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Procyanidin Dimers Are Metabolized by Human Microbiota with 2-(3,4-Dihydroxyphenyl)acetic Acid and 5-(3,4-Dihydroxyphenyl)-γ-valerolactone as the Major Metabolites

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Procyanidins (PCs) are highly abundant phenolic compounds in the human diet and might be responsible for the health effects of chocolate and wine. Due to low absorption of intact PCs, microbial metabolism might play an important role. So far, only a few studies, with crude extracts rich in PCs but also containing a multitude of other phenolic compounds, have been performed to reveal human microbial PC metabolites. Therefore, the origin of the metabolites remains questionable. This study included in vitro fermentation of purified PC dimers with human microbiota. The main metabolites identified were 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Other metabolites detected were 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, phenylvaleric acids, monohydroxylated phenylvalerolactone, and 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol. Metabolites that could be quantified accounted for at least 12 mol % of the dimers, assuming 1 mol of dimers is converted into 2 mol of metabolite. A degradation pathway, partly different from that of monomeric flavan-3-ols, is proposed.

KEYWORDS: Degradation pathway; human microbial metabolites; hydroxyphenylacetic acids; proanthocyanidins; valerolactones

INTRODUCTION

Intestinal microbiota have been shown to be important for the bioavailability (*1*, *2*) and bioactivity (*3*) of certain phenolic compounds that are present in many daily consumed food products, such as fruits, vegetables, and nuts (*4*). Proanthocyanidins (PAs) are some of the most abundant phenolic compounds in the human diet (*5*). They potentially are responsible for the cardioprotective properties of cocoa products and wine (*6*, *7*). PAs are thought to be poorly absorbable. Hence, microbiota of the colon play an important role in their digestion. However, knowledge on the fate of PAs in the body is still incomplete. The most common subclass of PAs is the procyanidins (PCs), which exclusively consist of (epi)catechin units and their galloyl derivatives. The monomeric units of PCs are linked through a C4−C8 or C4−C6 bond (B-type), which can coexist with an additional C2−O−C7 bond (A-type). Only PC dimers are believed to be absorbed intact (*8*−*10*), albeit at much lower efficiency than the monomeric units (*11*). Thus, high amounts of PCs will reach the colon intact as shown by Kahle and coworkers (*12*).

The monomeric units of PCs, (epi)catechin, are known to be degraded into several phenolic acids, namely, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, and hippuric acids (**Figure 1**), all mainly hydroxylated at the meta position (*13*−*16*). Furthermore, 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone have been identified as metabolites of both epicatechin and catechin in various species including man (*13*, *17*−*22*).

Ingestion of PC-rich products resulted in increased urinary excretion of most of the phenolic acids mentioned above (*14*−*16*, *23*, *24*). Therefore, phenolic acids seem to be major metabolites of PCs. However, often PC-rich mixtures were used, which does not exclude the possibility that the metabolites found actually originate from other phenolic compounds that are also present in those mixtures. The microbial metabolic pathways of PCs are still not completely characterized. Knowledge of the formation and identity of these metabolites is important as they could contribute to the potential health effects of PCs in, for example, cocoa and wine.

A limited number of in vivo and in vitro studies, mostly in rats, have been performed to characterize microbial metabolites

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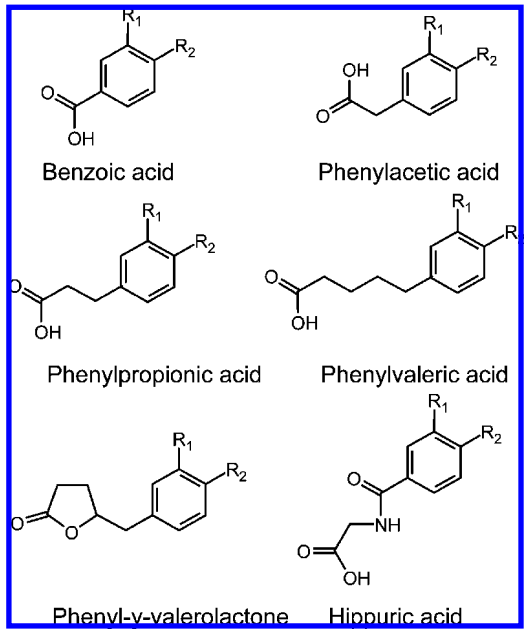


Figure 1. Basic structures of microbial metabolites from various species formed of (epi)catechin, which are usually hydroxylated at the positions indicated by R_1 and/or R_2.

of PCs (*14*, *15*, *23*−*26*). Studies performed with animals can result in substantially different bioconversions compared to humans, as has been shown for catechin and epicatechin (*19*, *27*). In vitro fermentations are easier to perform than in vivo studies. They have the additional advantage that relatively high concentrations of metabolites, not yet affected by absorption and metabolism, are present, which facilitates detection. So far, only one in vitro fermentation of PC oligomers with an average degree of polymerization (DP) of 7 has been performed with microbiota of one human subject (*28*). The use of large PC oligomers might inactivate the microbiota as they have pronounced antimicrobial properties (*29*, *30*), likely due to their high interaction with proteins (*31*, *32*). Therefore, in the present study, a well-defined and pure PC dimer fraction was subjected to an in vitro fermentation with human feces as a source of microbiota. Feces of four volunteers were pooled, as commonly done by others (*27*, *33*), to adjust for interindividual variations. The main objective of this study was to identify human microbial metabolites originating from PCs. On the basis of the metabolites that were detected a possible degradation route is proposed.

MATERIALS AND METHODS

Materials. All organic solvents used for HPLC analysis were of HPLC grade. Vitaflavan (DRT, Levita Chemical International NV, Antwerpen, Belgium), a commercially available grape seed extract, was used as PC dimer source. PC standards B1 (epicatechin-(4→8)-catechin), B2 (epicatechin-(4→8)-epicatechin), B3 (catechin-(4→8)-catechin), and B4 (catechin-(4→8)-epicatechin) were purchased from Apin Chemicals (Abingdon, U.K.). Standards of (−)-epicatechin and (+)-catechin and the phenolic acids 3-(3-hydroxyphenyl)propionic acid (3-HPP), 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diHPP), 2-(3-hydroxyphenyl)acetic acid (3-HPA), 2-(4-hydroxyphenyl)acetic acid (4-HPA), 2-(3,4-dihydroxyphenyl)acetic acid (3,4-diHPA), 3-(phenyl)propionic acid (PPA), and benzoic acid (BA) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Isolation and Characterization of Grape Seed Procyanidins. *Removal of Gallic Acid Substituents.* To enable preparative HPLC isolation of PC dimers, gallic acid substituents were enzymatically removed. Vitaflavan was dissolved (2 g/L) in a sodium acetate buffer (0.1 M, pH 5.0), after which tannase (Gamma Chemie, Darmstadt, Germany) was added (80 mg/L). The mixture was incubated at 30 °C for 24 h after which freshly prepared tannase was added. After another 24 h of incubation, free gallic acid was removed by SPE. The C18 cartridge (Sep-Pak Vac 200 cm^3, 5 g, Waters, Milford, MA) was conditioned following the supplier's instructions. The sample was loaded and washed with 2 column volumes (CV) of H_2O. Subsequently, PCs were eluted with methanol (2 CV). The methanol was evaporated under vacuum. The sample was dissolved in H_2O, frozen, and lyophilized. The proportion (mol %) of gallic acid that was removed was determined with thiolysis as described in the materials and methods of the Supporting Information.

Preparative Isolation of Procyanidin Dimers. Fractionation by DP was performed on a normal phase (NP) column, Inertsil PREP-SIL, 30 mm i.d. × 250 mm, with a 10 μm particle size (GL Sciences, Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector, and a UV fraction manager was used. The binary mobile phase consisted of (A) hexane and (B) acetone. The elution was as follows: 0−30 min, 40−60% B; 30−50 min, isocratic at 60% B; 50−70 min, 60−75% B, followed by a washing step at 98% B for 3 min, and reconditioning of the column. The flow rate was 27 mL/min, and PDA spectra from 210 to 300 nm were recorded. The tannase-treated PC fraction was dissolved in 10 mL (∼70 mg/mL) of acetone/hexane/ethanol (7:3:2) and injected. The eluate containing the dimers was collected (28.2−38.1 min), evaporated under vacuum, and lyophilized prior to further analysis.

Recovery of Phenolic Acids and PC Dimers from the Fermentation Buffer. Seven phenolic acids were used to determine their extraction efficiency from a fecal suspension: 3-HPP, 3,4-diHPP, 3-HPA, 4-HPA, 3,4-diHPA, PPA, and BA. A 25% (w/v) fecal suspension was made as described previously (*34*) with some alterations. Freshly passed feces were immediately suspended in the buffer and homogenized with a stomacher (Seward, Worthing, U.K.) operating for 60 s and sieved (1 mm). Part of it was autoclaved to inactivate the microbiota. The active and inactive suspensions (200 μL) were each combined with carbonate−phosphate buffer (pH 5.5) (*35*) containing the phenolic acids (800 μL) in a Kimax tube ($n = 3$ for both active and inactive suspension), resulting in a 5% (w/v) suspension and 200−400 μg/mL phenolic acids. Samples containing the active suspension were immediately acidified to a pH below 2 by adding 60 μL of HCl (12.1 M) and 5 mL of ethyl acetate to inactivate the microbiota and prevent conversions. The time between the collection of the feces and acidification did not exceed 10 min. Samples were vortexed vigorously and centrifuged at 14000 rpm (Eppendorf 5417C, rotor F45-30-11, Hamburg, Germany) for 10 min at room temperature. The ethyl acetate layer was pipetted into another Kimax tube. The remaining H_2O phase was re-extracted twice with 5 mL of ethyl acetate as described above. The ethyl acetate phases were individually evaporated with a TurboVap (Zymark, Russelsheim, Germany) operated at 50 °C under a stream of nitrogen. The samples were subsequently dissolved in 1 mL of methanol and diluted 40 times before analysis with HPLC-UV-ECD as described later. Extraction recoveries are shown in Supporting Information Table 1. The third extraction removed only ≤10% of each phenolic acid.

To test the stability of the dimers during extraction they were dissolved in carbonate−phosphate buffer (pH 5.5) (100 μg/mL) without microbiota and extracted twice as described above. The total ethyl acetate phase (10 mL) was evaporated, and the samples were dissolved in 500 μL of H_2O/ACN/MeOH (86:12:2), acidified with 8 μL of H_3PO_4 (14.8 M), and filtered over a 0.22 μm Acrodisc membrane (Pall Corp.). Samples were analyzed with HPLC-UV-ECD. Extraction recovery of dimers was 15−30%. Furthermore, degradation into catechin and epicatechin took place, which together accounted for ∼0.6 mol % of the originally added dimers. This was observed on the basis of injection of standards and MS analysis (described later). No other degradation products were detected. Catechin and epicatechin were stable in this system (*36*).

Fermentation of Procyanidin Dimers by Human Microbiota. *In Vitro Fermentation Process.* The dimeric fraction was subjected to fermentation with a pool of human microbiota obtained from four volunteers who usually ingested a normal diet, presented no digestive disease, and had not received antibiotics for at least 3 months as previously described (*34*). Freshly passed feces were immediately taken

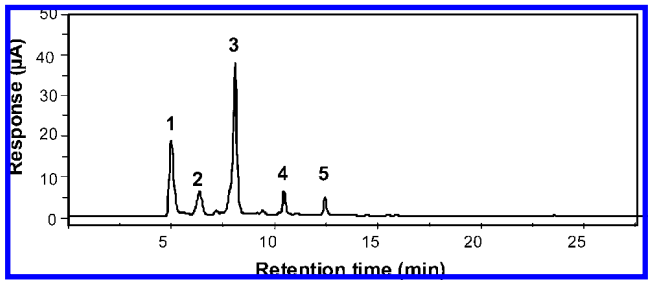


Figure 2. RP-HPLC profile of the dimer fraction purified by NP-HPLC. Dimers 1 (B1), 2 (B4), and 3 (B2 + B3) were identified with standards. Peaks 4 and 5 were also identified as PC dimers (*m*/*z* 577) but could not be identified further.

in an anaerobic chamber, pooled, and homogenized in carbonate−phosphate buffer (pH 5.5). The mixture of dimers (5 μmol in total) was fermented by 1% (w/v) and 5% (w/v) fecal suspensions (10 mL) at 37 °C. Samples were taken after 0, 1, 2, and 4 h for the 1% (w/v) suspension and after 0, 1, 2, 4, 6, 8, and 24 h for the 5% (w/v) suspension. The stability of the dimers to the applied conditions was studied by incubating them in the buffer without microbiota or with heat-inactivated microbiota. Furthermore, fermentations with active fecal suspensions without added dimers were conducted as negative controls for each time point. All incubations were performed in triplicate at all time points mentioned above. At each time point, the content of the fermentation bottle was divided over four Eppendorfs (2 mL each), frozen rapidly in liquid nitrogen, and stored at −20 °C.

Short-Chain Fatty Acid (SCFA) Analysis. Suppression of SCFA production in the presence of PC dimers could indicate their possible inhibitory effects on microbial enzymes involved in SCFA formation. Analysis of SCFA was performed with capillary gas chromatography as described previously (*37*). The sum of the amounts of acetic acid, propionic acid, 2-methylpropionic acid, and butyric acid was taken and expressed as total SCFA production.

Sample Preparation before HPLC Analysis. One Eppendorf for each time point was put in an ice bath, slowly thawed, transferred into a Kimax tube, acidified, and extracted with ethyl acetate twice as described for the analysis of the extraction recovery. The total ethyl acetate phase (10 mL) was evaporated under nitrogen to a small volume, transferred into HPLC vials, and evaporated to dryness. Samples were stored at −20 °C.

Analysis of Microbial Metabolites (a) HPLC-UV-ECD Analysis. A Hitachi system (Tokyo, Japan) composed of L-2100 pumps and an L-2200 autoinjector extended with a Spark Mistral column oven set at 30 °C, a UV, and a CoulArray detector (ECD) (ESA, Chelmsford, MA) was used. Analysis was performed on an Inertsil ODS-3, 4.6 mm i.d. × 150 mm, 5 μm, column (GL Sciences). The mobile phase was composed of (A) 10% ACN in sodium phosphate buffer (100 mM, pH 3.35) containing 10 mg/L SDS and (B) 60% ACN, 10% MeOH in sodium phosphate buffer (30 mM, pH 3.45) containing 15 mg/L SDS. The flow rate was 1 mL/min. Detection was performed at 270 nm and 50, 450, 600, and 700 mV. The elution was as follows: 0−15 min, 20−57.5% B; 15−20 min, 57.5−100% B; 20−30 min, isocratic at 100% B; 30−32 min, 100−20% B, and reconditioning of the column. Samples were dissolved in 500 μL of H_2O/ACN/MeOH (86:12:2), acidified with 8 μL of H_3PO_4 (14.8 M), and filtered over a 0.22 μm Acrodisc membrane (Pall). For each sample 10 μL was injected.

Phenolic acid concentrations were calculated on the basis of calibration curves (1−20 μg/mL) and corrected with the average amounts found in the active controls (microbiota without dimers) (20−80 nmol, except 4-HPA gave backgrounds of 0.03−0.14 μmol) and extraction recoveries (Supporting Information Table 1). Dimer contents were calculated on the basis of calibration curves (3−1000 μg/mL) and peak areas of dimer peaks 1, 3, and 4 (**Figure 2**) at 600 mV and dimer peak 2 at 450 mV. Dimer peak 5 could not be monitored because of coelution with other peaks. The amount of dimers detected at $t = 0$ h was expressed as 100%.

(b) MS Analysis. To screen the samples for the presence of phenolic acids and additional metabolites, such as valerolactones, HPLC coupled to an LCT Premier quadruple TOF spectrometer equipped with an ESI source (Waters/Micromass) was used. An Acquity Ultra Performance HPLC system (Waters) was used with the same gradient as used with ECD analysis. The eluents were adjusted for MS analysis. Sodium phosphate and SDS were omitted, and the pH was adjusted with formic acid. After passing the HPLC, the flow rate was reduced to 0.2 mL/min for MS analysis. The LCT Premier was operated in the negative mode at a resolution of 10500 (FHWM), with a source temperature of 120 °C, a desolvation temperature of 350 °C, a capillary voltage of 2250 V, and a sample cone voltage of 50 V. Measurements were performed in the W mode. Data were collected in the full-scan mode from *m*/*z* 100 to 750.

Table 1. Formation of the Phenolic Acids over Time Was Confirmed by MS/MS Analysis of Specific Product Ions Originating from Their Parent Ions[a]

phenolic compound	parent ion $[M - H]^-$	product ions $[M - H]^-$	coll (V)	cone (V)
2-(3,4-dihydroxyphenyl)acetic acid	166.9	122.9	10	20
		94.7	20	20
2-(3-hydroxyphenyl)acetic acid	150.9	106.9	10	20
		64.7	25	20
2-(4-hydroxyphenyl)acetic acid	150.9	106.9	10	20
		64.7	25	20
3-(3-hydroxyphenyl)propionic acid	164.9	121	10	25
		105.9	20	25
		76.7	10	20
procyanidin dimer	577.1	289	25	35
		124.9	30	35

[a] The collision energy used is indicated by coll and the cone voltage applied with cone.

A Waters Micromass Quattro Micro 3 system, equipped with an ESI source and controlled by Masslynx software, was coupled to an HPLC system (Agilent Technologies, 1200 series, Santa Clara, CA) with the same conditions as used with the LCT Premier quadruple TOF spectrometer. Measurements were performed in the negative mode with a capillary voltage of 2.0 kV, a capillary temperature of 300 °C, and a source temperature of 120 °C. The cone voltage and collision energy were optimized for the phenolic acids and dimers by infusion experiments of standards (**Table 1**). Selected samples were analyzed for the presence of phenolic acids and dimers in the MRM mode. Furthermore, daughter scans were made for the following selected masses, representing intermediate structures, valeric acids, and valerolactones: 581, 579, 475, 416, 414, 305, 291, 289, 275, 221, 209, 207, 193, 191, 175, and 125 at a cone voltage of 20 V and a collision energy value of 20%.

Statistical Analysis. Student's *t* test was used to test if the amount of dimers significantly reduced in time, if SCFA production was altered due to the presence of PC dimers, and if there were significant differences in phenolic acid recovery after extraction. A probability of <0.05 was considered to be significant. Error bars are depicted as mean ± SD.

RESULTS

Composition of the Procyanidin Dimer Substrate. Enzymatic removal of gallic acid substituents improved the resolution upon preparative NP-HPLC, especially between the dimers and trimers (data not shown). The proportion of gallic acid decreased from 14 to 1% (w/w). After preparative purification, the dimer fraction was free of monomeric flavan-3-ols and trimers as their masses, *m*/*z* 289 $[M - H]^-$ and *m*/*z* 865 $[M - H]^-$, respectively, were not detected by NP-HPLC-UV-MS. Three peaks of five could be identified on the basis of RP-HPLC with standards; that is, peak 1 was characterized as B1 and peak 2 as B4, and peak 3 consisted of both B2 and B3, on the basis of separation on a different RP column, that is, AtlantisC18 (unpublished results). PC dimer peaks 4 and 5 (*m*/*z* 577 $[M - H]^-$) could not be further identified (**Figure 2**). Only B-type dimers (*m*/*z* 577 $[M - H]^-$) were detected to be present in the mixture.

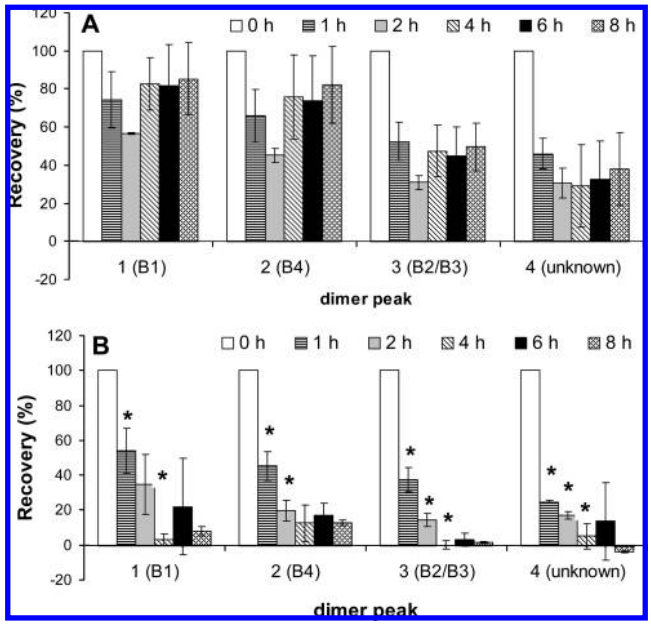


Figure 3. Recovery of dimers 1 (B1), 2 (B4), 3 (B2/B3), and 4 (unknown) (see **Figure 2**) from the buffer control (**A**) and the 5% active suspension (corrected for the concentrations detected in the control samples) (**B**) over 8 h of incubation. The amount detected at $t = 0$ h was expressed as 100%. The bars represent the fermentation time as indicated in the legend. Error bars represent the SD of triplicates, and a significant decline in comparison to the previous time point was calculated by Student's t test ($P < 0.05$) based on the concentrations and indicated by an asterisk.

Quantitative Analysis of Microbial Metabolites Originating from Procyanidin Dimers. PC dimers were not completely stable over 8 h of incubation in the absence of microbiota (buffer control) (**Figure 3A**). Peaks 1 and 2 were rather stable, but peaks 3 and 4 showed a decline of 40−60% compared to $t = 0$ h (not significant, based on concentrations). After 1 h, no further decline was detected. Although extraction recovery of dimers was low, a significant decrease in the dimer content in the presence of microbiota was detected for the first 4 h (**Figure 3B**). Whereas the dimers disappeared, 3,4-diHPA, 3-HPA, 3-HPP, and 4-HPA appeared (**Figure 4A**), which were identified on the basis of retention time and relative ECD response (at 450 and 600 mV) of standards. Except for 3,4-diHPA and an unknown peak (VI), which were better detected at 450 mV (**Figure 4B**), the phenolic acids were optimally detected at 600 mV. The identity of the four phenolic acids was confirmed with HPLC-MS/MS analysis (**Table 1**). The additional unknown product (VI) (**Figure 4B**) appeared after 1 h of fermentation with a maximum response around 4 h. The concentrations of the phenolic acids identified all increased during 4−6 h of fermentation, after which they decreased rapidly (**Figure 5**). After 6 h, the four identified phenolic acids accounted for ~1.2 μmol in a ratio of 1:6:12:21 (4-HPA/3-HPA/3-HPP/3,4-diHPA) originating from 5 μmol of PC dimers. BA proved to be absent, and the concentration of PPA was similar to that found in the active suspension without dimers. Additionally, both catechin and epicatechin were detected within the first 2 h of fermentation. However, their total amount was similar as detected at all time points of the buffer control, which was 1.5−2 mol % of the dimers added. This excludes the possibility that catechin and epicatechin were formed by microbial degradation of dimers during incubation. As described earlier, catechin and epicatechin were formed during the extraction in similar amounts as found

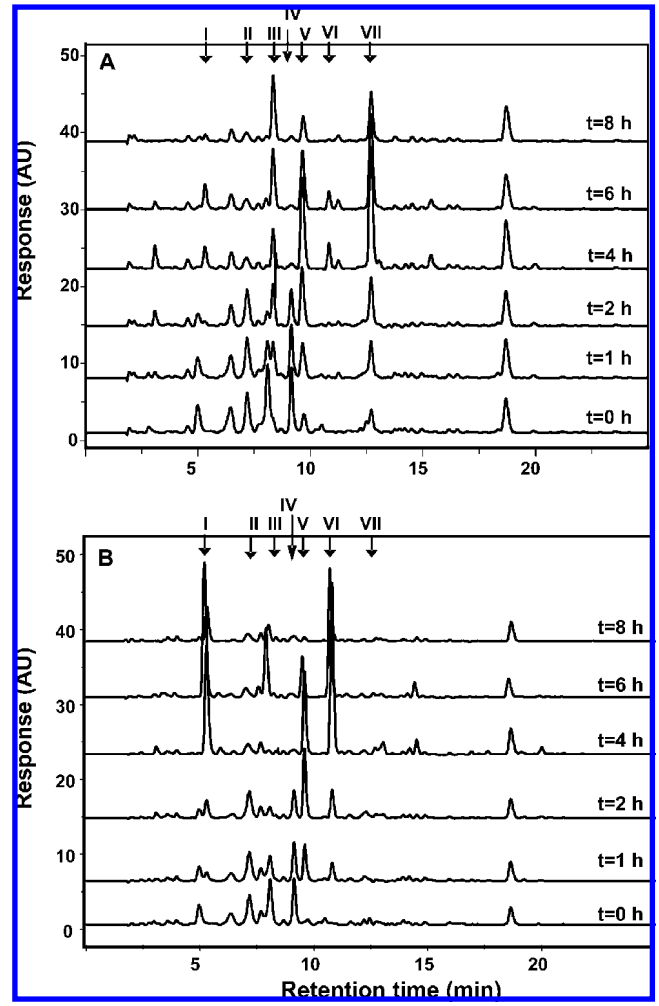


Figure 4. HPLC-ECD detection at 600 mV (**A**) and 450 mV (**B**) of the dimer mixture fermented with a 5% fecal suspension over 8 h. Several phenolic acids and flavonoids could be identified with standards, and one peak (VI) remained to be identified. Compounds are indicated by peaks I = 3,4-diHPA, II = catechin, III = 4-HPA, IV = epicatechin, V = 3-HPA, VI = ?, and VII = 3-HPP.

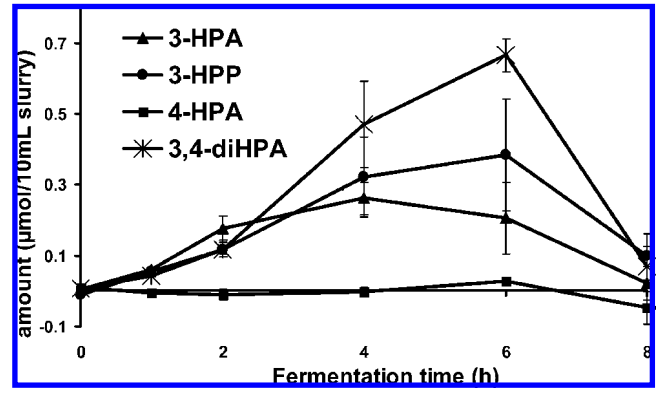


Figure 5. Formation of four phenolic acids over 8 h of fermentation of the dimer mixture with a 5% (w/v) human fecal suspension, corrected for the concentrations detected in the control samples. Error bars represent the SD of triplicates.

in the samples (total of ~0.06 mol %). No phenolic metabolites were detected in fermentations performed with a 1% (w/v) suspension.

SCFA analysis was performed to monitor the activity of the microbiota in the presence of dimers. Initially (1−2 h), the

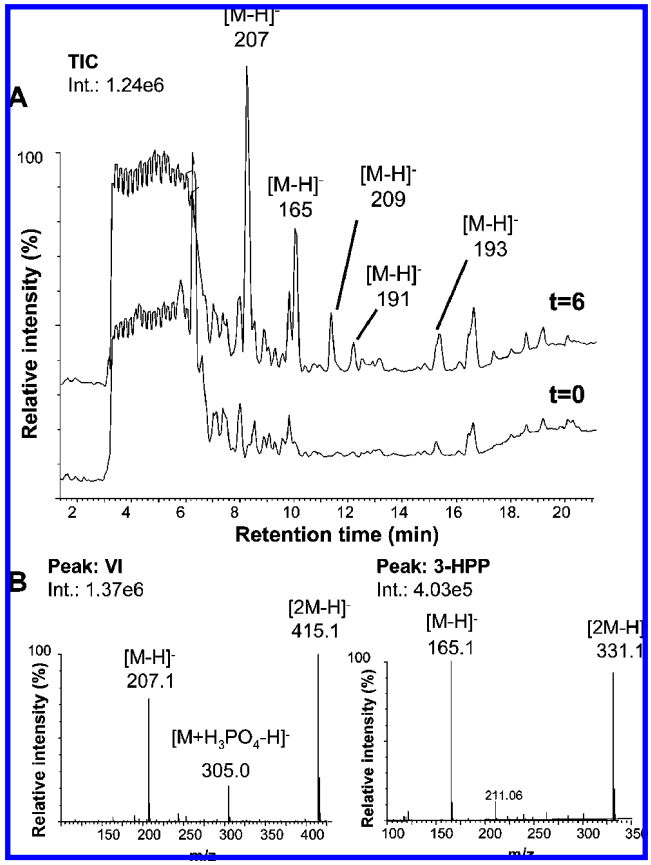


Figure 6. HPLC-qTOF analysis after 0 and 6 h of fermentation of the dimer mixture. The TICs of $t = 0$ and $t = 6$ h are compared (**A**), and spectra of metabolite VI (RT = 8.3 min) and 3-HPP (RT = 10 min) are given (**B**).

presence of dimers inhibited SCFA formation significantly in the 5% (w/v) suspension (Supporting Information Figure 1). However, no significant difference in maximum amounts of total SCFA produced was found in the presence or absence of dimers. In the 1% (w/v) suspension no significant differences in SCFA production were found, and levels were 6−7 times lower (data not shown) than those in the 5% (w/w) suspension.

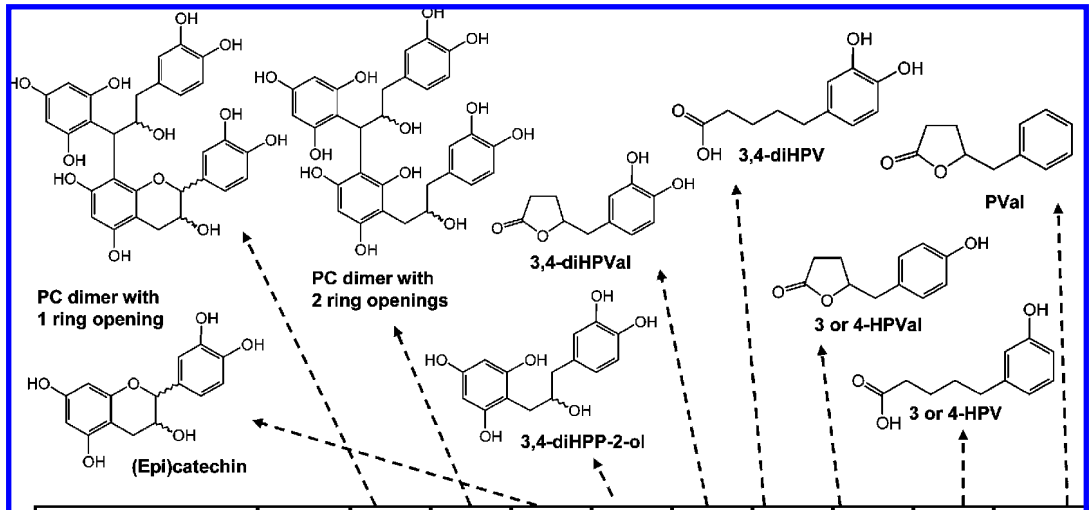
Identification of Microbial Metabolite VI as 5-(3,4-Dihydroxyphenyl)-γ-valerolactone. Additional analysis with HPLC-qTOF-MS was performed to identify the unknown peak VI. Two major peaks appeared in the total ion current (TIC) trace after 6 h at RT = 8.3 and 10.0 min along with some additional ones at RT = 11.4, 12.2 and 15.3, which were absent or small at $t = 0$ h (**Figure 6A**). In addition, these peaks were not detected in the active suspension without dimers (data not shown). A shift in retention times was observed with HPLC-qTOF-MS (**Figure 6A**) compared to the HPLC-UV-ECD profiles (**Figure 4**). On the basis of the retention time and mass analyzed for the standard, the peak at 10.0 min was identified as 3-HPP. On the basis of the relative elution order of the phenolic acids, (epi)catechin, and dimers, the peak at $t = 8.3$ min most probably was metabolite VI. The average mass spectrum recorded for metabolite VI consisted of three *m/z* values, namely, 207, 305, and 415 (**Figure 6B**). $[M - H]^-$ 207 pointed to 5-(3,4-dihydroxyphenyl)-γ-valerolactone (3,4-diHPVal), which has a mass of 208 Da and has been detected previously as a metabolite of catechin and epicatechin (*13, 17*−*20*). The elemental composition, calculated by Masslynx software, supported this hypothesis. *m/z* 415 corresponded to 208 plus 207, most likely an adduct of 207 and annotated as $[2M - H]^-$. Similar adducts were observed for most of the phenolic acid standards, including 3-HPP (**Figure 6B**). The adduct *m/z* 305 $[M + H_3PO_4 - H]^-$, showed a product ion of *m/z* 97 when analyzed with MS/MS and arises through the interference of phosphate originating from the samples. The MS/MS spectrum of *m/z* 207 analyzed with triple-quad MS contained *m/z* 163 as the main product ion, which is typical for 3,4-diHPVal (*17, 20*). Therefore, metabolite VI was annotated as 3,4-diHPVal, and its presence was confirmed at all time points except at $t = 0$ h (**Figure 7**). No 3,4-diHPVal was detected in the control samples.

Identification of Precursors of 5-(3,4-Dihydroxyphenyl)-γ-valerolactone and Other Metabolites. *Precursors.* 3,4-diHPVal has been described to be preceded by the formation of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (3,4-diHPP-2-ol) (MW 292) by reductive cleavage of the (epi)catechin C-ring (*19, 20*). HPLC-MS/MS of the samples taken at 0−8 h of the active suspension, incubated with and without dimers, were analyzed for the presence of these metabolites (*m/z* 291) (**Figure 7**). The monomeric units (epi)-catechin (*m/z* 289 $[M - H]^-$) were confirmed to be present between 0 and 2 h of fermentation (also based on RT of standards). As mentioned earlier, this was attributed to the extraction procedure. Furthermore, *m/z* 291 $[M - H]^-$ (3,4-diHPP-2-ol) was present after 1 and 2 h, but absent after 6 h.

Other Metabolites. Analysis with HPLC-qTOF indicated the presence of several metabolites other than 3,4-diHPVal (**Figure 6A**). At RT 11.4, 12.2, and 15.3 *m/z* values of 209, 191, and 193, respectively, were identified. The elemental compositions of these compounds were calculated by Masslynx software to represent mono- and dihydroxylated phenylvaleric acids (*m/z* 209 and 193) and monohydroxylated phenylvalerolactone (*m/z* 191). Results of daughter scans of these and other selected parent ions are given in **Figure 7**. No masses corresponding to a dimer that underwent reductive cleavage in one or both of the C-rings of the monomeric units (*m/z* 579 and 581 $[M - H]^-$) were detected. On the basis of qTOF data 3,4-diHPVal seemed to be dehydroxylated into 5-(3- or 4-hydroxyphenyl)-γ-valerolactone (3- or 4-HPVal, *m/z* 191 $[M - H]^-$) (**Figure 6**), which was confirmed by MS/MS analysis (**Figure 7**). No 5-phenyl-γ-valerolactone (PVal) (*m/z* 175 $[M - H]^-$) was detected. Both monohydroxylated phenylvaleric acid (3- or 4-HPV) and dihydroxylated phenylvaleric acid (3,4-diHPV) were present after 6 h of fermentation. None of the selected parent ions were detected in the active suspension without dimers. Other potential metabolites, phloroglucinol (*m/z* 125), a flavan-3-ol with a phloroglucinol substituent (*m/z* 414), 3,4-diHPP-2-ol with a phloroglucinol substituent (*m/z* 416), a flavan-3-ol with a carboxylated phloroglucinol substituent (*m/z* 475), a monohydroxylated HPP-2-ol (*m/z* 275), and a mono or dimethylated valerolactone (*m/z* 221 and 235), were not present in any sample.

DISCUSSION

The four quantified ethyl acetate soluble metabolites, 3,4-diHPA, 3-HPA, 4-HPA, and 3-HPP, accounted for 1.2 μmol originating from 5 μmol of B-type PC dimers (**Figure 5**). Additional metabolites found were various hydroxylated phenylvaleric acids and phenylvalerolactones and 3,4-diHPP-2-ol. However, these could not be quantified because of the lack of standards. The metabolite 3,4-diHPA has not been detected as a human metabolite in studies performed with pure catechin (*21, 36*). Therefore, a direct release of 3,4-diHPA from PC dimers is suggested, without preceding conversion of PCs into monomers.



	Time (h)	579	581	289	291	207	209	191	193	175
5% active + PC dimers	0	no	-	yes	no	no	-	-	-	-
	1	no	no	yes	yes	yes	-	no	-	-
	2	no	-	yes	yes	yes	-	-	-	no
	4	-	-	no	no	yes	no	no	no	-
	6	no	-	-	no	yes	yes	yes	yes	no
	8	no	-	-	no	yes	no	no	yes	no
5% active control	0	no	-	no	no	no	-	-	-	-
	1	no	-	no	no	no	-	no	-	-
	2	no	-	no	no	no	-	-	-	-
	4	-	-	no	no	no	no	no	-	-
	6	no	-	-	no	no	no	no	no	-
	8	no	-	-	no	no	no	no	no	-

Figure 7. Several metabolites were detected with HPLC-MS/MS in the negative mode. Daughter scans of the parent masses ($[M - H]^-$) of each metabolite were collected. Data of the fermented dimer mixture (only B-types) with a 5% (w/w) microbial suspension for 8 h were compared with the control samples. Samples that were not analyzed are indicated with "-"; "yes" and "no" indicate the presence or absence of the metabolite, respectively.

Quantification of Reaction Products. PC dimers (B-type) were converted into smaller molecules without preference for a specific dimer (**Figure 3B**) as they all decreased within the same time scale. It was estimated that the metabolites found after 6 h accounted for at least 12 mol % conversion, assuming that 1 mol of dimer is converted to 2 mol of metabolites. In vitro fermentation of PA oligomers performed by Deprez and co-workers (*28*) resulted in only 2.7% ethyl acetate soluble products, based on radioactive labeling. This difference might indicate that the dimers are more easily accessible for microbiota than higher oligomers. A high protein affinity of the oligomers might have contributed to low accessibility or antimicrobial effects.

Identified Metabolites Compared to the Literature. The most abundant human metabolite of PC dimers was 3,4-diHPA. In rats the urinary excretion of this metabolite also increased after the intake of PC dimer B3 (*15*). An in vitro fermentation of PA oligomers with human microbiota did not result in this metabolite (*28*). Also, ingestion of PC-rich grape seed tablets did not result in increased urinary excretions of 3,4-diHPA in humans (*24*), in contrast to ingestion of PC-rich chocolate, when 3,4-diHPA was observed in the urine of healthy human subjects (*16*). This variation in metabolism could have resulted from differences in composition (degree of galloylation and/or degree of polymerization) of the oligomers or extracts used. Furthermore, an in vitro fermentation with tea extracts rich in monomeric flavan-3-ols resulted in the formation of 3,4-diHPA. However, quercetin, which is also present in tea (*38*), is also known to be degraded into 3,4-diHPA (*34, 39*), and therefore it remains unclear where 3,4-diHPA, after fermentation of the tea samples, originates from. Studies performed with pure catechin and human microbiota (*21, 36*) did not result in 3,4-diHPA.

The formation of 3-HPP as a main metabolite is consistent with previous studies (*23, 28*). The high amount of 3-HPA in comparison to 4-HPA suggests that 3,4-diHPA is preferably dehydroxylated at the para position. A preference to dehydroxylate the para position of several flavonoids has been described previously for human microbiota (*34, 36, 40*). Therefore, the monohydroxylated phenylvalerolactone that was detected (**Figure 7**) is suggested to be 3-HPVal, formed from 3,4-diHPVal, through dehydroxylation at the para position.

Tentative Degradation Pathway. An important question is whether cleavage of the interflavan bond of dimeric PCs into monomeric flavan-3-ols is the first step in their metabolism. The detection of 3,4-diHPP-2-ol within 2 h suggests that dimers are cleaved into their monomeric units. However, no additional catechin or epicatechin other than that formed during extraction was detected. It might be argued that a slow conversion of dimers into flavan-3-ols followed by a rapid conversion into 3,4-diHPP-2-ol, phenylvalerolactones, and phenolic acids explains that the flavan-3-ol intermediates are not detected. Indeed, Aura and co-workers (*36*) found that monomeric flavan-3-ols are rapidly metabolized; 1 μmol of monomeric flavan-3-ols was completely fermented within 2 h by a 10 mL 5% fecal suspension within the same fermentation system. To our knowledge monomeric flavan-3-ols have never been detected as microbial metabolites of PCs in humans. Their absence (indicated by us and others) and our finding of 3,4-diHPA [not found as a common human metabolite in studies performed with pure monomeric flavan-3-ols (*21, 36*)] as the main metabolite

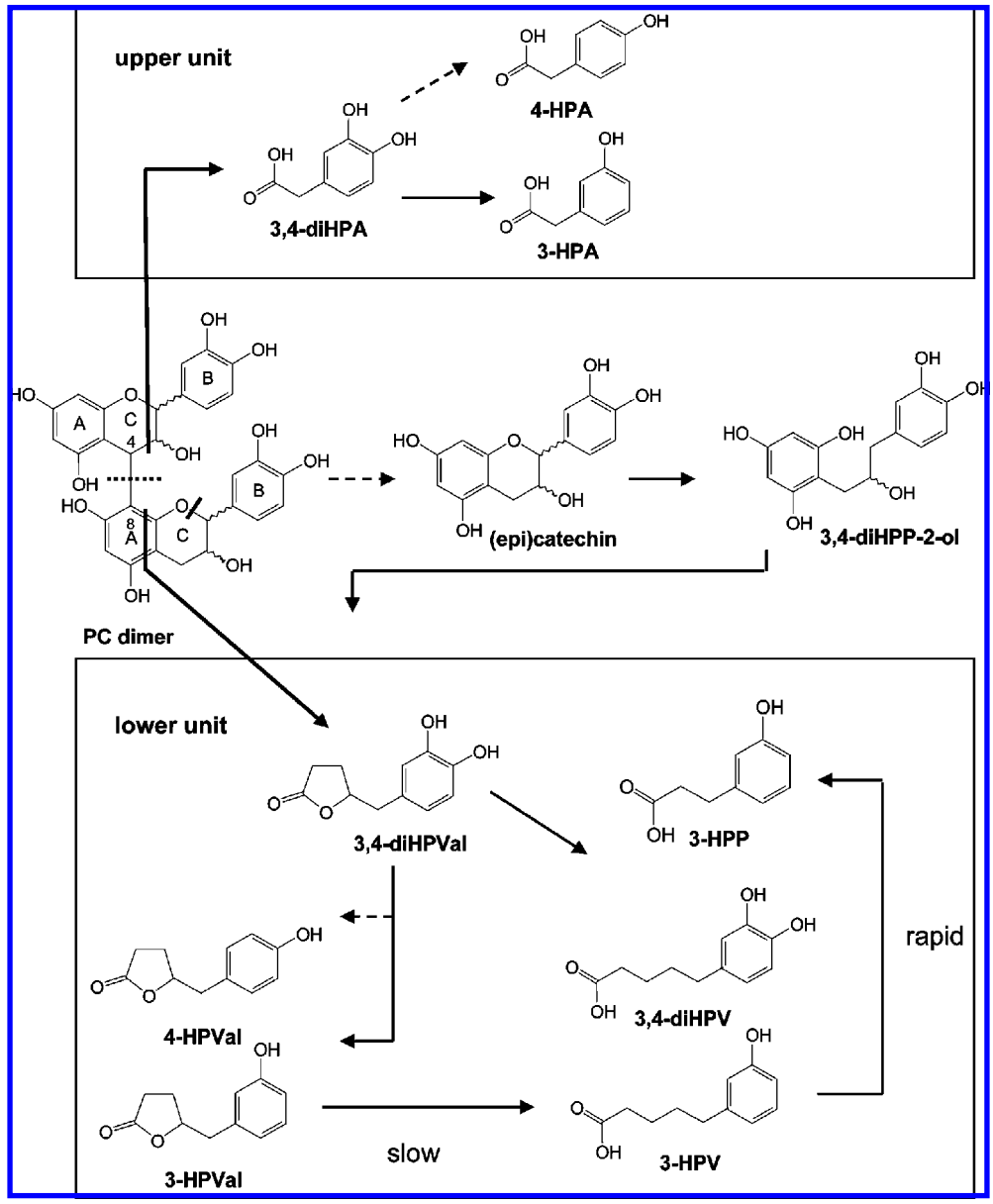


Figure 8. Tentative pathway for human microbial degradation of PC dimers (B-type). The preferred route is indicated with →, and metabolites derived from the upper and lower units are grouped within rectangles.

do not hint at conversion of dimers into monomeric flavan-3-ols as the main degradation route as previously suggested (*26*). An alternative route could be that the interflavan bond is split after reductive cleavage of one (MW 580) or both C-rings (MW 582) of the dimer, resulting in the release of 3,4-diHPP-2-ol. However, this route also seems unlikely as *m/z* 579 and 581 (**Figure 7**) were not detected.

Figure 8 shows a tentative degradation pathway for PC dimers (B-type). The formation of both phenolic acids and phenylvalerolactones suggests that cleavage of the C- and A-ring occurs at the points indicated in **Figure 8**. The C-ring cleavage has previously been shown to be an important mechanism for human microbial degradation of several flavonoids (*41*). Our data (**Figure 7**) imply that phenylvalerolactones are slowly degraded into phenylvaleric acids, which were detected only after 6 h of fermentation, whereas 3,4-diHPVal was already present after 2 h. The amount of phenylvaleric acid produced varies between species (*42*). It is not often detected in high amounts as a metabolite of monomeric flavan-3-ols or PCs, whereas 3-HPP is often detected as a main metabolite (*24*, *28*). Therefore, phenylvaleric acids seem to be rapidly converted into 3-HPP, which was present in high amounts. This fits with the results obtained by Meselhy and co-workers (*19*), who obtained phenylvaleric and phenylpropionic acids after fermentation of phenylvalerolactones with human microbiota. Previously, rat microbiota were shown to cleave (+)-[*ring A*-^{14}C]catechin through the A-ring to form phenylvalerolactone and CO_2. The valerolactone was again further metabolized into 3-HPP (*22*, *43*). Detection of phenylvalerolactones after the fermentation of PC dimers shows that human microbiota are also capable of cleaving the A-ring.

Human microbiota convert PC dimers into a number of smaller metabolites. Our findings suggest a direct degradation of dimers as depicted in **Figure 8** instead of cleavage into flavan-3-ols first. The health potential of these metabolites should be studied as the original PAs are present in plasma in only low amounts.

ABBREVIATIONS USED

PCs, procyanidins; CV, column volume; ECD, coularray detector; BA, benzoic acid; PPA, 3-(phenyl)propionic acid; 3,4-

diHPA, 2-(3,4-dihydroxyphenyl)acetic acid; 3,4-diHPP, 3-(3,4-dihydroxyphenyl)propionic acid; 3-HPP, 3-(3-hydroxyphenyl)-propionic acid; 3-HPA, 2-(3-hydroxyphenyl)acetic acid; 4-HPA, 2-(4-hydroxyphenyl)acetic acid; 3,4-diHPP-2-ol, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol; 3,4-diHPVal, 5-(3,4-dihydroxyphenyl)-γ-valerolactone; 3,4-diHPV, 5-(3,4-dihydroxyphenyl)valeric acid; HPP-2-ol, 1-(3′- or 4′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol; 3- or 4-HPVal, 5-(3- or 4-hydroxyphenyl)-γ-valerolactone; 3- or 4-HPV, 5-(3- or 4-hydroxyphenyl)valeric acid; PVal, 5-phenyl-γ-valerolactone.

Supporting Information Available: Method to determine the percentage of galloyation of PCs and recoveries of selected phenolic acids after extraction and the effect of dimers on the amount of SCFA produced. This material is available free of charge via the Internet at http://pubs.acs.org.

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