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Tetramethylated Dimeric Procyanidins Are Detected in Rat Plasma and Liver Early after Oral Administration of Synthetic Oligomeric Procyanidins

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Procyanidins (PC) are of great interest in nutrition because they account for a major fraction of the total flavonoids ingested in Western diets and have health benefits in humans. However, it remains unknown which species of PC, namely, monomers, oligomers, or aromatic acid derivatives of gut microflora, are responsible for their beneficial effects in vivo. The high molecular complexity of PC extracts and PC-rich foods is a major problem in absorption studies. To circumvent this difficulty, we have synthesized oligomeric PC consisting of (−)-epicatechin units linked by ethyl bridges. The synthetic PC (SPC) only contains dimers, trimers, tetramers, and nanomers. After oral gavage of this SPC (200 mg/kg body weight) to male Wistar rats, tetramethylated dimeric PC (TDPC) were detected in plasma and liver. TDPC were detected in plasma as soon as 1 h after intake, reaching maximum concentrations (14 mg/L) 2 h after gavage. At this time, liver contained as much as 15 μg of TDPC per gram of tissue. In conclusion, orally administered dimeric PC are rapidly absorbed and internally methylated in rats. To our knowledge, this is the first time that methylated dimeric PC have been detected in plasma and liver. We consider that plasma and liver concentrations of TDPC are sufficient to exert a hormone-like effect and, therefore, that PC dimers are good candidates as agents of the biological activities of PC extracts and PC-rich foods.

KEYWORDS: Flavonoids; procyanidin; in vivo; absorption; metabolism; methylated dimers; plasma; liver

INTRODUCTION

Procyanidins (PC) are flavonoids present in many fruits, cereals, and nuts, and the highest concentrations are found in apples, chocolate, and grapes/red wine (*1*). In foods and drinks, procyanidins are present as individual monomers (catechin and epicatechin) and, more commonly, as oligomers (2−10 units of monomers) (*1*). PC are of great interest in nutrition because they account for a major fraction of the total flavonoids ingested in Western diets (*1*) and exert health benefits in humans (*2*). Human intervention studies have shown that PC extracts and PC-rich foods increase plasma antioxidant activity (*3*), decrease platelet aggregation (*4*, *5*), decrease LDL cholesterol concentrations (*6*) and the susceptibility of LDL to oxidation (*7*, *8*), increase HDL cholesterol concentrations (*6*), and decrease blood pressure (*9*). Similar effects of PC have been shown in animal models (*10*−*14*). In a rat model for voluntary and moderate wine consumption (*10*), it was shown that red wine reduces cholesterol−LDL (*11*) and protects tissues against oxidation (*12*). In normolipemic rats, an acute oral dose of grape seed PC improves the atherogenic risk index, dramatically reduces plasma triglyceride and apo B concentrations, and modulates the expression of genes related with lipid and lipoprotein metabolism, such as CYP7a1 and SHP, in the liver (*13*). Also, the oral administration of grape seed PC has an antihyperglycemic effect in streptozotocin-induced diabetic rats that is partly due to an insulin mimetic activity in muscular and adipose cells (*14*).

To explain these metabolic effects of PC and to understand the mechanism by which PC act at the cellular level in vivo, it is essential to determine how PC are absorbed by the gastrointestinal tract and reach their target tissues. Absorption, bioavailability, and metabolism of monomers have been extensively studied in both animals (*15*−*21*) and humans (*21*−*23*), but little is known about the bioavailability of oligomeric PC, and the results are controversial. Some studies have demonstrated the presence of dimeric PC in the serum of humans (*24*, *25*) and rats (*26*, *27*) after PC oral intake. Also, results in vitro, using Caco-2 cells, suggest that PC dimers and trimers could be absorbed in vivo (*28*). Therefore, dimeric PC could reach and directly act on target tissues. On the other hand, other studies did not detect oligomeric PC in plasma and urine following PC ingestion, whereas monomers (*29*, *30*) and aromatic acids derived from PC metabolism by gut microflora (*31*−*34*) were detected in urine. In this case, the nutritional effects of PC would

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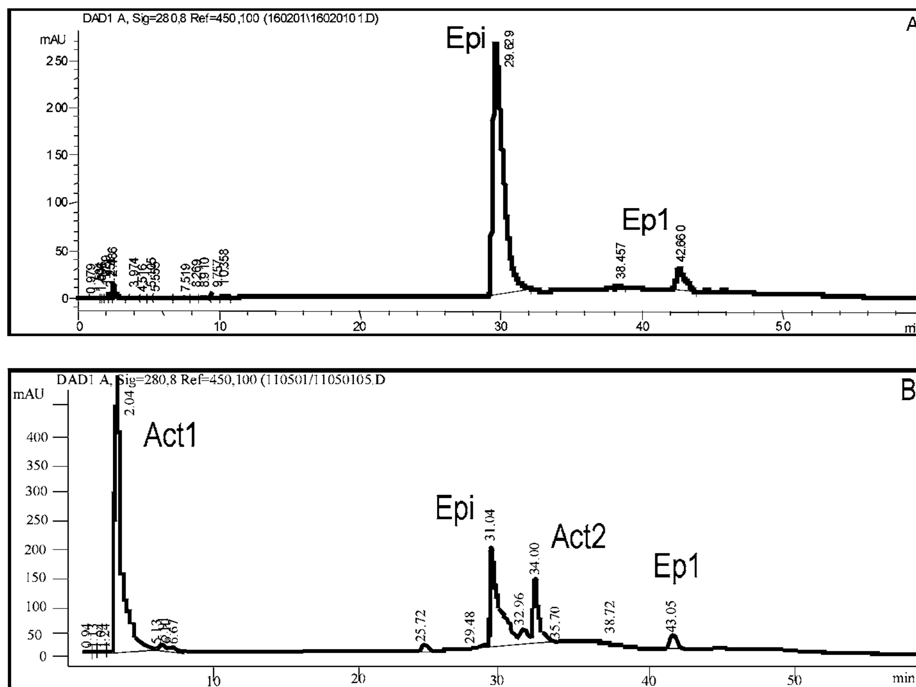

Figure 1. Representative HPLC chromatograms of samples of reaction media containing a mixture of (–)-epicatechin and acetaldehyde in molar deficiency (**A**) or molar excess (**B**). The reaction mixture consisted of 3% acetic acid, 10% ethanol, 0.04 M (–)-epicatechin, and either 0.024 M (A) or 2 M (B) acetaldehyde. Samples were taken at the end of the reactions and HPLC chromatograms were recorded at 280 nm. Chromatographic picks labeled Epi and Ep1 correspond to monomeric (–)-epicatechin and ethyl-bridged dimeric epicatechin, respectively. Act1 and Act2 correspond to residual acetaldehyde and an acetaldehyde dimer, respectively.

be a consequence of the absorbed monomers and aromatic acid and/or of the interaction of unabsorbed PC oligomers with components of the gastrointestinal tract system.

Indirect evidence suggests that at least some of the actions of PC are independent of their catabolization by colonic microflora. Changes in the pattern of liver gene expression in rats are observable as soon as 5 h after the oral administration of grape seed PC (*13*), and even 30 min after gavage there is a hypoglycaemic effect of PC in streptozotocin-diabetic rats (*14*). The speed of these responses suggests that they are triggered, a least in part, by the parent molecules of PC extracts and not by their catabolites. Moreover, some effects of PC extracts in vivo are reproduced in vitro, whereas monomers have no activity or even display an opposite effect (*35*−*37*). Taken together, these results suggest that oligomeric PC must exercise some effects directly in vivo.

The molecular complexity of PC extracts or PC-rich foods is a major problem in absorption studies. PC extracts from foods contain a great variety of oligomers (from monomer to 10 units or more), and each oligomer contains a variable number of isomers, which increases as the polymerization degree increases (*38*). Thus, the amount of each individual PC species is actually low in PC extracts and its concentration in plasma after absorption will be very low. Moreover, the efficiency of PC extraction from biological fluids is 50−70% (*39*), which makes it even more difficult to detect one particular PC in plasma. To simplify the system and facilitate the detection of dimeric PC forms in plasma and tissues, we have synthesized PC from (−)-epicatechin linked with ethyl bridges. The synthetic PC (SPC) obtained contains only dimers, trimers, tetramers, and nanomers, which allows a more controlled system than procyanidin extracts from foods. Using this SPC, it was demonstrated that, in rats, dimeric PC are rapidly absorbed, highly methylated, and found in physiologically relevant amounts in plasma and liver. To our knowledge, this is the first time that highly methylated dimeric PC have been detected in liver and plasma, which shows that these oligomeric compounds are absorbed and metabolized in the same way as the monomeric forms. These results provide evidence that oligomeric procyanidins are not depolymerized to monomers after ingestion and strengthen the hypothesis that oligomeric procyanidins exert their biological activities independently of their metabolization by the gut microflora.

MATERIALS AND METHODS

Synthesis of Procyanidin Oligomers. Oligomeric PC were synthesized by acetaldehyde-induced polymerization of (−)-epicatechin, as described by Es-Safi et al. (*40*). Briefly, the reaction mixture consisted of 3% acetic acid, 10% ethanol, 0.04 M (−)-epicatechin, and either 2 M (molar excess) or 0.024 M (molar deficiency) acetaldehyde (pH 2.2). Acetaldehyde was added last to initiate the polymerization reaction, which was allowed to proceed at room temperature. Aliquots of the reaction mixtures were taken at different time intervals and used to monitor epicatechin concentration and the appearance of new compounds by HPLC coupled to a diode-array and mass spectrometry detectors (*41*). The polymerization degree of the SPC obtained in each case was determined by gel permeation chromatography in the analytical laboratory of the company Les Dérivés Résiniques et Terpéniques (Dax, France)

Simulation of SPC Digestion under Gastrointestinal Conditions. SPC (1.5 g/L) were incubated either with HCl (pH = 0.8−1) at different concentrations (0.32, 0.16, or 0.8 M) or with 1.5% $NaHCO_3$ (pH = 8.4) at 37 °C with constant agitation. After 3 h, an aliquot of incubated SPC was filtered (0.45 μm, Millipore) and analyzed by HPLC/diode array to monitor epicatechin formation, as indicated in the HPLC−MS analysis section.

Animal Experiments. Two-month-old Male Wistar rats weighing 250 g were purchased from Charles River Laboratories (Barcelona, Spain). The Animal Ethics Committee of the Rovira i Virgili University approved all procedures. The animals were housed in animal quarters at 22 °C with a 12 h light/dark cycle (light from 8 a.m. to 8 p.m.) and were fed a standard diet (Panlab, Barcelona, Spain). Rats were fasted overnight and then fed an oral gavage of SPC in aqueous solution (200

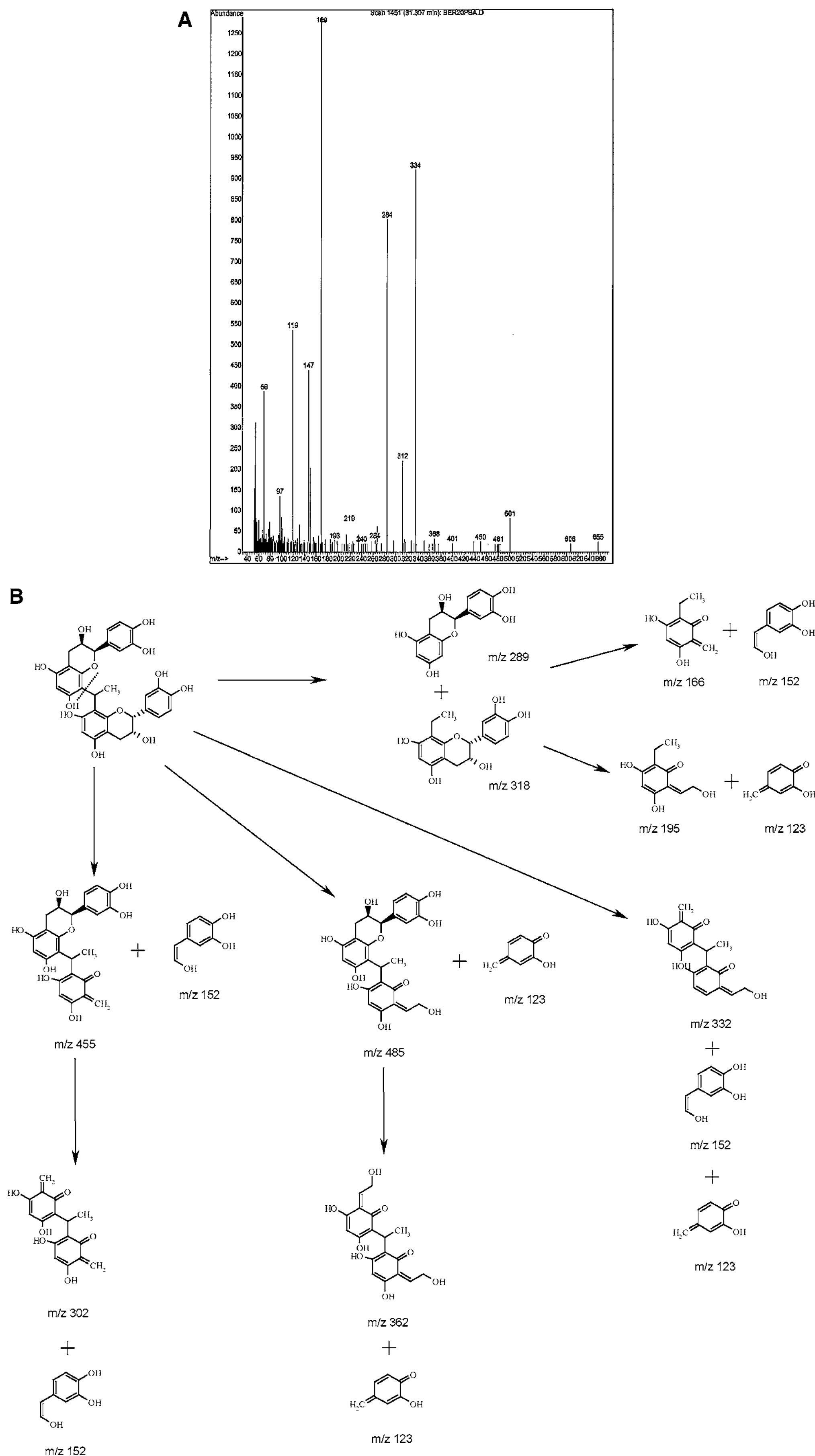

Figure 2. LC/MS spectra of Ep1 compound (**A**) and the structure of the major fragments generated from Ep1 (**B**). The structures of the fragments generated from Ep1 were deduced from those described for epicatechin and dimeric procyanidins (*42*). The *m*/*z* signals showed that the molecular weight of this compound was 606 and that its structure corresponds to two (–)-epicatechin units linked by an ethyl bridge.

Table 1. Polymerization Degree of the Synthetic Procyanidin Oligomers Obtained by the Reaction between Epicatechin and Acetaldehyde[a]

retention time (min)	% area	acetaldehyde concn in sample (M)	mol wt	polymerization degree
16.8	83.13	0.024[b]	2919	9
19.7	7.43		1126	4
21	6.91		963	3
23	2.53		606	2
15.8	96.36	2[c]	5075	16
20.5	3.64		963	3

[a] Aliquots of precipitates were taken at the end of the reaction and analyzed by gel permeation chromatography. [b] Represents a deficiency. [c] Represents an excess.

Table 2. Release of Epicatechin Monomers upon Incubation of Synthetic Procyanidin Oligomers with Hydrochloric Acid or Bicarbonate[a]

incubation conditions	% of released epicatechin monomer	incubation conditions	% of released epicatechin monomer
0.32 M HCl	0.6 ± 0.02	0.08 M HCl	0.43 ± 0.01
0.16 M HCl	0.52 ± 0.01	1.5% $NaHCO_3$	not detected

[a] Synthetic procyanidin oligomers (1.5 g/L) were incubated with either HCl or $NaHCO_3$ for 3 h at 37 °C and constant agitation ($n = 3$). Monomeric epicatechin in the medium was measured by HPLC/diode-array analysis and expressed as the percentage of total epicatechin subunits in the synthetic procyanidin oligomers. Values are means with their standard errors ($n = 3$).

mg/kg of body wt). One, two, three, and four hours after treatment (six animals/group), the rats were anaesthetized with sodium pentobarbital (60 mg/kg of body wt), and blood was collected from abdominal aorta using heparin as anticoagulant. Plasma was obtained by centrifugation and stored at −80 °C until analysis. Livers were excised, frozen immediately in liquid nitrogen, and stored at −80 °C until processing.

HPLC−MS Analysis. Plasma samples were first treated with 1000 units of β-glucuronidase type VII-A (*Escherichia coli*, Sigma) and 100 units of sulfatase type VIII (abalone entrails, Sigma) for 3 h at 37 °C with constant agitation and then extracted three times with ethyl acetate. The extracts were dried under nitrogen stream, dissolved in 20% aqueous ethanol, and filtered (0.45 μm, Millipore). Livers (1 g) were homogenized with 0.9% NaCl. The homogenates were filtered with gauze and then processed identically as plasma samples. Plasma and liver samples were analyzed by HPLC (Hewlett-Packard 1100 series) coupled to a diode-array (Hewlett-Packard 1315A) and a mass spectrophotometer (Hewlett-Packard 5989A). Samples (100 μL) were separated using a Spherisorb ODS-2 column (4.6 × 250 mm) with 5 μm particle size at room temperature. The mobile phase consisted of (A) formic acid (4.5%) in water and (B) formic acid (4.5%) in acetonitrile, at a flow rate of 1.5 mL/min. Procyanidin dimer B2 was used as a calibration standard. The estimated concentration of dimers is therefore quoted in B2 equivalents.

RESULTS

Synthesis of Procyanidin Oligomers. The acetaldehyde-induced polymerization of (−)-epicatechin was carried out using acetaldehyde either in molar excess or in molar deficiency with regard to epicatechin in order to investigate how this factor affects the SPC polymerization degree. In both cases, a visible, brownish precipitate was formed along with the progression of the polymerization reaction. **Figure 1** shows typical HPLC chromatograms obtained from aliquots of the reaction media with acetaldehyde in deficiency (**A**) and excess (**B**) taken at the end of the reactions. The molecular weights and nature of the compounds corresponding to different chromatographic peaks were determined by particle beam mass spectrometry. Peak Epi corresponds to residual epicatechin in the reaction medium. LC/MS spectra of the Ep1 compound and the structure of the major fragments generated, deduced from those previously described for epicatechin and dimeric procyanidins (*42*), are shown in **Figure 2**. *m/z* signals revealed that the molecular weight of this compound was 606 and that its structure corresponds to two (−)-epicatechin units linked by an ethyl bridge. Gel permeation chromatography analyses of the precipitates obtained showed that the polymerization degree of SPC generated was dependent upon the molar ratio between (−)-epicatechin and acetaldehyde (**Table 1**). When acetaldehyde was present in molar excess, the two major components of SPC were a polymer of 16 units of (−)-epicatechin (≈96%) and a trimer (≈4%). In contrast, acetaldehyde in molar deficiency generated SPC of lower polymerization degree. In this case, the most abundant compound synthesized was an oligomer of nine (−)-epicatechins (≈83%), followed by a tetramer (≈7.5%), a trimer (≈7%), and a dimer (≈2.5%). In this way, this SPC will be useful for studying the absorption and metabolism of procyanidins in vivo, since they are of a much lower complexity than PC mixtures extracted from natural sources. For subsequent experiments we used the SPC containing dimers, trimers, tetramers, and nanomers.

Absorption and Metabolism of SPC in Vivo. Prior to the in vivo absorption study, it was determined whether the SPC could be degraded into monomers by the pH conditions of the gastrointestinal tract. To determine the stability of SPC in the acidic pH of the stomach and in the basic pH of the intestine, SPC oligomers were incubated either with HCl or $NaHCO_3$. Because SPC does not contain epicatechin, the stability of the oligomers was measured by the presence of epicatechin in the incubated medium (**Table 2**). SPC was very stable at the basic pH of the intestine, since epicatechin was not detected. Also, the stability of SPC at the acidic pH of the stomach was very high. Epicatechin was detected in the three HCl concentrations studied, but it only represented a low percentage (maximum 0.6%) of the epicatechin contained in oligomeric forms.

Given the high chemical stability demonstrated by SPC at the acidic and basic pH values typical of the gastrointestinal tract, we conducted in vivo absorption studies of these oligomeric procyanidins by orally administering a single dose of SPC to male Wistar rats that had been deprived of food overnight. Using grape seed procyanidin extracts, the amount of SPC administered to the animals (200 mg/kg body weight) has been shown to be nontoxic and to exert metabolic effects in vivo, both on the plasma lipid state and on the liver gene expression profile (*13*). One, two, three, and four hours after SPC gavage, blood samples were analyzed for the presence of procyanidins and procyanidin-derived compounds, which would indicate that oligomeric SPC had been absorbed. Liver samples were collected 2 h after SPC gavage and analyzed in the same way to check whether procyanidins and/or procyanidin-derived compounds had reached this procyanidin-target tissue. **Figure 3** shows a representative diode-array chromatogram of plasma and liver samples of rats treated with SPC gavage. All major chromatographic peaks found in the HPLC/diode-array analyses of plasma and liver samples were further analyzed by mass spectrometry to check their possible procyanidin-like spectral characteristics. Only the compound with a retention time of 38 min in the HPLC/diode-array analysis of plasma and liver samples demonstrated flavanol-like spectral characteristics, as is shown in **Figure 4**A. This peak had an *m/z* of 663, corresponding to a dimeric structure made up of two dimethylated epicatechins linked by an ethyl bridge, and was thereafter

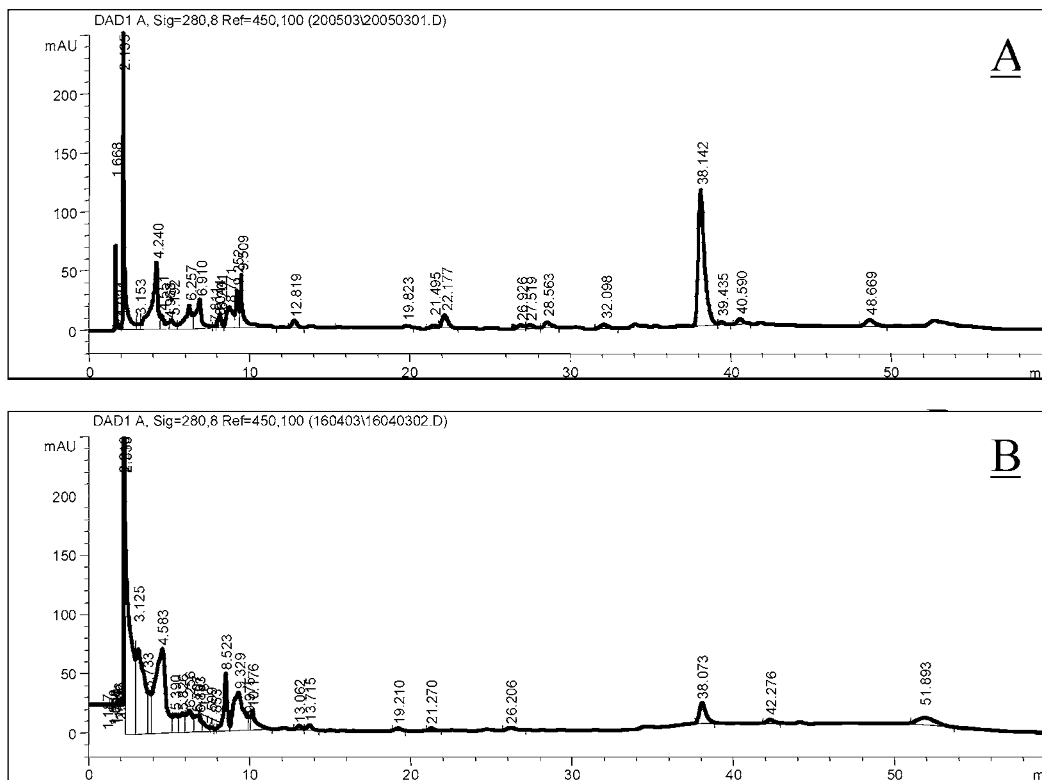

Figure 3. Representative chromatograms generated by HPLC/diode-array analysis of plasma (**A**) and liver (**B**) samples of rats treated with an oral gavage of synthetic procyanidins. Two hours after oral administration of synthetic procyanidin oligomers, rats were sacrificed. Plasma and homogenized liver samples were treated with glucuronidase and sulfatase. Procyanidins were extracted with ethyl acetate and analyzed by HPLC/diode array.

named tetramethylated dimeric procyanidin (TDPC) (**Figure 4B**). The structure of the main fragments of TDPC generated in the mass spectrometer was deduced from those previously described for epicatechin and dimeric procyanidins (*42*).

TDCP concentrations in plasma and liver were quantified using the commercial procyanidin dimer B2 (as a standard in the HPLC/diode-array assay), because no pure TDCP was available. The concentrations of TDPC expressed here are therefore equivalents of B2. TDCP was detected in plasma as early as 1 h after oral gavage of SPC, reached maximum concentrations 2 h after gavage, and was still noticeably present 4 h after SPC administration (see **Table 3**). Also, the livers of SPC-treated rats contained significant amounts of TDPC. These reached concentrations of up to 15 μg of B2 equivalents per gram of fresh tissue 2 h after gavage (data not shown). Thus, TDPC, originally from the oral intake of oligomeric procyanidins, were therefore present in liver before the already described effects on gene expression of this procyanidin-target tissue (*13*).

DISCUSSION

Many benefits for human health have been ascribed to PC-rich foods (*2*). However, which compounds present in PC, i.e., monomers, oligomers, or their aromatic derivatives produced by colon microflora, are responsible for their beneficial effects in vivo is still unknown. The different polymerization degree of PC, the large number of isomers within each degree, and the low efficiency of PC extraction from biological fluids are major problems in bioavailability studies of PC extracts or PC-rich foods. To overcome these problems, we have synthesized oligomeric PC from (−)-epicatechin linked exclusively with ethyl bridges (*40*). The SPC mixture obtained has several advantages over PC extracts for bioavailability studies:(I) it does not contain monomers; (II) it only contains dimers, trimers, tetramers, and nanomers; (III) each oligomer contains a low number of isomers; and (IV) if they are chemically unstable at the pH of the gastrointestinal tract, only epicatechin monomers will be produced. Although the SPC mixture was obtained in the laboratory, these kinds of oligomers are produced naturally during the winemaking process (*43*).

Chocolate PC have been shown to be stable during gastric transit in humans (*44*). However, some authors have shown that cocoa PC are degraded into monomers and dimers upon incubation with simulated gastric juice (*45*). Before analyzing the PC bioavailability in vivo, therefore, it was considered necessary to determine whether the SPC synthesized by us were stable at the pH of the gastrointestinal tract in order to determine whether monomers (epicatechin) could be generated. Incubating the SPC with simulated gastric and intestinal pH juices showed that they were very stable under both conditions: minimal amounts of epicatechin were generated only in the acidic milieu. It can therefore be hypothesized that SPC do not generate monomers during their transit through the gastrointestinal tract in vivo and are therefore suitable for studying the absorption and bioavailability of oligomeric PC in vivo. Despite the absence of epicatechin in the plasma of rats treated with the SPC, some cleavage of tetramers and nanomers under the actual conditions of gastrointestinal tract, which should contribute to dimers detected as bioavailable, cannot be ruled out.

HPLC-coupled mass spectrometry analysis of the plasma and liver of rats treated with an oral gavage of SPC indicated the presence of a tetramethylated dimeric PC (TDPC), whose structure is depicted in **Figure 3B**, which confirms that dimeric PC are absorbed. These results are in agreement with those of other authors, who detected procyanidin dimer B1 in the plasma of humans after intake of PC-rich grape seed extract (*24*) and procyanidin dimer B2 in the plasma and urine of rats after B2

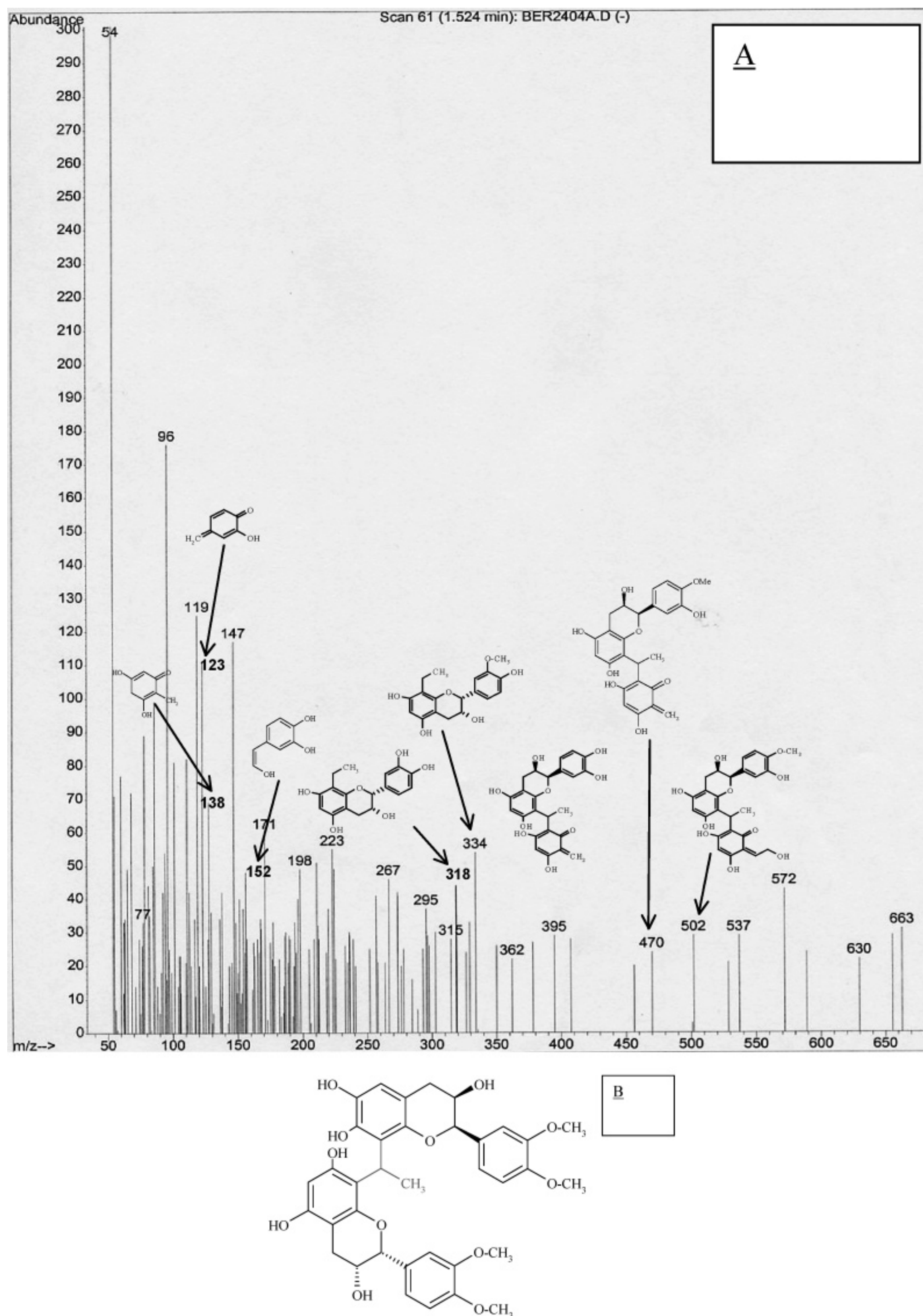

Figure 4. Mass spectra (**A**) and structure (**B**) of the compound corresponding to the elution peak at 38 min in the HPLC/diode-array chromatograms shown in **Figure 3**, parts **A** and **B**. The structures of the major fragments generated from the tetramethylated dimeric procyanidin in the mass spectrometer were deduced from those previously described for epicatechin and dimeric procyanidins (*42*).

administration (*26*). TDPC reached a maximum concentration in plasma 2−3 h after SPC administration, though it was already detectable as early as 1 h after the treatment. These results strongly suggest that dimeric PC are rapidly absorbed in the upper digestive tract after oral gavage. The absorption of bigger PC cannot be ignored, since current methods make it difficult to detect PC with a polymerization degree of over 3.

Flavonoids are substrates for several enzymes located in the small intestine and liver (*46*). Potential molecular sites of metabolic modification include methylation on the B-ring catechol group and glucuronidation/sulfation mainly on the A ring (*46*). The catechol-like structure of some flavonoids, such as quercetin and tea catechins, makes them prone to *O*-methylation by soluble catechol-*O*-methyltransferase (COMT) (*47*). Interestingly, the rate of *O*-methylation of quercetin and fisetin are up to 3 orders of magnitude higher than those of catechol estrogens and catecholamines (*48*). In accordance with this elevated propensity to methylation, unmethylated dimeric PC was not found in plasma and liver. This is in agreement with other studies, which have shown that the methylated metabolites of catechin (*18*, *19*), epicatechin (*19*), and epicatechin gallate (*15*) predominate over the original unmethylated

Table 3. Concentrations of Tetramethylated Procyanidin Dimer in Plasma of Rats after an Oral Gavage of Synthetic Oligomeric Procyanidins[a]

time (h) after oral gavage	dimer concn (mg/L)	time (h) after oral gavage	dimer concn (mg/L)
1	5.0 ± 1.0	3	12.5 ± 2.6
2	14.4 ± 3.0	4	9.9 ± 2.1

[a] Plasma samples of rats treated with synthetic oligomeric procyanidins were incubated with sulfatase-glucuronidase and extracted with ethyl acetate. Quantification of tetramethylated procyanidin dimer was made by HPLC/diode-array analysis and calculated as B2 equivalents. Values are means with their standard errors ($n = 6$).

forms in plasma. It has been reported that glucuronidation and sulfation of catechin and epicatechin are produced in rat liver and small intestine (*18*, *19*, *21*, *49*). We cannot rule out the possibility that the TDPC was also glucuronidated and sulfated, since plasma and liver samples were treated with sulfatase and glucuronidase prior to HPLC analysis.

The maximal plasma concentration of TDPC was about 20 μM, found after a single gavage of 200 mg of SPC, which contained 6 mg of dimer per kilogram of body weight. This value is higher than that reported for PC dimers in other studies. In rats (*26*), the maximal plasma concentration of B2 was 0.5 μM after oral gavage of 50 mg of B2 per kilogram of body weight. In humans (*24*), maximal plasma concentrations of B1 were 10 nM after ingestion of 2 g of PC containing 0.9% B1, which represents 0.25 mg of dimer per kilogram of body weight. Also in humans (*25*), maximal plasma concentrations of B2 were 40 nM after ingestion of 0.375 g of cocoa, which contained 3.63 mg of dimers per kilogram of body weight. These differences in the plasma concentrations of dimeric PC could be due to differences in dimer bioavailability caused by the quantity of dimeric PC per kilogram of body weight, by the composition of the PC mixture, and/or by the chemical structure of the dimer. In any case, the concentrations of dimeric PC found in plasma may be high enough to exert biological activity, since there is increasing evidence that flavonoids, beyond their antioxidant activity, act as modulators of cell signaling in vivo (*50*). In this context, it is worth noting that flavonoids can exert estrogenic and insulin-like effects (*14*, *51*) and that insulin and estrogens are found at nanomolar concentrations in the blood (*52*, *53*). In all these studies, dimeric PC have been detected in plasma soon after oral administration, and as early as 30 min for B2 in humans (*25*). Taken together, these observations strongly suggest that PC dimers are responsible for some of the biological effects attributed to PC extracts and PC-rich foods, regardless of the biological activity that might be exerted by the aromatic acid derivatives of PC generated by the colon microflora. Reinforcing this argument, TDPC was detected also in the liver, which suggests that the effects of PC upon gene expression and lipid metabolism in liver that we observed (*13*) may have been the result of a direct interaction of PC with cellular signal transduction pathways. In humans, the plasma concentration of dimeric PC after a standard meal is unknown, and only estimations of the intake of PC have been made for adult Americans; PC consumption has been estimated at 50−70 mg/day, 6−8 mg of which is PC dimer (*1*). Total PC intake may be enormously underestimated, since data on the PC contents in foods are not sufficient and in analyzed foods the PC quantity often is underestimated (*1*).

In summary, using synthetic oligomeric PC, we have demonstrated that, in rats, dimeric PC are rapidly absorbed and found in physiologically relevant amounts in plasma and liver as highly methylated forms. PC dimers are therefore good candidates for agents of the biological activities of PC extracts and PC-rich foods.

ABBREVIATIONS USED

PC, procyanidins; SPC, synthetic procyanidins; TDPC, tetramethylated dimeric procyanidin.

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