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Sorghum Bran in the Diet Dose Dependently Increased the Excretion of Catechins and Microbial-Derived Phenolic Acids in Female Rats

LIWEI GU,[‡,§] SUZANNE E. HOUSE,[‡] LLOYD ROONEY,[||] AND RONALD L. PRIOR*,[†,‡]

Arkansas Children's Nutrition Center, Agricultural Research Service, U.S. Department of Agriculture, 1212 Marshall Street, Little Rock, Arkansas 72202, Department of Physiology & Biophysics, University of Arkansas for Medical Sciences, 4301 West Markham Street, Little Rock, Arkansas 72205, and Cereal Quality Lab, Texas A&M University, 2474 TAMU, College Station, Texas 77843-2474

Sorghum bran is concentrated with procyanidins (predominately polymers), which may be beneficial for health in humans; however, the bioavailability of procyanidins is not well-understood. Female Sprague−Dawley rats were fed an AIN93G diet containing 0, 5, 10, 20, or 40% Hi-tannin sorghum bran ($n = 5-7$ for each group) for 50 days. Sorghum bran contained 23.3 mg/g of procyanidins. The urinary excretions of catechin, epicatechin, methylated catechins, and phenolic acids were analyzed using liquid chromatography−tandem mass spectrometry. Sorghum bran dose dependently increased the urinary excretion of catechin (0−2.2 nmol/day) and 3′-*O*-methylcatechin (0−9.5 nmol/day). Their serum concentrations also increased with dose (range of 0−14 nM for 3′-*O*-methylcatechin). Among the 14 phenolic acids analyzed, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, and 4-hydroxyphenylacetic acid dominated in the serum (1.8−8 μmol/L). In the urine, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid dominated and their excretion increased significantly with the level of sorghum bran in the diet. The summed phenolic acid excretion was 0.8 μmol/day in the control group and increased to 23 μmol/day for 40% sorghum bran group. The hippuric acid excretion ranged from 2.2 to 16.2 μmol/day and peaked in the 10% sorghum bran group. On the basis of chromic oxide, a nonabsorbable marker, total procyanidins and polymers disappeared progressively, and significant degradation occurred in the cecum and colon. Catechins and procyanidins in sorghum were bioavailable; however, bacteria-derived phenolic acids were the predominant metabolites of procyanidins. Procyanidins degraded in the gastrointestinal tract. Depolymerization was not observed.

KEYWORDS: Sorghum; procyanidins; catechin; epicatechin; phenolic acids

INTRODUCTION

Sorghum is the fifth most important cereal crop in the world after wheat, rice, corn, and barley. More than 35% of the sorghum is grown directly for human consumption. The United States is the largest producer and exporter of sorghum, accounting for 20% of the world production and almost 80% of the world sorghum exports during 2001−2002 (*1*).

Different varieties of sorghum can be roughly divided into two categories: the tannin-free sorghums, such as the white sorghum, and the tannin sorghums, such as the brown sorghum. Tannins are the most unique and important constituents in tannin sorghum because of their high concentrations and various effects on human health (*2*, *3*). Tannins in sorghum are almost exclusively procyanidins (*2*). Other proanthocyanidins with greater heterogeneity have previously been identified in sorghum (*4*), but they appear to be minor components. Procyanidins in sorghum are the oligomers and polymers of catechin or epicatechin. The sizes of procyanidins are defined by the degree of polymerization (DP). We have reported that the average DP of procyanidins in sorghum is 8.4 (*5*) and that they consisted of catechin (88%) and epicatechin (12%) as the chain-terminating units. The chain extension units were exclusively epicatechin (*3*, *5*). Procyanidins in sorghum are concentrated in the bran layer, which can be removed by abrasive milling (decortication) (*6*). Over 60% of procyanidins in sorghum are polymers with DP > 10 (*3*, *7*). Food processing, such as extrusion and baking, can affect the total procyanidin content as well as the proportion of oligomers and polymers in the total contents (*7*, *8*).

Procyanidins from other food sources are reported to have positive health effects in humans including cancer and cardiovascular disease prevention and antidiabetic effects. For ex-

* To whom correspondence should be addressed. Tel: 501-364-2747. Fax: 501-364-2818. E-mail: PriorRonaldL@uams.edu.
[†] U.S. Department of Agriculture.
[‡] Arkansas Children's Nutrition Center.
[§] University of Arkansas for Medical Sciences.
[||] Texas A&M University.

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Table 1. Retention Time and Molecular Weight of Phenolic Acids

peak #	phenolic acids	symbols	MW	R_t (min)
1	3,4-dihydroxybenzoic acid	C1: 3,4-diOH	154	8.9
2	3,4-dihydroxyphenylacetic acid	C2: 3,4-diOH	168	9.5
3	3,4-dihydroxyphenylpropionic acid	C3: 3,4-diOH	182	14.0
4	4-hydroxyphenylacetic acid	C2: 4-OH	152	14.7
5	vanillic acid (3-methoxy, 4-hydroxybenzoic acid)	C1: 3-M, 4-OH	168	16.6
6	3-methoxy, 4-hydroxyphenylacetic acid	C2: 3-M, 4-OH	182	16.7
7	caffeic acid (*trans*-3,4-dihydroxy cinnamic acid)	caffeic acid	180	16.8
8	3-hydroxyphenylacetic acid	C2:3-OH	152	17.2
9	3-hydroxybenzoic acid	C1: 3-OH	138	19.0
10	*trans*-*p*-coumaric acid	*p*-coumaric acid	164	24.9
11	3-hydroxyphenylpropionic acid	C3: 3-OH	166	25.0
12	ferulic acid (*trans*-3-methoxy,4-hydroxycinnamic acid)	ferulic acid	194	25.8
13	4-hydroxyphenylpropionic acid	C3: 4-OH	166	27.8
14	phenylpropionic acid	C3	150	34.1

ample, studies indicated that procyanidins from cocoa may prevent the development of atherosclerosis (*9*), inhibit the growth of human breast cancer cells (*10*), increase insulin sensitivity, and decrease blood pressure (*11*) (reviewed in *12*). The structure and contents of procyanidins in sorghum are comparable to those in cocoa. Surprisingly, there have been few studies on the health effects of sorghum. We postulated that sorghum, due to its higher procyanidin content, may also have health effects. The absorption of phytochemicals is an important factor to determine the efficacy. The absorption of procyanidin polymers depends on their molecular size (*13*). Because the bioavailability of procyanidins in sorghum has not been studied, in the first step of our studies, we evaluated the bioavailability of procyanidins from sorghum bran fed to female rats.

METHODS AND MATERIALS

Standards. (±)-Catechin and (−)-epicatechin were purchased from Sigma Chemical Co. (St. Louis, MO). 3′-*O*-Methylcatechin, 4′-*O*-methylcatechin, 3′-*O*-methylepicatechin, and 4′-*O*-methylepicatechin were synthesized by methylating (±)-catechin or (−)-epicatechin with methyl iodine according to a published method (*14*). They were purified on preparative high-performance liquid chromatography (HPLC), and the methylation site was confirmed with tandem mass spectrometry (MS/MS) (*15*). Procyanidin standards, including a composite oligomer standard (DP = 1−10) and a polymer standard (DP = 36.1), were prepared as described previously (*3*). Procyanidin dimer B1 was purchased from ChromaDex (St. Santa Ana, CA). Standards for phenolic acids were products of Sigma Chemical Co. The names and abbreviations of these phenolic acids are listed in **Table 1**, and their structures are depicted in **Figure 1**.

Animals and Diet. The protocol was approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Female Sprague−Dawley rats (200 g) were fed an AIN-93G diet containing 0, 5, 10, 20, or 40% sorghum bran by weight ($n = 5-7$ for each group) for 50 days. Diets were formulated according to **Table 2**. Sorghum bran was analyzed for amino acid content (Eurofins Scientific Inc., North Little Rock, AR), and methionine was added to provide 0.49% in all diets. Sorghum bran from the Hi-tannin variety was mixed with other ingredients, and the diet was pelleted. Pelleting did not change the total content or the profile of the procyanidins in sorghum bran. Diets for the last 5 days were mixed with 0.5% (w/w in diet) of chromic oxide as a nonabsorbable marker. Urine and feces were collected (24 h) for individual rats at day 49. Rats were killed on day 50 by decapitation. Trunk blood was collected and centrifuged at 3000 rpm for 15 min at 4 °C to collect the serum. Gut contents were collected from the stomach, duodenum, jejunum, ileum, cecum, and colon. All samples were stored at −20 °C prior to analyses.

Analytical Methods. *Procyanidins in Diet and Gut Contents.* Samples were extracted and analyzed on normal phase HPLC with fluorescent detection. The method was developed in our laboratory and has been described previously (*3*, *5*). Intestinal contents in the duodenum, jejunum, and ileum were pooled for five rats of the same diet group for procyanidin analyses because of the low amount.

Catechin and Epicatechin in Diet and Gut Contents. Catechin and epicatechin coeluted as monomers on normal-phase HPLC and, thus, could not be quantified separately. They were also analyzed using reverse-phase HPLC with fluorescent detection. Separation was performed on a Phenomenex Synergi Max-RP column (250 × 4.6, 4 μm, Phenomenex, Torrance, CA) using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). The solvent consisted of (A) 0.1% (v/v) of formic acid in water and (B) methanol. The 23.5 min linear gradient was as follows: 0−17.5−18−23−23.5 min, 22−35−80−80−22% of B, followed by 5 min of re-equilibration of the column before the next run. The flow rate was 0.8 mL/min. The fluorescent detector was set at 276 nm for excitation and 316 nm for emission. This method had a quantitation limit of 0.5 ng injected on column and an intra-assay variation of 3%.

Hippuric Acid. Urine was thawed overnight and centrifuged at 14000 rpm for 10 min. Urine (50 μL) was injected on a Phenomenex Synergi Max-RP column (250 × 4.6, 4 μm). UV detection was set at 240 nm (10 nm bandwidth) with 400 nm (30 nm bandwidth) as a reference. The solvent consisted of (A) 0.1% (v/v) of formic acid in water and (B) methanol. The 25 min linear gradient was as follows: 0−20−20.5−24.5−25 min, 20−35−80−85−20% of B, followed by 6 min of re-equilibration of the column before the next run. The flow rate was 1.0 mL/min. Hippuric acid eluted at 13.8 min.

Catechins and Phenolic Acids in Urine and Serum. Thawed urine (100 μL) was added into 6 mL screw-capped glass tubes. Four hundred microliters of ammonium acetate buffer (pH 5, 1 M) containing 80 units of sulfatase (type H2, from *Helix pomatia*, Sigma) and 0.5 mM ascorbic acid was added. Tubes were incubated in a 37 °C water bath for 45 min. Twenty-five microliters of 6 N HCl was added in the tube to adjust the pH to 2−3. Catechins and phenolic acids were extracted twice with 2.5 mL of ethyl acetate. To the combined ethyl acetate extracts, 50 μL of 100 mM ascorbic acid solution was added. The solvent was evaporated on a SpeedVac (SC210A; Thermo, Marietta, OH) at 25 °C. Dried extracts were reconstituted in 500 μL of 30% methanol containing 1 mM ascorbic acid. For serum, 400 μL was used for extraction. Acetic acid (100 μL) was used in the place of HCl to adjust the pH to avoid excessive precipitation of protein. After the incubation, tubes were extracted twice with 2.5 mL of hexane to remove the lipids before ethyl acetate extraction.

Catechins in urine or serum were analyzed on Agilent 1100 HPLC system coupled to an Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Separation was carried on a Phenomenex Synergi Max-RP column (250 × 4.6, 4 μm) using a flow rate of 0.8 mL/min. The solvent consisted of (A) 0.1% (v/v) of formic acid in water and (B) methanol. The 30 min linear gradient was as follows: 0−17.5−21−23−29.5−30 min, 30−40.5−55−80−90−30% of B, followed by 6 min of re-equilibration of the column before the next run. The mass spectrometer used an electrospray interface in positive ionization mode. Other parameters for the mass spectrometer included the following: nebulizer, 40 psi; dry gas, 10 L/min; dry temperature, 360 °C; capillary, −4000 V; skim 1, 15 V; isolate width, 4 mass units;

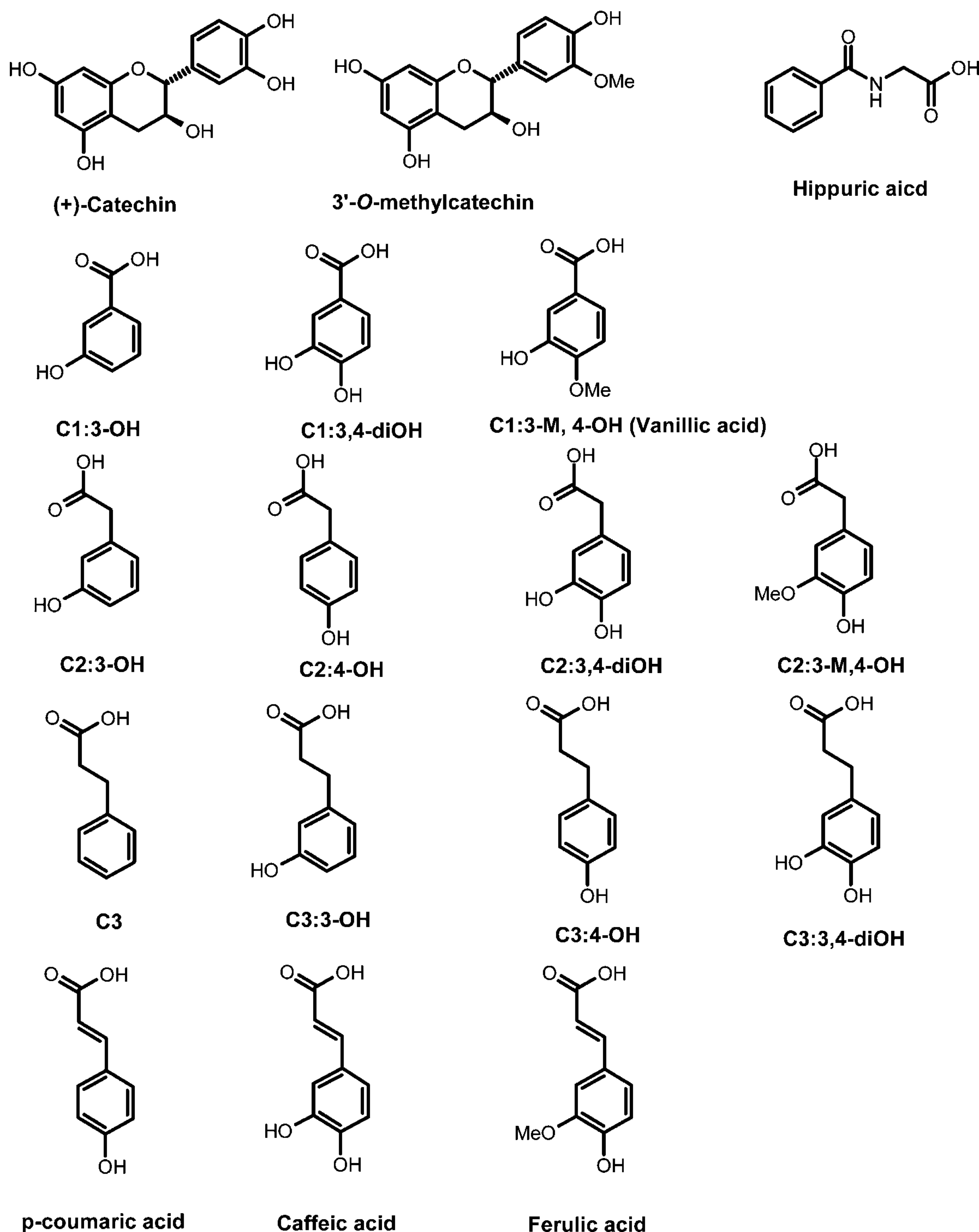

Figure 1. Chemical structures of catechins and phenolic acids. Names and symbols of all phenolic acids are listed in **Table 1**.

and fragmentation energy, 1.1 V. Catechins were quantified using their specific fragments: 291 > 139 *m*/*z* for (±)-catechin and (−)-epicatechin; 305 > 137 *m*/*z* for 3′-*O*-methylcatechin, 4′-*O*-methylcatechin, 3′-*O*-methylepicatechin, and 4′-*O*-methylepicatechin. Catechins were quantified against an external standard using quadratic curves. This method had a quantitation limit of 0.15 ng injected on column and an intra-assay variation of 7%.

Phenolic acids were analyzed on the same liquid chromatography−mass spectrometry (LC-MS) system, using the same column and solvents. The 40 min linear gradient was as follows: 0−20−21−30−37−39.5−40 min, 20−35−40−60−80−85−20% of B, followed by 6 min of re-equilibration of the column before the next run. The mass spectrometer used an electrospray interface in negative ionization mode. Other parameters for the mass spectrometer included the following: nebulizer, 40 psi; dry gas, 10 L/min; dry temperature, 360 °C; capillary, +4500 V; and skim 1, −15 V. Phenolic acids were monitored using their deprotonated ions and quantified against an external standard using quadratic curves. This method had a quantitation limit of 2 ng on column and an intra-assay variation of 7%.

Detection of Procyanidin Dimers. Serum and urine extracts were analyzed on an Agilent 1100 HPLC system coupled to an API 4000QTRAP linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA). Separation was carried on a Phenomenex Synergi Max-RP column (150 × 3.0, 4 μm) using a flow rate of 0.4 mL/min. The solvent consisted of (A) 0.1% (v/v) of formic acid in water and (B) methanol. The 15.5 min linear gradient was as follows: 0−7−10−15−15.5 min, 25−55−80−90−25% of B, followed by 7 min of re-equilibration of the column before the next run. The mass spectrometer used an electrospray interface in negative ionization mode. Other parameters for the mass spectrometer included the following:

Table 2. Composition of the Diets

ingredient	control	Hi-tannin sorghum bran 5%	10%	20%	40%
casein	200.0	196.5	193.1	186.1	172.3
L-cystine	3.0	3.0	3.0	3.0	3.0
L-methionine	0.0	0.033	0.066	0.133	0.265
L-tryptophan	0.0	0.0	0.0	0.0	0.048
sorghum bran	0.0	50.0	100.0	200.0	400.0
corn starch	392.5	355.0	317.6	244.9	99.5
maltodextrin	132.0	132.0	132.0	132.0	132.0
sucrose	100.0	100.0	100.0	107.7	107.7
corn oil	70.0	67.16	64.31	58.6	47.2
cellulose	50.0	43.73	37.47	24.93	0.0
mineral mix[a]	35.0	35.0	35.0	25.1[c]	20.4[d]
vitamin mix[b]	10.0	10.0	10.0	10.0	10.0
choline bitartrate	2.5	2.5	2.5	2.5	2.5
TBHQ (antioxidant)	0.014	0.014	0.013	0.012	0.01
chromic oxide	5.0	5.0	5.0	5.0	5.0

[a] AIN-93G-MX. [b] AIN-93-VX. [c] Mineral mix altered to decrease K by 0.08%. [d] Mineral mix altered to increase Ca by 0.08% and decrease P by 0.08%.

declustering potential, −130 V; collision energy, −36 V; certain gas, 20 psi; temperature, 450 °C; ion spray voltage, −4500 V; and dwelling time, 300 ms. Procyanidin dimers were detected using their specific fragments 577.2 > 288.8 *m/z*. Procyanidin dimer B1 eluted at 6.2 min and had a detection limit of 10 pg injected on column.

Chromium Analyses. Analyses were performed at the central analytical laboratory of the Poultry Science Department of the University of Arkansas (Fayetteville, AR). Briefly, gut contents were weighed on a piece of filter paper and dried in the hood overnight. The sample on the paper was ashed at 600 °C for 16 h in an ashing oven (Fisher Scientific, Pittsburgh, PA). The ashed samples were dissolved in nitric acid (5% in final solution) and analyzed on an inductively coupled plasma mass spectrometer (Spectro Instrument, Fitchburg, MA). This method had a detection limit of 0.5 ppm.

Statistics. Data are expressed as means ± standard deviations. Statistical analyses were performed on Sigma Stat (V 3.1, Jandal Scientific). Rats from multiple diet groups were compared by one-way analysis of variance (ANOVA) with Tukey pairwise posthoc test. Student's *t* test was performed where there were only two groups. A difference of $P < 0.05$ was considered significant.

RESULTS

The sorghum bran contained monomeric, oligomeric, and polymeric procyanidins (**Figure 2A** and **Table 3**). The total quantity of procyanidins in sorghum bran was 23.3 mg/g. Polymers contributed to 84.5% of the total. The amounts of monomers, dimers, and trimers were very low. Each accounted for less than 0.5% of the total procyanidins. Analyses on reverse-phase HPLC revealed that 94% of the monomers are catechin and only 6% of the monomers are epicatechin (**Figure 2B**). Sorghum bran also contained a very low amount of phenolic acids in soluble free forms, including 3,4-dihydroxybenzoic acid (10 μg/g), 3-methoxy-4-hydroxybenbenzoic acid (2.6 μg/g), caffeic acid (0.6 μg/g), *p*-coumaric acid (0.6 μg/g), and ferulic acid (0.4 μg/g).

Analyses of catechins in urine and serum took advantage of the high sensitivity of the HPLC-MS/MS. Catechin and 3′-*O*-methylcatechin were detected in rat urine. Other catechins were not detectable (**Figure 3A**,**B**). Urinary excretion of catechins increased with the dose of sorghum in the diet. They ranged from 0 to 2.2 nmol/day for catechin and from 0 to 9.5 nmol/day for 3′-*O*-methylcatechin (**Figure 4A**). Serum concentrations ranged from 0 to 1.8 nM for catechin and from 0 to 13.8 nM for 3′-*O*-methylcatechin, respectively (**Figure 4B**). The majority of absorbed catechin was metabolized into 3′-*O*-methylcatechin.

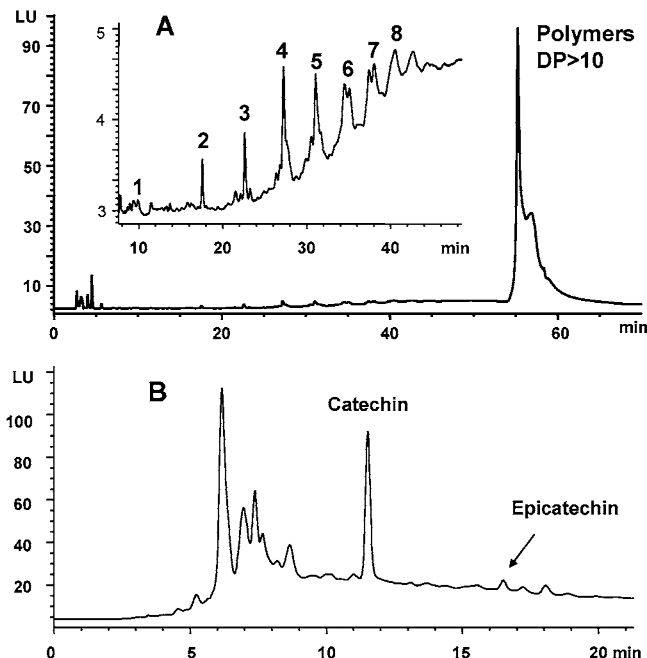

Figure 2. Fluorescent profile of procyanidins in the Hi-tannin sorghum bran on normal phase HPLC (**A**) and fluorescent profiles of catechin and epicatechin in sorghum bran on reverse-phase HPLC. Numbers on the peaks denote the DP of the procyanidins in the peak on the upper panel.

Table 3. Catechins and Procyanidin Contents of Hi-Tannin Sorghum Bran

monomers and oligomers	mg/g	oligomers and polymers	mg/g
catechin	0.06	heptamers	0.47
epicatechin	0.004	octamers	0.53
dimers	0.10	nonamers	0.68
trimers	0.11	decamers	0.37
tetramers	0.22	polymers (DP > 10)	19.8
pentamers	0.36		
hexamers	0.59	total	23.3

A dominance of the methylated catechins has been reported in rats fed grape seed extract (*16*). Only a small amount of epicatechin was detected in the serum of two rats fed the diet containing 20% sorghum bran. Procyanidin dimers were not detected in rat serum or urine.

Fourteen phenolic acids were analyzed by monitoring their deprotonated ions on LC-MS (**Figure 3C**). Nearly all of the phenolic acids were detectable in rat urine (**Figure 3D**). Among these, 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid, (**Figure 5A**), and 3-hydroxyphenylpropionic acid (**Figure 5B**) dominated and their excretion increased significantly with a dose of sorghum bran in the diet. Excretion of other minor phenolic acids, such as caffeic acid, ferulic acid, and *p*-coumaric acid, also increased with the dose of sorghum bran. The 3-hydroxyphenylpropionic acid increased sharply in rats fed 20 and 40% sorghum bran in the diet. On the other hand, the excretion of 4-hydroxyphenylacetic acid decreased in rats fed the high dose of sorghum bran. Propionic acid and 4-hydroxypropionic acid were not detectable in the urine. Three peaks eluted at 21.4, 28.0, and 33.5 min in urine had *m/z* 165, 163, and 193 (peeks u1, u2, and u3 in **Figure 3D**) were unknown metabolites.

The profile of phenolic acids in serum differed significantly from that in the urine (**Figure 6**). The 3,4-dihydroxyxbenzoic acid, 3-methoxy-4-hydroxybenzoic acid, and 4-hydroxypheny-

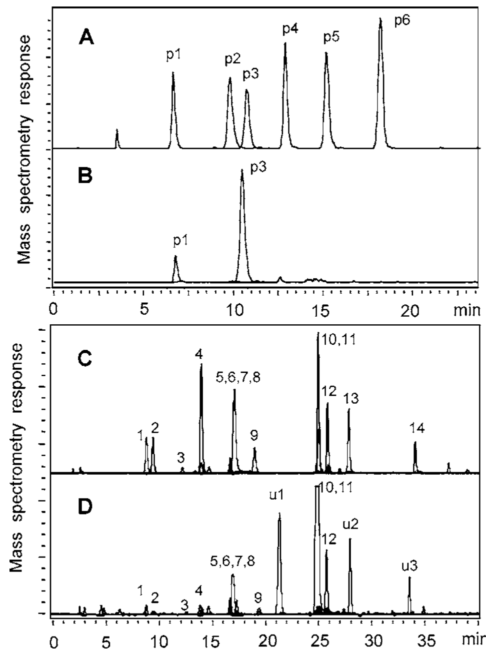

Figure 3. LC-MS/MS profile of catechin standards (**A**) and rat urine (**B**); LC-MS profile of phenolic acid standards (**C**) and rat urine (**D**). Peaks p1, p2, p3, p4, p5, and p6 are (±)-catechin, (–)-epicatechin, 3′-*O*-methyl catechin, 4′-*O*-methyl catechin, 3′-*O*-methyl epicatechin, and 4′-*O*-methyl epicatechin, respectively. The retention times of 14 phenolic acids are listed in **Table 1**. Peaks u1, u2, and u3 were unknown metabolites with $[M-H]^-$ at *m*/*z* 165, 163, and 193, respectively.

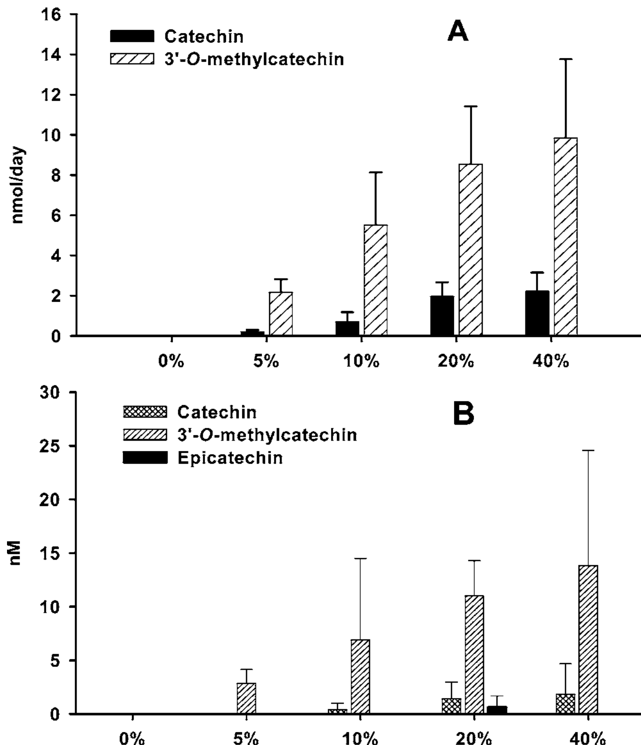

Figure 4. Urinary excretion (**A**) and serum concentrations (**B**) of catechin, 3′-*O*-methyl catechin, and epicatechin in rats fed 0, 5, 10, 20, and 40% of sorghum bran in diet. Epicatechin was not detected in urine.

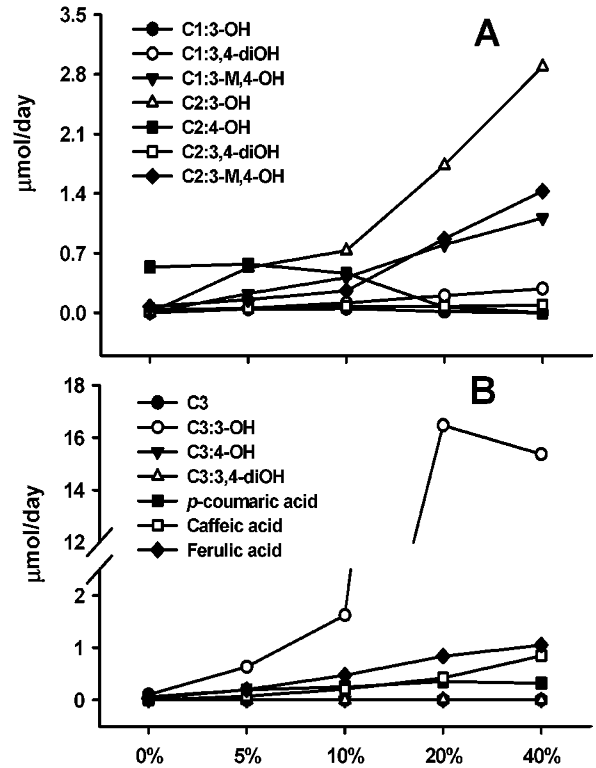

Figure 5. Urinary excretion of phenolic acids in rats fed 0, 5, 10, 20, and 40% of sorghum bran in diet. Names and symbols for phenolic acids are listed in **Table 1**.

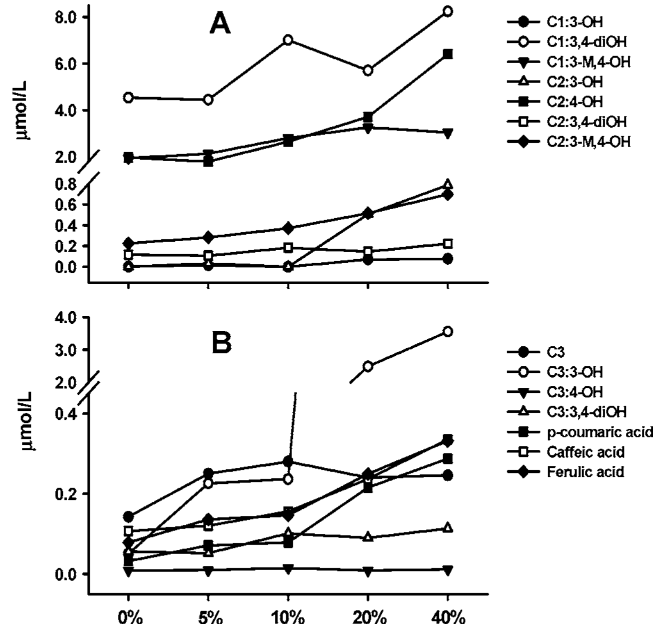

Figure 6. Serum concentration of phenolic acids in rats fed 0, 5, 10, 20, and 40% of sorghum bran in diet. Names and symbols for phenolic acids are listed in **Table 1**.

lacetic acid were the dominant phenolic acids. The serum content of 3-hydroxyphenylacetic acid was low in rats fed 0−10% sorghum bran but increased significantly in rats fed 20−40% sorghum bran. The serum content of 3-hydroxyphenylpropionic acid increased sharply in rats fed 20−40% sorghum bran (**Figure 6B**), which was consistent with the increased excretion in urine. Serum concentrations of most phenolic acids were much higher than those of catechin and 3-*O*-methylcatechin.

Hippuric acid has been reported as a universal metabolite of many flavonoids and phenol compounds (*17*, *18*). Hippuric acid excretion was 2.2 μmol/day in rats fed no sorghum bran. It increased to 16.2 μmol/day and peaked in the 10% sorghum bran group (**Figure 7**). Hippuric acid excretion in rats fed diets with 20 and 40% sorghum bran was not significantly higher

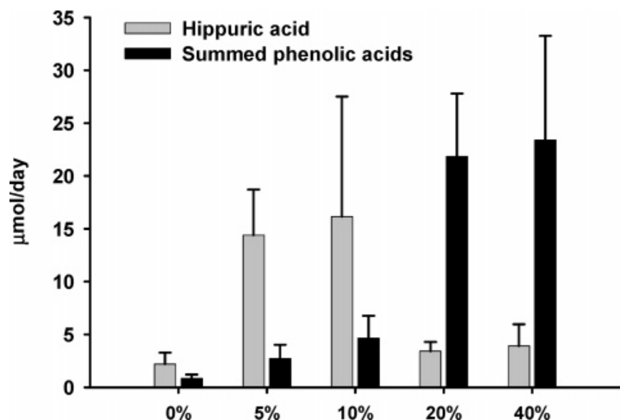

Figure 7. Hippuric acid excretion and summed phenolic acid excretion in rats fed 0, 5, 10, 20, and 40% of sorghum bran in diet.

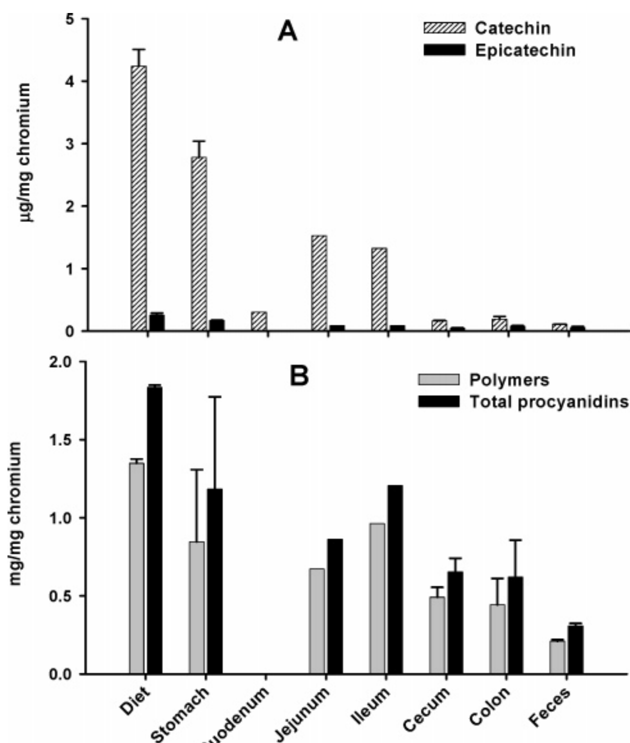

Figure 8. Proportion of catechin and epicatehcin over chromium (**A**) and proportion of procyanidins (total, polymers) over chromium (**B**) in the diet, feces, and gut contents from different segments of the GI tract. Data for rats on diet containing 20% sorghum bran are presented.

than the control. Summed phenolic acid excretion was 0.8 μmol/day in the control group and increased to 23 μmol/day for the 40% sorghum bran group. The urinary excretion of hippuric acid was higher than summed phenolic acids in rats fed 5 and 10% sorghum bran. Excretion of summed phenolic acids was significantly higher than hippuric acid in rats fed 20 and 40% sorghum bran. Excretion of hippuric acid and other phenolic acids was much higher (100-fold) than catechin and 3′-*O*-methylcatechin.

The predominant monomer in the diet and throughout the gastrointestinal (GI) tracts was catechin. This was consistent with the presence of catechin and 3′-*O*-methylcatechin in the urine. The content of catechin decreased along the GI tract on the basis of chromic oxide, a nonabsorbable marker (**Figure 8A**). Total procyanidins and polymers disappeared progressively in the GI tract. The ratio of polymers in total procyanidins appeared to be constant during the digestion process (**Figure 8B**). Data suggested that a significant degradation of procyanidins occurred in the cecum and colon. No depolymerization of polymers was apparent in this study.

DISCUSSION

This is the first study on the absorption and metabolism of sorghum procyanidins in animals after long-term feeding. Procyanidins in sorghum are predominantly high oligomers and polymers. Catechin and epicatechin can be absorbed (*19*), but their contents in sorghum are extremely low (0.06 mg/g). Catechin and epicatechin were known to be absorbed (*19*). Procyanidin dimers and trimers have been detected in rat serum after the administration of apple procyanidin oligomers (*20*). Our data demonstrated that the contents of catechins in serum were very low (0−14 nM). The urinary excretion of catechins was less than 15 nmol/day and indicative of the low bioavailability of sorghum procyanidins in the form of catechins. This was comparable with a previous study using ^{14}C-labeled procyanidins, which concluded that sorghum procyanidins were not absorbed from the digestive tract of chickens (*21*). Dimers and trimers were not detected in serum and urine, partly because dimers and trimers contributed less than 0.5% of the total procyanidins.

The bioavailability of polymeric procyanidins remains controversial. A previous study suggested that polymers may depolymerize into monomers and other low oligomers in gastric milieu and, thus, can be absorbed (*22*). A subsequent study in humans demonstrated that depolymerization did not occur and that procyanidins were stable during gastric transit (*23*). There is no information available to indicate whether depolymerization would occur in the small intestine and colon. The procyanidins in sorghum have a unique property in that catechin is only present on the chain termination units. Only 12% of the chain termination units were epicatechin, and the chain extension units were exclusively epicatechin (*3*, *5*). Furthermore, nearly all of the monomers in sorghum bran are catechin. This property gives a unique advantage to sorghum in studying the depolymerization of procyanidins in the gut. The catechin, epicatechin, and procyanidins were analyzed throughout the GI tract, and their contents were calculated on the basis of the chromic oxide. Depolymerization of sorghum procyanidins would yield a considerable amount of epicatechin. Yet, serum and urine data indicated that epicatechin was not absorbed, and epicatechin appeared to be a minor monomer throughout the GI tract. Data showed that total procyanidins degraded progressively along the GI tract; however, the proportion of polymers remained unchanged. This suggests that depolymerization of procyanidins to monomers and the depolymerization of the polymers is minimal. This conclusion is in agreement with a recent study on the absorption of procyanidins from grape seed extract in rats (*24*).

The chromic oxide marker indicated that over 80% of the ingested procyanidins disappeared in the GI tract. Significant degradation occurred in the cecum and colon. Colonic microflora have been shown to cause complete degradation of polymeric procyanidins in vitro in 48 h. The degradation products included phenylacetic, phenylpropionic, and phenylvaleric acids (*25*). Consistent with this observation, we detected 12 phenolic acids in rat urine. The excretion of phenolic acids increased with the level of procyanidins in the diet and was more than 100-fold higher than that of catechins. Five phenolic acids detected in urine were also the natural components of sorghum bran, and they may also be absorbed. However, the amount of phenolic acids ingested directly from sorghum bran appeared to be

negligible in the total urinary excretion. For example, the rats consuming a diet with 40% sorghum bran ingested about 0.1 μmol of 3-methoxy-4-hydroxybezoic acid and 0.01 μmol of caffeic acid or ferulic acid from sorghum bran daily. However, their excretions in the 24 h urine were 1.1, 0.8, and 1.0 μmol, respectively. Therefore, the microbial-derived phenolic acids are the predominant metabolites of procyanidins. Similar conclusions have been drawn by other research groups (*26*, *27*).

Phenolic acids contributed differently to the summed phenolic excretion. Among these, 3-methoxy-4-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid were the dominant compounds. Their excretion in urine increased significantly with the dose of sorghum bran in the diet. Of particular interest is 3-hydroxyphenylpropionic acid. Its excretion in the urine was extremely high in rats fed 20 or 40% sorghum bran (**Figure 5B**). On the other hand, the excretion of 4-hydroxyphenylacetic acid was high in rats fed no or low amounts of sorghum bran, but its excretion decreased in rats fed a higher amount of sorghum bran (20 and 40%) (**Figure 5A**). The high excretion of 3-hydroxyphenylpropionic acid is consistent with previous studies. Deprez et al. showed that 3-hydroxyphenylpropionic acid was the predominant product when the polymeric procyanidins were incubated with human colon microflora (*25*). It was also found as a major metabolite in the urine of rats fed wine polyphenols and humans who consumed grape seed extracts (*27*, *28*).

It is important to point out that although phenolic acids are the major metabolites of procyanidins and other polyphenols, they can also originate from other sources endogenously. The endogenous phenolic acids have not been stressed in any previous studies. Phenolic acids can originate from tyrosine or phenylalanine in the gut (*29*). Tyrosine can be degraded by gut microflora to 4-hydroxyphenylacetic acid, which can be converted to 3,4-dihydroxyphenylacetic acid by P450 enzymes. Tyrosine loses an amine group to form the 4-hydroxyphenylpropionic acid, which can be converted into 3,4-dihydroxyphenylpropionic acid. However, the amounts of these two phenolic acids were extremely low both in serum and in urine. Possible explanations are that (i) tyrosine deamination is a minor process in the gut or (ii) they are converted to 4-hydroyxbenzoic acid and 3,4-hydroxybenzoic acid after β-oxidation in the cell. The 3,4-hydroxybenzoic acid can be methylated into 3-methoxy-4-hydroxybenzoic acid in the cell by catechol-*O*-methyltransferase (*30*). Indeed, the 3,4-dihydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid were present at high concentrations in the serum. Thus, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, and 3,4-dihydroxybenzoic acid are primarily endogenous metabolites of amino acids. Serum concentrations of those phenolic acids were high in rats fed the high dose of sorghum bran, suggesting that polyphenols also contributed to a portion of these metabolites in the serum. On the other hand, 3-hydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid are primarily exogenous metabolites of polyphenols. They were the dominant ones excreted in the urine. Their excretion showed clear dose-dependent increases. These data suggest that the kidney can differentiate between phenolic acids produced endogenously from amino acids and phenolic acids that originated from polyphenols. Exogenous phenolic acids were excreted more efficiently from the body. This explains why the excretion of amino acid-derived phenolic acids did not increase with the dose of sorghum bran in the diet. It also explains the higher excretion of 4-hydroxyphenylacetic acid in rats fed no sorghum bran and the decreased excretion in rats fed a high dose of sorghum bran (20 and 40%). This may be associated with the decrease in digestibility of protein and tyrosine. Polymeric procyanidins have been well-known to bind proteins in the gut (*31*). However, the serum concentration of 4-hydroxyphenylacetic acid increased with the level of sorghum bran in the diet. The mechanism related to this was not clear.

Polyphenols and phenolic acids can be further degraded by gut microflora into benzoic acid. Benzoic acid can be conjugated with glycine in the cell to yield hippuric acid. It has been shown that urinary excretion of hippuric acid increased in human or animals after ingestion of polyphenols (*17*, *18*). In rats fed 0, 5, and 10% sorghum bran, the hippuric acid excretion was higher than the summed phenolic acids. Hippuric acid excretion decreased in rats fed 20 and 40% sorghum bran and was much lower than the summed phenolic acids. This unusual change in hippuric acid production and excretion suggests an alteration in the gut microflora population. Smith and Mackie (*32*) reported that procyanidins altered bacterial populations in the rat GI tract after 3 weeks of feeding, resulting in a shift in the predominant bacteria toward tannin-resistant Gram-negative *Enterobacteriaceae* and *Bacteroides* species. Our data suggested that alteration of the bacterial population may lead to a decrease in benzoic acid production and an increase in 3-hydroxypropionic acid production.

Procyanidins have been reported to be effective in preventing cancers and cardiovascular diseases (*12*). It is not known which metabolites have led to such effects. Epicatechin has been implied to be a major bioactive molecule for procyanidins in cocoa (*33*); however, the much more abundant phenolic acids may also be important. Indeed, phenolic acids have been shown to inhibit cyclooxygenase-2 and inhibit the proliferation of colon cancer cells (*34*, *35*), although it is worth noting that excreted phenolic acids that are high in the urine are not necessarily high in the circulation.

On the basis of diet consumption of the rats (20 g/day), approximately 0.7−1.4% (w/w) of ingested catechins was excreted in the 24 h urine in the form of catechin and 3′-*O*-methylcatechin. It should be noted that our data could not distinguish if absorbed catechins were from monomeric catechins or procyanidins. About 0.4−11% (w/w) of ingested total procyanidins was excreted in the 24 h urine in the form of phenolic acids and hippuric acids. About 80% (w/w) of the procyanidins disappeared in the GI tract on the basis of the nonabsorbable marker. Therefore, there are still over 50% (w/w) of the procyanidins unaccounted. This fraction of procyanidins may be metabolized into other phenolic acids or hydroxyphenyl-γ-valerolactone, which were not analyzed in our study, or some other unknown metabolites. A large range of fractional excretion of catechin had been reported in the literature. For example, one study reported that only 4.6% of the radioactivity was recovered in 24 h urine after rats were orally administered ^{3}H-labeled catechin (*36*), a ratio similar to our observation, whereas in another study, 32−43% of a catechin dose was reported to be excreted in 24 h urine in rats after a single dose of grape seed extract (*16*). The discrepancy may be due to the differences in the nature of the diet and feeding regiment (acute vs long-term).

In summary, catechins and procyanidins in sorghum bran were absorbed as catechin and 3′-*O*-methylcatechin in rats. The predominant metabolites are microbial-derived phenolic acids. 3-Hydroxyphenylacetic acid and 3-hydroxypropionic acid appeared to be unique metabolites of polyphenols. The excretion of metabolites in urine increased dose dependently. Depolymerization of procyanidins was not apparent throughout the GI

tract. Procyanidins were degraded by the gut microflora to form phenolic acids.

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