Journal of Pharmaceutical and Biomedical Analysis 233 (2023) 115477

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Development of a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for characterizing pomegranate extract pharmacokinetics in humans

Yan-Hong Wang, Goutam Mondal, Washim Khan, Bill J. Gurley, Charles R. Yates *

The National Center for Natural Products Research, University of Mississippi, Oxford, MS, USA

ARTICLE INFO

Keywords:
UHPLC-MS/MS
Pomegranate
Punicalagins
Ellagic acid
Urolithin
And human pharmacokinetics

ABSTRACT

Pomegranate extracts standardized to punicalagins are a rich source of ellagitannins including ellagic acid (EA). Recent evidence suggests that gut microbiota-derived urolithin (Uro) metabolites of ellagitannins are pharmacologically active. Studies have evaluated the pharmacokinetics of EA, however, little is known about the disposition of urolithin metabolites (urolithin A (UA) and B (UB)). To address this gap, we developed and applied a novel ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) assay for the characterization of EA and Uro oral pharmacokinetics in humans. Subjects (10/cohort) received a single oral dose (250 or 1000 mg) of pomegranate extract (Pomella® extract) standardized to contain not less than 30 % punicalagins, < 5 % EA, and not less than 50 % polyphenols. Plasma samples, collected over 48 h, were treated with β-glucuronidase and sulfatase to permit comparison between unconjugated and conjugated forms of EA, UA and UB. EA and urolithins were separated by gradient elution (acetonitrile/water, 0.1 % formic acid) using a C_{18} column connected to a triple quadrupole mass spectrometer operating in the negative mode. Conjugated EA exposure was ~5–8-fold higher than unconjugated EA for both dose groups. Conjugated UA was readily detectable beginning ~8 h post-dosing, however, unconjugated UA was detectable in only a few subjects. Neither form of UB was detected. Together these data indicate EA is rapidly absorbed and conjugated following oral administration of Pomella® extract. Moreover, UA's delayed appearance in the blood, primarily in the conjugated form, is consistent with gut microbiota-mediated metabolism of EA to UA, which is then rapidly converted to its conjugated form.

1. Introduction

The pomegranate fruit (*Punica granatum L.*) is considered to be part of the so-called Super Fruits group, which is a term used to highlight the excellent nutritional qualities and health-promoting phytochemicals of certain fruits. A wide variety of health-promoting antioxidants, representing different phytochemical classes, are found in pomegranates and these include gallic acid (3,4,5-trihydroxybenzoic acid) derivatives known as hydrolyzable tannins (*e.g.*, ellagitannin) such as pomellatannin, punicatannins A/B and punicalagin [1]. The most abundant ellagitannin, punicalagin, is tightly linked to the salutary health effects of pomegranate despite the fact that punicalagin plasma exposure (area under the curve, AUC) is barely detectable due to high oral clearance, viz., low oral bioavailability and/or high systemic clearance. This interesting paradox can be partially explained by the fact that intestinal microbiota catabolize punicalagin to form orally bioavailable phenolic metabolites including ellagic acid and various urolithin species [2] (Fig. 1). Consequently, there has been great interest in elucidating the oral pharmacokinetics and pharmacologic mechanisms of action of ellagic acid and its urolithin metabolites [3].

Early human pharmacokinetic studies revealed high interindividual variability in ellagic acid AUC following ingestion of pomegranate juice. Specifically, Seeram et al. [4] found that ellagic acid was rapidly absorbed whereas Cerdá et al. [5] failed to detect ellagic acid following oral administration of pomegranate juice. These data are consistent with the current hypothesis that interindividual variability in oral bioavailability and plasma AUC of ellagic acid is due to its poor aqueous solubility (low fraction absorbed, fa) and extensive metabolism (reduced fraction escaping gut metabolism, fg) in the gastrointestinal tract [6]. However, it is important to note that extensive hepatic metabolism of EA

* Correspondence to: Thad Cochran Research Center West National Center for Natural Products Research, Oxford, MS 38677–1848, USA.
E-mail address: cryates2@olemiss.edu (C.R. Yates).

https://doi.org/10.1016/j.jpba.2023.115477
Received 24 December 2022; Received in revised form 18 May 2023; Accepted 20 May 2023
Available online 23 May 2023

might also contribute to variable EA plasma exposure via first-pass hepatic metabolism (decreased fraction escaping hepatic metabolism) and clearance. Although little or nothing has been done regarding P450 metabolism of EA, much has been done regarding gut microbe-mediated metabolism of EA. Recently, it has been demonstrated that gastrointestinal microbiota (*e.g.*, *Gordonibacter* species) catabolize EA to urolithin metabolites urolithin A (UA), urolithin B (UB), and isourolithin A (Isouro-A) primarily in the distal colon region [2,7,8]. These urolithin metabolites were also detected in healthy human volunteer orally administered with pomegranate extract but not quantified [9]. Bacteria-mediated urolithin metabolite formation is particularly intriguing considering the fact that urolithins have recently been shown to exert potent anti-inflammatory and anti-proliferative effects [10].

Considering the potential pharmacologic importance of the urolithin metabolites, recent human pharmacokinetic studies consider the plasma exposure of both EA and key urolithin metabolites following oral administration of pomegranate extracts. For example, González-Sarrías et al. determined the relative oral bioavailability of EA using two different standardized pomegranate extracts (130 mg punicalagin + 524 mg EA or 279 mg punicalagin +25 mg EA) [6]. Importantly, EA plasma exposure was the same between the two cohorts despite the ≈ 20-fold difference in EA dose (524 vs 25 mg). The authors concluded that administration of higher oral EA doses increases urolithin formation without a commensurate increase in plasma EA exposure due to EA's limited aqueous solubility in the gastrointestinal tract. Interestingly, plasma urolithin levels remained undetectable until 24 h after extract administration. The potential pharmacologic importance of the urolithin metabolites necessitates development of sensitive bioanalytical techniques for a comprehensive assessment of urolithin plasma exposure following oral administration of pomegranate juice or extracts. To that end, our objective herein was to utilize the triple quadrupole mass spectrometer, the workhorse instrument used in pharmacokinetic studies, to develop and validate a bioanalytical assay for the characterization of pomegranate ellagitannin metabolites pharmacokinetics in humans.

2. Materials and methods

2.1. Reagents and chemicals

LC-MS grade methanol, acetonitrile, and formic acid (FA) used for chromatographic separation and extraction were procured from Thermo Fisher Scientific (Waltham, MA, USA). Water was purified using a Millipore Synergy UV Water Purification System (Millipore SAS, Molsheim, France). Ellagic acid (catalog number-14668), urolithin A (catalog number-SML1791), urolithin B (catalog number-SML1649), 6,7-Dihydroxycoumarin (catalog number-PHL80449), chrysin (catalog number-95082), and human plasma (product number-P9523) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

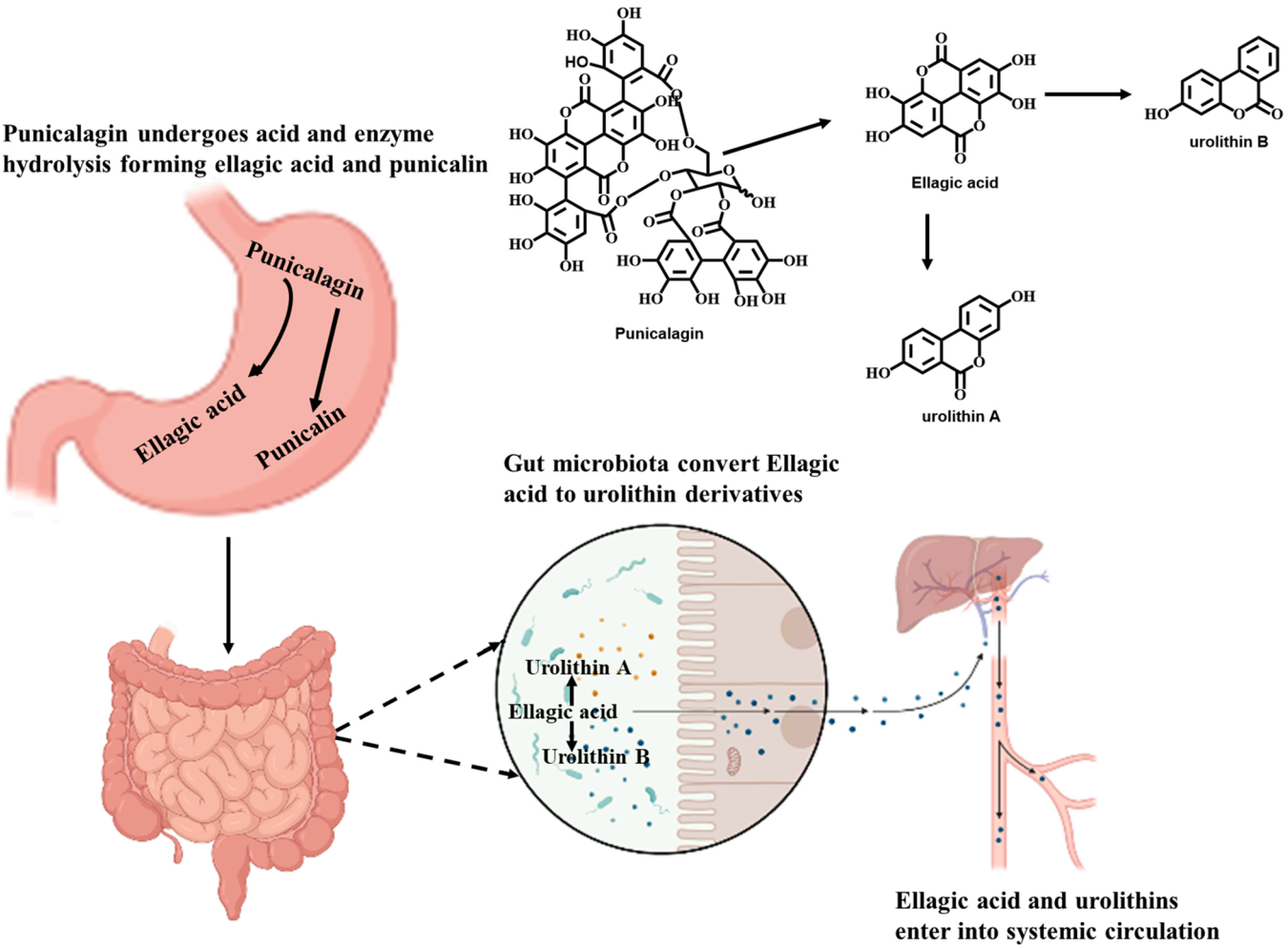

Fig. 1. Punicalagin undergo acid and enzyme hydrolysis to yield ellagic acid and it further converted to various urolithins by gut microbiota. Ellagic acid and urolithins are able to reach systemic circulation.

2.2. Preparation of calibration standards and quality controls

Individual stock solutions (100 μg/mL) of EA, UA, UB, 6,7-Dihydroxycoumarin (DHC, IS-1, for extraction method), and chrysin (IS-2, for instrument) was prepared by dissolving exactly weighed reference substances in methanol. For the calibration curve, a series of working solutions were prepared from the above stock solutions with acetonitrile containing 2 % formic acid to obtain the desired concentration range. An aliquot (700 μL) of the standard solution containing 0.55 ng/mL of EA, UA and UB and 14.28 ng/mL of DHC was added to drug-free human plasma (200 μL). Plasma containing EA, UA, UB, and DHC was vortex mixed for 5 min, bath sonicated for 10 min, and the mixture was then centrifuged for 15 min at 4 °C, circa 14,000 rpm. A supernatant aliquot (750 μL) was taken and evaporated to dryness by SpeedVac (12 h). The dried sample was resuspended in 150 μL of methanol (1 % formic acid) containing chrysin (50 ng/mL) as an IS, the resuspended mixture was vortex mixed for 5 min, sonicated for 10 min, and centrifuged at 15,294 *g* for 15 min. A supernatant aliquot (100 μL) was transferred to an LC vial for analysis to achieve the final concentration of EA, UA, and UB at 2.78 ng/mL and DHC (55.6 ng/mL). Similarly, EA, UA, and UB standard compounds were prepared as final concentration of 2.78, 5.6, 11.1, 27.8, 44.4, 55.6, 111.1, 277.8, 444.4, and 555.6 ng/mL for each analyte. Quality control (QC) samples were added a working solution of standards to drug free human plasma at final concentrations of 27.8, 111.1, 277.8 and 444.4 ng/mL. Lower limit of quantification (LLOQ) samples were added a working solution of standards to drug free human plasma at a final concentration of 2.78 ng/mL for UA and UB respectively, whereas for EA was 5.6 ng/mL. The final concentration of DHC and chrysin were 55.6 ng/mL and 50 ng/mL respectively in all prepared samples. Calibration curves for EA, UA, and UB in human plasma were derived from their peak area ratios relative to that of DHC from the linear regression with a weighting factor of 1/x for the three analytes. The LLOQ and QC samples were analyzed along with each batch of plasma samples to assess the intra- and inter-day precision and accuracy of the method. All prepared solutions were stored at 4 °C prior to analysis.

2.3. Sample preparation

Different liquid-liquid extraction methods were tested to optimize recovery of EA, UA, and UB from plasma. For the best recovery, frozen plasma samples were thawed on ice for about 3 h. From each sample, 200 μL of plasma was taken and placed in a microcentrifuge tube. Then, 700 μL of chilled acetonitrile (2% formic acid) containing 14.28 ng/mL DHC were added orderly and vortexed for 5 min after adding each solvent. The sample was then sonicated for 10 min and centrifuged for 15 min at 15,294 *g* at 4 °C. 750 μL from the resultant supernatant was collected and evaporated to dryness by SpeedVac for about 12 h. The dried sample was resuspended in 150 μL of methanol (1 % formic acid) which contained chrysin (50 ng/mL) as an internal standard. The resuspended mixture was vortexed for 5 min, sonicated for 10 min and centrifuged at 15,294 *g* for 15 min, then 100 μL of supernatant was transferred into a LC vial for analysis.

2.4. Deconjugation reaction

For deconjugation, plasma (148 μL) was incubated with bovine liver-derived β-glucuronidase (2000 U/mL) and *H. pomatia*-derived sulfatase (2000 U/mL) overnight in acetate buffer (0.2 M, pH 5.0, 37 °C). Negative control samples lacked β-glucuronidase and sulfatase. Samples were then centrifuged (1 min at 4 °C, 15,294 *g*) followed by the addition of chilled acetonitrile (700 μL, 2 % formic acid) containing DHC (10 ng). Samples were then sonicated (10 min) and centrifuged (15 min, 15,294 *g*, 4 °C). The resultant supernatant (750 μL) was evaporated to dryness by SpeedVac (≈12 h).

2.5. UHPLC-MS/MS parameters

The UHPLC-MS/MS system comprised an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a binary solvent manager, sample manager, and heated column compartment, and a 6470 triple quadrupole mass spectrometry detector. The instrument was controlled by Agilent MassHunter software. Chromatographic separation of EA, UA, UB, DHC, and chrysin was carried out using an Agilent ZORBAX Eclipse Plus C18 column (50 mm × 2.1 mm I.D., 1.8 μm), maintained at 40 °C for the column. The mobile phase consisted as A: water containing 0.1 % formic acid and B: acetonitrile with 0.1 % formic acid. The gradient elution was applied for analysis at a flow rate of 0.4 mL/min and programmed as 0–3.0 min, 11 % B to 70 % B; 3.0–4.0 min, 70 % B to 100 % B. The analysis was followed by a one-minute washing procedure with 100 % B and re-equilibration period of 3 min with initial condition. A wash solvent (1:1:1 methanol/isopropanol/water, *v/v/v*) and needle wash (3:1 methanol/water, *v/v*) were used for the autosampler and needle wash. The injection volume was 2 μL.

MS/MS analysis was performed on an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) that was connected to the UHPLC system via an electrospray ionization (ESI) interface. The ESI-MS/MS parameters were set in a negative mode as follows: capillary voltage: 2.5 kV; gas temperature: 325 °C; gas flow: 10 L/min; nebulize: 20 psi; sheath gas temp: 300 °C; sheath gas flow: 11 L/min. Nitrogen was used as the desolvation and cone gas. Nitrogen (99.99% purity) was introduced as the collision gas into the collision cell. The effluent was introduced into the 6470 triple quadrupole mass spectrometer for quantification of the analytes. Detection was obtained by Multiple Reaction Monitoring (MRM) mode including two MRMs for confirmation of the analytes. The quantification of EA, UA, UB, DHC and chrysin were acquired with transitions of key product ions at *m/z* 301→284 (dwell time 5 ms, collision cell accelerator voltage 4 V, and collision energy 34 eV), *m/z* 227→198 (dwell time 5 ms, collision cell accelerator voltage 4 V, and collision energy 38 eV), *m/z* 211→167.1 (dwell time 5 ms, collision cell accelerator voltage 4 V, and collision energy 30 eV), *m/z* 177→133.1 (dwell time 5 ms, collision cell accelerator voltage 4 V, and collision energy 18 eV), and *m/z* 253.1→143.1 (dwell time 5 ms, collision cell accelerator voltage 4 V, and collision energy 38 eV) respectively. Data acquisition was carried out using the Agilent MassHunter version 10.1 (Agilent Technologies, Santa Clara, CA, USA).

2.6. UHPLC-MS/MS method validation

Method validation was performed in accordance with the criteria suggested by the US Food and Drug Administration (FDA) Guidance for Industry – Bioanalytical Method Validation [11]. Method parameters such as specificity, linearity, sensitivity, precision, accuracy, and stability of ellagic acid, urolithins A (UA), and B (UB) were validated in human plasma. Method specificity was evaluated by comparing chromatograms of nine drug-free human blank plasma for interference at the retention times of EA, UA, UB, DHC (IS), and chrysin (IS).

2.6.1. Linearity and sensitivity

Calibration curves in human plasma were constructed by plotting the peak ratios of EA, UA, and UB, respectively, to the IS DHC against the nominal concentrations of the calibration standards at 2.78, 5.6, 11.1, 27.8, 44.4, 55.6, 111.1, 277.8, 444.4, and 555.6 ng/mL for the three analytes. Triplicate measurement of 10 concentrations of EA, UA, and UB were used for the calibration curve construction for every batch of samples. The linear least-squares regression of the calibration lines, slopes, intercepts, and correlation coefficients were obtained from the peak area ratios of EA, UA, and UB to IS DHC versus corresponding concentrations. Unknown sample concentrations of EA, UA, and UB were calculated from the linear regression with a weighting factor of 1/x

for the three analytes. The lower limit of quantification (LLOQ) for all analytes in plasma samples were defined as the lowest concentration with a signal-to-noise ratio of 10, respectively. While the lower limit of detection (LOD) for all analytes were calculated by from the lowest concentration of the calibration curve and it has been expressed as ($3 \times \sigma/S$), where S is the slope of the calibration curve and σ is the standard deviation of the lowest concentrations. For LLOQ, the acceptable accuracies of 80–120 % and sufficient precisions within 20 % were adopted and verified using seven replicate analyses.

2.6.2. Accuracy and precision

Intra-day precision and accuracy were determined by analyzing four different QC samples (27.8, 111.1, 277.8 and 444.4 ng/mL) on the same day. Inter-day precision and accuracy were also evaluated by analyzing four different QC samples on three different days. Each analytical run consisted of a plasma blank, 10 concentrations of calibration standards, and four different QC samples. Precision was expressed as the relative standard deviation (RSD, %) and the accuracy was expressed as [(mean observed concentration)/(nominal concentration)× 100 %]. However, absolute recoveries (n = 3) using QC samples were evaluated by comparison of the response ratio of extracted plasma QC samples with QC samples in acetonitrile.

2.6.3. Specificity, selectivity and matrix effect

The specificity and selectivity of the method were determined by comparing chromatograms of blank samples (solvent control and drug-free plasma sample) and spiked blank (analytes added to drug-free plasma sample). No interfering peaks at the retention times of EA (t_R 2.29 min), UA (t_R 2.87 min), and UB (t_R 3.42 min) were observed in the solvent control blank and drug-free blank samples. To check for matrix effects, we performed a recovery assay by spiking analytes into blank plasma. The spiked plasma was processed and quantified. The recovery of analytes was expressed as the percentage ratio of quantified values to the spiked values. To minimize the matrix effect, plasma spiked with analytes and IS were considered for linearity assay and quantification.

2.6.4. Stability

The stability of EA, UA, and UB in plasma were assessed by analyzing four different concentrations (27.8, 111.1, 277.8 and 444.4 ng/mL) for the three analytes under short-term storage (8 h at 22 ± 5 °C) and long-term storage (72 h at 4 °C and two months at −20 °C). The peak areas of the EA, UA, and UB, and IS DHC obtained from freshly prepared samples were considered as the reference to measure the relative stability at short-term and long-term points.

2.7. Pharmacokinetic study design

The study was conducted at the National Center for Natural Products Research Clinical Center at the University of Mississippi, School of Pharmacy. The Division of Research Integrity and Compliance Institutional Review Board, Office of Research and Sponsored Programs, University of Mississippi (IRB# 21–028) approved the study protocol and informed consent. The study was conducted in accordance with the Declaration of Helsinki and all study participants provided informed consent before study enrollment.

A total of ten healthy subjects (male and female), non-smokers, were enrolled into a two-cohort cross-over pharmacokinetic study. Subjects first received an oral dose of Pomella® Pomegranate extract standardized to 30 % punicalagins (single capsule, 250 mg). After a one-week washout period, subjects received an oral dose of Pomella® Pomegranate extract (four capsules, 1000 mg). Pomella® capsules were supplied by Verdure Sciences, Inc. (Noblesville, IN). After the administration of capsules, blood samples for pharmacokinetic study were collected in 9 mL heparinized Vacutainer® tubes at pre-dose, then at 30 min, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h. The tubes were immediately processed for centrifugation under refrigerated conditions (4 °C) to separate plasma. The plasma samples from individual volunteers were divided into aliquots, and transferred for storage at − 80 °C till further analysis. The aliquots of plasma samples were processed and analyzed using UHPLC-MS/MS for quantification of EA, UA and UB.

2.8. Pharmacokinetic data analysis

Plasma concentration versus time data were used to estimate the maximum concentration (C_{max}) and time corresponding to the C_{max} (T_{max}). The area under the plasma concentration-time curve from time 0 to time (AUC_{0-t}) was determined using the linear trapezoidal rule. For AUC determinations, plasma concentration values less than LLOQ, but greater than the limit of detection, were reported as LLOQ/2.

3. Results and discussion

3.1. Method development and qualification

DHC was selected as the IS due to its resemblance chemical structure, physicochemical property, and mass spectrometric characteristics to those of EA, UA, and UB. The mobile phase composition, retention time, flow rate, and suitable chromatographic column were assessed to optimize the best chromatographic settings. ZORBAX Eclipse Plus C18 column (50 mm × 2.1 mm I.D., 1.8 μm) could achieve the base-line separation for EA (t_R 2.29 min), UA (t_R 2.87 min), and UB (t_R 3.42 min) within a 5 min run time, which provided satisfying results in the shorter run time, the separation and peak shape. An acetonitrile-based mobile phase had lower background noise and system pressure than methanol. The addition of 0.1 % formic acid greatly enhanced the intensities of the peaks. Optimal chromatographic separation was observed with a solvent composition of acetonitrile with 0.1 % formic acid (*v/v*) and water containing 0.1% formic acid as the mobile phase. It was also observed that gradient elution at a flow rate of 0.4 mL/min and eluted as 0–3.0 min, 11 % B to 70 % B; 3.0–4.0 min, 70 % B to 100 % B significantly improved response intensity, resolution, and peak shape.

In order to achieve maximal sensitivity for analytes EA, UA, and UB, and IS DHC, IS chrysin, tandem mass parameters including targeted ion selection, capillary voltage, gas temperature, gas flow, nebulize, sheath gas temp, and sheath gas flow were optimized in ESI negative ionization modes for each analyte using a 1 μg/mL tuning solution in acetonitrile. When gas temperature, sheath gas temperature, and sheath gas flow rate set at 325 °C, 300 °C, and 11 L/min, respectively, the steady product ions of EA was found at *m/z* 284 which further yielded ions at *m/z* 229, the steady product ions of UA was found at *m/z* 198 which further yielded ions at *m/z* 182, the steady product ions of UB was found at *m/z* 167.1 which further yielded ions at *m/z* 139.1 (Fig. 2). Under the optimized condition, deprotonated ions of DHC at *m/z* 143.1 which generated product ions at *m/z* 63.1, whereas deprotonated ions of chrysin at *m/z* 133.1 which generated product ions at *m/z* 105.1. Two different multiple reaction monitoring (MRMs) were chosen to be quantifier and qualifier, respectively, for each analyte. The most intense peaks, which were used for quantification of corresponding analyte, were observed at *m/z* 284 for EA, 198 for UA, 167.1 for UB, 133.1 for IS DHC, and 63.1 for IS chrysin (Fig. 2). The MS parameters of quantifier and qualifier ions such as cone voltage and collision energy for all analytes are listed in Table 1. Sample preparation for bioanalysis was conducted using a simple protein precipitation protocol due to its simplicity and low cost. Method optimization initially focused on the choice of a suitable organic extraction and reconstitution solvent (methanol, acetonitrile, and isopropanol). Acetonitrile containing 2 % formic acid showed best analyte recovery extraction, methanol with 1% formic acid showed best analytes reconstitution after drying the extracted samples, and peak shape (data not shown).

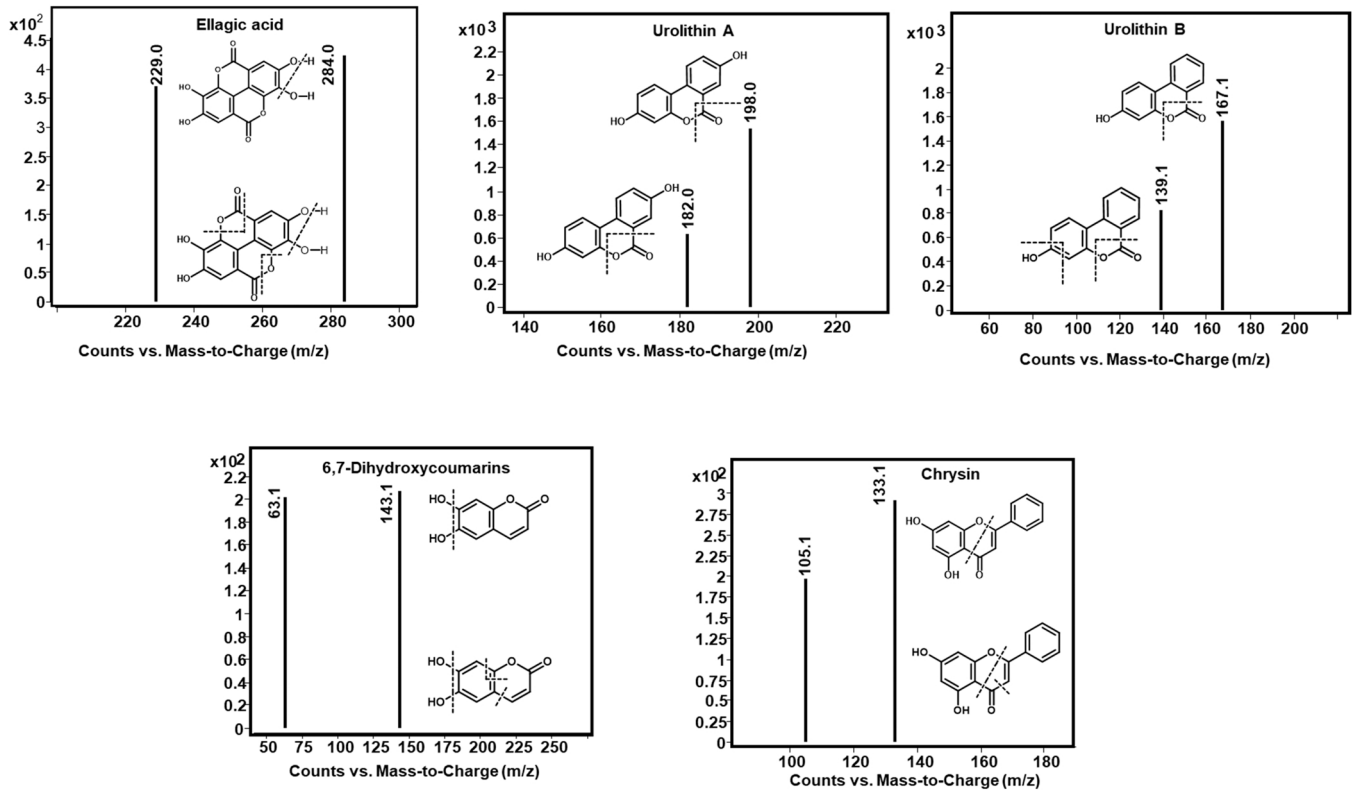

Fig. 2. MS/MS spectra of analytes used as quantifier and qualifier.

Table 1
Settings of MS parameters for the quantification of ellagic acid, urolithin A, and urolithin B.

Analyte	Target Ion (*m/z*)	Quantifier			Qualifier		
		Transition (*m/z*)	Cell ACC (V)	Collision energy (eV)	Transition (*m/z*)	Cell ACC (V)	Collision energy (eV)
Ellagic acid	301	284.0	4.0	34	229.0	4	30
Urolithin A	227	198.0	4.0	38	182.0	4	38
Urolithin B	211	167.1	4.0	30	139.1	4	34

Cell ACC: Collision cell accelerator voltage

3.2. Method validation

Selectivity, linearity, sensitivity, precision, accuracy, recovery, matrix effects and stability were evaluated for validation of developed method.

3.2.1. Selectivity

Assay selectivity was evaluated at the LLOQ using drug-free human plasma samples from different plasma lots. No interfering peaks at the retention times of EA (t_R 2.29 min), UA (t_R 2.87 min), UB (t_R 3.42 min), IS DHC (t_R 1.37 min), and IS chrysin (t_R 3.8 min) were observed in blank plasma. Representative chromatograms of drug-free human plasma spiked with 55.6 ng/mL IS DHC, and 50 ng/mL IS chrysin, a plasma QC sample (27.8 ng/mL EA, UA, and UB with 50 ng/mL IS chrysisn and 55.6 ng/mL IS DHC), and a plasma sample collected at 4 h after administration of Pomella® capsule was shown in Fig. 3.

3.2.2. Linearity and sensitivity

To minimize matrix effects, standard solutions containing IS were spiked into the drug-free human plasma and extracted using the optimized method. Calibration curves of EA, UA, and UB were linear over the concentration range of 2.78–555.6 ng/mL respectively. The coefficient of correlation (r^2) for EA, UA, and UB in human plasma was greater than 0.99 (n = 9). The calibration curves, linear ranges, and sensitivity for EA, UA, and UB are shown in Table 2.

3.2.3. Precision and accuracy

Intra-day precision and accuracy of the developed method were evaluated by analyzing triplicate (n = 3) QC samples at four concentrations (27.8, 111.1, 277.8 and 444.4 ng/mL for three analytes) on the same day (Table 3), while inter-day precision and accuracy of the developed method were evaluated by analyzing QC samples (27.8, 111.1, 277.8 and 444.4 ng/mL for three analytes) on three different days (Table 3). Both intra-day and inter-day precision and accuracy values in plasma were well within the 20 % acceptance range. The relative standard derivation (RSD, %) for intra- and inter-day precision values in plasma were below 10 %, and the intra- and inter-day accuracies values in plasma were 2.8–9.5 % for all analytes.

3.2.4. Stability

Stock solutions of EA, UA, UB, DHC, and chrysin in methanol solvent stored at − 20 °C were fairly stable up to two months without any changes of peak areas and didn't show the appearance of any extra peaks. The stability of short-term storage and post-treatment storage for EA, UA, and UB in human plasma didn't show any major degradation, very minor variations were noted for the test samples, but that was

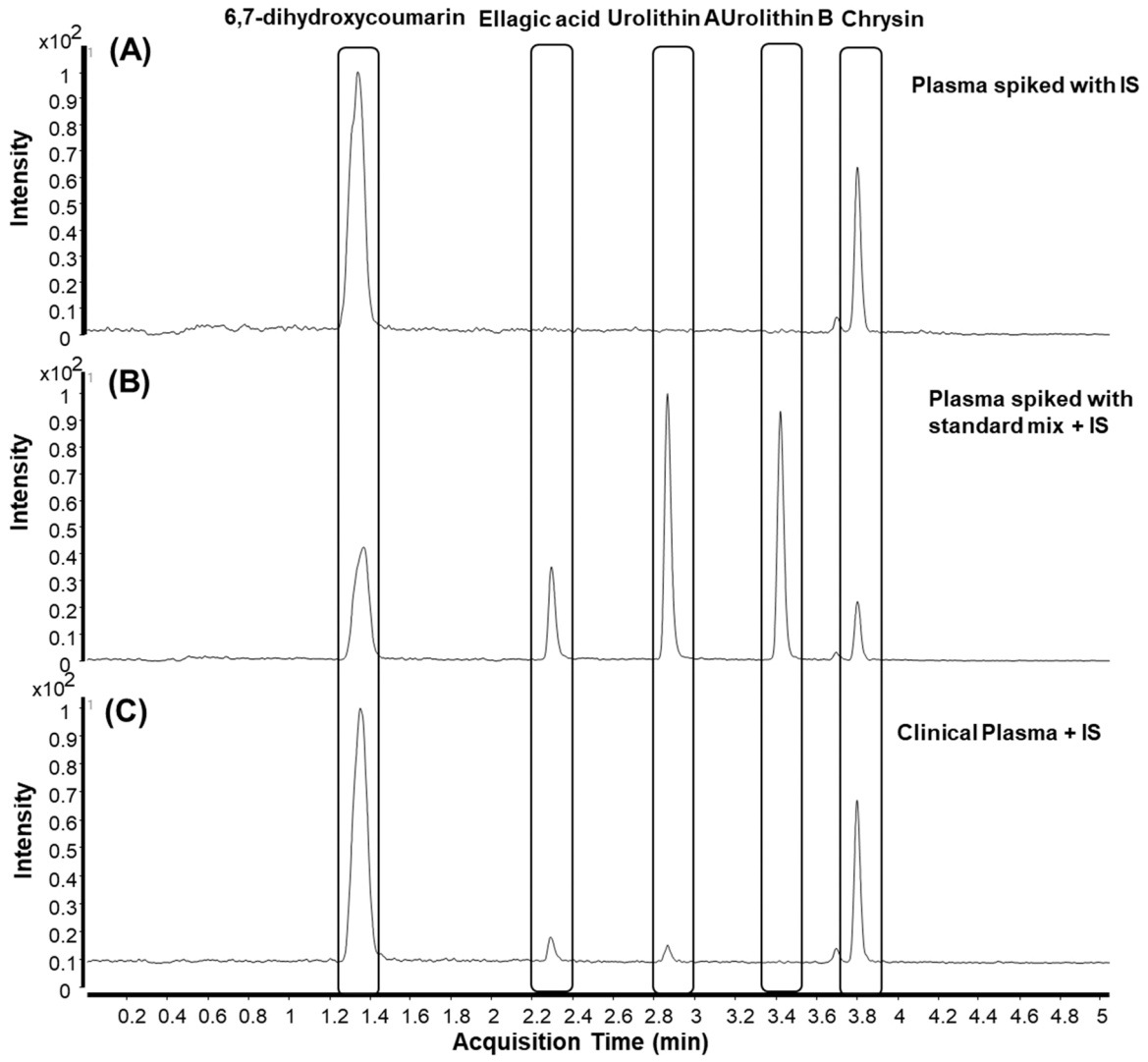

Fig. 3. Representative UHPLC-MS/MS chromatogram of all analytes in (A) blank plasma spiked with IS, (B) blank plasma spiked with standards and IS, and (C) clinical plasma with IS.

Table 2
Calibration curve, coefficient of determination, linearity range, and sensitivity of ellagic acid, urolithin A, and urolithin B.

Analyte	Calibration curve	R2	Linearity range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
Ellagic acid	Y = 0.002067 *X + 0.002177	0.99	5.6–554.8	1.6	5.6
Urolithin A	Y = 0.001661 *X + 0.023506	0.99	2.7–554.8	0.8	2.7
Urolithin B	Y = 0.079158 *X + 2.78376	0.99	2.7–554.8	0.8	2.7

within ± 15 % deviation between the predicted and nominal concentrations (Table 1S).

3.3. Pharmacokinetics

For the purposes of this study, we administered a standardized pomegranate extract (30 % punicalagin) containing 30 % punicalagins and < 5 % of EA per capsule (Pomella®). Importantly, intestinal microbiota are responsible for converting punicalagin to EA, which may in turn be transformed into various urolithin metabolites [3]. Thus, circulating EA and urolithins may derive from punicalagin or EA itself. Eight of ten subjects in both Cohorts I (250 mg) and II (1000 mg) had quantifiable free (unconjugated) EA levels at 1.5 h (C_{max}, 8.1 ± 2.3 ng/mL) and 2.0 h (C_{max}, 7.7 ± 1.1 ng/mL) after extract administration, respectively (Table 4). However, there was no quantifiable free EA in the other two subjects from each cohort. The fact that free EA C_{max} levels did not differ in a dose-dependent manner suggests that other mechanisms, e.g., absorption and/or pre-systemic metabolism, as opposed to either punicalagin or EA dose, determine free plasma EA levels.

In comparison to free EA, β-glucuronidase and sulfatase treatment yielded measurable EA plasma concentrations providing C_{max} (11 ± 2.6 vs 17 ± 2.4 ng/mL) and $AUC_{0\rightarrow t}$ (38 ± 7.8 vs 63 ± 9.0 h x ng/mL) values for Cohort I and II, respectively (Table 4). The preponderance of EA found in the plasma was conjugated implying rapid pre-systemic (intestinal and/or hepatic) metabolism of EA (Fig. 4). In addition, it should be noted that while there was a dose-dependent increase in conjugated EA, the increase was less than linear, i.e., 4-fold increase in punicalagin/EA dose with a corresponding ~2-fold increase in AUC. These data also support the notion that the pharmacologic effect of pomegranate constituents can potentially be attributed to conjugated forms of EA and/or downstream metabolites such as urolithins [3,10].

Next, we sought to determine levels of both free and conjugated

Table 3
Intra-day and Inter-day precision and accuracy of the developed analytical method.

Analyte	Nominal Conc. (ng/mL)	Detected Conc. (ng/mL) (Mean ±SD) Inter-day	RSD (%)	Accuracy (%)	Detected Conc. (ng/mL) (Mean ±SD) Intra-day	RSD (%)	Accuracy (%)
Ellagic acid	LLOQ (5.6 ng/mL)	5.48 ± 0.95	17.44	97.83	5.48 ± 0.51	9.47	97.86
	27.8	29.76 ± 2.85	9.59	107.1	29.77 ± 1.75	5.91	107.07
	55.6	56.71 ± 5.04	8.89	102	56.71 ± 4.09	7.22	102.00
	277.8	304.93 ± 21.07	6.91	109.8	304.93 ± 18.47	6.06	109.77
	444.4	442.51 ± 26.90	6.08	99.6	442.51 ± 12.70	2.87	99.58
Urolithin A	LLOQ (2.78 ng/mL)	2.60 ± 0.35	13.78	93.6	2.60 ± 0.21	8.24	93.53
	27.8	28.62 ± 2.01	7.03	103.0	28.36 ± 1.66	5.86	102.03
	55.6	57.07 ± 3.62	6.36	102.6	55.94 ± 3.92	7.01	100.62
	277.8	302.19 ± 19.76	6.54	108.8	302.19 ± 18.01	5.96	108.78
	444.4	443.36 ± 31.25	7.05	99.7	443.36 ± 16.22	3.66	99.77
Urolithin B	LLOQ (2.78 ng/mL)	2.80 ± 0.54	19.42	100.84	2.87 ± 0.21	7.47	100.84
	27.8	28.78 ± 1.95	6.79	103.5	28.78 ± 1.16	4.04	103.53
	55.6	57.39 ± 3.47	6.06	103.2	57.39 ± 2.72	4.75	103.23
	277.8	311.66 ± 21.41	6.87	112.2	311.66 ± 13.33	4.28	112.19
	444.4	458.1 ± 32.98	7.2	100.84	458.10 ± 12.73	2.78	103.08

Table 4
Plasma exposure parameters for ellagic acid and Urolithin A were determined after a single oral administration of Pomella® capsules for both Cohort I (250 mg of pomegranate extract) and Cohort II (1000 mg of pomegranate extract).

PK parameters	Cohort I		Cohort II	
	Free	Conjugated	Free	Conjugated
Ellagic acid				
C_{max} (ng/mL)	8.1 ± 2.3	11 ± 2.6	7.7 ± 1.1	17 ± 2.4
T_{max} (h)	1.5 ± 0.2	1.7 ± 0.3	2.0 ± 0.3	2.0 ± 0.3
AUC (h × ng/mL)	-	38 ± 7.8	-	63 ± 9.0
Urolithin A				
C_{max} (ng/mL)	-	35.3 ± 17.8	-	77.8 ± 28.1
T_{max} (h)	-	24.0 ± 0.0	-	31 ± 4.4
AUC (h × ng/mL)	-	848.5 ± 427.9	-	1738 ± 584.9

Data are expressed as Mean ± SEM (N = 8); AUC values represent AUC0→t

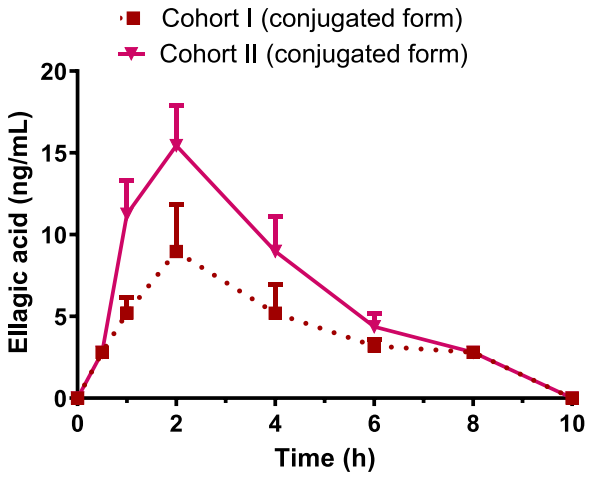

Fig. 4. Mean concentration of free and conjugated ellagic acid in single oral administration of pomegranate capsules for Cohort I (250 mg of pomegranate extract) and Cohort II (1000 mg of pomegranate extract). Data are expressed as mean with SEM (N = 8).

urolithins, specifically UA and UB, to determine the contribution of urolithins to the overall plasma exposure profile of Pomella®. Interrogation of plasma for free UA and UB up to 48 h after administration of Pomella® yielded no results. In contrast, after β-glucuronidase and sulfatase treatment, we were able to readily detect UA with C_{max} (35.3 ± 17.8 vs 77.8 ± 28.1 ng/mL) and $AUC_{0\rightarrow t}$ (848.5 ± 427.9 vs 1738 ± 584.9 h x ng/mL) values for Cohort I and II, respectively (Table 4). We were unable, however, to detect UB after deconjugation in either cohort. This result is not surprising as UB is found infrequently in generally healthy populations such as ours [10]. Moreover, appearance of UA conjugates after approximately eight hours is consistent with gut microbiota-mediated transformation of EA to UA and subsequent pre-systemic conjugation (Fig. 5).

The entirety of the UA plasma exposure in our study was attributed to the conjugated form. These data are consistent with Singh et al. who reported similar findings in humans following administration of pomegranate juice [12]. Interestingly, however, Singh et al. reported only 40 % of their subject population was able to produce UA while we detected UA formation in eight of ten (80 %) subjects. Colonic microbiota are primarily responsible for UA formation, which means UA conjugate appearance in the plasma is delayed relative to administration and is characterized by high inter-individual variability. Consequently, the large difference in UA producers between studies could simply reflect the fact that we measured UA levels up to 48 h after Pomella® administration whereas Singh et al. measured UA levels out to 24 h. Importantly, extending the sampling time out to 48 h allowed us to estimate the plasma half-life (≈24 h, Fig. 5) of UA conjugates for the first time in humans.

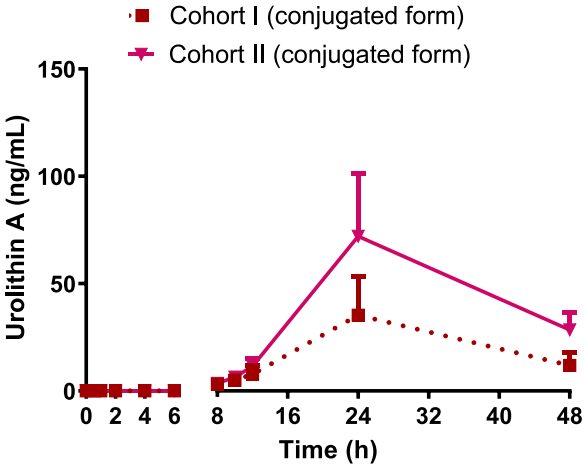

Fig. 5. Mean concentration of conjugated Urolithin A in plasma in single oral administration of pomegranate capsules for Cohort I (250 mg of pomegranate extract) and Cohort II (1000 mg of pomegranate extract). Data are expressed as mean with SEM (N = 8).

4. Conclusions

Pomegranate polyphenols including punicalagins and EA undergo extensive pre-systemic metabolism mediated by intestinal microbiota and hepatic Phase II metabolizing enzymes. As a result, conjugated urolithin forms (sulfate and glucuronide) dominate the pomegranate polyphenol plasma exposure following oral administration of pomegranate juice or extracts. In this study we applied UHPLC-MS/MS based quantification to characterize EA and urolithin exposure following oral administration of Pomella®, a unique pomegranate extract standardized to 30 % punicalagins. Key findings from our study include: 1) conjugated UA represented the major contributor to pomegranate polyphenol plasma exposure with minor contribution from conjugated EA, 2) eight of ten healthy subjects were determined to be Metabotype A, i.e., producers of UA, however, none were Metabotype B, i.e., producers of UB, and 3) UA conjugates were detectable up to 48 h post-administration and displayed a half-life of ≈ 24 h.

Funding

Funding for this work was provided by Verdure Sciences, Inc. and The National Center for Natural Products Research.

CRediT authorship contribution statement

Yan-Hong Wang: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review and Editing. **Goutam Mondal**: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review and Editing. **Washim Kim**: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review and Editing. **Bill Gurley**: Conceptualization, Writing – Review and Editing. **Charles R Yates**: Conceptualization, Formal Analysis, Writing – Original Draft, Writing – Review and Editing; Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Charles Ryan Yates reports financial support was provided by Verdure Sciences.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115477.

References

[1] Y. Liu, N.P. Seeram, Liquid chromatography coupled with time-of-flight tandem mass spectrometry for comprehensive phenolic characterization of pomegranate fruit and flower extracts used as ingredients in botanical dietary supplements, J. Sep. Sci. 41 (2018) 3022–3033, https://doi.org/10.1002/jssc.201800480. Liquid.

[2] R. García-Villalba, H. Vissenaekens, J. Pitart, M. Romo-Vaquero, J.C. Espín, C. Grootaert, M.V. Selma, K. Raes, G. Smagghe, S. Possemiers, J. Van Camp, F. A. Tomas-Barberan, Gastrointestinal simulation model TWIN-SHIME shows differences between human urolithin-metabotypes in gut microbiota composition, pomegranate polyphenol metabolism, and transport along the intestinal tract, J. Agric. Food Chem. 65 (2017) 5480–5493, https://doi.org/10.1021/acs.jafc.7b02049.

[3] R. García-Villalba, J.A. Giménez-Bastida, M.A. Ávila-Gálvez, F.A. Tomás-Barberán, J.C. Espín, A. González-Sarrías, Ellagitannins and their gut microbiota-derived metabolites: urolithins, in: Diet. Polyphenols, John Wiley & Sons, Ltd, 2020, pp. 319–364, https://doi.org/10.1002/9781119563754.ch9.

[4] N.P. Seeram, R. Lee, D. Heber, Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice, Clin. Chim. Acta 348 (2004) 63–68, https://doi.org/10.1016/J.CCCN.2004.04.029.

[5] B. Cerdá, C. Soto, M.D. Albaladejo, P. Martínez, F. Sánchez-Gascón, F. Tomás-Barberán, J.C. Espín, Pomegranate juice supplementation in chronic obstructive pulmonary disease: a 5-week randomized, double-blind, placebo-controlled trial, Eur. J. Clin. Nutr. 60 (2006) 245–253, https://doi.org/10.1038/sj.ejcn.1602309.

[6] A. González-Sarrías, R. García-Villalba, M.Á. Núñez-Sánchez, J. Tomé-Carneiro, P. Zafrilla, J. Mulero, F.A. Tomás-Barberán, J.C. Espín, Identifying the limits for ellagic acid bioavailability: a crossover pharmacokinetic study in healthy volunteers after consumption of pomegranate extracts, J. Funct. Foods 19 (2015) 225–235, https://doi.org/10.1016/j.jff.2015.09.019.

[7] M.V. Selma, F.A. Tomás-Barberán, D. Beltrán, R. García-Villalba, J.C. Espín, Gordonibacter urolithinfaciens sp. nov., a urolithin-producing bacterium isolated from the human gut, Int. J. Syst. Evol. Microbiol. 64 (2014) 2346–2352, https://doi.org/10.1099/ijs.0.055095-0.

[8] M.V. Selma, D. Beltrán, R. García-Villalba, J.C. Espín, F.A. Tomás-Barberán, Description of urolithin production capacity from ellagic acid of two human intestinal Gordonibacter species, Food Funct. 5 (2014) 1779–1784, https://doi.org/10.1039/C4FO00092G.

[9] S.U. Mertens-Talcott, P. Jilma-Stohlawetz, J. Rios, L. Hingorani, H. Derendorf, Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers, J. Agric. Food Chem. 54 (2006) 8956–8961, https://doi.org/10.1021/jf061674h.

[10] F.A. Tomás-Barberán, A. González-Sarrías, R. García-Villalba, M.A. Núñez-Sánchez, M.V. Selma, M.T. García-Conesa, J.C. Espín, 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. 61 (2017), 1500901, https://doi.org/10.1002/mnfr.201500901.

[11] USFDA, Office of Clinical Pharmacology in the Center for Drug Evaluation and Research and the Center for Veterinary Medicine at the Food and Drug Administration: Bioanalytical method validation: Guidance for Industry, 2018. https://doi.org/10.5958/2231–5675.2015.00035.6.

[12] A. Singh, D. D'Amico, P.A. Andreux, G. Dunngalvin, T. Kern, W. Blanco-Bose, J. Auwerx, P. Aebischer, C. Rinsch, Direct supplementation with Urolithin A overcomes limitations of dietary exposure and gut microbiome variability in healthy adults to achieve consistent levels across the population, Eur. J. Clin. Nutr. 76 (2022) 297–308, https://doi.org/10.1038/s41430-021-00950-1.