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Synthesis, Analytical Features, and Biological Relevance of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone, a Microbial Metabolite Derived from the Catabolism of Dietary Flavan-3-ols

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ABSTRACT: The physiological significance of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, an important metabolite derived from the catabolism of flavan-3-ols by gut microbiota, has been often overlooked due to the lack of the commercial standard. In the present work, this metabolite has been chemically synthesized, and its analytical parameters and antioxidant capacity have been determined in comparison to other chemical analogues [isomer 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone and γ-valerolactone] and other structurally related compounds [(+)-catechin, (−)-epicatechin, and 3-(3,4-dihydroxyphenyl)-propionic acid]. The synthesized compound was also used to perform a targeted analysis in samples collected during the in vitro fermentation of a grape seed flavan-3-ol extract with human fecal microbiota from three healthy volunteers. The time−course formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone revealed large interindividual differences among volunteers, with concentrations ranging from 3.31 to 77.54 μM at 10 h of fermentation. These results are further discussed in view of the scarce reports quantifying 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in in vitro fermentation studies, and pharmacokinetic and intervention studies.

KEYWORDS: Flavan-3-ols, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone, γ-valerolactone, gut microbiota

INTRODUCTION

Epidemiological and interventional studies suggest an association between polyphenol consumption and human health.[1] Flavan-3-ols are among the most abundant polyphenols in our diet, important sources being fruits (grapes, apples, and pears), legumes, cacao, and beverages such as wine, cider, tea, and beer.[2] In nature, these compounds occur as monomers, oligomers, and polymers, the latter also known as proanthocyandins or condensed tannins. (Epi)afzelechin, (epi)catechin, and (epi)gallocatechin are the most abundant monomeric structural units in dietary proanthocyanidins.[3] In the last decades, these polyphenols have attracted considerable interest because of their numerous health effects, including antioxidant, anticarcinogenic, cardioprotective, antimicrobial, and neuroprotective activities.[3]

Despite their abundance in nature, proanthocyanidins present poor absorption in the small intestine and reach the colon where they are catabolized by the intestinal microbiota into simple compounds that could be further absorbed and exert health effects.[4,5] Therefore, the bioactivity of these compounds is largely dependent on the microbiota activity. The catabolism of flavan-3-ols starts with the reductive cleavage of the heterocyclic C ring, resulting in the formation of diphenylpropan-2-ols, followed by the breakdown of the A ring and further lactonization into phenylvalerolactones derivatives, specifically into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in the case of (epi)catechin.[6−9] This metabolite is subsequently degraded into 5-phenylvaleric, 3-phenylpropionic, phenylacetic, and benzoic acids bearing a different hydroxylation pattern and side chain lengths. In spite of the fact that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone is an intermediate, it is considered as one of the most abundant and characteristic metabolites exclusively arising from the microbial catabolism of flavan-3-ols.[10] Recent metabolomic studies in humans indicated that this metabolite could be a potential biomarker of the intake flavan-3-ol-rich foods and that its occurrence and residence time in the human organism deserve consideration for possible local or systemic health effects.[11,12] Although the anti-inflammatory and antiproliferative activities of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone have also been reported,[13,14] the biological significance of this metabolite has been often overlooked due to lack of commercial standards to determine its real content in biological fluids.

Different approaches for the chemical synthesis of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone have been reported.[14−19] The aim of this study was to determine analytical features and physiological properties (i.e., in vitro antioxidant activity) of this metabolite that would allow us better understanding of its biological relevance. Initially, the metabolite was obtained by chemical synthesis using the protocol reported by Lambert et al.[14] The analytical features and ORAC (oxygen radical absorbance capacity) of the synthesized compound were determined and compared to those of other chemical analogues and structurally related polyphenol compounds (Figure 1). Also, quantitative determinations have been carried out by

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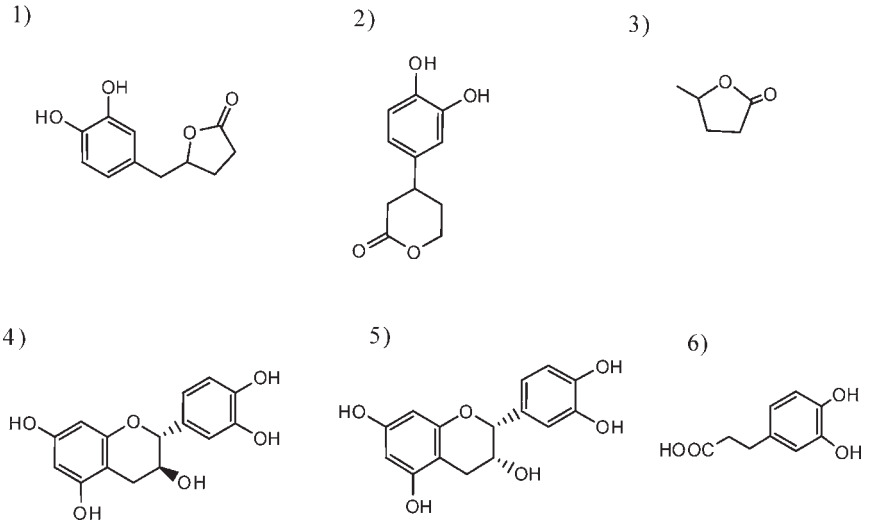

Figure 1. Chemical structures of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**1**), 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone (**2**), γ-valerolactone (**3**), (+)-catechin (**4**), (−)-epicatechin (**5**), and 3-(3,4-dihydroxyphenyl)-propionic acid (**6**).

ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS) in samples collected during the time course of the in vitro fermentation of a grape seed flavan-3-ol extract with human fecal microbiota, and interpretation of results based on the few previously reported data was made.

MATERIALS AND METHODS

Standards. (+)-Catechin and (−)-epicatechin, 3-(3,4-dihydroxyphenyl)-propionic acid γ-valerolactone, 4-pentenoic acid, and 4-chlorocatechol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). In addition, 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone, an isomer of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, was kindly provided by TransMIT GmbH (Giessen, Germany). All remaining products were analytical grade. Vitaflavan extract obtained from grape seeds was kindly provided by Les Dérives Resiniques & Terpéniques (DRT), S.A. (Dax Cedex, France). The extract contained the following: gallic acid (9.11 mg/g), (+)-catechin (74.57 mg/g), (−)-epicatechin (67.68 mg/g), (−)-epicatechin-3-*O*-gallate (26.21 mg/g), procyanidin B1 (60.99 mg/g), procyanidin B2 (45.13 mg/g), procyanidin B3 (20.39 mg/g), procyanidin B4 (15.04 mg/g), B2-3-*O*-gallate (1.80 mg/g), B2-3′-*O*-gallate (1.61 mg/g), procyanidin C1 (7.07 mg/g), and procyanidin T2 (6.81 mg/g).

Synthesis of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone. The synthesis of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was carried out following the protocol of Lambert et al.[14] Reactions were monitored by TLC (thin-layer chromatography, using hexane/EtOAc mixtures) using precoated silica gel aluminum plates containing a fluorescent indicator. Detection of compounds was done by UV (254 nm) followed by charring with sulfuric−acetic acid spray, 1% aqueous potassium permanganate solution, or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups, and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash chromatography was performed using silica gel 60 (230−400 mesh). The ^{1}H NMR spectra of the final synthesized compound were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as an internal standard.

Preparation of Standard Solutions. Individual stock solutions of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone [(1000 μg/mL in acetonitrile/water (1:4, v/v)] and of the different standards [(250 μg/mL in acetonitrile/water (1:4, v/v)] used for comparative purposes were first prepared. Dilutions were made to obtain 50 and 5 μg/mL solutions to optimize the MS/MS parameters and to initially assess the MS response of each compound under optimized conditions. According to their response, individual stock standard pool solutions were prepared ranging from 0.005 to 50 μg/mL, except for γ-valerolactone that was from 0.01 to 100 μg/mL, for the validation of quantitative parameters including limit of detection (LOD), limit of quantification (LOQ), and linearity range.

Fecal Fermentations. Fecal samples were collected from three healthy individuals. Each volunteer was informed about the study and gave a written informed consent before providing the samples. Samples were collected, on site, on the day of the experiment and were used immediately. The samples were diluted 1:10 (w/v) with anaerobic phosphate buffer (1 M; pH 7.2) and homogenized in a stomacher for 2 min. Resulting fecal slurries from each individual (i.e., fecal samples were not pooled) were used to inoculate the batch-culture vessels.

A basal nutrient medium containing peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $NaHCO_3$ (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.01 g/L), $CaCl_2 \cdot 6H_2O$ (0.01 g/L), Tween 80 (2 mL/L), hemin (50 mg/L), vitamin K (10 μL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), and resazurin (1 mg/L), pH 7.0, was prepared and autoclaved. The basal nutrient medium (135 mL) was dispensed into 300 mL vessels, and it was gassed overnight with O_2-free N_2. Before the addition of fecal slurry samples, the temperature of the basal nutrient medium was set to 37 °C, and the pH was maintained at 6.8. Vessels were inoculated with 15 mL of fecal slurry (1:10, w/v), and then, vitaflavan grape seed extract (600 mg/L) was added. Batch cultures were run under anaerobic conditions for a period of 48 h during which samples were collected at six time points (0, 5, 10, 24, 30, and 48 h) in sterile Eppendorf tubes (1.5 mL). Two different control experiments were conducted as follows: (a) incubations of the fecal microbiota in medium, but lacking the grape seed extract, to monitor catabolites arising from basal metabolism; and (b) incubations of the grape seed extract in medium but without fecal microbiota, to monitor changes due to the non-microbial chemical transformation of precursor compounds of the substrate. Samples were stored at −70 °C until required for the analysis of phenolic compounds by UPLC-diode array detector (DAD)-electrospray ionization (ESI)-TQ MS. Before injection, samples were defrosted, centrifuged (14926*g*, 20 °C, 10 min), and filtered through a 0.22 μm filter and finally diluted (1:1, v/v) with a mixture of water/acetonitrile (6:4, v/v).

UPLC-DAD-ESI-TQ MS. An UPLC system coupled to a Acquity PDA eλ photodiode array detector (PAD) and a Acquity TQD tandem quadrupole mass spectrometer equipped with Z-spray electrospray interfece (UPLC-DAD-ESI-TQ MS) (Waters, Milford, MA) was used. Separation was performed on a Waters BEH C18 column (2.1 mm × 100 mm; 1.7 μm) at 40 °C, as described by Sánchez-Patán et al.[20] A gradient composed of solvent A, water:acetic acid (98:2, v/v), and B, acetonitrile:acetic acid (98:2, v/v), was applied at flow rate of 0.5 mL/min as follows: 0−1.5 min, 0.1% B; 1.5−11.17 min, 0.1−16.3% B; 11.17−11.5 min, 16.3−18.4% B; 11.5−14 min, 18.4% B; 14−14.1 min, 18.4−99.9% B; 14.1−15.5 min, 99.9% B; 15.5−15.6 min, 99.9−0.1% B; and 15.6−18 min, 0.1% B. The PAD was operated in the 250−420 nm wavelength range at a 20 point/s rate and 1.2 nm resolution. The ESI parameters were as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; and cone gas (N_2) flow rate, 60 L/h. The ESI was operated in positive and negative modes. The MS/MS parameters (cone voltage and collision energy) of each analyte were initially optimized by direct infusion experiments using 10 μg/mL solutions at a flow rate of 5 μL/min. The most sensitive transition (precursor and product ions) was selected for quantification purposes using the multiple reaction monitoring (MRM) mode. Data acquisition and processing were carried out by the MassLynx 4.1 software.

Radical Scavenging Activity. The radical scavenging activity of the extracts was determined by the ORAC method using fluorescein as a fluorescence probe.[21] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 μL) contained fluorescein (70 nM), 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (12 mM), and antioxidant [Trolox (1−8 μM) or sample (at different concentrations)]. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 98 min. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.11−0) for fluorescence measurement. Black 96-well untreated microplates (Nunc, Denmark) were used. 2,2′-Azobis (2-methyl-propionamidine)-dihydrochloride and Trolox solutions were prepared daily, and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4).

All reaction mixtures were prepared in duplicate, and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\text{AUC} = 1 + \sum_{i=1}^{i=98} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$$

The regression equation between net AUC and antioxidant concentration was calculated. The ORAC value was calculated by dividing the slope of the latter equation by the slope of the Trolox line obtained for the same assay. Final ORAC values were expressed as mmol of Trolox equivalents/g of compound.

RESULTS AND DISCUSSION

On the basis of the precedents for the synthesis of the target molecule, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, we selected the method reported by Lambert et al.,[14] which we were able to reproduce as described using the same synthetic protocols. Compound **7** showed identical ^{1}H NMR data as reported by Lambert et al.[14] The MS/MS parameters, analytical features, and ORAC value of the synthesized compound have been determined and compared to other chemical analogues and other structurally related compounds. Optimized parameters were used for the quantification of the metabolite in samples collected during an in vitro fecal fermentation of a grape seed extract containing diverse flavan-3-ol structures (nongalloylated and galloylated flavan-3-ol monomers and procyanidins). To interpret the changes in 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone during the time course of the in vitro fermentations, we have summarized all previous analytical protocols and data of this metabolite reported using the corresponding standard, either isolated and purified from biological samples or chemically synthesized.

Optimization of MS/MS Parameters and Validation of Analytical Parameters of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone and Comparative Compounds. Table 1 summarizes the chromatography data, MS/MS parameters, and quantification parameters determined for the synthetic 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in comparison to (epi)catechin and some anologues such as γ-valerolactone and the isomer 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone. In addition, 3-(3,4-dihydroxyphenyl)-propionic acid, which is a product from the subsequent catabolism of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone,[4,5] was also considered for comparative purposes. It is important to note that the synthetic 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone is a racemic mixture, which would not be separated under our chromatographic conditions. As a result of microbial catabolism,

Table 1. Retention Time, MS/MS Parameters (Transition, Cone, and Collision), and Quantification Parameters (LOD, LOQ, Linear Concentration Range, and Calibration Curves) for Analysis of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone and Comparative Compounds

name	MW	R_t (min)	transition (m/z)[a]	cone (V)	collision (V)	LOD (μg/mL)	LOQ (μg/mL)	linear concentration range (μg/mL)	calibration curve; R^2
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	208	5.52	207 > 163	40	18	0.003	0.008	0.01−10	10430x + 9.98; 0.998
3-(3′,4′-dihydroxyphenyl)-δ-valerolactone	208	5.45	207 > 121	40	18	0.001	0.004	0.05−5	5458x + 28.5; 0.988
γ-valerolactone	102	2.31	101 > 55	25	7	0.035	0.08	0.1−20	1475x + 10.5; 0.993
(+)-catechin	290	3.84	289 > 245	40	16	0.005	0.016	0.1−10	2519x + 5.17; 0.997
(−)-epicatechin	290	5.27	289 > 245	38	14	0.010	0.025	0.05−10	2737x + 3.13; 0.992
3-(3,4-dihydroxyphenyl)-propionic acid	182	4.09	181 > 137	32	12	0.008	0.027	0.050−10	5716x + 10.9; 0.994

[a] Transitions were acquired in negative ion ESI with the exception of γ-valerolactone that was acquired in positive mode ESI.

the (*R*)-enantiomer is expected to arise from (−)-epicatechin and the (*S*)-enantiomer from (+)-catechin.

With the exception of γ-valerolactone, negative ion ESI showed better sensitivity than positive ion ESI for all compounds. The MS/MS parameters of the different analytes were optimized for each compound by performing direct infusion experiments, and the most sensitive transitions (parent and daughter ions, cone voltage, and collision energy) to be used in MRM mode for quantitative purpose were selected. Cone voltages ranged from 25 to 40 V, and collision energies ranged from 7 to 18 V among the different analytes. γ-Valerolactone required the lowest voltage values of all compounds (Table 1). It is of note that the fragmentation pattern of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (*m*/*z* 207/163) differed from that of its close isomer 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone (*m*/*z* 207/121), which may help to discriminate between both compounds even in the absence of standards (Figure 2).

The instrumental LODs and LOQs following the criterion of signal-to-noise ratio ≥3 (S/N ≥ 3) and S/N ≥ 8, respectively, were determined by the injection of the different standard

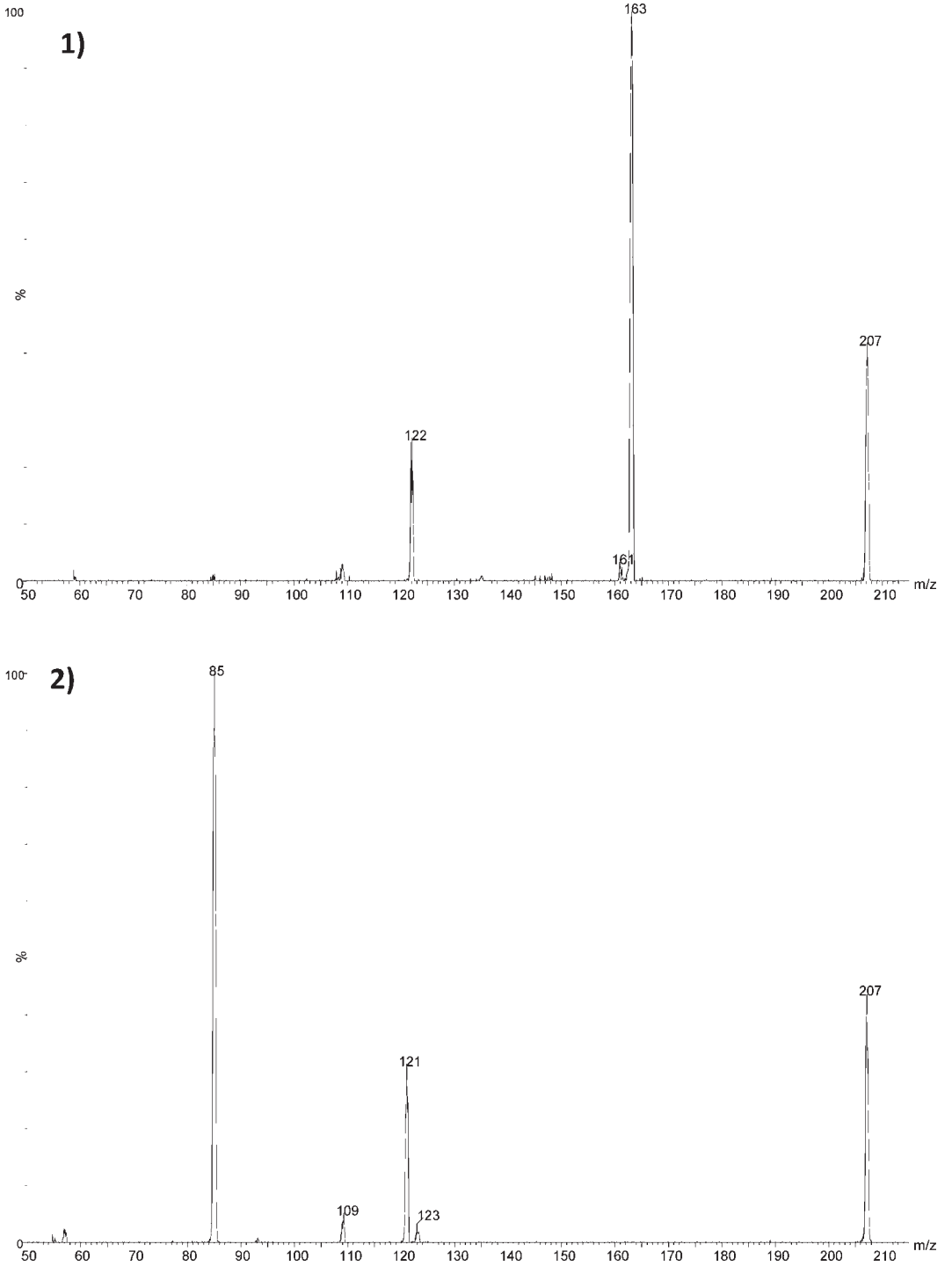

Figure 2. Mass spectrum of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**1**) and 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone (**2**).

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dilutions. γ-Valerolactone presented the highest LOD (0.035 μg/mL) of all compounds (Table 1). On the other hand, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and its isomer presented very similar LOD (0.003 and 0.001 μg/mL, respectively) and the lowest one among all analytes, followed by (+)-catechin (0.005 μg/mL) and finally by (−)-epicatechin and 3-(3,4-dihydroxyphenyl)-propionic acid (0.01 and 0.008 μg/mL, respectively). Our LOD results for (+)-catechin and (−)-epicatechin seem to be in line with those of Lee et al.,[22] who reported LODs in the range of 5−10 ng/mL (0.005−0.01 μg/mL) for (−)-epigallocatechin-3-*O*-gallate, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate, and (−)-epicatechin using a LC system coupled to an electrochemical detector. As expected from LODs, LOQs ranged from 0.004 [3-(3′,4′-dihydroxyphenyl-δ-valerolactone] to 0.080 μg/mL (γ-valerolactone).

Calibration curves were prepared by the method of external standard (Table 1). The linear concentration range slightly varied among the different analytes, with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone presenting the widest linear range. High correlation values (r^2 > 0.992) were obtained for all curves. It is also of note that the curve of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone presented the highest slope (i.e., response factor = Δ area/Δ concentration), the order of response among compounds being as follows: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone > 3-(3′,4′-dihydroxyphenyl)-δ-valerolactone and 3-(3,4-dihydroxyphenyl)-propionic > (+)-catechin and (−)-epicatechin > γ-valerolactone. In other words, the use of the chemical analogues and structurally related compounds for the quantification of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone will overestimate its concentration value in approximately 47, 45, 77, 76, and 86%, respectively, for the isomer, 3-(3,4-dihydroxyphenyl)-propionic acid, (+)-catechin, (−)-epicatechin, and γ-valerolactone.

Antioxidant Capacity of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone and Comparative Compounds. Among all compounds, (+)-catechin and (−)-epicatechin presented the highest ORAC values, as expected from their flavonoid structure[21] (Table 2). On the other hand, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone showed very similar ORAC values to its isomer and to 3-(3,4-dihydroxyphenyl)-propionic acid, but the activity was lost in γ-valerolactone, indicating the important contribution of the catechol moiety to the antioxidant activity of the molecule (Table 2).

There is only one previous report of the antioxidant activity of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.[13] Contrary to our results, using the nitroblue tetrazolium (NBT) reduction assay which is based on the scavenging of radicals generated by the hypoxanthine−xanthine oxidase system, the metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was found to be slightly more effective than (+)-catechin, ascorbic acid and Trolox. Similar results were obtained using the ferric-reducing antioxidant potential (FRAP) test.[13]

Table 2. Antioxidant Activity (ORAC Values) of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone and Comparative Compounds[a]

name	ORAC (mmol Trolox/g)
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	28.8 ± 2.6
3-(3′,4′-dihydroxyphenyl)-δ-valerolactone	28.0 ± 1.9
γ-valerolactone	<0.001
(+)-catechin	46.8 ± 3.0
(−)-epicatechin	44.0 ± 2.5
3-(3,4-dihydroxyphenyl)-propionic acid	29.8 ± 1.3

[a] Values represent means (n = 3) ± SDs.

Quantification of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone Samples during the in Vitro Fecal Fermentation of a Grape Seed Flavan-3-ol Extract. The catabolic pathway of flavan-3-ols by gut microbiota, which is still under elucidation, is very complex involving numerous reactions (hydrolysis, hydrogenation, α- and β-oxidation, dehydroxylation, demethoxylation, and decarboxylation) that result in the formation of a wide range of phenolic and aromatic catabolites, many of which may not have been identified yet. The first steps of the microbial degradation of flavan-3-ols involve the reductive cleavage of the heterocyclic C ring, resulting in the formation of diphenylpropan-2-ols, followed by the breakdown of the A ring and further lactonization to give rise to phenylvalerolactones derivatives [6,8,9] (Figure 3). In particular, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone arises from the microbial catabolism of (epi)catechin, whereas the trihydroxylated derivative, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, originates from (epi)gallocatechins, which by further microbial dehydroxylation also gives rise to the 3′,4′- or 3′,5′-dihydroxylated forms. Recently, another possible pathway has been proposed, suggesting that phenylvalerolactone derivatives may exclusively originate from the catabolism of the top unit of dimeric procyanidins[10] (Figure 3). In fact, it seems that distinct pathways may coexist depending on the intestinal microbiota composition of the different individuals.[8,9] An interconversion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone with the open form 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid has also been described.[8,9] Further steps of the catabolic pathway of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone involve dehydroxylation reactions to mono- and nonhydroxylated forms, as well as β-oxidation to 3-(3,4-dihydroxyphenyl)-propionic and 3,4-dihydroxybenzoic acids and α-oxidation to 3,4-dihydroxyphenylacetic acid[23] (Figure 3).

As an application of the use of the synthesized product, the metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and other structure-related compounds were targeted by UPLC-MS/MS in samples collected during the in vitro fermentation of a grape seed flavan-3-ol extract with feces collected from three healthy volunteers (V1, V2, and V3) (Figure 4). The metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone started to be registered between 0 and 6 h of fermentation increasing up to 10 h and declining afterward. However, large interindividual differences in concentration values at 10 h of fermentation [3.31, 28.2, and 77.5 μM for V1, V2, and V3, respectively (equivalent to 0.497, 4.24, and 11.6 μmol, respectively, or 0.103, 0.882, and 2.42 mg, respectively, or 0.688, 5.87, and 16.1 μg/mL, respectively)] were observed among volunteers. The formation of the monohydroxylated derivative [5-(3′-hydroxyphenyl)-γ-valerolactone], which was quantified using the synthesized dihydroxylated form, was also detected increasing up to 30−48 h in the case of V1 and V3. However, for V2, the formation of this metabolite occurred earlier (up to 24 h) and markedly decreased afterward, indicating possible differences in the metabolic activity (i.e., dehydroxalases) of the microbiota among these volunteers (Figure 4). Despite the differences in evolution pattern, it is of note that the peak values achieved by each volunteer for this metabolite were in keeping with that of its precursor compound, as could be observed from the values of the ratio of 5-(3′-hydroxyphenyl)-γ-valerolactone/5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (at 10 h of fermentation): 0.08, 0.04, and 0.02 for V1, V2, and V3, respectively.

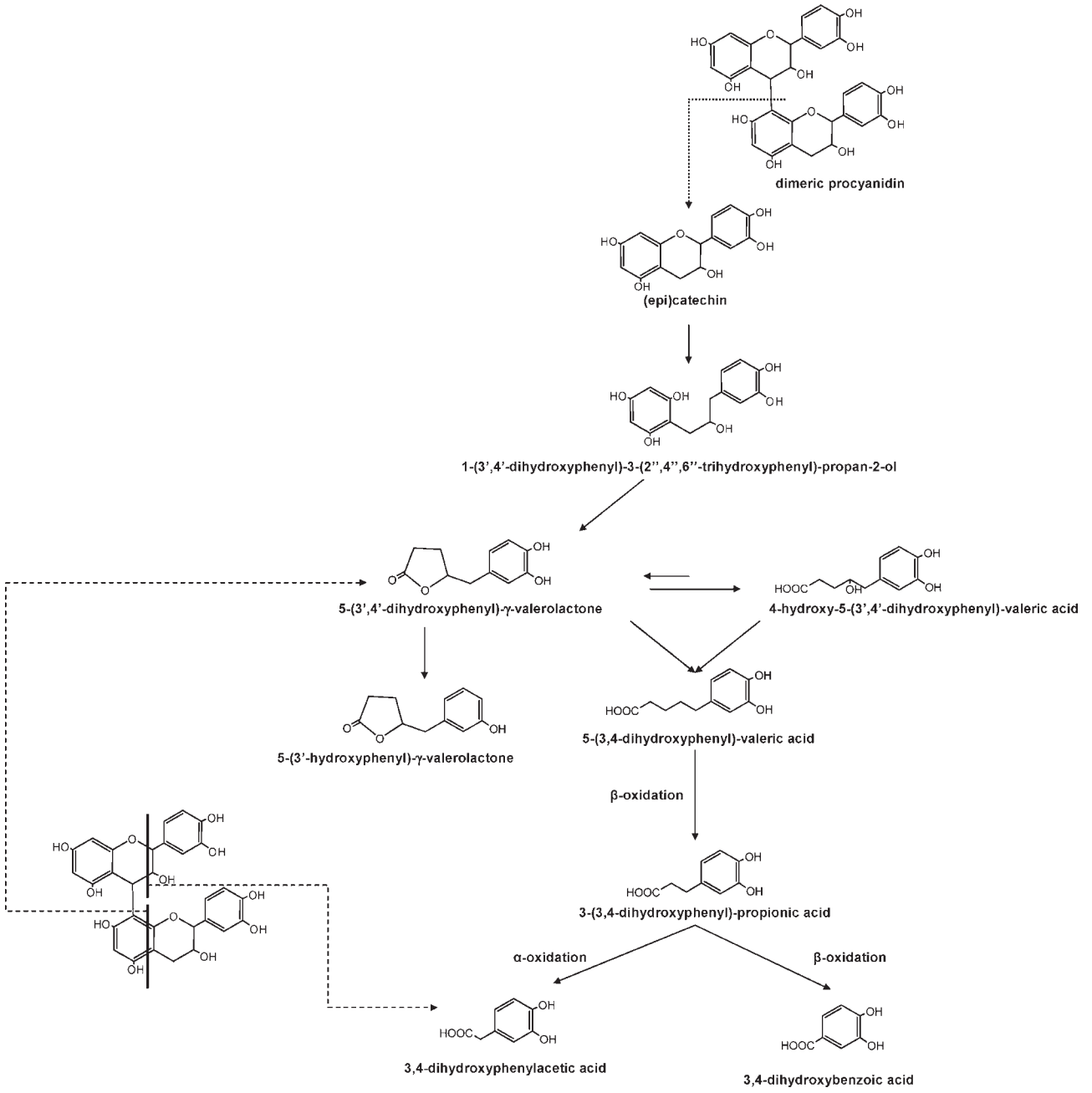

Figure 3. Simplified microbial catabolic pathway of flavan-3-ols and formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.

The nonhydroxylated derivative, 5-phenyl-γ-valerolactone, was also targeted, but it was not detected. However, the simple γ-valerolactone was detected, indicating that the breakdown of the phenyl moiety may also occur as a consequence of microbial catabolism (Figure 4). The profile of this metabolite for each volunteer was in line with that of the monohydroxylated form, indicating a concurrent formation from 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.

Data from in Vitro Fermentation Studies. Fermentations carried out with fecal microbiota from healthy donors ($n = 3$) and (−)-epicatechin (50 μmoL) yielded a maximun concentration of 5-(3′,4′dihydroxyphenyl)-γ-valerolactone ranging from 1.0 ± 0.6 to 19.1 ± 1.1 μmoL, the T_{max} varying between 6 and 30 h of fermentation, according to the fecal donor.[24] These data seem to be in line with our results, since the concentration range was 0.497−11.6 μmol at 10 h of fermentation. Recently, in a comparison between the in vitro biotransformation of (−)-epicatechin and procyanidin B2 (both at 0.45 mM) by human fecal microbiota, a maximum concentration of 27.1 ± 2.3% (relative abundance) of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was reported at 12 h for procyanidin B2, although it was detected much later (24 h) in the case of (−)-epicatechin, reaching a maximum content of 20.8 ± 2.3%.[9] These results indicated that the degree of polymerization of the precursor flavan-3-ol may affect both the T_{max} and the C_{max} of the microbial metabolite.

Data from Pharmacokinetic Studies. The study of Düweler and Rohdewald[17] constitutes one of the first bioavailability studies related to the intake of flavan-3-ol-rich source that reported the

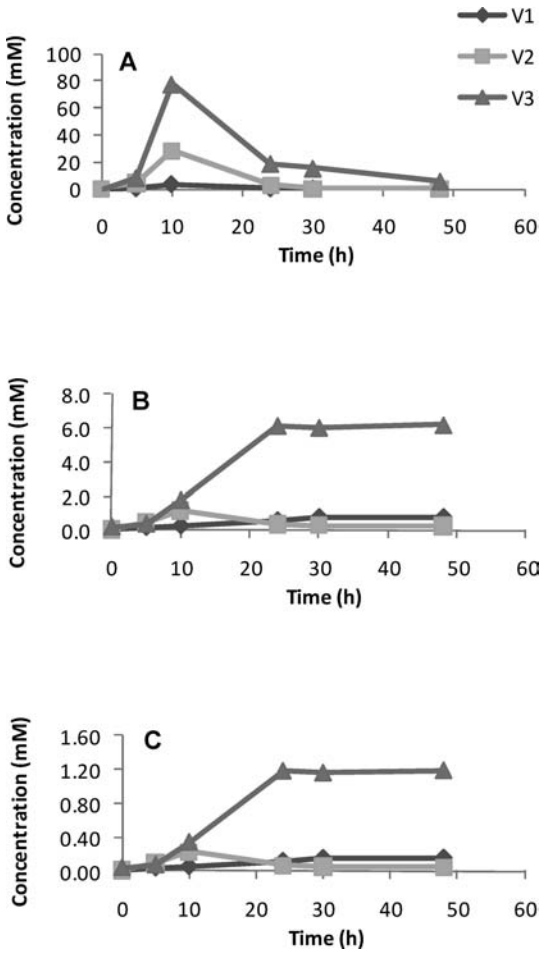

Figure 4. Formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (A), 5-(3′-hydroxyphenyl)-γ-valerolactone (B), and γ-valerolactone (C) during the time course of the fermentation of a grape seed polyphenol extract by human fecal microbiota from three healthy volunteers.

synthesis of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. The metabolite was detected in hydrolyzed urine (i.e., treatment with β-glucuronidase to eliminate the conjugated moeties) after the intake of different doses (5.28 and 1.06 g) of French maritime pine bark extract or 960 mg of an isolated procyanidin fraction by a healthy volunteer. Urinary peak levels of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, which were registered at 8−12 h after the intake, were 29.2, 9.6, and 17.6 mg, respectively, for the two different doses of pine bark and procyanidin fraction. These data indicated a dose-dependent relationship between the precursor compound and the microbial metabolite, and the flavan-3-ol source may have a profound effect on the C_{max} of metabolite.

Li et al.[25] isolated the metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone from human urine after the intake of green tea [1.2 g of green tea solids equivalents to 268.85 and 110.34 μmol of (−)-epigallocatechin-3-*O*-gallate and (−)-epicatechin, respectively, n = 5] in hydrolyzed urine. Cumulative amounts (0−47 h collection period) of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in urine ranged from 39.19 to 2.86 μmol. In another set of samples, the urinary levels ranged from 3.70 to 6.82 μg/mL (n = 2) at 3−8 h after the intake of green tea (20 mg/kg of body weight).[25] In a later study, Meng et al.[26] following the same sample preparation protocol and standard as above,[25] reported a maximum urinary excretion of 4.7 μM at 3−6 h after the intake of 200 mg of pure (−)-epigallocatechin-3-*O*-gallate by humans. However, after the ingestion of green tea (150 mg of green tea solids) by humans (n = 4), a maximum concentration of 31.2 ± 26.4 μM was attained at 8−24 h (total excretion = 0.6−10 mg). The peak plasma levels of 0.36 ± 0.13 μM were attained at 12 h after the intake. In the case of rats fed 0.6% of green tea, urinary levels ranged from 1.2 to 6.6 μM. These findings suggested once again the influence of the flavan-3-ol source on the formation of the metabolite but also the effect of the metabolic potential of the microbiota of rat versus human toward the catabolism of flavan-3-ols.

Similarly, Khori et al.[7] recovered 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone from rat urine collected after the intake of (−)-epigallocatechin-3-*O*-gallate and followed by further treatment with β-glucuronidase. The metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was found in plasma (214 pmol/mL) 6 h after the intake of (−)-epigallocatechin-3-*O*-gallate, reaching a maximum concentration at 24 h (562 ± 307 pmol/mL). Urinary excretion was detected in the 6−24 h fraction (58 ± 39 pmol/mL), reaching maximum content at 24−48 h (711 ± 168 pmol/mL). However, in contrast to the previous studies, samples were not hydrolyzed previous to analysis; therefore, metabolites were detected in the form of conjugates but quantified using the standard in the aglycone form.

In another study,[19] 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was obtained by chemical synthesis using the protocol first reported by Watanabe[18] and later by Düweler and Rohdewald.[17] A dose-dependent excretion of the metabolite was observed in the 24 h after a single dose of 20, 40, and 80 μmol of (−)-epicatechin to rats. Free (measured in not hydrolyzed sample) and total (measured in hydrolyzed sample) were determined using the synthesized standard. The urinary level of the free metabolite ranged between 0.7 and 0.2 μmol/rat for the dose of 20−80 μmol of (−)-epicatechin, whereas the total (free + conjugated) ranged between 0.7 and 1.7 μmol/rat, indicating than more than 82% of the 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone accounted for the conjugated form.

Finally, using the same standards synthesized by Düweler and Rohdewald,[17] the peak plasma concentration of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was found at 10 h (mean concentration 3.59 ng/mL) after the intake of a pine martime extract picnogenol (1800 mg) by humans (n = 11).[27]

Data from Human Intervention Studies. To date, only two human intervention studies have measured 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone as a biomarker of exposure and correlated it with biomarkers of biological effects. In a prospective study in the Shangai Chinese population, the association between urinary excretion of tea polyphenols metabolites, including 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and gastric/esophageal cancer was determined.[28] The urinary levels of 5-(3′,4′dihydroxyphenyl)-γ-valerolactone, expressed as geometric means, for control and gastric/esophageal cancer cases were 0.76 and 0.79 mg/g of creatinine, but no significant differences were found between both cases.

In another recent analysis within the Shangai Cohort Study, which was designed to study the effect of *COMT* (catechol-*O*-methyltransferase) genotype on the urinary excretion of tea polyphenols of daily green tea drinkers, levels of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone were significantly lower in persons presenting the heterozygous low activity (*LL*) gentoypes (geometric mean = 21.7 μmol/g creatine) than for the those presenting high activity *HH* and *HL* genotypes (33.7 and 36.8 μmol/g creatine, respectively), suggesting that the former

may retain more tea polyphenols and obtained greater health benefits from green tea intake.[29]

In summary, the present work provides relevant information on the analytical parameters and in vitro antioxidant activity of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in comparison to other chemical analogues and structurally related compounds that have been previously used for its estimation. Our in vitro fermentation experiments indicate that the metabolic potential of the microbiota toward the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone may vary considerably among volunteers, and this may have a profound influence on derived health effects. Despite the use of the correct standard, the method for the quantification of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in biological samples largely varies among different authors, making comparisons very difficult to established between different studies. Variables contributing to this variation include type of individuals (rat or humans), flavan-3-ols source (food, extract, or purified compound), structure and degree of polymerization, previous sample pretreatment protocol with β-glucuronidase/sulfatase, quantification of the aglycone or conjugated metabolite, and unit expression of results (μmol, μM, μmol/g creatinine, mg/g of creatinine, etc). Although some attempts have been made to measure 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone as biomarkers of exposure in intervention trials, to provide real physiological relevant data on 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, it is necessary to perform a larger number of in vitro fermentation studies to evaluate the metabolic potential of the microbiota among individuals to produce this metabolite and then correlate this potential with urinary levels and biomarkers of bioefficacy after in vivo consumption of flavan-3-ol rich sources.

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