Identification of Plasma and Urinary Metabolites and Catabolites Derived from Orange Juice (Poly)phenols: Analysis by High Performance Liquid Chromatography-High Resolution-Mass Spectrometry

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ABSTRACT: Orange juice is a rich source of (poly)phenols, in particular the flavanones hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside. Following the acute consumption of 500 mL of orange juice containing 398 μmol of (poly)phenols by 12 volunteers, 0-24 h plasma and urine samples were analysed by targeted high-performance liquid chromatography-high resolution-mass spectrometry in order to identify flavanone metabolites and phenolic acid and aromatic catabolites. A total of 19 flavanone metabolites which comprised di-*O*-glucuronide, *O*-glucuronide, *O*-glucuronyl-sulfate, and sulfate derivatives of hesperetin, naringenin and eriodictyol, and 65 microbial-derived phenolic catabolites, such as phenylpropanoid, phenylpropionic, phenylacetic, benzoic and hydroxycarboxylic acids and benzenetriol and benzoylglycine derivatives, including free phenolics and phase II sulfate, glucuronide and methyl metabolites, were identified or partially identified in plasma and/or urine samples. The data obtained provide a detailed evaluation of the fate of orange juice (poly)phenols as they pass through the gastrointestinal tract, and are absorbed into circulatory systems prior to renal excretion. Potential pathways for these conversions are proposed.

KEYWORDS**:** *orange juice, flavanones, HPLC-HR-MS identification, urinary and plasma metabolites; colon-derived phenolic catabolites, bioavailability, humans*

■INTRODUCTION

Human intervention trials have provided evidence for the protective effects against chronic diseases of a fruit and vegetable (poly)phenol-rich diet.[1,2] Fruit juices represent a further option for consumers to increase their intake of (poly)phenols. In particular, orange juice (OJ) is of interest due to its widespread consumption and high flavanone content, predominantly in the form of hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside[3]. Epidemiological prospective studies have shown consistent associations between the intake of flavanone-containing citrus fruit and prevention of various types of cancer,[4,5,6] and regular consumption of OJ has been linked to improved vascular function.[7]

Among the factors necessary to elucidate the mode of action underlying the potential protective effects of flavanones in humans, is an understanding of the absorption, disposition, metabolism and excretion (aka bioavailability) of flavanones in vivo. Following ingestion, OJ flavanones were, until recently, considered to be poorly bioavailable with only relatively small amounts of the ingested dose entering the systemic circulation, not as the parent compounds but as phase II metabolites, mainly glucuronide and sulfate derivatives, with studies reporting peak plasma concentration ranging from 63 to 1500 nM[8,9] and urinary recoveries <5-16% of intake.[10-13] Research with ileostomists has shown that ~70% of ingested flavanones pass to the colon[14] where they are degraded by the action of the microbiota giving rise, principally to small phenolic and aromatic acid catabolites[10-11] which are absorbed into the circulatory system. When urinary excretion of the phase II flavanone metabolites and the colonic catabolites are taken into account the bioavailability of OJ flavanones increases markedly.[10]

To date, a number of studies have investigated the absorption, metabolism and excretion of OJ (poly)phenols in humans[8-13,15,16] and most have focused on the analysis of flavanone metabolites in plasma and/or urine, without taking in consideration the major transformations mediated principally by the colonic microbiota. The aim of this study was to apply targeted HPLC-HR-MS methodology, which provides high sensitivity and selectivity, to obtain detailed and novel information on the identity of metabolites and colon-derived phenolic catabolites in plasma and urine 0-24 h after acute consumption of 500 mL of OJ by 12 physically-fit male volunteers.

■MATERIAL AND METHODS

Chemicals and Reagents. 4-Hydroxybenzoic acid, 3-hydroxybenzoic acid, ferulic acid, isoferulic acid, 4´-hydroxyphenylacetic acid, 3´-hydroxyphenylacetic acid, 3´,4´-dimethoxyphenylacetic acid, 3´-methoxy-4´-hydroxyphenylacetic acid (homovanillic acid), hippuric acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-methoxy-4-hydroxybenzoic acid (vanillic acid), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), 3-(3´-methoxy-4´-hydroxyphenyl)propionic acid (dihydroferulic acid), 3-(3´-hydroxy-4´-methoxyphenyl)propionic acid (dihydroisoferulic acid), 3-(3´-methoxy-4´-hydroxy)mandelic acid, 4´-hydroxymandelic acid, 3-(3´,4´-dihydroxyphenyl)propionic acid (dihydrocaffeic acid), 3-(3´,4´-dihydroxyphenyl)acetic acid (homoprotocatechuic acid), 3-(4´-hydroxyphenyl)propionic acid, sinapic acid, phenylacetic acid, 3-(phenyl)propionic acid, 1,3,5-trihydroxyphenol (phloroglucinol), 1,2,3-trihydroxyphenol (pyrogallol) and 1,2-dihydroxyphenol (catechol), 3´-hydroxycinnamic acid (*m*-coumaric acid), 4´-hydroxycinnamic

acid (*p*-coumaric acid) and *p*-sympatol were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). 3´-Hydroxyhippuric acid, 3-(3´-hydroxyphenyl)hydracrylic acid, hesperetin-7-*O*-glucuronide, naringenin-4´-*O*-glucuronide, and naringenin-7-*O*-glucuronide were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Coumaric acid-3´-*O*-glucuronide, coumaric acid-4´-*O*-glucuronide, caffeic acid-3´-*O*-glucuronide, caffeic acid-3´-sulfate, caffeic acid 4´-*O*-glucuronide, caffeic acid 4´-sulfate, ferulic acid-4´-*O*-glucuronide, ferulic acid-4´-sulfate, isoferulic acid-3´-*O*-glucuronide, 3-(3´-hydroxyphenyl)propionic acid-4´-*O*-glucuronide (dihydrocaffeic acid-4'-*O*-glucuronide), 3-(phenyl)propionic acid-4´-*O*-glucuronide, 3-(4´-hydroxyphenyl)propionic acid-3'-*O*-glucuronide (dihydrocaffeic acid-3'-*O*-glucuronide), 3-(3´-hydroxyphenyl)propionic acid-4´-sulfate (dihydrocaffeic acid-4'-sulfate), 3-(4´-hydroxyphenyl)propionic acid-3´-sulfate(dihydrocaffeic acid-3'-sulfate), 3-(3´-methoxyphenyl)propionic acid-4´-*O*-glucuronide (dihydroferulic acid-4´-*O*-glucuronide), 3-(4´-methoxyphenyl)propionic-acid-3´-*O*-glucuronide (dihydro-isoferulic acid-3´-*O*-glucuronide) and 3-(3´-methoxyphenyl)propionic-acid-4´-sulfate (dihydroferulic acid-4´-sulfate) were kindly provided by Denis Barron (Nestle Research Center, Lausanne, Switzerland) and Gary Williamson (School of Food Science and Nutrition, University of Leeds, UK). 4´-Hydroxyhippuric acid was obtained from Bachem (UK) Ltd (St Helens, UK). 3-(3'-Hydroxyphenyl)propionic acid was supplied by Fluorochem (Derby, UK). 3-(3′-Hydroxy-4′-methoxyphenyl)hydracrylic acid was isolated in a previous study.[10] Hesperetin-7-*O*-rutinoside (hesperidin), 4´-*O*-methyl-naringenin-7-*O*-rutinoside (didymin), and naringenin-7-*O*-rutinoside (narirutin) were obtained from Extrasyntheses (Genay, France). Hesperetin-3'-sulfate was a generous gift from Dr. Christine Morand (INRA/Clemont-Ferrand, France). Formic acid and HPLC-MS grade methanol were obtained from Panreac (Barcelona, Spain).

Study Design. Twelve men aged 31.8 ± 5.7 years, with a body mass index of 21.7 ± 1.8 kg/m^2 mean value ± SD) volunteered to participate in this study. All trained regularly, were fit, healthy normotensive non-smokers and were not taking any drug therapies or supplements. Other exclusion criteria included: history of gastrointestinal disease, eating disorders and being vegetarian. Written informed consent was provided by all participants. The study protocol was approved by the College of Medical, Veterinary and Life Sciences Ethics Committee of the University of Glasgow and registered at BioMed Central Ltd. (http://www.controlledtrials.com/ISRCTN04271658).

Before attending the clinic, participants were asked to follow a low (poly)phenol diet for 2 days by avoiding fruits and vegetables, nuts, high-fibre products, chocolate and beverages such as tea, coffee and fruit juices, as well as to abstain from consuming alcohol. On the morning of the feeding trial, volunteers reported to the metabolic suite between 08.00 and 09.00 h after a 12-h fast and brought their 12 h overnight urine. A venous cannula was inserted and after a 10 min interval a 10 mL baseline blood sample was obtained. Participants then consumed 500 mL of OJ (Tropicana "With Bits"), homogeneity of samples was ensured by mixing and freezing in bulk and, except for water intake to maintain adequate levels of hydration, no other food or drink was allowed for the next 4 h. Further 10 mL blood samples were obtained 1, 2, 3, 4, 5, 6, 7 and 8 h after OJ consumption. Four hours after collection of the first blood sample participants were provided with a white roll with butter. After blood collection at 8 h, the cannula was removed and participants were provided with a standard low (poly)phenol meal (a buttered white roll with ham and cheese and potato chips/crisps) after which they left the laboratory. They were instructed to continue the low (poly)phenol diet that evening and return to the laboratory the next morning in the fasted state, to give the last blood sample.

In addition, after OJ consumption all urine excreted over the following time periods: 0-5, 5-8, 8-10 and 10-24 h was collected and into sealable flasks kept on ice. The total volume of each urine fraction was recorded and 2 mL aliquots were stored at –80 °C prior to analysis. Blood samples were placed in potassium EDTA tubes (BD Vacutainer Systems, UK) and immediately placed on ice. Plasma, separated within 15 min of collection by centrifugation at 2000 *g* for 15 min at 4 °C (Sorval™ ST, Thermo Scientific, San Jose, CA) was stored at –80 °C prior to analysis.

Extraction of Orange Juice. The OJ used in the feeding study was extracted using the procedure described by Pereira-Caro.[10] Briefly, 5 mL aliquots of juice, previously homogenised using an Ultraturrax homogenizer, were extracted twice with 5 mL of methanol for 2 min and centrifuged at 2800 *g* for 15 min at 4 °C. The pellet was extracted in the same manner with 2 mL of methanol. The 2 supernatants were pooled and reduced to dryness in vacuo, redissolved in 6 mL of 50% aqueous methanol and stored at –80 °C prior to analysis.

Processing of Urine and Plasma. Urine samples were defrosted, 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 HPLC-HR-MS. The extraction of metabolites from the plasma samples has been carried out as described by Ludwig et al.[17] with some modifications. Briefly, plasma samples were defrosted, vortexed and 400 μL aliquots were mixed with 10 μL of ascorbic acid (10%, v/v), and 980 μL of 1% formic acid in acetonitrile. One μg of rutin was added to the samples as internal standard for plasma extraction efficiency. The samples were then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation at 16110 *g* for 15 min, supernatants were reduced to dryness in vacuo using a concentrator plus (Eppendorf, Hamburg, Germany) and resuspended in 150 μL of distilled water containing 1% formic acid and 50 μL of methanol,

which was then centrifuged at 16100 *g* for 10 min and 10 µL aliquots of the supernatant analysed by HPLC-HR-MS. The recoveries values of the internal standard were of 78 ± 14% (n = 20).

HPLC-HR-MS Analysis. Aliquots of OJ, plasma and urine were analysed using a Dionex Ultimate 3000 RS UHPLC system comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Thermo Scientific). The HPLC conditions were previously described by Pereira-Caro et al.[10]. Briefly, reverse phase separations were carried out using a 150 x 4.6 mm i.d. 5 µm 100Å C18 Kinetex column (Phenomenex, Macclesfield, UK) maintained at 40 °C and eluted at a flow rate of 1.0 mL/min with a 45 min gradient of 3-50% of 0.1% acidic methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate was split and 0.2 mL/min directed to an Exactive™ Orbitrap mass spectrometer fitted with a heated electrospray ionization probe (Thermo Scientific) operating in negative ionization mode. Analyses were based on scanning from 100 to 1000 *m/z*, with in-source collision-induced dissociation at 25.0 eV. The capillary temperature was 300 °C, the heater temperature was 150 °C, the sheath gas and the auxillary gas flow rate were both 20 units, the sweep gas was 3 and the spray voltage was 3.00 kv. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Identification of flavanones in OJ and flavanone metabolites and phenolic acid catabolites in plasma and urine samples were achieved by comparing the exact mass and the retention time with available standards. In the absence of standards, compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with the measured accurate mass of the molecular ion. Identifications were categorized according to the Metabolite Standards Initiative Metabolite Identification (MSIMI) levels.[18]

(Poly)phenols and *p*-sympatol in OJ were quantified on the basis of chromatographic peak areas acquired at 280 nm and 325 nm by reference to standards of hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, 4-*O*-methyl-naringenin-7-*O*-rutinoside , ferulic acid, *p*-coumaric , sinapic acid and *p*-sympatol. Hesperetin-*O*-glucosyl-*O*-rutinoside was quantified as hesperetin-7-*O*-rutinoside equivalents, and naringenin-7-*O*-glucosyl-*O*-rutinoside and eriodictyol-7-*O*-rutinoside were quantified as naringenin-7-*O*-rutinoside equivalents.

■ RESULTS

Analysis of (Poly)phenols in Orange Juice. HPLC-PDA-HR-MS analysis detected and quantified 9 compounds in the OJ in agreement with our previous analysis of OJ[10]. The main compounds were hesperetin-7-*O*-rutinoside (hesperidin)(246 μmol) and naringenin-7-*O*-rutinoside (narirutin) (62 μmol), followed by the flavone apigenin-6,8-*C*-diglucoside (vicenin-2) (35 μmol) and the hydroxycinnamates ferulic acid-4′-*O*-glucoside (16 μmol), coumaric acid-4′-*O*-glucoside (11 μmol) and the flavanone 4′-*O*-methyl-naringenin-7-*O*-rutinoside (didymin) (14 μmol). Small quantities of other flavanones such as hesperetin-7-*O*-rutinoside-3′-*O*-glucoside (4 μmol) and eriodictyol-7-*O*-rutinoside (eriocitrin) (4 μmol) were also present along with a sinapic acid-*O*-hexoside (6 μmol). A total of 398 μmol of (poly)phenols were present in 500 mL of OJ of which 250 μmol corresponded to the total amount of hesperetin derivatives (~ 63% of the total (poly)phenol content) and 76 μmol of naringenin derivatives (~19% of the total (poly)phenol content). In addition the juice contained 27 μmol of the amine *p*-sympatol. For structures of the fully identified OJ components see Figure 1.

Identification of Plasma and Urinary Metabolites and Phenolic Catabolites. A total of 19 flavanone metabolites and 65 phenolic and aromatic catabolites were identified or tentatively identified in plasma and urine collected 0-24 h after the consumption of 500 mL of OJ. Typical HPLC-HR-MS chromatograms are illustrated in Figures 2 and 3.

Flavanone metabolites. The HPLC-HR-MS characteristics such as the retention time, accurate mass, mDa error between mass found and the accurate mass and the molecular formula of the flavanone metabolites are summarized in Table 1.

Peaks **M1**, **M2** and **M3** (Rts 17.3, 18.1 and 20.2 min) all had a negative exact mass at *m/z* 623.1245 (+0.48 ppm). The low collision energy spectrum in negative mode showed high intensity ions at 447.0927 (+1.34 ppm), indicating a neutral loss of 176.0313 Da (corresponding to an elemental formula (EF) of $C_6H_8O_6$) (glucuronide moiety) and at 271.0605 (+1.48 ppm) (naringenin daughter ion) indicating a further loss of 176.0322 Da. This fragmentation pattern partially identifies these compounds as naringenin-*O*-diglucuronide isomers. Because there are only three hydroxyls on the naringenin skeleton, based on the likely HPLC elution order,[15] these metabolites are, respectively, the 4´,7-, 5,7- and 4´,5-*O*-diglucuronides. They were detected in urine but not plasma.

Peak **M4** (Rt 22.3 min) yielded a negative charge accurate mass at *m/z* 527.0490 (+0.18 ppm). The low collision energy spectrum in negative mode showed high intensity ions at 447.0927 (+1.34 ppm), indicating a neutral loss of 79.9563 Da (corresponding to an EF of a SO_3^-sulfate moiety), and the presence of SO_3^- in the fragmentation spectra (i.e. *m/z* 79.9562); and ions at 351.0171 (+0.56 ppm), indicating a neutral loss of 176.0319 Da (glucuronide

moiety). This metabolite, tentatively identified as a naringenin-*O*-glucuronyl-sulfate, has previously been detected in human urine after OJ intake.[10,13,16]

Peaks **M5** and **M6** (Rts 27.4 and 28.7 min) had a negative exact mass at 447.0927 (+1.34 ppm) which at low collision energy had a loss of 176.0322 Da (glucuronic acid moiety) yielding a daughter ion at m/z 271.0605 (+1.48 ppm) (naringenin). Co-chromatography with authentic standards established that **M5** and **M6** peaks were naringenin-4´-*O*-glucuronide and naringenin-7-*O*-glucuronide, respectively. These metabolites were present in both plasma and urine.

Peak **M7** (Rt 30.1 min) produced a negative exact mass at 351.0171 (0.56 ppm), which with the loss of 79.9566 Da (sulfate unit) yielded a fragment at 271.0605 (naringenin). This metabolite, tentatively identified as naringenin-4´-sulfate, has not been detected before in either urine or plasma after OJ consumption by humans. It has, however, recently been identified in stomach and colon lumen samples of mice after single-pass intestinal perfusion of naringenin.[19]

Peaks **M8**-**M10** (Rts 20.9, 21.8 and 25.9 min), which appeared in urine and plasma after OJ consumption, had a negative accurate mass at *m/z* 653.1355 (+ 1.07 ppm) which on low collision energy gave major fragments at *m/z* 477.1032 (+1.04 ppm) (loss of 176.0322 Da) (glucuronic acid moiety) and *m/z* 301.0710 (-1.33 ppm) (hesperetin) with further loss of 176.0322 Da. The three compounds showed the same pattern and were tentatively identified as hesperetin-*O*-diglucuronide derivatives. **M8** was tentatively identified as hesperetin-3´,7-*O*-diglucuronide and **M9** as hesperetin-5,7-*O*-diglucuronide, based on previously reported elution profiles[15]. **M10** is probably the 3´,5-*O*-diglucuronide.

Peak **M11** (Rt 24.8 min) produced a negative exact mass at *m/z* 557.0597 (+0.75 ppm) which on low collision energy yielded major fragments at 477.1032 (+1.04 ppm) loss of 79.9566 Da (sulfate unit) and *m/z* 301.0710 (-1.33 ppm) (hesperetin) with a loss of 176.0322 Da. This metabolite, which has been detected previously in human urine[10, 13,15,16], was tentatively identified as a hesperetin-*O*-glucuronyl-sulfate.

Peaks **M12**-**M14** (Rts 30.1, 30.5 and 33.6 min) had a negative exact mass at *m/z* 477.1032 (+1.04 ppm), which produced an ion at *m/z* 301.0710 (-1.33 ppm) (hesperetin) upon low collision energy. This loss of 176.0322 Da corresponded to cleavage of a glucuronic acid moiety. Co-chromatography with an authentic standard established that **M13** was hesperetin-7-*O*-glucuronide. Because there are only three hydroxyls on the hesperetin skeleton, **M12** is probably the 5-*O*-glucuronide which is a urinary metabolite while peak **M14** is probably the 3´-*O*-glucuronide. Peaks **M13** and **M14** were detected in plasma after intake of OJ.

Peaks **M15** and **M16** (Rts 24.8 and 31.7 min), occurred in urine and plasma, and produced a negative accurate mass at *m/z* 381.0279 (+1.31 ppm), which with the 79.9563 Da loss of a sulfate unit yielded a major fragment at 301.0710 (-1.33 ppm) (hesperetin). Co-chromatography with authentic standard established that **M16** was hesperetin-3´-sulfate. Because there are only three hydroxyls on the hesperetin skeleton, **M15** could be tentatively identified as either the 5- or the 7-sulfate.

Peak **M17** (Rt 23.7 min) had a negative exact mass at *m/z* 543.0808 (+0.92 ppm) with fragment with a loss of 162.0519 (+1.85 ppm) (corresponding to an EF $C_6H_8O_5$, hexose group) to produce a daughter ion at m/z 381.0279 (+1.31 ppm), which with an 79.9563 Da loss of a

sulfate unit yielded a fragment at 301.0710 (-1.33 ppm) (hesperetin). This metabolite was tentatively identified as a hesperetin-*O*-glucosyl-sulfate. It was found only in urine samples.

Peak **M18** (Rt 29.6 min), an urinary metabolite, produced a negative accurate mass at *m/z* 367.0122 (+1.08 ppm) which with low collision energy gave rise to a *m/z* 287.0553 (- 1.05 ppm) (eriodictyol moiety). The loss of 79.9569 Da indicates cleavage of a sulfate ion. This peak was, therefore, tentatively identified as an eriodictyol-sulfate.

Peak **M19** (Rt 22.1 min) had a negative exact mass at *m/z* 543.0447 (+1.47 ppm). The low collision energy spectrum in negative mode showed two major ions at 463.0876 (+1.07 ppm), indicating a neutral loss of 79.9571 Da (sulfate unit) and at 367.0122 (+1.08 ppm) indicating a loss of 176.0325 Da (glucuronide structure). This fragmentation pattern partially identifies this compound as an eriodictyol-*O*-glucuronide-sulfate not previously detected in human urine.

The structures of the identified flavanone metabolites are presented in Figure 4.

Phenolic and aromatic catabolites. The HPLC-HR-MS characteristics the phenolic and aromatic are summarized in Table 2. A total of 14 phenylpropanoid acid derivatives (**C1**-**C14**) were identified in human plasma and/or urine after OJ consumption, 4 of them were free phenolic acids (3´-hydroxycinnamic acid, 4´-hydroxycinnamic acid, ferulic acid and isoferulic acid) and 10 phase II metabolites (Table 2), mainly glucuronide and sulfate derivatives of coumaric, caffeic, ferulic and isoferulic acids. All, except peak **C5,** were identified by comparing the exact mass and the retention time with authentic standards. Peak **C5** was tentatively identified as coumaric acid-4´-sulfate as this peak presented a negative exact mass at *m/z*

242.9953 (+2.41 ppm) which with low collision energy gave rise to a *m/z* 163.0388 (+ 1.05 ppm) (coumarate moiety). The loss of 79.9564 Da indicated cleavage of a sulfate ion.

In addition, 20 phenylpropionic acid derivatives, 8 free phenolic acids and 12 conjugate derivatives (glucuronide and sulfate conjugates) were detected in plasma and urine. Peaks **C15**, **C17-27**, **C29**, **C30** and **C34** (Table 2) were identified based on comparisons with authentic standards. Peak **C16** had a negative accurate mass at *m/z* 211.0607 (-3.32 ppm) which with low collision energy gave rise to a *m/z* 181.0492 (+0.55 ppm) corresponding to a standard of 3-(3′-hydroxyphenyl)hydracrylic acid, with loss of 30.0115 Da (cleavage of a methoxy ion).

Peaks **C27** and **28** had a negative exact mass at *m/z* 275.0225 (-1.82 ppm) and a fragment at *m/z* 195.0657 (-2.64 ppm) (corresponding to a standard of 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid) indicating a further loss of 79.9568 Da (sulfate ion). The identity of peak **C2**7 was confirmed by reference to a standard of 3-(3′-methoxyphenyl)propionic acid-4′-sulfate. The later eluting **C28** was tentatively identified as 3-(4′-methoxyphenyl)propionic acid-3′-sulfate. This is in keeping with the sulfates of caffeic acid (**C7** and **C9**), 3-(hydroxyphenyl)propionic acid (**C21** and **C22**), and 3-(methoxyphenyl)propionic acid (**C27** and **C28**), where the 3′-derivative elutes after the 4'-isomer.

Peak **C31** had a negative exact mass at *m/z* 341.0874 (-2.03 ppm), yielding at low collision energy a fragment at *m/z* 165.0547 (-0.48 ppm). The loss of 176.0321 Da corresponded to a glucuronic acid moiety. This catabolite was putatively identified as 3-(phenyl)propionic acid-3′-*O*-glucuronide. Peaks **C32** and **C33** presented a negative accurate mass at *m/*z 245.0123 (-

3.53 ppm), which yielded an ion at m/z 165.0547 (-0.48 ppm) (corresponding to a standard of 3-(3′/4′-hydroxyphenyl)propionic acid) upon low collision energy. The loss of 79.9576 Da (sulfate ion) tentatively identified these two catabolites as 3-(phenyl)propionic acid sulfate isomers. As has been described above, the earlier eluting peak **C32** is tentatively identified as 3-(phenyl)propionic acid-4′-sulfate and **C33** as 3-(phenyl)propionic acid-3′-sulfate.

Peaks **C35**-**C46** corresponded to phenylacetic acid derivatives. Among them, peaks **C35**, **C39**, **C43**-**46** were identified by reference to standards and comprised a range of free phenolic acids (see Table 2). Peak **C36** had a negative exact mass at *m/z* 327.0719 (-2.75 ppm), yielding at low collision energy a fragment at *m/z* 151.0390 (-0.66 ppm) (hydroxyphenylacetic acid moiety). The loss of 176.0329 Da corresponded to a glucuronic acid group. This catabolite was putatively identified as a hydroxyphenylacetic acid-*O*-glucuronide. Peaks **C37** and **C38** presented negative exact masses at *m/z* 230.9964 (-3.03 ppm) and a fragment at *m/z* 151.0390 (-0.66 ppm) (corresponding to a standard of hydroxyphenylacetic acid) indicating a further loss of 79.9574 Da (sulfate ion). The HPLC elution order of the sulfates indicates that **C37** is probably 3′-hydroxyphenylacetic acid-4′-sulfate and **C38** is 4′-hydroxyphenylacetic acid-3′-sulfate.

Peak **C40** had a negative accurate mass at *m/z* 357.0828 (-3.30 ppm), yielding at low collision energy a fragment at *m/z* 181.0490 (+2.96 ppm) (hydroxymethoxyphenylacetic acid moiety). The loss of 176.0338 Da allowed the putative identification of this catabolite as a methoxyphenylacetic acid-*O*-glucuronide with glucuronic acid moiety at either the 3'- or 4'-positions. Peaks **C41**-**C42** had a negative exact mass at *m/z* 261.0073 (-3.64 ppm) and fragments at *m/z* 181.0490 (+2.96 ppm) indicating a further loss of 79.9583 Da, which indicates these compounds are methoxyphenylacetic acid sulfate isomers. In view of the

elution order **C41** was tentatively identified as 3'-methoxyphenylacetic acid-4'-sulfate and **C42** as 4'-methoxyphenylacetic acid-3'-sulfate.

Among the benzoic acid and hydroxycarboxylic acid derivatives identified, 7 corresponded to free phenolics (**C47**, **C51**-**54**, **C57**, **C58,** see Table 2) and have been identified based on their comparison with authentic standards. Peak **C48** presented a negative accurate mass at *m/z* 329.0513 (-3.04 ppm), yielding at low collision energy a fragment at *m/*z 153.0183 (-0.65 ppm) (corresponding to a standard of 3,4-dihydroxybenzoic acid). The loss of 176.0330 Da allowed the identification of this catabolite as hydroxybenzoic acid-*O*-glucuronide derivative. Peaks **C49**-**C50** and **C55**-**C56** presented negative exact masses at *m/z* 232.9756 (-2.58 ppm) and at *m/z* 216.9807 (-2.77 ppm), respectively, yielding at low collision energy fragments at *m/z* 153.0183 (-0.65 ppm) (corresponded to a standard of 3,4-dihydroxybenzoic acid) and 137.0230 (+2.19 ppm) (corresponded to a hydroxybenzoic acid standard), with the loss of 79.9583 Da, facilitating the tentative identification of peaks **C49**-**C50** as hydroxybenzoic acid-sulfate isomers with the elution order implying that **C49** is 3-hydroxybenzoic acid-4-sulfate and **C50** is 4-hydroxybenzoic acid-3-sulfate. Likewise the benzoic acid-sulfate isomers, **C55-C56,** are tentatively identified as benzoic acid-4-sulfate (**C55**) and benzoic acid-3-sulfate (**C56**).

Other groups of phenolic and aromatic acids identified in plasma and urine after OJ consumption by humans were the benzenetriol and benzoylglycine derivatives which comprises three hydroxyphenol derivatives (peaks **C59**-**C61,** see Table 2) and two hydroxyhippuric acid derivatives (peak **C63**-**C64**, see Table 2). Except for peak **C62**, all were identified based on the comparison of their exact mass and retention time with authentic standards. Peak **C62** had a negative accurate mass at *m/z* 370.0778 (-5.67 ppm) which with

329 low collision energy gave rise to a fragment at *m/z* 194.0455 (-4.12 ppm) (corresponding to a
330 standard of hydroxyhippuric acid). The loss of 176.0323 Da allowed the tentative
331 identification of this catabolite as hippuric acid-*O*-glucuronide. Finally, the fragmentation and
332 retention time of peak **C65** corresponded to those of a standard of hippuric acid (Table 2).

333

334 ■ DISCUSSION

335 This paper describes a comprehensive characterization of 19 flavanone metabolites and 65
336 colon-derived phenolic acid catabolites in human plasma and urine after OJ consumption.
337 Some hesperetin and narigenin metabolites have been detected in earlier studies. [8-13,15]
338 However, the current investigation identified 2 urinary flavanone metabolites, naringein-4´-
339 sulfate and an eriodictyol-*O*-glucuronyl-sulfate, and more than 40 phenolic catabolites that
340 had not previously been identified in human plasma and/or urine after OJ intake.

341 After ingestion and during transport through the small intestine, the 7-*O*-rutinosides of
342 hesperetin and naringenin undergo limited hydrolysis of the *O*-rhamnose-glucose unit and
343 release of the aglycone. As a consequence relatively little absorption and phase II metabolism
344 of the aglycone occurs in the enterocyte. The sugar moiety is a major determinant of the
345 absorption site and bioavailability of flavanones.[15,20] Flavanone monoglucosides are absorbed
346 in the small intestine after hydrolysis by lactase phlorizin hydrolase present in the gut lumen
347 brush border[21] and/or cytosolic β-glucosidase in the intestinal cells.[22] In contrast, substantial
348 amounts of flavanone rutinosides are not hydrolysed and substantial amounts reach the colon
349 intact[14] where the resident microbiota catalyse deconjugation of the sugar moiety [1]. A portion

of the released aglycones is absorbed and transformed by the phase II enzymes before entering in circulatory system.

In the current study 10 hesperetin, 7 naringenin and 2 eriodictyol metabolites were identified in urine after consumption of OJ while 8 hesperetin and 3 naringenin metabolites were detected in plasma (Table 1, Figure 4). None of the parent OJ flavanone glycosides were detected in either urine or plasma. These results are in keeping with earlier by HPLC-MS based studies which identified flavanone metabolites in human biological fluids. For instance, Mullen and co-workers[12] identified a hesperetin-*O*-diglucuronide, a naringenin-*O*-diglucuronide, naringenin-7-*O*-glucuronide and hesperetin-*O*-glucuronyl-sulfate in urine, and hesperetin-7-*O*-glucuronide in both urine and plasma after consumption of OJ by healthy subjects. In a further study, Bredsdorft et al.[15] identified hesperetin-3′,7-*O*-diglucuronide, hesperetin-5,7-*O*-diglucuronide, naringenin-4′-*O*-glucuronide, hesperetin-3′-*O*-glucuronide and hesperetin-3′-*O*-sulfate in urine after ingestion of OJ and an α-rhamnosidase-treated OJ. Two recent studies carried out by our group detected additional metabolites, namely naringenin-4′,7-*O*-diglucuronide, naringenin-5,7-*O*-diglucuronide, naringenin-4′,5-*O*-diglucuronide, hesperetin-3′,5-*O*-diglucuronide and hesperetin-*O*-glucosyl-sulfate and eriodictyol-sulfate in urine after ingestion of a pulp-enriched OJ[10] and after acute OJ intake with and without a microencapsulated probiotic.[13] As well as these metabolites, naringenin-4′-sulfate and an eriodictyol-*O*-glucuronyl-sulfate were detected in both plasma and urine in the current study. Further metabolites were detected in urine, namely hesperetin-5-*O*-glucuronide, a naringenin-*O*-glucuronyl-sulfate and an eriodictyol-*O*-glucuronyl-sulfate.

Not all the flavanone aglycones released in the distal GI tract are absorbed as sizable amounts undergo microbiota-mediated ring fission and yield a family of low molecular weight

phenolic catabolites which before being absorbed, in some instances, undergoing additional metabolism locally and/or in the liver and kidney before entering the systemic circulation and undergoing renal excretion.[10, 11] Analysis of urinary phenolic catabolites after OJ consumption has emphasized their involvement in the overall bioavailability of OJ (poly)phenols as well as subsequent hepatic conversions that lead to hippuric acid and its hydroxylated analogues[10,11,13]. Phase II metabolites of phenolic catabolites that have previously been identified in urine include coumaric acid-3´-*O*-glucuronide, coumaric acid-4´-sulfate, 3-(phenyl)propionic acid-4′-*O*-glucuronide, 3-(4´-hydroxyphenyl)propionic acid-4´-sulfate, a hydroxyphenylacetic acid-*O*-glucuronide and a hippuric acid-*O*-glucuronide[8]. In the current study with OJ, analysis of urine and plasma by HPLC-HR-MS enabled a much more comprehensive profile of phenolic catabolites to be obtained (Table 2). These findings, together with the results of earlier studies[10,13,14] and in vitro fecal incubations of OJ and OJ (poly)phenols[11, 23] have enabled us to propose up-dates of potential catabolic routes for the conversion of hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, ferulic acid-4´-*O*-glucoside and *p*-sympatol (Figures 5-6). For simplification the potential routes incorporate many, but not all, of the compounds listed in Table 2.

In the preparation of the proposed pathways illustrated in Figures 5 and 6, the following points were taken into consideration. The rupture of the flavanone skeleton is primarily a feature of the gut microflora while subsequent methylation and glucuronide, sulfate and glycine conjugation are mammalian in origin. Dehydroxylation and demethoxylation are almost certainly mediated by the gut microflora while demethylation and hydrogenation steps can be mediated by both microbial and mammalian enzymes. For convenience, the pathways in Figures 5 and 6 show C_6–C_3 catabolites being converted by two α-oxidations to C_6-C_1

compounds by microflora and/or mammalian enzymes. However, it is possible the C_6–C_3 catabolites progress directly to C_6–C_1 structures via β-oxidation and that C_6–C_2 catabolites arise by independently, possibly by α-oxidation. In reality, further complexity is introduced as there are multiple points at which catabolites might be absorbed. For example, a percentage of some C_6–C_3 catabolites could be absorbed and undergo β-oxidation and/or mammalian phase II conjugation while the balance is subjected to microbial hydrogenation and/β-oxidation prior to absorption and mammalian conjugation. Also for some catabolites mammalian conjugation either does not occur or is incomplete.

Figure 5 indicates that hesperetin, released through colonic bacteria-mediated deglycosylation, as well as being glucuronidated and sulfated, undergoes ring fission yielding 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid, 3-(3′-hydroxyphenyl)hydracrylic acid and isoferulic acid from the B-ring and phloroglucinol from the A-ring. It is noteworthy that 3-(3′-hydroxy-4′-methoxyhydroxyphenyl)hydracrylic acid has been reported as potential biomarker of the intake of OJ containing hesperetin-*O*-glycosides.[10,13] Urinary excretion of 3-(3′-hydroxyphenyl)hydracrylic acid, which also increases after OJ intake, has also been detected in urine after consumption of (+)catechin [24], green tea[25] and mixed wine and grape juice[26,27]. The hesperetin catabolite, isoferulic acid, is further converted to dihydro-isoferulic acid [3-(3′-hydroxy-4′-methoxyphenyl)propionic acid] which is demethylated yielding dihydrocaffeic acid [3-(3′,4′-dihydroxyphenyl)propionic acid]. Dihydro-isoferulic acid can also be degraded to 3′-hydroxy-4′-methoxyphenylacetic acid which is further converted to 3-hydroxy-4-methoxybenzoic acid via shortening of the side chain. Phase II metabolites of dihydro-isoferulic acid, in the form of dihydro-isoferulic acid-3′-sulfate, dihydro-isoferulic acid-3′-*O*-glucuronide, are also produced while dihydrocaffeic acid can be converted to sulfate

and glucuronide derivatives of 3-(hydroxyphenyl)propionic acid (Figure 5). Other products, potentially derived from dihydrocaffeic acid via successive dehydroxylations, include 3-(3′-hydroxyphenyl)propionic acid and 3-(phenyl)propionic acid, which could, respectively, contribute to the urinary pools of 3′-hydroxyhippuric acid and hippuric acid. In addition, dihydrocaffeic acid may be converted to 3-(4′-hydroxyphenyl)propionic acid which, via the pathways illustrated in Figure 5, could lead to the accumulation of 4′-hydroxyhippuric acid.

The proposed catabolic routes for naringenin begin with ring fission yielding phloroglucinol from the A-ring and 3-(4′-hydroxyphenyl)propionic acid from ring B. 3-(4′-Hydroxyphenyl)propionic acid undergoes dehydroxylation and shortening of the side chain to, respectively, produce 3-(phenyl)propionic acid and 4′-hydroxyphenylacetic acid, which, it is proposed, are ultimately converted to hippuric acid and 4′-hydroxyhippuric acid via the pathways shown in Figure 6. Other phenolics identified in plasma and/or urine after OJ consumption, such as 4′-hydroxycinnamic acid and dihydroferulic acid could be derived from ferulic acid, which occurs in OJ as ferulic acid-4′-glucoside. In addition, ferulic acid-4′-sulfate, ferulic acid-4′-*O*-glucuronide, dihydroferulic acid-4′-*O*-glucuronide and dihydroferulic acid-4′-sulfate were all identified in plasma and urine after OJ consumption. Catabolism of dihydroferulic acid via the network illustrated in Figure 6 leads to the formation of hippuric acid, 4′-hydroxyhippuric acid and benzoic acid-4-sulfate.

After OJ consumption, plasma and urine contain elevated levels of 4′-hydroxymandelic acid (Pereira-Caro, unpublished), the likely source of which is not flavanones but the amine *p*-sympatol (aka *p*-synephrine) which was present in the juice. Tritium-labeled *p*-sympatol has been shown to be converted to 4′-hydroxymandelic acid following ingestion by humans.[28] A potential pathway for the three step conversion of *p*-sympatol to 4’-hydroxymandelic acid is

442 shown in Figure 6. The plasma C_{max} of *p*-sympatol occurs ~1 h after OJ intake indicating that
443 the conversions are likely to be enterocyte/hepatic in origin consistent with findings using
444 tritated sympatol.[28]

445 It is noteworthy, that except for compounds originating from hesperetin, no methylated
446 flavanone metabolites were detected either in plasma or urine after OJ intake. This supports
447 the view that the methylation is less prevalent in the large intestine than the upper GIT [17], and
448 also implies that there is little or no hepatic methylation of flavanone metabolites. This may
449 be because, except for eriodictyol-7-*O*-rutinoside, a very minor component, OJ flavanones lack
450 a catechol group on the B-ring which is a requirement for catechol-*O*-methyltransferase
451 activity. The appearance of several methoxy-phenolic catabolites, such as **C27** and **C41**,
452 indicates the some degree of methylation of phenolic acids does take place.

453 A number of the phenolic acid catabolites, such as hippuric acid, were present in 0-24 h
454 urine collected before supplementation, however, earlier studies have identified those which
455 increase following OJ consumption,[10,11,13] and this was taken into account in the preparation of
456 the pathways illustrated in Figures 5 and 6. The detailed elucidation of metabolites and
457 catabolites appearing in the circulatory system and excreted in urine after OJ intake provides a
458 valuable foundation for time-course studies and quantitative analyses after supplementation.
459 This will help identify potentially bioactive compounds to test using in vitro models of human
460 cell lines in order to assist elucidation the mechanisms underlying the protective effects of OJ
461 consumption.

462

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■ ABBREVIATIONS USED

OJ, orange juice; HPLC-HR-MS, ultra-high performance liquid chromatography-high resolution mass spectrometry; MSIMI Metabolite Standards Initiative Metabolite Identification .

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562

563 ■ AUTHOR INFORMATION

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568 **Notes**

The authors declare no competing interest.

570 **Figure Legends**

571

572 **Figure 1.** Structures of compounds identified in orange juice.

573 **Figure 2**. HPLC-HR-MS traces of flavanone metabolites in urine samples collected 10-24h
574 after the consumption of orange juice. For peak identification see Table 1.

575 **Figure 3.** HPLC-HR-MS traces of selected flavanone catabolites identified in urine samples
576 collected 10-24h after the consumption of orange juice. For peak identification see Table 2.

577 **Figure 4.** Structures of metabolites of hesperetin and naringenin identified in
578 plasma/urine after consumption of orange juice.

579 **Figure 5.** Proposed pathways for the catabolism of hesperetin by colonic microbiota and
580 mammalian phase II metabolism. Red arrows indicate microbiota-mediated steps, blue
581 arrow represent mammalian enzyme-mediated conversions. Based on the data presented
582 in this paper and earlier orange juice feeding studies and in vitro anaerobic incubations of
583 fecal material with flavanones and orange juice.[9,10,22] GlcUA - glucuronide; * - potential
584 intermediate that did not accumulate in detectable amounts.

585 **Figure 6.** Proposed pathways for the catabolism of naringenin, ferulic acid and *p*-sympatol
586 by colonic microbiota and mammalian phase II metabolism. Red arrows indicate
587 microbiota-mediated steps, blue arrow represent mammalian enzyme-mediated
588 conversions. Based on the data presented in this paper and earlier orange juice feeding
589 studies and in vitro anaerobic incubations of fecal material with flavanones and orange
590 juice.[9,10,22] GlcUA - glucuronide; * - potential intermediates that did not accumulate in
591 detectable amounts.

Table 1. HPLC-HR-MS Based Identification of Flavanone Metabolites in Human Plasma and Urine Collected 0-24 h After Orange Juice Consumption.

Peak	Metabolites	Chemical Formula $[m/z]^-$	Theoretical mass $[m/z]^-$	Experimental mass $[m/z]^-$	Δ ppm	Rt (min)	Location[a]	MSI MI level[b]
Naringenin metabolites								
M1	Naringenin-4′,7-*O*-diglucuronide	C27H27O17	623.1248	623.1245	0.48	17.3	U	2
M2	Naringein-5,7-*O*-diglucuronide	C27H27O17	623.1248	623.1245	0.48	18.1	U	2
M3	Naringenin-4′,5-*O*-diglucuronide	C27H27O17	623.1248	623.1245	0.48	20.2	U	2
M4	Naringenin -*O*-glucuronyl-sulfate	C21H19O4S	527.0490	527.0491	0.18	22.3	U	2
M5	Naringenin-4′-*O*-glucuronide	C21H19O4	447.0921	447.0927	1.34	27.4	U, P	1
M6	Naringenin-7-*O*-glucuronide	C21H19O4	447.0921	447.0927	1.34	28.7	U, P	1
M7	Naringenin-4′-sulfate	C15H11O8S	351.0169	351.0171	0.56	30.1	U, P	2
Hesperetin metabolites								
M8	Hesperetin-3′,7-*O*-diglucuronide	C38H30O18	653.1348	653.1355	1.07	20.9	U, P	2
M9	Hesperetin-5,7-*O*-diglucuronide	C38H30O18	653.1348	653.1355	1.07	21.8	U, P	2
M10	Hesperetin-3′,5-*O*-diglucuronide	C38H30O18	653.1348	653.1355	1.07	25.9	U, P	2
M11	Hesperetin-*O*-glucuronyl-sulfate	C22H22O15S	557.0595	557.0597	0.75	24.8	U, P	2
M12	Hesperetin-5-*O*-glucuronide	C22H22O12	477.1027	477.1032	1.04	30.1	U	2
M13	Hesperetin-7-*O*-glucuronide	C22H22O12	477.1027	477.1032	1.04	30.5	U, P	1
M14	Hesperetin-3′-*O*-glucuronide	C22H22O12	477.1027	477.1032	1.04	33.6	U, P	2
M15	Hesperetin-sulfate	C16H14O9S	381.0274	381.0279	1.31	24.8	U, P	2
M16	Hesperetin-3′-sulfate	C16H14O9S	381.0274	381.0279	1.31	31.7	U, P	1
M17	Hesperetin-*O*-glucosyl-sulfate	C22H23O14S	543.0803	543.0808	0.92	23.7	U	2
Eriodictyol metabolites								
M18	Eriodictyol sulfate	C15H11O9S	367.0118	367.0122	1.08	29.6	U, P	2
M19	Eriodictyol-*O*-glucuronyl-sulfate	C21H19O15S	543.0439	543.0447	1.47	22.1	U, P	2

[a] U, urine; P, plasma.

[b] Metabolite standards initiative (MSI) metabolite identification (MI) levels[17]. Reference compounds were available for all compounds identified at MSI MI level 1

Table 2. HPLC-HR-MS Based Identifications of Phenolic Acid Catabolites in Human Plasma and Urine Collected 0-24 h After Orange Juice Consumption.

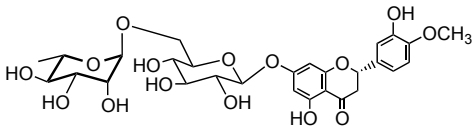
Peak	Rt (min)	Catabolites	Chemical Formula [*m/z*]-	Theoretical mass [*m/z*]-	Experimental mass [*m/z*]-	Δppm	Location[a]	MSI MI level[b]
		Phenylpropanoid acid derivatives						
C1	22.70	3'-Hydroxycinnamic acid	C9H7O3	163.0390	163.0388	1.05	U	1
C2	15.21	Coumaric acid-3'-*O*-glucuronide	C15H15O9	339.0711	339.0712	-0.42	U	1
C3	19.33	4'-Hydroxycinnamic acid	C9H7O3	163.0390	163.0388	1.05	U	1
C4	10.53	Coumaric acid-4′-*O*-glucuronide	C15H15O9	339.071	339.0712	-0.59	U	1
C5	12.32	Coumaric acid-4'-sulfate	C9H7O6S	242.9958	242.9953	2.41	U	2
C6	14.00	Caffeic acid-3'-*O*-glucuronide	C15H15O10	355.0660	355.0659	0.21	U	1
C7	11.94	Caffeic acid-3′-sulfate	C9H7O7S	258.9907	258.9917	-3.86	U, P	1
C8	11.03	Caffeic acid-4′-*O*-glucuronide	C15H15O10	355.0659	355.0659	0.00	U	1
C9	10.31	Caffeic acid-4′-sulfate	C9H7O7S	258.9907	258.9905	0.77	U, P	1
C10	22.42	Ferulic acid	C10H9O4	193.0495	193.0492	1.74	U, P	1
C11	13.88	Ferulic acid-4′-*O*-glucuronide	C16H17O10	369.0816	369.0825	-2.38	U, P	1
C12	13.99	Ferulic acid-4′-sulfate	C10H9O7S	273.0063	273.0074	-3.85	U, P	1
C13	24.32	Isoferulic acid	C10H9O4	193.0495	193.0496	-0.34	U,P	1
C14	18.37	Isoferulic acid-3′-*O*-glucuronide	C16H17O10	369.08162	369.0825	-2.38	U, P	1
		Phenylpropionic acid derivatives						
C15	8.74	3-(3′-Hydroxyphenyl)hydracrylic acid	C9H9O4	181.0493	181.0492	0.55	U, P	1
C16	11.32	3-(3′-Hydroxy-4′-methoxyphenyl)hydracrylic acid	C10H11O5	211.0600	211.0607	-3.32	U, P	1
C17	11.20	3-(3′,4′-Dihydroxyphenyl)propionic acid	C9H9O4	181.0495	181.0489	3.31	U, P	1
C18	11.59	3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	C15H17O10	357.0816	357.0816	0.00	U	1
C19	14.11	3-(Phenyl)propionic acid-4′-*O*-glucuronide	C15H17O9	341.0867	341.0874	-2.03	U	1
C20	12.57	3-(4′-Hydroxyphenyl)propionic acid-3-*O*-glucuronide	C15H17O10	357.0816	357.0816	0.00	U	1
C21	9.11	3-(3′-Hydroxyphenyl)propionic acid-4′-sulfate	C9H9O7S	261.0063	261.006	1.34	U, P	1

C22	9.48	3-(4′-Hydroxyphenyl)propionic acid-3′-sulfate	C9H9O7S	261.0063	261.0067	-1.34	U, P	1
C23	19.42	3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	C10H11O4	195.0652	195.0648	1.98	U, P	1
C24	14.85	3-(3'-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	C16H19O10	371.0973	371.0979	-1.69	U, P	1
C25	21.45	3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid	C10H11O4	195.0652	195.0657	-2.64	U, P	1
C26	17.44	3-(4′-Methoxyphenyl)propionic-3′-*O*-glucuronide	C16H19O10	371.0973	371.0967	1.54	U, P	1
C27	12.23	3-(3′-Methoxyphenyl)propionic acid-4′-sulfate	C10H11O7S	275.0220	275.0225	-1.82	U, P	1
C28	13.75	3-(4′-Methoxyphenyl)propionic acid-3′-sulfate	C10H11O7S	275.0220	275.0225	-1.82	U, P	2
C29	18.33	3-(3′-Hydroxyphenyl)propionic acid	C9H9O3	165.0546	165.0547	-0.48	U,	1
C30	16.78	3-(4′-Hydroxyphenyl)propionic acid	C9H9O3	165.0546	165.0547	-0.48	U, P	1
C31	12.64	3-(Phenyl)propionic acid-3′-*O*-glucuronide	C15H17O9	341.0867	341.0874	-2.03	U	2
C32	11.73	3-(Phenyl)propionic acid-4′-sulfate	C9H9O6S	245.0114	245.0123	-3.53	U	2
C33	12.32	3-(Phenyl)propionic acid-3′-sulfate	C9H9O6S	245.0114	245.0123	-3.53	U	2
C34	12.20	3-(Phenyl)propionic acid	C9H9O2	149.0597	149.0593	2.72	U,P	1
		Phenylacetic acid derivatives						
C35	7.72	3′,4′-Dihydroxyphenylacetic acid	C8H7O4	167.0338	167.0332	3.59	U	1
C36	7.97	Hydroxyphenylacetic acid-*O*-glucuronide	C14H15O9	327.071	327.0719	-2.75	U, P	2
C37	6.62	Hydroxyphenylacetic acid-4′-sulfate	C8H7O6S	230.9957	230.9964	-3.03	U	2
C38	7.21	Hydroxyphenylacetic acid-3’-sulfate	C8H7O6S	230.9957	230.9964	-3.03	U, P	2
C39	14.14	3′-Methoxy-4′-hydroxyphenylacetic acid	C9H9O4	181.0495	181.049	2.96	U	1
C40	7.23	Methoxyphenylacetic acid-*O*-glucuronide	C15H17O10	357.0816	357.0828	-3.30	U, P	2
C41	7.19	3'-Methoxyphenylacetic acid-4′-sulfate	C9H9O7S	261.0063	261.0073	-3.64	U, P	2
C42	8.65	4'-Methoxyphenylacetic acid-3′-sulfate	C9H9O7S	261.0063	261.0073	-3.64	U, P	2
C43	22.28	3′,4′-Dimethoxyphenylacetic acid	C10H11O4	195.0652	195.0653	-0.59	U	1
C44	12.53	3′-Hydroxyphenylacetic acid	C8H7O3	151.0389	151.0390	-0.66	U, P	1
C45	11.34	4′-Hydroxyphenylacetic acid	C8H7O3	151.0389	151.0390	-0.66	U, P	1
C46	16.84	Phenylacetic acid	C8H7O2	135.0440	135.0445	-3.70	U	1
		Benzoic acid derivatives						
C47	6.63	3,4-Dihydroxybenzoic acid	C7H5O4	153.0182	153.0183	-0.65	U, P	1
C48	3.85	Hydroxybenzoic acid-*O*-glucuronide	C13H13O10	329.0503	329.0513	-3.04	U	2
C49	4.83	3-Hydroxybenzoic acid-4-sulfate	C7H5O7S	232.9750	232.9756	-2.58	U	2

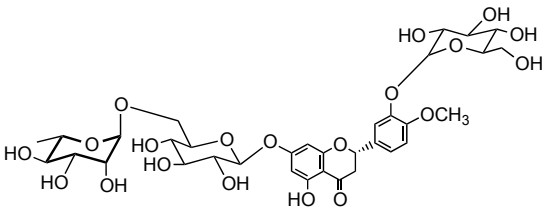
C50	5.50	4-Hydroxybenzoic acid-3-sulfate	C7H5O7S	232.9750	232.9756	-2.58	U	2
C51	13.51	3-Methoxy-4-hydroxybenzoic acid	C8H7O4	167.0339	167.0337	1.11	U	1
C52	15.00	3-Hydroxy-4-methoxybenzoic acid	C8H7O4	167.0339	167.0336	1.71	U	1
C53	12.14	3-Hydroxybenzoic acid	C7H5O3	137.0233	137.023	2.19	U	1
C54	9.83	4-Hydroxybenzoic acid	C7H5O3	137.0233	137.0227	4.38	U	1
C55	5.98	Benzoic acid-4-sulfate	C7H5O6S	216.9801	216.9807	-2.77	U, P	2
C56	7.41	Benzoic acid-3-sulfate	C7H5O6S	216.9801	216.9807	-2.77	U	2
		Hydroxycarboxylic acid derivatives						
C57	4.02	3´-Methoxy-4'-hydroxymandelic acid	C9H9O5	197.0444	197.0453	-4.57	U, P	1
C58	2.96	4´-Hydroxymandelic acid	C8H7O4	167.0338	167.034	-1.20	U, P	1
		Benzenetriol derivatives						
C59	2.98	1,3,5-Trihydroxyphenol	C6H5O3	125.0233	125.0234	-0.80	U	1
C60	3.36	1,2,3-Trihydroxyphenol	C6H5O3	125.0233	125.0236	-2.40	U	1
C61	6.63	1,2-Dihydroxyphenol	C6H5O2	109.0284	109.0282	1.83	U	1
		Benzoylglycine derivatives						
C62	9.44	Hippuric acid-*O*-glucuronide	C15H16NO10	370.0757	370.0778	-5.67	U, P	2
C63	7.50	3´-Hydroxyhippuric acid	C9H8NO4	194.0447	194.0455	-4.12	U, P	1
C64	6.84	4´-Hydroxyhippuric acid	C9H8NO4	194.0447	194.0455	-4.12	U, P	1
C65	10.76	Hippuric acid	C9H8NO3	178.0498	178.0495	1.68	U, P	1

[a]P, plasma; U, urine

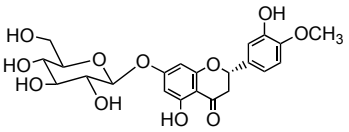
[b]Metabolite standards initiative (MSI) metabolite identification (MI) levels.[17] Reference compounds were available for all compounds identified at MSI MI level 1



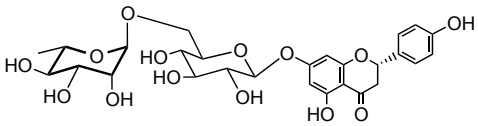
Hesperetin-7-*O*-rutinoside
(hesperidin)



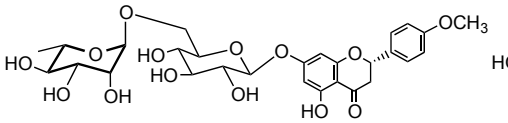
Hesperetin-7-*O*-rutinoside-3'-*O*-glucoside



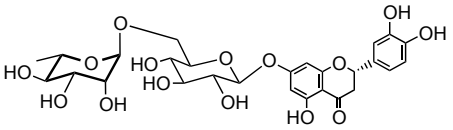
Hesperetin-7-*O*-glucoside



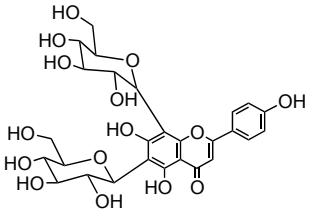
Naringenin-7-*O*-rutinoside
(narirutin)



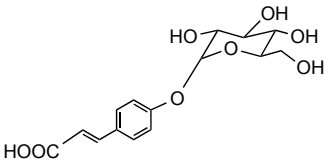
4'-*O*-Methyl-naringenin-7-*O*-rutinoside
(didymin)



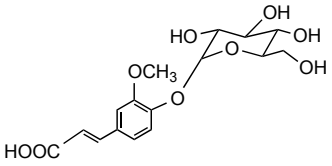
Eriodictyol-7-*O*-rutinoside
(eriocitrin)



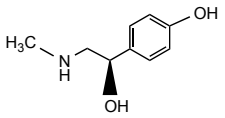
Apigenin-6,8-*O*-diglucoside
(vicenin-2)



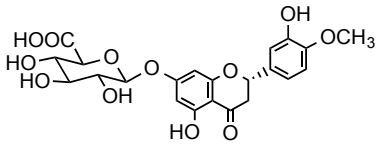
Coumaric acid-4´-*O*-glucoside



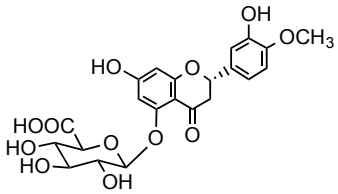
Ferulic acid-4'-*O*-glucoside



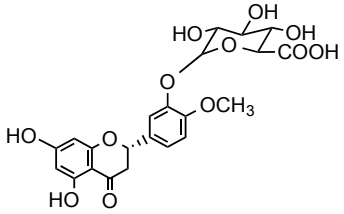
p-Sympatol



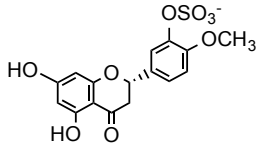
Hesperetin-7-*O*-glucuronide



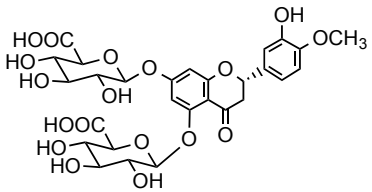
Hesperetin-5-*O*-glucuronide



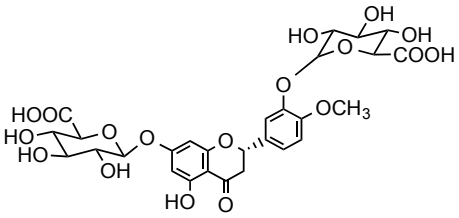
Hesperetin-3'-*O*-glucuronide



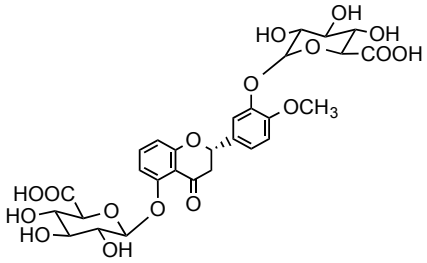
Hesperetin-3'-sulfate



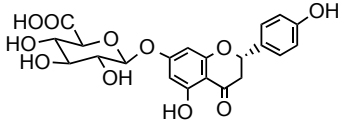
Hesperetin-5,7-*O*-diglucuronide



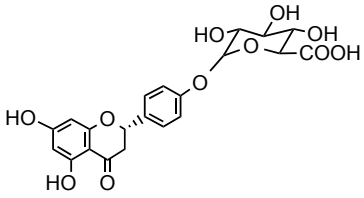
Hesperetin-3',7-*O*-diglucuronide



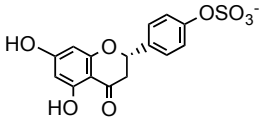
Hesperetin-3',5-*O*-diglucuronide



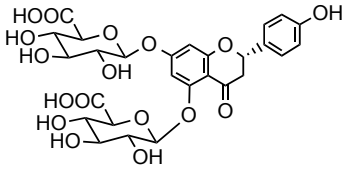
Naringenin-7-*O*-glucuronide



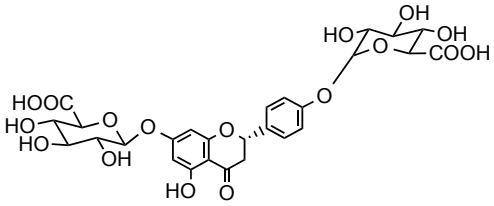
Naringenin-4'-*O*-glucuronide



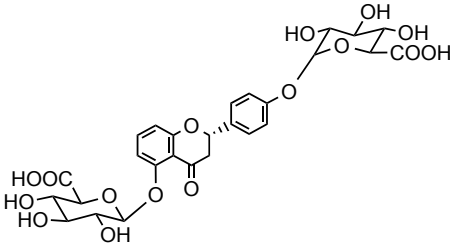
Naringen-4'-sulfate



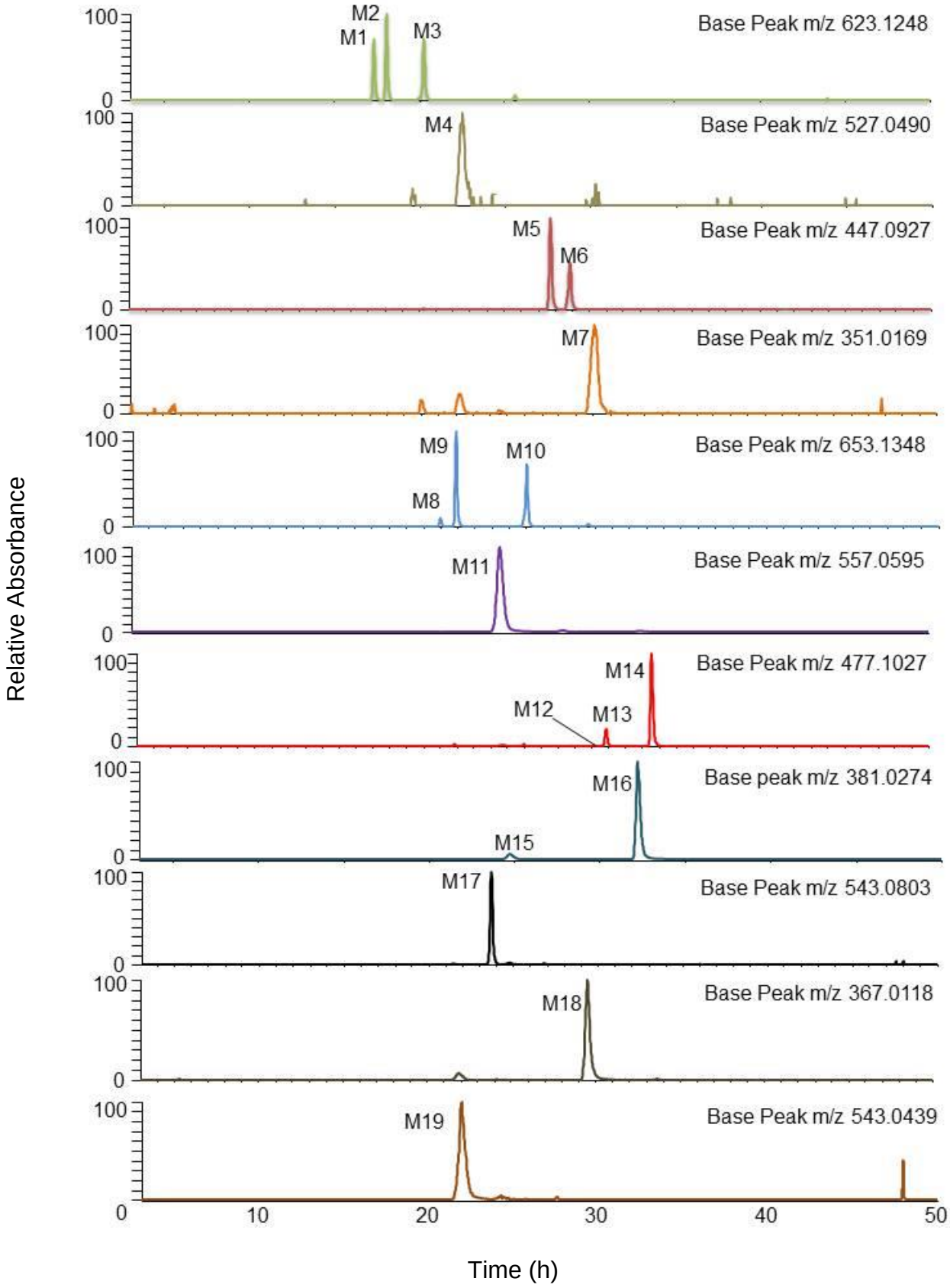
Naringenin-5,7-*O*-diglucuronide



Naringenin-4',7-*O*-diglucuronide



Naringenin-4',5-*O*-diglucuronide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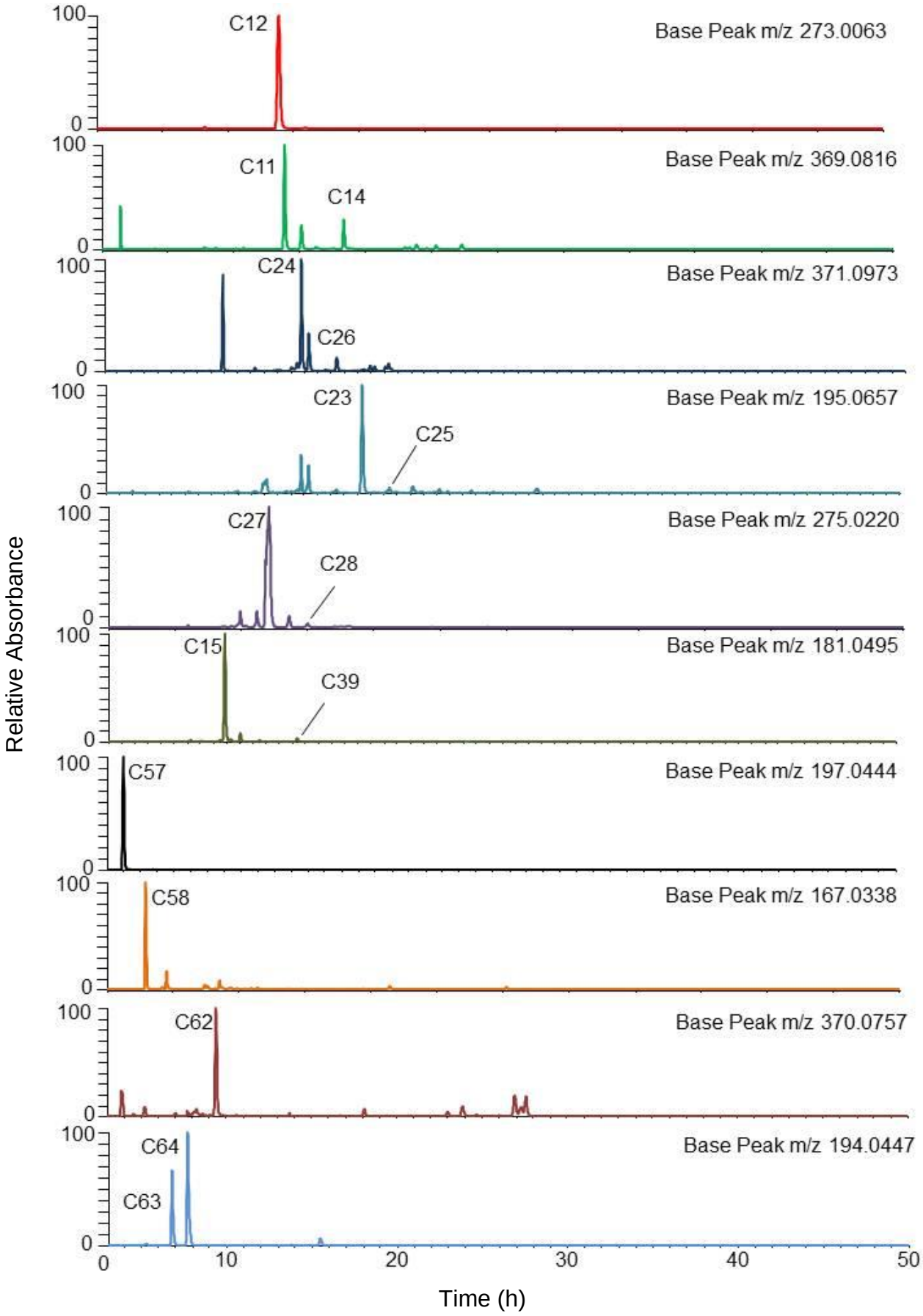
M1
M2
M3
Base Peak m/z 527.0490
M4
Base Peak m/z 447.0927
M5
M6
Base Peak m/z 351.0169
M7
Base Peak m/z 653.1348
M8
M9
M10
Base Peak m/z 557.0595
M11
Base Peak m/z 477.1027
M12
M13
M14
Base peak m/z 381.0274
M15
M16
Base Peak m/z 543.0803
M17
Base Peak m/z 367.0118
M18
Base Peak m/z 543.0439
M19
100
0
10
20
30
40
50
Time (h)


Relative Absorbance
100
0
C12
Base Peak m/z 273.0063
C11
C14
Base Peak m/z 369.0816
C24
C26
Base Peak m/z 371.0973
C23
C25
Base Peak m/z 195.0657
C27
C28
Base Peak m/z 275.0220
C15
C39
Base Peak m/z 181.0495
C57
Base Peak m/z 197.0444
C58
Base Peak m/z 167.0338
C62
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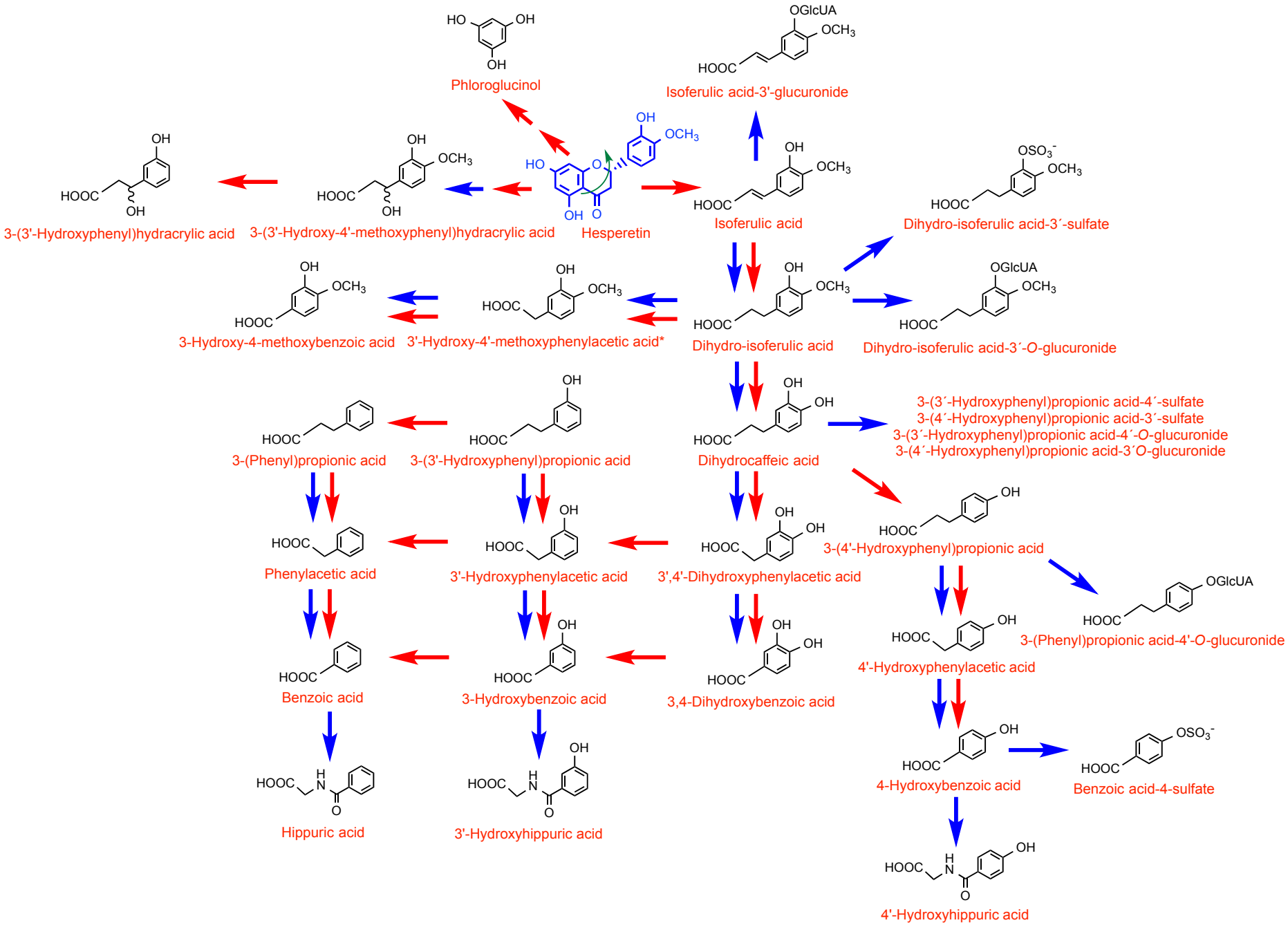
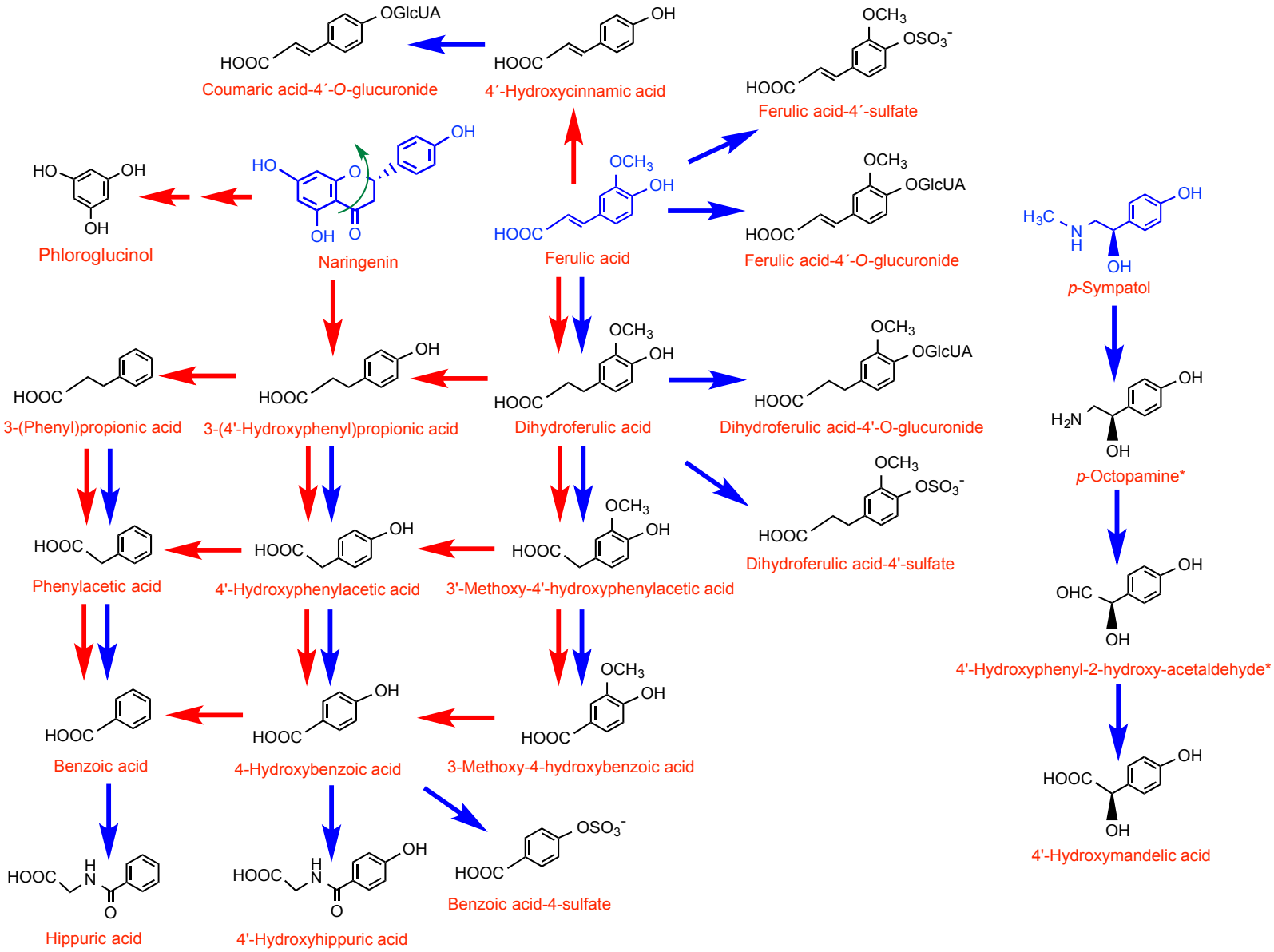
C64
C63
Base Peak m/z 194.0447
0
10
20
30
40
50
Time (h)


Phloroglucinol
Isoferulic acid-3'-glucuronide
3-(3'-Hydroxyphenyl)hydracrylic acid
3-(3'-Hydroxy-4'-methoxyphenyl)hydracrylic acid
Hesperetin
Isoferulic acid
Dihydro-isoferulic acid-3´-sulfate
3-Hydroxy-4-methoxybenzoic acid
3'-Hydroxy-4'-methoxyphenylacetic acid*
Dihydro-isoferulic acid
Dihydro-isoferulic acid-3´-O-glucuronide
3-(Phenyl)propionic acid
3-(3'-Hydroxyphenyl)propionic acid
Dihydrocaffeic acid
3-(3´-Hydroxyphenyl)propionic acid-4´-sulfate
3-(4´-Hydroxyphenyl)propionic acid-3´-sulfate
3-(3´-Hydroxyphenyl)propionic acid-4´-O-glucuronide
3-(4´-Hydroxyphenyl)propionic acid-3´O-glucuronide
Phenylacetic acid
3'-Hydroxyphenylacetic acid
3',4'-Dihydroxyphenylacetic acid
3-(4'-Hydroxyphenyl)propionic acid
3-(Phenyl)propionic acid-4'-O-glucuronide
Benzoic acid
3-Hydroxybenzoic acid
3,4-Dihydroxybenzoic acid
4'-Hydroxyphenylacetic acid
4-Hydroxybenzoic acid
Benzoic acid-4-sulfate
Hippuric acid
3'-Hydroxyhippuric acid
4'-Hydroxyhippuric acid


Coumaric acid-4´-O-glucuronide
4´-Hydroxycinnamic acid
Ferulic acid-4´-sulfate
Phloroglucinol
Naringenin
Ferulic acid
Ferulic acid-4´-O-glucuronide
p-Sympatol
3-(Phenyl)propionic acid
3-(4'-Hydroxyphenyl)propionic acid
Dihydroferulic acid
Dihydroferulic acid-4'-O-glucuronide
p-Octopamine*
Phenylacetic acid
4'-Hydroxyphenylacetic acid
3'-Methoxy-4'-hydroxyphenylacetic acid
Dihydroferulic acid-4'-sulfate
4'-Hydroxyphenyl-2-hydroxy-acetaldehyde*
Benzoic acid
4-Hydroxybenzoic acid
3-Methoxy-4-hydroxybenzoic acid
Hippuric acid
4'-Hydroxyhippuric acid
Benzoic acid-4-sulfate
4'-Hydroxymandelic acid

Plasma and Urinary Metabolites and Catabolites Derived from Orange Juice (Poly)phenols: Analysis by High Performance Liquid Chromatography-High Resolution-Mass Spectrometry

Gema Pereira-Caro, Iziar A. Ludwig, Thelma Polyviou, Dalia Malkova , Ada García, José Manuel Moreno-Rojas, and Alan Crozier