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

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Abbreviations:

DMAP, 4-Dimethylaminopyridine; **Glc**, glucuronic acid; **LLOQ**, lower limit of quantification; **TBS**, *tert*-Butyldimethylsilyl; **TCECS**, 2,2,2-trichloroethyl chlorosulfate; **TIPSO**F, Triisopropylsilyloxy-furan; **ULOQ**, upper limit of quantification.

Keywords: colonic metabolites; flavan-3-ol; green tea; phenolic compounds; stereoselective synthesis.

ABSTRACT:

Scope: The contribution of the gut microbiota to the metabolism of catechins and proanthocyanidins remains unclear. Phenyl- γ -valerolactones have been identified as the most representative metabolites of these dietary flavan-3-ols, but their accurate quantification has posed problems because of a lack of appropriate bioanalytical standards. This work aimed at synthesizing a novel set of sulphate- and glucuronide-conjugated phenyl- γ -valerolactones and at developing an analytical platform using UHPLC-ESI-MS/MS for their quantification in urine.

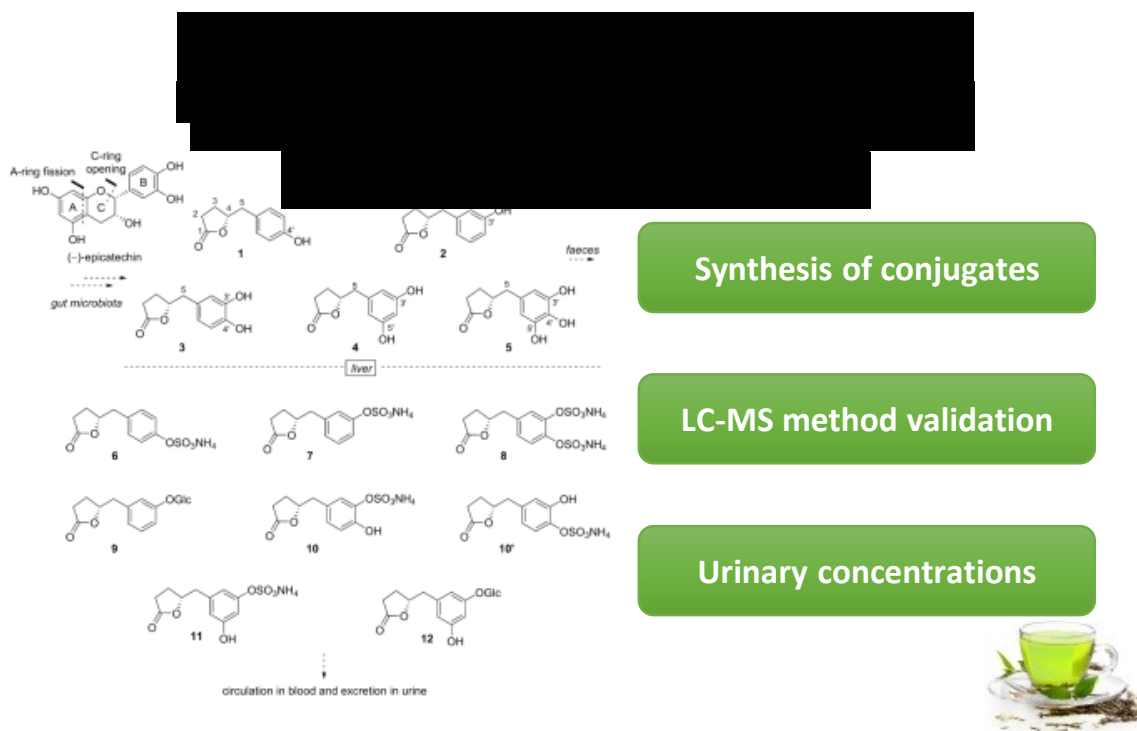
Methods and results: Eight glucuronide and sulphate conjugates of hydroxyphenyl- γ -valerolactones were synthesized and used as analytical standards, together with 5 phenyl- γ -valerolactone aglycones, for the development of a high-throughput, validated analytical method. Chromatographic and MS conditions 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.

Conclusions: 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.

Graphical abstract

This work described for the first time the synthetic procedure for 8 sulphate- and glucuronide-conjugated phenyl- γ -valerolactones. A quick, selective, sensitive, reproducible, validated UHPLC-ESI-MS/MS method allowing the quantification of up to 13 phenyl- γ -valerolactones in human urine was developed. 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.



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].

It is known that dietary polyphenols are extensively metabolized by the human body [1, 3]. In the case of flavan-3-ols, only a small fraction is absorbed from the gastrointestinal tract and is then rapidly modified by phase II enzymes. Most (usually more than two-thirds of the intake) of the ingested polyphenols 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]. Phenyl- γ -valerolactones have been identified as the main ring fission products produced by this colonic catabolism [12, 14], and these enter the bloodstream [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 a 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/microbiota 'superorganism', as they are valuable biomarkers of dietary consumption of flavan-3-ols, both monomers and proanthocyanidins [12, 16, 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. The current lack of reliable reference compounds of hydroxyphenyl- γ -valerolactones has restricted the use of colonic metabolites in bioavailability and *in vitro* bioactivity studies [26]. In the framework of the “joint venture” between chemical synthesis and analytical techniques, the development of a validated analytical method for the qualitative determination of these metabolites in biological fluids is needed.

Advances in asymmetric synthesis carried out by our research group have achieved the enantioselective synthesis of hydroxyphenyl- γ -valerolactone aglycones (compounds **1-5**, Figure 1) [27]. In this paper, the stereoselective synthesis of 2 *O*-glucuronide and 6 *O*-sulphate conjugated metabolites (compounds **6-12**, Figure 1) of (*4R*)-configured aglycones **1-4** 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 was developed and validated, and the viability of this method was tested by investigating 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 healthy volunteers.

2. Materials and methods

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2.1. Reagents

Dichloromethane (HPLC grade), was dried by distillation on CaH₂ according to standard procedures. THF dry, Et₂O 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 Supporting Information. 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 μ m particle size; Knauer, Berlin, Germany), Kinetex PFP (50 × 2.1 mm, 2.6 μ m particle size, Phenomenex, Macclesfield, UK), Ultra AQ C18 (100 × 2.1 mm, 3 μ m particle size; Restek, Bellefonte, PA, USA), Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μ m particle size, Waters, Milford, MA, USA), Kinetex EVO C18 (100 × 2.1 mm, 2.6 μ m particle size, Phenomenex), and Kinetex EVO C18 (100 × 2.1 mm, 1.7 μ m particle size, Phenomenex).

2.2. Synthesis of metabolites

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 (^{13}C). Spectra were referenced to tetramethylsilane (0.0 ppm, ^1H ; 0.0 ppm, ^{13}C , in CDCl_3). 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 ^{13}C NMR assignments were corroborated by 1D and 2D experiments (gCOSY, gHSQC, DEPT). Optical rotation data ($[\alpha]_{\text{D}}^{20}$) 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 $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$. All the compounds were synthesised according to known procedures [28, 31]. Details for the preparation and characterization of all synthesized compounds can be found in the Supporting Information. Most of these molecules are catalogued on the standards sharing platform FoodComEx (www.foodcomex.org).

2.3. Urine collection and processing

Urine samples for method validation were obtained from subjects consuming green tea. In particular, sixteen healthy adults aged 35 to 50 year 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 no 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.

Eight 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, fasting urine samples for their first 7-day treatment were collected into containers without any preservative and stored frozen ($-80\text{ }^{\circ}\text{C}$) 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 the same volume of 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 returned, in the fasting state, to the laboratory on day 8, when urine samples were again collected as described. Subjects then went through a 4-week washout period, after which the procedures of the 7 days' supplementation were repeated, with each subject crossed-over onto the other treatment. Pre- and post-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. There were no drop-outs, and compliance was satisfactory in all 16 volunteers.

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 thermostatted at 40°C.

The MS worked in negative ionization mode with capillary temperature at 270 °C, while the source was 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 spiked with the synthesized phenyl-γ-valerolactones and according to Food and Drug Administration (FDA)

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guidelines [32]. Blank urine samples were kindly provided by three healthy volunteers following a phenolic-free diet for 72 h.

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

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

2.6. Data and statistical analysis

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

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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.

O-Sulphated isomers **6** and **7** were synthesized using 2,2,2-trichloroethyl chlorosulphate (TCECS, **13**) as sulphate “donor” in presence of Et₃N, DMAP in CH₂Cl₂, starting respectively from the corresponding 5-(4'-hydroxyphenyl)-valerolactone **1** and its 3'-hydroxyphenyl congener **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% isolated yield, respectively, after two steps. Following the aforementioned procedure, 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 **2** was also conjugated with polybenzylated glucuronate “donor” **14** to give the protected glucuronidated adduct in 78% yield as an anomeric 0.4:1 α : β mixture. Finally, total debenylation with H₂ Pd/C in a 1:1 EtOH/AcOEt mixture afforded the desired metabolite **9** in 96% isolated yield (75% overall, 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 treated with

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HF •Py to unmask the phenolic group at the 3' position, which was then sulphated with TCECS **13** with a good 56% overall yield (Figure 2, eqs c). At this point, removal of the benzyl group with H₂, Pd/C in AcOEt and cleavage of TCE group with Zn dust-ammonium formate couple afforded metabolite **10** with a good 60% yield after two steps (34% overall). Following the same sequence of reactions outlined for compound **10** (desilylation, sulphation, protecting group cleavage), precursor **15'** was successfully converted into the 4'-O-sulphate derivative **10'** in an acceptable 29% overall yield (Figure 2, eq d).

Due to the chemical equivalence of the two phenolic groups in 3' and 5' position of valerolactone **4** (Figure 1), 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 NiCl₂, NaBH₄ in MeOH, giving the mono-protected compound **18** with an acceptable 54% of yield. The intermediate **18** represented a divergent point toward the final products **11** and **12**. In fact, sulphation and sequential reductive cleavage of the benzyl and TCE moieties afforded the final target **11** with 52% yield after three steps (Figure 2). On the other hand, coupling **18** with the trichloroacetimidate **14** gave the protected glucuronide derivative as anomeric 0.35:1- α/β mixture with a very good 85% isolated yield. Total cleavage of all benzyl groups with H₂, Pd/C yielded the targeted metabolite **12** in quantitative yield.

It is noted that, although synthesis of some phenyl- γ -valerolactone scaffolds has been reported previously [26, 27, 33], to the best of our knowledge this is the first time 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

Accepted Article

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 ESI 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-(3',4'-dihydroxyphenyl)- 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 developed 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 over 26-70 min [11, 13, 14, 18, 19, 35, 36], and in line with other UHPLC methods resolving a high number of phenolic metabolites for quantitation over 10-12 min [21-24].

3.3. Method validation

3.3.1. Selectivity

To determine whether 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-(3'-hydroxyphenyl)- 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-matched calibration. Different concentration levels, covering the anticipated 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 fitted linearly, but compounds **5**, **10**, and **10'** demonstrated quadratic calibration curves (Table 2). All the compounds showed R^2 higher than 0.987 (Table 2).

Concentration ranges, LODs, LLOQs, and UPLOQs varied widely 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-(3',4'-dihydroxyphenyl)- 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',4'-dihydroxyphenyl)- 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-(3',4'-dihydroxyphenyl)- 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-(3',4'-dihydroxyphenyl)- 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',4'-dihydroxyphenyl)- γ -valerolactone-4'-*O*-sulphate (**10**) and 5-(3',4'-dihydroxyphenyl)- 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-(4'-hydroxyphenyl)- γ -valerolactone-4'-*O*-sulphate (**6**) and 132,111 nM for 5-(dihydroxyphenyl)- 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'** (Supporting Information, Table 1). The most abundant compounds were **5**, **10/10'**, and **11**, 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, which may well relate to differences in microbiota patterns [3, 18, 26, 35, 39-44]. It is known that some steps in the degradation of flavan-3-ols monomers are mediated by specific bacteria [40]. Qualitative or quantitative differences in the microbiota composition might entail these differences in metabolite production. Nevertheless, the complexity behind gut microbiota-

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related inter-individual differences in the formation of these colonic metabolites needs further clarification.

The urinary concentrations recorded for some phenyl- γ -valerolactones, in particular **5**, **10/10'**, and **11** were quite high (reaching 132 μ M). Comparison of concentration with previous reports is avoided, since most 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, the accurate quantification of phenyl- γ -valerolactones with their respective reference compounds, as presented here, may lead to the redefinition of the recovery and bioavailability of flavan-3-ols in humans.

4. Concluding remarks

This work described for the first time the synthetic procedure for 8 sulphate- and glucuronide-conjugated phenyl- γ -valerolactones. A quick, selective, sensitive, 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, which likely need pre-analytical clean-up steps. However, this point requires further investigations since optimization of solid-phase extraction may be of critical importance. In addition, due to the information presented 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.

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The availability of phenyl- γ -valerolactone conjugates as authentic bioanalytical standards will also allow the use of these key flavan-3-ol metabolites in cell assays, which will shed light on their *in vitro* putative bioactivity. Moreover, the quantification of phenyl- γ -valerolactones in urine samples using authentic synthesized standards opens the door to improved study of bioavailability of flavan-3-ols and the real exposure of humans to flavan-3-ol sources. Overall, the present work provides valuable insights to facilitate future study of the fate of flavan-3-ols and phenyl- γ -valerolactones in the human body, as well as helping 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; metabolite synthesis: NB, CC, FZ; 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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Conflict of interest statement

The authors have declared no conflict of interest.

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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.

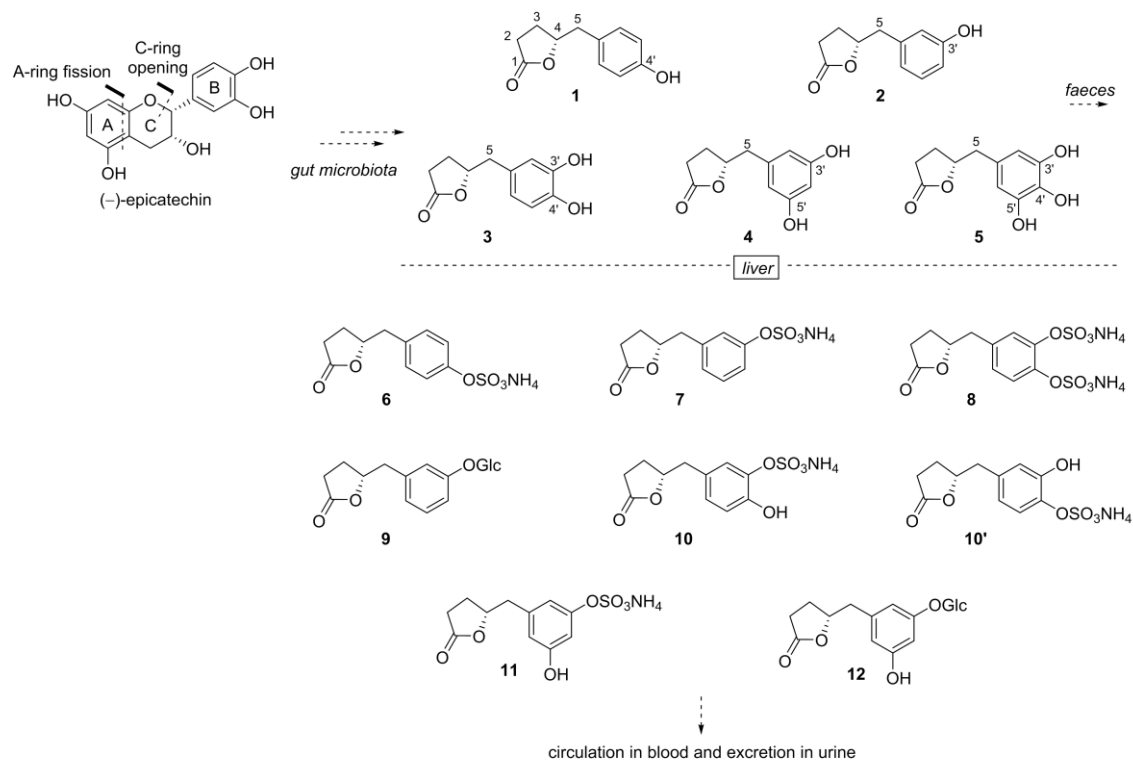


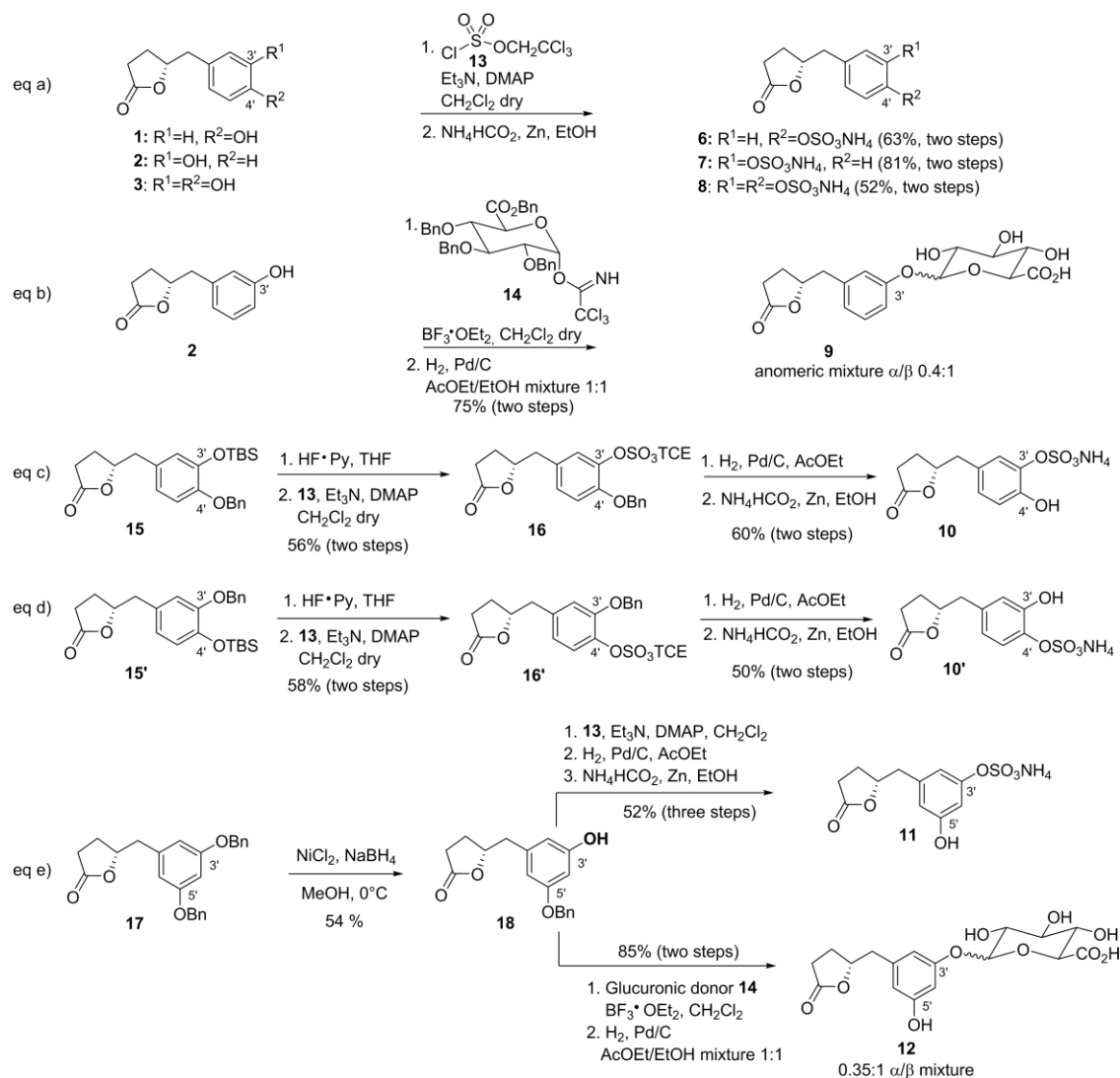
Figure 2. Synthetic routes towards phenyl- γ -valerolactone conjugates.

Table 1. Retention times and optimized SRM conditions for identification and quantification of phenyl- γ -valerolactones

No.	Compound	RT (min)	Parent ion (<i>m/z</i>)	S-lens	Quantifier		Qualifier	
					Product ion (<i>m/z</i>)	CE (V)	Product ion (<i>m/z</i>)	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-(4'-hydroxyphenyl)- γ -valerolactone-4'- <i>O</i> -sulphate	4.53	271	93	191	23	147	35
7	5-(3'-hydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -sulphate	4.71	271	92	191	23	106	48
8	5-(3',4'-dihydroxyphenyl)- γ -valerolactone-3',4'-di- <i>O</i> -sulphate	4.97	367	52	287	12	207	33
9	5-(3'-hydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -glucuronide	4.03	367	93	191	25	147	43
10	5-(3',4'-dihydroxyphenyl)- γ -valerolactone-4'- <i>O</i> -sulphate	4.46	287	96	207	23	163	34
10'	5-(3',4'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -sulphate	4.42	287	96	207	23	163	35
11	5-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -sulphate	3.83	287	96	207	23	163	35
12	5-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -glucuronide	2.11	383	87	207	24	163	40

CE: collision energy

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-(4'-hydroxyphenyl)- γ -valerolactone-4'- <i>O</i> -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-(3'-hydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -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-(3',4'-dihydroxyphenyl)- γ -valerolactone-3',4'-di- <i>O</i> -	$y = 131.85x$	0.993	1110	2220	100000	6.0	2.1	1.3	16.2	13.8	11.1	94.3

sulphate

9	5-(3'-hydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -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',4'-dihydroxyphenyl)- γ -valerolactone-4'- <i>O</i> -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-(3',4'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -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-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -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-(3',5'-dihydroxyphenyl)- γ -valerolactone-3'- <i>O</i> -glucuronide	$y = 11046x$	0.993	12.3	24.7	66667	17.8	14.3	0.1	19.5	9.7	0.3	92.8

Table 3. Urinary concentrations of phenyl- γ -valerolactones (nM) following an unrestricted diet with or without green tea consumption, at baseline and 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 \pm SD	CV	Median	Max.	NP	Mean \pm SD	CV	Median	Max.	NP	Mean \pm SD	CV	Median	Max.	NP	Mean \pm 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 \pm 77	120	54	322	25	66 \pm 87	133	22	288	50	95 \pm 91	96	68	338	20	130 \pm 273	210	44	1074	38
4	5-(3',5'-dihydroxyphenyl)- γ -valerolactone	28 \pm 76	276	0	249	88	173 \pm 288	167	0	908	64	60 \pm 178	296	0	666	87	64 \pm 123	193	0	408	75
5	5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone	7383 \pm 17454	236	0	67510	75	8474 \pm 14969	177	0	54681	57	2795 \pm 5426	194	0	18375	73	7849 \pm 13508	172	0	49686	56
6	5-(4'-hydroxyphenyl)- γ -valerolactone-4'-O-sulphate	nd	-	0	0	100	36 \pm 94	258	0	335	71	39 \pm 133	339	0	515	87	33 \pm 71	213	0	199	69
7	5-(3'-hydroxyphenyl)- γ -valerolactone-3'-O-sulphate	65 \pm 142 ab	219	0	528	63	137 \pm 213 a	156	47	682	36	13 \pm 28 b	210	0	83	73	15 \pm 33 ab	211	0	119	69
9	5-(3'-hydroxyphenyl)- γ -	122 \pm 306	251	0	1230	56	433 \pm 769	178	41	2230	43	111 \pm 306	275	0	1179	73	33 \pm 78	235	0	275	81

valerolactone-3'-O-glucuronide																				
5-(3',4'-dihydroxyphenyl)-10' 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
5-(3',5'-dihydroxyphenyl)-11 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
5-(3',5'-dihydroxyphenyl)-12 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

Values are 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 (%). Means within a row followed by different letters are significantly different at $p < 0.05$.