Synthetic and analytical strategies for the quantification of phenyl-γ-valerolactone conjugated metabolites in human urine

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Running title: Synthesis and analysis of valerolactones

ABSTRACT:

The contribution of the gut microbiota to the metabolism of catechins and proanthocyanidins remains still unclear. Although phenyl-γ-valerolactones have been pointed out as the most representative metabolites of these flavan-3-ols, their accurate quantification has not been addressed because of a lack of appropriate bioanalytical standards. This work aimed at synthesizing a set of sulphate- and glucuronide-conjugated phenyl-γ-valerolactones and at developing an analytical UHPLC-ESI-MS/MS method for their quantification in urine samples. Eight glucuronide and sulphate conjugates of hydroxyphenyl-γ-valerolactones were synthesized for the first time. They were used as analytical standards, together with 5 phenyl-γ-valerolactone aglycones, for the development of a high-throughput, validated method. Chromatographic and MS conditions were optimized. The method validation showed acceptable linearity, intra-day and inter-day repeatability, and accuracy, with the analytical range, limit of detection (LOD), and lower limit of quantification (LLOQ) varying notably among compounds. The method was used to calculate the excretion of phenyl-γ-valerolactones in healthy subject consuming green tea, providing novel information on the real concentrations of phenyl-γ-valerolactones in urine. This work opens the door to better studying the bioavailability of flavan-3-ols and the real exposition to flavan-3-ol sources, as well as to define the bioactivity of these colonic metabolites in cell assays.

Key words: colonic metabolites, flavan-3-ol, green tea, phenolic compounds, polyphenols, synthesis.

1. Introduction

Flavan-3-ols or flavanols are a class of polyphenols widely found in green tea, apples, pears, grapes, red wine, cocoa-based products, and some nuts and berries. They range from single monomers (such as (+)-catechin, (-)-epicatechin, (–)-epigallocatechin, and their galloyl substituted derivatives) to oligomers and polymers (also known as proanthocyanidins or condensed tannins) [1-3]. Flavan-3-ols are among the most largely consumed phenolic compounds in Western populations [4, 5], and evidence has established their role in the prevention of chronic diseases such as cardiovascular- and diabetes-related pathologies and neurodegenerative disorders [1, 6-10].

Nowadays, it is well known that phenolic compounds are extensively metabolized by the human body [1, 3]. In the case of flavan-3-ols, once ingested, they reach the gastrointestinal tract, where only a small fraction is absorbed and modified by phase II enzymes. The largest part (usually more than two-thirds of the intake) is not absorbed in the small intestine and reaches the large intestine, where flavan-3-ols are subjected to colonic microbial breakdown and are converted to phenolic and aromatic acids [3, 11-13]. In the microbial catabolism of flavan-3-ols, phenyl-γ-valerolactones have been identified as the main ring fission products [12, 14], later entering the bloodstream and being excreted in urine [1, 15]. Once absorbed, phenyl-γ-valerolactones may be further metabolised by phase II conjugation reactions occurring at the intestinal epithelium level and/or in the liver, leading to *O*-glucuronidated, *O*-sulphated, and *O*-methylated conjugates or a combination thereof [3, 11-14]. These colonic metabolites remain in circulation for an relatively long period of time before being excreted in large quantities in urine. These facts account for the importance of studying colonic metabolites, and phenyl-γ-valerolactones in particular, to state the absorption and metabolic fate of flavan-3-ols in the human superorganism [12, 16]. Therefore, they can also be regarded as valuable biomarkers of dietary consumption of flavan-3-ols, both monomers and proanthocyanidins [12, 17].

Several studies, mostly through LC-MS or NMR analytical techniques, have confirmed 5-

(hydroxyphenyl)-*γ*-valerolactone structures as the main products derived from flavan-3-ols metabolism [11-14, 18] and a variety of methods have been developed aiming at targeting flavan-3-ol metabolites in biological fluids [11, 19-24]. However, without appropriate reference standards, most of the analyses remain only qualitative or semi-quantitative [25], as the complex composition of biological samples, the possible regioisomeric forms, and the likely low MS ionization of some derivatives, among other factors, may hamper the unambiguous identification and absolute quantification of these metabolites.

Actually, the lack of reliable reference compounds of hydroxyphenyl-γ-valerolactones has restricted their use in both analytical methods and *in vitro* bioactivity assays, so far [26]. Nevertheless, progresses in asymmetric synthesis carried out by our research group have overcome this situation and now the enantioselective synthesis of hydroxyphenyl-γ-valerolactone aglycones has been reported (compounds **1**-**5**, Figure 1) [27]. In the framework of the "joint venture" between chemical synthesis and analytical techniques, the development of a validated analytical method for the quali-quantitative determination of these metabolites in biological fluids is really needed.

In this paper, the synthesis of 8 *O*-glucuronide and *O*-sulphate conjugated metabolites (compounds **6-12**, Figure 1) of aglycones **1-5** or from suitable protected valerolactone precursors, is reported for the first time. Furthermore, using the synthesized compounds as chemically unambiguous authentic standards, an analytical UHPLC-ESI-MS/MS method to quantify phenyl-γ-valerolactone metabolites in urinary samples has been successfully developed. The viability of this method was tested by evaluating the urinary excretion of phenyl-γ-valerolactones after consumption of green tea, one of the most popular beverages worldwide and one of the major dietary sources of flavan-3-ols, in a population of 16 volunteers.

2. Materials and methods

2.1. Reagents

Dichloromethane (HPLC grade), was dried by distillation on CaH_2 according to standard procedures. THF dry, Et_2O dry were distilled by Na/Benzophenone. Solvents for chromatography and filtration including hexane, ethyl acetate, dichloromethane, anhydrous ethanol, methanol, DMF, toluene and 2-propanol were ACS or HPLC grade and used as received. Petroleum ether for flash chromatography was ACS grade (bp≥90% 40-60 °C) and was used as such without further purifications. Ammonia-methanol mixture was prepared by bubbling liquid ammonia in methanol at 0 °C for 30 min. Reagents were obtained from commercial sources without further purification. Valerolactone aglycones **1-5** and valerolactone precursors **15**, **15′**, and **17** were prepared in house using the synthetic strategy previously outlined by Curti et al. [27]. Denmark's chiral bis-phosphoramides (R,R) (352310-87-3), and (S,S) (873306-78-6) were commercially available, and were used as such, without further purifications. 2,2,2-trichloroethyl chlorosulphate **13** (TCECS) [28], benzyl 2,3,4-tri-*O*-benzyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate **14** [29], Triisopropylsilyloxy-furan (TIPSOF) [30] were prepared according to reported procedures. For preparation of 3-benzyloxy-4-(*tert*-butyldimethylsilyloxy)benzaldehyde see Supplementary Material. All solvents and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

The HPLC columns used for method development were: BlueOrchid C18 (50 × 2 mm, 1.8 μm particle size; Knauer, Berlin, Germany), Kinetex PFP (50 × 2.1 mm, 2.6 μm particle size, Phenomenex, Macclesfield, UK), Ultra AQ C18 (100 × 2.1 mm, 3 μm particle size; Restek, Bellefonte, PA, USA), Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μm particle size, Waters, Milford, MA, USA), Kinetex EVO C18 (100 × 2.1 mm, 2.6 μm particle size, Phenomenex), and Kinetex EVO C18 (100 × 2.1 mm, 1.7 μm particle size, Phenomenex).

2.2. Synthesis of metabolites

General Experimental Procedure: Unless otherwise noted, all reactions were performed in oven-dried or flame-dried glassware under an atmosphere of nitrogen or argon. Air-sensitive reagents and solutions were transferred via syringe or cannula and were introduced to the apparatus through rubber septa. NMR spectra were recorded at 300 MHz or 400 MHz (1H) and 75 MHz or 100 MHz (13C). Spectra were referenced to tetramethylsilane (0.0 ppm, 1H; 0.0 ppm, 13C, in CDCl3). Chemical shifts (δ) are reported in parts per million (ppm), and multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), sext (sextet), sept (septet), dd (double doublet), m (multiplet), and b (broad). Coupling constants, *J*, are reported in Hertz. 1H and 13C NMR assignments were corroborated by 1D and 2D experiments (gCOSY, gHSQC, DEPT). Optical rotation data ($[\alpha]_D^{20}$) were obtained on a digital Perkin Elmer polarimeter at 589 nm (NaD) and 20 °C using a 100 mm cell with a 1 mL capacity and are given in units of 10^{-1} deg cm^2 g^{-1}. Details for the preparation and characterization of all synthesized compounds can be found in the Supplementary Material.

Representative Procedure for *O*-sulphate γ-valerolactones synthesis: Synthesis of compound 6

Representative procedure 1 for the preparation of the 2,2,2-trichloroprotected γ-valerolactone sulphates

According to a known procedure [28], a solution of (***R***)-**1** (10.0 mg, 0.05 mmol) in DCM dry (3 mL) was added Et_3N (8.4 μL, 0.06 mmol, 1.2 equiv), DMAP (6.1 mg, 0.05 mmol, 1 equiv) and 2,2,2-trichloroethyl chlorosulphate (TCECS) (74.4 mg, 0.3 mmol, 6 equiv). The solution was stirred at room temperature for 16 h. The resulting white suspension was diluted with EtOAc (6 mL) and washed with H_2O (6 mL), 1 N HCl (6 mL), and brine (6mL). The organic layer was dried (Na_2SO_4), and concentrated under vacuum. The residue was chromatographed on silica gel (elution by gradient from 75:25 to 65:35 Petroleum Ether/EtOAc) to give pure the corresponding protected sulphate intermediate **I** (15.1 mg, 75%) as a pale yellow resin.

Representative procedure 2 for the removal of 2,2,2-trichloroethyl group to generate γ-

valerolactone sulphates

According to a known procedure [28], ammonium formate (15.1 mg, 0.24 mmol, 6 equiv) and Zn dust (5.2 mg, 0.08 mmol, 2 equiv) was added to a solution of protected intermediate valerolactone **I** (15.1 mg, 0.04 mmol) in absolute EtOH (2 mL). The solution was stirred until all of reagent was consumed as determined by TLC (2 h). The reaction was filtered through Celite and the supernatant was concentrated in vacuum. The residue was subjected to flash chromatography (EtOAc/MeOH 80/20) to give the desired sulphated valerolactone **6** as amorphous solid (9.7 mg, 84%). TLC: R_f = 0.19 (90/10 ethyl acetate/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −19.0 (c 0.7 g/100mL,CH_3OH); ^{1}H NMR (400 MHz, MeOD): δ 7.22 (m, 4H, Ar), 4.71 (dddd, *J* = 6.8, 6.8, 6.8, 6.8 Hz, 1H, H4), 3.03 (dd, *J* = 14.0, 6.5 Hz, 1H, H5a), 2.90 (dd, *J* = 14.0, 6.0 Hz, 1H, H5b), 2.51 (ddd, *J* = 17.7, 9.4, 9.4 Hz, 1H, H2a), 2.40 (ddd, *J* = 17.7, 9.4, 4.5 Hz, 1H, H2b), 2.27 (dddd, *J* = 12.8, 9.7, 6.7, 4.5 Hz, 1H, H3a), 1.97 (dddd, *J* = 12.8, 9.3, 9.3, 7.5 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): 180.3 (Cq, C1), 153.0 (Cq, Ar), 134.5 (Cq, Ar), 131.4 (2C, CH, Ar), 122.7 (2C, CH, Ar), 83.2 (CH_2, C4), 41.6 (CH, C5), 29.7 (CH_2, C2), 28.2 (CH_2, C3).

***O*-Sulphate 7:** Colourless resin; R_f = 0.20 (90/10 ethyl acetate/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −15.7 (c 0.6 g/100mL,MeOH); ^{1}H NMR (400 MHz, MeOD): δ 7.29 (dd, *J* = 7.8, 7.8 Hz, 1H, Ar), 7.24 (dd, *J* = 1.8, 1.8 Hz, 1H, Ar), 7.21 (ddd, *J* = 8.0, 2.1, 1.2 Hz, 1H, Ar), 7.10 (ddd, *J* = 7.5, 1.3, 1.3 Hz, 1H, Ar), 4.82 (dddd, *J* = 6.8, 6.8, 6.8, 6.8 Hz, 1H, H4), 3.06 (dd, *J* = 14.0, 6.6 Hz, 1H, H5a), 2.97 (dd, *J* = 14.0, 6.0 Hz, 1H, H5b), 2.54 (ddd, *J* = 17.8, 9.4, 9.4 Hz, 1H, H2a), 2.43 (ddd, *J* = 17.8, 9.4, 4.6 Hz, 1H, H2b), 2.30 (dddd, *J* = 12.7, 9.7, 6.7, 4.5 Hz, 1H, H3a), 2.00 (dddd, *J* = 12.7, 9.2, 9.2, 7.6 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): 180.3 (Cq, C1), 154.2 (Cq, Ar), 139.3 (Cq, Ar), 130.3 (CH, Ar), 127.2 (CH, Ar), 123.7 (CH, Ar), 123.0 (CH, Ar), 83.0 (CH, C4), 42.0 (CH_2, C5), 29.6 (CH_2, C2), 28.2 (CH_2, C3).

***O*-Sulphate 8**: White amorphous solid; R_f = 0.21 (80/20 AcOEt/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −12.1 (c 0.9 g/100mL, MeOH); ^{1}H NMR (300 MHz, MeOD): δ 7.50 (d, *J* = 8.4 Hz, 1H, H5′), 7.48 (d, *J* = 1.9, 1H, H2′), 7.05 (dd, *J* = 8.4, 2.2, 1H, H6′), 4.79 (dddd, *J* = 6.6, 6.6, 6.6, 6.6 Hz, 1H, H4),

3.02 (dd, *J* = 14.1, 6.5 Hz, 1H, H5a), 2.93 (dd, *J* = 14.1, 6.1 Hz, 1H, H5b), 2.54 (ddd, *J* = 17.8, 9.4, 9.4 Hz, 1H, H2a), 2.43 (ddd, *J* = 17.8, 9.2, 4.5 Hz, 1H, H2b), 2.29 (dddd, *J* = 12.7, 9.5, 6.6, 4.5 Hz, 1H, H3a), 1.99 (dddd, *J* = 12.7, 9.3, 9.3, 7.6 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): 180.3 (Cq, C1), 145.4 (Cq, Ar), 144.3 (Cq, Ar), 135.1 (Cq, Ar), 127.3 (CH, Ar), 124.8 (CH, Ar), 123.7 (CH, Ar), 83.0 (CH, C4), 41.6 (CH_2, C5), 28.7 (CH_2, C2), 28.2 (CH_2, C3).

***O*-Sulphate 10**: White amorphous solid; R_f = 0.16 (90/10 AcOEt/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −7.8 (c 0.4 g/100mL, MeOH); ^{1}H NMR (400 MHz, MeOD): δ 7.20 (d, *J* = 2.1 Hz, 1H, H2′), 6.93 (dd, *J* = 8.3, 2.1 Hz, 1H, H6′), 6.83 (d, *J* = 8.3 Hz, 1H, H5′), 4.75 (dddd, *J* = 6.9, 6.9, 6.9, 6.9 Hz, 1H, H4), 2.97 (dd, *J* = 14.0, 6.1 Hz, 1H, H5a), 2.84 (dd, *J* = 14.0, 6.5 Hz, 1H, H5b), 2.50 (ddd, *J* = 17.8, 9.2, 9.2 Hz, 1H, H2a), 2.38 (ddd, *J* = 17.7, 9.4, 4.7 Hz, 1H, H2b), 2.26 (dddd, *J* = 12.8, 9.7, 6.8, 4.7 Hz, 1H, H3a), 1.98 (dddd, *J* = 12.8, 9.2, 9.2, 7.4 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): 180.3 (Cq, C1), 149.6 (Cq, Ar), 141.4 (Cq, Ar), 129.3 (Cq, Ar), 128.4 (CH, C6′), 125.3 (CH, C2′), 118.5 (CH, C5′), 83.0 (CH, C4), 41.3 (CH_2, C5), 29.6 (CH_2, C2), 28.1 (CH_2, C3).

***O*-Sulphate 10′**: White amorphous solid; R_f = 0.15 (90/10 AcOEt/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −15.9 (*c* 1.0 g/100mL, CH_3OH); ^{1}H NMR (400 MHz, MeOD): δ 7.21 (d, *J* = 8.2 Hz, H5′), 6.82 (d, *J* = 2.1 Hz, 1H, H2′), 6.71 (dd, *J* = 8.2, 2.1 Hz, H6′), 4.75 (dddd, *J* = 6.6, 6.6, 6.6, 6.6 Hz, 1H, H4), 2.96 (dd, *J* = 14.0, 6.4 Hz, 1H, H5a), 2.86 (dd, *J* = 14.0, 6.0 Hz, 1H, H5b), 2.52 (ddd, *J* = 17.8, 9.4, 9.4 Hz, 1H, H2a), 2.41 (ddd, *J* = 17.7, 9.4, 4.5 Hz, 1H, H2b), 2.26 (dddd, *J* = 12.8, 9.7, 6.7, 4.5 Hz, 1H, H3a), 1.96 (dddd, *J* = 12.8, 9.3, 9.3, 7.7 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): δ 180.3 (Cq, C1), 150.6 (Cq, Ar), 140.2 (Cq, Ar), 136.1 (Cq, Ar), 124.2 (CH, C5′), 122.0 (CH, C6′), 119.5 (CH, C2′), 83.1 (CH, C4), 41.7 (CH_2, C5), 29.7 (CH_2, C2), 28.2 (CH_2, C3).

***O*-Sulphate 11**: Pale yellow resin; R_f = 0.23 (85/15 AcOEt/MeOH); Opt. Rot. $[\alpha]_D^{20}$ −13.4 (*c* 0.5 g/100mL, MeOH); ^{1}H NMR (400 MHz, MeOD): δ 6.72 (dd, *J* = 1.8, 1.8 Hz, 1H, H2′), 6.70 (dd, *J* = 2.1, 2.1 Hz, 1H, H4′), 6.54 (dd, *J* = 1.8, 1.8 Hz, 1H, H6′), 4.79 (dddd, *J* = 6.8, 6.8, 6.8, 6.8 Hz, 1H, H4), 2.98 (dd, *J* = 13.9, 6.4 Hz, 1H, H5a), 2.86 (dd, *J* = 13.9, 6.2 Hz, 1H, H5b), 2.54 (ddd, *J* = 18.0, 9.4, 9.4 Hz, 1H, H2a), 2.44 (ddd, *J* = 17.8, 9.4, 4.6 Hz, 1H, H2b), 2.29 (dddd, *J* = 12.7,

9.7, 6.7, 4.6 Hz, 1H, H3a), 2.00 (m, dddd, *J* = 12.8, 9.2, 9.2, 7.6 Hz, 1H, H3b). ^{13}C NMR (100 MHz, MeOD): δ 180.3 (Cq, C1), 159.4 (Cq, Ar), 155.0 (Cq, Ar), 139.7 (Cq, Ar), 114.7 (CH, C2′), 114.2 (CH, C6′), 108.3 (CH, C4′), 83.0 (CH, C4), 42.2 (CH_2, C5), 29.6 (CH_2, C2), 28.2 (CH_2, C3).

Representative Procedure for *O*-Glucuronide γ-valerolactones synthesis: Synthesis of compound 9

Representative procedure 3 for glucuronidation

According to a known procedure [31], a solution of valerolactone **2** (10.4 mg, 0.05 mmol, 1 equiv) in CH_2Cl_2 (1 mL) was added dropwise to a solution of α-D-Glucuronide Trichloroacetimidate **14** (69.9 mg, 0.1 mmol, 2 equiv) in CH_2Cl_2 (3 mL). The mixture was cooled to 0 °C, follow up by the addition of $BF_3{\cdot}OEt_2$ (1.5 µL, 0.01 mmol, 0.2 equiv). After 2 h, Et_3N was added and the solution was concentrated under vacuum. The residue was purified by flash chromatography on silica gel (from 85:15 to 80:20 Petroleum ether/EtOAc) to afford related protected glucuronide (28.8 mg, 79%) as a mixture of anomers α : β in ratio 0.40:1.0.

Representative Procedure 4 for Benzyl removal of protected glucuronide

To a solution of the abovementioned anomeric mixture benzylated glucoronide (21.9 mg, 0.03 mmol, 1.0 equiv) in a degassed EtOAc/EtOH 1:1 (15 mL), was added Pd (10% on carbon, 10 mg). To this black suspension H_2 was flushed and kept sealed under pressure for 6 h. After this period, the H_2 was removed under vacuum, the resulting suspension was filtered in EtOAc/EtOH mixture, and the residue concentrated to yield targeted compound **9** (10.5 mg, quantitative yield, anomeric 0.4:1 α:β mixture) as white amorphous solid. Rf = 0.15 (80/20 AcOEt/MeOH, 2% AcOH). ^{1}H NMR (400 MHz, MeOD): δ 7.23-7.39 (m, 1.4 H, Hα Ar, Hβ Ar), 6.95-7.09 (m, 4.2 H, 3Hα Ar, 3Hβ Ar), 5.56 (d, *J* = 3.6 Hz, 0.4 H, H1″α), 4.98 (d, *J* = 7.7 Hz, 1H, H1″β), 4.82 (m, 1.4 H, H4α, H4β), 4.15 (d, *J* = 9.9 Hz, 0.4H, H5″α), 4.01 (d, *J* = 9.7 Hz, 1H, H5″β), 3.88 (dd, *J* = 9.2, 9.2 Hz, 0.4H, H3″α), 3.59-3.66 (m, 1.8H, H2″α, H4″α, H4″β), 3.51 (m, 2H, H2″β, H3″β), 3.04 (dd, *J* = 14.0, 7.0 Hz, 0.4H, H5aα), 3.03 (dd, *J* = 13.8, 6.4 Hz, 1H, H5aβ), 2.96 (dd, *J* = 13.9, 5.8

Hz, 1H, H5bβ), 2.95 (dd, *J* = 13.9, 5.6 Hz, 0.4H, H5bα), 2.34-2.58 (m, 2.8H, H2α, H2β), 2.24-2-32 (m, 1.4H, H3aα, H3aβ), 1.94-2.04 (m, 1.4H, H3bα, H3bβ). ^{13}C NMR (100 MHz, MeOD): δ 180.3 (2C, Cq, C1α, C1β), 159.4 (2C, Cq α,β), 158.7 (2C, Cq α,β), 139.7 (Cq, Ar α), 139.4 (Cq, Ar β), 130.7 (CH, Ar α), 130.6 (CH, Ar β), 125.0 (CH, Ar β), 124.9 (CH, Ar α), 119.3 (2C, CH, Ar α, β), 116.7 (CH, Ar α), 116.6 (CH, Ar β), 102.6 (CH, C1″β), 99.3 (CH, C1″α), 83.1 (CH, C4α), 82.9 (CH, C4β), 77.8 (CH, Glc-β) 76.3 (CH, Glc-α), 74.8 (2C, CH, Glc-α, Glc-β), 74.8 (CH, Glc-α), 73.9 (CH, Glc-α), 73.9 (CH, Glc-β), 73.1 (CH, Glc-β), 42.2 (CH_2, C5α), 42.1 (CH_2, C5β), 29.6 (2C, CH_2, C2α, C2β), 28.3 (CH_2, C3α), 28.1 (CH_2, C3β).

Glucuronide 12. White amorphous solid. Rf = 0.10 (80/20 AcOEt/MeOH, 2% AcOH). ^{1}H NMR (400 MHz, MeOD): δ 6.60 (dd, *J* = 1.8, 1.8 Hz, 0.36H, Ar α), 6.53 (m, 1.30 H, H Ar β, H Ar α), 6.48 (dd, *J* = 2.2, 2.2 Hz, 1H, H Ar β), 6.41 (m, 1.31H, H Ar β), 5.50 (d, *J* = 3.6 Hz, 0.35H, H1″ α), 4.90 (d, *J* = 7.6 Hz, 1H, H1″ β), 4.79 (dddd, *J* = 6.9, 6.9, 6.9, 6.9 Hz, 1.5H, H4α, H4β), 4.06 (d, *J* = 10.0 Hz, 0.37H, H5″α), 3.90 (d, *J* = 9.6 Hz, 1H, H5″β), 3.87 (dd, *J* = 9.2, 9.2 Hz, 0.36H, H3″α), 3.57-3.61 (m, 1.77H, H4″β, H4″α, H2″α), 3.46-3.53 (m, 2H, H3″β, H2″β), 2.97 (dd, *J* = 14.3, 6.6 Hz, 0.36H, H5aα), 2.95 (dd, *J* = 14.0, 6.2 Hz, 1H, H5aβ), 2.86 (dd, *J* = 14.1, 6.2 Hz, 1H, H5bβ), 2.84 (dd, *J* = 14.0, 6.3 Hz, 0.38H, H5bα), 2.34-2.56 (m 2.75H, H2α, H2β), 2.23-2.32 (m, 1.38H, H3aα, H3aβ), 1.94-2.04 (m, 1.37H, H3bα, H3bβ). ^{13}C NMR (100 MHz, MeOD): δ 180.3 (2C, Cq, C1α, C1β), 160.3 (2C, Cq α, Cq β), 159.8 (Cq α), 159.7 (Cq β), 159.6 (2C, Cq α, Cq β), 140.3 (Cq α), 140.0 (Cq β), 112.2 (CH, Ar β), 112.1 (CH, Ar α), 110.6 (CH Ar, α), 110.4 (CH, Ar β), 104.0 (CH, Ar α), 103.9 (CH, Ar β), 102.5 (CH, C1″β), 99.2 (CH, C1″α), 83.1 (CH, C4α), 82.9 (CH, C4β), 77.7 (CH, Glc-β), 76.6 (CH, Glc-α), 74.8 (2C, CH, Glc-β), 74.7 (CH, Glc-α), 73.8 (CH, Glc-α), 73.4 (CH, Glc-β), 73.1 (CH, Glc-α), 42.3 (CH_2, C5α), 42.2 (CH_2, C5β), 30.9 (CH_2, C2α), 29.6 (CH_2, C2β), 28.3 (CH_2, C3α), 28.1 (CH_2, C3β).

2.3. Urine collection and processing

Urine samples for method validation were obtained from subjects consuming green tea. In

particular, sixteen healthy adults aged between 35 to 50 were recruited. Subjects were non-smokers with no previous history of chronic diseases, did not take regularly (daily) green tea or vitamin/herbal supplements, and had not special dietary preferences, e.g. vegetarianism. They were not under long-term medication, had not been hospitalised in the previous 12 months, and had not received medical care in the past three months. Subjects with Body Mass Index (BMI) higher than 27 kg/m^2 were excluded. Written consent was obtained and all procedures complied with the Declaration of Helsinki. The study was approved by the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University.

Subjects were assigned to have either tea or water first on a randomised, single-blinded basis. On day 1 of each subject's participation, baseline urine samples for their 7-day treatment (supplementation study) were collected into containers without any preservative and stored frozen (−80 °C) until used. From day 1, all subjects were required to drink either 200 mL of 1% w/v green tea (pre-rain Loong-cheng tea leaves, kindly provided by Ying Kee Tea House, HKSAR) or hot water twice a day (preferably, in the morning and at night) for seven consecutive days (tea bags of green tea were supplied), and they would return to the laboratory on day 8, when urine samples were collected as previously reported. Subjects then went through a 4-week washout period, after which the procedures of 7 days' supplementation were repeated, with each subject crossed-over onto the other treatment. Urine samples were collected again. Compliance was assessed by counting up the number of tea bags returned from green tea supplementation group and by inquiry to both groups. A compliance >80% was regarded as satisfactory.

Urine samples were defrosted, vortexed, diluted in 0.1% formic acid in water (1/4, v/v), centrifuged at 18000 *g* for 5 min, and filtered through 0.22 μm nylon filters prior to the analysis by UHPLC-ESI-MS/MS.

2.4. UHPLC-ESI-QqQ-MS/MS

All synthesized standards and samples were analysed by UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.).

Separations were performed with a Kinetex EVO C18 (100 × 2.1 mm), 2.6 μm particle size (Phenomenex). For UHPLC, mobile phase A was 0.2% formic acid in water and mobile phase B was acetonitrile containing 0.2% formic acid. The gradient started with 5%B, keeping isocratic conditions for 0.5 min, reaching 95%B at 7 min, followed by 1 minute at 95% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 mL/min, the injection volume was 5 μL, and the column was thermostated at 40°C.

The MS worked in negative ionization mode with capillary temperature at 270 °C, while the source at 300 °C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra high-purity argon gas was used for collision-induced dissociation (CID). Each synthesized compound was directly infused into the ESI source (5 μg/mL at a flow rate of 10 μL/min) in combined mode with a background mode of 70/30 v/v of phase A/phase B at 0.3 mL/min. Characteristic MS conditions (S-lens RF amplitude voltage and collision energy) were optimized for each phenyl-γ-valerolactone. The applied method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in the “selected reaction monitoring” (SRM) mode. Two molecular transitions were used to qualify and quantify phenyl-γ-valerolactone conjugates. Data processing was performed using Xcalibur software from Thermo Scientific.

2.5. Method validation

The method was validated for selectivity, calibration curve, range, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), intra-day and inter-day precision, and accuracy. Method validation was carried out on diluted blank urine samples

298 spiked with the synthesized phenyl-γ-valerolactones and according to Food and Drug
299 Administration (FDA) guidelines [32]. Blank urine samples were kindly provided by three
300 healthy volunteers following a phenolic-free diet for 72 h.

301 Compounds were individually dissolved in dimethyl sulfoxide at 10 mM and individual
302 stock solutions were diluted and pooled to obtain a standard solution at 200 μM in 1 mL 0.1%
303 formic acid in acetonitrile. Working dilutions of phenyl-γ-valerolactones from the standard pool
304 solution were prepared in 0.1% formic acid in water/blank urine (4/1, v/v), with concentrations
305 ranging from 0.1 nM to 133 μM. Compound **10** was prepared individually following the same
306 procedure. A minimum of 14 concentration levels were used.

307 Selectivity was assessed by analysing diluted blank urine samples spiked or not with
308 phenyl-γ-valerolactones at the LLOQ. The evaluation of the range of calibration curves was
309 based on data fitting to linear or quadratic regressions, prioritizing linear fitting. Acceptable
310 fitting was estimated by using the coefficient of determination (R^2). The LOD and LLOQ for
311 each compound were determined as the concentration in which the quantifier transition showed a
312 signal-to-noise (S/N) ratio ≥3 and ≥10, respectively. The intra-day precision (repeatability) and
313 inter-day precision (semi-reproducibility) of the method, reported as the relative standard
314 deviation (% RSD), was evaluated at the LLOQ of each compound (L1) and at two higher
315 concentration levels (5xLOQ, L2, and 10xLOQ, L3). Each solution was injected randomly three
316 times per day in three different days. The acceptance criteria was RSD <20% for L1 and <15%
317 for both L2 and L3. Accuracy was calculated in terms of recovery rate for the L2 concentration
318 level of each compound, as the ratio between the mean recorded concentration and the spiked
319 concentration, multiplied by 100.
320

321 **2.6. Data and statistical analysis**

322 All analyses were performed in triplicate for method validation. Data are reported as mean
323 ± standard deviation (SD). Statistical analysis was carried out using the IBM SPSS Statistics 23.0

software package (IBM, Chicago, IL, USA). Non-parametric Kruskal-Wallis test was performed and, when significant (p<0.05), the Mann-Witney *U* test was applied to define specific differences in the urinary excretion of phenyl-γ-valerolactones.

3. Results and discussion

3.1. Synthesis of conjugated phenyl-γ-valerolactones

Considering previous studies accounting for the transformation into conjugated phenyl-γ-valerolactones of flavan-3-ols by gut microbiota and human phase II enzymatic pools [11, 14, 18], different glucuronide and sulphate conjugates were synthesized. Only the main steps of the synthetic strategy are presented in the main body of this work; details are presented at Supplementary Material.

O-Sulphated isomers **6** and **7** were synthesized using 2,2,2-trichloroethyl chlorosulphate (TCECS, **13**) as sulphate "donor" in presence of Et_3N, DMAP in CH_2Cl_2, starting respectively from the corresponding 5-(hydroxyphenyl)-valerolactone **1** and **2** (Figure 2, eq a). The next removal of TCE moiety with Zn dust and ammonium formate afforded the target products as ammonium salts, with 63% and 81% of yield, respectively, after two steps. Following the aforementioned synthetic way, the di-*O*-sulphated metabolite **8** was prepared starting from the related aglycone (**3)** with a 52% overall yield (Figure 2, eq a).

The phenolic group of aglycone (*R*)-**2** was also conjugated with benzyl glucuronate "donor" **14** to give the protected glucuronidated adduct as an anomeric 0.4:1 α:β mixture with 78% of yield. Finally, total debenzylation with H_2 Pd/C in a 1:1 EtOH/AcOEt mixture afforded the desired metabolite **9** in quantitative yield (Figure 2, eq b).

The two monosulphate regioisomers **10** and **10′** of 5-(3,4-dihydroxyphenyl)-γ-valerolactone (**3**) were synthesized starting from orthogonally protected precursors **15** and **15′**, as depicted in Figure 2 (eqs c, d). For the synthesis of 3′-*O*-sulphated metabolite **10**, precursor **15** was desilylated by HF ▪Py unmasking the phenolic group at the 3′ position, which was sulphated

with compound **13** with a good 70% yield (Figure 2, eqs c). At this point, removal of the benzyl group with H_2, Pd/C in AcOEt and the cleavage of TCE group with Zn dust, ammonium formate afforded metabolite **10′** with a good 60% yield after two steps. The same treatment of desilylation, sulphation, benzyl and TCE cleavages converted the regioisomer precursor **15′** into the target molecule **10′** in a nice 38% overall yield (Figure 2, eq d).

Due to the chemical equivalence of the two phenolic groups in 3′ and 5′ position of valerolactone **4**, the synthesis of mono-conjugated metabolites **11** and **12** did not require the orthogonal protection strategy. As shown in Figure 2 (eq e), the dibenzylated valerolactone scaffold **17** was subjected to a mild deprotection with $NiCl_2$, $NaBH_4$ in MeOH, giving the mono-protected compound **18** with an acceptable 52% of yield. The intermediate **18** represented a divergent point toward the final products **11** and **12**. In fact, the sulphation and sequential reductive cleavage of benzyl and TCE moieties afforded the final target **12** with 52% yield after three steps (Figure 2). On the other hand, coupling **18** with the trichloroacetimidate **14** gave the protected glucuronide as anomeric 0.35:1- α/β mixture with a very good 85% yield. Treatment of this polybenzylated mixture with H_2, Pd/C afforded in only one step the total cleavage of all benzyl groups yielding the targeted metabolite **12** in quantitative yield.

Despite the synthesis of some phenyl-γ-valerolactone scaffolds has already been reported [26, 27, 33], this is the first time, to the best of our knowledge, that the synthesis of authentic bioanalytical standards of sulphate- and glucuronide-conjugated phenyl-γ-valerolactones is reported.

3.2. Development and optimization of the UHPLC-ESI-MS/MS method

One of the aims of this work was to develop a quick method to quantify phenyl-γ-valerolactones in human urine. Six UHPLC columns (Knauer BlueOrchid C18, Restek Ultra AQ C18, Waters Acquity UPLC HSS T3, Phenomenex Kinetex PFP, Phenomenex Kinetex EVO C18 2.6 μm, and Phenomenex Kinetex EVO C18 1.7 μm) often used for the separation of phenolic

metabolites were utilized. Column length was a critical characteristic to allow the separation of isomers **6** and **7**, for which long columns were required (100 mm). Both Kinetex EVO C18 and the Acquity HSS T3 columns provided the best peak resolutions at their optimal flow rates, but the EVO C18 2.6 μm was preferred since lower operating pressures were achieved due to its higher particle size (2.6 μm in comparison with 1.8 μm of the Acquity and 1.7 μm of the other Kinetex EVO C18). Despite the Restek Ultra AQ C18 was similar to the EVO C18 2.6 μm in terms of length and particle size, peak shape for sulphated derivatives was poor in the former after repeated analyses. Flow rates under 0.4 mL/min resulted in poor peak resolution. Regarding mobile phase solvents, acetonitrile but not methanol improved peak shape for sulphated conjugates (approximately 35%). All phenyl-γ-valerolactones eluted within 12 minutes and all compounds, including isomers, were well separated under the above described chromatographic conditions. However, this method did not succeed to separate co-eluting isomers **10** and **10'**, and analysis times longer than 30 minutes were required to achieve an acceptable separation. This fact had been previously observed by other authors using longer gradients [18].

The MS/MS related parameters were optimized for each individual compound separately, by performing direct infusion experiments (Table 1). A greater sensitivity was reached in negative ionization condition for all the compounds. In general, sulphated compounds responded better to ES ionization conditions with respect to their glucuronidated counterparts and to free forms of phenyl-γ-valerolactones. As it had been previously reported [13], the deprotonated aglycone ions of phenyl-γ-valerolactone conjugates were always the predominant peaks in the fragment ion MS spectra. Two selective SRM transitions were used for each metabolite, making a robust qualitative and quantitative information easily achievable [34].

A particular behaviour in terms of peak resolution and ionization was observed for 5-phenyl-γ-valerolactone-3′,4′-di-*O*-sulphate (**8**). This compound showed an asymmetric peak shape, characterised by a severe peak tail, and also showed a limited ionization, characterized by the co-presence of four different molecular ions: the doubly-charged molecular ion ($[M-2H]^{2-}$) at

m/z 183 (100% of relative abundance), two in-source fragments of one and two sulphate moieties yielding molecular ions at *m/z* 287 and 207 (30% of relative abundance for both ions), and the single molecular ion ($[M-H]^-$) at *m/z* 367 (10% of relative abundance). Unfortunately, it was not possible to improve its chromatographic and ionization features, despite multiple efforts.

Despite the feasibility of analysing different classes of flavan-3-ol metabolites, this method was exclusively developed for phenyl-γ-valerolactones because the lack of authentic standards has hindered accurate calibration and absolute quantification of these phenolic metabolites so far [25]. The method allowed the simultaneous resolution and quantification of 12 authentic standards of phenyl-γ-valerolactones within 12 minutes. The analysis time was short if compared to other methods that detected phenyl-γ-valerolactones as well as other flavan-3-ol metabolites in 26-70 min [11, 13, 14, 18, 19, 35, 36], and in line with other UHPLC methods resolving a high number of phenolic metabolites in 10-12 min [21-24].

3.3. Method validation

3.3.1. Selectivity

To determine whether endogenous peaks from human urine or other sample components co-eluted with the analytes of interest, selectivity was evaluated in diluted blank matrix spiked or not with phenyl-γ-valerolactones. In all cases, no interference signals from the matrix at the specific SRM transitions were observed. The concomitant presence of 5-(3′-hydroxyphenyl)-γ-valerolactone (**2**) and 5-phenyl-γ-valerolactone-3′-*O*-sulphate (**7**) in the sample caused a loss of selectivity for the former due to the in-source fragmentation of the latter. For all the other analysed compounds, the method was characterized by a high selectivity.

3.3.2. Linearity, limit of detection and limits of quantification

Calibration curves were established using diluted blank urine for matrix-match calibration. Different concentrations levels, covering the expected range for each compound and

ranging from its LLOQ to its UPLOQ were used. Calibration curves were forced to pass through the origin and the regression line best fitting data (linear or quadratic) was used. Most of the compounds were fitted linearly, but compounds **5**, **10**, and **10′** fitted quadratic calibration curves (Table 2). All the compounds showed R^2 higher than 0.987 (Table 2).

Concentration ranges, LODs, LLOQs, and UPLOQs varied largely among the different analytes (Table 2), with most of the compounds displaying analytical ranges along 3-5 orders of magnitude, with the exception of unconjugated mono- and trihydroxy-phenyl-γ-valerolactones (**1**, **2**, and **5**) and 5-phenyl-γ-valerolactone-3′,4′-di-*O*-sulphate (**8**). LOD values varied from 0.2 to 1,113 nM and the median LOD was 6.2 nM. With respect to the LLOQ, it ranged from 0.6 to 2,227 nM and the median LLOQ was 12.4 nM. UPOQ varied between 66,667 and 133,333 nM, with median values for 1,000,000 nM. LODs of compounds **1**, **2**, **4**, **5**, and **8** were above 20 nM, mostly because of their poor ionization. On the contrary, mono-sulphated hydroxy- and dihydroxy-phenyl-γ-valerolactones (compounds **6**, **7**, **10** and **10′**) had LODs and LLOQs below 1.5 and 10 nM, respectively. These LOD and LLOQ values, in the low nM range, were in agreement or even lower than those reported for 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**3**), 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulphate (**10**), and other phenolic metabolites [19, 23, 24, 37, 38].

3.3.3. Precision and accuracy

The intra-day and inter-day precision, calculated as the respective relative standard deviation (% RSD), was determined at three concentrations (L1-L3) (Table 2). The intra-day precision was lower than 15% for all the compounds at L2 and L3, while it was within 20% for most of the compounds at the LLOQ (L1). Average intra-day precision values (%) were 10.6 ± 6.7, 7.7 ± 4.3, and 2.2 ± 3.0 for L1, L2, and L3, respectively. The values of the inter-day precision were lower than 20% at L1 and fell within 15% at L2 and L3 for most of the compounds. Average values for inter-day precision (%) were 12.1 ± 6.6, 8.6 ± 3.7, and 6.1 ± 2.8 for L1, L2,

and L3, respectively. The accuracy was excellent for most of the compounds, with values ranging from 86.6% for 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone to 121.9% for 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate (Table 2). Average accuracy (%) was 101.4 ± 10.9. Overall, the method met the acceptance criteria of FDA for intra- and inter-day precision, and accuracy [32].

3.4. Method application: urinary excretion of phenyl-γ-valerolactones after consumption of green tea

Of the 13 metabolites targeted within the present UHPLC-ESI-MS/MS method, 10 compounds were identified and quantified in urine samples of subjects consuming green tea and following an unrestricted diet. In this set of analyses, 5-(3′-hydroxyphenyl)-γ-valerolactone (**2**) and 5-phenyl-γ-valerolactone-3′,4′-di-*O*-sulphate (**8**) were not detected, and their absence could be related to their intrinsic poor selectivity and resolution, respectively. Nevertheless, their absence in the samples could not be completely ruled out. On the other hand, it was impossible to distinguish between 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulphate (**10**) and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate (**10′**) due to their chromatographic behaviour. These isomers were quantified using **10′** as reference compound.

There were no statistically significant differences in the excretion of most of the phenyl-γ-valerolactones between the water control and the green tea supplementation periods ($p>0.05$) (Table 3). This fact could be linked to the limited contribution of normal dosages of green tea to the total pool of circulating phenyl-γ-valerolactones under free-living conditions (with no dietary restrictions). However, green tea supplementation guaranteed the presence of phenolic scaffolds allowing the formation of 5-(3′,4′,5′-trihydroxyphenyl)-phenyl-γ-valerolactone (**5**), since it is mainly produced by the colonic catabolism of (–)-epigallocatechin (EGC) and (–)-epigallocatechin-3-gallate (EGCG) [11], contained in green tea but not in other flava-3-ol rich sources, like cocoa or red wine. Maximum urinary concentrations varied between 515 nM for 5-

phenyl-γ-valerolactone-4′-*O*-sulphate (**6**) and 132,111 nM for 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate isomers (**10**/**10′**) (Table 3). In terms of absolute excretion, maximum values ranged from 84 nmol/mmol creatinine for metabolite **6** to 15,697 nmol/mmol creatinine for isomers **10**/**10′** (Supplementary Material, Table 1). The most abundant compounds were **4** and **10**/**10′**, although the relative contribution of each phenyl-γ-valerolactone to the total urinary excretion varied notably among subjects. With respect to minimum urinary concentrations, it should be noted that some phenyl-γ-valerolactones were not produced/excreted by some volunteers (Table 3), and this can be related to the large inter-individual variability existing in the production of these colonic metabolites [3, 18, 26, 35, 39-44].

The urinary concentrations recorded for some phenyl-γ-valerolactones, in particular **4** and **10**/**10′**, were quite high (reaching 132 μM). Comparison with other works is avoided, since most of them quantified phenyl-γ-valerolactones without using synthesised exact standards, or because the analysed samples were hydrolysed by using sulphatase and β-glucuronidase enzymes before analysis [14, 35, 36]. In this sense, these data and the accurate quantification of phenyl-γ-valerolactones with their respective reference compounds may lead to the redefinition of the recovery and bioavailability of flavan-3-ols.

4. Conclusions

This work described for the first time the synthetic procedure for 8 sulphate- and glucuronide-conjugated phenyl-γ-valerolactones. A quick, selective, sensitive, and reproducible validated UHPLC-ESI-MS/MS method allowing the quantification of up to 13 phenyl-γ-valerolactones in human urine was also developed. Moreover, the analytical challenges faced when dealing with some of these molecules were reported to save researchers in the field from further future unsuccessful attempts. The analytical method allowed, for the first time, the accurate quantification of 10 phenyl-γ-valerolactones in urine samples of subjects consuming green tea, by using exact reference compounds. Additional efforts are needed to extrapolate the

application of this analytical method to other biological samples, such as plasma and faeces, likely by using clean-up steps. However, this point requires further investigations since optimization of solid-phase extraction may be of critical importance. In addition, thanks to the information on the ionization properties of the synthesized compounds, the method could be extended to other phenyl-γ-valerolactones for which their pure forms are still lacking.

The availability of phenyl-γ-valerolactone conjugates as authentic bioanalytical standards will also allow the use of these key flavan-3-ol metabolites in cells assays, in order to shed light on their *in vitro* putative bioactivity. Moreover, the quantification of phenyl-γ-valerolactones in urine samples by comparison with authentic synthesized standards will open the door to better studying the bioavailability of flavan-3-ols and the real exposure of populations to flavan-3-ol sources. Overall, the present work can provide valuable insights in the future study of the fate of flavan-3-ols and phenyl-γ-valerolactones in the human body, as well as help in the understanding of their potential role in the prevention of chronic diseases.

Author contributions

Study conception and design: PM, CC, DDR; Acquisition of data: NB, PM, CC, IB, S-WC; Analysis and interpretation of data: NB, PM, LC, CC, DDR; Drafting of manuscript: NB, PM, IB; Critical Revision: LC, IB, FZ, FB, CC, DDR.

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Figure legends

Figure 1. Panel of thirteen phenyl-γ-valerolactone metabolites (aglycones and conjugates) **1**-**12** used in this work for analytical method development. Sulphate-containing molecules 6, 7, 8, 10, 10', and 11 do not contain amine groups in circulation or dissolved.

Figure 2. Main steps of the synthetic routes towards phenyl-γ-valerolactone conjugates

667 **Table 1.** Retention times and optimized SRM conditions for identification and quantification of

668 phenyl-γ-valerolactones

No.	Compound	RT (min)	Parent ion (*m/z*)	S-lens	Quantifier		Qualifier	
					Product ion (*m/z*)	CE (V)	Product ion (*m/z*)	CE (V)
1	5-(4′-hydroxyphenyl)-γ-valerolactone	5.26	191	70	147	13	106	31
2	5-(3′-hydroxyphenyl)-γ-valerolactone	4.81	191	70	147	20	106	31
3	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	3.94	207	75	163	20	122	25
4	5-(3′,5′-dihydroxyphenyl)-γ-valerolactone	3.54	207	75	163	18	123	20
5	5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	2.19	223	78	179	21	138	26
6	5-phenyl-γ-valerolactone-4′-*O*-sulphate	4.53	271	93	191	23	147	35
7	5-phenyl-γ-valerolactone-3′-*O*-sulphate	4.71	271	92	191	23	106	48
8	5-phenyl-γ-valerolactone-3′,4′-di-*O*-sulphate	4.97	367	52	287	12	207	33
9	5-phenyl-γ-valerolactone-3′-*O*-glucuronide	4.03	367	93	191	25	147	43
10	5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulphate	4.46	287	96	207	23	163	34
10’	5-(4′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	4.42	287	96	207	23	163	35
11	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	3.83	287	96	207	23	163	35
12	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*-glucuronide	2.11	383	87	207	24	163	40

669 CE: collision energy

670

671 **Table 2.** Parameters for quantification of phenyl-γ-valerolactones in human urine samples by HPLC-ESI-MS/MS

No.	Compound	Calibration curve	R^2	LOD (nM)	LLOQ (nM)	ULOQ (nM)	Precision intra-day (% RSD)			Precision inter-day (% RSD)			Accuracy (%)
							L1	L2	L3	L1	L2	L3	
1	5-(4′-hydroxyphenyl)-γ-valerolactone	y = 2982x	0.989	556	1113	100000	5.2	3.9	4.2	6.9	7.5	4.3	112.6
2	5-(3′-hydroxyphenyl)-γ-valerolactone	y = 891x	0.989	1113	2227	100000	8.6	6.3	3.0	7.7	8.6	6.9	99.8
3	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	y = 93122x	0.991	6.2	12.4	100000	16.8	0.1	1.8	16.1	1.8	3.4	102.6
4	5-(3′,5′-dihydroxyphenyl)-γ-valerolactone	y = 6245x	0.996	61.6	123	99852	18.9	5.1	0.1	21.7	7.8	3.6	96.5
5	5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	$y = 39.4x^2 + 76.9x$	0.998	1112	2223	66695	23.6	12.5	8.8	17.2	15.2	5.3	86.6
6	5-phenyl-γ-valerolactone-4′-*O*-sulphate	y = 94304x	0.990	1.2	2.5	133333	2.8	10.2	1.0	3.3	7.6	6.8	106.7
7	5-phenyl-γ-valerolactone-3′-*O*-sulphate	y = 341669x	0.996	1.2	6.2	100000	6.6	9.3	0.2	10.8	4.4	7.0	117.3
8	5-phenyl-γ-valerolactone-3′,4′-di-*O*-sulphate	y = 131.85x	0.993	1110	2220	100000	6.0	2.1	1.3	16.2	13.8	11.1	94.3
9	5-phenyl-γ-valerolactone-3′-*O*-glucuronide	y = 16626x	0.993	2.5	12.4	66722	8.2	13.0	0.0	17.6	12.8	7.8	87.9
10	5-(3′-hydroxyphenyl)-γ-valerolactone-4′-*O*-sulphate	$y = -2231x^2 + 396261x$	0.995	0.2	0.6	100000	12.9	7.8	7.9	13.6	5.8	6.5	94.7
10’	5-(4′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	$y = -1856x^2 + 428010x$	0.988	0.6	1.2	100000	4.9	5.6	0.1	1.0	8.5	6.8	121.9
11	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	y = 136366x	0.989	6.2	12.3	100000	4.9	10.0	0.2	5.5	8.9	9.7	104.6
12	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*- glucuronide	y = 11046x	0.993	12.3	24.7	66667	17.8	14.3	0.1	19.5	9.7	0.3	92.8

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674

675 **Table 3.** Urinary concentrations of phenyl-γ-valerolactones (nM) following an unrestricted diet with or without green tea consumption, at baseline and

676 after a 7-day supplementation period (n=16).

No.	Compound	Green tea supplementation											Hot water control										
		Day 0						Day 7						Day 0						Day 7					
		Mean ± SD		CV	Median	Max.	NP	Mean ± SD		CV	Median	Max.	NP	Mean ± SD		CV	Median	Max.	NP	Mean ± SD		CV	Median	Max.	NP
1	5-(4′-hydroxyphenyl)-γ-valerolactone	6480			6480	6480	94	nd		-	0	0	100	nd		-	0	0	100	10014			10014	10014	94
3	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	64 ± 77		120	54	322	25	66 ± 87		133	22	288	50	95 ± 91		96	68	338	20	130 ± 273		210	44	1074	38
4	5-(3′,5′-dihydroxyphenyl)-γ-valerolactone	28 ± 76		276	0	249	88	173 ± 288		167	0	908	64	60 ± 178		296	0	666	87	64 ± 123		193	0	408	75
5	5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	7383 ± 17454		236	0	67510	75	8474 ± 14969		177	0	54681	57	2795 ± 5426		194	0	18375	73	7849 ± 13508		172	0	49686	56
6	5-phenyl-γ-valerolactone-4′-*O*-sulphate	nd		-	0	0	100	36 ± 94		258	0	335	71	39 ± 133		339	0	515	87	33 ± 71		213	0	199	69
7	5-phenyl-γ-valerolactone-3′-*O*-sulphate	65 ± 142	ab	219	0	528	63	137 ± 213	a	156	47	682	36	13 ± 28	b	210	0	83	73	15 ± 33	ab	211	0	119	69
9	5-phenyl-γ-valerolactone-3′-*O*-glucuronide	122 ± 306		251	0	1230	56	433 ± 769		178	41	2230	43	111 ± 306		275	0	1179	73	33 ± 78		235	0	275	81
10’	5-(4′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	13724 ± 23592		172	5136	73092	0	13118 ± 17955		137	8956	66074	0	14414 ± 23039		160	6016	85864	0	18078 ± 36051		199	2495	132111	0
11	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*-sulphate	5658 ± 13970		247	381	46444	19	12895 ± 22031		171	4584	81979	0	1444 ± 1721		119	1386	6310	20	5871 ± 14914		254	172	46615	19
12	5-(5′-hydroxyphenyl)-γ-valerolactone-3′-*O*-glucuronide	1981 ± 5468		276	137	21023	44	2421 ± 3236		134	1063	9929	0	400 ± 329		82	330	1242	7	1373 ± 3538		258	75	12210	44

677 Values are in reported in nM, except for CV and NP, which are reported as %. Max, maximum concentration; nd, non-detected or below the LLOQ; NP, non-producer subjects (%).

678 Means within a row followed by different letters are significantly different at $p<0.05$.