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UV and MS Identification of Urolithins and Nasutins, the Bioavailable Metabolites of Ellagitannins and Ellagic Acid in Different Mammals

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Supporting Information

ABSTRACT: Urolithins are microbial metabolites produced from ellagic acid after the intake of dietary ellagitannins by different animals. Urolithin metabolites have distinct UV spectra that enable their detection and differentiation by HPLC coupled with UV photodiode array detectors. Correlations between structural characteristics, including conjugation, with the UV spectra and retention times are established. The production of urolithin derivatives in different animals feeding on ellagitannins, including rodents (rats and mice), humans, pigs, squirrels, beavers, sheep, bull calves, birds, and insects, was investigated. All mammals produced urolithins, and their glucuronyl and sulfate conjugates were the main metabolites detected in plasma and urine. Unconjugated urolithins were detected in feces, ruminal content, and beaver castoreum. Different urolithin hydroxylation patterns were observed for different animal species, suggesting that the microbiota responsible for the metabolism of ellagitannins in each animal species produces dehydroxylases for the removal of specific hydroxyls from the ellagic acid residue. Metabolites were characterized using HR HPLC-TOF-MS and ion trap MS/MS. Insects and birds feeding on ellagitannin-containing foods did not produce urolithins, although they released ellagic acid. Beavers and pigs were able to produce dehydroxyellagic acid derivatives (nasutin A), showing that in some cases the removal of hydroxyl groups from the ellagic acid nucleus can be carried out before the lactone ring is opened to produce urolithins.

KEYWORDS: Ellagic acid, dibenzopyranones, metabolism, HPLC-MS, UV spectra

INTRODUCTION

Urolithins include a family of metabolites of the 6*H*-dibenzo-[*b*,*d*]pyran-6-one structure with different phenolic hydroxylation patterns. They are produced in different animals after the intake of ellagitannins.[1−3] They are also produced from ellagic acid and their conjugates, which are often found in different food products including berries, walnuts and pecans, pomegranates, and oak-aged wines among others.[4] Urolithins are relevant for dietary reasons as they are bioavailable, circulate in plasma at micromolar concentrations as methyl, glucuronide, and/or sulfate conjugates, and are excreted in urine, whereas the original ellagitannins are not bioavailable and ellagic acid is only slightly absorbed.[3,5] Urolithins remain in the body for long periods of time due to enterohepatic recirculation and slow microbial metabolism in the colon and are found in urine up to 4 days after consumption of ellagitannin-containing foods.[5,6] Due to the high concentrations of urolithin conjugates in plasma, and the long time they remain in the body, urolithins have been proposed as responsible for the biological activity and the systemic health effects related to the intake of ellagitannins.[3,5,7] Studies of the metabolism of ellagitannins following the intake of different foods (pomegranates, berries, nuts, medicinal plants, wood-aged products, etc.) have shown the production of different urolithin metabolites.[3,8,9] Production and analysis of urolithins is therefore critical in the evaluation of the biological activity of ellagitannin-containing foods, as is the case of pomegranate juice and walnuts, because urolithins are not always produced to the same extent in different volunteers,[3] probably due to differences in the gut microbiota.[10] This interindividual variability could explain the weak statistical significance of many nutritional studies of ellagitannin-containing foods.[11,12] In addition, ellagitannins are considered to be antinutritional for animals, and their potentially toxic effects could be related to the ability to metabolize ellagitannins in the rumen.[13,14] On the other hand, a recent study showed that dietary supplementation with fresh pomegranate peels, rich in ellagitannins, increased weight gain in bull calves.[15] The identification and characterization of urolithins is therefore also relevant for animal nutrition studies.

The UV spectra of phenolic compounds have been used for identification purposes. Thus, the UV spectra are generally used for the identification of flavonoid compounds[16] and provide a straightforward method to help in the identification of the phenolic compounds in plant extracts, foods, and biological fluids after the intake of these products.

In the present study, the UV spectra of the urolithin metabolites are studied to establish structure−spectra relationships that can be used in future studies. These UV spectra together with MS

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analyses and HPLC chromatographic behavior are particularly useful in studies of the biological activity of these ellagitannin metabolites. In addition, these analytical methods have been applied to the study of the occurrence of these metabolites in different biological materials of animals, including mammals, birds, and insects, feeding on ellagitannin-containing foodstuffs.

MATERIALS AND METHODS

Chemicals and Reagents. Ellagic acid (EA) was purchased from Sigma (St. Louis, MO). Methanol (MeOH), acetonitrile, and formic acid (99.9%, HPLC grade) were supplied by Merck (Darmstadt, Germany). Urolithins A and B and 8-hydroxyurolithin were chemically synthesized and provided by Kylolab (Murcia, Spain). Urolithins M5, M6, and M7 were previously isolated from rat feces after the intake of *Geranium* ellagitannins.[8] Ultrapure water was obtained using a Milli-Q system (Millipore Corp., Bedford, MA) throughout this study.

Biological Samples. Experiments followed protocols approved by the local animal ethics committee and the local government. All experiments achieved were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Young Brown Swiss bulls and sheep were fed oak (*Quercus pyrenaica*) leaves and tannin extracts, respectively.[14] Ruminal content and urine and feces samples of bull calves and sheep were collected. Mice received a standard diet supplemented with pomegranate (*Punica granatum* L.) extracts.[17] Greenfinches (*Carduelis chloris*) were fed for 2 weeks with blackberries, and the feces were collected. Rats were fed *Geranium* sp. as reported previously.[8] Feces of the beetle *Thorectes lusitanicus* feeding on acorns were collected and dried. Castoreum samples were collected in Norway from wild European beavers. *Pteropi* feces were purchased in a local Chinese Pharmacy in Hong Kong. All samples were immediately frozen after collection, at −30 °C, and stored at this temperature until analysis.

Analysis of Phenolic Compounds and Their Metabolites in Biological Fluids and Other Biological Samples. *Feces.* Feces samples were defrosted and extracted using a method developed by Espín et al.[3] Briefly, feces samples were defrosted and homogenized with MeOH/HCl/water (79.9:0.1:20, v/v/v) using an Ultra-Turrax homogenizer for 1 min at 24000 rpm. The mixture was centrifuged at 5000*g* for 5 min at room temperature and the supernatant filtered through a 0.45 μm membrane filter Millex-HV13 before analysis by HPLC-DAD-MS/MS.

Urine. Urine samples were defrosted, vortexed, centrifuged at 14000*g* for 10 min at 4 °C, filtered, and analyzed by HPLC-DAD-MS/MS.

Castoreum. Samples were processed as reported previously[3] with some modifications. Briefly, 3.5 g of each sample was homogenized with 10 mL of MeOH/HCl/water (79.9:0.1:20, v/v/v) using an Ultra-Turrax homogenizer for 1 min at 24000 rpm and centrifuged at 5000*g* for 5 min at room temperature. The supernatant was reduced to dryness under nitrogen flow at room temperature. The remaining aqueous phase was supplemented with an additional 10 mL of distilled water plus 10 μL of 6 M HCl and loaded onto a preconditioned Sep-Pak C_{18} cartridge, which was washed with 10 mL of distilled water before elution with 2 mL of methanol. The methanolic phase was reduced to dryness under nitrogen flow at room temperature. The dry samples were resuspended in 100 μL of MeOH plus 100 μL of 1% aqueous formic and filtered before analysis by HPLC-DAD-MS/MS.

HPLC-DAD. HPLC measurements were made using an Elite LaChrom-Hitachi HPLC system with a pump model 2130, a photodiode array detector model 2455, and an autosampler model 2200 (VWR-Hitachi, Barcelona, Spain). The column used was a 250 mm × 4 mm i.d., 5 μm LiChroCART RP18, with a 4 mm × 4 mm i.d. guard column of the same material (Merck). The mobile phases were water with 5% formic acid (solvent A) and acetonitrile (solvent B) at flow rate of 1 mL/min.

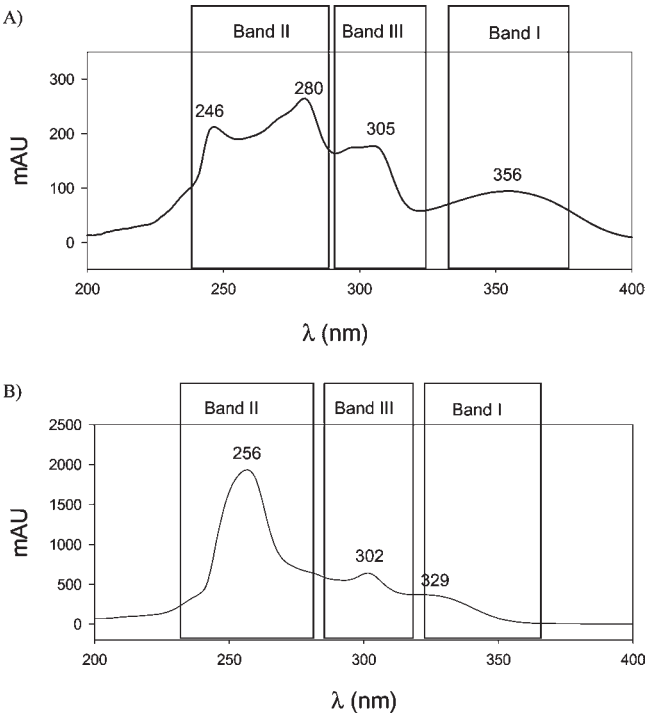


Figure 1. UV spectra in methanol of urolithin A (3,8-dihydroxyurolithin) (A) and isourolithin A (3,9-dihydroxyurolithin) (B).

Elution was performed with a gradient starting with 1% B in A to reach 25% B at 20 min, 55% B at 30 min, and 90% for 5 min and then returning to the initial conditions for 10 min (1% B in A). UV chromatograms of samples were recorded at 280, 305, and 360 nm. Urolithins were identified according to their UV spectra and retention times by chromatographic comparisons with authentic standards, when available, and also by their absorbance spectra based on data previously reported.[2,3,8]

HPLC-DAD-MS/MS. The HPLC system was equipped with an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312A), an autosampler (G1313 A), a degasser (G1322A), and a photodiode array detector (G1315B) controlled by software (v. A08.03). The ion trap mass spectrometer (G2445A) was fitted with an electrospray ionization (ESI) source operating in negative ionization mode and controlled by software (v. 4.1). The nebulizer gas was nitrogen; the pressure and the flow rate of the dryer gas were set at 65 psi and 11 L/min, respectively. Analyses were carried out using full-scan mass and data-dependent MS^2 scanning from m/z 100 to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50% with voltage ramping cycles from 0.3 up to 2 V. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively.

Chromatographic separations of biological samples were carried out in the same column and guard column as detailed above with 1% formic acid (A) and acetonitrile (B) as solvents. The gradient employed for this analysis was the same as detailed above. UV chromatograms of samples were recorded at 280, 305, and 360 nm. Comparison with authentic standards, when available, absorbance spectra, and mass spectra, using MS^2, was used to confirm the identity of compounds previously reported in the literature.[2,3,8]

HPLC-TOF-MS/MS. Identification of the compounds was performed using an HPLC-TOF-MS Agilent 6220 system equipped with an HPLC system Agilent 1200 series diode array detector (Agilent Technologies). The HPLC system consisted of a binary pump (G1312 A), an autosampler (G1329 A), a degasser (G1322 A), a thermostated column

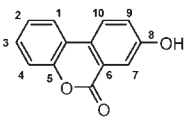
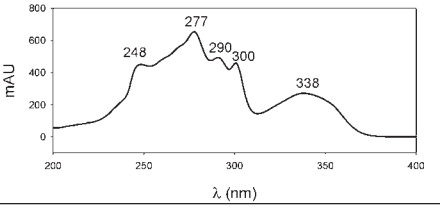
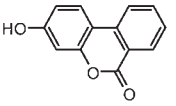
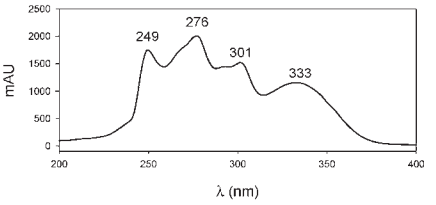
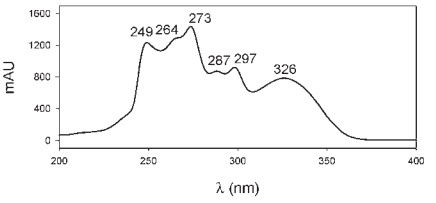
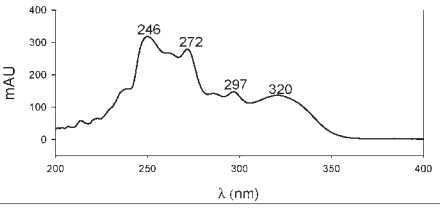
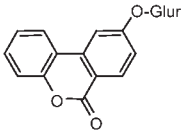
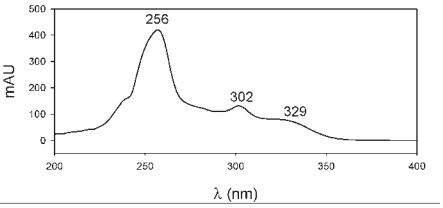
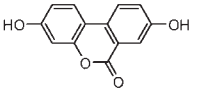
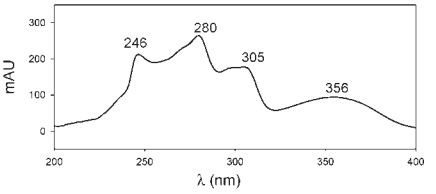
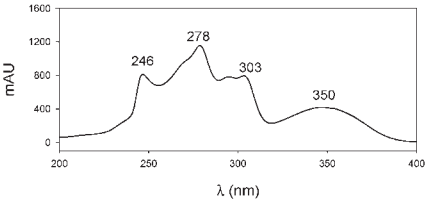
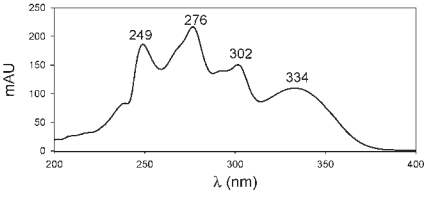
Nº	Rt (min)	Compound	Structure	MS [M-H]⁻	UV
1	29.9	8-hydroxy-urolithin (synthesis)		211	
2	29.3	3-hydroxy-urolithin (Urolithin B)		211	
11	22.2	Urolithin B-glucuronide		387	
13	35.1	Urolithin B-sulfate		291	
12	24.0	9-hydroxy-urolithin-glucuronide		387	
4	24.8	3,8-dihydroxy-urolithin (Urolithin A)		227	
14	16.7	Urolithin A-glucuronide		403	
19	25.2	Urolithin A-sulfate		307	

Figure 2. Continued

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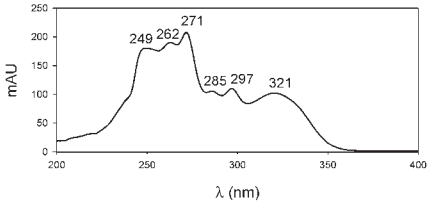
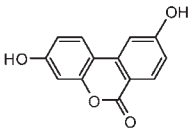
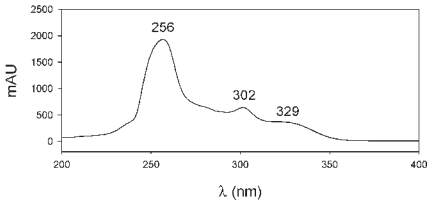
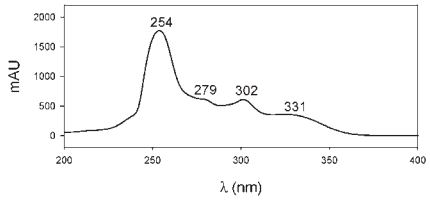
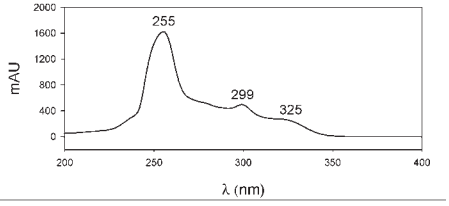
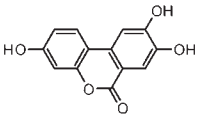
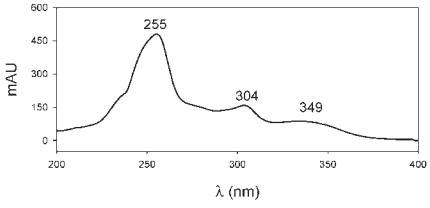
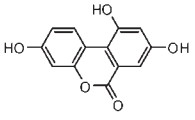
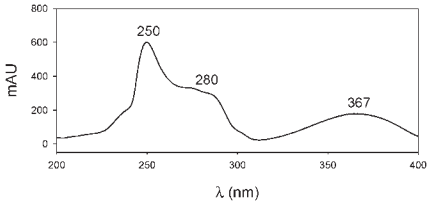
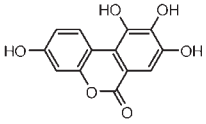
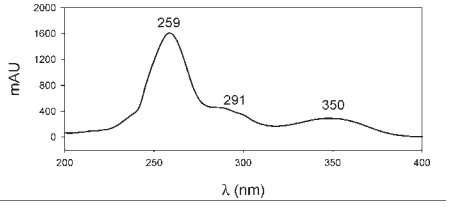
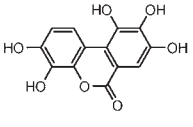
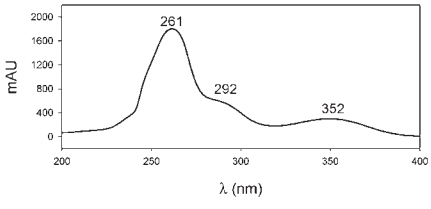
N°	Rt (min)	Compound	Structure	MS [M-H]⁻	UV
15	20.5	Urolithin A-sulfate-glucuronide		483	
5	24.2	3,9-dihydroxy-urolithin (Isourolithin A)		227	
17	16.2	3,9-dihydroxy-urolithin 3-glucuronide		403	
18	17.1	3,9-dihydroxy-urolithin 9-glucuronide		403	
6	20.5	Urolithin C 3,8,9-trihydroxy-urolithin		243	
7	21.1	Urolithin M7 3,8,10-trihydroxy-urolithin		243	
9	18.8	Urolithin M6 3,8,9,10-tetrahydroxy-urolithin		259	
10	15.2	Urolithin M5 3,4,8,9,10-pentahydroxy-urolithin		275	

Figure 2. Continued

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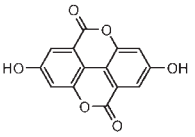
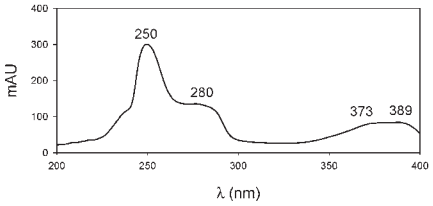
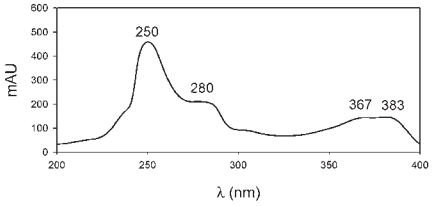
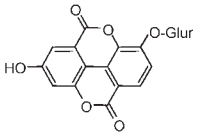
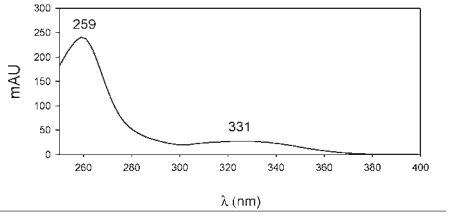
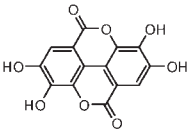
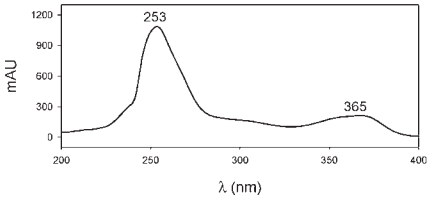
Nº	Rt (min)	Compound	Structure	MS [M-H]⁻	UV
23	25.6	Nasutin A (4,9-dehydroxy-ellagic acid)		269	
24	17.4	Nasutin A glucuronide		445	
26	20.5	Isonasutin A glucuronide		445	
22	18.7	Ellagic acid		301	

Figure 2. UV spectra, $[M - H]^-$, and HPLC retention time of different urolithin and nasutin conjugates.

compartment (G1316A), and a photodiode array detector (G1315 B) controlled by software (v. A08.03). The mass detector was a TOF spectrometer equipped with an electrospray ionization (ESI) interface operating in negative ion mode using a capillary voltage of 14 kV. The other optimum values of the ESI-MS parameters were as follows: temperature, 350 °C; drying gas flow, 11 L/min; and nebulizing gas pressure, 55 bar. Mass scan (MS) mode and MS/MS fragments were measured from m/z 50 to 1000. Mass spectrometry data were acquired in negative ionization mode. The accurate mass data of the molecular ions were processed by Data Analysis MASS HUNTER software (Agilent Technologies). In this case, the mobile phases used were water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile (solvent B), and the chromatographic separation and elution were performed with the same gradient and column as detailed above. The flow rate of 0.7 mL/min and the UV chromatograms of samples were recorded at 305 and 360 nm.

RESULTS AND DISCUSSION

Identification of Urolithin Derivatives. Urolithin derivatives, both aglycones and conjugates, were studied in plasma, urine, ruminal fluid, and feces of different animals after the intake of ellagitannins and ellagic acid (see the Supporting Information). Enzyme hydrolysis (glucuronidase and sulfatase) was not used as the originally occurring metabolites were analyzed. In most cases, the isolation of the different compounds was not possible as they were present in very small amounts and in biological materials that were difficult to obtain in large amounts. For these reasons the different metabolites were characterized using high-resolution HPLC-TOF-MS, which allowed the determination of the empirical formulas of the different metabolites. The locations of the different hydroxyls on the benzopyranone nucleus were assigned after a study of their UV spectra and comparisons with synthetic metabolites, when available, as was the case of 3-hydroxyurolithin (urolithin B), 8-hydroxyurolithin, 3,8-dihydroxyurolithin (urolithin A), urolithin B-glucuronide, urolithin A 3-glucuronide, and ellagic acid. In addition, authentic samples of urolithin C and urolithins M7, M6, and M5 were also available isolated and identified from rat feces after the intake of ETs from *Geranium*.[8] Nasutin A (**23**) showed a UV spectrum similar to that of ellagic acid (**22**) but with significant differences in agreement with previously published UV spectra.[18,19]

UV Spectra of Urolithin Derivatives. The UV spectra of urolithins and ellagic acid derivatives in methanol exhibit two major absorption peaks in the region of 240−400 nm (Figure 1). These two peaks can be referred to as band I (usually 300−380 nm) and band II (usually 240−280 nm) following the nomenclature generally accepted for flavones.[16] In most cases, an additional band III between 280 and 300 nm is observed. Unlike flavones, bands I and II cannot be associated with a specific ring of the urolithin nucleus, as urolithins with hydroxyls in only one or in both rings show similar UV spectra plots (Figure 2).

Two families of UV spectra of urolithin derivatives can be clearly distinguished after a study of the plots of the spectra: those

Table 1. Effect of Hydroxylation Number and Position on the Wavelength of Band I and Band II Absorption Maxima of the Urolithin Derivatives

hydroxylation pattern	9-hydroxyl	non-9-hydroxyl
	Band I	
one hydroxyl	329sh	338, 333
two hydroxyls	329sh	356
three hydroxyls	349	356
four hydroxyls	350	367
five hydroxyls	352	
ellagic acid[a]	353	389[a]
	Band II	
one hydroxyl	256	277, 276
two hydroxyls	256	280
three hydroxyls	255	250 and 280sh
four hydroxyls	259	
five hydroxyls	261	
ellagic acid	253	250 and 280sh[a]

[a] Comparison of ellagic acid (**22**) and nasutin (**23**).

of compounds with a hydroxyl in the 9-position and those of urolithins without hydroxylation in the 9-position (Figures 1 and 2). The occurrence of the hydroxyl at position 9, which is in the para-position of the carbonyl residue of the urolithin nucleus (Table 1), produces a hypsochromic shift (shift to shorter wavelengths) in band I and an increase in the absorption of band II, showing a maximum around 256 nm, which is the main absorption band of the spectrum of urolithins with a hydroxyl in the 9-position (Figure 1). This effect can be illustrated with the following pairs: urolithin B (**2**)/isourolithin B (**3**); urolithin A (**4**)/isourolithin A (**5**), and urolithin M7 (**7**)/urolithin C (**6**) (Figure 2). In the same way urolithin M6 (**9**) and urolithin M5 (**10**) also show a predominant absorption band II at 259 and 261 nm, respectively (Table 1). These UV features are consistent with those observed when the UV spectrum of ellagic acid (**22**) is compared with that of nasutin A (**23**), in which a hypsochromic and a hypochromic effect (decrease in absorbance intensity) in band I is observed when hydroxyls in the para-position of the carbonyl group are present as in ellagic acid (Figure 2). In addition, band II is more relevant in the UV spectrum of ellagic acid than in that of nasutin A. The strong influence of the hydroxyl in the 9-position in the UV spectrum of urolithins and ellagic acid derivatives is also illustrated when the UV spectra of 9-hydroxyurolithin (isourolithin B) and 3,9-dihydroxy urolithin (isourolithin A) are compared, as their UV spectra are virtually identical, showing little contribution of the hydroxyl in the 3-position to the UV spectra of urolithins.

Effect of Conjugation on the UV Spectra of Urolithins and Ellagic Acid Derivatives. Urolithins and EA are usually present in the plasma and urine as glucuronide, sulfate, and/or methyl conjugates, and therefore it is relevant to know the effect of conjugation on their UV spectra. When hydroxyls in the urolithin or EA nucleus are methylated or glycosylated (glucuronidated), hypsochromic shifts are observed. The shifts associated with the conjugation can also be linked to the position where they are located. Glucuronidation produces hypsochromic shifts in the UV maxima of the spectra of urolithins. This is illustrated by looking at the UV maxima of urolithin B (**2**) and its glucuronide (**11**), in which shifts of 7 and 3 nm were observed for bands I and

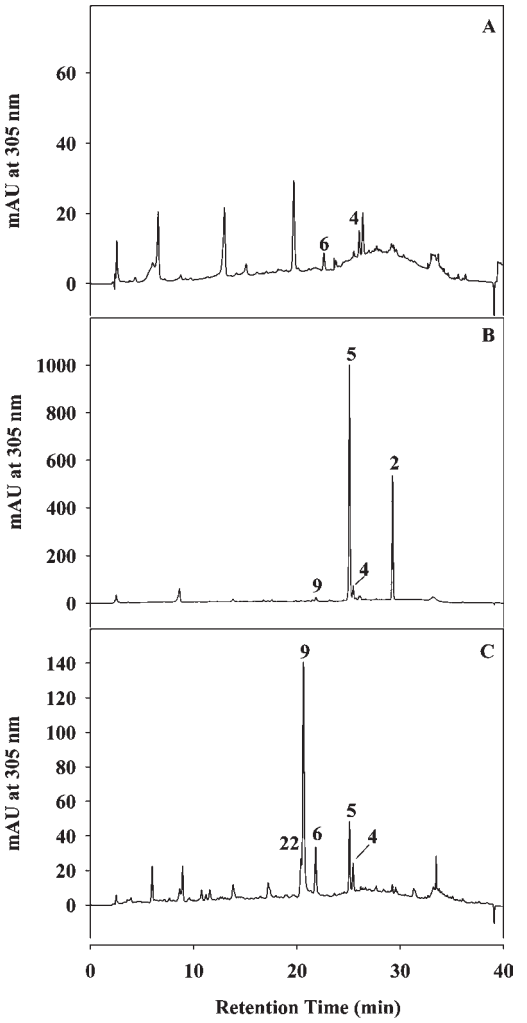


Figure 3. HPLC chromatograms at 305 nm of feces samples: (A) mouse feces; (B) *Pteropi* feces (squirrel); (C) bull calf feces. Peaks: 2, urolithin B; 4, urolithin A; 5, isourolithin A; 6, urolithin C; 9, urolithin M6; 22, ellagic acid.

II, respectively (Figure 2). This hypsochromic shift is even more pronounced in the case of sulfate conjugation as 13 and 4 nm shifts were observed for bands I and II, indicating that the kind of conjugation can have a significant effect in the UV spectrum, and this could be used for diagnostic structural purposes. The dramatic effect of sulfation is supported by the reported effect of acylation of phenolic hydroxyls in flavonoids that is known to nullify their contribution to the UV spectrum of flavones.[16]

Similar effects were observed for urolithin A glucuronidation, which produced a hypsochromic shift of 6 or 2 nm in bands I and II, respectively. Sulfation again produces a more dramatic effect, showing shifts of 18 and 4 nm for bands I and II. A double conjugation including a glucuronidation and a sulfation on urolithin A produced even more significant hypsochromic shifts of 35 and 9 nm for bands I and II, respectively (Figure 2).

In the case of isourolithin A (**5**), glucuronidation produced only a slight hypsochromic effect on band I (6 nm), whereas band II was not affected. The influence of the 9-hydroxyl in para-position of the carbonyl of the urolithin nucleus seems to be higher than the effect of the conjugation, and therefore the conjugation produces no effect on band II. In this case two

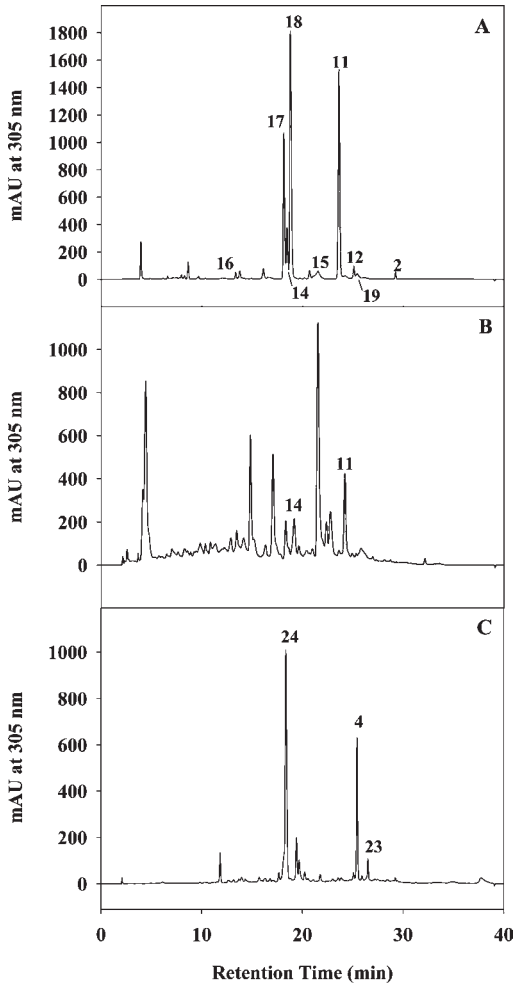


Figure 4. HPLC chromatograms at 305 nm of bull and sheep urine and castoreum samples: (A) bull calf urine; (B) sheep urine; (C) castoreum of European beaver. Peaks: 2, urolithin B; 4, urolithin A; 11, urolithin B-glucuronide; 12, isourolithin B-glucuronide; 14, urolithin A-3 glucuronide; 15, urolithin A-sulfate glucuronide; 16, urolithin A-diglucuronide; 17, isourolithin A-3 glucuronide; 18, isourolithin A-9 glucuronide; 19, urolithin A-sulfate; 23, nasutin A; 24, nasutin A-glucuronide.

glucuronic acid conjugates were observed, the 3-glucuronide (**17**) and the 9-glucuronide (**18**). This contrasts with the detection of only the 3-glucuronide in the case of urolithin A, suggesting that both the 3- and 9-positions are easily conjugated with glucuronic acid, whereas the 8-position is not so favored. This can be associated with the acidity of the different phenolic hydroxyls.

In the case of nasutin A (**23**), glucuronidation also produced a hypsochromic shift, but in this case this was seen only in band I as in the case of isourolithin A (6 nm).

Identification of Urolithins and Ellagic Acid Metabolites Produced in Different Animals following Consumption of Ellagitannins. HPLC-DAD-MS/MS was applied for the identification of urolithins in biological fluids of different animals after the intake of ellagitannins or EA. Urolithins have already been reported to be present in biological materials of different mammals after the intake of ellagitannin-containing foods. These have been previously reported in plasma, feces, and urine of humans after the intake of pomegranates, walnuts, strawberries, raspberries, and oak-aged red wines, which contain ellagitannins and ellagic acid.[2,23−26] Urolithins have also been reported in the feces, plasma, and urine of rats after the rats had fed on pomegranate husk[1,27−29] and ellagitannin-containing medicinal plants (*Geranium*),[8] and in the feces, plasma, bile, and urine of Iberian pigs after the intake of oak acorns.[3] Previous studies on EA and ellagitannin metabolism in mice reported only the occurrence of ellagic acid conjugates in plasma and urine.[20] In the present study the occurrence of urolithins in the feces of mice after having pomegranate ETs in the diet was evaluated. This study has shown that mice feces contain urolithin C (**6**) and urolithin A (**4**) after the intake of punicalagin and EA present in pomegranate extracts (Figure 3A).

Pteropi feces is a medicinal product used in traditional Chinese medicine, which consists of the feces of the squirrel *Trogopterus xanthipes*, which naturally feeds on ellagitannin-rich materials (woods, acorns, nuts, etc.).[21] Feces were extracted and analyzed (Figure 3B) showing that ellagic acid (**22**), urolithin M6 (tetrahydroxyurolithin) (**9**), urolithin C (**6**), urolithin A (**4**), and isourolithin A (**5**) were present.

In ruminants, two experiments showed the occurrence of unconjugated urolithins in ruminal fluids and feces, after the intake of ellagitannin-containing food products. This was demonstrated in bull calves feeding on young oak leaves and in lactating sheep after the intake of ellagitannin-containing fodder. In bull feces isourolithin A (**5**) and urolithin B (**2**) were the main urolithin metabolites detected, whereas urolithin C (**6**) and urolithin A (**4**) were also detected in minor amounts (Figure 3C).

In urine and plasma samples, the glucuronyl and sulfate conjugates are known to be the main metabolites present. The analysis of bull calf urine showed a complex metabolic profile (Figure 4A) in which two isomers of isourolithin A-glucuronide (the 3- and the 9- glucuronide) (**17**, **18**) and urolithin B-glucuronide (**11**) are the main compounds detected. In addition, other minor metabolites such as urolithin A-glucuronide (**14**), isourolithin B-glucuronide (**12**), urolithin A-sulfate (**19**), urolithin A-glucuronide sulfate (**15**), and urolithin A-diglucuronide (**16**) were also detected. In sheep urine urolithin A-glucuronide, urolithin B-glucuronide, and a small amount of urolithin B were detected (Figure 4B). Clear differences in the metabolites produced by both ruminants were observed as bull calves produced mainly isourolithin (9-hydroxylated) derivatives, whereas sheep produced mainly the main urolithins found in humans, rats, and pigs.

Urolithins were also studied in the European beaver (*Castor fiber*) castoreum. This is the exudate from the beaver sacs that are used for perfume, cosmetics, and medicinal purposes.[22] Castoreum contains nasutin A-glucuronide (**24**) and aglycone (**23**) and urolithin A aglycone (**4**) (Figure 4C). The occurrence of these EA metabolites in beaver excretions is not unexpected, as it feeds on barks and other plant materials that are rich in ellagitannins.

The Iberian pig was previously used as a model to evaluate the bioavailability and metabolism of ellagic acid and ellagitannins as it feeds naturally on oak acorns rich in ellagitannins.[3] Different urolithins were detected in feces, urine, plasma, and bile. During this study these biological materials were analyzed again, and some new metabolites have been identified when studying the HPLC-DAD-MS analysis with chromatograms recorded at 360 nm (Figure 5). Pig feces contained nasutin A (**23**) in addition to urolithin A (**4**). Nasutin A-glucuronide (**24**) was also detected in the bile samples, and in urine and plasma, in addition to urolithin A-glucuronide. The different metabolites were characterized by HPLC-DAD-MS/MS, and the extracted ion chromatograms for

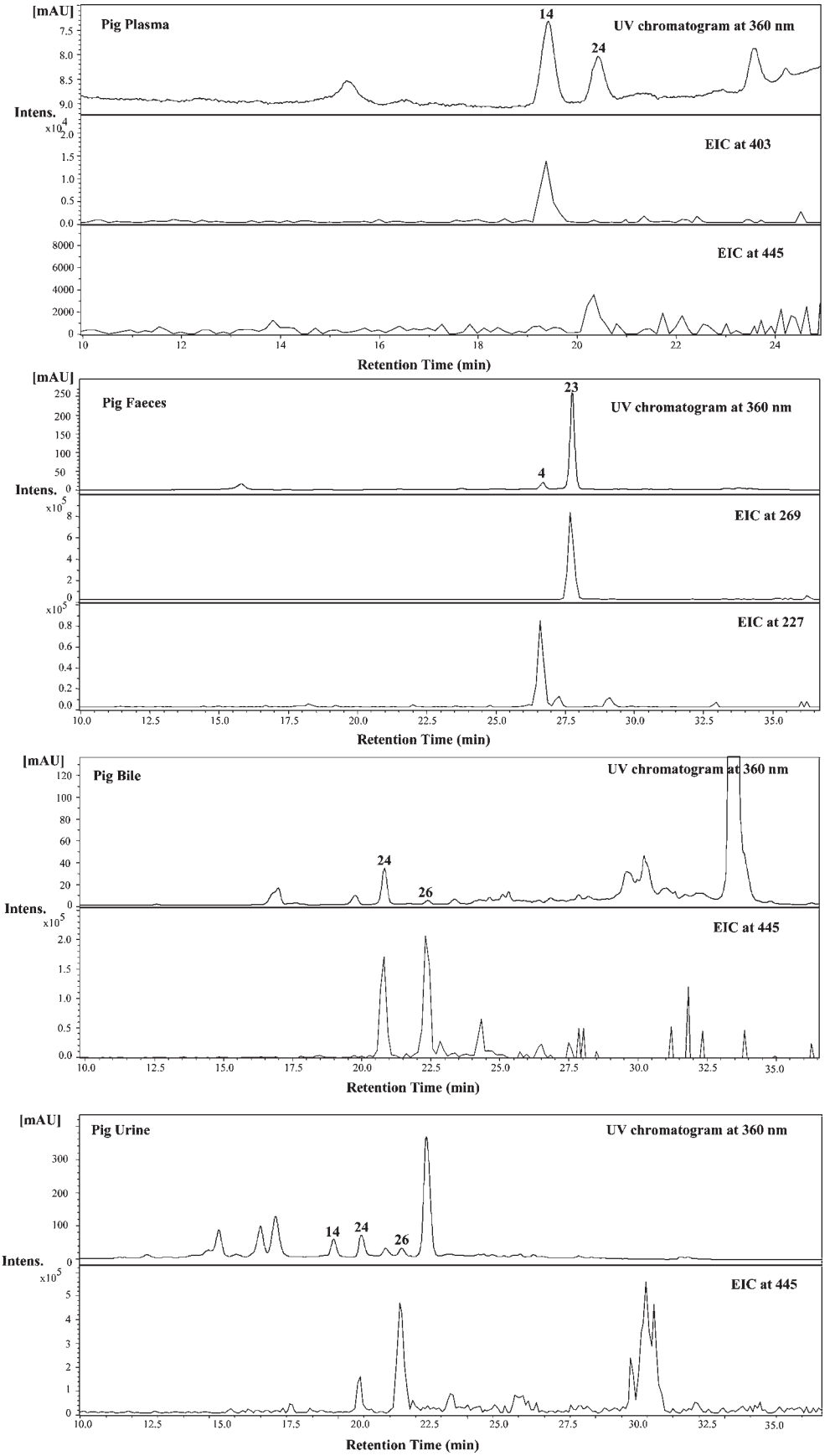


Figure 5. HPLC-DAD-MS/MS analyses of Iberian pig feces, bile, urine, and plasma, after the intake of oak acorns. Chromatograms were recorded at 360 nm to highlight the occurrence of nasutin derivatives. Peaks: 4, urolithin A; 14, urolithin A-glucuronide; 23, nasutin A; 24, nasutin A-glucuronide; 26, isonasutin A-glucuronide.

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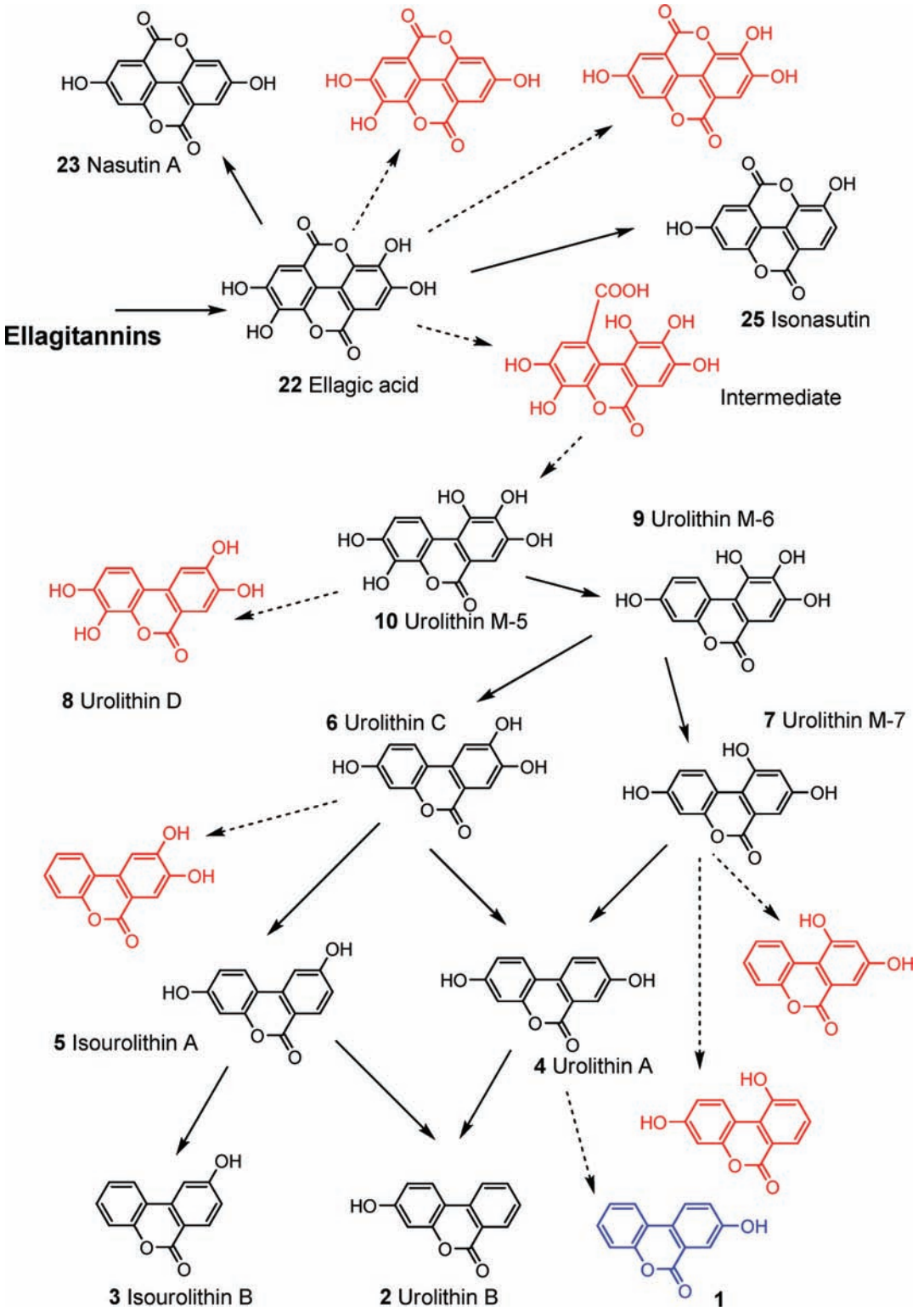

Figure 6. Postulated transformations of microbial metabolism pathways from ellagitannins to urolithins: in black, compounds identified in different biological fluids; in red, the potential metabolites not yet found in biological fluids; in blue, the synthetic compound. Solid lines represent metabolic transformations leading to compounds already detected in nature. Dashed lines represent potential transformations leading to metabolites not yet identified in nature.

the relevant ions (m/z at 445 for nasutin A-glucuronide, m/z at 403 for urolithin A-glucuronide, and m/z at 269 for nasutin A) confirmed the characterization of these metabolites in the samples (Figure 5). It is interesting that an isomer of nasutin A-glucuronide (**24**) was observed in pig urine.[3] Its UV spectrum clearly shows that it is not a positional isomer of the glucuronide and that this is probably isonasutin A-glucuronide (**26**) This is because the spectrum has the characteristics of a 9-hydroxylated

derivative and shares the same differences between nasutin A and isonasutin A that are observed between urolithin A and isourolithin A. Although the production of nasutin A by human microbiota cannot be ruled out, it was not detected in any of the human samples analyzed.

Some birds eat berries that contain ellagitannins, and for this reason the occurrence of urolithins and EA metabolites was explored in the feces. Greenfinch feces were analyzed after the intake of blackberries for 2 weeks, and no urolithin derivative was detected. Only traces of EA released from ellagitannins were found in the extracts. However, the berry ellagitannins castalagin, sanguiin H6, lambertianin-C, lambertianin-A, and lambertianin-D were detected, suggesting that the bird microbiota was not able to metabolize ellagitannins to urolithins.

Some insects also feed on foodstuffs containing ellagitannins and, therefore, they are also possible urolithin producers. For this reason the feces of the acorn beetle (*Torecthes lusitanicus*), which is known to feed on oak acorns, were analyzed. In this case no urolithins were found, and only free EA was detected, showing the hydrolysis of ellagitannins to release EA and the lack of the microbiota responsible for the transformation of EA into urolithins. Previous studies have reported the presence of nasutin A[18] in the hemolymph of some Australian termites of the genus *Nasutitermes*. This was also reported to be present in fresh termite feces but was not detected in saliva. This, however, was not present in all of the termite species analyzed. The occurrence of these ellagitannin metabolites in termites has to be associated with the intake of wood by this xylophage insect. It seems, though, that insect gut microbiota do not have the ability to decarboxylate ellagic acid, which leads to urolithin formation, whereas they do have some dehydroxylating enzymes that remove hydroxyls from EA or from the hexahydroxydiphenic acid residue of the ellagitannins, leading to nasutin A (Figure 6).

These results show that the production of urolithins is a general metabolic trend in mammals after the intake of dietary ellagitannins. These are produced in the rumen of ruminants and are further metabolized during the transport through the intestinal tract. Ruminal microbiota is already able to produce the transformation of ellagitannins to urolithins by opening one of the lactone rings and further decarboxylation and some specific dehydroxylations. This metabolic transformation also occurs in rodents, squirrels, beavers, pigs, and humans. This, however, is not a general trend in nature, as birds and beetles do not produce urolithins after ellagitannin intake.

Gastrointestinal Microbiota Metabolism of Ellagitannins and Ellagic Acid. The metabolites of EA detected in different animals and different biological fluids and tissues provide relevant information regarding the metabolic routes leading to the different metabolites and the enzymatic activities necessary to produce the urolithin metabolites and the EA metabolites. Both decarboxylations (opening of the lactone ring and removal of the carboxyl residue) and specific dehydroxylations (removal of hydroxyl groups from different specific positions of the EA nucleus) lead to the whole range of metabolites shown in Figure 6.

Removal of ellagic acid phenolic hydroxyls without opening the lactone ring (or, alternatively, in the ellagitannin prior to hydrolysis) gives rise to a number of metabolites that can be relevant in castoreum and in pigs fed oak acorns. These metabolites were first reported in termites (not all species). In addition to nasutin A (the equivalent to urolithin A), isonasutin A (equivalent to isourolithin A) was also detected, in which the hydroxyl in the para-position of the carbonyl produces effects in the UV spectrum of ellagic acid similar to those observed in urolithins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Table 1S, HPLC HR-MS TOF analysis of urolithins and conjugates; Table 2S, Occurrence of urolithins in animal biological fluids and organs. This material is available free of charge via the Internet at http://pubs.acs.org.

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