | OCH3 | OSO3 | H | OH | 247.9991 |  |
| Isovanillic acid-3-*O*-sulfate | OSO3 | OCH3 | H | OH |
| Vanillic acid-4-*O*-glcrnd | OCH3 | OGlcrnd | H | OH | 344.0743 |  |
| Isovanillic acid-3-*O*-glcrnd | OGlcrnd | OCH3 | H | OH |
| Gallic acid | OH | OH | OH | OH | 170.0215 |  |
| Gallic acid-1-*O*-glcrnd | OGlcrnd | OH | OH | OH | 346.0536 |  Χ |
| Gallic acid-2-*O*-glcrnd | OH | OGlcrnd | OH | OH |
| 4-*O*-Methylgallic acid | OH | OCH3 | OH | OH | 184.0372 |  |
| 4-*O*-Methylgallic acid-3-*O*-sulfate | OSO3 | OCH3 | OH | OH | 263.9940 |  |

Legend: Glcrnd - Glucuronide

Table 3. Structure of metabolites of caffeic and ferulic acids, and presence in human urine using the Orbitrap LC-MS.



|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Metabolites** | **R1** | **R2** | **Exact mass** | **Detected in urine** |
| Caffeic acid | OH | OH | 180.0423 |  |
| Caffeic acid-3-*O*-sulfate | OSO3 | OH | 259.9991 |  |
| Caffeic acid-4-*O*-sulfate | OH | OSO3 |
| Caffeic acid-3-*O*-glcrnd | OGlcrnd | OH | 356.0743 |  |
| Caffeic acid-4-*O*-glcrnd | OH | OGlcrnd |
| Ferulic acid | OCH3 | OH | 194.0579 |  |
| Ferulic acid-4-*O*-sulfate | OCH3 | OSO3 | 274.0147 |  |
| Ferulic acid-4-*O*-glcrnd | OCH3 | OGlcrnd | 370.0900 |  |
| Isoferulic acid | OH | OCH3 | 194.0579 |  |
| Isoferulic acid-3-*O*-sulfate | OSO3 | OCH3 | 274.0147 |  |
| Isoferulic acid-3-*O*-glcrnd | OGlcrnd | OCH3 | 370.0900 |  |

Legend: Glcrnd - Glucuronide

Table 4. Structure of metabolites of dihydrocaffeic acid and dihydroferulic acid, and presence in human urine using the Orbitrap LC-MS.



|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Metabolites** | **R1** | **R2** | **Exact mass** | **Detected in urine** |
| DHC acid | OH | OH | 182.0579 | Χ |
| DHC acid-3-*O*-sulfate | OSO3 | OH | 262.0147 |  |
| DHC acid-4-*O*-sulfate | OH | OSO3 |
| DHC acid-3-*O*-glcrnd | OGlcrnd | OH | 358.0900 |  Χ |
| DHC acid-4-*O*-glcrnd | OH | OGlcrnd |
| DHF acid | OCH3 | OH | 196.0736 |  Χ |
| DHF acid-4-*O*-sulfate | OCH3 | OSO3 | 276.0304 |  |
| DHF acid-4-*O*-glcrnd | OCH3 | OGlcrnd | 372.1056 |  |

Legend: Glcrnd - Glucuronide

Table 5. Structure of metabolites of (-)-epicatechin and presence in human urine using the Orbitrap LC-MS.



|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Metabolites** | **R1** | **R2** | **R3** | **R4** | **R5** | **Exact mass** | **Detected in urine** |
| (-)-Epicatechin | OH | OH | OH | OH | OH | 290.0790 |  |
| (-)-Epicatechin-3-*O*-sulfate | OSO3 | OH | OH | OH | OH | 370.0359 |  |
| (-)-Epicatechin-5-*O*-sulfate | OH | OSO3 | OH | OH | OH |
| (-)-Epicatechin-7-*O*-sulfate | OH | OH | OSO3 | OH | OH |
| (-)-Epicatechin-3'-*O*-sulfate | OH | OH | OH | OSO3 | OH |
| (-)-Epicatechin-4'-*O*-sulfate | OH | OH | OH | OH | OSO3 |
| (-)-Epicatechin-3-*O*-glcrnd | Oglcrnd | OH | OH | OH | OH | 466.1111 |  |
| (-)-Epicatechin-5-*O*-glcrnd | OH | Oglcrnd | OH | OH | OH |
| (-)-Epicatechin-7-*O*-glcrnd | OH | OH | Oglcrnd | OH | OH |
| (-)-Epicatechin-3'-*O*-glcrnd | OH | OH | OH | Oglcrnd | OH |
| (-)-Epicatechin-4'-*O*-glcrnd | OH | OH | OH | OH | Oglcrnd |

Legend: Glcrnd - Glucuronide

Table 6. Structure of metabolites of catechol and pyrogallol, and presence in human urine using the Orbitrap LC-MS.



|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Metabolite** | **R1** | **R2** | **R3** | **R4** | **Exact mass** | **Detected in urine** |
| Catechol | OH | OH | H | H | 110.0368 |  |
| Catechol-1-*O*-sulfate | OSO3 | OH | H | H | 189.9936 |  |
| Catechol-1-*O*-glcrnd | OGlcrnd | OH | H | CH3 | 286.0689 |  |
| 4-Methylcatechol | OH | OH | H | CH3 | 124.0524 |  |
| 4-Methylcatechol-1-*O*-sulfate | OSO3 | OH | H | CH3 | 204.0092 |  |
| 4-Methylcatechol-2-*O*-sulfate | OH | OSO3 | H | CH3 |
| 4-Methylcatechol-1-*O*-glucrnd | OGlcrnd | OH | H | CH3 | 300.0845 |  |
| 4-Methylcatechol-2-*O*-glucrnd | OH | OGlcrnd | H | CH3 |
| Pyrogallol | OH | OH | OH | H | 126.0317 |  |
|  Pyrogallol-1-*O*-sulfate | OSO3 | OH | OH | H | 205.9885 |  |
| Pyrogallol-2-*O*-sulfate | OH | OSO3 | OH | H |
| Pyrogallol-1-*O*-Glcrnd | OGlcrnd | OH | OH | H | 302.0638 |  |
| Pyrogallol-2-*O*-Glcrnd | OH | OGlcrnd | OH | H |  |
| 1-*O*-Methylpyrogallol | OCH3 | OH | OH | H | 140.0473 |  Χ |
| 2-*O*-Methylpyrogallol | OH | OCH3 | OH | H |
| 1-*O*-Methylpyrogallol-3-*O*-sulfate1-*O*-Methylpyrogallol-2-*O*-sulfate | OCH3OCH3 | OHOSO3 | OSO3OH | HH | 220.0042 |  |
| 2-*O*-Methylpyrogallol-1-*O*-sulfate | OSO3 | OCH3 | OH | H |
| 1-*O*-Methylpyrogallol-3-*O*-glcrnd1-*O*-Methylpyrogallol-2-*O*-glcrnd | OCH3OCH3 | OHOGlcrnd | OGlcrndOH | HH | 330.0587 |  Χ |
| 2-*O*-Methylpyrogallol-1-*O*-glcrnd | OGlcrnd | OCH3 | OH | H |

Legend: Glcrnd - Glucuronide

Figure 1. LC-MS chromatograms of enzymically produced compounds *in vitro*. The [M-H]- ion chromatograms were selected for: (A) m/z 345.1, gallic acid-*O*-glucuronides; (B) m/z 301.1, vanillic acid-*O*-glucuronides; (C) m/z 233.1, proctocatechuic acid-*O*-sulfate; (D) m/z 465.1, (-)-epicatechin-*O*-glucuronides.

