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Iberian Pig as a Model To Clarify Obscure Points in the Bioavailability and Metabolism of Ellagitannins in Humans

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Ellagitannin-containing foods (strawberries, walnuts, pomegranate, raspberries, oak-aged wine, etc.) have attracted attention due to their cancer chemopreventive, cardioprotective, and antioxidant effects. Ellagitannins (ETs) are not absorbed as such but are metabolized by the intestinal flora to yield urolithins (hydroxydibenzopyran-6-one derivatives). In this study, Iberian pig is used as a model to clarify human ET metabolism. Pigs were fed either cereal fodder or acorns, a rich source of ETs. Plasma, urine, bile, lumen and intestinal tissues (jejunum and colon), feces, liver, kidney, heart, brain, lung, muscle, and subcutaneous fat tissue were analyzed. The results demonstrate that acorn ETs release ellagic acid (EA) in the jejunum, then the intestinal flora metabolizes EA sequentially to yield tetrahydroxy- (urolithin D), trihydroxy- (urolithin C), dihydroxy- (urolithin A), and monohydroxy- (urolithin B) dibenzopyran-6-one metabolites, which were absorbed preferentially when their lipophilicity increased. Thirty-one ET-derived metabolites were detected, including 25 urolithin and 6 EA derivatives. Twenty-six extensively conjugated metabolites were detected in bile, glucuronides and methyl glucuronides of EA and particularly urolithin A, C, and D derivatives, confirming a very active enterohepatic circulation. Urolithins A and B as well as dimethyl-EA-glucuronide were detected in peripheral plasma. The presence of EA metabolites in bile and in urine and its absence in intestinal tissues suggested its absorption in the stomach. Urolithin A was the only metabolite detected in feces and together with its glucuronide was the most abundant metabolite in urine. No metabolites accumulated in any organ analyzed. The whole metabolism of ETs is shown for the first time, confirming previous studies in humans and explaining the long persistency of urolithin metabolites in the body mediated by an active enterohepatic circulation.

KEYWORDS: Ellagitannin; ellagic acid; bioavailability; tissue distribution; metabolism; bile; gall bladder; colon; intestine; urolithin

INTRODUCTION

Ellagitannins (ETs) and ellagic acid (EA) are phenolic compounds that have received attention during the past few years due to their high in vitro antioxidant activity (*1*, *2*) and the biological properties associated with the intake of ET-containing products that are related to the prevention of cardiovascular diseases (*3*) and cancer (*4*).

These compounds include free EA itself and glycosidic combinations with different sugars (glucose, rhamnose, and arabinose) or acylated sugars (acetylglucose) (*5*). ETs are hexahydroxydiphenoyl esters of sugars that upon hydrolysis release hexahydroxydiphenic acid, which spontaneously suffers an internal lactone formation to give EA. Examples of ETs are punicalagin from pomegranates, sanguiin H-6 from strawberries and raspberries, sanguiin H-5 from muscadine grapes, pedunculagin from walnuts, and vescalagin and castalagin from oak wood (and oak-aged wines and spirits). All of these compounds show high radical scavenging and antioxidant activities in vitro.

Food products containing ETs and EA conjugates include pomegranates, strawberries, raspberries, blackberries, and several nuts including walnuts, chestnuts, oak acorns, pistachios, and pecans. Muscadine grapes are also a relevant source of ETs. In addition, these compounds are also present in oak wood, and

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from this they can pass to drinks that have been aged in oak barrels. This is the case of wines and some spirits.

The bioavailability and metabolism of these polyphenols have been studied during recent years. Previous studies indicate that large ETs are generally not absorbed as such. Only in one study using rats as animal model, and after the intake of large amounts of pomegranate ETs, in a toxicologically oriented study, were traces of punicalagin (MW 1084) found in plasma and urine (*6*). All of the studies with human subjects have shown no absorption of the native ETs after the intake of ET-containing foods (*7–9*). We have reported that these compounds are partially hydrolyzed to EA under physiological conditions as shown in human cell lines (*10*). Processing can also increase the release of EA from ETs (*5*). EA, however, has been reported to be poorly absorbed in humans after the intake of 'Wonderful' pomegranate juice or that of black raspberry freeze-dried powder and detected in plasma as such (not conjugated) within 30 min−1 and 2 h of the intake, showing absorption in the stomach (*11, 12*).

In previous studies we showed for the first time in humans extensive transformation of ETs to dibenzopyranones (urolithins A and B) that circulate in plasma as glucuronide conjugates and are excreted in urine. These metabolites are present in significant concentrations in plasma and urine, reaching the highest concentrations after 24–48 h, and they are present in urine as long as 48–72 h after intake (*7–9*). These results were later confirmed by other authors (*13, 14*). These studies suggested that benzopyranones were the product of intestinal microflora metabolism as was, in fact, later demonstrated (*15*). The persistency of the metabolites in urine for such a long time suggested a low clearance of these metabolites due to the presence of an enterohepatic circulation, but this has not been demonstrated so far.

These previous studies, however, did not show where the metabolites are produced in the intestine, how are they absorbed and conjugated, and the distribution in different target tissues. These issues are relevant in terms of establishing the biological activity of these polyphenols. We demonstrated for the first time the disposition of urolithin derivatives in the rat, where metabolites were detected in liver and kidney, whereas these metabolites, EA, or ETs were not detected in other rat tissues (*16*).

The pig has been used as a monogastric animal model physiologically closer to humans to test tissue distribution of flavonoids (*17*). Iberian pigs, which are usually fed oak acorns, provide an excellent model to test the metabolism and bioavailability of ETs, as acorns are very rich in these polyphenols (*18*). In addition, it was previously reported that Iberian pig meat and fat, when the pigs were fed on acorns, was particularly resistant to rancidity and oxidation, suggesting that some constituents of the diet could be responsible for this particular resistance (*19*).

The aim of this study was the evaluation of the bioavailability and metabolism of ETs in pigs as well as their distribution in different tissues in order to provide information on some obscure points of the ET bioavailability in humans.

MATERIALS AND METHODS

Chemicals and Reagents. Ellagic acid, β-glucuronidase from bovine liver (G-0251, EC 3.2.1.31; 1000 units/g of solid), and sulfatase from *Helix pomatia* (S-9626, EC 3.1.6.1; 10000 units/g of solid) were purchased from Sigma (St. Louis, MO). Methanol (MeOH), diethyl ether, hydrochloric acid, phosphoric acid, and hexane were obtained from Merck (Darmstadt, Germany). Urolithins A and B were chemically synthesized and provided by Kylolab (Murcia, Spain). Ascorbic acid was obtained from Aldrich (Steinheim, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment.

Table 1. Chemical Compositions of Acorns and Formulated Diet[a]

major nutrient	acorns	feed
dry matter (DM) (g/kg)	660.0	891.1
crude protein (g/kg of DM)	54.0	141.3
crude fat (g/kg of DM)	56.1	31.1
crude fiber (g/kg of DM)	57.2	45.0
nitrogen- free extractives (g/kg of DM)	818.0	770.3
ash (g/kg of DM)	14.7	12.3
fatty acids (g/100 g of total fatty acids)		
C14:0	0.1	0.3
C16:0	18.4	18.1
C17:0	0.1	0.5
C18:0	3.4	3.5
C16:1 n-7	0.1	4.1
C18:1 n-9	56.7	44.3
C18:2 n-6	18.5	25.5
C18:3 n-3	1.7	0.8
C20:0	0.3	0.6

[a] Check reference Cantos et al. (*18*) for detailed polyphenolic composition of acorns.

Acorns, Pigs, and Sampling Procedure. All assays were carried out by fulfilling the Declaration of Helsinki related to animal welfare and experimental guidelines. Iberian barrows of the Torbiscal line (El Dehesón del Encinar, Junta de Comunidades de Castilla-La Mancha, Oropesa, Toledo, Spain) of the same age (approximately 10 months) with an average initial live weight of 100.1 kg (SEM = 1.9 kg) were randomly distributed into two groups of 16 pigs each. The pigs were located in confinement in individual cages. One group was regularly fed fresh acorns from *Quercus ilex* and *Quercus suber* collected from a Mediterranean forest located in Oropesa (Toledo, Spain) during 117 days. The average daily amount of acorns given to pigs was 4.04 kg. Acorn-fed pigs did not eat anything else. The phenolic compound composition of acorns was analyzed as previously reported (*18*) and showed that the acorns were rich in EA and ETs. The nutritional composition of acorns is shown in **Table 1**. The other (control) group of pigs was fed during the same period of time (117 days) a diet containing (per kilogram) 475 g of barley, 400 g of wheat, 80 g of soybean meal, 20 g of lard, 8 g of calcium carbonate, 12 g of calcium phosphate, 3 g of sodium chloride, and 2 g of vitamin−mineral mix. The diet had a calculated energy content of 13.3 mJ of ME/kg. The experiment was conducted during two consecutive campaigns in 2004–2005 and 2005–2006.

To obtain preprandial and postprandial plasma samples, blood was collected by jugular puncture from immobilized pigs. After food deprivation for 24 h (preprandial), blood samples were taken from each experimental pig. Then they were refed, and blood samples were taken after 3 h (postprandial). Blood was collected in heparinized tubes and immediately centrifuged. Plasma samples were frozen in liquid nitrogen and stored at −80 °C until required.

Pigs (nonfasted) were slaughtered at a local slaughterhouse following the legal procedure at an average weight of 146.5 ± 3.7 kg. Samples of digesta taken from the terminal jejunum, urine, bile, brain, kidney, liver, Longissimus dorsi muscle, and subcutaneous fat at the level of the last rib were taken immediately after slaughter, frozen in liquid nitrogen, and stored at −80 °C until required.

Processing of Biological Fluids and Tissue Samples. *Plasma.* This was processed as previously described (*20*) with some modifications. Plasma samples (900 μL) were mixed with 333 μL of a water solution containing ascorbic acid (0.2 g/L) and EDTA (1 mg/mL). To break possible polyphenol−plasmatic protein linkages, 18 μL of orthophosphoric acid was added. Samples were homogenized and centrifuged in a Sigma 1-13 microcentrifuge (Braun Biotech. International, Melsungen, Germany) at 14000*g* at 4 °C. The supernatants were filtered through a reverse phase C_{18} Sep-Pak cartridge (Millipore Corp.), and these were washed with distilled water and polyphenol metabolites eluted with methanol. The cartridges were previously activated with 10 mL of

Table 2. Ellagitannins and Ellagic Acid Metabolites Detected in Pig after Acorn ET Intake

structure[a]	no.	t_R (min)	MS (M − H)	MS/MS	UV[b]	occurrence[c]
urolithin A diglucuronide	**1**	12.8	579	403, 227, 175	D	Bi
urolithin C diglucuronide	**2**	13.5	595	419, 243, 175	C_a	Bi
urolithin C methyl ether diglucuronide	**3**	14.0	609	433, 257, 242, 175	C_a	Bi
urolithin C diglucuronide	**4**	15.0	595	419, 243, 175	C_a	Bi
urolithin C methyl ether diglucuronide	**5**	15.7	609	433, 257, 242, 175	C_a	Bi
urolithin D glucuronide *N*-acetyl-cysteine	**6**	16.4	597	421, 259, 175	B	Bi
urolithin D diglucuronide	**7**	16.9	611	435, 259, 175	B	Bi
urolithin D glucuronide	**8**	18.2	435	259, 175	B	Bi
urolithin C glucuronide	**9**	18.6	419	243, 175	C_a	Bi
urolithin D glucuronide	**10**	18.9	435	259, 175	B	Bi
ellagic acid methyl ether diglucuronide	**11**	19.3	667	491, 315, 175	A	Bi
urolithin A glucuronide	**12**	19.7	403	227, 175	D	Bi, P, T
urolithin D methyl ether glucuronide	**13**	20.1	449	273, 258, 175	B	Bi
urolithin C methyl ether glucuronide	**14**	20.2	433	257, 243, 175	C_a	Bi
urolithin C methyl ether glucuronide	**15**	20.4	433	257, 242, 175	Ca	Bi
ellagic acid methyl ether glucuronide	**16**	20.4	491	315, 300	A	Bi
ellagic acid	**17**	20.9	301	315, 300	A	L, T
ellagic acid dimethyl ether glucuronide	**18**	21.5	505	329, 315, 300, 175	A	U, Bi
urolithin D (tetrahydroxydibenzopyranone)	**19**	21.7	259	241, 231, 205	B	L, T, Bi
urolithin D methyl ether glucuronide	**20**	21.9	449	273, 259, 175	B	Bi
urolithin C (trihydroxydibenzopyranone)	**21**	22.9	243	225, 197, 181	C_a	L, T, P, Bi
urolithin C isomer	**22**	24.3	243	199, 183, 163	C_b	L, T
urolithin C methyl ether glucuronide sulfate	**23**	24.5	513	337, 257, 175	C_a	Bi
ellagic acid methyl ether	**24**	24.7	315	300	A	Bi
urolithin B glucuronide	**25**	24.8	389	211, 175	E	U, P
urolithin D methyl ether	**26**	25.6	273	258	B	Bi
urolithin C methyl ether	**27**	26.0	257	243	Ca	Bi
urolithin A	**28**	26.2	227	210, 199, 183, 160	D	L, T, U, F, Bi
urolithin C methyl ether	**29**	26.7	257	242, 211, 197	C_a	L, T
ellagic acid dimethyl ether	**30**	27.7	329	315, 300	A	Bi
urolithin B	**31**	30.1	211	167	E	U

[a] Compounds were tentatively identified according to their UV and MS/MS spectra (with the exception of the aglycones urolithins A and B as well as their corresponding glucuronides, which have been characterized in previous papers (*7*–*9*). [b] For UV spectra type see **Figure 1**. [c] Ocurrence: (Bi) bile; (U) urine; (P) plasma; (L) intestinal lumen; (T) intestinal tissue; (F) feces.

MeOH and 10 mL of water. The eluted methanolic fraction (1 mL) was taken to dryness under nitrogen flow at room temperature, and the extract was redissolved in 200 μL of methanol/HCl (99.9:0.1) and analyzed (100 μL) by HPLC-MS/MS.

Urine. Urine samples (3.5 mL) from pigs and from the volunteer who consumed strawberry (200 g of fresh strawberries and the urine volume of 24 h was collected) were acidified with 20 μL of 6 M HCl and filtered through reversed-phase C_{18} Sep-Pak cartridges, washed with distilled water (10 mL), and further eluted with methanol (1 mL). A sample of 100 μL was analyzed by HPLC-DAD-MS/MS.

Bile. Bile samples (1.5 mL) were homogenized in a vortex with 1.5 mL of MeOH/HCl/water (94.9:0.1:5) and exposed to ultrasound for 5 min. The mixture was centrifuged in the Sigma 1-13 microcentrifuge at 7000*g* at 4 °C for 5 min. The supernatant (approximately 1.5 mL) was vigorously homogenized with 1.5 mL of hexane and the mixture centrifuged in the microcentrifuge at 14000*g* at 4 °C for 10 min. The methanolic phase was further centrifuged in the same conditions, the supernatant was filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp.), and a sample of 100 μL was analyzed by HPLC-DAD-MS/MS.

Intestinal Lumen. The contents of both jejunum and colon were collected separately. Samples of 2 g of each portion of intestine were homogenized in a vortex with 10 mL of MeOH/HCl/water (79.9:0.1:20), exposed to ultrasound for 10 min, and centrifuged in the microcentrifuge at 14000*g* at 4 °C for 10 min. The methanolic phase was evaporated under nitrogen flow at room temperature. The remaining aqueous phase was supplemented with an additional 10 mL of water plus 20 μL of 6 M HCl and filtered through an activated Sep-Pak cartridge. The cartridge was washed with 10 mL of distilled water, and the sample was eluted with 1 mL of MeOH. A sample of 100 μL was analyzed by HPLC-DAD-MS/MS.

Feces. Samples (1 g) were homogenized in Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24000 rpm for 1 min with 10 mL of MeOH/HCl/water (79.9:0.1:20). The mixture was centrifuged in the microcentrifuge at 14000*g* at 4 °C for 10 min. The supernatant was filtered through a 0.45 μm membrane filter Millex-HV_{13}, and a sample of 100 μL was analyzed by HPLC-DAD-MS/MS.

Organs and Tissues. Jejunum and colon tissues (3.5 g) as well as liver, kidney, heart, brain, lung, and muscle (10 g each) were processed as previously described for intestinal lumen. Tissues and organs were thoroughly washed with physiological buffer. In the case of jejunum and colon tissue, special attention was paid to remove any luminal content adhering. Subcutaneous fat (20 g) was vigorously homogenized with 200 mL of hexane, exposed to ultrasound for 10 min in a water bath at 37 °C, and further filtered through cheesecloth. Afterward, 100 mL of MeOH/HCl/water (79.9:0.1:20) was added to the filtered solution. The mixture was vigorously stirred for 5 min and transferred to a decantation funnel. The hydroalcoholic phase was extracted with ethyl acetate (1:1, v/v) twice and taken to dryness under nitrogen flow at room temperature, and the final residue was redissolved in 200 μL of methanol/HCl (99.9:0.1) and analyzed (100 μL) by HPLC-MS/MS.

Enzyme Hydrolysis. Samples from the different fluids, organs, and tissues were also treated simultaneously with 40 units/mL β-glucuronidase from bovine liver (1000 units/g of solid, Sigma-Aldrich) and 0.3 unit/mL sulfatase from *H. pomatia* (10000 units/g of solid, Sigma-Aldrich) to determine the possible conjugation to sulfate and/or glucuronide moieties. Samples (1 mL of bile, plasma, or urine) plus 144 μL of acetate buffer (0.1 M, pH 5) and the enzymes were incubated overnight. 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 HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) was equipped with a mass detector in series (Agilent). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system (capillary voltage, 4 kV; dry temperature, 350 °C). Mass scan (MS) and MS/MS daughter spectra were measured from *m*/*z* 75 to 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium

as collision gas, and the collision energy was set at 25%. Mass spectrometry data were acquired in the negative ionization mode.

Chromatographic separations of samples were carried out on a reverse phase C_{18}LiChroCART column (25 cm × 0.4 cm, particle size = 5 μm, Merck) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 1% B in A to reach 25% B in A at 20 min, 55% B in A at 30 min, and 90% B in A at 31 min. UV chromatograms of extracts were recorded at 280, 305, and 360 nm. Ellagic acid, urolithins A and B, and the derived metabolites were identified according to their UV and MS spectra as well as MS/MS fragments.

RESULTS

ET Metabolite Identification. Thirty-one ET metabolites were detected and characterized in the present study (**Table 2**). They are conjugates of EA and their microbial degradation metabolites belonging to the dibenzopyranone group and known as urolithins. Conjugates with glucuronic acid, methyl ether, and sulfates were detected in different biological fluids and tissues. The metabolites were characterized by their UV spectra and MS/MS analyses. The small amount of metabolites in the biological fluids and tissues prevented their isolation and full characterization by other spectroscopic means. The MS analysis, however, allows, together with the UV spectrum, a tentative identification of most of the metabolites.

In previous studies in vitro, we have shown that EA (**17**) is released from ETs under the physiological conditions of the gastrointestinal tract (GI) and that this release can take place spontaneously under the pH conditions of the small intestine without the need of enzymes (*10*). In addition, the microbial metabolism of EA to produce urolithins A (**28**) and B (**31**) has been reported in humans and rats (*7–9, 16*). In the present study, both EA and urolithins A and B were detected in pig biological fluids. In addition, intermediate metabolites in the degradation pathway from EA to urolithin A were detected. Thus, tetrahydroxydidenzo[*b*,*d*]pyran-6-one (urolithin D, **19**) and two trihydroxydibenzo[*b*,*d*]pyran-6-one isomers (urolithin C, **21** and **22**) were also characterized (**Figure 1**). These compounds were identified by their mass and UV spectra (**Table 2**). The characteristic UV spectra of these metabolites are shown in **Figure 1**. All UV spectra show two main absorption bands (BII, 250–280 nm; and BI, 330–380 nm), and in most cases a third band is also observed (BIII, 280–320 nm). It is clear that, as could be expected, the higher the number of hydroxyls on the aromatic rings, the higher the wavelength of the BI maximum. EA and urolithins are mainly detected in conjugated forms with glucuronic acid and methyl ethers, and seldom as sulfates (only one sulfated metabolite has been detected) (**23**). The different conjugate combinations were characterized using the MS/MS fragmentation of isolated ions (ion trap) (**Table 2**).

Free EA (**17**), EA methyl ether (**24**) and dimethyl ether (**28**), EA methyl ether glucuronide (**16**), and EA dimethyl ether glucuronide (**18**) were detected (**Table 2**). All showed UV spectra similar to that of EA (**Figure 1**), and the conjugates after MS/MS rendered the corresponding EA (m/z^- 301), EA methyl ether (m/z^- 315), and EA dimethyl ether (m/z^- 329) aglycones, after separation of the glucuronyl residue.

Tetrahydroxydibenzo[*b*,*d*]pyran-6-one (urolithin D, **19**) (m/z^- 259) and the corresponding monomethyl ether (**26**) (*m*/*z* 273) were also detected as well as different combinations with glucuronic acid (**6**, **7**, **8**, and **10**).

Two trihydroxydibenzo[*b*,*d*]pyran 6-one isomers (urolithin C **21**, **22**) (*m*/*z* 243) were detected, and these showed quite different UV spectra that indicate a very different substitution pattern of the hydroxyls on the benzopyranone nucleus. Two monomethyl ethers (m/z^- 257) of **21** (same UV spectrum) (**27** and **29**) were also detected, as well as conjugates of **21** (**2**, **4**, and **10**) and the methyl ethers **27** and **29** (**3**, **5**, **14**, **15**, and **24**) with glucuronic acid. The last one is a glucuronide-sulfate conjugate of trihydroxydibenzo[*b*,*d*]pyran-6-one methyl ether, this being the only sulfate conjugate detected in this study.

Urolithin A (3,8-dihydroxydibenzo[*b*,*d*]pyran 6-one) (m/z^- 227) (**28**) and urolithin B (3-hydroxydibenzo[*b*,*d*]pyran-6-one) (m/z^- 211) (**31**) were also produced, but in this case no methyl ether derivatives were detected. This is due to the lack of catechol (*o*-dihydroxy) groupings in the molecules, and therefore they are not substrates of the enzyme COMT (catechol *o*-methyl transferase). Glucuronides of both compounds have been detected (**1**, **12**, and **25**).

The HPLC-MS/MS analyses show the sequential loss of glucuronic residues and methyl residues to render the corresponding aglycones. In bile samples, some mass spectra showed a molecular weight 23 mass units higher than that expected, and one of the main fragments was that obtained after the loss of 198 mass units (glucuronic + sodium). This suggests that the compound is the sodium adduct of a glucuronide or a diglucuronide (these adducts were not included in **Table 2**).

In addition, in some samples metabolic products derived from tergallic acid, which is present in the acorns as different combinations, were also detected. Thus, one of these metabolites was detected at a retention time of 28.0 min in bile extracts, with m/z^- 537. This coincided with a trihydroxytergallagic degradation metabolite, conjugated with glucuronic acid. The loss of the glucuronyl residue from the M − H ion rendered the aglycone at m/z^- 361 and a fragment for the glucuronyl residue at m/z^- 175.

Distribution of ET Metabolites in Different Tissues and Biological Fluids. *ET Metabolites in Peripheral Plasma.* The analyses of plasma samples obtained from fasting control pigs (fed only cereal fodder) showed that none of the metabolites from ETs was detected. When control pigs were fed 2 kg of acorns and plasma samples were taken 3 h after consumption, their HPLC analyses showed neither microflora metabolites (urolithins) nor EA derivatives (**Figure 2**), either after enzyme treatment or as crude plasma. On the contrary, the analyses of plasma samples obtained from fasting pigs regularly fed acorns clearly showed the occurrence of urolithin A (**28**) after enzyme treatment of plasma with glucuronidase and sulfatase, and the same metabolite was also observed in plasma of control pigs obtained 24 h after the intake of 2 kg of acorns.

The acorns used to feed the pigs were analyzed by HPLC-DAD-MS/MS as described previously (*18*). The chromatograms were characterized by large amounts of free EA and gallic acid, several isomers of the ET trigalloylhexahydroxydiphenoyl glucose (M − H, *m*/*z* 937), and EA dimers and pentosyl derivatives. In addition, gallotannins such as digalloyl-, trigalloyl-, and tetragalloylglucose (M − H at *m*/*z* 483, 635, and 787, respectively) were also observed (data not shown).

The analyses of samples from control pigs after a single acorn intake showed no metabolites in peripheral plasma at 3 h after intake, whereas urolithin A and B metabolites were detected after 24 h, showing that the metabolites were produced in the distal portion of the pig GI tract. In **Figure 2** it is shown that both urolithin A and B glucuronides (**12** and **25**) (m/z^- 403 and m/z^- 389, respectively) were detected in plasma, and the enzyme treatment with glucuronidase and sulfatase released the corresponding aglycones urolithins A and B (m/z^- 227 and 211, respectively) (**28** and **31**). Even if free EA is a relevant

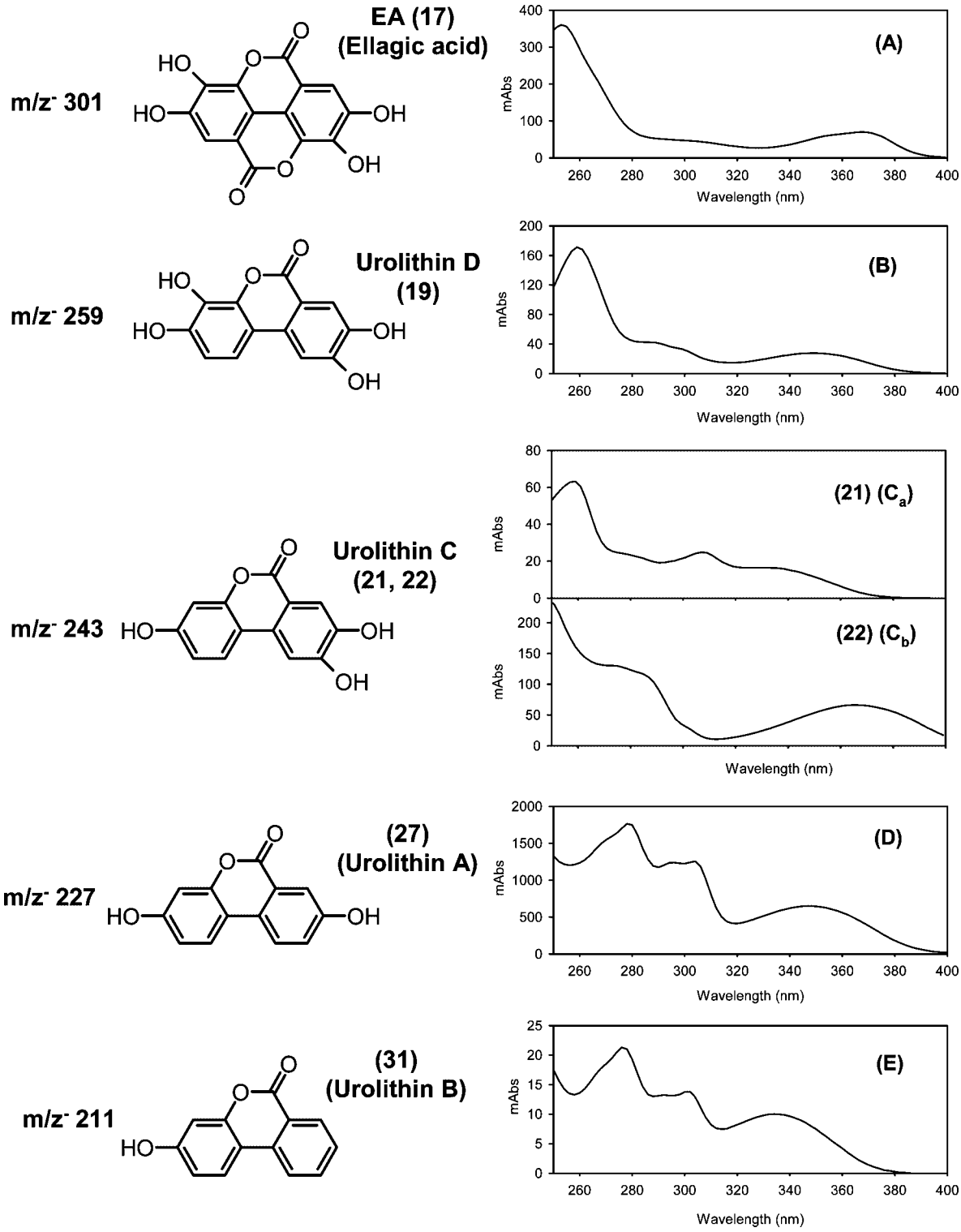


Figure 1. Ellagic acid bacterial metabolites and their UV spectra.

constituent in acorns, this compound or its conjugated forms were not detected in plasma at 2 h after intake (below the limit of detection).

Changes in the Intestinal Tract. The pig provided an excellent model to evaluate the fate of ETs in the intestinal tract, as the content and tissues of different intestinal portions were obtained in control animals (fed cereals) and those fed acorns. The feces were also evaluated to follow the metabolites excreted by this way.

The original acorn phenolics were present only as traces in some of the jejunum content samples after HPLC analyses. Most of the samples contained aglycone metabolites with EA (**17**) being the main one, with significant amounts of urolithin D (tetrahydroxydibenzopyranone) (m/z^- 259) (**19**) and urolithin C isomers (trihydroxydibenzopyranone) (m/z^- 243) (**21** and **22**). Isomer **21** showed the characteristic spectrum of the trihydroxy derivatives (**Figure 1**), whereas **22** showed a UV spectrum similar to that of urolithin D (**19**). This means that different substitution patterns for the three hydroxyls were present in both isomers. Urolithin A (dihydroxydibenzopyranone) (**28**) was present in much smaller amounts, and urolithin B (hydroxydibenzopyranone) (**31**) was not detected here (**Figure 3**). Traces of urolithin C methyl ether (m/z^- 257) (**27**, **29**) were also

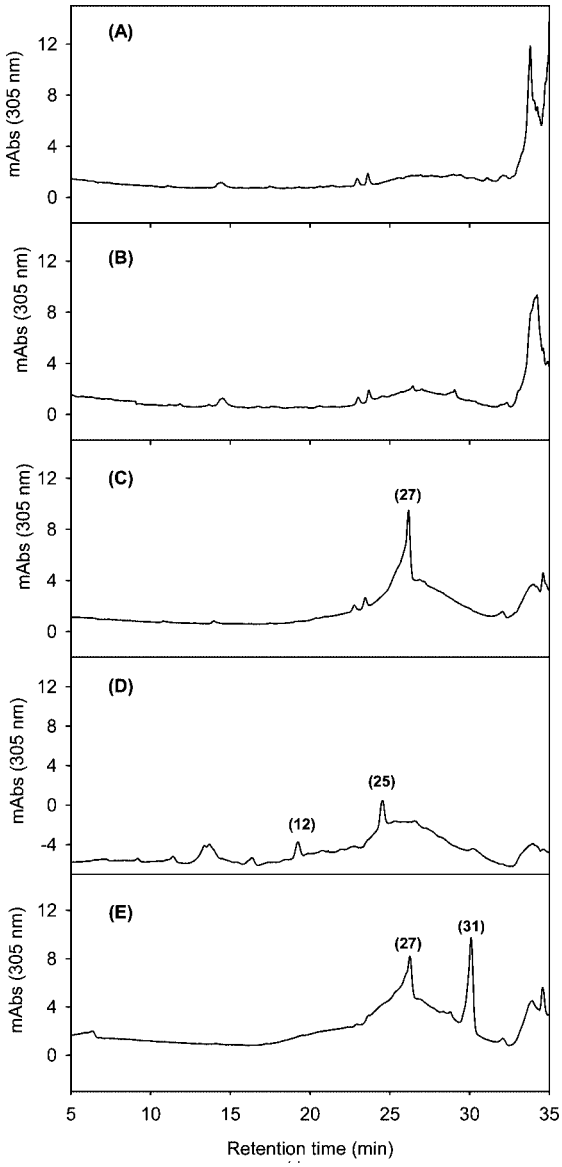


Figure 2. HPLC analyses of peripheral plasma samples: (**A**) fasting control pigs (fed cereals); (**B**) control pigs 2 h after acorn intake; (**C**) fasting pigs regularly fed acorns; (**D**) control pigs 24 h after acorn intake (plasma not treated with enzymes); (**E**) control pigs 24 h after acorn intake (plasma hydrolyzed with enzymes). Peaks: (12) urolithin A glucuronide; (25) urolithin B glucuronide; (27) urolithin A; (31) urolithin B. All samples were treated with enzymes (glucuronidase and sulfatase) before analysis except that shown in panel **D**.

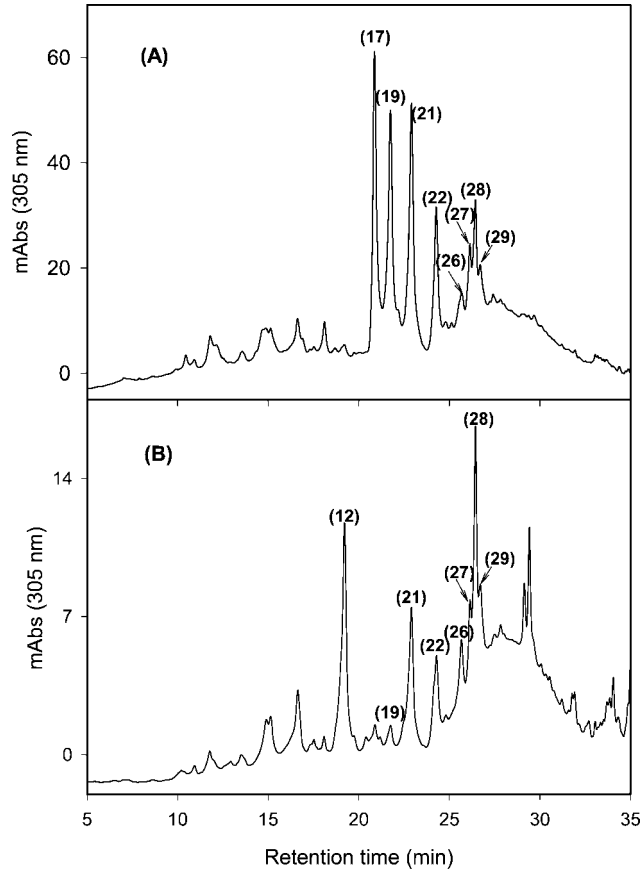


Figure 3. ET metabolites in the small intestine (jejunum): (**A**) small intestine lumen content; (**B**) small intestine tissue. Peaks: (12) urolithin A glucuronide; (17) EA; (19) urolithin D (tetrahydroxydibenzopyran-6-one); (21, 22) urolithin C (trihydroxydibenzopyran-6-one); (26) urolithin D methyl ether (tetrahydroxydibenzopyran-6-one methyl ether); (27, 29) urolithin C methyl ether (trihydroxydibenzopyran-6-one methyl ether); (28) urolithin A.

observed. No glucuronide or sulfate derivatives were detected in the lumen of the small intestine (**Figure 3**).

When the tissue of the small intestine was extracted and analyzed, most of the aglycones present in the lumen were also detected in the tissue, with the exception of EA, the main compound found in the lumen that was not detected in the intestinal tissue. The main metabolites detected in the tissue were urolithin A (**28**) and its glucuronic conjugate (**12**) (**Figure 3**). It seems that the ET metabolite uptake by the intestinal tissue is larger when the number of hydroxyl residues in the molecule decreases (increasing the lipophilic character). Thus, EA is poorly absorbed, the tetrahydroxydibenzopyranone metabolite (urolithin D, **19**) is detected in the tissue but at a low concentration, and the trihydroxydibenzopyranone metabolites (urolithin C) are quite well absorbed (**21**, **22**), whereas the dihydroxydibenzopyranone (urolithin A) (**28**) and methyl trihydroxydibenzopyranones (**27**, **29**) are very well absorbed and are detected in quite significant amounts in the tissues.

This shows that most food ETs are transformed to EA during the digestion in the first part of the gastrointestinal tract. In addition, this demonstrates that the bacteria present in the jejunum are able to metabolize EA into a number of degradation metabolites, which are then absorbed (they are detected in the intestinal cells). The bacterial metabolism follows a degradation order,and different intermediates with decreasing number of phenolic hydroxyls are detected. The intestinal uptake is more facilitated for those metabolites that are more lipophilic. No monohydroxydibenzopyranone (urolithin B) (**31**) was detected in the small intestine, uggesting that the last steps of the intestinal metabolism of EA occur in the large intestine.

When the lumen content of the colon and the content of the colon tissues were examined, only small amounts of urolithin A (**28**) and urolithin B (**31**) were detected, whereas the other metabolites or conjugates were not observed. This confirms that the bacterial metabolism of the ETs continues in the large intestine, to end with the final formation of urolithin B.

An analysis of the feces of pigs fed acorns shows the occurrence of urolithin A as the main ET metabolite (**Figure 4**). In addition, smaller amounts of the tetrahydroxydibenzopyranone and trihydroxydibenzopyranone metabolites were also detected, whereas no urolithin B (**31**) was found in feces, showing the high uptake of this metabolite by the intestinal tissues. In addition, feces contained small amounts of trigal-

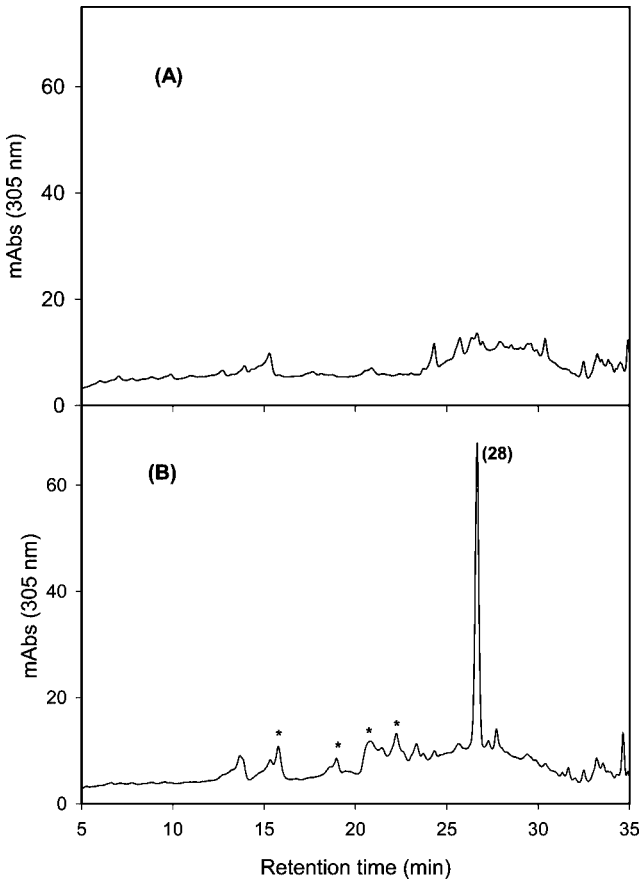


Figure 4. HPLC analyses of pig feces: (**A**) control pigs (fed cereals); (**B**) pigs fed acorns. Peaks: (28) urolithin A; (∗) acorn ETs. Samples were not treated with enzymes.

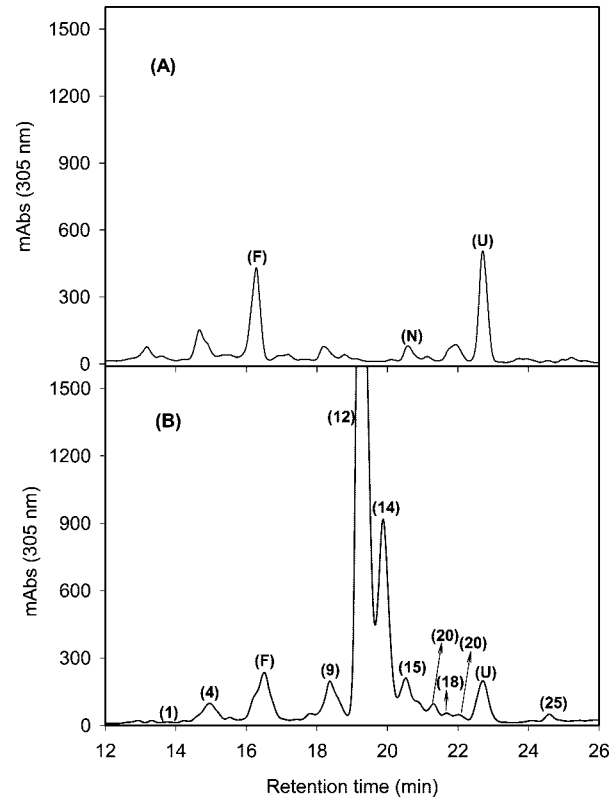


Figure 5. HPLC analyses of urine samples: (**A**) control urine (pigs fed cereals); (**B**) urine of pigs fed acorns. Peaks: (U) urine pigment; (F) feruloylglycine; (N) naringenin glucuronide; (1) urolithin A diglucuronide; (4) urolithin C diglucuronide; (9) urolithin C glucuronide; (12) urolithin A glucuronide; (14, 15) urolithin C methyl ether glucuronide; (18) EA dimethyl ether glucuronide; (20) urolithin D methyl ether glucuronide; (25) urolithin B glucuronide.

loylhexahydroxydiphenoylglucose (m/z^- 937, 769, 617, 301) and pentagalloylglucose-derived molecules (m/z^- 861, 787, 465, 301), showing that the metabolism of the hydrolyzable tannins present in the ingested acorns was not complete.

Urine Excretion. The urine of control pigs fed ocereal fodder showed the characteristic compounds for this kind of feeding, which includes feruloylglycine (M − H, *m/z* 250; MS/MS at *m/z* 206, 191, 177, 163) and traces of naringenin glucuronide (m/z^- 447 and MS/MS at m/z^- 271 and 175; probably from citrus byproducts used to aromatize the fodder) (**Figure 5**). No ET metabolites were detected as could be expected. On the contrary, pigs regularly fed acorns showed urine chromatograms in which glucuronides and diglucuronides of the EA metabolites (dibenzopyranones) were detected. Thus, urolithin A and trihydroxydibenzopyranone diglucuronides (**1**, **4**), as well as glucuronides of tetrahydroxymethyl- (**20**), trihydroxy- (**9**), trihydroxymethyl- (**14**, **15**), urolithin A (**12**), and urolithin B (**25**) were detected. In addition, small amounts of EA dimethyl ether glucuronide (m/z^- 505, MS/MS 329, 314, 175) were detected (**18**). The urine of pigs regularly fed cereal fodder, which was supplemented with acorns 24 h before sample collection, showed a fodder characteristic chromatogram in which small amounts of acorn ET metabolites (urolitin A and B glucuronides) were detected (data not shown).

Bile Metabolites. The gall bladder was removed and the bile content extracted and analyzed. Bile from pigs fed acorns and those fed cereal fodder were compared. Control bile samples were lacking the ET metabolites, and the main phenolic metabolite detected was feruloylglycine (**Figure 6A**), a biliary metabolite of ferulic acid present in cereal products and in agreement with the metabolites found in urine. On the contrary, the bile of pigs fed acorns showed a wide range of ET metabolites (**Table 2**; **Figure 6B**) including urolithin A, trihydroxydibenzopyranone, trihydroxydidenzopyranone methyl ether, tetrahydroxybenzopyranone, tetrahydroxybenzopyranone methyl ether, and EA methyl and dimethyl ether glucuronides and diglucuronides. In these analyses, the presence of sodium adducts of the glucuronides and diglucuronides was observed. In all cases the sodium adducts coeluted with the corresponding glucuronides in the HPLC chromatograms. Only one sulfated metabolite was detected (**23**) (trihydroxydibenzopyranone methyl ether, glucuronide, sulfate), showing that the main metabolites produced were the glucuronides. No urolithin B related metabolite was detected in bile, although it was one metabolite readily identified in peripheral plasma and urine. Some minor metabolites were observed in which the tetrahydroxybenzopyranone or trihydroxybenzopyranone metabolites were linked to glucuronic acid (176 loss) and an additional loss of 161. This could be associated with the combination with *N*-acetylcysteine. The identification of these metabolites is under study.

After enzyme hydrolysis with glucuronidase and sulfatase, the aglycones were obtained and coincided with those found in the lumen of the small intestine with the exception of ellagic acid, which was not detected in bile (**Figure 6C**). Some phenolic metabolites (feruloylglycine and tetrahydroxydibenzopyranone *N*-acetylcysteine) did not suffer hydrolysis with these enzymes, showing the different nature of these metabolites (**Figure 6C**).

Tissue Distribution of the Metabolites. The distribution of ellagitannin metabolites in different tissues was evaluated. Muscle, adipose (subepidermal and visceral), lung, liver, heart,

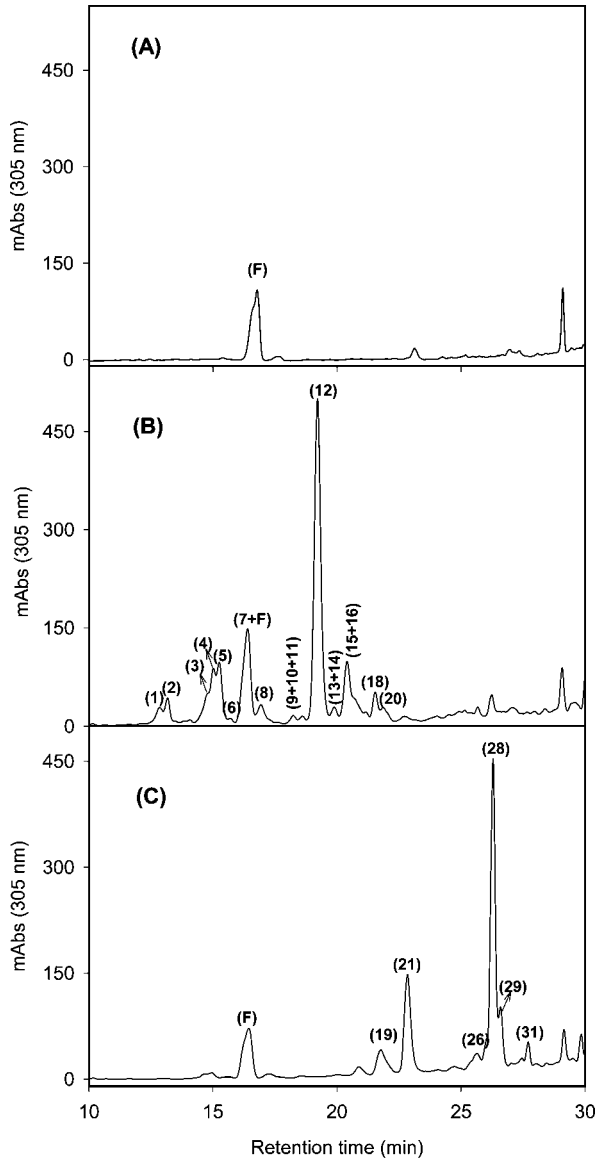


Figure 6. HPLC analyses of bile: (**A**) control pig bile (fed cereals); (**B**) acorn-fed pig bile; (**C**) acorn-fed pig bile after enzyme hydrolysis. Peaks: (F) feruloylglycine; (1) urolithin A diglucuronide; (2, 4) urolithin C diglucuronide; (3, 5) urolithin C methyl ether diglucuronide; (6) urolithin D glucuronide *N*-acetylcysteine; (7) urolithin D diglucuronide; (8, 10) urolithin D glucuronide; (9) urolithin C glucuronide; (11) EA methyl ether diglucuronide; (12) urolithin A glucuronide; (14, 15) urolithin C methyl ether glucuronide; (16) EA methyl ether glucuronide; (18) EA dimethyl ether glucuronide; (19) urolithin D; (20) urolithin D methyl ether glucuronide; (21) urolithin C; (26) urolithin D methyl ether; (28) urolithin A; (29) urolithin C methyl ether.

and kidney tissues were extracted and analyzed by HPLC-MS/MS. None of the metabolites was detected in the crude extracts. After hydrolysis with sulfatase and glucuronidase, no metabolite aglycone was detected, showing that these tissues are not targets for the accumulation of ellagitannin metabolites.

DISCUSSION

In previous studies the metabolism of different ETs to yield urolithins was described in humans upon the intake of pomegranate juice, walnuts, strawberries, raspberries, and oak-aged wine. The main metabolites detected in plasma and urine were urolithins A and B (*7*, *8*). The production of urolithins in the

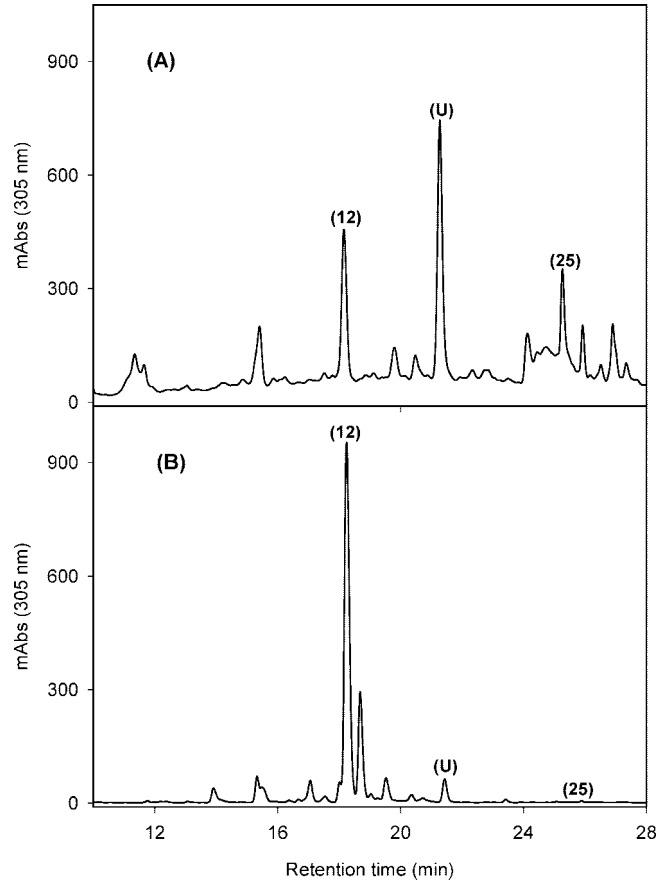
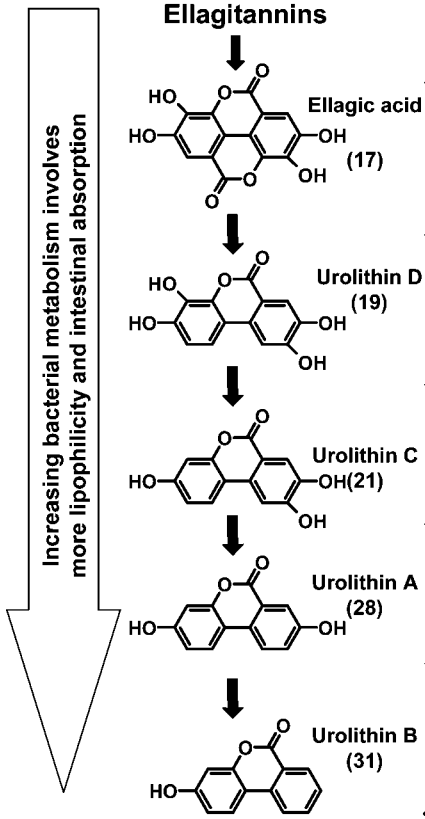


Figure 7. Comparison of pig and human urine HPLC profiles: (**A**) human urine (24 h urine volume) after the intake of 225 g of fresh strawberries; (**B**) pig urine after oak acorn intake. Peaks: (U) urinary pigment; (12) urolithin A glucuronide; (25) urolithin B glucuronide.

complex-toothed flying squirrel *Trogopterus xanthipes* fed different wood sources has been also reported (*21*). In all cases, independently on the ET nature, the final metabolites detected were urolithins. In this context, our hypothesis was that Iberian pigs fed acorns, an ellagitannin-rich source (*18*), would be able to produce urolithins. In addition, the pig's digestive system is nearly identical to that of a human, which would offer the possibility to explore the disposition of metabolites in fluids and tissues to complete the current knowledge on the metabolism of ellagic acid-related molecules using this suitable model.

The extensive metabolism of ETs observed in previous studies suggested a potential local (cancer chemopreventive) effect in the GI tract, whereas the bioavailable metabolites (urolithins) could exert systemic effects due to their persistent excretion in urine for various days and high plasmatic concentrations (*7*, *8*, *22*). These metabolites started to appear in biological fluids several hours after the ET intake and reached a maximum level after 24–48 h depending on the individual (*8*). This was explained by suggesting that ETs were extensively metabolized in the large intestine and that enterohepatic circulation is most probably occurring. However, there were some unanswered questions such as the follwoing: Are the ETs metabolized in the small intestine and to what extent? Is there a gradual degradation of ellagic acid into urolithins? Are therefore the metabolites absorbed in the small intestine and transported to the liver for further metabolism? What metabolites are present in the bile? Does the enterohepatic circulation explain the long persistence of urolithins in the body prior to their excretion?

The chromatographic profiles of pig peripheral plasma and urine samples are similar to those previously found in humans



Plasma	Jejunum Lumen	Jejunum Tissue	Colon Lumen	Colon Tissue	Bile	Urine	Feces
-	+	-	Traces	-	-	-	+
+ (a)	++++	-	-	-	+ (a)	+ (a)	Traces
-	+++	+	+	+	+ (b,c)	-	-
+ (b)	++	++ (b)	-	-	+++ (b,c)	Traces	-
+++ (b)	+	++++ (b)	++++	+	++ (b,c)	++++ (b)	++
++ (b)	-	-	+	Traces	-	+++ (b)	-

Figure 8. Metabolism of ellagitannins to urolithins; tissue and biological fluids distribution: (**a**) dimethyl glucuronide; (**b**) glucuronides; (**c**) methyl glucuronides. Crosses designate the relative abundance of metabolites (not for quantitative purposes) in the different samples analyzed from pigs, "+" being the lowest and "++++" the highest values. "Traces" indicate that metabolites were detected only in some samples and always in very small amount (close to the limit of detection).

after the intake of ET-rich food (strawberries, raspberries, walnuts, pomegranates, oak-aged wines; *7*−*9*), suggesting that the metabolism in both cases is quite similar (**Figure 7**). This therefore confirms that the Iberian pig fed acorns is a suitable model to study ET metabolism and tissue distribution in humans.

Therefore, this study shows that ETs release EA under physiological conditions in vivo and that EA is then gradually metabolized in the intestine, starting in the jejunum, to produce urolithin D (tetrahydroxydibenzopyranone), urolithin C (trihydroxydibenzopyranone) and to end with urolithin A (dihydroxydibenzopyran-6-one) and urolithin B (monohydroxydibenzopyran-6-one). This last compound is mainly produced at the distal parts of the intestine. In addition, analyses of the intestinal tissues show that the metabolites are absorbed preferentially when their lipophilicity increases (**Figure 8**).

It is relevant that the pig is capable of producing the microflora metabolites as soon as 24 h after the intake of ellagitannins, even if they have been regularly fed cereal fodder and the microflora is not used to metabolize ETs. This is remarkable because we observed in a previous study that rats needed some days to be able to produce urolithins upon repeated oral administration of pomegranate extracts (*16*).

Once the metabolites are absorbed, the first glucuronidation seems to occur in the intestinal cells and this is most probably the conjugated form found mainly in portal vein plasma. Once in the liver, the metabolites are further metabolized to produce diglucuronides, and/or sulfates, to give a whole combination of metabolites secreted in the bile (**Figure 6**). The metabolite profile in peripheral plasma and in urine is, however, much simpler (**Figure 5**), containing mainly the urolithin A and B glucuronides and ellagic acid dimethyl ether glucuronide (the latter, in much lesser extent), showing that only those metabolites that are sufficiently metabolized are excreted in urine, whereas the tetrahydroxy- (urolithin D) and trihydroxy- (urolithin C) metabolites are absorbed earlier in the intestine, suffering enterohepatic circulation and preventing them from entering the peripheral plasma and urine until they are further metabolized to reduce the number of phenolic hydroxyls. The metabolite urolithin B (**31**) seems to be the last degradation product in the metabolism of EA by intestinal flora (**Figure 8**). In this case, this molecule can be glucuronidated only once (only one hydroxyl group), and this seems to occur in the intestinal cells, immediately after absorption, which could explain the lack of urolithin B in the bile because no further conjugations can occur in this molecule prior to its urine excretion. In the case of the other metabolites, including urolithin A, more conjugations can take place, justifying the need for enterohepatic circulation. Urolithin A is the main ET-derived metabolite detected in feces in both pigs and humans (*15*). No urolithin B is found in feces, which supported its rapid absorption prior to the excretion via feces.

The presence of EA metabolites in bile and in urine, and the lack of EA in intestinal tissues, suggests that it can be directly absorbed in the first portions of the GI tract (stomach), and this will explain the findings reporting absorption of free EA in short times (from 30 min to 1 h) after intake (*11*, *12*).

The absence of acorn polyphenols or derived metabolites in the organs analyzed, including muscle and fat tissues, suggests that the well-known resistance to rancidity claimed for meat products prepared from acorn-fed pigs would not be explained by the direct accumulation of polyphenolic compounds or derived metabolites in these tissues. However, the presence of antioxidant ETs in the GI tract could prevent the oxidation of other (antioxidant) acorn compounds such as vitamin E, which could be involved in the resistance to meat rancidity.

We reported in a previous study that humans showed a large interindividual variability in the metabolism of ETs identifying "high and low urolithin excreters" (*8*). In the present study, this variability was also observed in the pigs that showed different amounts of urolithins in bile and urine in pigs regularly fed acorns (results not shown). In addition, some pigs were able to produce more urolithin B than A in accordance with that observed in some human individuals (*7*–*9*).

Therefore, we report in the present study the most advanced knowledge regarding the bioavailability, metabolism, and disposition of ETs and ellagic-related molecules described so far. In addition, the use of the pig as a suitable model to clarify obscure points related to these issues for dietary phytochemicals is strongly encouraged.

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