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Quantification of phenolic-sulfates as colonic metabolites in plasma of human volunteers after ingestion of a mixed berry fruit puree

Rui C. Pimpão[1,2], M. Rita Ventura[a], Ricardo B. Ferreira[1,3], Gary Williamson[4], Claudia N. Santos[1,2,*]

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, EAN, 2781-901 Oeiras, Portugal.

[2] Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal.

[3]Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal.

[4]School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, United Kingdom.

* Corresponding author: Dr. Claudia Santos, Tel: +351214469651; Fax: +351214433644; Email address: csantos@itqb.unl.pt

List of abbreviations: ACN, acetonitrile; CA, caffeic acid; Cat, catechol; DHCA, Dihydrocaffeic acid; DHFS, Dihydroferulic acid; FA, Ferulic acid; GA, Gallic acid; MePyr, Methylpyrogallol; PA, protocatechuic acid; Pyr, Pyrogallol; Sulf, Sulfate; 4-MeCat, 4-Methylcatechol; 4-MeGA, 4-methylgallic acid.

Keywords: Berry fruits/ Bioavailability/ Polyphenols/ Conjugates/ Sulfates

Abstract

Bioavailability studies are vital to assess the potential impact of bioactive compounds on human health. Although conjugated phenolics from colonic metabolism have been identified in urine, the quantification and appearance of these compounds in plasma is less well studied, but important when assessing potential activity in vivo. To address this gap, a crossover intervention with ingestion of fruit puree and a standard (poly)phenol-free meal by thirteen volunteers was conducted, and plasma metabolites identified by HPLC-MS/MS. Sulfated-standards were chemically synthesized to facilitate quantification. There was a fast absorption of gallic and caffeic acid-conjugates, reaching a maximum concentration between 1 and 2 h. Sulfated-metabolites resulting from colonic degradation of more complex (poly)phenols started to increase in plasma from 4 h, and reached 5-20 μM at 6 h for pyrogallol-sulfate and catechol-sulfate. In conclusion, phenolic-sulfates were quantified in plasma and reached much higher concentrations than their parent compounds. They have potential use as biomarkers of (poly)phenol intake and their biological activities need to be considered.

Introduction

Berry fruits assume great importance for daily consumption of (poly)phenols, due to the high contents of compounds such as phenolic acids and flavonoids, particularly anthocyanins [1, 2]. Even in populations where there is only a small consumption of berries, the contribution of these fruits to the overall ingestion of (poly)phenols can be high, in comparison to other food sources [3]. Epidemiological and experimental studies suggest that (poly)phenols reduce the risk of developing several pathological conditions [4-6]. In vitro studies are important to elucidate the mechanism of action of (poly)phenols in this regard. However, for the credibility of these studies, it is essential to use compounds that can actually be found in the human body and use them in physiologically-relevant concentrations. The role of intervention studies in calculating bioavailability and understanding the metabolic fate of the ingested compounds is very important [7, 8].

Recently, the idea has emerged that quantification of (poly)phenols in biological samples collected from humans and animals might be underestimated, since many metabolites formed from catabolism of (poly)phenols by colon microbiota can still be absorbed into the blood, and are usually not accounted for [9, 10]. Many of these compounds can undergo further metabolism and be conjugated by phase II enzymes, to form sulfated, glucuronidated and methylated compounds [11]. We previously identified several phenolics in conjugated form in the urine of healthy human volunteers, after ingestion of a puree composed of five different berry fruits [12]. Some of these compounds were indentified in humans and associated with the consumption of (poly)phenols for the first time. It is now important to calculate the plasma appearance of these metabolites and assess the physiological concentration they can reach, in order to account for total and combined (poly)phenol bioavailability.

In this study, the sulfated compounds previously identified were chemically synthesized and used as standards for quantification in plasma of human subjects, after ingestion of the berry puree. A crossover study in which participants ingested either the fruit puree or a standard (poly)phenol-free meal was also conducted to assess any endogenous or non-(poly)phenol dietary origin of the studied metabolites.

Material and methods

Reagents and reference materials

All chemicals used in this investigation were purchased from Sigma-Aldrich, unless stated otherwise and purchased reference standards were all HPLC grade (>95%). ACN (LS-MS grade) was purchased from Fisher Scientific Ltd. (Leicestershire, UK). LC-MS grade water was produced by an Elix/MilliQ purification system (Millipore, Waterford, UK). 4-Methylgallic acid and 2-methylpyrogallol were obtained from Apin chemicals (Oxon, UK). Taxifolin was obtained from Extrasynthese. Hydroxycinnamic acid-conjugates were chemically synthesized and characterized as described previously [13] and were kindly provided by Prof. Denis Barron, NIHS, Lausanne, Switzerland; protocatechuic acid-3-O-sulfate and protocatechuic acid-4-O-sulfate were kindly provided by Dr. Paul Needs, IFR, Norwich, UK.

Fruit puree

The fruit puree was prepared on the day of the study and its composition has been described previously [12]. Briefly, 100 g of each of five fruits were used: 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 on a domestic food processor, for 1 min at room temperature.

Subjects and study design

Twenty two volunteers (4 of them male), aged between 22 and 54, with an average BMI of 22.6 kg/m^2 were recruited for this study. Individuals were all considered healthy by a medical questionnaire and standard blood tests. 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.

The study was divided in two groups. In the first group, nine volunteers followed a (poly)phenol-free diet for 2 days before the study and throughout the day of the study. 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. Blood samples were collected into EDTA containing tubes before ingestion of the puree and after at 0.5, 1, 2, 4 and 6 h. The second group, containing thirteen volunteers, participated in a cross-over controlled trial. The procedure was similar as the first group, however the two stages, which occurred two weeks apart, varied with ingestion of the puree or a standard breakfast (poly)phenols free, where the puree was substituted by plain yogurt. Blood samples were collected into EDTA containing tubes before ingestion of the fruit puree and at 2 and 4 h after ingestion.

The compliance with the food restriction was confirmed through a questionnaire. The study protocol was in accordance with the Declaration of Helsinki of 1975, as revised in 1983, 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.

Sample treatment

After collection, blood samples were immediately centrifuged at 2200 g for 15 min, and 1 mL plasma samples were stored in cryotubes with addition of 30 µL of formic acid (50 % v/v). Samples were kept at -80 ℃ prior to analysis.

Protein precipitation was based on a modification of a previously described method [14]. Briefly, to 380 µL of plasma were added 20 uL ascorbic acid 4 mg/mL (final concentration of 1 mM) and taxifolin as internal standard to a final concentration of 250 nM. Sample was combined with 1 mL hexane, homogenized and centrifuged for 10 min at 17 000 g. The aqueous phase was recovered and added dropwise to 1200 µL of ACN. The mixture was vortexed for 2 min and centrifuged for 10 min at 17 000 g. Supernatant was recovered and pellet was reconstituted with 400 µL ACN and after centrifugation, ACN supernatants were combined and dried on a centrifugal evaporator. Samples were reconstituted in 100 µL of water containing 1 µM of sinapic acid as second internal standard. Samples were centrifuged at 17 000 g for 10 min and the supernatants were filtered prior to HPLC-MS/MS analysis.

HPLC-MS/MS analysis

Plasma sample analysis was conducted on an Agilent HPLC 1200 series comprising a 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) connected with a 6410a triple-quadrupole LC-MS-MS. Separation was achieved on an Atlantis T3 Column, 100 Å, 3 µm, 2.1 mm ID x 100 mm HPLC column (Waters, Hertfordshire, UK) at a flow rate of 0.26 mL/min over a gradient of 100% solvent A (95% H_2O, 5% ACN with 0.5% (v/v) formic acid) for 10 min, reaching 15% B (95% ACN, 5% H_2O 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 interface was set 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 optimized using the reference standards (see Table 1). The system was controlled and data processed by Agilent MassHunter software (version B.01.03). The m/z transitions optimized for each standard were previously optimized [12]. Quantification of metabolites in plasma was obtained using calibration curves of the available standards. At least 8 concentrations, ranging from 0.05 to 20 µM, were constructed from analytical standards and each point was injected in triplicate. Standard curves were all linear within the concentration range and linearity was ensured as R^2=0.997-1.000 (Table 1).

Statistical analysis

Pharmacokinetic profile of plasma 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 99%. For the cross-over study comparisons were made between consumption of fruit puree and the control breakfast for each time point and the same test was used.

Synthesis of sulfated compounds

The products were synthesized by a treatment of the initial aglycones (500 mg) with sulfor trioxide pyridine [pyridine:SO_3]. The aglycones (500 mg) and pyridine:SO_3 (1 equivalent for all compounds with exception of vanillic acid where 2 equivalents were used) were dissolved in 10 mL of anhydrous pyridine and kept at 65 ºC with constant stirring for 24 h. Reaction was stopped by addition of water. Solvents were dried in vacuo and the residue was dissolved in water. The unreacted aglycones were separated

with ethyl acetate and the product in the water phase was purified with a Dowex 50W-X8 ion-exchange column loaded with Na^{+}. Purified compounds were then dried in vacuo and characterized by ^{1}H and ^{13}C NMR. NMR chemical shifts are reported using the residual solvent peak as reference. Peak assignments were based on COSY and HMQC experiments.

Catechol-O-sulfate [(2-hydroxyphenyl)-oxidanesulfonic acid] : 674 mg, 66 % yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.85 (s, 1H, OH), 7.12 (dd, 1H, J 1.6, 7.96 Hz), 6.96 (ddd, 1H, J1.64, 7.96, 9.26 Hz), 6.83 (dd, 1H, J 1.6, 8 Hz), 6.75 (ddd, 1H, J 1.68, 7.92, 9.24 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 149.21, 140.92, 124.88, 123.13, 119.25, 117.24.

Pyrogallol-O-sulfate: 677 mg, 75 % yield; mixture of two compounds in approximate similar proportion; pyrogallol-2-O-sulfate [(2,6-dihydroxyphenyl)-oxidanesulfonic acid]:^{1}H NMR (400MHz, D_2O): δ 6.97 (t, 1H, J 8.24 Hz, H-4), 6.51 (d, 2H, J 8.28 Hz, H-3 + H-5) and pyrogallol-O-sulfate [(2,3-dihydroxyphenyl)oxidanesulfonic acid]: ^{1}H NMR (400MHz, D_2O): δ 6.90 – 6.86 (m, 1H, H-5), 6.79 – 6.74 (m, 2H, H-4 + H-6); ^{13}C NMR (100 MHz, D_2O): δ 149.84, 145.42, 139.76, 127.19, 120.34, 114.31, 113,80, 108.93.

2-Methylpyrogallol-O-sulfate [(3-hydroxy-2-methoxyphenyl)-oxidanesulfonic acid]: 450 mg, 44 % yield, having 11 % contamination with the disulfated byproduct. ^{1}H NMR (400MHz, DMSO-d_6): δ 8.97 (s, 1H, OH), 6.97 (dd, J 1.52, 8.32 Hz, H-6), 6.74 (t, 1H, J 8.2 Hz, H-5), 6.51 (dd, 1H, J 1.52, 8.08 Hz, H-4), 3.34 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.57, 147.20, 139.10, 122.33, 112.30, 110.89, 60.09.

1-Methylpyrogallol-O-sulfate: 500 mg, 58 % yield; (2-hydroxy-6-methoxyphenyl)-oxidanesulfonic acid and (2-hydroxy-3-methoxyphenyl)-oxidanesulfonic acid, mixture of both compounds in approximate equal amounts (56%:44%). ^{1}H NMR (400MHz, DMSO-d_6): δ 9.20 (s, 1H, OH), 8.52 (s, 1H, OH), 6.92 (t, 1H, J 8.60 Hz), 6.77-6.68 (m, 3H), 6,52-6.44 (dd, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.39, 150.93, 149.17, 141.35, 139.07, 124.86, 118.07, 115.65, 110.08, 108.64, 104.11, 55.87, 55.72

4-Methylcatechol-O-sulfate: 604 mg, 66 % yield. Mixture of the two compounds, (2-hydroxy-4-methylphenyl)-oxidanesulfonic acid and 2-hydroxy-5-methylphenyl)-oxidanesulfonic acid, not being possible to distinguish them, One is present at 64%, ^{1}H NMR (400MHz, DMSO-d_6): δ 8.76 (s, 1H, OH), 6.96 (d, 1H, J 8.08 Hz), 6.64 (d, 1H, J 1.76 Hz), 6.55 (dd, 1H, J 2.04, 8.6 Hz), 2.20 (s, 3H, CH$_3$). The other being 36% of the sample, ^{1}H NMR (400MHz, DMSO-d_6): δ 8.62 (s, 1H, OH), 6.93 (d, 1H, J 1.84 Hz), 6.77 (dd, 1H, J 1.56, 8.04 Hz), 6.71 (d, 1H, J 8.12 Hz), 2.19 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 148.87, 146.80, 140.70, 138.80, 134.20, 128.14, 125.27, 123.58, 122.94, 119.87, 117.77, 116.96, 20.06, 20.47.

4-Methylgallic-3-O-sulfate [3-hydroxy-4-methoxy-5-(sulfooxy)benzoic acid]: 485 mg, 57 % yield, having a contamination of 13% of the disulfate and 6% of the starting material;^{1}H NMR (400MHz, D$_2$O): δ 7.46 (d, 1H, J 2.04 Hz, H-2), 7.32 (d, 1H, J 2 Hz, H-6), 3.88 (s, 3H, CH$_3$) ; ^{13}C NMR (100 MHz, D$_2$O):δ 172.07, 149.19, 143.99, 142.85, 129.61, 115.42, 114.84, 61.29.

Vanillic acid-4-O-sulfate [3-methoxy-4-(sulfooxy)benzoic acid]: 924 mg, quantitative yield. ^{1}H NMR (400MHz, DMSO-d_6): δ 7.57 (d, 1H, J 8.16 Hz, H-2), 7.50-7.47 (m, 2H, H-5+H-6), 3.79 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.39, 149.51, 148.4, 146.41, 121.96, 119.33, 112.71, 55.48.

Results

Composition of fruit puree

Detailed identification and quantification of (poly)phenols in the fruit puree have been described previously [12]. The fruit puree (500 mL) contained anthocyanins (636 ±19 mg) and caffeoylquinic acids (5-caffeoylquinic and 3-caffeoylquinic acids, 135.9 ± 2.1 mg). Aglycones were quantified after hydrolysis by the use of gycosidases from *Aspergillus niger* as previously published [15]. Gallic acid (GA) was the most abundant aglycone (425.9 ± 14.0 mg/500 mL fruit puree). Caffeic acid (CA) was also abundant (140.4 ± 2.2 mg/500 mL fruit puree), resulting from hydrolysis of the caffeoylquinic acids and conjugated glycosides. After hydrolysis, several other phenolic acids were detected and quantified including the flavanols (-)-epicatechin and (+)-catechin, and the flavonols quercetin, myricetin and kaempferol. The presence of vanillic (VA), ferulic (FA) and protocatechuic (PA) acids were also observed in the hydrolyzed extract.

Chemical synthesis of sulfated phenolics

(Poly)phenol metabolites were identified previously in urine samples from human volunteers after ingestion of the fruit puree [12]. In this work, we focused on quantification of sulfated-metabolites in plasma. Some were chemically synthesized for this purpose (Table 1). Synthesized compounds were mostly pure as determined by NMR, except for 2-methylpyrogallol-O-sulfate (89% pure due to a double-sulfate subsituted compound) and 4-methylgallic acid-3-O-sulfate (81 % pure due to the presence of some aglycone and a double-sulfate substituted derivative). The LC-MS/MS chromatographic profile of the synthesized standards is shown in Table 1, with most compounds resulting in single peaks. However, for the compounds 4-methylcatechol (4-MeCat) and 1-methylpyrogallol (1-MePyr), two different sulfated metabolites were obtained after synthesis as can be observed by NMR, although they co-eluted as only one chromatographic peak. Protocatechuic acid-3-O-sulfate (PA-3-sulf) and protocatechuic acid-4-O-sulfate (PA-4-sulf) also co-eluted when run together on HPLC-MS/MS.

Plasma appearance of phenolics

Previously, several metabolites were identified in the urine of volunteers after ingestion of fruit puree, and although only a relative quantification was performed, sulfated-phenolics were more abundant than glucuronidated-phenolics [12]. In this work, plasma of human volunteers was analysed after ingestion of the fruit puree for confirmation of the presence of those sulfated-metabolites by LC-MS/MS (Fig 1). Gallic acid was identified, as well as its conjugated metabolites 4-O-methylgallic acid (4-MeGA) and 4-O-methylgallic acid-3-O-sulfate (4-MeGA-Sulf). Sulfated-metabolites of VA, FA, dihydroferulic (DHFA) and dihydrocaffeic (DHCA) acids were also confirmed in plasma. PA-O-sulfate (PA-sulf) was also present, but it was not possible to distinguish between the isomers PA-3-O-sulf and PA-4-O-sulf, since they co-eluted.

Although one peak of sulphated DHCA was found in plasma at low levels, it is possible that this could be two sulfated metabolites, since they co-eluted (Table 2). The presence of sulfated metabolites of catechol (Cat), 4-MeCat, 1-MePyr and 2-O-methylpyrogallol (2-MePyr) was also confirmed. Two sulfated-metabolites of pyrogallol (Pyr) were found, corresponding to the synthesized standards.

Plasma quantification of phenolic metabolites

The synthesized standards or pure aglycones were used for the quantification of phenolic metabolites (Table 1). For the co-eluting compounds in plasma (sulfates of PA, 1-MePyr and 4-MeCat) quantification was done as corresponding to a single compound. Measurement of plasma metabolites was performed between 0 and 6 h following the ingestion of 500 mL of fruit puree. The pharmacokinetic profile of each metabolite is shown in Fig. 2 and an average maximum concentration of the metabolites when present in six or more volunteers is presented in Table 2. GA, despite being present in all volunteers, was mostly found under the limit of quantification, only being quantifiable in three out of nine volunteers, reaching a maximum concentration of 840±340 nM at 1 h after ingestion of the fruit puree. However, its conjugated metabolites 4-O-methylgallic (4-MeGA) and 4-O-methylgallic-3-O-sulfate clearly peaked at 2 h. DHFA-O-sulf was detected in the plasma of volunteers, however it was under the limit of quantification. DHCA-O-sulfate was only quantifiable in four volunteers at 6h where it reached 151±18 nM.

The metabolites catechol-O-sulfate (Cat-sulf), 4-MeCat-sulf, pyrogallol-O-sulfates and O-methylpyrogallol- O-sulfates were found at baseline in several volunteers. All these metabolites, with exception of 4-MeCat-sulf, had a statistically significant increase over

the collection period time in comparison to the baseline. Interestingly, the compounds Cat-sulf and Pyr-sulf even reached concentrations up to 20 µM in some volunteers. However, variability between plasmatic concentrations between the volunteers was high for all the quantified metabolites.

Comparison between ingestion of fruit puree and (poly)phenol-free meal

Confirmation of the provenance of the analysed metabolites was assessed by the ingestion of a (poly)phenol-free meal (control) as opposed to the ingestion of fruit puree by the same volunteers. Comparative analysis was done in plasma samples collected at baseline, at 2 h and at 4 h and is summarized in Fig 3. At baseline, no differences were observed between ingestion of the fruit puree or the (poly)phenol-free meal. However, for most metabolites, a statistically significant increase was observed after ingestion of the fruit puree at a certain time point. The only exceptions were observed for PA-sulf where an increase was observed both after ingestion of the fruit puree and the control meal and for VA-sulf which at 4 h had a higher increase in plasma after ingestion of the control meal than for the fruit puree.

For Pyr-sulf, although a small increase was observed in plasma of volunteers 2 h after the ingestion of the control meal, at 4 h there was a marked increase in the plasma of volunteers ingesting the fruit puree.

Discussion

Conjugation reactions, particularly sulfation, glucuronidation and methylation, are known to be involved in the metabolism of phenolics in the human body, generally resulting in stabilisation and increased water-solubility, and therefore modifying their distribution and excretion [16, 17]. Sulfate conjugation is one of the major metabolic pathways for endogenous and exogenous phenolic compounds [18]. Previously, we demonstrated that sulfated phenols were found in urine after ingestion of a fruit puree containing 5 berry fruits [12]. In the present study, some of those compounds were chemically synthesized and used for quantification purposes in plasma collected from volunteers. Confirmation of their provenance was also achieved by comparing the amounts in plasma of volunteers consuming the puree or a (poly)phenol-free standard meal.

GA, a compound present as aglycone in high amounts in the fruit puree, was previously quantified in human plasma as well as its methylated metabolite 4-MeGA after ingestion of GA tablets and black tea [19]. Its kinetics were similar to the present study, reaching a maximum concentration near 1.5 h. Identified for the first time in rats urine by Yasuda and co-workers [20], 4-MeGA-3-sulf was quantified in human plasma for the first time in the present study and was undoubtedly derived from compounds within the fruit puree (Fig. 3). Its kinetic profile was similar to GA and 4-MeGA reaching a maximum concentration at 2 h (Figure 2, Table 1), suggesting that absorption and metabolism of GA was fast, with the 4-hydroxyl group being regioselectively preferred for methylation and the 3-hydroxyl group for sufation.

Previous studies suggested PA as one of the major metabolites from degradation of cyanidin [21, 22]. Of the compounds studied here, PA-sulf, VA-sulf and FA-sulf were previously identified in plasma as metabolites resulting from the degradation of ^{13}C-labeled cyanidin-3-glucoside in the digestive tract [9]. However, in the present study, only at 6 h was there a significant increase of PA-sulf and VA-sulf compared to the baseline, and an increase of these metabolites was registered also in the control meal. It is unlikely that (poly)phenols are present in the control meal, and so phenolics present after ingestion of the control meal are likely to be derived from endogenous metabolism. A comparable example is dopamine metabolism, where homovanillic acid is a known metabolite, which would probably exist as homovanillic acid-sulfate [23].

Hydroxycinnamic acid metabolism has been widely studied in relation to coffee consumption and our results are consistent with these data. Sulfation is dominant over glucuronidation for most hydroxycinnamic acids [24], but methylation also appears to have a important role, resulting in FA and isoferulic acid [25]. CA-3-O-sulfate and FA-4-O-sulfate clearly resulted from ingestion of the puree (Fig. 3) and were quantified in plasma, having similar values and absorption kinetics. These results were in accordance with previous studies proposing rapid absorption kinetics for CA and FA metabolites in plasma [25, 26]. DHCA and DHFA-conjugates were not quantifiable in plasma, being mostly under the limit of quantification. However, these are derived from the action of colonic bacterial reductases [25], and so would only start to appear at 5 hours and have a T_{max} between 7 and 11 hours [27].

Previously we identified conjugated-phenolic metabolites possibly resulting from catabolism of other (poly)phenols [12]. Their colonic origin is suggested since some unconjugated phenolics have been identified in faeces after ingestion of (poly)phenols [10, 28]. However, our data suggest that absorption in the colon might also occur and these compounds can subsequently be found conjugated in plasma. The origin of these phenolic-conjugates such as catechol, pyrogallol, 2-methylpyrogallol and 3-methylpyrogallol-O-sulfated metabolites was a consequence of consuming the fruit puree, and to our knowledge this is the first time that these compounds have been quantified in human plasma and associated with (poly)phenol metabolism. Clearly, this class of metabolites has been neglected in bioavailability studies and, since they reached considerable amounts in the plasma, further studies are needed to access their biological significance. Recently, the importance of colonic catabolism of (poly)phenols and several metabolites has been appreciated. Due to the ability of the colonic microbiota to catalyse reactions such as O- and C-deglycosylation, ester and amide hydrolysis and deglucuronidation, flavonoids, once considered to show poor bioavailability, might result in smaller phenolics with a much higher absorption than the parent compounds [29]. Additionally, new classes of metabolites have been specifically assigned to colonic metabolites of flavonoids, such as valerolactones from catabolism of catechins and urolithins from catabolism of ellagic acid and ellagitannins [28, 30]. These metabolites of colonic origin might be of great importance for the health effects commonly associated to the ingestion of polyphenols.

Glucuronidation has been described as being a major route for conjugation of phenolics such as acetaminophen [31], quercetin [32], protocatechuic and vanillic acids [33] and other substances with a 1,2-dihydroxybenzene group [34]. However, sulfation is the primary conjugation route of the hydroxycinnamic acids CA, FA and DHCA [24] and many other sulfated phenolics have been reported, including sulfates of protocatechuic and vanillic acids [35]. Sulfation of phenolics is carried out by cytosolic sulfotransferases (SULTs) which catalyze the transfer of a sulfonate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a substrate containing a hydroxyl group. The isoforms SULT1A1, SULT1A3/4, SULT1B1, SULT1E1 and SULT2A1 were considered the most relevant in (poly)phenol metabolism in human adults [18]. Besides sulfation, methylation, carried out by catechol O-methyltransferase (COMT), also has great importance in metabolism of phenolic compounds [17]. This activity may be responsible for the conversion of pyrogallol to 1- and 2-methylpyrogallol, GA to 4-MeGA, CA to FA, DHCA to DHFA and PA to VA. Additionally, all of these compounds could be sulfated, and this dual conjugation of compounds will affect re-activation of conjugated compounds via deconjugation reactions [17].

In summary, our results confirm the provenance of several phenolic-conjugates resulting from the ingestion of a (poly)phenol-rich berry fruit puree by humans, since they increase in plasma in comparison to either the baseline or after ingestion of a (poly)phenol-free control meal. Metabolism of fruit (poly)phenols results in methylated, sulfated and some dual conjugated compounds. We highlight the importance of catabolism in the colon, generating simple phenols that can then be absorbed from the colon and circulate in conjugated form in the blood. Therefore, they contribute indirectly to the bioavailability of food (poly)phenols, and can potentially also be used as markers for (poly)phenol intake.

Table 1- HPLC-MS/MS parameter for the quantification of phenolic metabolites in human plasma after ingestion of 500 mL of fruit puree

Compound name	Compound abbreviation	MRM parent ion	MRM daughter ion	Calibration curve range (μM)	R^2
Gallic acid	GA	169.1	124.9	0.4-20	0.998
4-Methylgallic acid	4-MeGA	183.1	167.8	0.1-8	0.997
4-methylgallic acid-3-O-sulfate	4-MeGA-sulf	163.1	183.1	0.29-14.7	0.998
Protocatecuic acid-O-sulfate	PA-sulf	233.1	153.1	0.2-16	0.999
Vanillic acid-4-O-sulfate	VA-sulf	147.1	167.1	0.18-18.5	1.000
Caffeic acid-O-sulfate	CA-sulf	259	179	0.1-16	1.000
Ferulic acid-4-O-sulfate	FA-sulf	273	193	0.05-8	1.000
Isoferulic acid-3-O-sulfate	IA-sulf	273	193	0.05-8	1.000
Dihydrocaffeic acid-O-sulfate	DHCA-sulf	261	181	0.1-8	0.999
Dihydroferulic acid-4-O-sulfate	DHFA-sulf	275	195	0.1-8	0.999
Catechol-O-sulfate	Cat-sulf	189.1	109.1	0.2-20	0.999
4-Methylcatechol-O-sulfate	4-MeCat-sulf	203.1	123.1	0.1-20	0.999
Pyrogallol-O-sulfate	Pyr-sulf	205.1	125.1	0.2-20	0.998
1-Methylpyrogallol-O-sulfate	1-MePyr-sulf	219.1	139.1	0.34-17	1.000
2-Methylpyrogallol-1-O-sulfate	2-MePyr-sulf	219.1	139.1	0.2-20	0.999

Table 2 –Concentration in plasma of phenolic conjugated-metabolites and presence in volunteers after ingestion of 500 mL of fruit puree

Metabolite	Cmax (nM)[†]	Tmax (hours)*	Number of volunteers present
4-MeGA	300 ± 138	2	7
4-MeGA-sulf	2028 ± 1095	2	9
VA-sulf	1345 ± 1310	4	6
PA-sulf	1055 ± 972	4	6
CA-3-sulf	181 ± 127	1	8
FA-sulf	188 ± 107	1	9
Cat-sulf	12194 ± 5860	6	9
4-MeCat-sulf	636 ± 469	6	7
Pyr-sulf (1)	652 ± 328	6	7
Pyr-sulf (2)	11430 ± 6678	6	8
1-mePyr-sulf	2879 ± 1807	6	9
2-MePyr-sulf	1969 ± 982	6	8

[†]C_{max}, peak plasma concentration calculated from the average concentration in volunteers where it was present.

*T_{max}, time to reach C_{max} of the metabolites for the times of collection. It is no to exclude higher concentration of some metabolites after 6h.
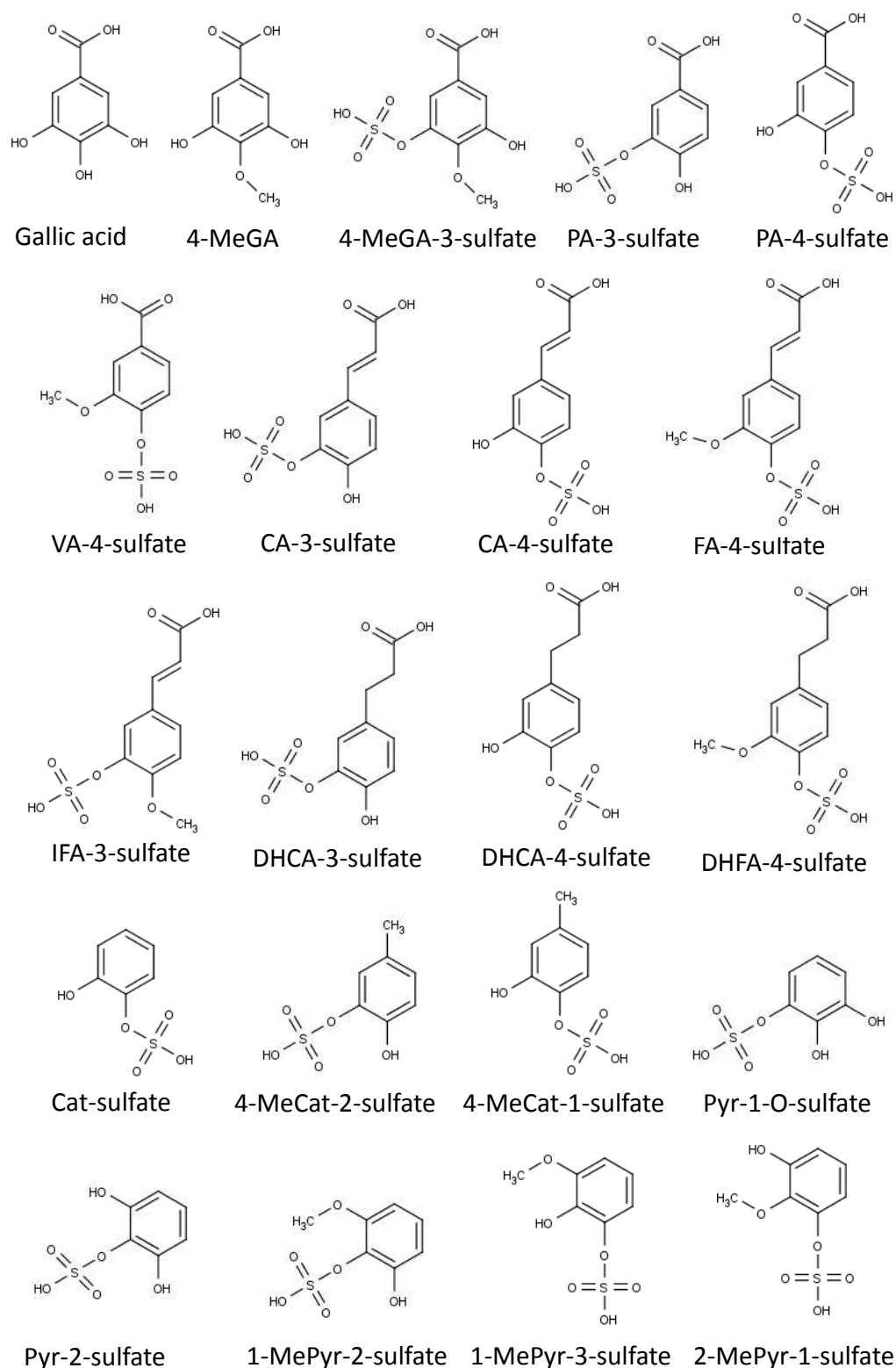
Fig. 1. Structure of phenolic metabolites searched for in the plasma from human volunteers:. 4-MeGA, 4-methylgallic acid; PA, protocatechuic acid; VA, vanillic acid; CA, Caffeic acid; FA, ferulic acid; IFA, isoferulic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; Cat, catechol; 4-MeCat, 4-methylcatechol; Pyr, Pyrogallol; MePyr, Methylpyrogallol.
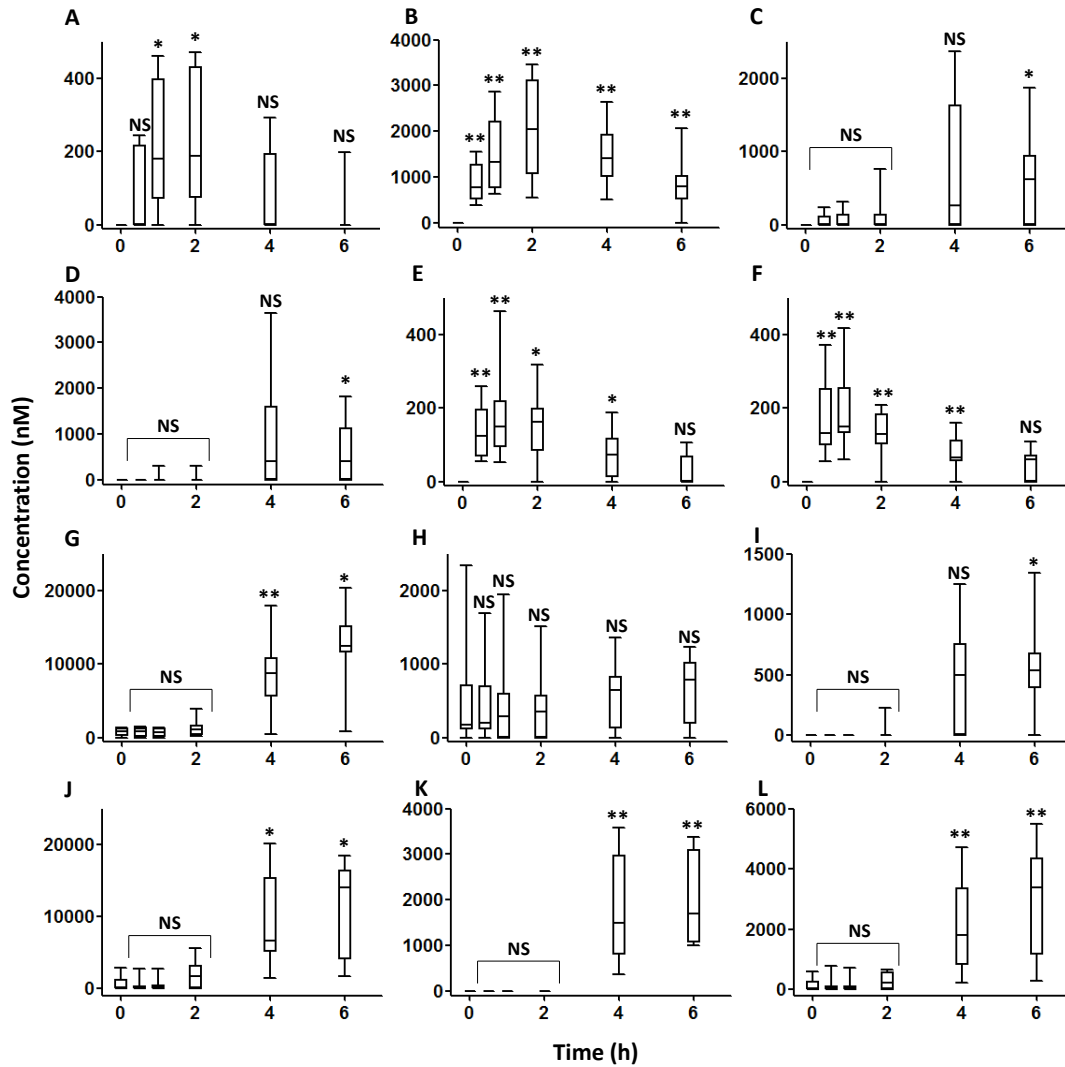
Fig. 2

Figure 2. Plasma quantification of phenolic metabolites at baseline, and at 0.5, 1, 2, 4 and 6 h after ingestion of 500 mL of fruit puree. A – 4-MeGA, B – 4MeGA-sulf, C – PA -sulf, D – VA-sulf, E – CA-sulf, F – FA-sulf, G – Cat-sulf, H – 4-MeCat-sulf, I – Pyr-sulf (1),   J – Pyr-sulf (2), K – 2-MePyr-sulf, L – 1-Mepyr-sulf.   Statistical comparison is in relation to baseline. No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.
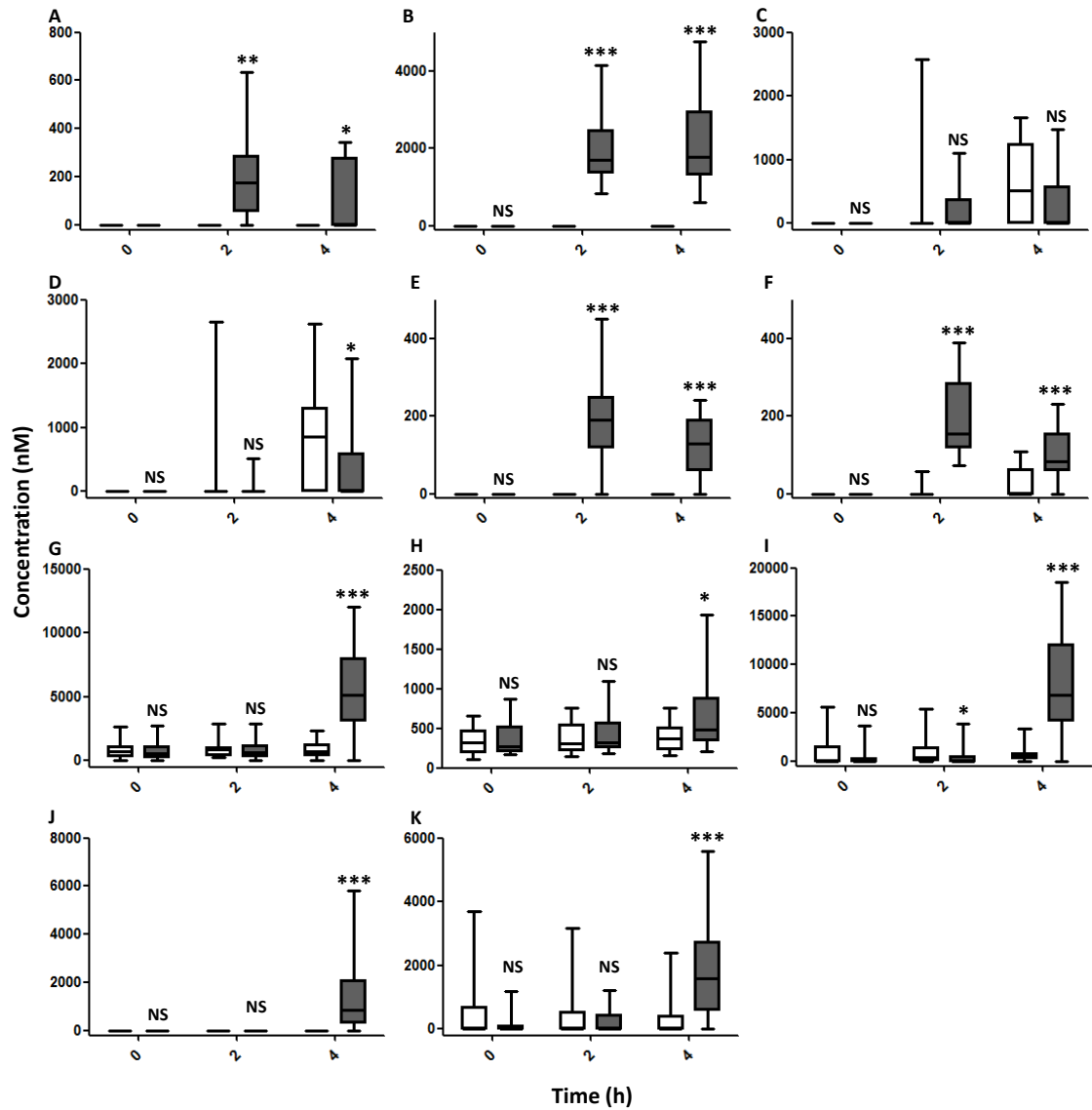
Fig. 3

Figure 3. Plasma quantification of phenolic metabolites at baseline, and at 2 and 4h after ingestion of the fruit puree (■) or standard breakfast (□). A – 4-MeGA, B –4-MeGA-sulft, C – PA-sulf, D – VA-sulf, E – CA-sulf, F – FA-sulf, G – Cat-sulf, H – 4-MeCat-sulf, I – Pyr-sulf (2), J – 2-MePyr-sulf, K – 3-MePyr-sulf. Statistics compare plasma concentrations of each volunteer after ingestion of the fruit puree or standard breakfast. No star, not significant; *, $p < 0.05$; **, $p < 0.01$, $p < 0.005$ relative to time zero.

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