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Metabolites in Contact with the Rat Digestive Tract after Ingestion of a Phenolic-Rich Dietary Fiber Matrix

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ABSTRACT: Grape antioxidant dietary fiber (GADF) is a phenolic-rich dietary fiber matrix. The aim of this work was to determine which phenolic compounds come into contact with colonic epithelial tissue after the ingestion of GADF. By use of HPLC-ESI-MS/MS techniques phenolic metabolites were detected in feces, cecal content, and colonic tissue from rats. Free (epi)catechin (EC) was detected in all three sources, and more than 20 conjugated metabolites of EC were also detected in feces. Fourteen microbially derived phenolic metabolites were also identified in feces, cecal content, and/or colonic tissue. These results show that during transit along the digestive tract, proanthocyanidin oligomers and polymers are depolymerized into EC units. After ingestion of GADF, free EC and its conjugates, as well as free and conjugated microbially derived phenolic metabolites, come into contact with the intestine epithelium for more than 24 h and may be partly responsible for the positive influence of GADF on gut health.

KEYWORDS: grape antioxidant dietary fiber, polyphenols, proanthocyanidins, epicatechin, metabolites, bioavailability, mass spectrometry, feces, cecal content, colonic tissue

INTRODUCTION

There is mounting evidence that gut physiological status, considered to be the result of the interaction between the epithelial tissue and its associated microbiota, decisively influences the health of whole organisms.[1] In particular, intestinal microbiota may modulate the incidence of certain kinds of cancer such as colon cancer[2] and possibly other cancers that affect organs far from the intestinal tract through the synthesis and absorption of bioactive molecules. Similarly, the recently proposed "gut−heart axis" hypothesis suggests that metabolites formed in the colon may play a significant role in the prevention of cardiovascular disease.[3] Therefore, food components and their metabolites that come into contact with the gut wall and interact with the intestinal microbiota for up to several hours may exert an influence on an organism's overall health that is more important than previously thought.[4]

Dietary fiber is of particular interest in this respect because it is transported largely unaltered along the small intestine all the way to the colon, where it is partially hydrolyzed and absorbed. Dietary fiber consists of two fractions: soluble dietary fiber is constituted mainly of soluble pectins, β-glucans, and gums and may be fermented by intestinal microbiota, thus releasing several beneficial short-chain fatty acids; insoluble dietary fiber is constituted of insoluble pectins, lignin, cellulose, and hemicelluloses and mainly contributes to the bulking effect of dietary fiber.

Interestingly, certain dietary fiber matrices carry putatively bioactive functional components embedded in them, antioxidant compounds in particular, which are gradually released in the intestinal lumen and partly absorbed into gut epithelial cells;[5] such matrices have been called antioxidant dietary fibers.[6] Oligomeric and polymeric proanthocyanidins (PAs) are major components associated with the fiber matrix (celluloses and hemicelluloses) through either weak (hydrophilic/hydrophobic)[7,8] or possibly strong (covalent) interactions[9] and are an object of microbial degradation. The metabolic transformation of PAs appears to be mediated by several bacterial species[10] and to yield the same main metabolites in both rats and humans.[11,12]

Grape antioxidant dietary fiber (GADF), a byproduct of wine production, is a model of a phenolic-rich dietary fiber matrix. Whereas some of the PAs present in GADF are associated with the fiber matrix through weak interactions, and therefore can be extracted by solvents (extractable PAs, or EPAs), most of them remain associated with the fiber matrix after extraction (nonextractable PAs, or NEPAs).[13] Studies in rats have shown that GADF exerts several protective effects on colonic health, including modulation of colonic mucosa apoptosis, modifications in colonic crypts, and increase of cecal free radical scavenging capacity.[14−16] Grape pomace, which is quite similar to GADF, has also been shown to stimulate the growth of beneficial *Lactobacillus acidophilus*.[17]

In previous work[18] we described the metabolic fate of GADF in rat urine, showing that some EPA components are partially depolymerized during transit through the intestinal tract. Furthermore, we reported that the bulkier polymers (NEPAs and the remaining EPAs) are degraded by the intestinal microbiota into smaller compounds such as phenolic acids, which pass into the bloodstream and are finally excreted in urine. As both dietary fiber and phenolic-rich materials have been related to the

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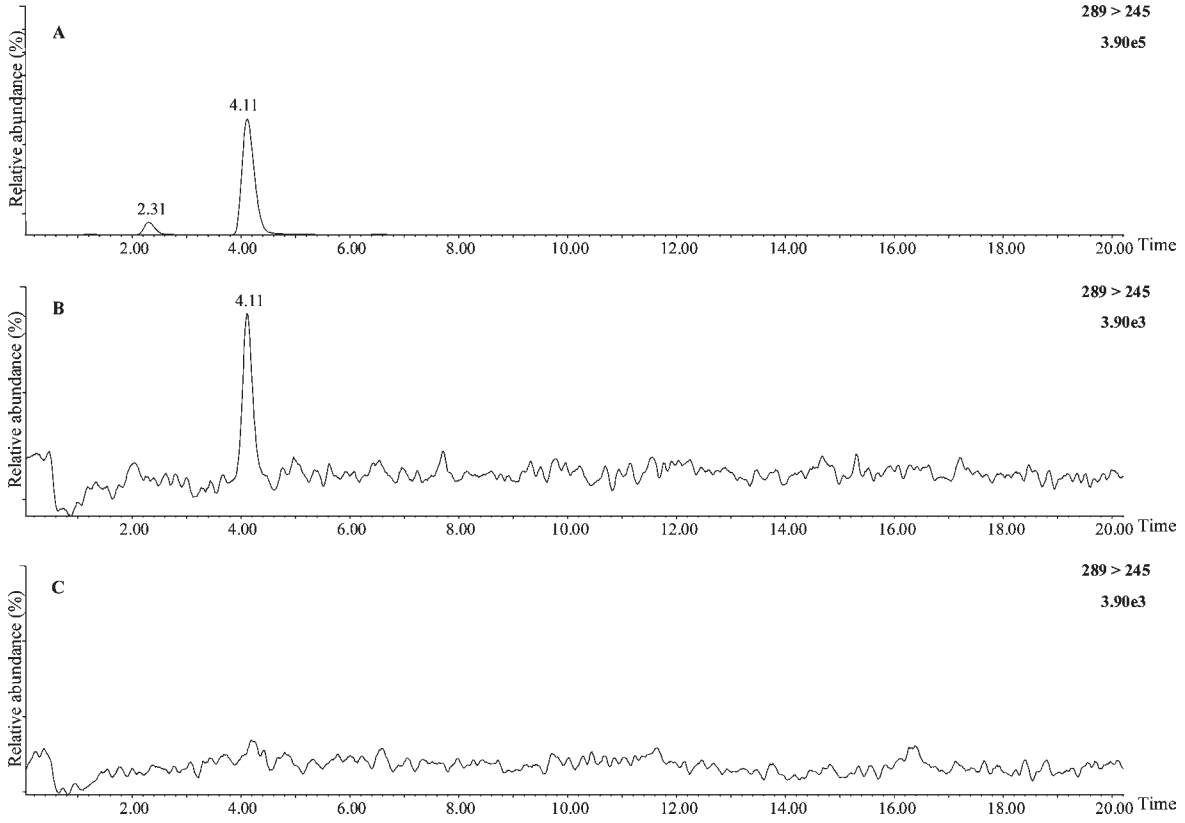

Figure 1. HPLC-ESI-MS profile corresponding to the detection of EC in colonic tissue samples from rats fed (A) EC, (B) GADF, or (C) water. Detection was by multiple reaction monitoring (MRM), transition 289 → 245.

prevention of colorectal cancer[19,20] and may indirectly affect other types of cancer by modulating the gut microbiota, it is important to study the metabolites in feces, cecal content, and colonic tissue as an indication of the type of compounds that come into contact with the colonic epithelial tissue for several hours after GADF ingestion. A number of previous studies have examined the metabolic fate of polyphenols in urine and plasma samples, but they did not directly search for the metabolites within the materials that come into contact with the colon or that can be found in the colonic tissue itself.

In the present work we examine the phenolic composition of feces, cecal content, and colonic tissue in rats 24 h after GADF ingestion. Polyphenols and their metabolites were identified by a combination of mass spectrometry (MS) modalities (multiple reaction monitoring, or MRM, and product ion scan MS/MS experiments) on a triple-quadrupole apparatus. We provide new information regarding components that are in contact with the gut wall and may partly explain the activity of GADF on epithelial cells and on the intestinal microbiota. This will help to complete our understanding of the influence that intestinal transformations of polyphenols have on intestine health and on the status of other parts of the organism.

MATERIALS AND METHODS

Chemicals and Reagents. GADF was obtained from red grapes ('Cencibel' variety) harvested in the vintage year 2005 in the La Mancha region in Spain, as described in a published patent.[21] The percentage composition of GADF used in this work was as follows: dietary fiber, 73.48 ± 0.79 (soluble 15.53 ± 0.11, insoluble 57.95 ± 0.78); polymeric PA associated with insoluble dietary fiber, 14.81 ± 0.19 (measured by the cyanidin method[22]); fat, 7.69 ± 0.49; protein, 11.08 ± 0.46; ash, 5.25 ± 0.19. (−)-Epicatechin (EC) monomer content in GADF was <0.01%.[18] More than 100 phenolic compounds (not associated with dietary fiber) have been detected in GADF.[23] Standards of EC (≥97%), 3- and 4-hydroxyphenylacetic acid (≥98%), 3,4-dihydroxyphenylacetic acid (≥98%), 3- and 4-hydroxybenzoic acid (≥97%), vanillic acid (≥97%), caffeic acid (≥95%), 3,4-dihydroxyphenylpropionic acid (>98%), 4-hydroxyphenylpropionic acid (>98%), protocatechuic acid (≥97%), caffeic acid (≥98%), ferulic acid (≥99%), isoferulic acid (≥97%), *p*-coumaric (≥98%), *m*-coumaric (≥97%), and taxifolin (≥85%) were obtained from Sigma-Aldrich (St. Louis, MO). Methanol (analytical grade) and 37% hydrochloric acid were purchased from Panreac (Castellar del Vallès, Barcelona, Spain). Acetonitrile (HPLC grade) and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified by using a Milli-Q plus system from Millipore (Bedford, MA) to a resistivity of 18.2 MΩ cm.

Animal Experiments. Female Sprague−Dawley rats (SD, n = 12, body weight = 233 ± 9.3 g, 12 weeks of age) provided by Harlan Interfauna Ibérica SL (Barcelona, Spain) were fed a polyphenol-free diet (TD94048) (from Harlan Interfauna Ibérica SL) and maintained in plastic cages at room temperature (22 ± 2 °C) and 55 ± 10% relative humidity, with a 12 h light/dark cycle for 1 week, in accordance with European Union regulations. The experimental design was essentially the same as that used in our previous study of urine metabolites.[18] The rats were divided into three groups (n = 4) (GADF group, EC group, and control group). The number of animals per group was adequate for qualitative and semiquantitative comparative purposes. Because most PA metabolites are similar to those derived from EC and due to the absence of commercial standards for them, the EC group was included in

Table 1. (Epi)catechin and Conjugated Metabolites in Feces from Rats Fed GADF[a]

metabolite[b]	MRM[c] parent	MRM daughter	MS/MS ions	relative area[d] (%)
(−)-epicatechin[e,f]	289 → 245			16.1
monoconjugated metabolites				
Gluc- EC-1[e]	465 → 289	289 → 245	465; 289; 113	25.7
Gluc- EC-2[e]	465 → 289	289 → 245	465; 289; 245; 205; 143; 125,113	3.2
Gluc- EC-3	465 → 289	289 → 245	465; 327; 289; 245; 203; 175; 151; 113	1.6
Sulf- EC-1[e]	369 → 289	289 → 245	369; 289; 245; 217; 205; 203;151; 137	2.6
Sulf- EC-2[e]	369 → 289	289 → 245	369; 289; 245; 231; 203; 179; 151;137	6.9
Sulf- EC-3	369 → 289	289 → 245	369; 289; 245; 231; 203;179; 151; 137	5.0
GHS EC	594 → 289	289 → 245		0.6
diconjugated metabolites				
Me-Gluc-EC[e]	479 → 303		479.0; 375; 313;303;285;240;235;113	0.2
Me-Sulf-EC-1[e]	383 → 289		383; 321; 303; 285; 217; 137	5.1
Me-Sulf-EC-2[e]	383 → 289		383; 303; 285; 270; 259; 244; 217; 202; 165; 151; 137	1.1
Me-Sulf-EC-3[e]	383 → 289		383; 303; 289; 285; 259; 244; 219; 204; 179; 137	4.9
Me-Sulf-EC-4[e]	383 → 289		383; 303; 285; 259; 245; 219; 204; 165; 137	5.0
di-Gluc-EC	641 → 289			0.4
di-Me-EC	318 → 289			16.6
triconjugated metabolites				
di-Me-Sulf-EC	387 → 289		387;369; 307; 289; 263; 245; 161	1.4
di-Me-Gluc-EC[e]	493→ 289		493.0; 469; 379; 303; 285; 267; 259; 233; 199; 137	0.5
Me-Gluc-Sulf-EC-1[e]	559 → 289		559;313; 289; 231	0.1
Me-Gluc-Sulf-EC-2	559 → 289		625; 335; 289; 193; 175; 159; 113	0.2
Gluc-disulf-EC	625 → 289		625; 335; 289; 193; 175; 159	2.0
di-Gluc-Me-EC-1	655 → 289	289 → 245		0.2
di-Gluc-Me-EC-2	655 → 289	289 → 245		0.4
tri-Sulf-EC[e]	529 → 289			0.3

[a] Metabolites detected only in the GADF group or detected as signals at least 10-fold stronger than those in the control group. [b] EC, (epi)catechin; GHS, glutathione; Gluc, glucuronidated; Me, methylated; Sulf, sulfated. [c] MRM, multiple reaction monitoring. [d] Peak areas from the chromatograms generated by MRM experiments. [e] Metabolites also detected in urine from rats fed GADF.[18] [f] Compound identified by retention time of a standard.

the study to clearly identify the metabolite signals. The animals were housed individually in metabolic cages, and after food deprivation for 12 h with free access to water, the rats were administered the corresponding feed by oral gavage as follows: GADF group, a saturated GADF suspension in tap water (1 g in 6 mL) as a single dose of 1.6 g/kg body weight; EC group, an EC solution in tap water (1 g in 6 mL) as a single dose of 1 g/kg body weight; control group, tap water as a single dose of 10 mL/kg body weight. To facilitate the detection of PA metabolites, the dose of GADF was the highest possible considering both the recommended volume administered via oral gavage on laboratory animals and the dispersibility of GADF in water. Feces were collected over a period of 24 h after administration. Then the animals were killed by an overdose of anesthesia (isoflurane gas) and the colons removed. The cecal contents were collected and weighed, and the colonic wall was washed three times with saline solution (0.9% NaCl in water) to eliminate any residue of cecal contents and weighed. The samples were stored at −80 °C until extraction and analysis. These experimental protocols were approved by the Experimental Animal Ethical Research Committee of the University of Barcelona (Permission DMA3123) in accordance with current regulations for the use and handling of experimental animals.

Sample Processing. The colonic tissue samples (500 mg) were homogenized in 4 mL of cold phosphate-buffered saline (PBS, 0.1 M, pH 7) using an Ultraturrax homogenizer (IKA, Staufer, Germany). After centrifugation (3000*g*, 10 min) and collection of the supernatant, acetonitrile (1.5 mL) was added to precipitate interfering proteins. The pellet was removed and the internal standard (taxifolin, 50 ppm final concentration) added. Samples were concentrated by nitrogen stream, and the residue was taken up in water (1.5 mL) and vortexed.

Samples were then subjected to solid-phase extraction (SPE) to isolate phenolic compounds. An Oasis HLB cartridge from Waters (Milford, MA) was activated with 1 mL of methanol and 2 mL of 1 mM HCl. To remove interfering components, the sample was washed with 9 mL of acid water. Then, samples were eluted with 1 mL of methanol. The eluate was evaporated under nitrogen and the residue reconstituted with 1 mL of 5% methanol in water. The temperature of evaporation was kept under 30 °C to avoid deterioration of the phenolic compounds. The samples were then filtered through a polytetrafluoroethylene (PTFE) 0.45 μm membrane from Waters into amber vials for HPLC-MS/MS analysis.

In the case of feces and cecal content, 500 mg of sample was homogenized in 4 mL of cold PBS using an Ultraturrax homogenizer and centrifuged at 3000*g* for 10 min. Then the same SPE procedure was followed as for the colonic tissue samples. The number of replicates was limited by the amount of sample available (colonic tissue, feces, and cecal content). Four replicate samples (one from each animal) were processed per group.

HPLC-ESI-MS/MS Analysis. A Waters Quattro LC triple-quadrupole mass spectrometer with an electrospray source was used in negative mode to obtain MS and MS/MS data. Liquid chromatography was performed on an Alliance 2695 system from Waters equipped with a Phenomenex (Torrance, CA) Luna C18 (50 × 2.1 mm i.d.) 3.5 μm particle size column and a Phenomenex Securityguard C18 (4 × 3 mm i.d.)

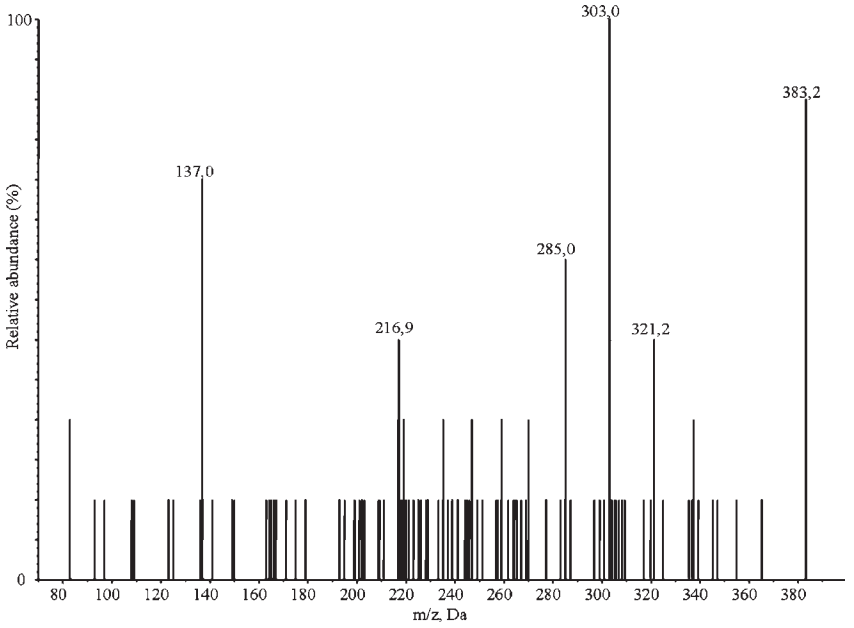

Figure 2. HPLC-ESI-MS/MS product ion scan spectrum of Me-Sulf-EC-1 (m/z 383).

column. Gradient elution was performed with a binary system consisting of (A) 0.1% aqueous formic acid and (B) 0.1% formic acid in acetonitrile. The following increasing linear gradient (v/v) of B was used [t (min), %B]: 0, 8; 10, 23; 15, 50; 20, 50; 21, 100. This was followed by a re-equilibration step.

Metabolites in feces, cecal content, and colonic tissue were identified by (i) MRM transitions of the putative metabolites using a dwell time of 100 ms and (ii) product ion scan MS/MS experiments. The cycle time used was 1.11 s. Cone energy and collision energy in MRM mode were optimized for each group of metabolites: 30 V and 10 eV for taxifolin, 30 V and 15 eV for phenolic acid derivatives, 30 V and 20 eV for EC and PA dimers, and 40 V and 20 eV for EC derivatives. Capillary energy was 2.8 kV. Source temperature and desolvation temperature were 150 and 350 °C, respectively.

The analytical strategy adopted to analyze the samples has been fully described previously.[18] Briefly, the metabolic outcome of GADF in rat feces, cecal content, and colonic tissue was expected to be mainly oligomeric and polymeric EC, so that the fragmentation patterns obtained with pure EC would help to assign the weaker signals from GADF. The search was focused on free EC, conjugated EC metabolites (glucuronidyl, methyl, and sulfate derivatives) and microbially derived phenolic metabolites, mainly phenolic acids (free and conjugated) from intestinal fermentation of EC and PAs.

RESULTS

EC and Its Phase II Metabolites. Free EC (MRM transition 289 → 245) was detected in feces, cecal content, and colonic tissue from the GADF group. Figure 1B shows the HPLC-ESI-MS profile in MRM mode corresponding to the detection of free EC in the colons of the GADF group. No signals corresponding to EC dimers (577 → 289) or to EC dimers with an open ring (581 → 289) were detected in any of the samples.

Most EC in the fecal samples was present as several derivatives, indicating that after absorption (either in the small intestine or in the colon) and hepatic conjugation, some of these metabolites returned to the colon via bile.[24] The conjugates we found included mono-, di-, and triconjugated derivatives resulting from combinations of glucuronidyl, methyl, and sulfate moieties. Table 1 lists all of the EC conjugates detected by HPLC-MS and HPLC-MS/MS in feces from the GADF group. The relative areas of the corresponding MS peaks are given as a preliminary estimation of their relative concentrations. Monoconjugated metabolites detected in the feces of the GADF group included three glucuronidated metabolites (465 → 289) previously detected in urine and three sulfated metabolites of EC (369 → 289), one of them not detected in urine.[18] Additionally, a monoconjugated metabolite with glutathione (GHS) (594 → 289) was detected. No monomethylated EC metabolites were detected in feces. With regard to diconjugated metabolites, the four Me-Sulf-EC metabolites (383 → 289) and the Me-Gluc-EC derivative (479 → 303) previously detected in urine[18] were also identified in feces. Two other peaks were tentatively assigned to other diconjugated (diglucuronidated and dimethylated) metabolites (with MRM transitions of 641 → 289 and 318 → 289, respectively). Several triconjugated metabolites were also detected in feces. These demonstrated the extensive phase II metabolization suffered by EC after absorption. They included EC conjugated with two methyl and one sulfate moieties (di-Me-Sulf-EC) (387 → 289), an EC conjugated with two methyl and one glucuronide moieties (di-Me-Gluc-EC) (493 → 289), a dimethylated glucuronidated sulfated derivative (559 → 289), a glucuronidated disulfated conjugate (625 → 289), and two diglucuronidated methylated derivatives (655 → 289). Additionally, trisulfated EC (529 → 289), previously detected only in urine from the EC group,[18] was identified, although it could not be confirmed either by a second MRM transition or MS/MS fragments. EC metabolites were not detected in either the cecal content or the colonic tissue of the GADF group. Comparison of the MS/MS fragmentation patterns confirmed that some of the metabolites detected in feces were those previously detected in urine. Thirteen EC conjugates detected in the feces of the EC group and seven in their cecal content were not detected in the GADF group.

The MS/MS fragments of EC metabolites (Table 1) provided supporting information on their structures. Besides the characteristic

Table 2. Microbially Derived Phenolic Metabolites in Feces, Cecal Content, and Colonic Tissue from Rats Fed GADF[a]

metabolite	MRM	compound confirmation	feces	relative area[b] (%)	colonic tissue	relative area[b] (%)	cecal content	relative area[b] (%)
valerolactones								
3- or 4-hydroxyphenylvalerolactone	191 → 147	second transition: 191 → 106	X	1.0				
dihydroxyphenylvalerolactone	207 → 163	MS/MS ions: 207; 163; 148; 121; 109	X	47.0			X	49.4
lignans								
enterolactone	297 →253	MS/MS ions: 297; 253; 189; 165; 145; 133; 121; 107	X	25.2				
phenylvaleric acids								
3- or 4-hydroxyphenylvaleric acid[c,d]	193 → 175	MS/MS ions: 193;175;147; 119;107	X	0.3			X	3.9
dihydroxyphenylvaleric acid[e]	209 → 137	MS/MS ions: 209; 163; 144; 117	X[d]	0.04				
phenylpropionic acids								
Sulf-3,4-dihydroxyphenylpropionic acid[c,e]	261 → 181	MS/MS ions: 261; 199; 185; 137	X	0.4				
phenylacetic acids								
3-hydroxyphenylacetic acid[c,d]	151 → 107	standard retention time	X	0.3	X	50.0		
4-hydroxyphenylacetic acid	151 → 107	standard retention time	X	0.2				
homovanillic acid[e]	181 → 121	standard retention time	X	3.4			X	15.7
benzoic acids								
gallic acid[e]	169 → 125	standard retention time	X	13.0			X	30.9
4-*O*-Me-gallic acid[e]	183 →169	MS/MS ions: 183; 124; 106; 95; 78	X	0.2				
sulf-3 or 4-hydroxybenzoic acid	217 → 137	MS/MS ions: 217; 137; 93	X	0.03				
cinnamic acids								
caffeic acid[c]	179 → 135	standard retention time	X	2.4				
m-coumaric acid	163 → 119	standard retention time	X	5.8	X	29.9		
p-coumaric acid[c,e]	163 → 119	standard retention time	X	0.7	X	20.0		

[a] Metabolites not detected in control group or detected at a concentration at least 10-fold higher. [b] Peak areas from the chromatograms generated by MRM experiments. [c] Previously detected in urine from rats fed GADF.[18] [d] Previously detected in supernatant from in vitro fermentation of GADF.[45] [e] Metabolites not detected in samples from rats fed EC.

glucuronide fragment at m/z 113, Gluc-EC-3 yielded a fragment at m/z 327 corresponding to a B-ring fragment plus the glucuronide moiety resulting from retro-Diels−Alder fission of the EC ring and therefore indicating that glucuronidation took place on the B-ring. Sulf-EC-1 yielded sulfated fragments from the A-ring at m/z 217 and 137, whereas the fragment detected at m/z 231 from Sulf-EC-2 and Sulf-EC-3 indicated that in these two metabolites sulfation took place on the B-ring. With regards to the disubstituted metabolites, Me-Sulf-EC-1 and Me-Sulf-EC-2 yielded fragments at m/z 217 and 137, which correspond to sulfation on the A-ring. This fragmentation can be seen in Figure 2, which shows the MS/MS spectrum for Me-Sulf-EC-1. In contrast, Me-Sulf-EC-4 yielded fragments at m/z 245 and 165, corresponding to a B-ring fragment with Me and Sulf moieties and to a B-ring fragment with Me substitution, respectively; this indicates that these substitutions took place on the B-ring.

Microbially Derived Phenolic Metabolites. Both nonconjugated and conjugated microbially derived phenolic metabolites were identified in feces from the GADF group, whereas nonconjugated metabolites were present in their cecal content and colonic tissue. Free microbially derived metabolites come from colonic fermentation of PAs, whereas the conjugated metabolites come from free metabolites that have previously been absorbed, conjugated in the liver, and excreted via bile. Table 2 shows the complete list of microbially derived phenolic metabolites identified in the feces, cecal content, and colonic tissue of the GADF group, including their relative areas from the chromatograms generated by MRM experiments. The assignments were confirmed by the use of standards and/or product ion scan experiments and corresponded to species that were either not detected in control animals or detected at a concentration at least 10-fold lower. We detected some phenolic acids described in the literature as being metabolites of EC (e.g., 3-HBA) that are not included in the list because the signals they yielded were not significantly stronger than those from the control samples obtained from nonsupplemented animals.

Fourteen microbially derived phenolic metabolites were detected in the feces of the GADF group, only 8 of which were detected in the rats fed monomeric EC. The most abundant free phenolic acids previously detected in urine[18] were also detected in feces, including direct products of fermentation, such as hydroxyphenylvaleric acid (193 → 175) and hydroxyphenylacetic acid (151 → 107), as well as acids derived from further transformations in the liver, such as caffeic acid (179 → 135) and

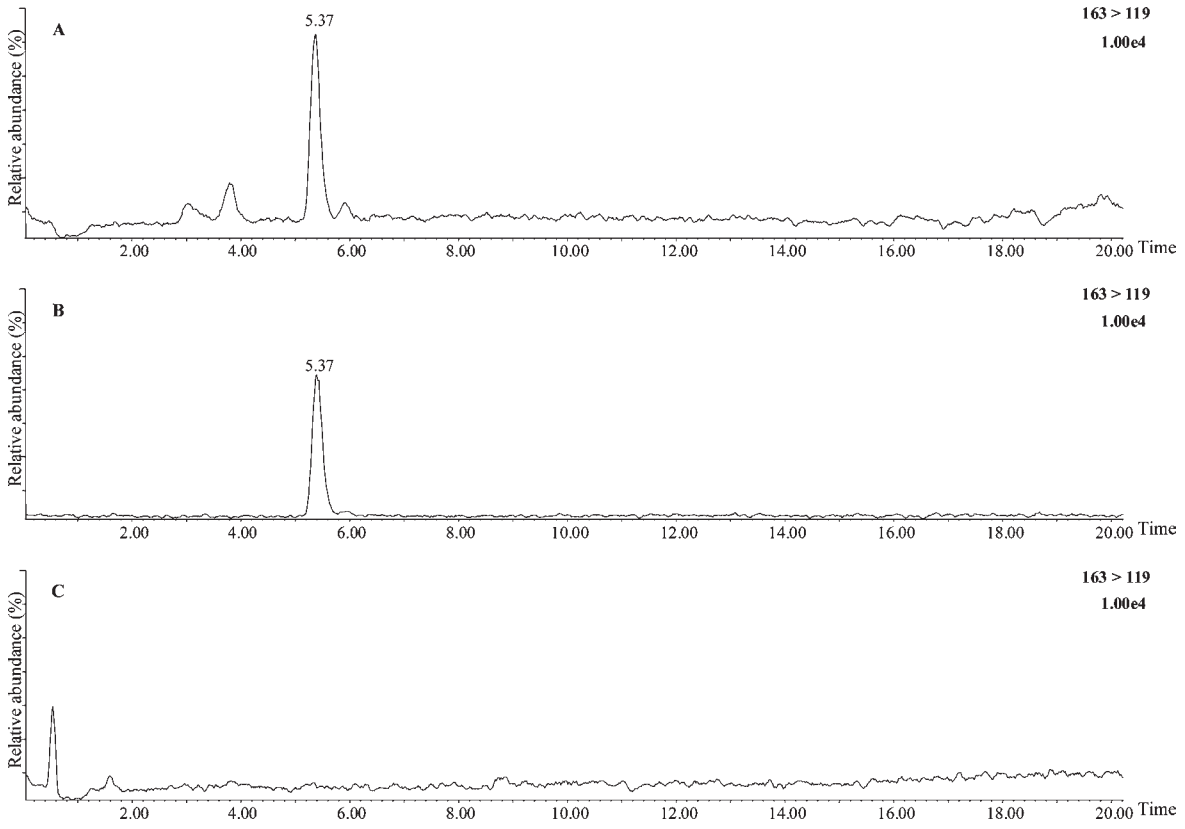

Figure 3. HPLC-ESI-MS profile corresponding to (A) *p*-coumaric acid standard, (B) feces from rats fed GADF, and (C) feces from rats fed EC. Detection was by multiple reaction monitoring (MRM), transition 163 → 119.

m-coumaric acid (163 → 119). Microbial metabolites were excreted via bile after conjugation, as shown by the detection of the derivatives Sulf-3,4-dihydroxyphenylpropionic acid (261 → 181) and Sulf-3- or 4-hydroxybenzoic acid (217 → 137). The metabolites detected only in feces from the GADF group were dihydroxyphenylvaleric acid (209 → 137), Sulf-3, 4-dihydroxyphenylpropionic acid (261 → 181), homovanillic acid (181 → 121), *p*-coumaric acid (163 → 119) (Figure 3), gallic acid (169 → 125), and 4-*O*-methylgallic acid (183 → 169).

Four microbially derived phenolic metabolites, namely, dihydroxyphenylvalerolactone (207 → 163), 3- or 4-hydroxyphenylvaleric acid (193 → 175), homovanillic acid (181 → 121), and gallic acid (169 → 125), were detected in the cecal content of the GADF group, indicating that fermentation of PAs was still taking place 24 h after the intake of the fiber matrix. The nonconjugated phenolic acids 3-hydroxyphenylacetic acid (151 → 107), *m*-coumaric acid (163 → 119), and *p*-coumaric acid (163 → 119) were detected in colonic tissue from the GADF group.

One microbially derived phenolic metabolite detected in the feces of the EC group, five in their colonic tissue, and six in their cecal content were not detected in the GADF group.

MS/MS fragments helped to confirm the identity of some of the microbially derived metabolites. Dihydroxyphenylvalerolatone (*m*/*z* 251), for instance, generated fragments at *m*/*z* 207 and 163, which correspond to the successive loss of two CO_2 molecules. MS/MS spectra of Sulf-3- or -4-hydroxybenzoic acid (*m*/*z* 217) provided signals at *m*/*z* 137 and 93, corresponding to the successive loss of Sulf and CO_2.

Most of the metabolites reported in a given group were detected in all four animals in that group.

DISCUSSION

GADF is a model of a dietary-fiber-rich matrix with associated PAs. It contains a complex mixture of polyphenols including monomers, oligomers, and polymers of EC (PAs), anthocyanins, flavonols, and hydroxycinnamic acids. These are associated with a fiber matrix of both soluble and insoluble polymers such as polysaccharides and lignins, which may influence the absorption of the putatively bioactive GADF components.[13,23] GADF has been used as a supplement in several animal and human studies.[25] It has been shown to have positive effects on colonic health, probably through local action of the dietary fiber and PAs. Moreover, positive modulation of cardiovascular disease risk factors by GADF[13] may also be mediated by metabolites derived from colonic fermentation. We focus here on PAs as the most abundant phenolic constituents of GADF.[13]

After ingestion, PAs are believed to undergo two main processes in the intestinal tract: (1) partial depolymerization into their constituent units, for example, EC, mainly by the gastric milieu[26] coupled with absorption of the free monomers and small oligomers (dimers, trimers) in the small intestine;[27,28] and (2) direct extensive fermentation by the intestinal microbiota into smaller metabolites such as phenolic acids and their subsequent absorption.[29,30] The species absorbed are conjugated into glucuronidyl, methyl, or sulfate derivatives in the small intestine and liver and are transferred either to the bloodstream or back to the colon via the bile.[11,24,31] It is generally accepted that PAs are not depolymerized into their monomeric units in the intestinal tract prior to the microbial transformations. However, this assumption comes from studies of small oligomers or by

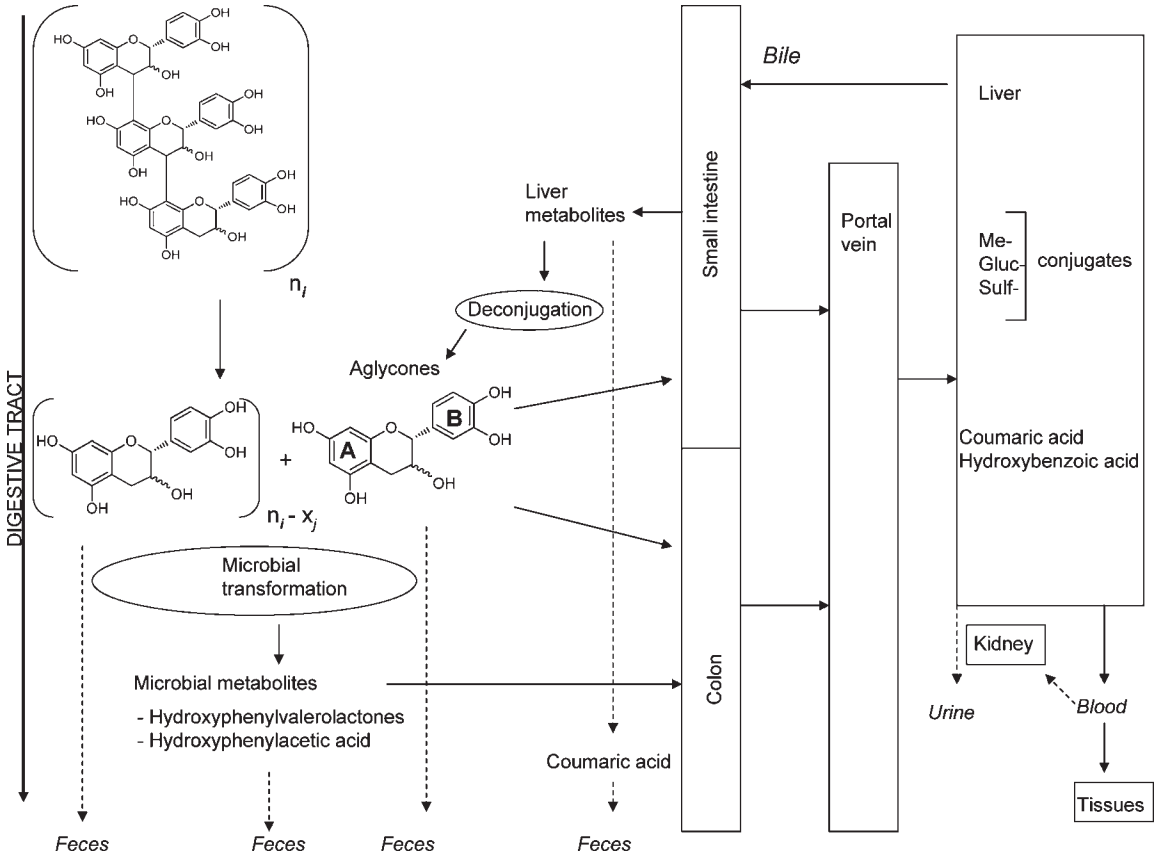

Figure 4. Metabolization of polymeric proanthocyanidins from GADF.

examining the microbial end products of purified oligomers.[11,29] Recently, we have provided indirect evidence that polymeric PAs are depolymerized in the intestinal tract,[18] because the wide variety of EC conjugates that were clearly detected in urine from rats fed GADF could not come only from the minute amounts of free EC present in GADF. By examining the metabolites in feces, cecal content, and colonic tissue, we present here further evidence that PAs are depolymerized during their transit along the intestinal tract. Furthermore, the present work provides novel information about PA-derived species in contact with the intestinal wall and within colonic tissue itself. This information may help to explain the local action of PAs in the colon as well as a possible prolonged systemic action of PA metabolites that results from the host organism and its associated microbiota, causing the slow release of polymers from the fiber matrix.

The feces of the GADF group contained a variety of EC phase II metabolites (Table 1). This corroborates the hypothesis that EC metabolites come from the depolymerization of oligomeric PAs and not only from EC monomers, because the intensities of the MS signals corresponding to EC metabolites are too high to come only from the small amounts (<0.01%) of monomeric EC in GADF.[23] The presence of the same metabolites in both urine and feces indicates that, once EC is conjugated in the liver, a fraction of the phase II metabolites passes into the bloodstream, whereas another fraction returns to the intestine via the bile. Conjugated forms of quercetin, another flavonoid, have been shown to have anti-inflammatory effects,[32] preventive effects on endothelial dysfunction,[33] and effects on apoptosis.[34] Similarly, at least some of the EC derivatives are expected to exhibit activities associated with intact EC, including scavenging activity.[18] No EC derivatives were detected in colonic tissue, indicating that there was no reabsorption of the conjugates. Nonconjugated EC was detected in feces, cecal content, and colonic tissue (Table 1; Figure 1). This again indicates that PAs from GADF are depolymerized and that the monomers that are released are absorbed during transit along the intestine and corroborates our previous suggestion[18] that intact EC is in contact with the intestinal tissue for hours after intake of GADF. Moreover, PAs in the process of being depolymerized must also expose free phenolic groups to the intestinal epithelium.

We also detected a variety of smaller phenolic species in the feces. These products of microbial fermentation included three groups of metabolites: (1) metabolites directly released from PA fermentation, such as 3- or 4-hydroxyphenylvalerolactone, dihydroxyphenylvalerolactone, 3- or 4-hydroxyphenylvaleric acid, dihydroxyphenylvaleric acid, 3- and 4-hydroxyphenylacetic acid, and homovanillic acid; (2) phenolic acids such as caffeic acid and *p*-coumaric acid derived from transformations in the liver of compounds from the aforementioned group after absorption; and (3) conjugated metabolites such as Sulf-3,4-dihydroxyphenylpropionic acid and Sulf-3- or -4-hydroxybenzoic acid generated in the liver by the action of phase II enzymes on compounds from group 1 after absorption. We detected more phenolic acids (e.g., dihydroxyphenylvaleric acid, Sulf-3,4-dihydroxyphenylpropionic acid, homovanillic acid, and *p*-coumaric acid) in the feces of the GADF group than in the feces of the EC group. This indicates that these compounds came from direct fermentation

of PA oligomers and polymers. Similar results were obtained by in vitro fermentation of procyanidin dimer B2 with human microbiota.[35] Most of the products of direct fermentation (group 1) detected in feces were also detected in urine,[18] which means that they are absorbed and bioavailable before being excreted. The metabolites with the longest carbon chains, corresponding to the initial stages of EC fermentation by ring cleavage of EC moieties, that is, hydroxyphenyvalerolactone and dihydroxyphenylvalerolactone, were not detected in urine. This means that these initial products of intestinal fermentation are quickly transformed into smaller units (phenylvaleric, phenylpropionic, and phenylacetic acids), by successive β-oxidations. *p*-Coumaric acid was detected in feces from the GADF group. Because *p*-coumaric acid is quickly absorbed in the small intestine,[36] we concluded that it came from PA fermentation and not directly from GADF. The intestinal metabolites would have been absorbed and further transformed in the liver, and the resulting small species (e.g., *p*-coumaric acid) would have been excreted back to the intestine via bile. Finally, nonconjugated gallic acid and its main metabolite 4-*O*-methylgallic acid were also detected in feces from the GADF group and not in those from the EC group, probably due to the release of gallic acid from gallate esters of catechins. We also detected phenolic acids derived from intestinal fermentation in the cecal content and colonic tissue of the GADF group, which indicates that the process of fermentation, release, and absorption of putatively bioactive compounds was still going on 24 h after the intake of the PA-rich fiber matrix.

The biological activities of the products of microbial fermentation of PAs have not yet been systematically tested, except for a few reports that products of colonic degradation of flavonoids exhibit anti-inflammatory effects[37,38] and antioxidant capacity.[39] It has been pointed out that the scavenging effect of polyphenols in vivo is negligible because they suffer extensive conjugation and subsequent excretion. Whereas studies of the capacity of polyphenols to modify the redox homeostasis of a complex living organism have provided contradictory results,[40−42] local concentrations of intact polyphenolic substructures (catechols, pyrogallols, gallates), for example, in the intestine, may have a significant redox effect, which may be free radical scavenging or generating, depending on the nature and concentration of the polyphenol.[43,44] This might be the case with GADF, which is the object of extensive transformations in the intestinal tract, which include depolymerization into EC units and ring cleaving that lead to smaller phenolic structures, resulting in the release of a large number of putatively bioactive substances over a long period of time.

The picture that arises from our results on GADF metabolization (Figure 4) is that of PA polymers being gradually cleaved into monomeric ECs and smaller phenolics along the intestinal tract for more than 24 h after ingestion. This slow and persistent process provides a variety of phenolics, both free and conjugated, that are in contact with the intestinal epithelial tissue, as proven by the different EC conjugates and microbially derived metabolites found in the feces, cecal content, and/or colonic tissue of rats after GADF supplementation. Many of these phenolic compounds can be absorbed and may exert their action in the colon as well as in other target tissues after absorption.

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ABBREVIATIONS USED

EC, (epi)catechin; EPA, extractable proanthocyanidin; GADF, grape antioxidant dietary fiber; GHS, glutathione; Gluc, glucuronide; Me, methyl group; MRM, multiple reaction monitoring; NEPA, nonextractable proanthocyanidin; PA, proanthocyanidin; PBS, phosphate-buffered saline; PTFE, polytetrafluoroethylene; Sulf, sulfate.

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