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Metabolism of Antioxidant and Chemopreventive Ellagitannins from Strawberries, Raspberries, Walnuts, and Oak-Aged Wine in Humans: Identification of Biomarkers and Individual Variability

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Ellagitannins (ETs) are dietary polyphenols, containing ellagic acid (EA) subunits, with antioxidant and cancer chemopreventive activities that might contribute to health benefits in humans. However, little is known about their metabolic fate. We investigate here the metabolism of different dietary ETs and EA derivatives in humans. Forty healthy volunteers were distributed in four groups. Each group consumed, in a single dose, a different ET-containing foodstuff, i.e., strawberries (250 g), red raspberries (225 g), walnuts (35 g), and oak-aged red wine (300 mL). After the intake, five urine fractions (F) were collected at 8 (F1), 16 (F2), 32 (F3), 40 (F4), and 56 (F5) h. Neither ETs nor EA were detected in urine after LC-MS/MS analysis. However, the microbial metabolite 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (urolithin B) conjugated with glucuronic acid was detected along the fractions F3−F5 in all of the subjects, independently of the consumed foodstuff. The mean percentage of metabolite excretion ranged from 2.8 (strawberries) to 16.6% (walnuts) regarding the ingested ETs. Considerable interindividual differences were noted, identifying "high and low metabolite excreters" in each group, which supported the involvement of the colonic microflora in ET metabolism. These results indicate that urolithin B (a previously described antiangiogenic and hyaluronidase inhibitor compound) is a biomarker of human exposure to dietary ETs and may be useful in intervention studies with ET-containing products. The antioxidant and anticarcinogenic effects of dietary ETs and EA should be considered in the gastrointestinal tract whereas the study of potential systemic activities should be focused on the bioavailable urolithin B derivatives.

KEYWORDS: Ellagitannin; ellagic acid; polyphenol; biomarker; cancer chemopreventive; metabolism; colonic microflora; dihydroxy-6H-dibenzo[*b,d*]pyran-6-one; urolithin B; walnut; raspberry; strawberry; oak-aged wine

INTRODUCTION

Epidemiological data support that dietary habits rich in plant-derived food products could be related to a decrease in the mortality by cardiovascular diseases and certain types of cancer (*1*, *2*). This health-promoting effect has been mainly attributed to the content of polyphenols, plant secondary metabolites ubiquitously found in commonly consumed fruits, vegetables, and derived foodstuffs such as juices, wine, and tea (*3*−*5*). However, there are some uncertainties in relation to the dietary intake assessment methods currently used in the epidemiological studies. In fact, reliable biomarkers for the intake of dietary polyphenols are needed to get better insight into their health effects (*6*, *7*). Consequently, there has been an increasing interest in the study of the bioavailability and metabolism of naturally occurring polyphenols to properly ascertain their in vivo activity (*6*−*8*).

Ellagitannins (ETs) are polyphenols included within the so-called "hydrolyzable tannins" in which hexahydroxydiphenic acid forms diesters with sugars (most often β-D-glucose) (**Figure 1**). ETs can occur as complex polymers reaching molecular weights up to 4000. These polymers can be hydrolyzed with acids or bases to yield ellagic acid (EA), which can be used to indirectly quantify ETs (*9*) (**Figure 1**). The occurrence of ETs has been reported, among others, in hazelnuts, walnuts, pomegranates (fruit and juice), persimmon, oak-aged wines (leakage of ETs from oak barrel to wine), strawberries, raspberries, blackberries (and their derivatives such as juices, jams, and jellies), peach, plum, muscadine grape and wine, etc. (*10*−*14*).

ETs and EA have been reported to show different antitumorigenic and antipromoting activities in mice (*15*, *16*). Furthermore, the dietary administration of ET-containing foods such

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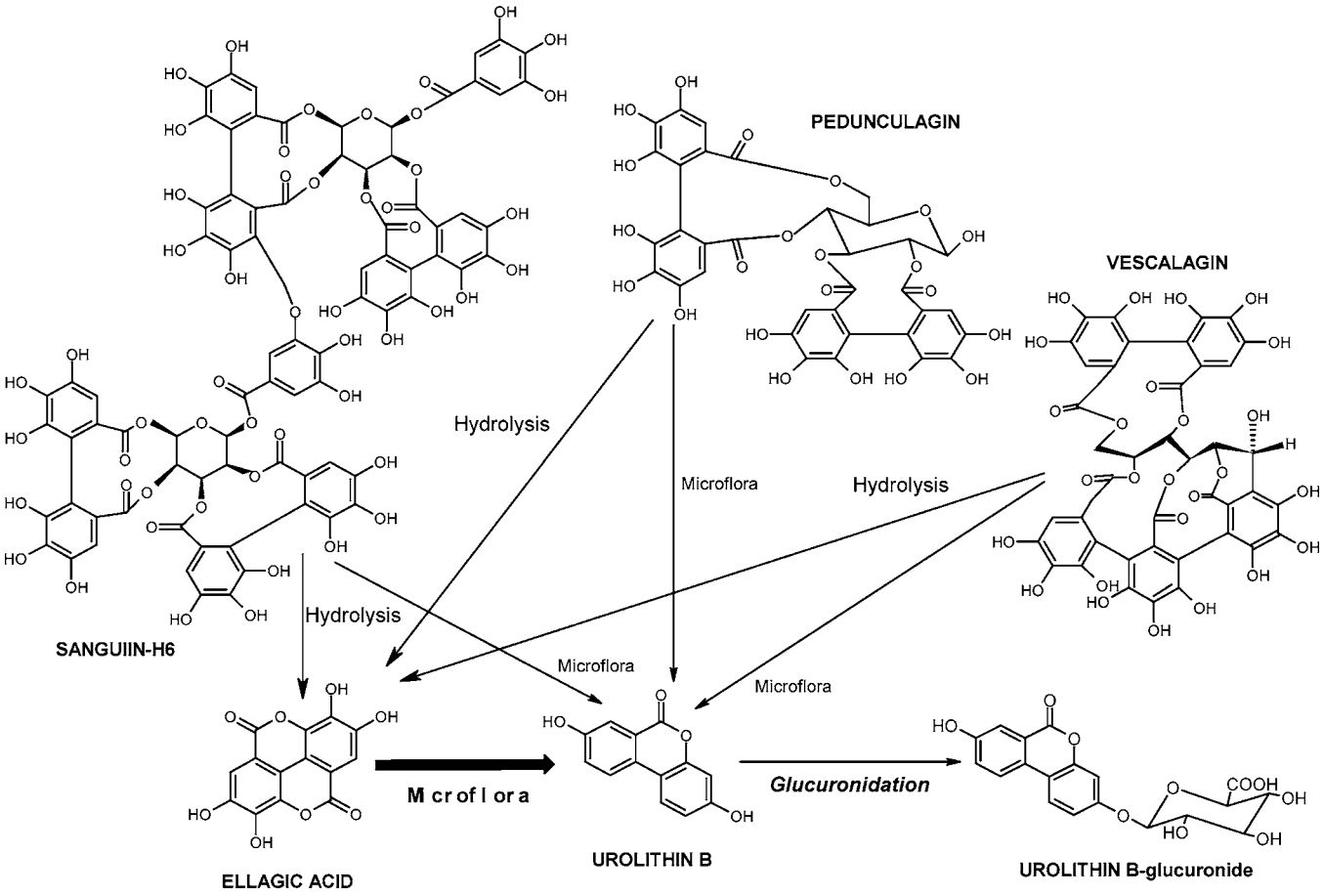

Figure 1. Structures of some representative dietary ETs and proposed pathways to yield urolithin B derivatives. Sanguiin H-6 (raspberry and strawberry); pedunculagin (walnut); and vescalagin (oak-aged wine). The pathway that implies a direct conversion from ellagitannins to urolithin B by microflora is still under research.

as strawberries and raspberries to rats has proved to inhibit events associated with both the initiation and the promotion/progression of chemically induced esophageal cancer (*17*−*19*). In addition, ETs and EA have also been studied because of their in vitro antioxidant properties (*13*, *20*−*22*). However, the huge output of studies suggesting the health benefits of many polyphenols (including ETs and EA) based on in vitro experiments may need a profound reevaluation. This is the case of ETs and EA in which their bioavailability and metabolism have been poorly studied (*10*).

The above is important because molecules appearing in blood or excreted in urine can be very different from those ingested (*23*) so that the identification of specific biomarkers of polyphenol metabolism could be a valuable tool in epidemiological studies to investigate the role of dietary polyphenols on human health (*6*−*8*). Some biomarkers of consumption of polyphenols have been identified. For instance, isoferulic acid was proposed as a biomarker of the metabolism of caffeic acid derivatives in vivo (*24*). The metabolites hydroxyphenylacetic acid derivatives, hydroxyphenylpropionic acid derivatives, vanillic, homovanillic, and hydroxyhippuric acids have also been proposed as biomarkers of consumption of different polyphenols including flavonoids and hydroxycinnamic acids (*25*, *26*). Isoflavonoid and lignan phytoestrogens have been proposed as dietary biomarkers for soy and food-containing fiber, respectively (*27*), and the total excretion of flavonoids in urine as biomarkers for fruit and vegetable intake (*6*, *28*).

The metabolism of EA by the colonic microflora to yield 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one was previously reported in rats (*29*). This microbial metabolite (the so-called urolithin B) was further identified in the feces of the complex-toothed flying squirrel *Trogopterus xanthippes* (*30*). The feces of this squirrel has long been used in Chinese traditional medicine as angiostatic and hyaluronidase inhibitor drug to treat pains due to blood stasis and allergy, respectively (*30*, *31*). Actually, there is an U.S. patent application that includes *Trogopterus* feces in its formulation (*32*).

Recent studies regarding the metabolism of the pomegranate ET punicalagin in rats described the presence of different metabolites (including urolithin B), EA derivatives, and ETs in urine, plasma, and feces (*33*, *34*). This ET, punicalagin, has been reported to be a potent in vitro antioxidant and cancer chemopreventive compound (*13*, *35*). For example, punicalagin-enriched extract from the plant *Terminalia catappa* is a popular folk medicine used in many parts of the world for preventing hepatoma (*36*). However, we have also reported for the first time that punicalagin is metabolized into bioavailable, but very poor antioxidant, urolithin B derivatives after consumption of pomegranate juice by healthy subjects (*37*). This study identified two urolithin B-related metabolites in plasma and urine. Although the "high" in vitro antioxidant activity attributed to the ingested ETs was lost in vivo, other possible cancer chemopreventive properties (antiangiogenic) attributed to urolithin B should not be ruled out. These previous results prompted us to confirm whether urolithin B derivatives were also produced upon consumption of other common ET-containing foodstuffs such as strawberries, raspberries, walnuts, and oak-aged wine in humans. This will be important to establish possible biomarkers of the bioavailability and metabolism of ETs in humans and to compare the in vitro antioxidant and cancer chemopre-

Table 1. Schematic Design of the Protocol Followed by the Volunteers

range of time	day 0	day 1	day 2	day 3
(23 to 7 h) 8 h		CF (16 h)	F2 (8 h)	F4 (8 h)
(7–15 h) 8 h		intake	F3	F5
(15–23 h) 8 h	CF (8 h)	F1 (8 h)	(16 h)	(16 h)

ventive (precursor) polyphenols ingested, which can be in vivo transformed into other metabolites with significantly different activities. In this context, the results presented here may be useful in intervention studies with ET-containing foodstuffs.

MATERIALS AND METHODS

Chemicals and Reagents. EA, β-glucuronidase from bovine liver (G-0251, EC 3.2.1.31; 1000 units/g solid), and sulfatase from *Helix pomatia* (S-9626, EC 3.1.6.1; 10000 units/g solid) were purchased from Sigma (St. Louis, MO). Methanol (MeOH), diethyl ether, acetyl chloride, hydrochloric acid, acetone, and hexane were obtained from Merck (Darmstadt, Germany). Ascorbic acid was obtained from Aldrich (Steinheim, Germany). Milli-Q system (Millipore Corp., United States) ultrapure water was used throughout this experiment.

ET-Containing Foodstuffs. Strawberries, raspberries, walnuts, and red wine were purchased in a local supermarket. The red wine (grape varieties: 80% Tempranillo and 20% Garnacha) used in this study was a Gran Reserva (3 years in American oak plus 2 more years in bottle) from the Rioja Spanish Appellation of Origin.

Study Design. Forty healthy volunteers (20 males and 20 females) with a mean age of 29 years were recruited for this "single dose" trial. Subjects were nonsmokers, no vegetarians, without a history of gastrointestinal disease or any chronic disease and not involved in a weight-reducing dietary regimen. The ingestion of either dietary supplements or medication was forbidden. Female volunteers were not either pregnant or lactating. All of the subjects filled out a questionnaire about their common dietary habits prior to participation in the study, i.e., weekly intake of milk, cheese, vegetables, yogurt, fruits, coffee, wine, olive oil, cereals, etc. The study complied with the Helsinki Declaration, and the protocol was fully explained to the volunteers who gave their written consent prior to participation.

The volunteers followed a 4 days protocol. **Table 1** shows schematically the study design. The intake of ET-containing sources such as berries (strawberry, raspberry, blackberry, etc., and derived foodstuffs such as jams), pomegranates, chocolate, nuts, and wine was strictly forbidden for 1 week before the experiment. In addition, volunteers followed a controlled diet during the 4 days protocol in which the intake of the rest of polyphenol-containing sources (fruits, vegetables, tea, coffee, juices, and olive oil) was also restricted.

Volunteers were randomly divided into four groups (five males and five females per group). Subjects consumed the samples in a single dose (**Table 1**). The raspberry group (RG) consumed 225 g of frozen red raspberries, the strawberry group (SG) consumed 250 g of fresh strawberries, the walnut group (WG) consumed 35 g of walnuts (weight without hull), and the red wine group (RWG) consumed 300 mL of oak-aged red wine.

The volume of 24 h of control urine was collected prior to the intake of the samples (**Table 1**, CF). After consumption of the selected ET-containing foodstuffs (intake, **Table 1**), the urine fractions collected were divided as follows: F1 (8 h), F2 (8 h), F3 (16 h), F4 (8 h), and F5 (16 h) (**Table 1**). Urine samples were collected in plastic bottles containing 1 g/L ascorbic acid and immediately stored at -70 °C until the analysis.

Preparation of Foodstuffs. Three grams of walnuts was homogenized in Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24000 rpm for 1 min with 20 mL of MeOH:water (80:20). The extracts were centrifuged at 5000*g* for 10 min in a Centromix centrifuge (Selecta, Barcelona). MeOH was evaporated, and the aqueous phase was extracted with hexane (1:1) and further filtered through a Sep-Pak solid phase extraction cartridge (a reverse phase C-18 cartridge; Waters Millipore, United States). The cartridges were previously activated with 10 mL of MeOH and 10 mL of water. After the sample volume was eluted, the cartridge was washed with 10 mL of water. The remaining volume in the cartridge was eluted with 2 mL of MeOH. The MeOH fraction was filtered through a 0.45 μm membrane filter Millex-HV_{13} (Waters Millipore) and then analyzed by liquid chromatography−tandem mass spectrometry (LC-MS/MS).

Two grams of strawberries and raspberries was homogenized with 20 mL of acetone using the Ultraturrax equipment. The extract was centrifuged as described above, and the acetone phase was removed under reduced pressure at 40 °C. The aqueous phase was further centrifuged at 11300*g* in a Sigma 1−13 microcentrifuge (Braun Biotech. International, Germany), and the supernatant was filtered through a Sep-Pak cartridge, which was previously prepared as described above. The cartridge was washed with 10 mL of water, and the polyphenols were eluted with 2 mL of MeOH. The MeOH fraction was filtered through a 0.45 μm filter and then analyzed by LC-MS/MS.

The ethanol content of 100 mL of wine was removed under reduced pressure at 80 °C. Forty milliliters of the remaining aqueous fraction was filtered through a Sep-Pak cartridge, prepared as described above. The cartridge was washed with 10 mL of water, and the phenolic fraction was eluted with 2 mL of MeOH. The MeOH fraction was filtered through 0.45 μm filter and then analyzed by LC-MS/MS.

Hydrolysis of Foodstuffs. The hydrolysis was carried out following the procedure of Häkinnen et al. (*9*) with some modifications. Briefly, wine (4 mL) and strawberries, raspberries, and walnuts (1 g each) were homogenized in 5 mL of 2 M HCl (8 mL in the case of wine). The mixtures were placed in a heater for 20 h at 85 °C. Afterward, the solutions were vigorously homogenized with diethyl ether three times. The organic phases were pooled and evaporated to dryness, and the residue was redissolved in MeOH (1 mL), filtered through a 0.45 μm filter, and then analyzed by LC-MS/MS.

Urine Samples. Forty milliliters of urine from each fraction and volunteer was filtered through a Sep-Pak cartridge prepared as described above. Then, the cartridge was washed with 10 mL of water, and the phenolic fraction was eluted from the cartridge with 2 mL of MeOH. A sample of 100 μL of the methanolic fraction was analyzed by LC-MS/MS.

For the enzymatic treatment, 60 mL of urine was incubated for 20 h at 37 °C with 2440 units of β-glucuronidase and with 20 units of sulfatase. Afterward, the samples were extracted with diethyl ether three times. The organic phases were pooled and evaporated under reduced pressure until dryness and redissolved in MeOH. A sample of 100 μL of this methanolic fraction was analyzed by LC-MS/MS.

LC-MS/MS Analysis. The high-performance liquid chromatography (HPLC) system equipped with both a photodiode array detector and a mass detector in series consisted of a HPLC binary pump, autosampler, and degasser controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent) equipped with an electrospray ionization (ESI) system (capillary voltage, 4 kV; dry temperature, 350 °C) for the analysis of foodstuffs extracts and glucuronides in urine samples. In the analysis of enzymatic-treated urine samples, the mass detector was equipped with an atmospheric pressure chemical ionization (APCI) system (capillary voltage, 4 kV; dry temperature, 350 °C; crown voltage, 4 kA; APCI temperature, 375 °C). Mass scan (MS) and MS/MS daughter spectra were measured from *m*/*z* 150 up to *m*/*z* 500 for urine samples and from *m*/*z* 200 up to *m*/*z* 4000 for foodstuffs extracts. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the positive ionization mode for enzyme-treated urine samples (with APCI) and in the negative ionization mode for nontreated urine samples and foodstuffs extracts (with ESI).

Chromatographic separations of foodstuffs extracts and urine samples were carried out on a reverse phase C_{18} LiChroCART column (25 cm $\times$ 0.4 cm, particle size 5 μm, Merck, Darmstadt, Germany) using water: formic acid (99.9:0.1, v/v) (A) and MeOH (B) as the mobile phases at a flow rate of 1 mL/min. The gradient for foodstuffs extracts started with 10% B in A, to reach 40% B in A at 15 min and 95% B in A at

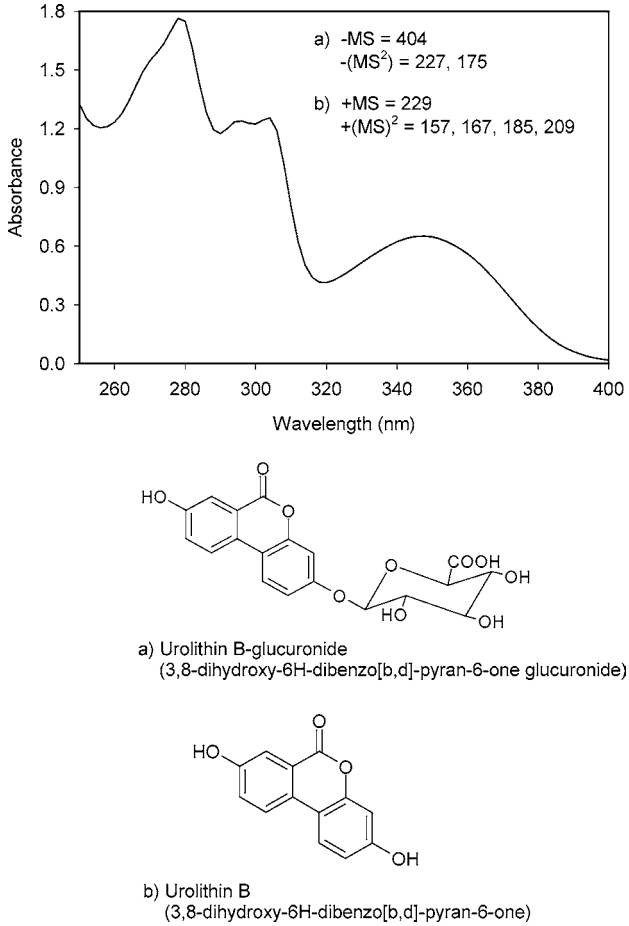

Figure 2. Characteristic spectrum of urolithin B derivatives and their ion mass (MS) and daughter fragments (MS^2).

40 min. UV chromatograms of extracts were recorded at 255, 280, 360, and 510 nm. The gradient for urine samples started with 20% B in A to reach 70% B in A at 25 min and 95% B in A at 30 min. UV chromatograms of urine samples were recorded at 255, 305, and 360 nm.

Quantification of ETs and Urolithin B Derivatives. The most representative ETs from foodstuffs were identified according to their ion mass (MS) and daughter fragments (MS/MS) using ion traps (*14*, *38*, *39*). Identification of EA was carried out by chromatographic comparisons (UV and MS) with a pure standard of EA. ETs were quantified as free EA at 360 nm after the hydrolysis described above (*9*). Urolithin B-glucuronide and its aglycone were identified according to their UV spectra, retention times, ion mass, and MS/MS daughter fragments using the corresponding purified metabolites previously isolated from human urine (*37*). Quantification of metabolites (glucuronide and aglycone) was carried out at 305 nm and by extracting their ion mass, 404 m/z^- with ESI for the glucuronide derivative and 229 m/z^+ with APCI for the aglycone, and using the corresponding purified metabolites previously isolated from human urine as standards (*37*).

Statistics and Graphs. Graphs of the experimental data and their statistic analysis were carried out by using the Sigma Plot 6.0 program for Windows.

RESULTS

The foodstuffs consumed by the subjects were selected on the basis of their different content of ETs and also because strawberries, raspberries, walnuts, and oak-aged wines are common products consumed in the western diet. The MS analysis of the extracts was critical to distinguish between the daughter ion fragments at m/z^- 301 that are coincident for the polyphenol flavonol quercetin (MS^2 ions at m/z^- 151 and 179) and EA (MS^2 ions at m/z^- 257 and 229) (*14*). Raspberry and strawberry extracts mainly contained the ET sanguiin H-6 (**Figure 1**; m/z^- at 1869), as well as various EA derivatives (*14*). Walnut extract contained, among others, pedunculagin (**Figure 1**; m/z^- at 784), valoneic acid dilactone (m/z^- at 469), and casuarictin (m/z^- at 936) (*38*). The ET vescalagin (**Figure 1**; m/z^- at 933) was detected in wine although other ETs such as castalagin and roburin E have also been previously described in oak-aged wines (*40*). Subjects consumed different ETs amounts (quantified as free EA) depending on the group; that is, the SG consumed 190 mg of EA, the RG consumed 422 mg of EA, the WG consumed 191 mg of EA, and the RWG consumed 5.4 mg of EA.

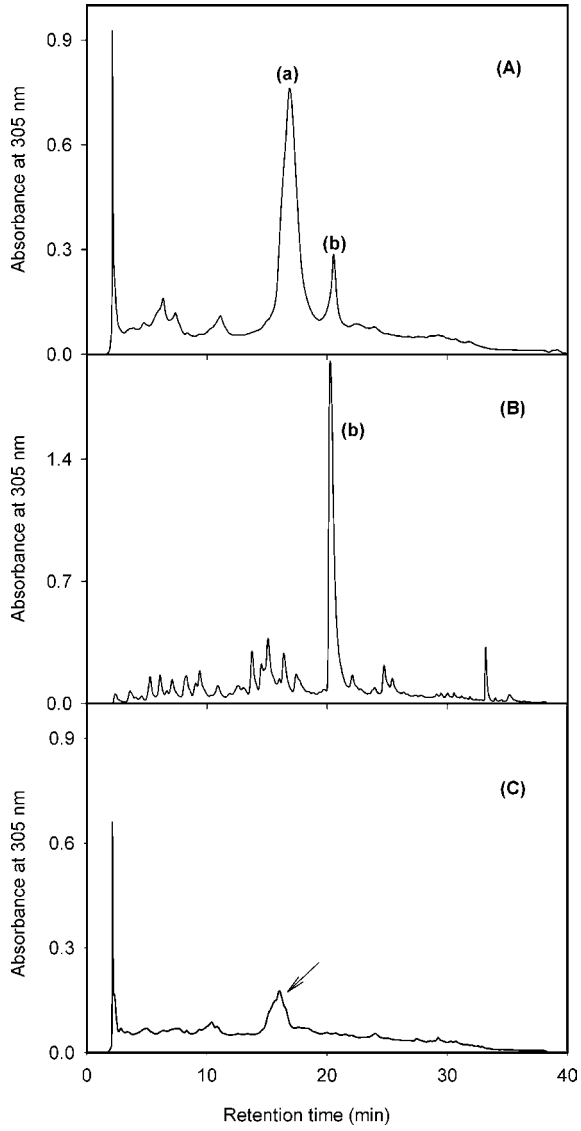

Figure 3. HPLC profile of urine (fraction 5, F5) from one volunteer after consumption of walnuts (35 g). (**A**) Nonenzymatic-treated urine; a, urolithin B-glucuronide; b, urolithin B. (**B**) The same urine but treated with glucuronidase + sulfatase showing the corresponding aglycone urolithin B. (**C**) Control urine (the arrow designates another urine compound not related with ET consumption).

The molecule urolithin B conjugated with glucuronic acid (**Figures 1** and **2**) was by far the most abundant metabolite detected in human urine upon consumption of strawberry, oak-aged red wine, walnut, and raspberry (**Figure 3A**, peak a). In some volunteers, the molecule was also detected in its aglycone form (**Figure 3A**, peak b). The MS analysis of urine samples

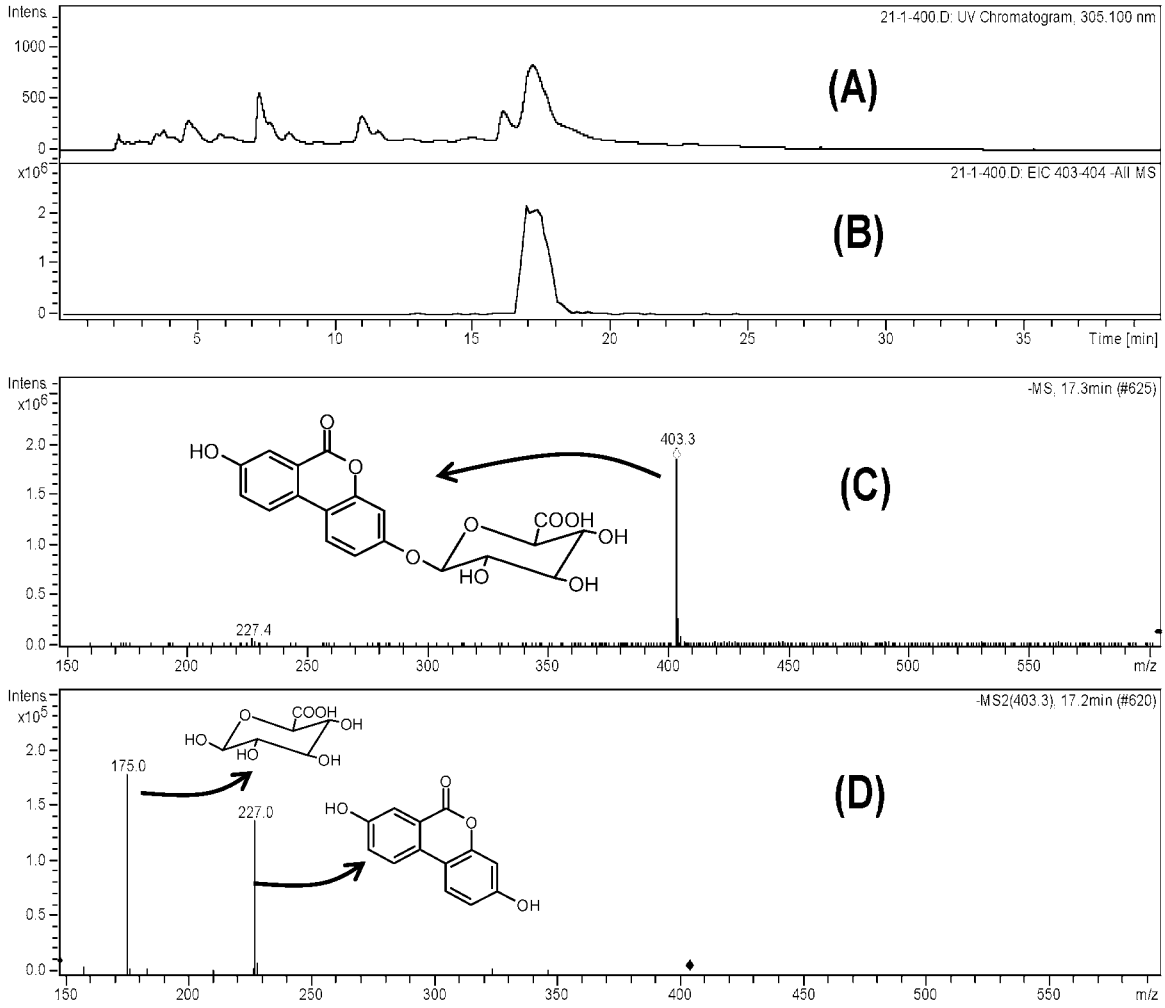

Figure 4. LC-MS/MS analysis of urine (fraction 4, F4) from one volunteer after consumption of raspberries (225 g). (**A**) UV chromatogram at 305 nm. (**B**) Extracted ion chromatogram (EIC) at m/z^- 404 showing the metabolite urolithin B-glucuronide. (**C**) MS scan at the retention time of the above EIC. (**D**) MS/MS daughter spectra from the glucuronide m/z^- 404 showing the glucuronyl residue (m/z^- at 175) and the aglycone part (urolithin B) (m/z^- at 227).

corroborated the presence of urolithin B-glucuronide with m/z^- ion at 404 (**Figures 2** and **4**) that suffered the loss of a glucuronyl residue (m/z^- at 175) to give a daughter ion fragment at m/z^- 227 (**Figures 2** and **4**). When the urine samples were previously treated with both glucuronidase and sulfatase, the corresponding aglycone with m/z^+ ion at 229 was observed (**Figures 2**, **3B** and **5**). These metabolites were unequivocally linked to the consumption of the ET-containing foodstuffs because the metabolites did not appear in any control sample before the intake of these foodstuffs (**Figure 3C**). It should be stressed that neither ETs nor EA derivatives (free or conjugated) were detected in any urine sample. In addition, the urolithin B derivative (monohydroxy-6H-dibenzo[*b*,*d*]pyran-6-one) previously detected in plasma and urine of humans after administration of pomegranate juice (*37*) was not found in the present study.

Volunteers excreted the metabolite along the fractions F3, F4, and F5 (**Tables 1** and **2**), and only trace amounts were detected in fractions F1 and F2 in some volunteers who consumed walnuts or raspberries (**Table 2**). This meant that the excretion started, in most of cases, 16 h after the intake (F3; **Tables 1** and **2**) and lasted during the following 40 h (F3−F5; **Tables 1** and **2**). The complete clearance of ET metabolism could not be estimated because the excretion was still very high in F5 in most of the volunteers (**Tables 1** and **2**). The amount of metabolite excreted greatly depended on the subjects. In fact, high and low metabolite excreters were detected in each group yielding high standard deviation values when the mean total excretion was calculated (**Table 2**). The mean highest excretion was observed in WG and the lowest in RWG (**Table 2**). The maximum excretion values detected were 12 mg in SG (6.3% excretion), 155 mg in WG (81% excretion), 32 mg in RG (7.6% excretion), and 0.75 mg in RWG (7.4% excretion) (**Table 2**). Some volunteers (including high and low excreters) from each group (except RWG) ingested a double amount of foodstuff to compare the ratio of ET consumed and amount of metabolite excreted. A higher metabolite excretion was detected in these volunteers although the excretion was not directly proportional to the amount of ET consumed (results not shown).

DISCUSSION

The studies concerning metabolism of naturally occurring polyphenols are greatly encouraged because of the increasing evidence suggesting that dietary polyphenols with reported in vitro health-promoting (including antioxidant and cancer chemopreventive) activities do not necessarily impart these properties in vivo. This has been recently reported for apple polyphenols where despite the high in vitro antioxidant capacity of individual apple polyphenols the ingestion of large amounts of apples by

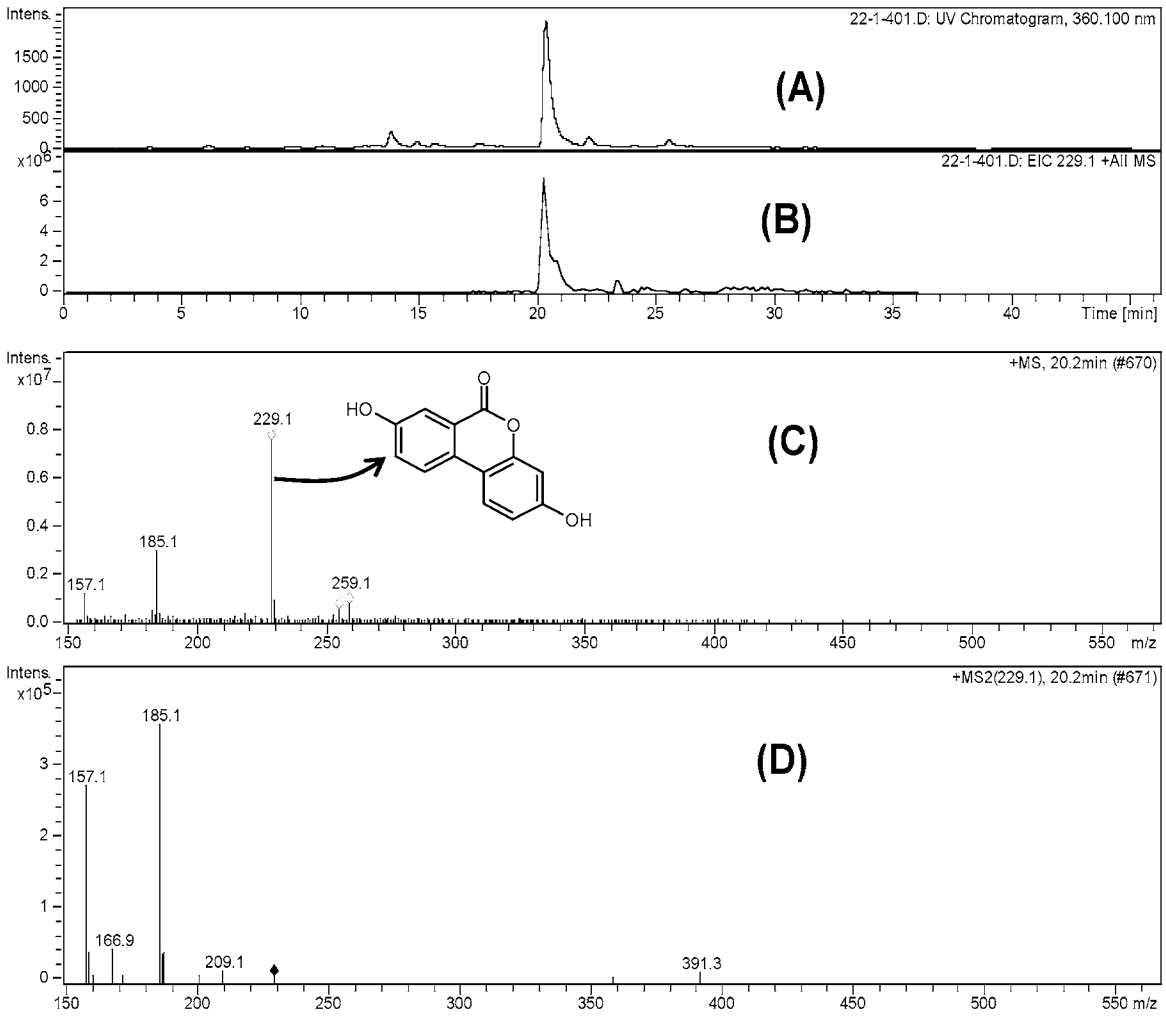

Figure 5. LC-MS/MS analysis of human urine (F4) shown in **Figure 4** but treated with glucuronidase + sulfatase (**A**) UV chromatogram of treated urine at 305 nm. (**B**) EIC at m/z^+ 229 showing the aglycone dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one (urolithin B). (**C**) MS scan at the retention time of the above EIC. (**D**) MS/MS daughter spectra from the aglycone m/z^+ 229 showing the main fragments (m/z^- at 157, 167, 185, and 209).

Table 2. Excretion (mg) of Urolithin B Derivatives (Glucuronide or Aglycone[a]) by Volunteers ($n = 40$, 10 per Group) after Intake of Different ET-Containing Foodstuffs

	strawberry	walnut	raspberry	oak-aged red wine[a]
		urine fractions		
F1	ND[b]	ND	ND	ND
F2	ND	tr	tr	ND
F3	1.8 ± 2.9	8.3 ± 18.4	2.8 ± 2.9	0.17 ± 0.12
F4	1.3 ± 1.6	10.7 ± 20.4	3.3 ± 3.4	0.11 ± 0.13
F5	2.2 ± 3.8	12.6 ± 14.5	8.3 ± 12.1	0.07 ± 0.06
total excretion[c]	mean: 5.3 ± 8.3	mean 31.6 ± 53.3	mean: 14.4 ± 18.4	mean: 0.35 ± 0.31
	max: 12	max: 155	max: 32	max: 0.75
	min: ND	min: 2.3	min: ND	min: ND
percentage of excretion[d]	mean: (2.8 ± 4.4)%	mean: (16.6 ± 28)%	mean: (3.4 ± 4.4)%	mean: (6.5 ± 5.7)%
	max: 6.3%	max: 81%	max: 7.6%	max: 7.4%
	min: 0.05%	min: 1.2%	min: 0.21%	min: 1.8%

[a] Samples were treated with glucuronidase + sulfatase since no glucuronides could be detected. [b] ND, not detected; tr, trace amount (detected but not quantified). A metabolite concentration under 0.025 μg/mL in urine could not be quantified using the protocols specified in the Materials and Methods. [c] Total excretion (mean, maximum, and minimum values) for all urine fractions (F1 + F2 + F3 + F4 + F5) and each volunteer was calculated. The total urine volume for each volunteer was considered. [d] Percentage of excretion in volunteers (mean, maximum, and minimum values) with respect to the amount of ETs consumed. The total ET consumed (quantified as free EA) was as follows: SG, 190.1 mg of EA; RG, 422 mg of EA; WG, 190 mg of EA; and RWG, 5.4 mg of EA.

humans did not appear to result in equivalent in vivo antioxidant effects of apple polyphenols (*41*). This could be explained because the biological activity of the in vivo-generated metabolites (such as those produced by colonic microflora) could not be coincident with that of the ingested polyphenol precursors (*42*, *43*).

This is also the case of dietary ETs whose metabolism has been poorly investigated so far despite their reported in vitro

and in vivo biological activities (*13*, *17*−*19*). Our group has recently described that urolithin B derivatives were bioavailable metabolites detected in plasma and excreted in urine in both rats and humans after oral administration of pomegranate, a rich source in the antioxidant ET punicalagin (*33*, *34*, *37*). In addition, we have also detected these molecules in plasma and urine of free-ranged Iberian pigs fed with acorns (unpublished results), a rich source in different ETs (*39*). ETs and EA (free or conjugated) have been described as molecules that impart in vitro antioxidant and cancer chemopreventive properties to ET-containing foodstuffs such as pomegranates, walnuts, raspberries, etc. (*13*, *20*, *21*, *38*). However, our previous study reported that neither ETs nor EA were detected in plasma or urine in humans upon consumption of pomegranate juice (1 L per day for 5 days) equivalent to the daily intake of 4.4 g of punicalagin ET (*37*). Punicalagin has been proved to be a potent in vitro antioxidant polyphenol (*13*, *37*), but on the contrary, the products of ET metabolism, the bioavailable urolithin B derivatives, did not show this activity (*37*).

The volunteers of the present study consumed different types and amounts of ETs depending on the foodstuff (sanguiin H-6, pedunculagin, casuarictin, EA-pentose conjugates, etc.). According to the results obtained, urolithin B derivatives were excreted independently of the ET consumed. A common monomeric moiety in the ETs consumed was EA (m/z^- at 301), which could indicate that this subunit belonging to ET molecules was the critical molecule to produce urolithin B derivatives in accordance with previous studies that defined urolithin B derivatives as microbial metabolites in rats fed with EA (*29*) and in flying squirrels fed with acorns and ET-containing berries (*30*). In fact, the hydrolysis of ETs to yield EA has been reported to occur in vivo (*16*, *44*) (**Figure 1**).

The results of the present study indicated that the metabolism of ETs was not critically related to either the gender or the dietary habits of volunteers. The amount of ET consumed was not proportionally related to the amount of metabolite excreted although the lowest excretion was observed in RWG (**Table 2**) that consumed the lowest amount of ETs (5.4 mg of EA). The highest excretion was observed in the volunteers from WG (**Table 2**) although other groups consumed the same (SG) or more (RG) ETs (**Table 2**).

The large variability in the metabolism of ETs among volunteers was evidenced by the presence of high and low metabolite excreters in each group. This was in accordance with our recent study on the bioavailability and metabolism of the pomegranate ET punicalagin in humans (*37*). In that study, the excretion of urolithin B derivatives with respect to the punicalagin consumed ranged from 0.7 to 52.7%, and in the present study, the excretion also showed very different interindividual values ranging from 0.05 to 6.3% in SG, from 1.2 to 81% in WG, from 0.21 to 7.6% in RG, and from 1.8 to 7.4% in RWG (**Table 2**).

The presence of high and low metabolite excreters has also been previously identified in the metabolism of isoflavones by humans using the terms equol producer and nonequol producer to describe those subjects who were able (or not) to produce the metabolite equol from isoflavones and the high variability among volunteers was attributed to their different colonic microflora (*45*). Therefore, epidemiological studies on the impact of isoflavone intake on health should also consider the in vivo-generated isoflavone-derived metabolites as previously suggested (*46*). Other studies have also stressed the important role of the colonic microflora in the metabolism of polyphenols (*26*, *42*, *47*). For instance, the polymeric proanthocyanidins are converted by human colon microflora into low molecular weight phenolic acids (*47*) and also the above-mentioned isoflavones (daidzein and genistein) that are mainly converted into the metabolites equol and *o*-desmethylangolesin (*48*, *49*).

The results presented here, together with the apparent parallelism with isoflavone metabolism by colonic microflora (presence of high and low excreters), suggest that urolithin B derivatives are qualitative biomarkers of human exposure to ETs and that the colonic microflora are critically involved in the metabolism yielding high variability among subjects in the excretion of the metabolite. However, the food matrix and possible interactions of ETs with other dietary constituents could also be involved in the bioavailability and metabolism of ETs. Whether these metabolites can be formed directly from ETs or the presence of free EA is a necessary step needs further research, although our preliminary observations (unpublished results) suggest that human fecal samples can directly convert pure ETs to urolithin B derivatives (**Figure 1**).

Taking into account the metabolism of ETs, the assignment of health-promoting properties (such as antioxidant and cancer chemopreventive activities) based on in vitro assays of the ET-containing foodstuffs should be taken with precaution. In this case, special attention should be made on their possible activity in the gastrointestinal tract rather than on their systemic effect, which preferably should be investigated for the bioavailable urolithin B derivatives metabolites, which have been previously described as antiangiogenic and hyaluronidase inhibitors (*30*). Our results on ET metabolism in humans establish the theory that depending on the colonic microflora of each individual, high or low ET metabolism can occur, and thus, high or low amount of urolithin B derivatives can be formed. This could be important in epidemiological and intervention studies with ET-containing foodstuffs because depending on the individual microflora, a different biological activity could be expected.

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