Strawberry Processing Does Not Affect the Production and Urinary Excretion of Urolithins, Ellagic Acid Metabolites, in Humans

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Strawberry Processing Does Not Affect the Production and Urinary Excretion of Urolithins, Ellagic Acid Metabolites, in Humans

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ABSTRACT: The study of fruit and vegetable processing and its effects on the levels of health-promoting constituents and their bioavailability and metabolism is very relevant to understanding the role of these constituents in human health. Strawberry polyphenols, and particularly ellagitannins and ellagic acid, have been associated with the health benefits of this berry for humans. These compounds are transformed into urolithins by the gut microbiota, and these metabolites exert several biological activities that could be responsible for the health effects of strawberries. Processing potentially increases the extraction of ellagitannins from the strawberry achenes and the release of ellagic acid from ellagitannins. It is of interest to evaluate the effect of processing on strawberry ellagitannin microbial metabolism compared with fresh strawberries. This study shows that no significant differences in the production and excretion of urolithins were found between the intake of fresh strawberries and that of a thermally processed strawberry puree containing the same amount of strawberries. Processing increases the amount of free ellagic acid 2.5-fold, but this had no effect on the transformation in urolithins by the gut microbiota or in the excretion of urolithin metabolites (urolithin glucuronides) in urine, showing that the release of ellagic acid from ellagitannins is not a relevant factor affecting the microbial metabolism. All of the volunteers produced urolithin A, but only 3 of 20 volunteers produced and excreted urolithin B. It is confirmed that some volunteers were efficient producers of urolithins, whereas other produced much lower amounts. These results show that processing does not modify the potential health effects of strawberry polyphenols.

KEYWORDS: *strawberry, metabolism, urolithin, food processing, ellagitannin, ellagic acid*

INTRODUCTION

Urolithins are metabolites produced by the gut microbiota from the polyphenols ellagic acid and ellagitannins.[1] After the ingestion of foods rich in ellagic acid or ellagitannins such as strawberries, raspberries, pomegranates, or walnuts, these polyphenols are poorly absorbed and transformed by the gut microbiota into urolithins.[2] In fact, urolithins, and particulary urolithin A, were first described as markers of the intake of foods rich in ellagic acid and ellagitannins.[3] The distribution of urolithins and their metabolites in different organs and tissues has been deeply investigated in pigs fed acorns. In this animal model, 31 ellagitannin-derived metabolites were detected, including 25 urolithin and 6 ellagic acid derivatives.[4] The most abundant urolithin after ellagitannin ingestion is urolithin A, but other urolithins (B, C, and D) have also been described.[4] The production of urolithins after the intake of ellagitannins has also been investigated in different animals, including rodents (rats and mice), squirrels, beavers, sheep, bull calves, birds, and insects, leading to the conclusion that all mammals studied produced urolithins.[5] Although ellagitannins have a high antioxidant capacity in vitro,[6] the antioxidant capacity of urolithins A and B, into which they are converted, is very low.[2] However, independent of their low antioxidant activity, multiple biological effects have been described for urolithins. In vitro, urolithins A and B have shown antiestrogenic and antiaromatase activities in breast cancer cells[7,8] and inhibition of the Wnt signaling pathway,[9] which is implied in the majority of colon cancers.[10] Urolithins also act as antiglycative agents in vitro[11] and inhibit MMP-9 expression in leukemia cells stimulated with TNF.[12] Urolithin glucuronides are able to reach the human prostate,[13] where they could have some action against prostate cancer, as in vitro urolithin aglycones inhibit the activity and expression of cytochrome CYP1B1, a well-known target in prostate cancer chemoprevention.[14] Urolithins induce the expression and activity of CYP1A1 and UGTA10 in Caco-2 cells and in rat colon[15] and regulate the expression of multiple genes involved in colon cancer both in vitro and in vivo.[16,17] In rats, the administration of pomegranate extract or urolithin A has been shown to enhance the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. and to decrease intestinal inflammation markers.[17] For all of these reasons it could be interesting to promote the production of urolithins in the gut and as circulating metabolites.

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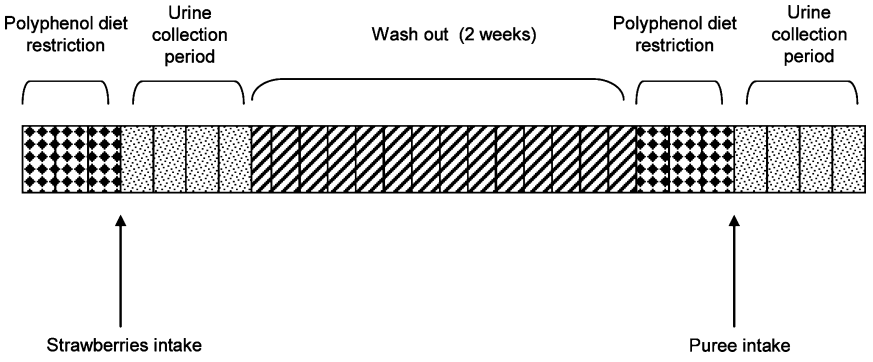

Figure 1. Scheme showing the study design.

Strawberries are an important source of ellagitannins and ellagic acid.[18] The processing of strawberries may increase the amount of free ellagic acid in the final product,[19,20] by the release of ellagic acid from the achenes and from the hydrolysis process undergone by ellagitannins present in the achenes and in the flesh.[21] The aim of our study was to evaluate whether strawberry puree containing the same amount of total ellagic acid as fresh strawberries, but a higher quantity as free ellagic acid, could lead to an increase in the production of urolithins that might result in additional health benefits to consumers. For this purpose we manufactured a strawberry puree that was given to healthy volunteers against fresh strawberries, and the levels of urolithins produced were assessed in urine.

MATERIALS AND METHODS

Reagents and Standards. Ellagic acid was purchased from Sigma (St. Louis, MO). Urolithin A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one; ≥95% purity, HPLC) and urolithin B (3-hydroxy-6*H*-dibenzo-[*b*,*d*]pyran-6-one; ≥98% purity, HPLC) were chemically synthesized by Kylolab S.A. (Murcia, Spain). Methanol (MeOH), diethyl ether, chloride acid, and acetone were obtained from Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this study.

Fresh Strawberries and Strawberry Purees. Fresh strawberries (*Fragaria* × *ananassa* Duchesne) of the cultivar Camarosa and strawberry puree were kindly provided by Hero S.A. Co. (Alcantarilla, Murcia, Spain). Strawberry puree was produced by microcrushing and slight thermal treatment at 80 °C for 5 min. Puree ingredients were 98.64% strawberries, 0.6% pectin, 0.6% citric acid, 0.1% potassium sorbate, 0.06% acesulfame K, and 0.003% neohesperidin chalcone.

Extraction and Hydrolisis. Polyphenolic compounds from fresh strawberries and strawberry puree were processed, extracted, and submitted to hydrolysis according to the methodology previously reported by Cerdá et al.[3] The hydrolysis process of fresh strawberries and strawberry puree was carried out to quantify the total ellagic acid content administered.

Subjects. Healthy volunteers (n = 20, 8 men and 12 women) aged between 25 and 30 years having body mass index within the normal range (18.5−24.99) gave their written consent and participated in the study. Normal functions for gut, liver, and kidney were assumed. Subjects agreed to refrain from consuming pomegranates, berries, nuts, and wine or use any medication 72 h before each intervention and, specifically, antibiotics 1 month before the study. The experimental design, included in the Spanish Research National Project AGL2003-02195, was in accordance with the Helsinki Declaration and approved by the Ethics Committee from CSIC (Madrid, Spain).

Study Design. Volunteers were enrolled in a 4 week randomized study and received either 200 g of fresh strawberries or the equivalent dose of strawberry puree with a 2 week washout interval. During the study, volunteers consumed their usual diet. A urine sample was taken (time point = 0) before the consumption of fresh strawberries or strawberry puree, and then urine was collected in a urine flask in five different fractions: 0−8, 8−32, 32−56, 56−80, and 80−92 h after the intake of fresh strawberries or strawberry puree (Figure 1). Strawberry or strawberry puree intake was monitored under supervision.

Urine Samples. Urine flasks were collected, and the volume of each fraction was quantified. Urine samples were processed and analyzed the same day of collection. Fifty milliliters of urine from each fraction and volunteer was filtered through a Sep-Pak solid phase extraction cartridge. Each cartridge was washed with 10 mL of water, and the phenolic fraction was eluted with 2 mL of MeOH. The methanolic fractions of each cartridge were collected and filtered through a 0.45 μm filter, Millex-HV13 (Millipore), and then analyzed by LC-MS/MS.

Analysis of Strawberry Polyphenols and Urolithins by LC-MS/MS. All LC-MS/MS analyses were achieved using an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany) with the methodology described by Buendía et al.[22] for foodstuffs. Urine urolithins levels were analyzed according to the method of Gonzalez-Sarrías et al.[13] Ellagic acid from fresh strawberries and strawberry puree was identified by chromatographic comparison (UV and MS) with pure commercial standard. Total ellagic acid was quantified as free ellagic acid at 360 nm after hydrolysis.[3] Strawberry polyphenols were identified according to the methods of Buendía et al.[22] and Seeram et al.[23] Ellagitannins were quantified as ellagic acid at 280 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 and MS fragments ions to confirm the identity of compounds previously reported in the literature. Urolithin A and B derivatives were quantified using urolithin A or B, respectively, as standard.[4] The limits of detection and quantification were 0.05 and 0.15 μM for urolithin A and 0.6 and 2 μM for ellagic acid, respectively. The repeatability value was 99.9%.

Pharmacokinetic Analysis. Urolithin and urolithin metabolite concentration−time data were analyzed by noncompartmental pharmacokinetic analysis. Pharmacokinetic parameters were estimated using the WinNonlin software package (WinNonlin Professional version 5.2.1, Pharsight Corp., Mountain View, CA). WinNonlin model 200 was used for the analysis. The area under the urinary excretion rate curve (AURCall) from time 0 to the last time with measurable concentration was estimated using a linear/log trapezoidal approximation. Other pharmacokinetic parameters obtained were the lag time (t_{lag}), the time prior to the first measurable (nonzero) concentration, and the midpoint of collection interval associated with the maximum observed excretion rate (t_{max} rate).

Statistical Analysis. Data on urolithin levels are expressed as the mean value ± standard error (SE), n = 20. Subjects were divided into tertiles according to their urolithin A glucuronide production level. When appropriate, data were subjected to statistical analysis of variance for repeated measures. Differences between pharmacokinetic parameters were tested by Wilcoxon's signed rank procedure. A P value of <0.05 was considered to be significant. Statistical analysis was performed using the SPSS package (version 19.0).

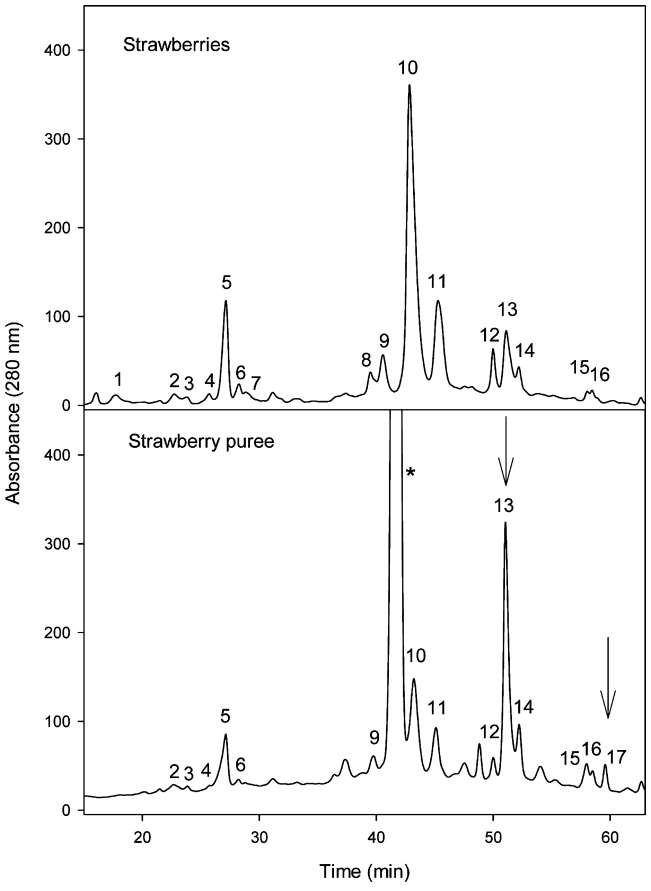

Figure 2. HPLC-DAD chromatograms obtained at 280 nm for fresh strawberries and strawberry puree. Peaks: 1, unknown ellagitannin; 2, unknown ellagitannin; 3, procyanidin dimer; 4, epicatechin; 5, *p*-coumaroyl-glucoside; 6, hydroxycinnamic acid; 7, unknown ellagitannin; 8, cyanidin-3-*O*-glucoside; 9, galloyl-bis-HHDP-glucose; 10, pelargonidin-3-*O*-glucoside; 11, pelargonidin-3-*O*-rutinoside; 12, ellagic acid rhamnoside; 13, free ellagic acid; 14, quercetin-3-*O*-glucuronide; 15, kaempferol-3-*O*-glucoside; 16, kaempferol-3-*O*-glucuronide; 17, methyl ellagic acid rhamnoside; * furfural derivative.

Table 1. Maximum Concentration of Urolithin A Glucuronide for Each Tertile and Percentage of Urine Recovery[a]

	tertile	urolithin A glucuronide (mg/L)	urine recovery (%)
fresh strawberries	T1 (n = 6)	2.28 (0, 5.29)	7.66 ± 7.46
	T2 (n = 7)	13.00 (7.73, 17.66)	45.35 ± 14.31
	T3 (n = 7)	23.95 (19.77, 27.46)	98.84 ± 38.33
strawberry puree	T1 (n = 6)	5.24 (0, 8.33)	14.46 ± 12.44
	T2 (n = 7)	11.53 (9.88, 14.66)	34.71 ± 12.31
	T3 (n = 7)	21.22 (16.05, 25.77)	80.43 ± 41.33

[a]Data values for urolithin A glucuronide are the mean, and within parentheses the range is specified. Percentage of urine recovery is calculated as the sum of all metabolites detected divided by the total amount of ellagic acid ingested (62 mg).

RESULTS AND DISCUSSION

Ellagic Acid Content. The content of free ellagic acid (**13**) in fresh strawberries was 1.79 ± 0.30 mg/100 g of fresh weight, similar to those previously found in the same cultivar[18] and in Senga Sengana cultivar.[20] When strawberries were processed to puree, all the achenes were completely crushed and the content of free ellagic acid (**13**) significantly increased 3-fold ($P < 0.05$) (Figure 2). The increase observed was accompanied by a decrease in the total content of ellagitannins (**1**, **2**, **7**, **9**) and a reduced level of ellagic acid-rhamnoside (**12**) (Figure 2). These changes can be related to the mechanical process by which the achenes are crushed and also to the slight thermal treatment that produces a depolymerization of the larger ellagitannins.[24,25] This result is in line with those published by Zafrilla et al.,[24] who reported a 2.5-fold increase of free ellagic acid after raspberry processing into jam; with those reported by Aaby et al.,[20] who found a 20% of increase in the content of free ellagic acid and a subsequent decrease in ellagitannins and ellagic acid glycosides in puree made from whole strawberries; and with those reported by Hager et al.,[25] who described a 1.4-fold increase of total ellagitannins after blackberry pureeing. In addition to increased free ellagic acid, methylellagic acid rhamnoside (**17**), which was not present in fresh strawberries, was probably released from the achenes and detected in the puree (Figure 2). The total content of ellagic acid, as a result of the hydrolysis process, was 31 mg/100 g of fresh strawberries or strawberry puree.

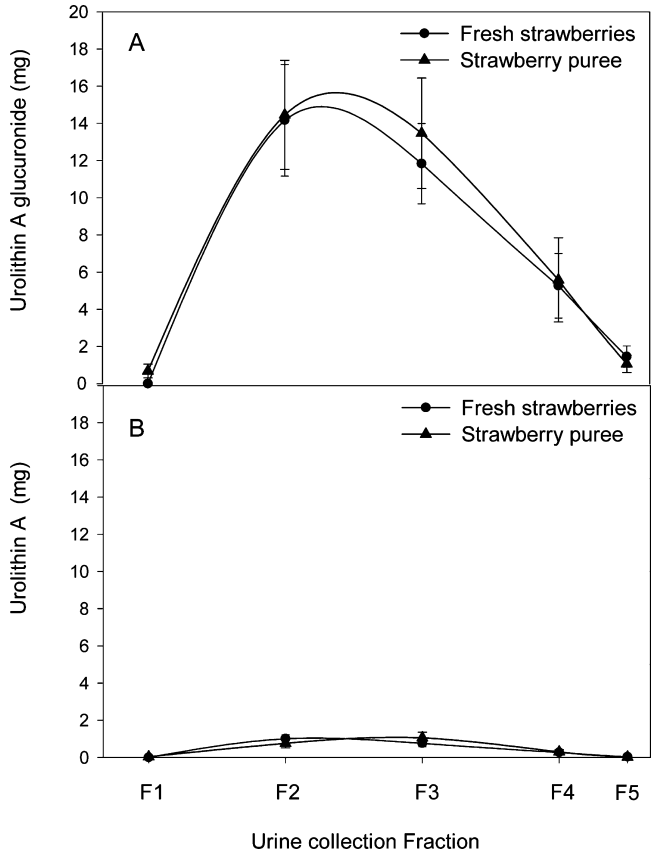

Figure 3. Excretion for each fraction of urolithin A glucuronide (A) and urolithin A (B). Results are expressed as milligrams in the total volume of urine collection. F1, 0−8 h; F2, 8−32 h; F3, 32−56 h; F4, 56−80 h; F5, 80−92 h.

Urinary Excretion of Urolithins. All subjects completed the study. Urolithins were not detected in the baseline urine (t = 0). In accordance with previous studies[2,3,26] the predominant metabolite excreted in urine after ellagitannin-rich food administration was urolithin A glucuronide. There was a high interindividual variation in urolithin A and urolithin A

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Table 2. Pharmacokinetic Parameters for Urine Excretion of Urolithin A Glucuronide and Urolithin A after a Single Oral Administration of Fresh Strawberries and Strawberry Puree[a]

		t_{lag} (h)	t_{max} rate (h)	AURCall (mg·h/mL)	amount recovered (mg)
urolithin A glucuronide	fresh strawberries	12.78 ± 1.22	28.05 ± 3.36	0.52 ± 0.09	36.35 ± 6.30
	strawberry puree	10.79 ± 1.02	31.84 ± 3.23	0.53 ± 0.11	33.40 ± 6.69
urolithin A	fresh strawberries	12.07 ± 1.07	29.65 ± 3.14	0.04 ± 0.006	2.75 ± 0.44
	strawberry puree	13.26 ± 1.75	45.55 ± 4.59	0.04 ± 0.008	2.73 ± 0.49

[a]Results are expressed as the mean ± SE.

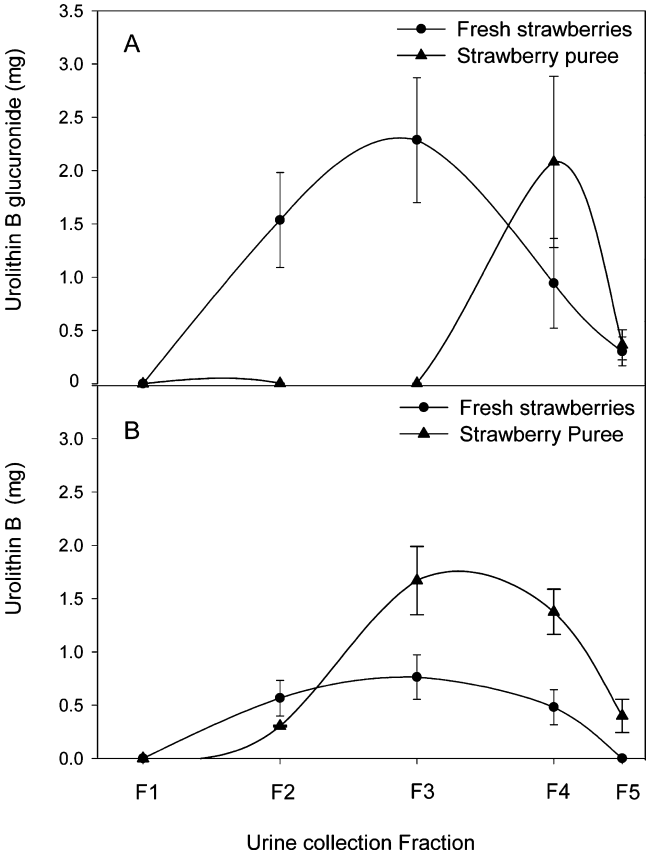

Figure 4. Excretion for each fraction of urolithin B glucuronide (A) and urolithin B (B). Results are expressed as milligrams in the total volume of urine collection. F1, 0–8 h; F2, 8–32 h; F3, 32–56 h; F4, 56–80 h; F5, 80–92 h.

glucuronide excretion levels. When subjects were classified into tertiles according to their level of total urolithin A glucuronide excretion, the participants ranking from 4 to 18 mg of urolithin A glucuronide excretion were classified as low producers, participants ranking between 20 and 30 mg of urolithin A glucuronide excretion were classified as medium producers, and those that excreted >34 mg of urolithin A glucuronide were classified as high producers (Table 1). The high interindividual variability in the production of urolithins, which has been described in previous studies,[2,3,13] seems to be due to the gut microbiota that is responsible for the conversion of ellagic acid into urolithins; thus, subjects, depending on their microbiota profile, produce more or less quantity of urolithins. Currently, the microbiota responsible for the synthesis of urolithins is unknown, and further studies are required. In vitro and in vivo studies[17,27] suggest that urolithins could modify the microbiota, which would indicate a two-way urolithin–microbiota interaction that could be involved in the beneficial effects that have been described for the consumption of ellagitannin-rich foods.[28]

Table 4. Urolithin A, Urolithin B, Urolithin A Glucuronide, and Urolithin B Glucuronide Recovery Levels over 92 h of Urine Collection in the Three Volunteers (V1, V2, and V3) Who Produce Both Types of Urolithins after Fresh Strawberry and Strawberry Puree Ingestion

		urolithin A (mg)	urolithin B (mg)	urolithin A glucuronide (mg)	urolithin B glucuronide (mg)
fresh strawberries	V1	3.18	4.37	46.35	13.48
	V2	0.29	0.26	6.80	0.76
	V3	2.40	2.61	22.51	5.95
strawberry puree	V1	0.68	5.60	2.44	0.02
	V2	7.67	5.03	97.30	12.56
	V3	2.28	0.61	30.35	0.04

The excretion profiles of urolithin A glucuronide after fresh strawberry or strawberry puree administration were very similar (Figure 3A). When the pharmacokinetic parameters t_{lag}, t_{max} rate, AURCall, and total excretion amount of urolithin A glucuronide were compared between both treatments, no significant differences were found in any of the parameters analyzed (Table 2). Urolithin A and urolithin A glucuronide appeared in urine in the second fraction of urine collection (from 8 to 32 h), and these data are consistent with previous results[3,23] and corroborate the data about the microbial origin of urolithins.

Table 3. Pharmacokinetic Parameters for Urine Excretion of Urolithin B Glucuronide and Urolithin B after a Single Oral Administration of Fresh Strawberries and Strawberry Puree[A]

		t_{lag} (h)	t_{max} rate (h)	AURCall (mg·h/mL)	amount recovered (mg)
urolithin B glucuronide	fresh strawberries	16.35 ± 5.34	52.05 ± 8.05	0.06 ± 0.05	4.78 ± 3.95
	strawberry puree	37.70 ± 13.34	80.05 ± 7.99	0.06 ± 0.05	4.21 ± 4.18
urolithin B	fresh strawberries	11.00 ± 0	52.05 ± 7.99	0.03 ± 0.02	2.42 ± 1.19
	strawberry puree	11.00 ± 0	60.05 ± 19.28	0.06 ± 0.02	3.75 ± 1.57

[A]Results are expressed as the mean ± SE.

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Urolithin A was under detectable levels in five subjects that consumed fresh strawberries and in four subjects that consumed strawberry puree. These subjects were all in the group classified as low urolithin producers. There was a significant correlation ($P < 0.01$) between the amounts of urolithin A glucuronide and urolithin A excreted in urine ($R = 0.790$ and $R = 0.662$, coefficients of correlation of Pearson for strawberry puree and fresh strawberries, respectively). The amount of urolithin A glucuronide excreted in urine was 13-fold higher than the level of aglycone for both treatments (Figure 3), which suggests that there is a high glucuronidation rate of urolithin A and an efficient absorption.

Urolithin B and urolithin B glucuronide were detected in only four volunteers (20% of the total population) who consumed fresh strawberries and in three volunteers (15%) who consumed strawberry puree; these three volunteers were the same ones who produced urolithin B metabolites after fresh strawberry consumption. This fact could suggest that only a portion of the population synthesizes urolithin B; therefore, only a part of the population could have the microbiota needed to metabolize ellagic acid into urolithin B, in agreement with previous studies.[2−4] The excretion profiles of urolithin B were very similar for both treatments (Figure 4B), and when the pharmacokinetic parameters for urolithin B and urolithin B glucuronide were analyzed (Table 3), no significant differences were found. With regard to the appearance of urolithin B glucuronide in urine (Figure 4A), although there is a delay in the appearance of urolithin B glucuronide in the urine of volunteers who had ingested the strawberry puree compared to those who had ingested the fresh strawberries, no significant differences were found. Probably, the different profile observed is due to the very small sample size; further experiments selecting a urolithin B producer population could elucidate this point. The total level of urolithin B glucuronide excreted in urine was 2-fold higher than the level of urolithin B when fresh strawberries were ingested and 1.12-fold higher in the case of strawberry puree (Table 3). In the urine profile of volunteers who produced both urolithins (Table 4), levels of both aglycones were similar; however, urolithin A glucuronide levels were higher (between 3.4- and 122-fold) than urolithin B glucuronide levels, which would indicate a lower rate of glucuronidation of the urolithin B aglycone. Hence, our data suggest that the glucuronidation rate of urolithin B could be less efficient than those for urolithin A. Dimethylellagic acid and urolithin C or D described in previous studies[4,13] were not detected or were detected only in traces.

Mean urinary excretion of urolithin conjugates for 92 h reached 58 ± 48% (fresh strawberries) and 57 ± 52% (strawberry puree) of the amount of total ellagic acid administered (62 mg) (Table1). This is the highest recovery described, probably because this is the first time that urine has been recovered for such an extended period (92 h). Cerdá et al.[3] found a mean recovery of 16.6 ± 28% in urine collected for 56 h after the intake of walnuts and observed that the clearance was not complete. A 12.4% metabolite recovery over 72 h of urine collection has been described in rats fed the ellagitannin geraniin, and the authors indicated that after 72 h of collection, the presence of urolithin metabolites in urine was still increasing.[29] In the present study at 92 h, the urinary excretion of urolithins was almost complete, which indicates that urolithins and their metabolites persisted in the body for a long time after a single dose of ellagitannin-rich food. This observation is important to establish the systemic effects and possible target organs of urolithins and urolithin metabolites, as the half-life in plasma of urolithins and urolithin conjugates is shorter than in urine,[13] which supports an intensive enterohepatic recirculation of urolithins.[2] In summary, a 3-fold increase in free ellagic acid over the total content of ellagic acid and a food matrix in which the ellagitannins are more accessible does not affect an increased production of urolithins by the gut microbiota.

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Author Contributions

[‖]These two authors have contributed equally to the present work.

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