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Chromatographic and spectroscopic characterization of urolithins for their determination in biological samples after the intake of foods containing ellagitannins and ellagic acid

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ABSTRACT

Ellagitannins and ellagic acid (EA) are metabolized by the gut microbiota to produce urolithins that could be responsible for the health effects attributed to ellagitannin-containing food products. Several urolithin aglycones could be present in fecal samples while glucuronide and sulphate conjugates are mainly found in plasma and urine. So far, the lack of available standards has made difficult their correct identification and quantification. In the present study, UV and MS spectra characteristics of urolithins and their phase II metabolites have been determined using different systems based on liquid chromatography (LC) coupled with diode-array or mass spectrometer detectors with different analyzers (triple quadrupole (QqQ) and quadrupole time-of-flight (QTOF)). Chromatographic separation was achieved on a reversed-phase Poroshell C18 column (3 × 100 mm, 2.7 μm). Elution order, characteristic UV spectra, and relative response factors (RRFs) with respect to their parental compound (EA) and the most common metabolite urolithin A (Uro-A) were determined. This contribution, along with the most important mass spectra characteristics (MRM transitions, qualifier/quantifier ratio, accurate mass and fragmentation pattern) will allow the determination of urolithin metabolites in different biological samples and their quantification even if not all metabolites are commercially available. The methods developed in the three systems have been fully validated in terms of linearity, sensitivity, precision, recovery, matrix effect, selectivity and stability. After that, they were successfully applied to complex biological matrices (urine, feces and plasma) from two human studies in which volunteers consumed ellagitannin-containing foods, such as walnuts and pomegranate extracts.

1. Introduction

Urolithins constitute a family of metabolites produced from ellagic acid (EA) and ellagitannins (ETs) by gut microbiota [1,2]. ETs are often found in different food products including berries (strawberries, raspberries, blackberries), pomegranate, tropical fruits (camu-camu), nuts (walnuts, chestnuts, almonds, oak acorns, pistachios, pecans), oak barrel aged wines and spirits, and tea [3,4]. It seems that ETs release EA, which is then transformed into a polyhydroxylated dibenzopyranone, the 3,4,8,9,10-pentahydroxy-6H-dibenzo[*b,d*]pyran-6-one (Uro-M5) and then undergoes sequential dehydroxylation to tetrahydroxy- (Uro-M6 and Uro-D), trihydroxy- (Uro-M7 and Uro-C), dihydroxy- (Uro-A and IsoUro-A) and monohydroxy- (Uro-B) dibenzopyranones [1,2,5]. Once produced these catabolic metabolites can be absorbed, circulate in plasma and accumulate in urine as glucuronide and sulphate conjugates or can be directly excreted in feces as aglycones [6,7]. Their absorption and metabolism have been investigated both in animal models [6,8,9] and humans [10–12] following the intake of different ellagitannin-rich foods. They have also been found in human colonic tissues [13] and reach systemic organs such as the human prostate [14]. During the past few years, urolithins have received special attention because of their potential health promoting effects [1]. Different *in vitro* and preclinical studies have reported their anti-inflammatory and cancer chemopreventive activities [15–17]. The study of the nature of the metabolites and their concentration in different biological compartments is necessary to understand the health effects of ellagitannin-containing foods. Therefore, the development of sensitive and accurate methods for the determination of urolithins has become a critical factor.

Several methodologies have been described in literature to identify and quantify urolithins in biological samples, mainly based

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in the coupling of liquid chromatography to UV and MS detection. Unfortunately, due to the limited availability of the majority of urolithin metabolites these methodologies have not been fully validated and have mainly focused in the most common metabolites (Uro-A and Uro-B). Considering the characteristic UV spectra of these compounds [8], most authors use UV chromatograms for their identification, as glucuronides in plasma and urine [11,18–21] and as aglycones in feces and bacterial cultures [2,22,23]. Indirect quantification has been often applied in different studies in which it was assumed that the UV response for the conjugates was the same as that of the commercially available metabolites Uro-A and Uro-B [11,18,20,22,24,25]. Enzymatic hydrolysis of glucuronide and sulphate conjugates has also been used for quantification, although in this case relevant information about the naturally occurring metabolites is missing [12,19]. Some authors have described the presence of other intermediate catabolic compounds (Uro-M5, Uro-M6, Uro-M7, Uro-C, Uro-D) and their conjugates with the help of mass spectrometers [6,8,26,27]. However, information in literature on the MS characteristics of these metabolites is scarce, and only qualitative studies were carried out and relative quantitations assuming the same MS response for the unavailable metabolites and the commercial standards led to inaccurate results. The presence of several isomeric forms of Uro-A and Uro-A glucuronide (Uro-A glur) has also been described [8,18,22]. Unfortunately, the determination of the substitution position was not possible solely based on UV and MS data without the appropriate standards. In general, authentic standards are costly and their synthesis is time-consuming. Therefore, most of urolithin metabolites are actually not well characterized and this hampers their identification and correct quantitation.

The aim and novelty of the present study was to establish the most important LC, UV and MS features of a wide variety of urolithins, using synthesized and purified standards, so they could be used in future works where no commercial standards are available. For this purpose, three analytical solutions based on liquid chromatography coupled to different detectors: DAD and two mass spectrometers (QqQ and QTOF) were used. The methods were fully validated and successfully applied to determine urolithins in biological samples after the intake of different ellagitannin-containing foods.

2. Materials and methods

2.1. Chemical and reagents

Standards of urolithins, urolithin A 3-glucuronide (Uro-A 3-glur) (1), urolithin A 8-glucuronide (Uro-A 8-glur) (2), isourolithin A 9-glucuronide (IsoUro-A 9-glur) (3), isourolithin A 3-glucuronide (IsoUro-A 3-glur) (5), urolithin B glucuronide (Uro-B glur) (10), urolithin M6 (Uro-M6) (6), urolithin M7 (Uro-M7) (9), isourolithin A (IsoUro-A) (11), urolithin A (Uro-A) (13), and urolithin B (Uro-B) (14) were chemically synthetized and purified by Villapharma Research S.L. (Parque Tecnológico de Fuente Alamo, Murcia, Spain). Urolithin D (Uro-D) (4) and urolithin C (Uro-C) (8) were purchased from Dalton Pharma Services (Toronto, Canada). Urolithin A sulphate (Uro-A sulphate) (7) and urolithin B sulphate (Uro-B sulphate) (12) were obtained as described elsewhere [28]. The structures and purity of all compounds were determined by spectroscopic methods (NMR). Fig. 1 shows the chemical structures of the compounds studied. Stock solutions of individual standards were prepared by dissolving each compound in dimethyl sulphoxide (DMSO) to a final concentration of 10 mM. Standard stock mixtures were prepared every six months at a concentration of 200 μM dissolved in methanol and working solutions were prepared by appropriate dilutions of the stock solutions with methanol. Internal standards, 6,7-dihydroxycoumarin (DHC) and chrysin, were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in methanol to a final concentration of 2000 ppm (11.2 mM). All solutions were stored at −20 °C.

Methanol "MeOH" and acetonitrile were purchased from J. T. Baker (Deventer, The Netherlands). Formic acid and acetic acid were from Panreac (Barcelona, Spain). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment. All chemicals and reagents were of analytical grade.

2.2. Samples

Urine, feces and plasma samples were obtained from two pilot human studies after consumption of different ellagitannin-containing foods. Studies were approved by the CSIC Ethics Committee (Madrid, Spain) and all volunteers gave their written informed consent.

In the first study, 10 healthy volunteers aged between 21 and 55 years consumed 30 g of peeled walnuts per day for 3 days (5.1 mg/g free EA after hydrolysis). After the last intake, 24 h urine samples and feces were collected and stored at −20 °C.

In the second study, 10 healthy volunteers aged between 18 and 23 years ingested four capsules of pomegranate extract in a single intake (450 mg/g capsule; 122 mg/g free EA after hydrolysis). Plasma samples were collected 24 h after the intake and were stored at −20 °C.

Samples from volunteers consuming their usual diet but avoiding ellagitannin-containing food products were used as blank sample for validation studies.

2.3. Sample extraction

Urine samples were thawed, vortexed for 30s, centrifuged at 14,000 × *g* (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany) for 10 min and filtered through a 0.22 μm PVDF filter (Millipore). Samples were diluted 1:2 with water containing 0.1% formic acid before analysis by HPLC-DAD or 1:5 before analysis by UPLC-QqQ and UPLC-QTOF.

Feces samples (1 g) were defrosted and homogenized with 10 mL of MeOH/H_2O (80:20) with 0.1% HCl using an Ultra-Turrax for 1 min at 24,000 rpm. The mixture was centrifuged at 4000 × *g* for 10 min at room temperature and the supernatant filtered through a 0.22 μm PVDF filter. Samples were diluted 1:5 in methanol before analysis by UPLC-QqQ and UPLC-QTOF.

Plasma samples (200 μL) were thawed and extracted with 600 μL acetonitrile:formic acid (98:2, v/v) by vortexing for 2 min and ultrasonic bath for 10 min. The mixture was centrifuged at 14,000 × *g* for 10 min and the supernatant was reduced to dryness in the speed vacuum concentrator (Savant SPD121P, ThermoScientific, Alcobendas, Spain). The dried samples were re-suspended in 100 μL of MeOH and filtered through a 0.22 μm PVDF filter before analysis.

Two internal standards (DHC and chrysin) were added to each sample. DHC was added before sample preparation to control the extraction efficiency and chrysin was added immediately prior to analysis to monitor variability into analytical instrument (injection, ionization, detection, etc.). Final concentrations of both internal standards were 1 ppm when samples were analyzed by HPLC-DAD and 0.1 ppm when QqQ or QTOF were used as detectors.

2.4. Analysis by liquid chromatography coupled to photodiode array and mass spectrometry detectors

Three different analytical platforms have been used for the complete characterization of urolithins. In general, they consisted of a reversed phase liquid chromatography system coupled on line to a photodiode array and/or a mass spectrometer detector