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ARTICLE

Phenolic Metabolites of Grape Antioxidant Dietary Fiber in Rat Urine

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Grape antioxidant dietary fiber (GADF) combines the putative health benefits of fiber and polyphenols. Polyphenolic metabolites may play a key role in the overall biological effects of this supplement. We identified phenolic GADF metabolites in rat urine at different times after oral administration, using HPLC−ESI-MS/MS techniques. The phenolic metabolic outcome of GADF is essentially an array of mono- and polyconjugated epicatechins and free or conjugated smaller phenolic acids, some of them never reported before. We have detected 18 mono-, di-, and triconjugates of epicatechin with glucuronide, methyl and sulfate moieties and small phenolic acids both free and conjugated. The results suggest that the procyanidin oligomers are both depolymerized in the digestive tract into epicatechin conjugates and degraded by the colonic microbiota into phenolic acids and their conjugates. For several hours after ingestion of GADF, a great variety of phenolic species, including some with an intact catechol group, are in contact with the digestive tract tissues before, during and after metabolization, and many of them are systemically bioavailable before being excreted.

KEYWORDS: Grape antioxidant dietary fiber; polyphenols; procyanidins; epicatechin; metabolites; bioavailability; mass spectrometry; urine

INTRODUCTION

Dietary fiber is believed to play an important role in maintaining the functional integrity of the gastrointestinal tract. A high fiber intake is associated with body weight control (*1*) and a reduced risk of diseases such as colon cancer (*2*) and atherosclerosis (*3*). However, not all fibers are equally good, and the benefits of a fiber-rich diet greatly depend on the solubility and fermentability of the fiber as well as on other functional constituents, particularly antioxidant polyphenols (*4*, *5*). Polyphenols are scavengers of reactive oxygen species (ROS) and appear to interact with biologically significant proteins.

Grape antioxidant dietary fiber (GADF) is a particularly interesting nutritional supplement obtained from grape pomace (*6*). GADF is superior to other fibers in terms of digestibility and intestinal fermentation (*7*). Most promisingly, GADF may contribute to cardiovascular health, mainly by lowering blood pressure and levels of cholesterol (*8*). GADF is 58% insoluble fiber, 16% soluble fiber, and it contains a large proportion (up to 20%) of polyphenolic compounds, including phenolic acids and flavonoids, mainly oligomeric and polymeric procyanidins ((epi)catechin based) (*8*, *9*). Oligomeric procyanidins, rather than other polyphenols, are reported to be responsible for lowering blood pressure in humans (*10*). Therefore, the cardiovascular benefits of GADF may very well be due to the procyanidins it contains, or perhaps more precisely to their metabolites.

Procyanidins are extensively metabolized in the intestinal tract and liver, as are other polyphenols. It therefore follows that the putatively beneficial effects of polyphenols at the systemic level should be attributed to the metabolites rather than to the parent compounds. Flavonoid metabolites are formed by phase I (cleavage/degradation) and phase II (derivatization) metabolism to give an array of structures. Some of them are shown in **Figure 1**. The number of possible metabolites is enormous, and the amount of each individual metabolite may be small. That is why most studies in the literature use enzymes to strip off the derivatizing moieties and detect only the hydroxylic form of the metabolite (*11*, *12*). However, in this way valuable information on what particular active compounds may actually be bioavailable is lost. The most accurate accounts in terms of epicatechin (EC) derivatization (glucuronates, sulfates, methyl ethers) lack crucial information on phenolic acids (*13*), whereas the reports on colonic metabolites (phenolic acids) miss the variability of derivatized species (*11*, *14*).

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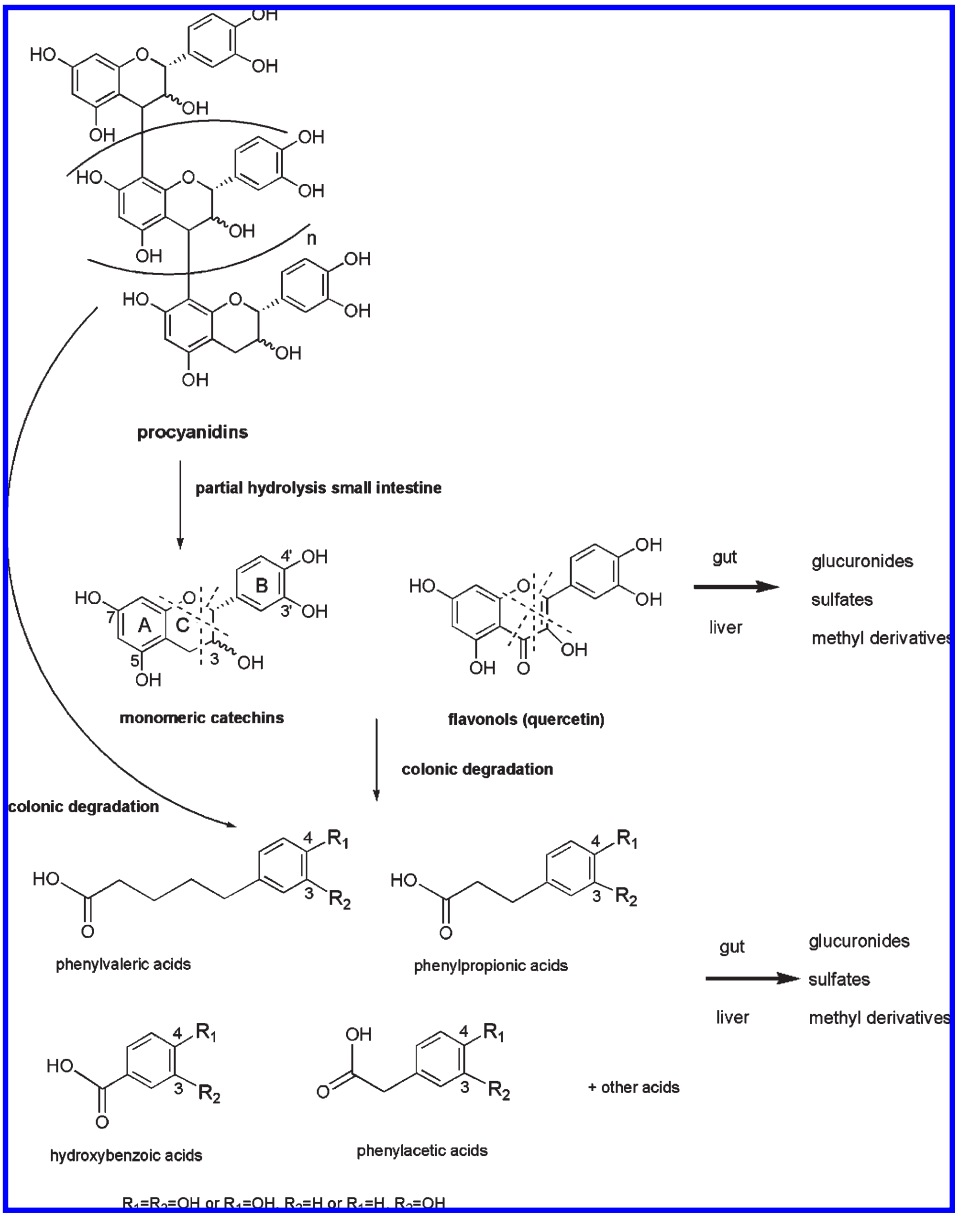

Figure 1. Metabolization of procyanidins and other flavonoids.

The aim of this study is to identify the main phenolic metabolites present in rat urine after GADF ingestion, using a combination of mass spectrometry (MS) techniques (multiple reaction monitoring (MRM); neutral loss (NL) experiments; precursor ion (PI) experiments and product ion experiments) on a triple quadrupole apparatus. The studies of polyphenol metabolites referenced above examined the metabolic fate of pure products (e.g., EC) or simple plant extracts (e.g., purified procyanidins). The complex GADF we examined is a much more faithful model for dietary intake of whole fruit. The information provided here may help explain the biological reactions to GADF and the possible beneficial effects of a diet rich in fruit and vegetables.

MATERIALS AND METHODS

Chemicals and Reagents. GADF was obtained from red grapes (the *Cencibel* variety) harvested in the vintage year 2005 in *La Mancha* region in Spain, as described in a published patent (*6*). 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); proanthocyanidins (PA), 14.81 ± 0.19 (total PA measured by the cyanidine method); fat, 7.69 ± 0.49; protein, 11.08 ± 0.46; ash, 5.25 ± 0.19. The insoluble fiber includes as major components part of the polymeric PA (nonextractable PA, NEPA), nonstarch polysaccharides, resistant protein and lignin. Standards of (−)-epicatechin (≥97%), (+)-catechin (≥98%), 3- and 4-hydroxybenzoic acid (≥97%), vanillic acid (≥97%), caffeic acid (≥95%), 3,4-dihydroxyphenylpropionic acid (DHPhPA; >98%), 4-hydroxyphenylpropionic acid (HPhPA; >98%), protocatechuic acid (≥97%), *p*-coumaric (≥98%), *m*-coumaric (≥97%) and taxifolin (≥85%) were obtained from Sigma Chemical (Saint 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 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) were provided by Harlan Interfauna Ibérica S.L (Barcelona, Spain). The animals were fed with a polyphenol-free diet (TD94048) purchased also from Harlan Interfauna Ibérica S.L and maintained in plastic cages at room temperature (22 ± 2 °C) and 55 ± 10% relative humidity, with a 12 h light/dark cycle for one week, according to European Union Regulations. The rats were randomly divided into three groups (n = 4) (GADF group, EC group, and control group) and then housed individually in metabolic cages. The first group was administered a GADF suspension in tap water (1 g/6 mL), and

the second group an (−)-epicatechin solution also in tap water (1 g/6 mL), both administered by oral gavage in a single dose of 1 and 1.6 g/kg body weight, respectively. Tap water was administered orally to the rats of the control group at a dose of 10 mL/kg body weight for blank urine collections. Prior to the administration, the rats were deprived of food for 12 h, with free access to water. Urine samples were collected and acidified with HCl (~5 μL) at 2, 4, 6, 8, 10, and 24 h after administration. Blood was taken by cardiopuncture from anesthetized rats 24 h after GADF administration, and plasma was obtained by centrifugation at 3000*g* for 10 min. The samples were stored at −80 °C until extraction and analysis. Finally, the animals were killed by an overdose of anesthetic (isoflurane gas). These experimental protocols were approved by the Experimental Animal Ethical Research Committee of the University of Barcelona (permission number: DMA3123) in accordance with current regulations for the use and handling of experimental animals.

Sample Processing. Urine samples were concentrated by nitrogen stream and then resuspended in 2 mL of acid water. Then, taxifolin (50 μL of a 50 ppm solution) of taxifolin was added to each sample as an internal standard (IS), to obtain a final concentration of 5 ppm. An Oasis HLB (60 mg) cartridge from Waters Corp. (Mildford, MA) was used for the solid phase extraction (SPE). The cartridge was activated with 1 mL of methanol and 2 mL of acid water (10^{-3} M HCl), and the samples were loaded onto the cartridge. To remove interfering components, the sample was washed with 9 mL of acid water. The phenolic compounds were then eluted with 1 mL of methanol. The eluate was evaporated under nitrogen and the residue reconstituted with 500 μL of the HPLC starting mobile phase ([A], see below). The temperature of evaporation was kept under 30 °C to avoid deterioration of the phenolic compounds. The samples were filtered through a polytetrafluoroethylene (PTFE) 0.45 μm membrane from Waters Corp. into amber vials for HPLC−MS/MS analysis. Plasma samples (700 μL) were treated with CH_3CN (1.5 mL) to precipitate interfering proteins. Then, after adding the IS (taxifolin, 5 ppm) the mixtures were centrifuged (3000*g*, 10 min), the supernatant was evaporated by nitrogen stream and the pellet was taken up in water (1.5 mL) and homogenized on a vortex. The samples were submitted to SPE and workup process as described for urine samples.

HPLC−ESI-MS/MS Analysis. An Applied Biosystems (PE Sciex, Concord, Ontario, Canada) API 3000 triple quadrupole mass spectrometer with a TurboIon spray source was used in negative mode to obtain mass spectrometry (MS) and MS/MS data. Liquid chromatography separations were performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany) liquid chromatograph 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.) column. Gradient elution was performed with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN. An increasing linear gradient (v/v) of [B] was used, [*t*(min), % B]: 0,8; 10,23; 15,50; 20,50; 21,100; followed by a re-equilibration step.

Metabolites in urine samples were identified by (i) MRM transitions of the putative metabolites using a dwell time of 100 ms, (ii) NL and PI scans and posterior comparison of the results with the retention time obtained by MRM, and (iii) product ion scan experiments to confirm the identity of each metabolite. The cycle time used was 2 s. The declustering potential (DP) and collision energy (CE) used in the product ion scan mode were −80 and −35 V respectively.

RESULTS

Analytical Strategy. In previous studies, we observed that GADF is particularly rich in (epi)catechin ((−)-epicatechin and (+)-catechin) polymers (procyanidins) (*8*, *9*). The monomeric (epi)catechin content of GADF is less than 0.01% by HPLC−UV. Consequently we expected the metabolic outcome of GADF in rat urine to be mainly that of oligomeric and polymeric (epi)catechin. In the present work we compare the metabolites generated after GADF ingestion with those generated by pure monomeric EC. Because GADF consisted mostly of polymeric EC, the fragmentation patterns obtained with pure EC helped to assign the weaker signals from GADF. With the tools provided by HPLC−MS/MS we searched for intact flavonoids (catechins and others) and phenolic acids, as well as glucuronidyl, methyl and sulfate conjugates of (epi)catechin, conjugates with other flavonoids and metabolites generated by ring fission (**Figure 1**). To gain information on the kinetics of the metabolization/absorption/metabolization and excretion, urine samples were collected at different times (2, 4, 6, 8, 10, 24 h) after ingestion. Blood was collected at the end of the experiment (24 h). The samples were first analyzed using an HPLC−MS system in the full-scan mode (FS). Some compounds detected in samples collected after ingestion of GADF and EC were also present in the controls and therefore were not considered GADF metabolites. We also detected metabolite conjugates with chloride coming from the HCl added to the samples before freezing. All the metabolites conjugated with chloride were also detected in the nonchlorinated form. We did not detect any significant metabolite signal in plasma after 24 h. The identity of the putative metabolites found in the FS mode was corroborated by MRM, which yields the highest selectivity and sensitivity in HPLC−MS/MS (*15*). MRM transitions in urine samples obtained after GADF and EC ingestion were compared in detail with the blank samples. To eliminate false positives and to obtain further structural information, NL experiments and PI experiments were run. NL experiments helped to identify the glucuronides (M − 176) and sulfates (M − 80). PI scanning helped to detect the main metabolites of the flavonoid aglycons which had been previously found in the GADF soluble fraction (*9*). Finally, the identity of putative GADF metabolites detected in the previous experiments was confirmed by product ion scan MS/MS experiments on the molecular ions and/or fragment ions.

Epicatechin Metabolites. EC oligomers appeared to be extensively but not completely depolymerized before absorption. Nonconjugated EC dimers were present in urine samples taken 2 h after ingestion of GADF. Most of the EC identified in urine samples occurred as conjugates. Nonconjugated EC (*m*/*z* 289) was only detected in samples collected early after EC ingestion. No signals corresponding either to conjugated dimers or to products resulting from the opening of the C-ring ($[M - H]^-$ at 579 and 581) (*14*, *16*) were recorded. We detected more EC conjugates than those previously reported (*13*, *17*). The conjugates include monoglucuronidated and monosulfated EC as well as diconjugates and triconjugates resulting from combinations of glucuronidyl, methyl and sulfate moieties. **Table 1** lists all the EC conjugates detected by HPLC−MS and HPLC−MS/MS in urine samples coming from rats fed both GADF and EC. A table with the times at which all these metabolites were detected is provided as Supporting Information.

Monoconjugated Metabolites of EC. The MRM transition 465 → 289 corresponding to EC glucuronides (Gluc-EC) under negative mode revealed the presence of three compounds: 1, 2, and 3. The identity of these metabolites with molecular ions at *m*/*z* 465 was corroborated by the NL (M−176) and PI (*m*/*z* 289) experiments. These glucuronidated metabolites of epicatechin appeared in samples collected at all the different times from rats fed GADF or pure EC. In the product ion scan mode, each Gluc-EC generated the corresponding molecular ion at *m*/*z* 465 ($[M - H]^-$) that released a main MS/MS (MS^2) fragment at *m*/*z* 289 (EC after loss of the glucuronide moiety) and the characteristic fragments of the glucuronide moiety at *m*/*z* 175 and 113 (*18*). Other MS^2 fragments from these metabolites were useful for establishing the position of the glucuronide moiety on the EC molecule. Ions at *m*/*z* 245 (loss of CO_2 from the EC moiety) and an intense signal at *m*/*z* 137 (a fragment of the A-ring) suggested that the glucuronide moiety in compounds 1 and 3 was attached to the A-ring. Indeed, it has been reported that, in rats, glucuronidation of (−)-epicatechin occurs at position 7 of

Table 1. Conjugated Metabolites of EC in Urine from Rats Fed with GADF or EC

metabolite	number	MRM	NL	PI	MS^2 fragments (% relative abundance)
Gluc-EC-1	1	465 → 289	176	289	465 (1); 289 (100); 245 (25); 175 (6); 113 (7)
Gluc-EC-2	2	465 → 289	176	289	465 (12); 327 (9); 289 (100); 245 (31); 203 (12); 175 (16); 151 (12); 113 (10)
Gluc-EC-3	3	465 → 289	176	289	465 (1); 289 (100); 245 (25); 203 (9); 179 (20); 137 (9)
Sulf-EC-1[a]	4	369 → 289	80	289	369 (37); 289 (100); 245 (80); 217 (33); 203 (44); 137 (62)
Sulf-EC-2[a]	5	369 → 289	80	289	369 (16); 289 (100); 245 (45); 231 (23); 151 (15); 137 (30)
Me-Sulf-EC-1	6	383 → 303	80		383 (13); 303 (100); 259 (22); 245 (36); 220 (24); 204 (15); 165 (18); 151 (6); 137 (11)
Me-Sulf-EC-2	7	383 → 303	80		383 (15); 303 (100); 285 (20); 244 (13); 217 (16); 137 (50)
Me-Sulf-EC-3	8	383 → 303	80		383 (39); 303 (100); 244 (20); 217 (17); 137 (33)
Me-Sulf-EC-4	9	383 → 303	80		383 (25); 303 (79); 217 (25); 137 (100)
Gluc-Me-EC-1	10	479 → 303	176		479 (5); 303 (100); 285 (13); 244 (10); 165 (6); 137 (7); 113 (15)
Gluc-Me-EC-2	11	479 → 303	176		479 (10); 303 (100); 285 (9); 259 (15); 175 (10); 137 (10); 113 (30)
Gluc-Me-EC-3	12	479 → 303	176	289	479 (1); 313 (5); 303 (20); 289 (100); 245 (35); 203 (11); 137 (10)
Gluc-Me-EC-4	13	479 → 303	176	289	313 (7); 303 (1); 289 (100); 245 (28); 203 (10); 179 (8)
Gluc-Sulf-EC-1	14	545 → 369	176; 80	289	545 (5); 465 (22); 369 (10); 289 (20); 271 (100); 151 (15)
di-Sulf-EC-1	15	449 → 369	80		369 (100); 289 (15); 245 (15); 231 (19); 217 (13)
di-Gluc-EC-1	16	641 → 465	176		641 (90); 465 (50); 289 (20)
di-Me-Gluc-EC-1	17	493 → 289	176	289	493 (6); 475 (8); 317 (11); 313 (10)289 (100); 245 (25); 205 (11); 137 (16)
di-Me-Gluc-EC-2	18	493 → 289		289	313 (3); 303 (6); 289 (100); 245 (25); 205 (10); 179 (6)
di-Me-Gluc-EC-3	19	493 → 289	176		317 (6); 303 (100); 259 (11); 244 (17); 151 (10); 137 (23)
Me-di-Sulf-EC-1	20	463 → 383	80		383 (100); 303 (40); 245 (35); 217 (17); 165 (9); 137 (10)
Me-di-Sulf-EC-2	21	463 → 383	80		383 (80); 303 (23); 273 (100); 245 (35); 231 (45); 217 (25); 189 (16); 137 (20)
Gluc-Me-Sulf-EC-1	22	559 → 383			625 (2); 335 (100); 289 (4); 193 (12); 175 (52); 159 (91); 113 (35)
Gluc-Me-Sulf-EC-2	23	559 → 383			559 (100); 313 (11); 289 (70); 245 (9); 231 (29); 203 (6); 149 (6)
tri-Sulf-EC-1[a]	24	529 → 289			449 (25); 369 (100)

[a] Only detected in the EC group.

the A-ring (*19*). Thus, Gluc-EC-3 (compound 3), which gave the most intense peaks in urine samples from both groups, may be assigned to 7-Gluc-EC, and Gluc-EC-1 (compound 1) to 5-Gluc-EC. Gluc-EC-2 (compound 2) gave one fragment at m/z 327 corresponding to a B-ring fragment plus the glucuronide moiety (151 + 176), resulting from the retro-Diels−Alder (RDA) fission of the EC C-ring (*20*). This indicates that, in this case, glucuronidation occurred at position 3′ or 4′ of the B-ring. Peak 2 is nearly as intense as peak 3 in the urine of rats fed GADF, in contrast to the urine from rats fed EC (**Figure 2**). This suggests that the catechol moiety (B-ring) may be bioavailable as a combination of intact and conjugated forms.

Two peaks (compounds 4 and 5) were assigned to sulfates of EC (Sulf-EC) which were only found in urine from rats fed EC (**Table 1**). Product ion scans of these compounds generated common fragments at m/z 369 ($[M - H]^-$) and 289 (EC, loss of sulfate moiety) and at m/z 245 (EC − CO_2). Metabolite Sulf-EC-1 (compound 4) produced fragments at m/z 217 (sulfate + A-ring fragment by RDA fission) and m/z 137 (A-ring fragment) indicating that the sulfate occurred at position 5 or 7 of the A-ring. Sulf-EC-2 (compound 5) produced fragments at m/z 151 and at m/z 231 corresponding to B-ring and "B-ring plus sulfate" fragments at positions 3′ and 4′ respectively. We did not detect any monomethylated metabolite in the urine samples.

Diconjugated Metabolites. Four sulfated and methylated EC conjugates (Me-Sulf-EC; compounds 6, 7, 8, 9) were detected in low quantities in urine samples from rats fed GADF by MRM transition 383 → 303 (**Table 1**). They were identified by comparison with stronger signals detected in samples from animals fed EC. The four Me-Sulf-ECs yielded a molecular ion at m/z 383. However, two different fragmentation patterns were observed. Compound 6 produced specific ions at m/z 245 and m/z 165, which were not present in the fragmentation of the other isomers. The fragment at m/z 165 corresponds to the characteristic B-ring fragment at m/z 151 plus a methyl moiety, and the fragment at m/z 245 corresponds to the same fragment (151) plus methyl and sulfate. These two ions and the absence of the sulfated A-ring fragment (m/z 217, 137 + 80) are clear evidence that methylation and sulfation of this metabolite occurred on the B-ring. In contrast, compounds 7−9 all gave the same product ion fragments at m/z 303, 217 and 137, which indicates that sulfation occurred on the A-ring for all of them.

By monitoring the transition 479 → 303 we observed three intense peaks (compounds 10, 11, 12) corresponding to methylated and glucuronidated EC (Gluc-Me-EC). Another Gluc-Me-EC (compound 13) was later detected through the product ion scan (**Figure 3**). Compound 13 was not detected by MRM because the molecular ion at m/z 479 produced an intense signal at m/z 289 instead of 303. Moreover, the NL of 176 Da (Gluc) and PI of 289 Da (EC) ruled out false positive identifications. The molecular ions of Gluc-Me-EC-1 and Gluc-Me-EC-2, corresponding to peaks 10 and 11, generated MS^2 fragments at m/z 303 (loss of a glucuronide moiety), which suggests that the glucuronide moiety was attached at position 3′ of the EC skeleton, said to be the most labile position (*21*). Compounds 12 and 13 showed MS^2 fragment ions at m/z 313 and m/z 289 (intense). Fragments at m/z 313 correspond to an A-ring fragment from RDA fission plus one glucuronide moiety (137 + 176). The different fragmentation pattern suggests that the glucuronidation position is different for 10 and 11 on the one hand, and 12 and 13 on the other.

Gluc-Me-EC-1 (compound 10) was clearly present in samples collected from the GADF group at all the different times (**Figure 3**). In samples from rats fed EC, this metabolite first appeared later (after 6 h). In contrast, peak 12, corresponding to Gluc-Me-EC-3, was clearly more intense in samples from the EC group.

Glucuronidated and sulfated EC (compound 14) was detected by monitoring the transition 545 → 369 (**Table 1**). The product ion scan of this metabolite generated fragments at m/z 465 (M − Gluc), 369 (M − Sulf) and 289 (EC), as well as an ion giving a strong signal at m/z 271, which could correspond to a posterior loss of water (289 − 18). This metabolite was clearly present in urine samples from the EC group, and only traces were detected in samples from the GADF group. Disulfated EC (compound 15) was detected in samples from the GADF group. The two sulfation sites are probably located on two different rings of the

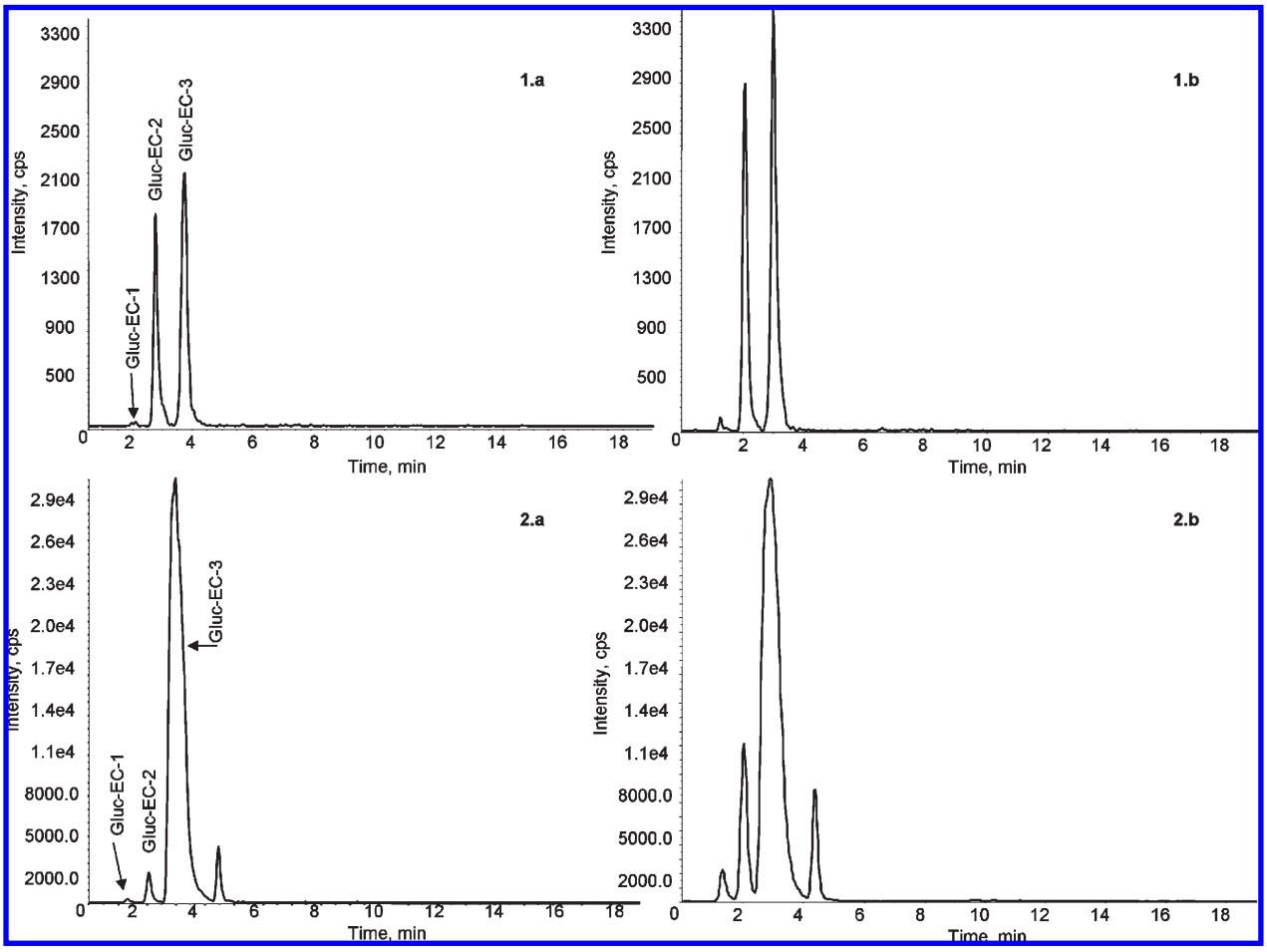

Figure 2. HPLC−ESI-MS/MS profile corresponding to the detection EC glucuronides in urine samples after feeding: (**1**) GADF and (**2**) EC. Samples collected at 2 h (**a**) and 6 h (**b**). Detection in the multiple reaction monitoring (MRM) mode, transition 465 → 289.

EC moiety. The ions at *m*/*z* 231 and *m*/*z* 217 correspond to a sulfated B-ring (151 + 80) and A-ring (137 + 80), respectively.

EC diglucuronide (compound 16) produced MS^2 fragments at *m*/*z* 465 and *m*/*z* 289 from the molecular ion $[M - H]^-$ at *m*/*z* 641. In this case we did not have enough experimental evidence to suggest a glucuronidation position. Again, only traces of this metabolite were found in samples from animals fed GADF, while it was a main component of the samples from the EC group.

Triconjugated Metabolites. EC conjugated with two methyl and one glucuronide moieties (compounds 17, 18 and 19) were detected by MRM transition 493 → 289. Compounds 17 and 18 yielded a main MS^2 fragment at *m*/*z* 289 (EC) and a second fragment at *m*/*z* 313, which indicates that the glucuronide moiety was located on the A-ring (137 + 176). In contrast, compound 19 yielded a main MS^2 ion at *m*/*z* 303 and minor ions at *m*/*z* 259 and 244. The absence of a fragment at *m*/*z* 313 indicates that the Gluc moiety was not attached to the A-ring. Methylated disulfate conjugates of EC (compounds 20, 21) were detected as very weak signals in urine samples from rats fed GADF. The identity of these metabolites was established by comparison with the samples from rats fed EC. These gave strong signals at all collection times. The product ion spectrum of the molecular ion $[M - H]^-$ at *m*/*z* 463 showed MS^2 fragments at *m*/*z* 383 $[M - H - Sulf]^-$ and *m*/*z* 303 $[M - H - 2Sulf]^-$ in both cases. Other MS^2 fragments provided further structural information. Me-di-Sulf-EC-1 (compound 20) gave characteristic peaks at *m*/*z* 245 (B-ring fragment + sulfate + methyl), *m*/*z* 217 (A-ring fragment + sulfate), and *m*/*z* 165 (B-ring fragment + methyl). This suggests that the two sulfate moieties were attached to different rings, and the methyl moiety was attached to the B-ring. The presence of the fragments at *m*/*z* 245 (B-ring + sulfate + methyl), *m*/*z* 231 (B-ring + sulfate) and *m*/*z* 217 (A-ring + sulfate) from the molecular ion of Me-di-Sulf-EC-2 (compound 21) suggested that this isomer also incorporated one sulfate on the A-ring, together with the methyl group and the other sulfate on the B-ring. Although compounds 20 and 21 have the same moieties on each ring, the substitution positions must be different because they elute at different retention times in HPLC−MS/MS.

Trisubstituted ECs with glucuronide, methyl and sulfate (compounds 22 and 23) were also detected in minute amounts in urine samples from rats fed GADF by the MRM transition 559 → 383 (**Table 1**). The identity of the metabolites was established thanks to the samples from rats fed EC. Product ion scans of metabolite 22 showed fragments at *m*/*z* 559 (molecular ion), 479 (559 − Sulf), 461 (479 − H_2O), 369 (EC + Sulf), 303 (loss of a glucuronide moiety from 479, Me − EC), 289 (EC), 217 (which is the characteristic ion of RDA fragmentation with an attached sulfate moiety; 137 + Sulf) and 137 (which typically results from the A-ring after RDA fission). Again, the sulfate moiety was attached to the A-ring as shown by the MS^2 fragments at *m*/*z* 217 and 137. The conjugation positions of the glucuronide and methyl groups could not be established for this metabolite. The glucuronidation position could be established for metabolite 23 from the information generated by the product ion scan. The MS^2 fragments were *m*/*z* 313 (glucuronide moiety attached to an A-ring fragmentation product; 137 + 176), 289 (EC), 245 (B-ring fragment + sulfate + methyl), and 231 (corresponding to a sulfate moiety attached to a B-ring fragment;

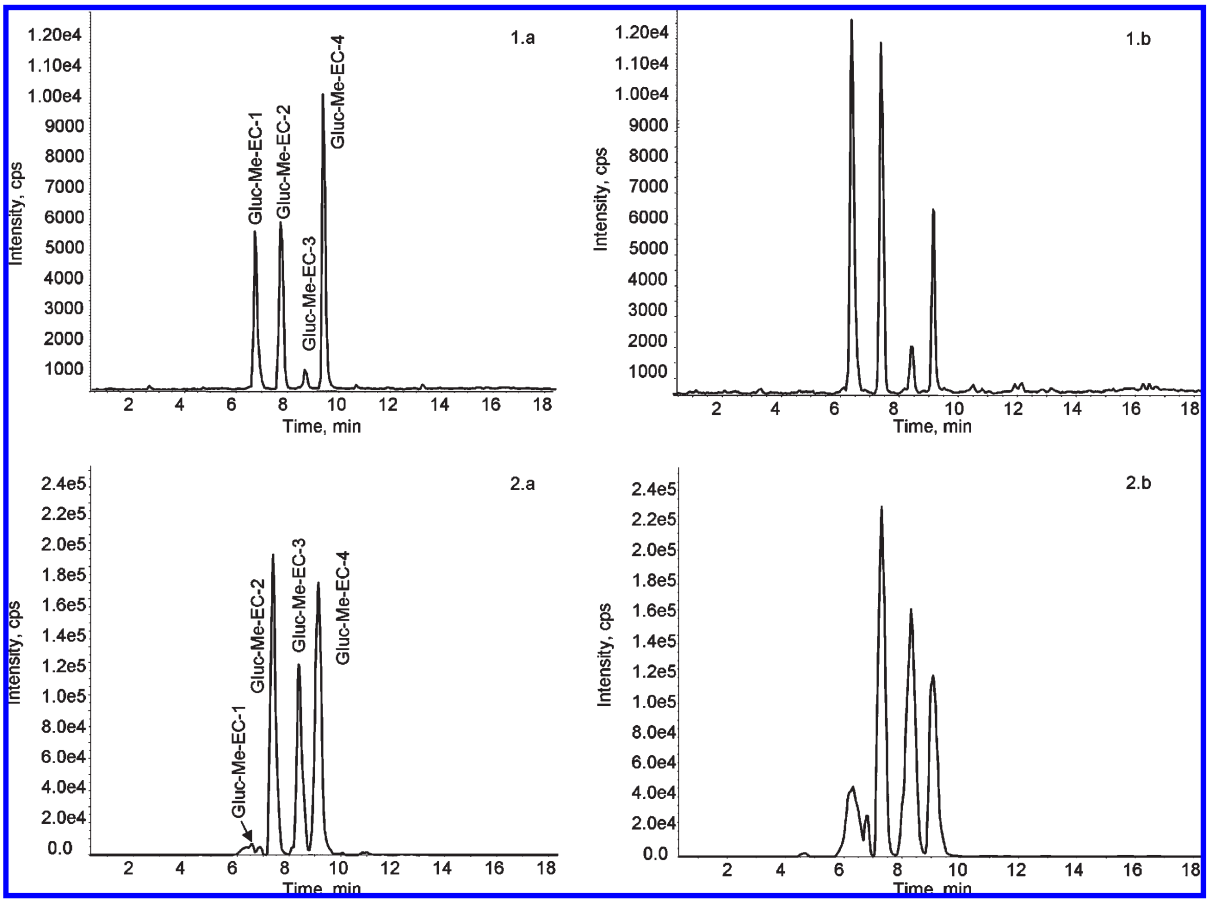

Figure 3. HPLC−ESI-MS/MS product ion scan profiles at *m*/*z* 479 of urine samples after feeding: (**1**) GADF and (**2**) EC. Samples collected at 2 h (**a**), 6 h (**b**).

151 + 80). So we concluded that the glucuronide was attached to the A-ring, and the methyl and sulfate moieties were attached to the B-ring. Finally, we report one more trisubstituted metabolite: tri-Sulf-EC (compound 24). This EC conjugated with three sulfate moieties generated two fragments at *m*/*z* 449 and *m*/*z* 369, which correspond to the successive loss of sulfate moieties. A fragment at 289 (EC) was also detected by PI scan.

Phenolic Acids. Both nonconjugated and conjugated phenolic acids were detected and identified in urine samples from rats fed GADF. Only a limited number of nonconjugated acids were detected in samples from the EC group. The results are summarized in **Table 2**. The species listed were confirmed by the use of standards and gave MS signals that were clearly stronger than those from the control animals. Some phenolic acids described in the literature as metabolites of EC (e.g., 3-HBA) are not included in the list because the signals they yielded were no stronger than those from the control samples obtained from nonsupplemented animals. More sensitive GC−MS experiments might clarify this point. A table with the times at which all these metabolites were detected is provided as Supporting Information.

Phenolic Acids Derived from GADF. Hydroxyphenylvaleric acid (HPhVA) detected at 24 h was one of the main metabolites in rats fed GADF. *p*-Coumaric and *m*-coumaric were detected and identified in GADF samples collected at all times. *p*-Coumaric signals were more intense than *m*-coumaric signals in all the samples, and were clearly stronger at 8 and 10 h. 3,4-Dihydroxyphenylacetic acid (DHPhAcA) was detected at 24 h by MRM transition 167 → 123. Hydroxyphenylacetic acid (HPhAcA) and 4-hydroxybenzoic acid (4-HBA) were also detected from 6 to 24 h.

Conjugated phenolic acids were detected in urine samples from the GADF group by MRM and product ion scan on parent/daughter pairs of ions. 3,4-DHPhPA conjugated with either a sulfate (**Figure 4a**) or a glucuronide moiety gave the transitions 261 → 181 plus 181 → 137, and 357 → 181 plus 181 → 137, respectively, and were confirmed by the product ion scan. We also detected sulfated coumaric acid (243 → 163 plus 163 → 119) as early as 2 h after ingestion of GADF and sulfated HPhPA (245 → 165) some time later (from 8 to 24 h) (**Figure 4b**).

Phenolic Acids Derived from EC. The metabolization of pure EC by the microbiota gave different phenolic acids with the three-carbon atom scaffold. Caffeic acid (179 → 135) gave signals of increasing intensity from 8 to 24 h. 3,4-DHPhPA, also called dihydrocaffeic acid (181 → 137), was only detected after 24 h. 4-HPhPA was detected from 8 to 24 h. *p*-Coumaric and *m*-coumaric acids gave signals of increasing intensity from 8 to 24 h.

We also detected shorter phenolic acids after EC ingestion. 3-HPhAcA and 4-HBA appeared at 10 and 24 h. We also observed MRM signals corresponding to sulfated caffeic acid, which were confirmed by a second transition of the free acid at the same retention time. We do not include this metabolite in **Table 2** because the less sensitive product ion scan did not yield any fragment.

DISCUSSION

Food polyphenols such as monomeric (epi)catechins and phenolic acids are rapidly glucuronidated and methylated in the small intestine (*22*), absorbed into the bloodstream and further conjugated (glucuronidated, sulfated) in the liver of both rats and humans (*23*−*26*). Those species that are not absorbed in the small intestine (e.g., polymeric (epi)catechins) reach the colon, where they are fermented by the colonic microbiota and absorbed in the

Table 2. Identification and Phenolic Acids in Urine from Rats Fed with GADF or EC

metabolite	MRM parent	MRM daugther	MS^2 fragments (% relative abundance)
3-hydroxyphenylvaleric acid (3-HPhVA)	193 → 175	—	—
3,4-dihydroxyphenylpropionic acid (DHPhPA)	181 → 137	—	181 (5); 137 (35); 135 (47); 121 (73); 109 (3)
4-hydroxyphenylpropionic acid (4-HPhPA)	165 → 121	—	165 (30); 121 (56); 93 (100); 77 (100)
caffeic acid	179 → 135	—	135 (100); 107 (9); 89 (8)
p-coumaric acid	163 → 119	—	163 (1); 119 (100); 93 (4); 91 (2)
m-coumaric acid	163 → 119	—	163 (3); 119 (100); 93 (5); 91 (10)
3,4-dihydroxyphenylacetic acid (DHPhAcA)	167 → 123	—	123 (100); 105 (7); 95 (17)
3-hydroxyphenylacetic acid (3-HPAcA)	151 → 107	—	107 (100); 91 (23)
4-hydroxybenzoic acid (4-HBA)	137 → 93	—	137 (57); 119 (8); 108 (100); 93 (64)
Sulf-dihydroxyphenylpropionic acid (Sulf-DHPhPA)	261 → 181	181 → 137	261 (10); 199 (37); 181 (85); 137 (100)
Gluc-dihydroxyphenylpropionic acid (Gluc-DHPhPA)	357 → 181	181 → 137	181 (58); 166 (100); 121 (9)
Sulf-hydroxyphenylpropionic acid (Sulf-HPhPA)	245 → 165	165 → 121	245 (5); 165 (35); 121 (100); 119 (40); 106 (18)
Sulf-coumaric acid	243 → 163	163 → 119	243 (5); 163 (15); 119 (100)

form of smaller phenolic acids (*27*). These smaller species may in turn be conjugated in the liver. The metabolites found in urine are the result of all these processes and may have exerted their biological effect during the period while they crossed membranes and circulated in the bloodstream.

GADF contains a complex mixture of polyphenols including monomers, oligomers and polymers of EC, anthocyanins, flavonols and hydroxycinnamic acids associated with a fiber matrix of both soluble and insoluble polymers such as polycaccharides and lignins (*8*, *9*) which may influence the absorption of the putatively bioactive GADF components. We report here that the phenolic metabolites in rat urine after the ingestion of GADF are mainly conjugates of (−)-epicatechin and phenolic acids. An important question is where the different polyphenolic components of GADF are absorbed and metabolized, as the putative protective effects of the polyphenols will be exerted where they are bioavailable. During the metabolization/absorption/metabolization process, different phenolic mixtures are in contact with the different organs. We believe that the EC metabolites in rat urine must come from the partial depolimerization of the GADF's oligomeric procyanidins, probably already in the small intestine. The extent to which procyanidins are processed in the small intestine is unclear in the literature. Some studies suggest that oligomers of EC are poorly absorbed (*27*, *28*) and that all the (epi)catechin found in rat tissues after the ingestion of grape seed extract must come from the monomeric fraction (*13*). However, others show that procyanidin dimers and trimers may be absorbed and excreted via urine intact (*13*) or as conjugates (*29*, *30*). Still others report that EC oligomers may be hydrolyzed to EC units and metabolized (*31*). Our results suggest that most of the array of EC derivatives detected in urine after GADF ingestion come from (epi)catechin-based soluble oligomers (i.e., procyanidins) which are depolymerized, derivatized and absorbed in the digestive tract, and further processed in the liver. The amount and variety of EC derivatives (**Table 1**) cannot come exclusively from the monomeric EC present in GADF (less than 0.01%). As there seems to be agreement that depolymerization into EC monomeric units is not mediated by the colonic microbiota (*14*, *27*, *32*) depolymerization must take place somewhere in the digestive tract before the EC rings are directly cleaved into smaller phenolic acids. This might occur already in the small intestine. We also detected EC dimers in urine samples collected 2 h after GADF ingestion. These dimers may come directly from GADF, or they may be the result of partial depolymerization of procyanidins. The hypothesis that EC metabolites do not come from EC monomers in GADF is corroborated by comparing the profile of EC conjugates excreted by animals fed GADF to that of animals fed only EC. Almost all the conjugates detected in the EC group samples also appear in the GADF group samples, but the intensity of the MS signals differs significantly for many of the metabolites. This is exemplified in **Figures 2** and **3** for the case of monoglucuronidated ECs and glucuronidated and methylated ECs respectively. Moreover, the intensity profiles reveal differences between the GADF and EC groups in the time at which the EC metabolites first appear. Here, we would like to point out that, even after extensive metabolization at the different levels, intact dimers and conjugated species with intact catechol moieties on the B-ring were detected in urine. **Figure 2** also shows this. Note that intact catechol containing compounds such as Gluc-EC-3 persist not only after an overdose of monomer (**Figure 2b**), but also after intake of a much lower amount of monomeric and oligomeric catechins (GADF, **Figure 2a**). As the catechol group is a necessary structural requirement for the free radical scavenging activity of polyphenols this observation suggests that some scavenging activity is bioavailable for several hours to different tissues after GADF ingestion.

The EC metabolites probably came from oligomers with low degree of polymerization (dimers, trimers). Bulkier oligomers and polymers are associated with proteins (*33*). The protein-associated material, as well as both soluble and insoluble procyanidins more or less associated with the fiber matrix (polysaccharides and lignin) and maybe the remaining monomers, eventually reach the colon, where the colonic microbiota break them into smaller compounds (phenolic acids) as described for both rats (*27*) and humans (*11*, *34*). It is believed that procyanidins are directly degraded to phenolic acids, without previous depolymerization into (epi)catechin units (*14*). In agreement with the observations from other authors working with purified oligomers (*14*, *27*, *32*), we found that the urine samples from animals fed GADF contained more phenolic acids than the samples from animals given only monomeric EC. The GADF colonic metabolites (**Table 2**) most probably come from the colonic fermentation of polymeric EC. The main MS signals corresponded to 3-HPhVA, 4-HPhPA and 3-HPhAcA. All these metabolites may be generated by ring cleavage of the EC moieties within the procyanidin polymers (*14*, *32*). *m*- and *p*-coumaric acids were detected in samples from both the GADF and EC groups. A small proportion of the coumaric acids may come directly from the fiber supplement, as they are minor components of GADF (*9*). Since coumaric acids have been shown to be absorbed rapidly in the small intestine (*35*), they may account for some of the phenolics detected soon after ingestion (2 h). The rest must be generated by degradation of the EC skeleton. We detected sulfated and glucuronidated conjugates of hydroxyphenyl acids only in the GADF group samples. These metabolites (e.g., Sulf-DHPhPA and Sulf-HPhPA, **Figure 4**) had not been detected from procyanidins before and may be produced in the liver from the colonic degradation products. Again, as for the EC dimers and EC

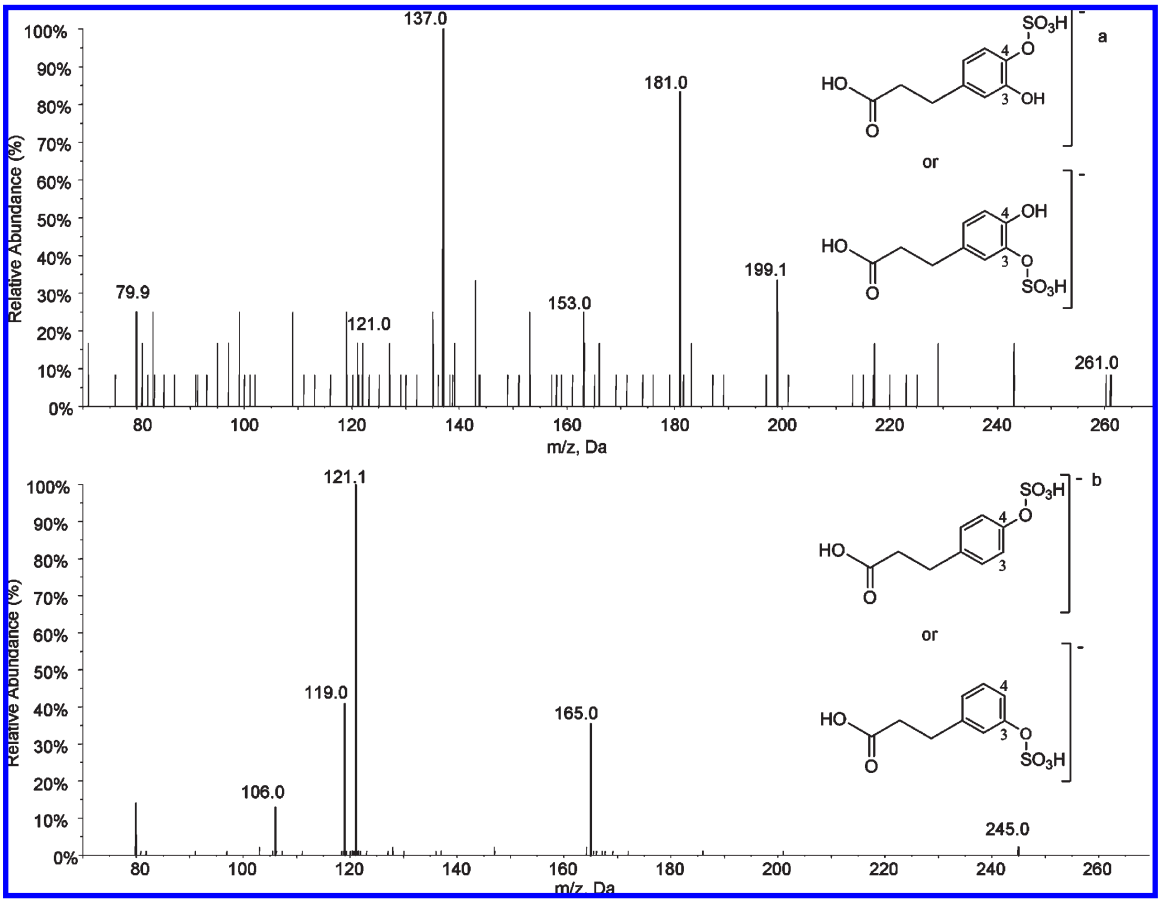

Figure 4. HPLC−ESI-MS/MS product ion scan spectra of sulfated dihydroxyphenylpropionic acid (*m*/*z* 261) (**a**) and sulfated hydroxyphenylpropionic acid (*m*/*z* 245) (**b**).

metabolites with intact catechol moieties, some of the phenolic acids existed in their nonconjugated form and may provide scavenging potential for several hours after GADF ingestion. However, it is evident that GADF polyphenols are extensively conjugated and the levels of free catechols may be too low to have any significant effect. In fact, many authors believe that the scavenging capacity of plant polyphenols is negligible "in vivo". While we have to agree in part, we certainly do not think that this means that plant polyphenols, in particular procyanidins, exert no significant antioxidant effect. Apart from the observation that the circulating free catechol may not be negligible, the extent of metabolization shows how polyphenols are treated by the living organism as harmful. In fact, it is known that polyphenols may be pro-oxidant depending on their nature and concentration. Thus, they may stimulate the endogenous antioxidant systems by acting as mild pro-oxidants (hormesis). Similarly, calorie restriction and moderate exercise are weak pro-oxidant insults that may result in antioxidant responses "in vivo" (*36*, *37*). The antioxidant/pro-oxidant balance provided by polyphenols and their metabolites may result in an overall antioxidant effect of GADF, and of fruit and vegetables in general.

For several hours after GADF ingestion, a great variety of phenolic species are in contact with the gut tissues before, during and after metabolization, and many of them (mainly mono- and polyconjugated epicatechin and free or conjugated smaller phenolic acids) are systemically bioavailable before being excreted. Some of these metabolites include the free catechol moiety. The metabolic outcome of GADF essentially corresponds to the degradation products of epicatechin oligomers and polymers.

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Supporting Information Available: Tables summarizing the occurrence of the metabolites at the different times. This material is available free of charge via the Internet at http://pubs.acs.org.

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