

Urolithins in Human Breastmilk after Walnut Intake and Kinetics of *Gordonibacter* Colonization in Newly Born: the Role of Mothers' Urolithin Metabotypes

Adrián Cortés-Martín^δ, Rocío García-Villalba^δ, Izaskun García-Mantrana[#], Ana Rodríguez-Varela^ψ, María Romo-Vaquero^δ, María Carmen Collado[#], Francisco A. Tomás-Barberán^δ, Juan Carlos Espín^δ, and María Victoria Selma^{δ,*}

^δ Laboratory of Food & Health, Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, Murcia, Spain.

[#] Group of Lactic Bacteria and Probiotics, Department of Biotechnology, IATA-CSIC, Valencia, Spain.

^ψ Primary Health Care Center of Bétera, Valencia, Spain

***Corresponding author:** María Victoria Selma, Ph.D., Laboratory of Food & Health, Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, Campus de Espinardo N°25, 30100 Murcia, Spain. **E-mail:** mvselma@cebas.csic.es; **Fax:** +34-968-396213.

"This document is the Accepted Manuscript version of a Published Work that appeared in final form in Journal of Agricultural and Food Chemistry, copyright ©2020 American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see 10.1021/acs.jafc.0c04821"

ABSTRACT: The maternal-infant transmission of several urolithins through breastmilk and the gut colonization of infants by urolithin-producing bacterium *Gordonibacter* during their first year of life were explored. Two trials (Proof of concept study: $n = 11$; Validation study: $n = 30$) were conducted where breastfeeding mothers consumed walnuts as a dietary source of urolithin precursors. An analytical method was developed and validated to characterize the urolithin profile in breastmilk. Total urolithins ranged from 8.5 to 176.9 nM while they were not detected in breastmilk of three mothers. The mothers' urolithin-metabotypes governed the urolithin profile in breastmilk, which might have biological significance on infants. A specific qPCR method allowed monitoring the gut colonization of infants by *Gordonibacter* during their first year of life, and neither breastfeeding nor vaginal delivery was essential for this. The pattern of *Gordonibacter* establishment in babies was conditioned by their mother's urolithin-metabotype, probably because of mother-baby close contact.

KEYWORDS: polyphenols, ellagitannins, ellagic acid, Coriobacteriia, Eggerthellaceae, microbial metabolism, urolithin-producing bacteria.

▪ INTRODUCTION

Walnuts are a substantial source of vitamins, minerals, proteins, unsaturated fats, fiber, and bioactive phenolic compounds such as ellagitannins (ETs) and ellagic acid (EA). ETs and EA present in nuts, pomegranates, berries and oak-aged wine among other food, are metabolized by characteristic colonic bacteria producing specific bioactive metabolites known as urolithins.¹ A recent study has shown that walnuts intake modulates gut microbial communities, including an increase of urolithin-producing bacterium *Gordonibacter*.² Although it is speculated that part of urolithins could be further metabolized in the gut, these are absorbed and circulate as glucuronide and sulfate conjugates, and as such, are detected in plasma, urine, and tissues such as the prostate, colon, and mammary gland.^{3–5} Urolithins show anti-inflammatory, antioxidant, anticarcinogenic, antimicrobial, cardioprotective and neuroprotective effects *in vitro* and in animal models.^{1,6,7} The evidence in human studies is still limited although the improvement of mitochondrial and cellular health in elderly individuals by urolithin consumption has recently been described.⁸ Differences in health effects observed *in vivo* after consumption of ETs-rich foods could be explained, at least partially, by the large inter-individual variability in the production of urolithins, derived from differences in the gut microbiota composition.⁷ Thus, the beneficial effects are not only a question of production vs. non-production of urolithins but also related to the type of urolithins produced. This variability allows stratifying the population into three groups, called urolithin metabolotypes (UMs).⁹ Metabotype A (UM-A) individuals produce only urolithin A (Uro-A; 3,8-dihydroxy-urolithin). Metabotype B (UM-B) is characterized by the production of urolithin B (Uro-B; 3-hydroxy-urolithin) and(or) isourolithin A (IsoUro-A; 3,9-dihydroxy-urolithin) in addition to Uro-A. Finally, individuals from metabotype 0 (UM-0) are those urolithin non-producers. While UM-A and UM-B can change over age, 10% of the population remains constant as UM-0 from 5 to 90 years.¹⁰ UMs are associated with differences in the gut microbiota profile.^{11–13} Furthermore, the genus *Gordonibacter* can produce urolithins and has been positively correlated with UM-A and Uro-A production in humans versus either

UM-0 or UM-B.^{9, 14–16} Uro-A, recently reported as a Generally Recognized as Safe (GRAS), is the predominant metabolite detected in the plasma and urine of urolithin producers (UM-A and UM-B). Several health benefits have been attributed to Uro-A, primarily intestinal anti-inflammatory properties, while Uro-B and IsoUro-A, although less studied, are also bioactive metabolites with similar effects than Uro-A.⁷

The acquisition and colonization of the microbiome in the first weeks of life play a crucial role in the development of the immune system, and set the stage for the adult microbiome.^{17,18} This process is shaped by many factors including infant feeding (breastfed or formula-fed), type of delivery (vaginal or caesarean section), gestational age at birth, maternal lifestyle, geographical location, intake of antibiotics and other medications, and host genetics.^{17,19} Although 90% of children aged between 5 and 10 years old can produce urolithins, according to a study with a Caucasian cohort (5–90 years, n = 839),¹⁰ it is unknown when and how gut colonization by urolithin-producing bacteria, such as *Gordonibacter*, occurs in infancy. Breastfeeding is one of the most significant factors associated with gut microbiome establishment during the first year of life.²⁰

The determination of dietary phenolics in breastmilk, their metabolizing bacteria and the metabolites produced, has still been little explored. There are only a few studies, and most of them focused on isoflavones^{21–25} and other flavonoid metabolites.^{26–30} Little is known about the existence of urolithins or urolithin producing bacteria in breastmilk. Only the presence of glucuronide conjugates of Uro-A and Uro-B have been reported in human breastmilk^{31,32} from only two and one volunteers, respectively. Unlike other biological samples (urine, feces, blood, etc.) for which different methodologies have been validated,³³ an optimal and validated method for determining these compounds in breastmilk matrix has not been established so far. Besides, essential aspects such as the influence of the mother's UM on the urolithin composition of breastmilk or the *Gordonibacter* colonization in infants have not been considered.

Therefore, this work aimed to: i) evaluate urolithin production in breastmilk after walnut intake and whether mothers' UMs determine the urolithin profile, and ii) explore possible relationships between maternal UMs, and type of feeding (breastfed or formula-fed) and delivery (vaginal or caesarean) with the colonization of the urolithin-producing bacteria *Gordonibacter* in infants.

▪ MATERIAL AND METHODS

Chemical and Reagents. Urolithin A 3-glucuronide (Uro-A 3-glur; 8-hydroxy-urolithin 3-glucuronide), isourolithin A 3-glucuronide (IsoUro-A 3-glur; 9-hydroxy-urolithin-3-glucuronide), urolithin B glucuronide (Uro-B glur; urolithin-3-glucuronide), urolithin A sulfate (Uro-A sulfate; 8-hydroxy-urolithin-3-sulfate), urolithin B sulfate (Uro-B sulfate; urolithin-3-sulfate), urolithin M6 (Uro-M6; 3,8,9,10-tetrahydroxy-urolithin), urolithin M7 (Uro-M7; 3,8,10-trihydroxy-urolithin), isourolithin A (IsoUro-A; 3,9-dihydroxy-urolithin), urolithin A (Uro-A; 3,8-dihydroxy-urolithin), and urolithin B (Uro-B; 3-hydroxy-urolithin) were chemically synthesized and purified by Villapharma Research S.L. (Parque Tecnológico de Fuente Álamo, Murcia, Spain). Urolithin D (Uro-D; 3,4,8,9-tetrahydroxy-urolithin) and urolithin C (Uro-C; 3,8,9-trihydroxy-urolithin) were purchased from Dalton Pharma Services (Toronto, Canada). Stock solutions (10 mM) of individual urolithins were prepared in dimethyl sulfoxide (DMSO), and standards mixtures were prepared in methanol at a concentration of 200 µM. Standard of EA and the internal standards, 6,7-dihydroxycoumarin (DHC), and chrysin were from Sigma-Aldrich (St. Louis, MO, USA). Their stock solutions were prepared in methanol. All standard solutions were stored at -20 °C.

Methanol and acetonitrile were purchased from J.T.Baker (Deventer, The Netherlands). Formic acid and acetic acid were from Panreac (Barcelona, Spain). Milli-Q system

(MilliporeCorp., Bedford, MA, USA) ultrapure water was used throughout this experiment. All chemicals and reagents were of analytical grade.

Packs of peeled walnuts were kindly provided by Borges International Group, S.L. (Reus, Tarragona, Spain). Peeled walnuts were subjected to acid hydrolysis and analyzed by HPLC-DAD-MS/MS³⁴ to quantify free EA (4.1 ± 0.6 mg/g fresh weight) as the primary precursor of urolithins.

Study Design and Participants. Two trials (Trial 1: Proof of concept study; Trial 2: Validation study) were carried out according to the Helsinki Declaration and its amendments. Ethics committees from the Servicio Valenciano de Salud (reference 52327) (Valencia, Spain) and the Spanish National Research Council (CSIC, Spain) (reference AGL2015-64124-R) authorized the protocols. In both trials, the exclusion criteria were: consumption of medication, antibiotics, or dietary supplementation such as probiotics or prebiotics one month before and during the study. Ellagitannins-containing foods (pomegranate, walnuts, strawberries, etc.) were avoided during the week before starting the intervention with walnuts. All the participants were informed and gave written, informed consent for study participation.

Trial 1 was a pilot study to determine the time required to detect urolithins in breastmilk after starting walnut intake and to develop a method for urolithins extraction in human breastmilk samples. Healthy women ($n = 11$) after delivering a healthy newborn and with breastfeeding were recruited at different postpartum stages (between 2 weeks and 24 months after giving birth) because breastmilk composition generally differs over time. Ten mothers consumed 30 g of peeled walnuts daily for three days as previously described for urolithin detection in urine.⁸ They kindly provided breastmilk at 0 h, 24 h, 48 h, and 72 h and urine samples at 72 h from starting walnuts intake, respectively. One of the mothers did not consume the walnuts for 3 days, and her breastmilk sample was used as a blank for the validation studies.

Trial 2 was a more extensive study to characterize the urolithin profile in breastmilk depending on the UMs of the donors and evaluate the colonization of *Gordonibacter* in infants during the first year after birth. For this purpose, healthy women ($n = 30$) were selected from a

cohort of 40 mothers whose gut microbiota and anthropometric profiles during the first year postpartum were previously described.³⁵ In this *Trial 2*, three weeks after delivering a healthy baby by vaginal (70%) or caesarean (30%) delivery, the mothers consumed 30 g of peeled walnuts daily for 3 days, and they kindly provided breastmilk and urine samples at 72 h from starting walnuts intake. Finally, only 27 volunteers could provide breastmilk samples. Mothers ($n = 30$) also provided stool samples from their babies four times during their first year of life (1, 4, 6 and 12 months) to study when and how the baby's intestine is colonized by *Gordonibacter* and the relation with the mother's UM.

Sample Collection. Infant feces and breastmilk samples were collected following a standardized protocol provided. Women self-collected both samples at home. Infant feces were collected in sterile containers directly from the diaper. Breastmilk samples were collected in parallel. In brief, the protocol included a breast cleaning step with water and soap. Then, breastmilk was collected from one breast by use of a sterile pumper in sterile bottles to normalize the breastmilk collection protocol. Morning collection was recommendable. Feces and breastmilk samples were aliquoted and stored at -80°C until further analysis.

Determination of Urolithin Metabotypes of the Mothers. Urine samples at 72 h from starting walnuts intake were used to stratify the mothers of both trials by UMs, analyzing the production of urolithins and their phase II metabolites. Samples were vortexed for 30 sec, centrifuged at $14,000 \times g$ for 10 min, and filtered through a $0.22 \mu\text{m}$ polyvinylidene fluoride (PVDF) filter (Millipore, Fisher Scientific, Madrid, Spain). Samples were diluted 1:2 with water containing 0.1% formic acid. The analyses were carried out using an Agilent 1200 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a photodiode array detector and a single quadrupole (single Q) mass spectrometer detector (HPLC-DAD-ESI-Q (MS)), following the protocol described elsewhere.³³

Breastmilk Sample Preparation for Urolithins Extraction. Two different strategies for milk sample pre-treatment and extraction of original urolithins were evaluated: protein precipitation

with various reagents, and separation using solid-phase extraction. Blank samples spiked with 0.5 μ M of a mixture of urolithins were subjected to different extraction protocols.

Acetonitrile (ACN), methanol (MeOH), and a pre-treatment with *n*-hexane were tested to remove proteins and lipids. ACN and MeOH were acidified with formic acid (0.5, 1, and 2%) or hydrochloric acid (1.5%) to enhance the separation of phases after centrifugation. Besides, different solvents [MeOH, MeOH/ACN (50/50, v/v) and ACN] were also assayed to re-dissolve the samples after the evaporation of the supernatant.

Regarding solid-phase extraction, 1 mL of clarified milk (previously centrifuged) was diluted with water acidified with different percentages of formic acid, and filtered through a C18 Sep-Pack cartridge. After washing with 5 mL of water, compounds were eluted with 2 mL of MeOH. We also tested hybridSPE-phospholipid technology (Sigma-Aldrich, San Luis, MO, USA) for removing phospholipids and proteins from the milk matrix. The final protocol optimized for urolithins extraction is further detailed in the Results Section.

UPLC-ESI-QTOF MS Analysis. Samples were analyzed using an Agilent 1290 Infinity UPLC system coupled to a 6550 Accurate-Mass Quadrupole time-of-flight (QTOF) (Agilent Technologies, Waldbronn, Germany). The chromatographic and mass spectrometric conditions tested were those previously optimized for the quantification of urolithins in other biological samples (urine, plasma, and feces).³³ Briefly, the separation was achieved on a reversed-phase Poroshell 120 EC-C18 column using as mobile phases: water plus 0.1% formic acid (phase A) and ACN plus 0.1% formic acid (phase B) in a gradient mode. The flow rate was 0.5 mL/min, and the injection volume was 5 μ L. Spectra were acquired in negative polarity with m/z range 100-1100. Data were processed using the MassHunter Qualitative Analysis software (version B.10, Agilent Technologies, Waldbronn, Germany).

Method Validation. The method was validated in terms of linearity, sensitivity, repeatability, matrix effects, and recovery based on the validation guidance of analytical methods.³⁶

Calibration curves of all available urolithins were prepared in MeOH and the milk matrix from 0.001 μ M to 5 μ M. For the latter, blank samples, taken for a volunteer from *Trial 1* not consuming ellagitannin-rich products, were extracted as described in the result section and spiked after the extraction with the mixture of urolithins at different concentrations (post-spike samples) to take into account possible matrix effect. An internal standard (dihydroxycoumarin (DHC)) was added in each sample before sample preparation in order to control extraction efficiency.

Limits of detection (LOD) and quantification (LOQ) were calculated using the most diluted standard solutions prepared in the milk matrix, considering a signal-to-noise ratio (S/N) of 3 for the LOD and of 10 for the LOQ (IUPAC method).³⁷

Repeatability was evaluated by injecting three concentrations of the urolithin mixture prepared in the matrix, three times on the same day (intra-day repeatability) and different days (inter-day repeatability).

Matrix effect (%ME) for each urolithin was calculated comparing the slopes of calibration curves in MeOH and in matrix (post-spiked sample): %ME= ((slope matrix-slope MeOH)/slope MeOH)*100. If the ratio is within $\pm 15\%$, no matrix effect was considered.³³

Recovery was evaluated by spiking blank samples with urolithins at three concentration levels: 0.01, 0.1, and 0.5 μ M, each in triplicate. After vortexing, the samples were extracted as described in the result section. Recovery was calculated by comparing the peak area of target compounds in the samples spiked before the extraction with those spiked after the extraction.

Determination of *Gordonibacter* in Breastmilk and Infant Stool Samples. Bacterial genomic DNA was extracted from 2 mL-breastmilk samples as described before,³⁸ and from infant stool samples following the manufacturer's instructions of the NucleoSpin® Tissue DNA Purification Kit (Macherey-Nagel, Dueren, Germany). NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA) was used to determine DNA concentration. In breastmilk and stool samples, a touch-down real-time qPCR was carried out for *Gordonibacter* DNA

amplification as described elsewhere¹⁴ with an ABI 7500 Real-Time PCR system. Samples were analyzed in triplicate and compared with a standard curve of genomic DNA (6-fold serial dilutions of *Gordonibacter pamelaee* DSM 19378^T).

Statistical Analysis. Statistical analysis was performed using the SPSS Software, version 23.0 (SPSS Inc., Chicago, IL, USA). The kinetics of urolithin excretion in breastmilk over 72 h of walnut consumption was evaluated by repeated measures analyses of variance (ANOVA) followed by Bonferroni-corrected *t*-test (for post-hoc analysis) (*Trial 1*). Mothers were grouped into UMs while babies, depending on the analysis, into their mothers' UMs, feeding mode, type of delivery, or presence/absence of *Gordonibacter* in the feces (*Trial 2*). A multinomial logit model was applied to evaluate differences in *Gordonibacter* gut colonization in babies over the first year after childbirth (1, 4, 6, and 12 months), according to mothers' UMs or type of delivery. Statistical significance was set at $P < 0.05$.

▪ RESULTS

Optimization of a Method for Urolithins Extraction in Human Breastmilk Samples. ACN showed a better effect on protein precipitation compared to MeOH, which yielded lower efficiency (from 30-60%, depending on the metabolite). The acidification improved the precipitation of proteins, but hydrochloric acid hampered the recovery of the conjugates (glucuronides and sulfates) by promoting their hydrolysis to the corresponding free forms. Best recoveries and fewer matrix effects was observed with 1% of formic acid. A previous wash with *n*-hexane decreased the recovery of some urolithins, especially those unconjugated metabolites. Regarding solid-phase extraction, sample acidification seemed to affect the recovery of the compounds, and hydrolysis of the conjugates, especially sulfates, was observed when using high percentages of formic acid. In general, good recoveries for most metabolites were obtained with less interference from the co-eluted endogenous matrixes, although with poorly reproducible

results. When hybridSPE-phospholipid technology was used, recoveries for most free forms were high ($\geq 85\%$). However, the most polar compounds, such as the conjugates and some polyhydroxylated free forms (Uro-D, Uro-M6, and Uro-C), showed recoveries below 40%.

Finally, in the optimized method, breastmilk samples (1 mL) were mixed with 3 mL of ACN:formic acid (99:1, v/v), and 20 μ L of internal standard (2 ppm 6,7-dihydroxycoumarin) was added. Samples were vortexed for 1 min, incubated under agitation in an ultrasonic bath for 10 min, and centrifuged twice at 18,000 $\times g$ for 30 min at 4 °C. The resulting supernatant was dried in a speed vacuum concentrator (Savant SPD121P, ThermoScientific, Madrid, Spain). The evaporated samples were dissolved in 200 μ L of methanol and filtered through a 0.22 μ m PVDF filter. A second internal standard (0.1 ppm chrysin) was added just before the analysis by UPLC-ESI-QTOF. This method was rapid, simple, and allowed a good recovery by minimizing the possibility to lose analytes during the sample clean-up process.

Method Validation. The parameters of the validation method are shown in **Table 1**. Recovery values ranged between 62 and 76% for glucuronide and sulfate conjugates of Uro-A and Uro-B, respectively, whereas recovery values for free forms were higher, between 66% for Uro-M7 and 92% for Uro-B. Acceptable recoveries were obtained for all the compounds considering the complexity of the matrix (**Table 1**). All calibrations curves showed good linearity ($r^2 \geq 0.995$) in a wide range of concentrations from LOQ to 5 μ M. The LOD was very low for glucuronide conjugates (6.0 nM for Uro-A glur, 5.8 nM for IsoUro-A glur, and 4.2 nM for Uro-B glur) and a bit higher for sulfate conjugates (7.3 nM for Uro-A sulfate and 10.3 nM for Uro-B sulfate). Lower LOD values were found for the unconjugated forms, 1.5 nM for Uro-A, 1 nM for IsoUro A, and 5 nM for Uro-B. LODs and LOQs in breastmilk for these urolithins were in the same range that those found in urine. Results of intra- and inter-day repeatability showed values of relative standard deviation (RSD) for the peak area ratios below 4.8% for intra-day and below 8.6% for inter-day repeatability, showing a good level of precision. The values of matrix effects were within a tolerance range of $\pm 15\%$, and indicated that there were no matrix effects for these

compounds.^{39,40} The matrix effect was higher than -15% for other analytes, reflecting moderate suppression of the signal. In any case, to reduce the matrix effect, a matrix-matched standard curve was used for quantification.

The kinetics of Urolithins Excretion in Breastmilk. Breastmilk samples from volunteers of the pilot study (*Trial 1*) were used to identify the presence of urolithins over time as donors were in different lactation stages and to evaluate their excretion kinetics in breastmilk over 72 h. Chemical structure and extracted Ion Chromatograms (EICs) of the main urolithins detected in human breastmilk are represented in **Figures 1** and **2**, respectively. Walnut intake led to the excretion of glucuronide and sulfate conjugates of Uro-A, IsoUro-A, and Uro-B [Uro-A glur (**1**), IsoUro-A glur (**2**), Uro-A sulfate (**3**), Uro-B glur (**4**) and Uro-B sulfate (**5**)]. Besides, peaks of the unconjugated forms IsoUro-A (**6**), Uro-A (**7**), and Uro-B (**8**) were also detected (**Figure 2**). Urolithins were found in urine samples from all mothers who consumed walnuts in *Trial 1* ($n = 10$). In contrast, in three of them ($n = 1$ from UM-A and 18-month-old baby; $n = 2$ from UM-B and 2- and 5-month-old babies, respectively), urolithins were not detected in breastmilk at any time, because they were not present or were below the LOD. In the rest of the volunteers ($n = 7$), Uro-A glur was the predominant metabolite in breastmilk, whereas other urolithins were detected in only a limited number of samples. Although urolithin concentrations were always in the nanomolar range (data not shown), high inter-individual variability was observed in the urolithins profile in milk. Volunteers, with detectable urolithins in breastmilk during walnut intake, were grouped into UM-A ($n = 4$) and UM-B ($n = 3$) (**Figure 3**). The kinetics of urolithins excretion showed a progressive increase of urolithins in breastmilk over time ($P < 0.001$). The maximum value was reached at the last sampling point (24 h after the last walnut intake) with no significant difference between UMs (UM-A mean value = 28.3 ± 12.1 nM and UM-B mean value = 28.8 ± 14.6). For this reason, breastmilk sampling for urolithins determination in *Trial 2* (which included more volunteers than *Trial 1*) was selected at 72 h.

Quantification of Urolithins in the Breastmilk of Mothers with Different UMs.

The UMs of the mothers in *Trial 2* ($n = 30$) were determined by analyzing their urine samples 72 h after walnut intake started. From the 27 mothers who finally provided breastmilk samples, 12 were UM-A (44%) and 15 UM-B (55%). As occurred in the preliminary study (*Trial 1*), none of the mothers belonged to the UM-0. The urolithin concentrations in both UMs, and each mother individually, are shown in **Tables 2** and **3**, respectively. There were no significant differences in urolithin concentration between breastmilk samples from *Trial 1* (established lactation: > from 1 month) and *Trial 2* (early lactation: 3 weeks).

Breastmilk samples of UM-A mothers were characterized by the presence of glucuronide and sulfate conjugates of Uro-A. In contrast, breastmilk samples of UM-B mothers contained conjugates of IsoUro-A and(or) Uro-B in addition to those of Uro-A (**Tables 2** and **3**). Among urolithins, Uro-A glur, a common compound in both UM-A and UM-B, was the predominant metabolite (it was detected in 26 out of the 27 volunteers, with a detection rate of 92%) with an average concentration of 27.6 nM for UM-A and 31.1 nM for UM-B (**Tables 2** and **3**). No significant differences were observed in the amount excreted of Uro-A-glur between both UMs. Uro-A sulfate was only detected in four volunteers (33%) within UM-A, and quantified in two of them (average concentrations 7.9 nM). In contrast, a larger occurrence was observed in mothers with UM-B (80%) with higher levels (17.2 nM). Some mothers with UM-B also excreted IsoUro-A glur and(or) Uro-B glur (53 and 67% of the mothers, respectively), but the concentration of most of these concentrations were below the LOQ (6.2 and 3.7 nM, respectively). The average levels were 14.8 nM for IsoUro-A glur and 19.8 nM for Uro-B glur. Uro-B sulfate was only detected in 2 mothers with UM-B, but the concentrations were below the LOQ (10.1 nM). Regarding unconjugated urolithins, their concentration in breastmilk was very low (≤ 5 nM). Uro-A was detected in both UM-A and UM-B (detection rate of 42 and 33%, respectively), and average concentrations of 4.7 and 3.3 nM for UM-A and UM-B, respectively. Six mothers with

UM-B (40%) excreted IsoUro-A (average concentration of 2.7 nM), and only one excreted Uro-B in milk (4.3 nM) (**Tables 2 and 3**).

Infant Gut Colonization by the Urolithin-Producing Bacteria *Gordonibacter*. Most of the newly born ($n=29$) who completed the one-year study ($n = 30$) consumed breastmilk during the first month of their life. A gradual and significant reduction ($P < 0.001$) in breastfeeding from 1 to 12 months after giving birth was observed. Thus, most of the 12-month-old babies (82%) were no longer breastfed. The presence of *Gordonibacter* in infant fecal samples was analyzed during first year of life (1, 4, 6, and 12 months). The concentration of *Gordonibacter* in infants ranged from below the detection limit (<2 log copies/g) to 8.4 log copies/g feces with a mean value of 4.4 ± 2.3 log copies/g. *Gordonibacter* was already detected in 45% of the 1-month-old babies. Interestingly, *Gordonibacter* was also detected in the fecal samples of the only baby who never was breastfed, even when he was 1-month-old. No associations between feeding type and presence of *Gordonibacter* in babies were found in the first year of life (results not shown). Furthermore, *Gordonibacter* was below the limit of detection (<2 log copies/mL) in breastmilk samples.

Infants were clustered depending on their mother's UM, and the presence of *Gordonibacter* in fecal samples was analyzed (**Figures 4A, 4B**). In babies born from UM-A mothers, *Gordonibacter* was detected in 47% of one-month-old babies. The presence of *Gordonibacter* significantly increased ($P = 0.025$) until 78% in four-month-old babies, and it was maintained without significant changes until the end of the study (12 months from birth) (**Figure 4A**). In contrast, in babies born from UM-B mothers, the presence of *Gordonibacter* was detected in 53% of one-month-old babies, and the increase in *Gordonibacter* incidence was not produced until 12-month-old babies, being later than in babies born from UM-A mothers (**Figure 4B**). Once the presence of *Gordonibacter* was detected in the babies, its abundance was significantly unchanged over time in most of the babies regardless of their mothers' UMs.

Infant clustering according to the type of delivery revealed that 46% and 40% of one-month-old babies born by vaginal or caesarean delivery, respectively, presented *Gordonibacter* in their feces (**Figures 4C, 4D**). The colonization kinetics by *Gordonibacter* was nearly identical in both types of delivery. At the last stage, 84% and 86% of twelve-month-old babies born by vaginal or caesarean delivery, respectively, presented *Gordonibacter* in their feces. Therefore, the type of delivery did not affect the increase of *Gordonibacter* prevalence in babies during their first year of life.

▪ DISCUSSION

Breastfeeding contributes to the protection of the health of the newly born because breastmilk contains nutrients, vitamins, minerals, hormones, antibodies, immune cells, and beneficial bacteria such as bifidobacteria as well as bioactive metabolites (postbiotics) produced by maternal gut microbiota after consumption of some foods.^{26,41,42} Walnuts are a very rich source of ellagitannins and therefore an excellent precursor for urolithin production. Previous studies have shown that the intake of 30 g walnuts for three days provides enough ellagitannins to enable the characterization of urolithin metabotypes in humans.^{2,43,44} Walnuts are a more optimum source of human urolithin precursors compared to other EA containing foods such as pomegranate. Indeed, a previous study showed that the walnut-matrix rich in fat favors the availability of EA-related compounds to urolithin production by the human gut microbiota compared to pomegranate.⁴⁵ During breastfeeding, urolithins could confer additional anti-inflammatory activity to human breastmilk, but their identification in this matrix has been poorly explored. The complexity of the milk matrix explains why the determination of polyphenol-derived metabolites such as urolithins in breastmilk has been hardly investigated and why an optimal and validated method has not been developed until now. It is essential to remove milk protein and fat content before analysis as previously suggested.³⁰ Most previous studies have

determined polyphenols in human milk after enzymatic hydrolysis and subsequent extraction with ethyl acetate or diethyl ether.^{21–23,25,27,29} Protein precipitation with ACN has also been used in previous works to quantify total phenolic compounds in breastmilk using the Folin-Ciocalteu method³⁰ and isoflavones after enzymatic hydrolysis.²⁴ However, in these cases, relevant information about the naturally occurring metabolites is missing. In other work, solid-phase extraction was applied to determine phase II metabolites of epicatechin.²⁶ In the present study, ACN was selected versus MeOH because of the better recoveries. Similarly, ACN was also used to extract isoflavones from human breastmilk.²⁴ Centrifugation was selected to improve the precipitation of protein and lipids as previously described.^{24,27,30} A more extended extraction protocol combining protein precipitation with ACN followed by solid-phase extraction on C18 cartridges was used to extract Uro-A gluc in human breastmilk samples after pomegranate juice consumption.³² No information about recoveries and validation parameters was provided to compare those results with our study. For the first time in the present study, a method to quantify urolithins in breastmilk has been fully optimized and applied in two trials. LOD and LOQ differed between the different urolithins. The main reason is that compounds with similar structure can have different response in the mass spectrometer. Good sensitivity was obtained with this methodology, showing LOD for Uro-A gluc in breastmilk (1.8 nmol/L) in the same range and even lower as that shown previously (4.95 nmol/L).³²

In *Trial 1*, the excretion kinetics of total urolithins in the breastmilk of 10 mothers was evaluated during 72 h after starting walnut intake as previously optimized for urolithin detection in urine.⁴⁴ Maximum urolithin concentrations in breastmilk was found on day 4 (72 h after the start of walnuts intake). This accumulation over time, even on day 4 where mothers did not consume walnuts, is due to the fact that urolithins remain in circulation for 12 to 72 h after consumption.^{1,46} Enterohepatic circulation is responsible for the long clearance of these metabolites, which persist in urine for 48-72 h after ellagitannin-containing food intake. Subsequent intervals were not analyzed because mothers did not continue consuming walnuts

beyond three days. The qualitative urolithin profile of human milk was similar to that previously found in plasma and urine, with glucuronide and sulfate conjugates as the predominant compounds, and the free forms present at much lower concentrations.^{9,33} Therefore, urolithins can be excreted in breastmilk regardless of their chemical structure. Blood metabolites, including those derived from the metabolism of dietary (poly)phenols by the gut microbiota, are excreted into breastmilk by passive and active processes, as described for enterolignans in different animals where the participation of the ABCG2 transporter is critical.^{47,48} In the case of urolithins, their absence in some human breastmilk samples could be due to the large inter-individual variability in urolithins production, i.e., high vs. low urolithin producers, which could prevent the occurrence of detectable urolithins in the milk in those urolithin low-producers.³ In the present study, the levels of Uro-A glur detected in breastmilk (from 8.7 to 90.9 nM) were in the same order of those detected in plasma (around 110 nM) after the intake of walnuts.³ Regarding breastmilk, these values are also in the order of those found in the breastmilk of two mothers after the intake of pomegranate juice (from 21.5 to 36.3 nM).³² However, these coincident values are somewhat unexpected, taking into account that Uro-A glur levels were estimated by MS using Uro-A as standard, which has a very different signal-response from Uro-A glur in MS.³³ Nanomolar urolithin concentrations in breastmilk were also in agreement with the breastmilk ranges of other polyphenols previously reported. In a recent study, the phytoestrogens genistein, daidzein, equol, and enterolactone were detected with total average concentrations of 270 nM.²⁵ Franke et al. also reported daidzein and genistein levels in human breastmilk at 80-110 and 30-50 nmol/L, respectively, after the consumption of soy-rich diets.^{21,23} The presence of different flavan-3-ols (epicatechin, epicatechin gallate, gallic acid, and gallic acid gallate) and other flavonoids (naringenin, kaempferol, hesperetin, and quercetin) in human milk samples was also reported at nanomolar levels.^{26,28,29} Although all these values are in the nanomolar range, differences in the concentration of the different polyphenol metabolites in breastmilk

may be the result of several factors, including dietary exposure, the efficiency of absorption and transfer from plasma to human milk, among others.²⁹

In *Trial 2*, the contribution of UMs of 27 mothers in the composition of their breastmilk was considered. Thus, the type of urolithins excreted in the breastmilk depended on the maternal UMs and were consistent with those found in urine. For the first time, we describe that the urolithin profile in breastmilk was governed by the maternal UMs. Therefore, as in the case of plasma and urine, the urolithins profile in breastmilk indirectly reflects the maternal gut microbiota composition because UMs have been associated with different gut microbiota profiles.¹¹

In the present study, the distribution of mothers' UMs was 44% UM-A and 56% UM-B, as previously described in the postpartum period.³⁵ This distribution differs from that reported for a healthy population,¹⁰ and closer to that reported for overweight-obese subjects with mild to high cardiometabolic risk.^{43,49} Uro-A glur was the predominant metabolite present in most breastmilk samples (detection rate 96%) in both UMs. In contrast, IsoUro-A glur and Uro-B glur were only detected in volunteers with UM-B with 53 and 67% detection rate, respectively. These differences in the type of urolithins to which infants are exposed via breastmilk could produce differences in the benefits of breastfeeding associated with urolithins since different health benefits have been attributed to each urolithin type.⁷ Besides, the prebiotic effect of Uro-A has also been previously described in a colitis rat model.⁵⁰ Therefore, the consumption of urolithins through breastfeeding could also contribute to the gut microbiota modulation of infants. However, further research about the direct and indirect health effects of urolithins in breastfed infants, especially against infant colic, should be explored.

Comparison of both trials did not show significant differences in urolithin concentration between breastmilk of *Trial 1* (established lactation samples: more than 1 month from delivery; $n = 7$) and *Trial 2* (early lactation samples: 3 weeks from delivery; $n = 27$). According to the lactation stage, a previous study also showed no difference in the concentration of metabolites

from other polyphenols (flavonoids) in breastmilk. In contrast, other non-polyphenolic compounds such as carotenoids decreased significantly from weeks 1 to 13 of lactation.²⁹ In the present study, most breastmilk samples were obtained 3 weeks from delivery and only 7 from later stages of lactation. Therefore, further research should be done to confirm that urolithin content is stable during different stages of lactation.

Gut colonization in infancy by the urolithin-producing bacteria *Gordonibacter* has not been previously explored. This genus is essential for the production of bioactive urolithins, which can not be produced by 10% of the population of any age.¹⁰ It is known that the richness and diversity of the fecal microbiome of newly born are lower than that of adults. Besides, 16S rRNA Illumina sequencing is less sensitive than real-time quantitative PCR (qPCR). Therefore, only a specific qPCR method¹⁴ allowed us to detect and quantify *Gordonibacter* in infant feces for the first time. Indeed, *Gordonibacter* was present in 45% of 1-month-old babies. It is known that the type of feeding (breastfed or formula-fed) and delivery (vaginal or caesarean section) are two of the most important factors, among others, involved in establishing the gut microbiota in neonatal and infant periods.^{17,19} However, our results show that neither breastfeeding nor vaginal delivery was an essential condition for the colonization of *Gordonibacter*. Thus, *Gordonibacter* was detected in fecal samples from some 1-month-old babies delivered by caesarean section and the only baby that was not ever breastfed. This agrees with a previous study, which showed that within the first 6 weeks of life, the infant microbiota undergoes substantial reorganization, primarily driven by body site and not by type of delivery.⁵¹

It is still unknown the precise beginning of the colonization of *Gordonibacter* since our follow-up started four weeks after delivery. This colonization increased in babies from one to twelve months, regardless of the type of delivery. Interestingly, *Gordonibacter* occurrence sharply increased up to 78% in 4-months-old babies from UM-A mothers despite their sources of bacterial colonization are scarce because at that age they still do not crawl. In contrast, in breastfed infant from UM-B mothers, the colonization was delayed for 6 months, and

Gordonibacter occurrence only increased from 6 to 12 months after birth. Furthermore, the relative abundance (%) of *Gordonibacter* in the gut was significantly higher in UM-A mothers than UM-B during the first year postpartum, as previously reported.³⁵ Therefore, close contact of mothers with their babies during their four months of life may favor colonization probability with *Gordonibacter*, especially in babies born from UM-A mothers.

In the present study, a method has been optimized and successfully applied to characterize the metabolic profiling of urolithins in breastmilk by LC-MS, and to explore, by using qPCR, the colonization of the newly born by *Gordonibacter*. Overall, this combined method allowed the identification of 8 different urolithin metabolites in breastmilk after walnut intake and described the colonization kinetics by *Gordonibacter* in the first year of the babies' life. Although urolithins are excreted into breastmilk at nanomolar concentrations, they could contribute to breastfeeding's anti-inflammatory properties and healthy properties. Besides, it was confirmed that breastmilk resembles the UMs of the mothers, which might have biological significance for the infants. Therefore, our study opens new research scenarios to explore the role of walnuts and breastfeeding with urolithin-enriched milk on infants' health and microbiota modulation. *Gordonibacter* colonization in infants occurs through their first year of life, and neither breastfeeding nor vaginal delivery was an essential condition for this. However, the kinetics of *Gordonibacter* colonization in babies is conditioned by their mother's UM.

AUTHOR INFORMATION

Corresponding author

Research Group on Quality, Safety, and Bioactivity of Plant Foods, Laboratory of Food & Health; Dep. Food Science and Technology, CEBAS-CSIC, 30100 Campus de Espinardo, Murcia, Spain. E-mail: mvselma@cebas.csic.es; Phone: +34-968396200. Fax: +34-968396213.

475

476 **Funding**

477 This research work was supported by the Projects AGL2015-64124-R, AGL-2015-73106-EXP
478 (MINECO, Spain), and 20880/PI/18 (Fundación Séneca de la Región de Murcia, Spain). A. C. M. is
479 the holder of a predoctoral grant from MINECO (Spain).

480

481 **Notes**

482 The authors declare no competing financial interest.

■ REFERENCES

- (1) Espín, J. C.; Larrosa, M.; García-Conesa, M. T.; Tomás-Barberán, F. Biological Significance of Urolithins, the Gut Microbial Ellagic Acid-Derived Metabolites: The Evidence So Far. *Evid. Based Complement. Altern. Med.* **2013**, 270418, DOI: 10.1155/2013/270418.
- (2) García-Mantrana, I.; Calatayud, M.; Romo-Vaquero, M.; Espín, J. C.; Selma, M. V.; Collado, M. C. Urolithin Metabotypes Can Determine the Modulation of Gut Microbiota in Healthy Individuals by Tracking Walnuts Consumption over Three Days. *Nutrients* **2019**, *11* (10), 2483, DOI: 10.3390/nu11102483.
- (3) González-Sarrías, A.; Giménez-Bastida, J. A.; García-Conesa, M. T.; Gómez-Sánchez, M. B.; García-Talavera, N. V.; Gil-Izquierdo, Á.; Sánchez-Álvarez, C.; Fontana-Compiano, L. O.; Morga-Egea, J. P.; Pastor-Quirante, F. A.; Martínez-Díaz, F.; Tomás-Barberán, F. A.; Espín, J. C. Occurrence of Urolithins, Gut Microbiota Ellagic Acid Metabolites and Proliferation Markers Expression Response in the Human Prostate Gland upon Consumption of Walnuts and Pomegranate Juice. *Mol. Nutr. Food Res.* **2010**, *54* (3), 311–322, DOI: 10.1002/mnfr.200900152.
- (4) Núñez-Sánchez, M. A.; García-Villalba, R.; Monedero-Saiz, T.; García-Talavera, N. V.; Gómez-Sánchez, M. B.; Sánchez-Álvarez, C.; García-Albert, A. M.; Rodríguez-Gil, F. J.; Ruiz-Marín, M.; Pastor-Quirante, F. A.; Martínez-Díaz, F.; Yáñez-Gascón, M. J.; González-Sarrías, A.; Tomás-Barberán, F. A.; Espín, J. C. Targeted Metabolic Profiling of Pomegranate Polyphenols and Urolithins in Plasma, Urine and Colon Tissues from Colorectal Cancer Patients. *Mol. Nutr. Food Res.* **2014**, *58* (6), 1199–1211, DOI: 10.1002/mnfr.201300931.
- (5) Ávila-Gálvez, M. Á.; García-Villalba, R.; Martínez-Díaz, F.; Ocaña-Castillo, B.; Monedero-Saiz, T.; Torrecillas-Sánchez, A.; Abellán, B.; González-Sarrías, A.; Espín, J. C. Metabolic Profiling of Dietary Polyphenols and Methylxanthines in Normal and Malignant Mammary Tissues from Breast Cancer Patients. *Mol. Nutr. Food Res.* **2019**, *63* (9), e1801239, DOI: 10.1002/mnfr.201801239.
- (6) Larrosa, M.; García-Conesa, M. T.; Espín, J. C.; Tomás-Barberán, F. A. Ellagitannins, Ellagic Acid and Vascular Health. *Mol. Aspects Med.* **2010**, *31* (6), 513–539, DOI: 10.1016/j.mam.2010.09.005.
- (7) Cortés-Martín, A.; Selma, M. V.; Tomás-Barberán, F. A.; González-Sarrías, A.; Espín, J. C. Where to Look into the Puzzle of Polyphenols and Health? The Postbiotics and Gut Microbiota Associated with Human Metabotypes. *Mol. Nutr. Food Res.* **2020**, *64* (9), e1900952. DOI: 10.1002/mnfr.201900952.
- (8) Andreux, P. A.; Blanco-Bose, W.; Ryu, D.; Burdet, F.; Ibberson, M.; Aebischer, P.; Auwerx, J.; Singh, A.; Rinsch, C. The Mitophagy Activator Urolithin A Is Safe and Induces a Molecular Signature of Improved Mitochondrial and Cellular Health in Humans. *Nat. Metab.* **2019**, *1* (6), 595–603, DOI: 10.1038/s42255-019-0073-4.
- (9) Tomás-Barberán, F. A.; González-Sarrías, A.; García-Villalba, R.; Núñez-Sánchez, M. A.; Selma, M. V.; García-Conesa, M. T.; Espín, J. C. Urolithins, the Rescue of “Old” Metabolites to Understand a “New” Concept: Metabotypes as a Nexus among Phenolic Metabolism, Microbiota Dysbiosis, and Host Health Status. *Mol. Nutr. Food Res.* **2017**, *61* (1), 1500901, DOI: 10.1002/mnfr.201500901.
- (10) Cortés-Martín, A.; García-Villalba, R.; González-Sarrías, A.; Romo-Vaquero, M.; Loria-Kohen, V.; Ramírez-de-Molina, A.; Tomás-Barberán, F. A.; Selma, M. V.; Espín, J. C. The Gut Microbiota Urolithin Metabotypes Revisited: The Human Metabolism of Ellagic Acid Is Mainly Determined by Aging. *Food Funct.* **2018**, *9* (8), 4100–4106, DOI: 10.1039/c8fo00956b.
- (11) Romo-Vaquero, M.; Cortés-Martín, A.; Loria-Kohen, V.; Ramírez-de-Molina, A.; García-Mantrana, I.; Collado, M. C.; Espín, J. C.; Selma, M. V. Deciphering the Human Gut Microbiome of Urolithin Metabotypes: Association with Enterotypes and Potential

- Cardiometabolic Health Implications. *Mol. Nutr. Food Res.* **2019**, 63 (4), e1800958, DOI: 10.1002/mnfr.201800958.
- (12) Selma, M. V.; Beltrán, D.; Luna, M. C.; Romo-Vaquero, M.; García-Villalba, R.; Mira, A.; Espín, J. C.; Tomás-Barberán, F. A. Isolation of Human Intestinal Bacteria Capable of Producing the Bioactive Metabolite Isourolithin from Ellagic Acid. *Front. Microbiol.* **2017**, 8, 1521, DOI:10.3389/fmicb.2017.01521.
- (13) Beltrán, D.; Romo-Vaquero, M.; Espín, J. C.; Tomás-Barberán, F. A.; Selma, M. V. *Ellagibacter isourolithinifaciens* gen. nov., sp. nov., a New Member of the Family Eggerthellaceae, Isolated from Human Gut. *Int. J. Syst. Evol. Microbiol.* **2018**, 68 (5), 1707–1712, DOI: 10.1099/ijsem.0.002735.
- (14) Romo-Vaquero, M.; García-Villalba, R.; González-Sarrías, A.; Beltrán, D.; Tomás-Barberán, F. A.; Espín, J. C.; Selma, M. V. Interindividual Variability in the Human Metabolism of Ellagic Acid: Contribution of *Gordonibacter* to Urolithin Production. *J. Funct. Foods* **2015**, 17, 785–791, DOI:10.1016/j.jff.2015.06.040.
- (15) Selma, M. V.; Tomás-Barberán, F. A.; Beltrán, D.; García-Villalba, R.; Espín, J. C. *Gordonibacter urolithinifaciens* sp. nov., a Urolithin-Producing Bacterium Isolated from the Human Gut. *Int. J. Syst. Evol. Microbiol.* **2014**, 64 (Pt 7), 2346–2352, DOI:10.1099/ijms.0.055095-0.
- (16) Selma, M. V.; Beltrán, D.; García-Villalba, R.; Espín, J. C.; Tomás-Barberán, F. A. Description of Urolithin Production Capacity from Ellagic Acid of Two Human Intestinal *Gordonibacter* Species. *Food Funct.* **2014**, 5 (8), 1779–1784, DOI:10.1039/c4fo00092g.
- (17) Tamburini, S.; Shen, N.; Wu, H. C.; Clemente, J. C. The Microbiome in Early Life: Implications for Health Outcomes. *Nat. Med.* **2016**, 22 (7), 713–722, DOI:10.1038/nm.4142.
- (18) Segata, N. No Bacteria Found in Healthy Placentas. *Nature* **2019**, 572 (7769), 317–318, DOI: 10.1038/d41586-019-02262-8.
- (19) Milani, C.; Duranti, S.; Bottacini, F.; Casey, E.; Turrioni, F.; Mahony, J.; Belzer, C.; Delgado-Palacio, S.; Arboleya-Montes, S.; Mancabelli, L.; Lugli, G. A.; Rodríguez, J. M.; Bode, L.; de Vos, W.; Gueimonde, M.; Margolles, A.; van Sinderen, D.; Ventura, M. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol. Mol. Biol. Rev.* **2017**, 81 (4), e00036-17, DOI:10.1128/mmb.00036-17.
- (20) Stewart, C. J.; Ajami, N. J.; O'Brien, J. L.; Hutchinson, D. S.; Smith, D. P.; Wong, M. C.; Ross, M. C.; Lloyd, R. E.; Doddapaneni, H.; Metcalf, G. A.; Muzny, D.; Gibbs, R. A.; Vatanen, T.; Huttenhower, C.; Xavier, R. J.; Rewers, M.; Hagopian, W.; Toppari, J.; Ziegler, A. G.; She, J. X.; Akolkar, B.; Lernmark, A.; Hyoty, H.; Vehik, K.; Krischer, J. P.; Petrosino, J. F. Temporal Development of the Gut Microbiome in Early Childhood from the TEDDY Study. *Nature* **2018**, 562 (7728), 583–588, DOI: 10.1038/s41586-018-0617-x.
- (21) Franke, A. A.; Custer, L. J. Daidzein and Genistein Concentrations in Human Milk after Soy Consumption. *Clin. Chem.* **1996**, 42 (6 Pt 1), 955–964, DOI: 10.1093/clinchem/42.6.955.
- (22) Franke, A. A.; Custer, L. J.; Tanaka, Y. Isoflavones in Human Breast Milk and Other Biological Fluids. *Am. J. Clin. Nutr.* **1998**, 68 (6 Suppl), 1466S-1473S, DOI: 10.1093/ajcn/68.6.1466S.
- (23) Franke, A. A.; Halm, B. M.; Custer, L. J.; Tatsumura, Y.; Hebshi, S. Isoflavones in Breastfed Infants after Mothers Consume Soy. *Am. J. Clin. Nutr.* **2006**, 84 (2), 406–413, DOI: 10.1093/ajcn/84.2.406.
- (24) Zhou, W.; Wu, H.; Wang, Q.; Zhou, X.; Zhang, Y.; Wu, W.; Wang, Y.; Ren, Z.; Li, H.; Ling, Y.; Zhang, F.; Li, P. Simultaneous Determination of Formononetin, Biochanin A and Their Active Metabolites in Human Breast Milk, Saliva and Urine Using Salting-out Assisted Liquid-Liquid Extraction and Ultra High Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrum. *J. Chromatogr. B Anal. Technol.*

- Biomed. Life Sci.* **2020**, *1145*, 122108, DOI: 10.1016/j.jchromb.2020.122108.
- (25) Min, J.; Wang, Z.; Liang, C.; Li, W.; Shao, J.; Zhu, K.; Zhou, L.; Cheng, J.; Luo, S.; Yu, L.; Wu, Y.; Xie, M.; Hu, X. Detection of Phytoestrogen Metabolites in Breastfed Infants' Urine and the Corresponding Breast Milk by Liquid Chromatography-Tandem Mass Spectrometry. *J. Agric. Food Chem.* **2020**, *68* (11), 3485–3494, DOI: 10.1021/acs.jafc.9b08107.
- (26) Khymenets, O.; Rabassa, M.; Rodríguez-Palmero, M.; Rivero-Urgell, M.; Urpí-Sardá, M.; Tulipani, S.; Brandi, P.; Campoy, C.; Santos-Buelga, C.; Andrés-Lacueva, C. Dietary Epicatechin Is Available to Breastfed Infants through Human Breast Milk in the Form of Host and Microbial Metabolites. *J. Agric. Food Chem.* **2016**, *64* (26), 5354–5360, DOI: 10.1021/acs.jafc.6b01947.
- (27) Romaszko, E.; Wiczowski, W.; Romaszko, J.; Honke, J.; Piskula, M. K. Exposure of Breastfed Infants to Quercetin after Consumption of a Single Meal Rich in Quercetin by Their Mothers. *Mol. Nutr. Food Res.* **2014**, *58* (2), 221–228, DOI: 10.1002/mnfr.201200773.
- (28) Romaszko, E.; Marzec-Wróblewska, U.; Badura, A.; Buciński, A. Does Consumption of Red Grapefruit Juice Alter Naringenin Concentrations in Milk Produced by Breastfeeding Mothers? *PLoS One* **2017**, *12* (10), e0185954, DOI: 10.1371/journal.pone.0185954.
- (29) Song, B. J.; Jouni, Z. E.; Ferruzzi, M. G. Assessment of Phytochemical Content in Human Milk during Different Stages of Lactation. *Nutrition* **2013**, *29* (1), 195–202, DOI: 10.1016/j.nut.2012.07.015.
- (30) Vázquez, C. V.; Rojas, M. G.; Ramírez, C. A.; Chávez-Servín, J. L.; García-Gasca, T.; Ferriz-Martínez, R. A.; García, O. P.; Rosado, J. L.; López-Sabater, C. M.; Castellote, A. I.; Montemayor, H. M.; de la Torre-Carbot, K. Total Phenolic Compounds in Milk from Different Species. Design of an Extraction Technique for Quantification Using the Folin-Ciocalteu Method. *Food Chem.* **2015**, *176*, 480–486, DOI: 10.1016/j.foodchem.2014.12.050.
- (31) Zhang, X.; Sandhu, A.; Edirisinghe, I.; Burton-Freeman, B. An Exploratory Study of Red Raspberry (*Rubus Idaeus* L.) (Poly)Phenols/Metabolites in Human Biological Samples. *Food Funct.* **2018**, *9* (2), 806–818, DOI: 10.1039/c7fo00893g.
- (32) Henning, S. M.; Wallenstein, M. B.; Weigel, N.; Johnson, C.; Yang, J.; Lee, R.-P.; Korn, M.; Scala, M.; Stevenson, D.; Ben-Nisaan, D.; Heber, D.; Li, Z. Appearance of Ellagic Acid Metabolites from Pomegranate Juice in Breast Milk: A Case Report. **2019**, *4*, 1738.
- (33) García-Villalba, R.; Espín, J. C.; Tomás-Barberán, F. A. Chromatographic and Spectroscopic Characterization of Urolithins for Their Determination in Biological Samples after the Intake of Foods Containing Ellagitannins and Ellagic Acid. *J. Chromatogr. A* **2016**, *1428*, 162–175, DOI: 10.1016/j.chroma.2015.08.044.
- (34) García-Villalba, R.; Espín, J. C.; Aaby, K.; Alasalvar, C.; Heinonen, M.; Jacobs, G.; Voorspoels, S.; Koivumäki, T.; Kroon, P. A.; Pelvan, E.; Saha, S.; Tomás-Barberán, F. A. Validated Method for the Characterization and Quantification of Extractable and Nonextractable Ellagitannins after Acid Hydrolysis in Pomegranate Fruits, Juices, and Extracts. *J. Agric. Food Chem.* **2015**, *63* (29), 6555–6566, DOI: 10.1021/acs.jafc.5b02062.
- (35) Cortés-Martín, A.; Romo-Vaquero, M.; García-Mantrana, I.; Rodríguez-Varela, A.; Collado, M. C.; Espín, J. C.; Selma, M. V. Urolithin Metabotypes Can Anticipate the Different Restoration of the Gut Microbiota and Anthropometric Profiles during the First Year Postpartum. *Nutrients* **2019**, *11* (9), 2079, DOI: 10.3390/nu11092079.
- (36) Guideline on Bioanalytical Method Validation. *Eur. Med. Agency.* **2011**.
- (37) Currie, L. A. Nomenclature in Evaluation of Analytical Methods , Including Detection and Quantification Capabilities. *Pure Appl. Chem.* **1995**, *67* (10), 1699–1723.
- (38) Boix-Amorós, A.; Collado, M. C.; Mira, A. Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. *Front. Microbiol.* **2016**, *7*, 492, DOI: 10.3389/fmicb.2016.00492.

- (39) Svoboda, P.; Vlčková, H.; Nováková, L. Development and Validation of UHPLC – MS/MS Method for Determination of Eight Naturally Occurring Catechin Derivatives in Various Tea Samples and the Role of Matrix Effects. *J. Pharm. Biomed. Anal.* **2015**, *114*, 62–70, DOI: 10.1016/j.jpba.2015.04.026.
- (40) Caban, M.; Migowska, N.; Stepnowski, P.; Kwiatkowski, M.; Kumirska, J. Matrix Effects and Recovery Calculations in Analyses of Pharmaceuticals Based on the Determination of β -Blockers and β -Agonists in Environmental Samples. *J. Chromatogr. A* **2012**, *1258*, 117–127, DOI: 10.1016/j.chroma.2012.08.029.
- (41) Jost, T.; Lacroix, C.; Braegger, C.; Chassard, C. Impact of Human Milk Bacteria and Oligosaccharides on Neonatal Gut Microbiota Establishment and Gut Health. *Nutr. Rev.* **2015**, *73* (7), 426–437, DOI: 10.1093/nutrit/nuu016.
- (42) Gómez-Gallego, C.; García-Mantrana, I.; Salminen, S.; Collado, M. C. The Human Milk Microbiome and Factors Influencing Its Composition and Activity. *Semin. Fetal Neonatal Med.* **2016**, *21* (6), 400–405, DOI: 10.1016/j.siny.2016.05.003.
- (43) Selma, M. V.; González-Sarrías, A.; Salas-Salvadó, J.; Andrés-Lacueva, C.; Alasalvar, C.; Örem, A.; Tomás-Barberán, F. A.; Espín, J. C. The Gut Microbiota Metabolism of Pomegranate or Walnut Ellagitannins Yields Two Urolithin-Metabotypes That Correlate with Cardiometabolic Risk Biomarkers: Comparison between Normoweight, Overweight-Obesity and Metabolic Syndrome. *Clin. Nutr.* **2018**, *37* (3), 897–905, DOI: 10.1016/j.clnu.2017.03.012.
- (44) Tomás-Barberán, F. A.; García-Villalba, R.; González-Sarrías, A.; Selma, M. V.; Espín, J. C. Ellagic Acid Metabolism by Human Gut Microbiota: Consistent Observation of Three Urolithin Phenotypes in Intervention Trials, Independent of Food Source, Age, and Health Status. *J. Agric. Food Chem.* **2014**, *62* (28), 6535–6538, DOI: 10.1021/jf5024615.
- (45) Romo-Vaquero, M.; García-Villalba, R.; González-Sarrías, A.; Beltrán, D.; Tomás-Barberán, F. A.; Espín, J. C.; Selma, M. V. Interindividual Variability in the Human Metabolism of Ellagic Acid: Contribution of Gordonibacter to Urolithin Production. *J. Funct. Foods* **2015**, *17*, 785–791, DOI: 10.1016/j.jff.2015.06.040.
- (46) Seeram, N. P.; Henning, S. M.; Zhang, Y.; Suchard, M.; Li, Z.; Heber, D. Pomegranate Juice Ellagitannin Metabolites Are Present in Human Plasma and Some Persist in Urine for up to 48 Hours. *J. Nutr.* **2006**, *136* (10), 2481–2485, DOI: 10.1093/jn/136.10.2481.
- (47) Otero, J. A.; Miguel, V.; González-Lobato, L.; García-Villalba, R.; Espín, J. C.; Prieto, J. G.; Merino, G.; Álvarez, A. I. Effect of Bovine ABCG2 Polymorphism Y581S SNP on Secretion into Milk of Enterolactone, Riboflavin and Uric Acid. *Animal* **2016**, *10* (2), 238–247, DOI: 10.1017/S1751731115002141.
- (48) García-Mateos, D.; García-Lino, A. M.; Álvarez-Fernández, I.; Blanco-Paniagua, E.; de la Fuente, Á.; Álvarez, A. I.; Merino, G. Role of ABCG2 in Secretion into Milk of the Anti-Inflammatory Flunixin and Its Main Metabolite: In Vitro-in Vivo Correlation in Mice and Cows. *Drug Metab. Dispos.* **2019**, *47* (5), 516–524, DOI: 10.1124/dmd.118.085506.
- (49) González-Sarrías, A.; García-Villalba, R.; Romo-Vaquero, M.; Alasalvar, C.; Örem, A.; Zafrilla, P.; Tomás-Barberán, F. A.; Selma, M. V.; Espín, J. C. Clustering According to Urolithin Metabotype Explains the Interindividual Variability in the Improvement of Cardiovascular Risk Biomarkers in Overweight-Obese Individuals Consuming Pomegranate: A Randomized Clinical Trial. *Mol. Nutr. Food Res.* **2017**, *61* (5), 1600830, DOI: 10.1002/mnfr.201600830.
- (50) Larrosa, M.; González-Sarrías, A.; Yáñez-Gascón, M. J.; Selma, M. V.; Azorín-Ortuño, M.; Toti, S.; Tomás-Barberán, F.; Dolara, P.; Espín, J. C. Anti-Inflammatory Properties of a Pomegranate Extract and Its Metabolite Urolithin-A in a Colitis Rat Model and the Effect of Colon Inflammation on Phenolic Metabolism. *J. Nutr. Biochem.* **2010**, *21* (8), 717–725, DOI: 10.1016/j.jnutbio.2009.04.012.
- (51) Chu, D. M.; Ma, J.; Prince, A. L.; Antony, K. M.; Seferovic, M. D.; Aagaard, K. M. Maturation of the Infant Microbiome Community Structure and Function across

690 Multiple Body Sites and in Relation to Mode of Delivery. *Nat. Med.* **2017**, 23 (3), 314-
691 326, DOI: 10.1038/nm.4272.
692
693

FIGURE CAPTIONS

Figure 1. Urolithin metabolites detected in human breastmilk after the intake of walnuts.

(1) 8-Hydroxy-urolithin-3-glucuronide (urolithin A-glucuronide); (2) 9-Hydroxy-urolithin-3-glucuronide (isourolithin A-glucuronide); (3) 8-Hydroxy-urolithin-3-sulfate (urolithin A-sulfate); (4) Urolithin 3-glucuronide (urolithin B-glucuronide); (5) Urolithin 3-sulfate (urolithin B-sulfate); (6) 3,9-Dihydroxy-urolithin (isourolithin A); (7) 3,8-Dihydroxy-urolithin (urolithin A); (8) 3-Hydroxy-urolithin (urolithin B).

Figure 2. Extracted Ion Chromatograms (EICs) of the main urolithins detected in human breastmilk after the intake of walnuts. 1) Uro-A glur; 2) IsoUro-A glur; 3) Uro-A sulfate; 4) Uro-B glur; 5) Uro-B sulfate; 6) IsoUro-A; 7) Uro-A; 8) Uro-B.

Figure 3. Excretion kinetics of total urolithins in breastmilk of UM-A and UM-B mothers from *Trial 1* during walnut consumption. Statistical significance was evaluated by repeated measures analyses of variance (ANOVA) followed by post-hoc Bonferroni-corrected *t*-test. **The three volunteers without detectable urolithins in breastmilk were not included.*

Figure 4. (A) Presence of *Gordonibacter* in fecal samples from babies born from UM-A mothers, or (B) born to UM-B mothers, by (C) vaginal delivery, or (D) caesarean delivery.

Table 1. Validation parameters for the quantification of urolithins in human breastmilk.

	Recovery (%)	LOD (nM) a/b	LOQ (nM) a/b	Precision (RSD%)		ME (%)
				(intra-day)	(inter-day)	
Uro-A 3-glur	67 ± 2	6.0/1.8	20.0/6.0	2.0	2.6	-6.3
IsoUro-A 3-glur	62 ± 1	5.8/1.9	19.2/6.2	4.8	3.7	-20.2
Uro-A sulfate	76 ± 1	7.3/1.9	24.3/6.4	2.6	8.1	-18.1
Uro-B glur	76 ± 8	4.2/1.1	14.1/3.7	4.5	7.3	+5.7
Uro-B sulfate	68 ± 4	10.3/3.0	34.5/10.1	2.0	4.3	-25.1
IsoUro-A	72 ± 3	1.0/0.3	3.4/0.9	4.1	6.0	-36.8
Uro-A	76 ± 6	1.5/0.4	5.0/1.3	3.5	4.5	-29.0
Uro-B	92 ± 3	5.0/1.1	16.7/3.6	2.5	8.6	-8.5
Uro-C	84 ± 7	0.8/0.2	2.8/0.7	4.0	2.6	-16.9
Uro-M7	66 ± 4	12.0/3.6	39.7/12.0	0.2	4.3	-29.8
Uro-D	79 ± 9	1.3/0.3	4.4/1.1	1.5	7.4	-18.5
Uro-M6	76 ± 9	1.4/0.4	4.6/1.2	0.7	8.3	-23.2

LOD: Limit of detection; LOQ: Limit of quantification; a) LOD and LOQ of the instrument, calculated in the sample injected; b) LOD and LOQ in the original sample, before extraction and injection; ME: Matrix effect; RSD: Relative Standard Deviation.

Table 2. Mean concentration (nM) of urolithins in breastmilk of mothers ($n = 27$, Trial 2) grouped by urolithin metabotypes (UMs).

Urolithins	UM-A mothers ($n = 12$)		UM-B mothers ($n = 15$)	
	Mean \pm SD; (range)	Samples ^a	Mean \pm SD; (range)	Samples ^a
Uro-A glur	27.6 \pm 22.9; (8.7 – 77.9)	10/11	31.1 \pm 24.8; (9.5 – 90.9)	12/15
Uro-A sulfate	7.9 \pm 1.8; (6.6 – 9.2)	2/4	17.2 \pm 11.8; (6.5 – 40.4)	11/12
IsoUro-A glur	<LOD	0	14.8 \pm 8.6; (6.3 – 23.4)	3/8
Uro-B glur	<LOD	0	19.8 \pm 21.6; (4.5 – 51.7)	4/10
Uro-B sulfate	<LOD	0	<LOQ	0/2
Uro-A	4.7 \pm 1.0; (3.9 – 5.4)	2/5	3.3 \pm 2.6; (1.4 – 7.8)	5/5
IsoUro-A	<LOD	0	2.7 \pm 1.4; (1.7 – 5.1)	6/6
Uro-B	<LOD	0	4.3	1/1

^a Samples represent the number of volunteers where each metabolite was quantified/detected; <LOQ (Limit of quantification): metabolites are detectable but the exact amount cannot be quantified; <LOD (Limit of detection): metabolites are not detectable.

Table 3. Concentration (nM) of urolithins in breastmilk of mothers (*n* = 27, *Trial 2*) grouped by urolithin metabotypes (UMs).

Volunteer	UMs	Uro-A glur	Uro-A sulfate	IsoUro-A glur	Uro-B glur	Uro-B sulfate	Uro-A	IsoUro-A	Uro-B
M2N1	UM-A	14.4	Det.	-	-	-	-	-	-
M4N1	UM-A	77.9	9.2	-	-	-	5.4	-	-
M6N1	UM-A	20.0	Traces	-	-	-	-	-	-
M7N1	UM-A	8.7	-	-	-	-	-	-	-
M10N1	UM-A	13.5	Traces	-	-	-	Det.	-	-
M20N1	UM-A	14.7	Traces	-	-	-	-	-	-
M26N1	UM-A	23.5	Det.	-	-	-	Det.	-	-
M35N1	UM-A	Det.	-	-	-	-	-	-	-
M38N1	UM-A	23.0	-	-	-	-	-	-	-
M39N1	UM-A	61.0	6.6	-	-	-	3.9	-	-
M40N1	UM-A	19.0	-	-	-	-	-	-	-
M50N1	UM-A	-	-	-	-	-	Det.	-	-
M1N1	UM-B	23.3	8.6	-	-	-	2.6	-	-
M9N1	UM-B	9.5	6.7	Det.	Det.	-	Traces	1.8	-
M11N1	UM-B	9.9	Det.	Det.	-	-	-	-	-
M14N1	UM-B	Det.	-	-	Det.	-	-	-	-
M15N1	UM-B	24.7	36.8	14.8	Det.	-	-	5.1	-
M18N1	UM-B	90.9	14.5	-	51.7	7.7	7.8	-	4.3
M22N1	UM-B	34.7	19.9	Det.	Det.	-	1.6	1.7	-
M23N1	UM-B	11.7	19.3	6.3	13.3	Traces	-	2.5	-
M25N1	UM-B	Det.	-	-	-	-	-	-	-
M28N1	UM-B	31.5	6.5	-	9.9	Traces	1.4	-	-
M32N1	UM-B	17.7	20.0	Det.	-	-	-	1.7	-
M33N1	UM-B	15.3	7.3	Det.	Det.	5.9	-	-	-
M42N1	UM-B	37.5	9.4	-	4.5	-	3.3	-	-
M43N1	UM-B	Det.	-	-	-	-	-	-	-
M44N1	UM-B	67.1	40.4	23.4	Det.	-	-	3.3	-

*Det., Detected but not quantified (below LOQ). Traces means that these metabolites are detected close to the limit of detection (LOD).

Figure 1.

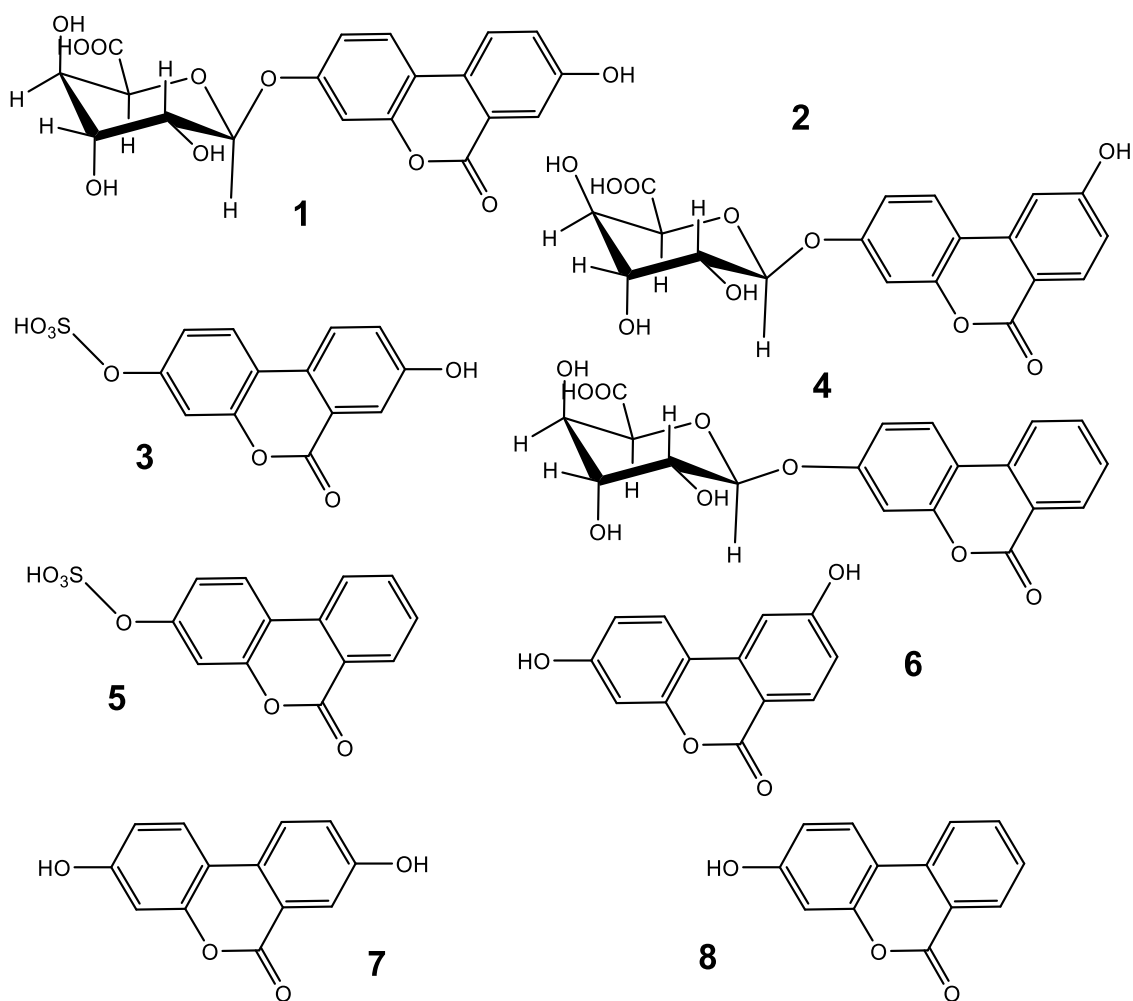


Figure 2.

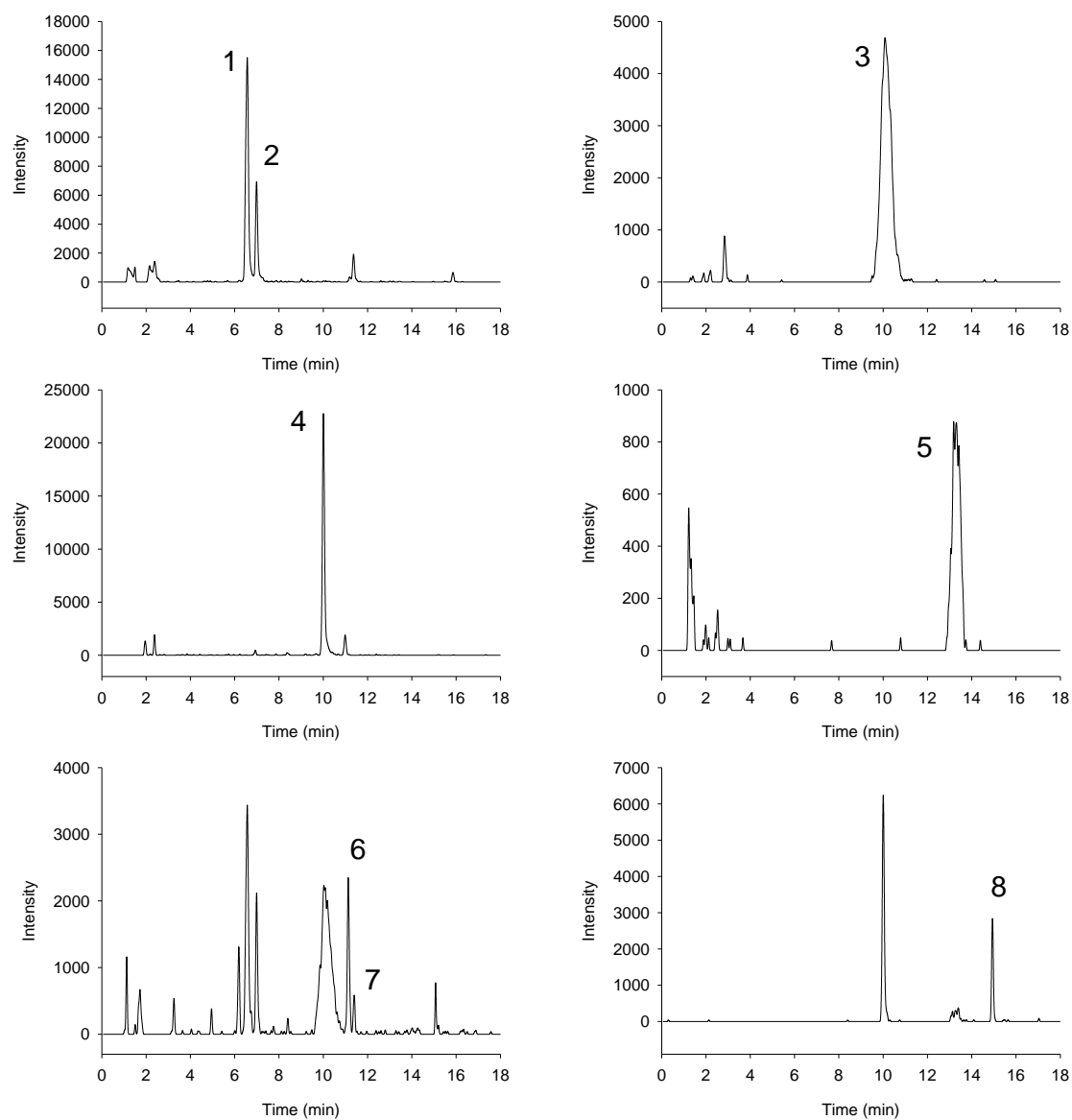


Figure 3.

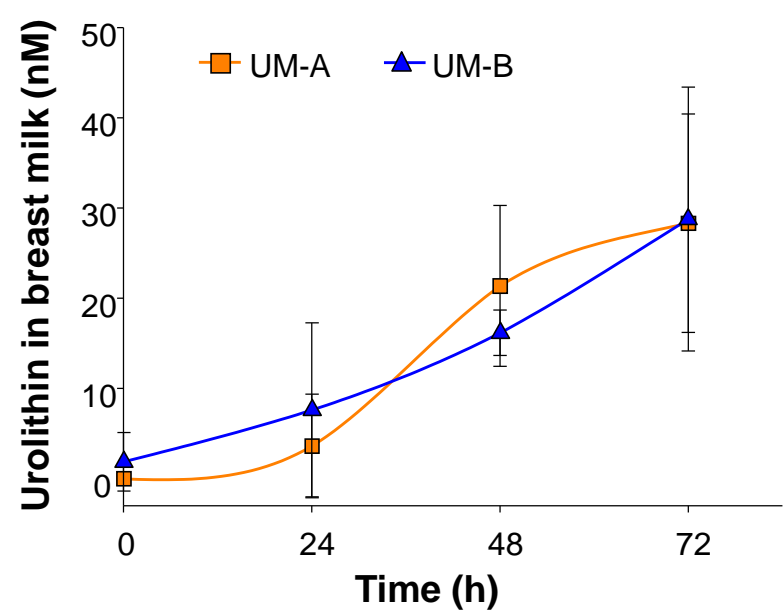
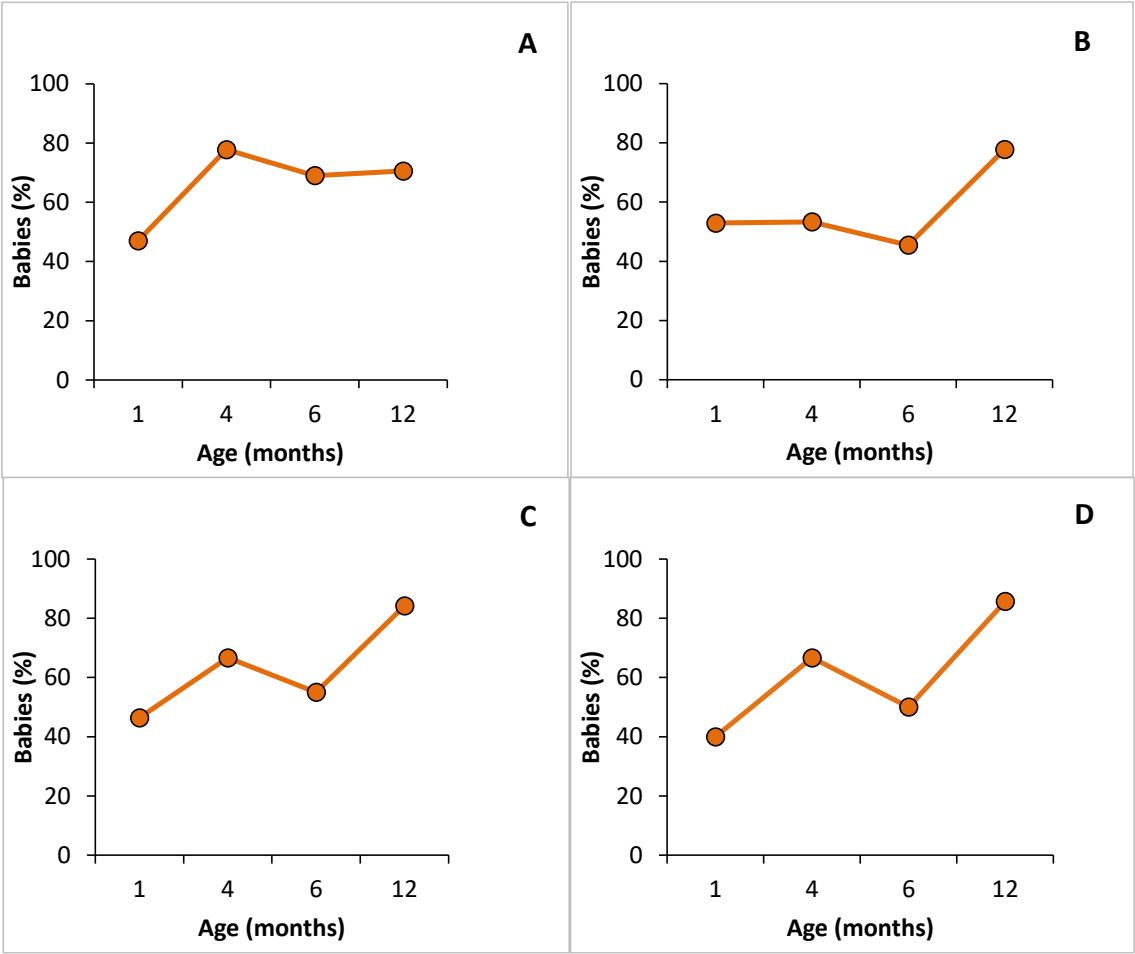


Figure 4.



TOC GRAPHIC

