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ORIGINAL CONTRIBUTION

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The potent *in vitro* antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans

Summary *Background* The antiatherogenic activity of pomegranate juice has been attributed to its antioxidant polyphenols. The most potent *in vitro* antioxidant polyphenol from this juice is the ellagitannin punicalagin. However, the bioavailability of ellagitannins, including punicalagin, has not been previously described in humans. *Aim of the study* The present work aims to evaluate, in healthy humans, the bioavailability and metabolism of pomegranate juice ellagitannins, to assess their effect on several blood parameters (including cardiovascular risk disease markers) and to compare the antioxidant activity of punicalagin with that of the *in vivo* generated metabolites. *Design* Six healthy subjects (four men and two women) consumed 1 L of pomegranate juice daily (5.58 g/L polyphenols, including 4.37 g/L punicalagin isomers) for 5 days. The polyphenols and the *in vivo* generated metabolites were measured by HPLC-DAD-MS-MS. Fourteen haematological and twenty serobiochemical parameters including LDL, HDL and VLDL as well as cholesterol and triglycerides in each lipoprotein were evaluated. *In vitro* antioxidant activity of plasma (ABTS and FRAP assays) and urine (ABTS and DPPH) were determined. *Results* Neither punicalagin nor ellagic acid present in the juice were detected in both plasma and urine. Three microbial ellagitannin-derived metabolites were detected: 3,8-dihydroxy-6H-dibenzo[*b,d*] pyran-6-one glucuronide, an unidentified aglycone (tentatively, trihydroxy-6H-dibenzo[*b,d*]pyran-6-one) and hydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide. These metabolites could reach up to 18.6 μM in plasma, although a large inter-individual variability was observed. In urine, the same metabolites and their corresponding aglycones became evident after 1 day of juice consumption. Total urine excretion of metabolites ranged from 0.7 to 52.7 % regarding the ingested punicalagin. No relevant effect was observed on any blood parameter. The metabolites did not show significant antioxidant activity compared to punicalagin from pomegranate juice. *Conclusion* The potential systemic biological effects of pomegranate juice ingestion should be attributed to the colonic microflora metabolites rather than to the polyphenols present in the juice.

Key words bioavailability – antioxidant – metabolism – hydroxy-6H-dibenzo [*b,d*] pyran-6-one – ellagitannin – polyphenol – punicalagin – microbial metabolites – pomegranate juice – *Punica granatum*

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Introduction

Pomegranate (*Punica granatum*, L.) is a fruit mainly cultivated in areas of the Near East, India, Spain (South-East), Israel and USA (California). It is consumed fresh or processed to obtain juice. Pomegranate and derived products such as juice have been acknowledged with health-beneficial effects from ancient times [1]. In fact, nowadays, there is a huge number of pomegranate-based patents which claim for the health-promoting properties of this fruit.

Pomegranate juice is very rich in polyphenols including mainly ellagitannins, gallotannins, and anthocyanins [2]. The anthocyanins include delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides. Among the ellagitannins, punicalagin isomers are the main compounds. Punicalagin is a water-soluble ellagitannin that renders one molecule of gallagic acid, one molecule of ellagic acid and one molecule of glucose. The punicalagin isomers have been reported to be mainly responsible for the high *in vitro* antioxidant capacity of pomegranate juice [2]. These compounds impart the characteristic yellow color of pomegranate husk, and are extracted with the juice during processing [2].

Pomegranate juice has been reported to be antiatherogenic by decreasing the serum angiotensin converting enzyme activity [3] and by reducing the oxidation rate of low-density lipoproteins *in vitro* [4].

Very little is known about the bioavailability of food ellagic acid derivatives and ellagitannins [5, 6]. The metabolic transformation of ellagic acid by the colonic microflora into the bioavailable metabolite 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one has been previously reported in rats [7]. However, other studies on the disposition of ellagic acid in mice reported the lack of these microflora metabolites, being excreted as ellagic acid derivatives in urine instead [8, 9]. In a recent paper, we have reported for the first time the fate of ellagitannins from pomegranate in rats [10]. In this study, a number of colonic microflora metabolites, ellagic acid derivatives and ellagitannins were detected in rat urine, plasma and faeces. However, to our knowledge, nothing is known on the metabolism and bioavailability of ellagitannins in humans [5].

The major purpose of the present work is to study for the first time the bioavailability and metabolism of pomegranate juice ellagitannins in healthy humans. In addition, the antioxidant activity of punicalagin from the juice will be compared with that of the *in vivo* generated metabolites. Moreover, several biochemical and haematological parameters, including cardiovascular risk disease markers, will be also evaluated upon the daily ingestion of 1 L of pomegranate juice for 5 days in healthy humans.

Subjects and methods

Chemicals

Pure punicalagin standard was obtained from pomegranate peel according to the protocol of Cerdá et al. [10]. Ellagic acid, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), 2,4,6-tripyridyl-*s*-triazine (TPTZ), β-glucuronidase (EC 3.2.1.31; 100,000 units) from bovine liver and sulfatase (EC 3.1.6.1; 10,000 units) from *Helix pomatia* were purchased from Sigma (St Louis, USA). Methanol, diethyl ether, hydrochloric acid, acetic acid, hexane, and ethyl acetate were obtained from Merck (Darmstadt, Germany). Ascorbic acid was obtained from Aldrich (Steinheim, Germany). Amberlite® XAD-2 was obtained from Supelco (Bellefonte, USA). Silica gel 60 F_{254} TLC aluminum sheets (20×20 cm sheets, thickness 0.2 mm) were obtained from Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., USA) ultra pure water was used throughout this experiment.

Subjects

Six healthy volunteers (four men and two women) with a mean age of 27 years (25–28 years old) and a mean body mass index (BMI) of 21.2 ± 2.5 kg/m^2 (17.6–25.0) were recruited for the study. The volunteers were non-smokers, no vegetarians, without history of gastrointestinal disease or any chronic disease and not involved in a weight-reducing dietary regimen. The ingestion of either dietary supplement or medication was forbidden. Female volunteers were neither pregnant nor lactating.

Ethical permission was obtained from the "Virgen de La Arrixaca" Hospital Committee of Clinical Investigation. The protocol study was fully explained to the volunteers who gave their written consent prior to participation.

Study design

The complete experiment lasted 13 days. Fig. 1 shows schematically the study design. During the 13-day period of the experiment, the volunteers followed a controlled diet in which ellagitannin-containing sources such as berries (strawberry, raspberry, blackberry, etc., and derived foodstuffs such as jams), pomegranates, chocolate, nuts, and wine were strictly forbidden. The intake of the rest of polyphenol-containing sources (fruits, vegetables, tea, coffee, juices and olive oil) was also restricted. The diet was the same during the "control days" and the "study days".

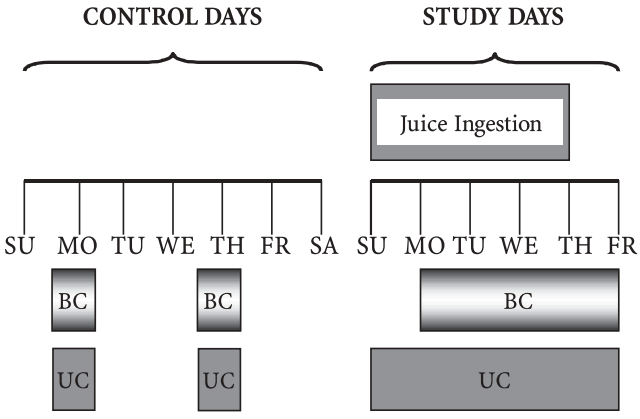

Fig. 1 Scheme of the study design. BC, blood collection. UC, urine collection

Collection of blood was carried out in the "Virgen de La Arrixaca" Hospital of Murcia (two separate extractions during the control week and daily blood collection during the study days; Fig. 1). Blood extraction was carried out in the morning before breakfast. Urine samples of 24 h were collected in plastic bottles containing 1 g/L ascorbic acid. Urine collection was carried out during 2 days in the control week (Fig. 1) and every day during the study days, starting after the first ingestion of juice until the next morning, prior to the corresponding blood extraction (Fig. 1). Urine samples were stored at –70 °C until the analysis.

During the study days, the volunteers ingested 1 litre of pomegranate juice distributed in five doses of 200 mL each throughout the day, starting after the blood samples were taken. The juice was supplied to the volunteers every day. The volunteers kept the juice refrigerated at home during the day to diminish polyphenol degradation.

Pomegranate juice preparation

Pomegranate juice was obtained from fresh pomegranates (*Punica granatum*, cv Mollar de Albatera) by using a laboratory pilot press (ZumoNat C-40, Barcelona, Spain). The juice was homogenised and bottled quickly at 4 °C, and then stored at –20 °C until consumed by the volunteers. The juice was filtered through a 0.45 µm membrane filter Millex-HV_{13} (Millipore Corporation, USA) and samples of 20 µL were analysed by HPLC-DAD-MS-MS.

Plasma samples

Blood was collected in heparinised tubes and immediately centrifuged in a Sigma 1–13 microcentrifuge (Braun Biotech. International, Germany) at 14,000 g for 15 min at 4 °C to obtain the plasma. The plasma (containing acetic acid 10 mM to prevent degradation of polyphenol-related metabolites) was stored at –70 °C until the analyses. The plasma was homogenised with MeOH:0.2 M HCl (1:1, v:v). The mixture was vortexed for 30 s and centrifuged at 14,000 g for 2 min at 4 °C. The supernatant was filtered through a 0.45 µm filter, and directly analysed by HPLC-DAD-MS-MS.

For the enzymatic treatment, the plasma was incubated for 18 h at 25 °C in the presence of 2440 units of β-glucuronidase and 20 units of sulfatase. Incubation conditions were as described elsewhere [10]. Afterwards, the samples were homogenised with MeOH:0.2 M HCl (1:1, v:v), vortexed for 30 s and centrifuged at 14,000 g for 2 min at 4 °C. The supernatant was filtered through a 0.45 µm filter, and analysed by HPLC-DAD-MS-MS.

For the antioxidant assays (ABTS and FRAP assays), plasma was treated with methanol (MeOH) (1 plasma:4 MeOH, v:v), centrifuged and the supernatant assayed.

Urine samples

Untreated urine samples were centrifuged at 14,000 g for 4 min at 4 °C, and the supernatant was filtered with a 0.45µm filter, and directly analysed by HPLC-DAD-MS-MS.

For the antioxidant assays (DPPH and ABTS assays), urine was treated with MeOH (1 urine:4 MeOH, v:v).

Purification of metabolites from urine

Two litres of urine were mixed with the resin Amberlite® XAD-2 and stirred at room temperature for 12 h to retain phenolic compounds [11]. The resin was packed into a 55 x 4 cm column and washed with 4.5 L of distilled water. Adsorbed phenolics were eluted with 1.5 L of MeOH. Methanol was removed under reduced pressure at 40 °C and the remaining aqueous fraction was further extracted with diethyl ether three times. The organic phases were pooled and evaporated until dryness and the pellet re-dissolved in MeOH. The methanol fraction was applied on silica gel 60 F_{254} TLC aluminium sheets at one of the extremes to run the thin layer chromatography (TLC). A mixture of hexane-ethyl acetate [1:1] was used as the mobile phase. The detection was carried out under UV light at 254 nm and 360 nm. The bands were scraped, suspended in MeOH, stirred for 5 min, and centrifuged at 14,000 g for 4 min at 4 °C. The supernatant corresponding to each band was filtered through a 0.45 µm filter, and analysed by HPLC-DAD-MS-MS.

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^{1}H NMR assays

The ^{1}H NMR spectra were recorded with a Bruker 350 MHz instrument (Bruker Biospin Corp., MA, USA) with the isolated metabolites dissolved in deuterated methanol and using tetramethyl sylane as an internal standard.

Antioxidant assays

Antioxidant activity (ABTS, DPPH and FRAP) was determined before ("control days") and after ("study days") pomegranate juice intake. In addition, the most potent antioxidant polyphenol from pomegranate juice, punicalagin, as well as the main *in vivo* generated metabolite 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one were also assayed. The diet ingested by volunteers was the same during the "control days" and "study days", so that the possible effect of the diet on antioxidant activity was carefully considered.

DPPH$^\cdot$ assay

Free radical scavenging activity using the free radical DPPH$^\cdot$ dissolved in methanol was evaluated by measuring the decrease in absorbance at 515 nm in a UV-1603 Shimadzu spectrophotometer (Tokyo, Japan) after 1 h of reaction in parafilm®-sealed glass cuvettes at 25 °C [12]. This assay was carried out to determine the antioxidant activity of urine, punicalagin (0.18 mM in methanol) and the metabolite 3,8-dihydroxy-6H-dibenzo[*b,d*] pyran-6-one (3.5 mM dissolved in dimethylsulfoxide). The samples volume assayed was 10 µL to avoid dilution in the cuvette (final volume of 1 mL). No effect of dimethylsulfoxide on DPPH$^\cdot$ was detected. No turbidity was observed. Antioxidant activity was expressed in µM as the Trolox Equivalent Antioxidant Capacity (TEAC), following the nomenclature of Rice-Evans and Miller [13]. DPPH$^\cdot$ assay was repeated three times. Coefficient of variation was always less than 5 %.

ABTS$^{\cdot+}$ assay

Aqueous solution of the radical cation ABTS$^{\cdot+}$ was chemically obtained with MnO_2, from ABTS, as described elsewhere [14]. Antioxidant activity of urine, plasma, punicalagin (92 mM dissolved in water) and 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (0.4 mM dissolved in methanol) was evaluated by measuring the disappearance of absorbance at 414 nm after 1 h of reaction at 25 °C [14]. No turbidity was observed. No effect of methanol on ABTS$^{\cdot+}$ was detected. The sample volume assayed of stock solutions was 10 µL (final volume of 1 mL). Antioxidant activity was expressed as in the DPPH$^\cdot$ assay. ABTS$^{\cdot+}$ assay was repeated three times. Coefficient of variation was always less than 5 %.

FRAP assay

The "Ferric Reduction Ability Power" (FRAP) assay was performed according to Llorach et al. [15]. The freshly made up FRAP solution contained 25 mL of 0.3 M acetate buffer (pH 3.6) plus 2.5 mL of 10 mM TPTZ solution in 40 mM HCl (previously prepared) and 2.5 mL of 20 mM ferric chloride. This solution was used as a blank. A 970 µL volume of warmed (37 °C) FRAP solution was mixed with 30 µL of plasma (treated as described above). The ferric reducing ability of plasma was measured by monitoring the increase of absorbance at 593 nm for 60 min.

HPLC-DAD-MS-MS

Chromatographic separations of pomegranate juice, plasma and urine were carried out on a reverse phase C_{18} LiChroCART column (25 x 0.4 cm, particle size 5 µm, Merck) using water/formic acid (99:1, v:v) (A) and MeOH (B) as the mobile phases at a flow rate of 1 mL/min. The linear gradient started with 20 % (B), 25 min 70 % (B), 30 min 95 % (B), 30 min 40 % (B), 36 min 95 % (B), 37 min 20 % (B), 45 min 20 % (B). UV chromatograms were recorded at 255, 330, 360 and 510 nm.

The HPLC system equipped with both a DAD and mass detector in series consisted of a HPLC binary pump, autosampler, degasser and photo-diode array detector controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent Technologies) equipped with an electrospray ionisation (ESI) system and controlled by software. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and MS-MS daughter spectra were measured from *m/z* 100 up to *m/z* 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas and the collision energy was set at 50 %. Mass spectrometry data were acquired in the alternative positive/negative ionisation mode.

Quantification of pomegranate juice polyphenols and related metabolites

Polyphenols and derived metabolites were identified according to their UV spectra, retention times, ion mass (MS) and MS-MS daughter fragments after isolation using ion trap. Quantification of ellagic acid and its derivatives was carried out by chromatographic comparisons with pure standard of ellagic acid. Ellagic acid derivatives were quantified as ellagic acid at 360 nm. Punicalagin isomers were quantified at 360 nm using an external standard of punicalagin previously isolated [10]. Antho-

cyanins were quantified as cyanidin-3-glucoside at 510 nm [2]. Pomegranate juice ellagitannin-derived metabolites (6H-dibenzopyran-6-one derivatives) were quantified as 3,8-dihydroxy-6Hdibenzo[*b,d*]pyran-6-one using the corresponding standard purified from urine as reported above. The metabolite 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one and its corresponding glucuronide derivative were quantified at 360 nm. The metabolite hydroxy-6H-dibenzo[*b,d*]pyran-6-one and its glucuronide were quantified at 330 nm.

Haematology and clinical chemistry

Biochemical parameters were determined in serum using an automated biochemical auto-analyser HITACHI Modular D+P (Roche Diagnostics, Switzerland). The parameters analysed were: glucose, urea, creatinine, uric acid, total proteins, albumin, calcium, phosphorus, total bilirubin, cholesterol, HDL-cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamil transferase (GGT), cholinesterase, and ferritin. Homocystein was analysed by fluorescence polarization immunoassay (FPIA) using an IMX automated analyser (Abbott Diagnostics, USA). Haematological parameters were determined in EDTA-treated blood using an automated haematological analyser (Cell-Dyn 3700 and 4000, Abbott, IL, USA). The parameters analysed were: red blood cells, haemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets, mean platelet volume (MPV), leucocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

Lipoproteins ultracentrifugation

HDL, LDL and VLDL fractions were separated by ultracentrifugation as reported elsewhere [16]. Briefly, sera were adjusted to 1.21 kg/L density with KBr. Tubes containing sera were filled with a 1.006 kg/L density KBr solution and centrifuged at 416,000 g for 55 min at 10 °C by using an Optima L-80 preparative ultracentrifuge with a vertical tube rotor VTi 65.2 fix angle (Beckman Instruments Inc., Palo Alto, USA).

Lipidogram

The separation of lipoproteins in human serum was carried out in agarose gels by using the Paragon Lipoprotein Electrophoresis Kit (Beckman). After electrophoresis, the lipoproteins were fixed in the gel with a mixture of ethanol:water:glacial acetic acid:MeOH [59:31:7:3]. Afterwards, the gel was dried and the lipoprotein pattern visualised by using the Sudan Black B Stain-containing Paragon Lipo Stain (0.07 %; w/w) (Paragon®). Lipidogram allowed the determination of chylomicrons, α-lipoprotein, pre-β-lipoprotein and β-lipoprotein.

Statistics and graphs

Before beginning the experiment, a statistical analysis was carried out in order to determine the minimum number of subjects (sample size) required to establish significantly statistical conclusions. The number of subjects was based on the construction of a confidence interval [17]. The required sample size to get a confidence interval of 95 % was determined for each parameter analysed. The mean of the sample size was 5.54 (e. g. a sample size of six subjects would allow getting a confidence interval of 95 %).

Results were expressed as mean ± SD (standard deviation) of both control and study days. The normal values range (NVR) establishes the limit between normal and pathological values. In the present study, the NVR were those reference values used by the Hospital's laboratory. Biochemical and haematological parameters were analysed with an analysis of variance (ANOVA) between the control days and study days. Differences were considered statistically significant if $P \leq 0.05$. Graphs of the experimental data and their statistic analysis were carried out by using the Sigma Plot™ 6.0 program for Windows™.

Results

Phenolic analysis in pomegranate juice

The prepared pomegranate juice was very rich in ellagitannins, especially punicalagin isomers (gallagyl-hexahydroxydiphenoyl glucose derivatives), free ellagic acid, and ellagic acid glycosides. In addition, six anthocyanin pigments were identified as delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides (results not shown). This juice contained 4.37 g/L punicalagin isomers, 0.61 g/L free ellagic acid, 0.60 g/L ellagic acid glycosides (glucoside, arabinoside and rhamnoside but quantified as ellagic acid) and 0.49 g/L anthocyanins. This makes a total of 5.58 g/L of phenolic compounds that could render ellagic acid under physiological conditions [18]. Regarding a molar basis, one molecule of punicalagin yields one molecule of ellagic acid and a molecule of gallagic acid (an ellagic acid dimmer), and this means that every punicalagin molecule can potentially release three ellagic acid equivalents *in vivo* (Fig. 2). The punicalagin concentration of this pomegranate juice was 4.03 mM that rendered 12.08 mM ellagic acid equivalents. The juice content of free ellagic acid and glycosidic derivatives was 1.21 g/L which meant

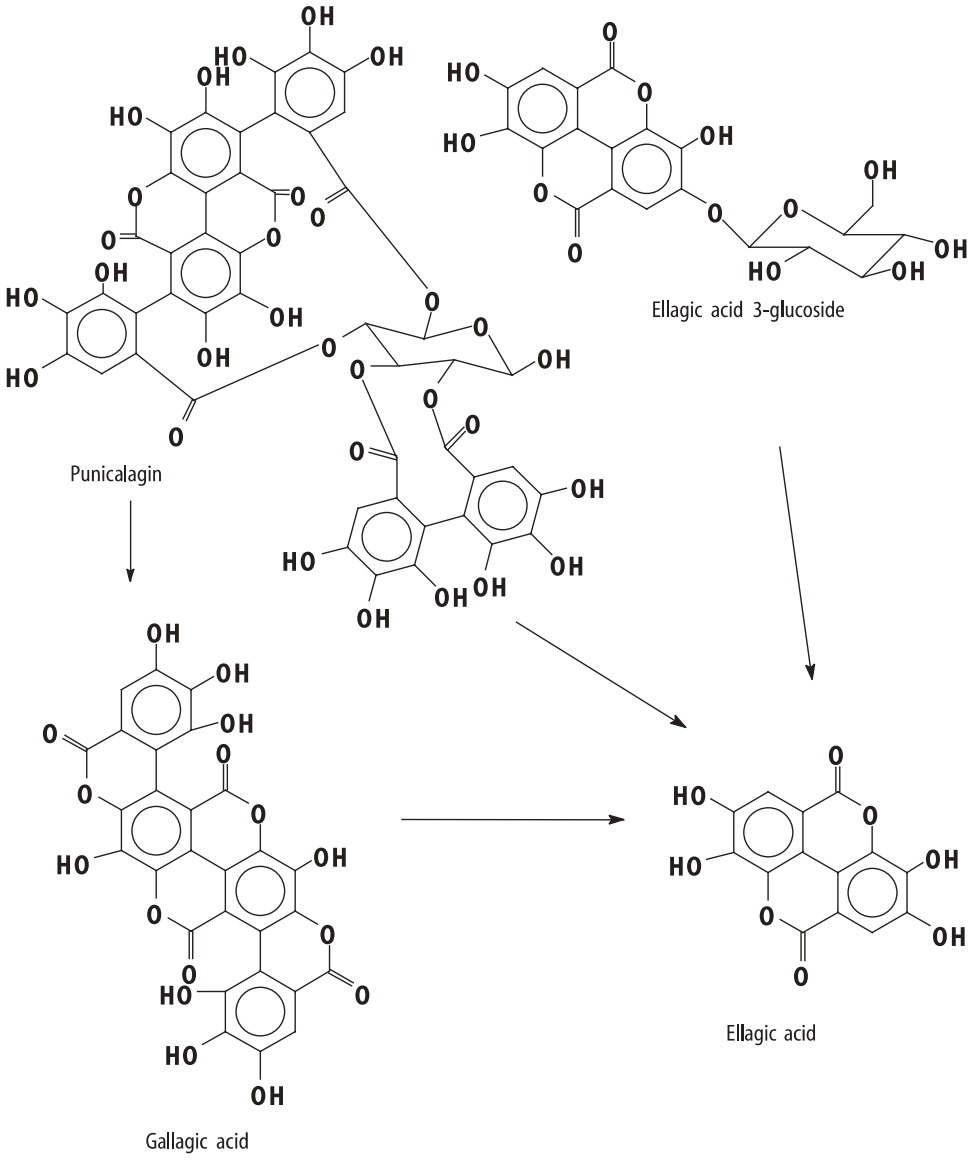

Fig. 2 Possible pathways to yield ellagic acid by hydrolysis from ellagitannins and ellagic acid derivatives precursors

4.0 mM concentration of free ellagic acid equivalents. As a whole, the dietary supplementation with 1 litre of pomegranate juice provided a daily intake of 16.09 mmoles of ellagic acid equivalents. This supplementation was carried out during 5 days involving a total intake during this experiment of 80.45 mmoles ellagic acid equivalents (24.3 g ellagic acid equivalents).

Pomegranate phenolics bioavailability and metabolism

In a first preliminary experiment (results not shown), one volunteer ingested 1 litre of pomegranate juice and blood samples were taken every hour during the following four hours. Neither pomegranate juice polyphenols nor their metabolites were detected in plasma samples during this preliminary experiment. However, similar metabolites to those previously detected in rat urine after pomegranate tannin ingestion [10] were detected in the urine of the same volunteer after 24 h of the pomegranate intake. This suggested that pomegranate polyphenols could be metabolised and absorbed in the colon. Neither anthocyanins nor related compounds were detected either in plasma or urine. Then, a second experiment was carried out with the scheme shown in Fig. 1 in which 1 litre of pomegranate juice was ingested daily for 5 days, blood samples were taken every day and urine was also collected.

Identification of plasma metabolites after pomegranate juice supplementation

In the second experiment (Fig. 1), neither ellagic acid nor conjugated metabolites, punicalagin or anthocyanin derivatives were detected in plasma in any of the 5 days in which blood samples were drawn. The presence of different metabolites was checked with a DAD detector (UV) and also with the MS detector (searching for specific ions, or for fragments obtained after compound breakdown under the MS conditions) in a similar manner to the analytical conditions used in a previous work on ellagitannin absorption and metabolism in rats [10].

Three main metabolites (*a*, *b* and *c*) were detected in plasma that were associated to pomegranate juice ingestion (not observed during the control sampling) (Fig. 3). Their UV spectra recorded with a Diode Array detector and MS/MS analysis allowed their tentative identification (Figs. 4 and 5). Compound *a* was the main ellagitannin metabolite in plasma of most volunteers and showed an M-H peak at *m/z*$^-$ 405 and MS/MS fragment at *m/z*$^-$ 227, corresponding to a glucuronide derivative of 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (Fig. 4a). This compound was previously reported in plasma of rats after the ingestion of pomegranate husk ellagitannins [10]. This compound released the corresponding aglycone *d* after glucuronidase treatment (result not shown). Compound *c* showed an M+H ion at m/z$^+$ 389 and MS-MS fragment at m/z$^+$ 213 (Fig. 4c) that suggested a compound similar to *a* but with a mass 16 units smaller (loss of a hydroxyl residue), being tentatively identified as a glucuronide of hydroxy-6H-dibenzo [*b,d*]pyran-6-one. The UV spectrum of compound *a* coincided with that reported for the same metabolite found in rat plasma [10] (Fig. 5), and that of compound *c* was similar, but with shorter wavelengths for the absorption maximal as could be expected for a metabolite with one less phenolic hydroxyl (Fig. 5). Compound *b* showed a UV spectrum of the same type as those of *a* and *c* (Fig. 5), with an M+H at m/z$^+$ 245 and MS-MS fragment at m/z$^+$ 227 suggesting a compound similar to the aglycone of compound *a* (compound *d*) but with an additional hydroxyl (Fig. 4b). However, its UV spectrum was different from that previously reported for 3,8,10-trihydroxy-6H-dibenzo[*b,d*]pyran-6-one detected in rat plasma after pomegranate tannin ingestion, suggesting that this could be another isomer with different hydroxyl substitution. Compound *b* was not detected in the glucuronide form (no change after glucuronidase treatment) and was only a minor metabolite in human plasma.

A large inter-individual variability was observed among the volunteers (Table 1) as it has been previously reported for the bioavailability of some phenolic metabolites in humans [19, 20]. In some volunteers, the plasma concentration of these three metabolites was below the detection limit, as is the case of volunteers V4 and V5, while in other individuals the ellagitannin metabolites were detected in plasma from the first day of the supplementation experiment. The concentration of metabolites found in plasma was quite variable, with values between 0.5 and 18.6 μM in those volunteers in which the metabolites were detected (Table 1). Regard-

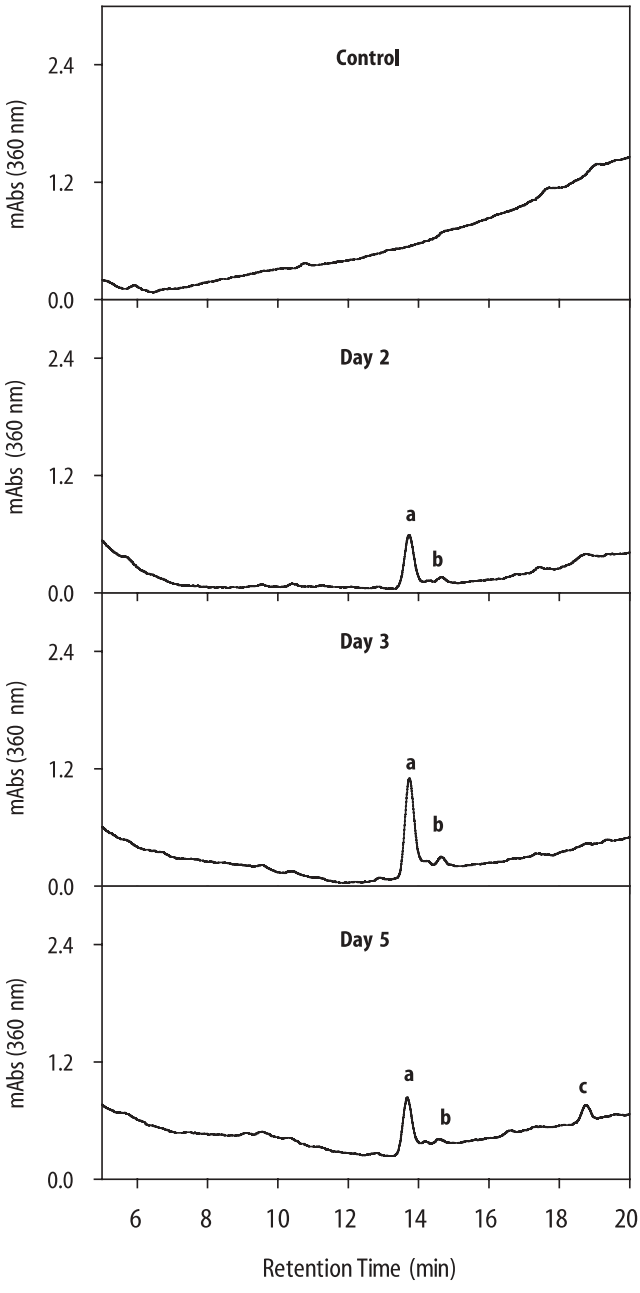

Fig. 3 HPLC profile of plasma, at different days, from one volunteer after pomegranate juice supplementation. **a**, 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide (MS = 406); **b**, unidentified aglycone metabolite, tentatively trihydroxy-6H-dibenzo[*b,d*]pyran-6-one derivative (MS = 244); **c**, hydroxy-6H-dibenzo [*b,d*]pyran-6-one glucuronide (MS = 390)

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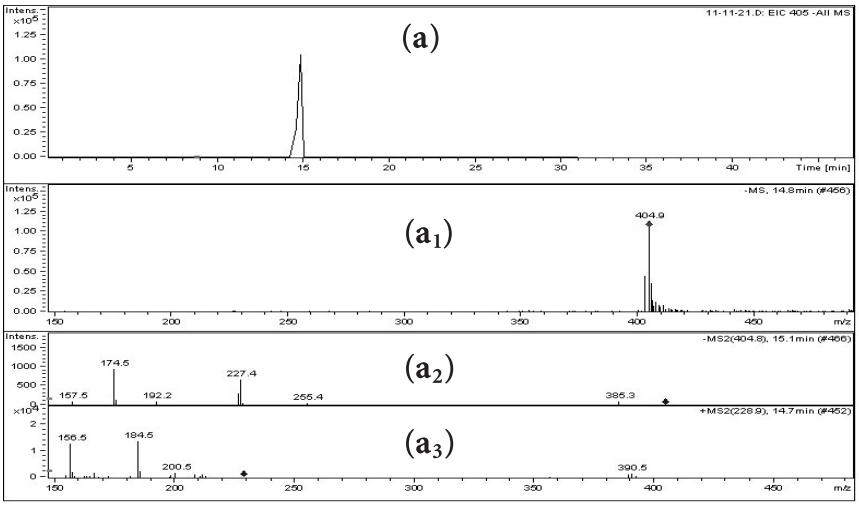

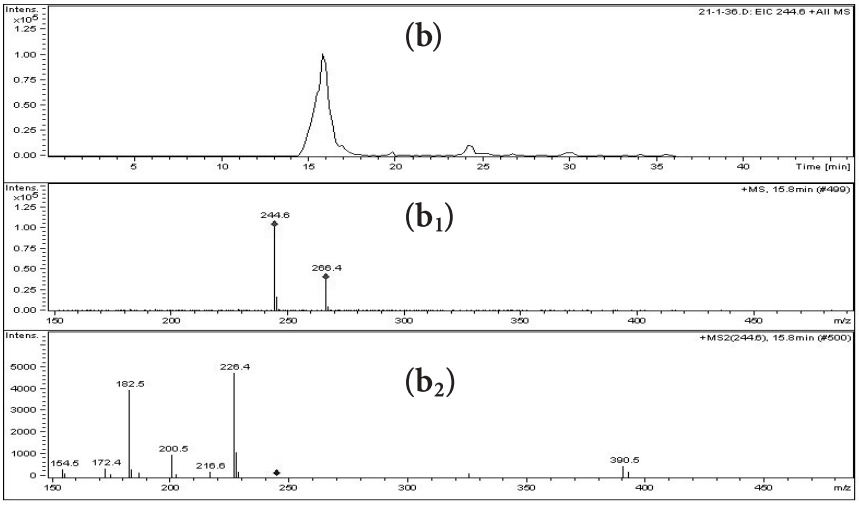

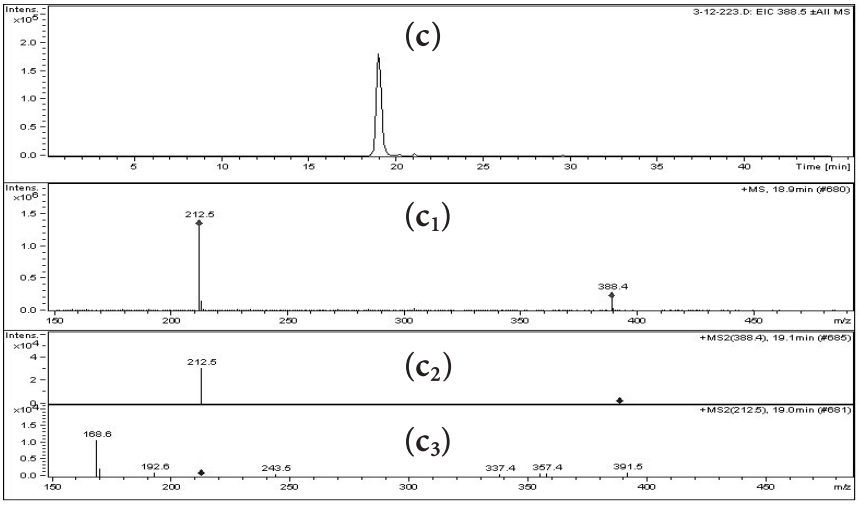

Fig. 4 LC-MS/MS analysis of the main detected metabolites in humans after pomegranate juice supplementation. (**a**) Extracted Ion Chromatogram (EIC) at m/z^- 405; (**a_1**) MS scan at the retention time of the above EIC; (**a_2**) MS/MS daughter spectra from the glucuronide m/z^+ 405; (**a_3**) MS/MS daughter spectra from the aglycone m/z^- 227; (**b**) EIC at m/z^+ 245, (**b_1**) MS scan at the retention time of the EIC at m/z^+ 245; (**b_2**) MS/MS daughter spectra from m/z^+ 245; (**c**) EIC at m/z^+ 389; (**c_1**) MS scan at the retention time of the EIC at m/z^+ 389; (**c_2**) MS/MS daughter spectra from the glucuronide m/z^+ 389; (c3) MS/MS daughter spectra from the aglycone m/z^+ 213

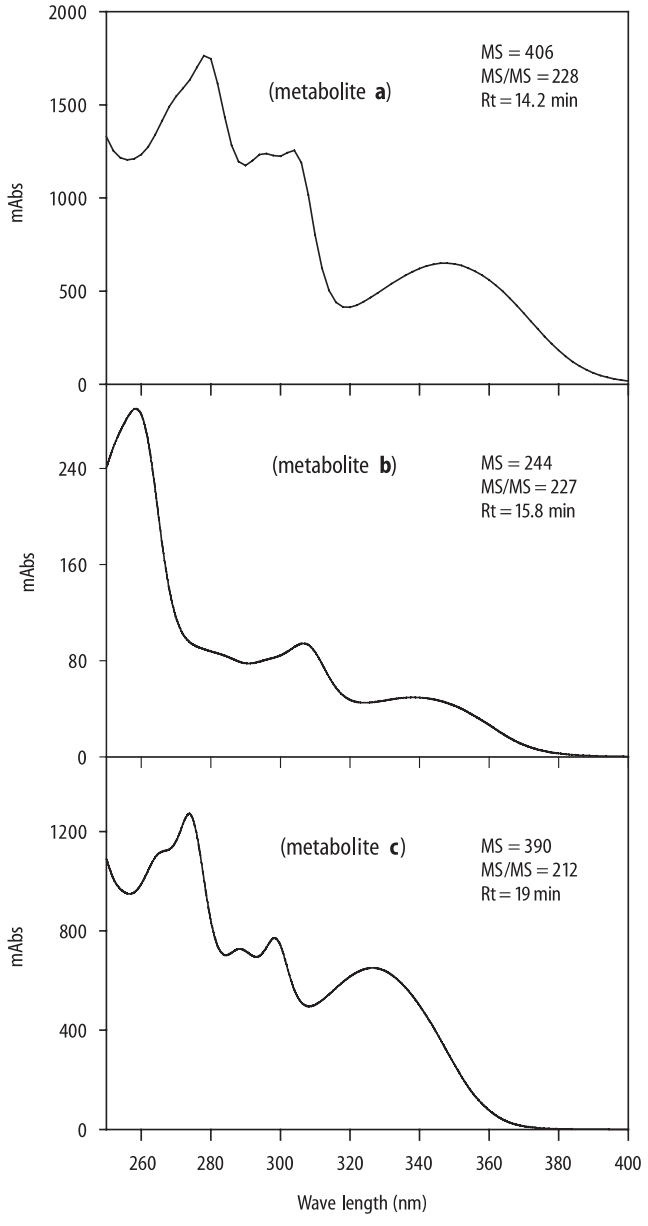

Fig. 5 Spectra of the colonic microflora ellagitannin-derived metabolites detected in humans after pomegranate juice supplementation. (Metabolite **a**) 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide; (metabolite **b**), tentatively, trihydroxy-6H-dibenzo[*b,d*]pyran-6-one derivative; (metabolite **c**) hydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide

ing the different metabolites, there were some individuals that produced both *a* and *c* in the same extent and timing (e. g. V2 and V3), while others did not produce compound *c* (Table 1). The main metabolite produced that was detected in the plasma of most volunteers was compound *a*. Compound *c* only became evident after day 5 in V1, while it was present most of the days in V2 and V3. Metabolite *b* (m/z$^+$ 245) was detected in the plasma of three volunteers (V1, V3 and V6), but was not detected in V2 that produced large amounts of the other two metabolites (Table 1).

Table 1 Plasma concentration of ellagitannin metabolites

Volunteers (V) and metabolites	Day 1	Day 2	Day 3	Day 4	Day 5
Metabolite **a** (MS = 406)					
V1	1.0	3.7	6.0	4.1	3.4
V2	n. d.	2.7	3.0	6.2	4.3
V3	n. d.	4.4	**	6.7	5.9
V4	n. d.	n. d.	1.1	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.
V6	t	2.6	3.2	t	1.9
Metabolite **b** (MS = 244)					
V1	n. d.	0.2	1.4	0.2	0.3
V2	n. d.	n. d.	n. d.	n. d.	n. d.
V3	n. d.	1.0	**	1.1	0.4
V4	n. d.	n. d.	n. d.	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.
V6	n. d.	1.4	1.8	0.5	1.0
Metabolite **c** (MS = 390)					
V1	n. d.	n. d.	n. d.	n. d.	2.4
V2	n. d.	n. d.	1.1	3.0	1.6
V3	n. d.	7.3	**	10.8	6.4
V4	n. d.	n. d.	n. d.	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.
V6	n. d.	n. d.	n. d.	n. d.	n. d.
Total					
V1	1.0	3.9	6.4	4.3	3.7
V2	n. d.	2.7	4.1	9.2	5.9
V3	n. d.	12.7	**	18.6	12.7
V4	n. d.	n. d.	1.1	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.
V6	t	4.0	5.0	0.5	2.9
Mean ± SD	0.17 ± 0.41	3.9 ± 4.7	3.3 ± 2.7	5.4 ± 7.4	4.2 ± 4.7

Values are plasmatic concentrations (μM) of ellagic acid metabolites (quantified as 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one); Values are the mean of three replicates; ** This sample was not available; *n. d.* not detected; *t* traces; Total = **a** + **b** + **c**

Enzyme hydrolysis of plasma metabolites with glucuronidase and sulphatase yielded the corresponding aglycones (*d* and *e*). Although no sulphates were detected in the HPLC-MS analysis, the presence of this type of metabolite cannot be ruled out as the concentration of the aglycones after enzyme treatment was always higher than that of the glucuronides, suggesting that some of the resulting aglycones could occur in the non-detected sulphate conjugates. It is well documented that sulphated phenolic metabolites give broad peaks under HPLC analytical conditions and this makes their detection in plasma especially difficult when only very small amounts are present [21].

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Urine analysis

Six different metabolites (*a*–*f*) associated to pomegranate juice supplementation were detected in urine (Fig. 6). Compounds *a*, *b* and *c* coincided with those found in plasma, while the minor urine metabolites (*d*, *e* and *f*) were not detected in plasma, probably because they were present in concentrations below the detection limit. Compound *d* (MS = 228) corresponded to the aglycone of *a* and compound *e* (MS = 212) to the aglycone of *c*, and, therefore, tentatively identified as 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one and hydroxy-6H-dibenzo [*b,d*]pyran-6-one, respectively. Compound *f* showed identical mass and UV spectra as those of *b* (Figs. 4 and 5) and suggested a similar type of structure (trihydroxy-6H-dibenzo[*b,d*]pyran 6-one derivative), but with a different hydroxyl substitution, which could explain its higher retention time. The MS-MS analyses of urine metabolites were identical to those found for the compounds present in plasma, and confirmed their chemical structures.

In some volunteers, the metabolites became evident in the urine collected during the first day of juice supplementation, although in very small concentrations (Table 2). The total amount of excreted ellagitannin metabolites reached a maximum between day 3 and 4 and then decreased slightly. Day 5 was the last day in which pomegranate juice was supplemented and the metabolites were also detected in the urine collected during the next day when the supplementation experiment had finished, although the concentration decreased considerably.

The same inter-individual variability observed in plasma samples was also observed in urine. The volunteers V4 and V5, in which the metabolites were not detected in plasma, only showed very small amounts of ellagitannin metabolites in urine (Table 2). A particular case was that of V2 that excreted the highest concentration of metabolites, although the plasma concentrations were not the highest.

Regarding the individual metabolites excreted in urine, compound *a* was the main metabolite excreted and was found in all the volunteers (Table 2) and the corresponding aglycone *d* was also present in most urine samples although in smaller amounts, while compound *c* and its corresponding aglycone *e* were only detected in V2 and V3 who were the volunteers that showed this metabolite in plasma.

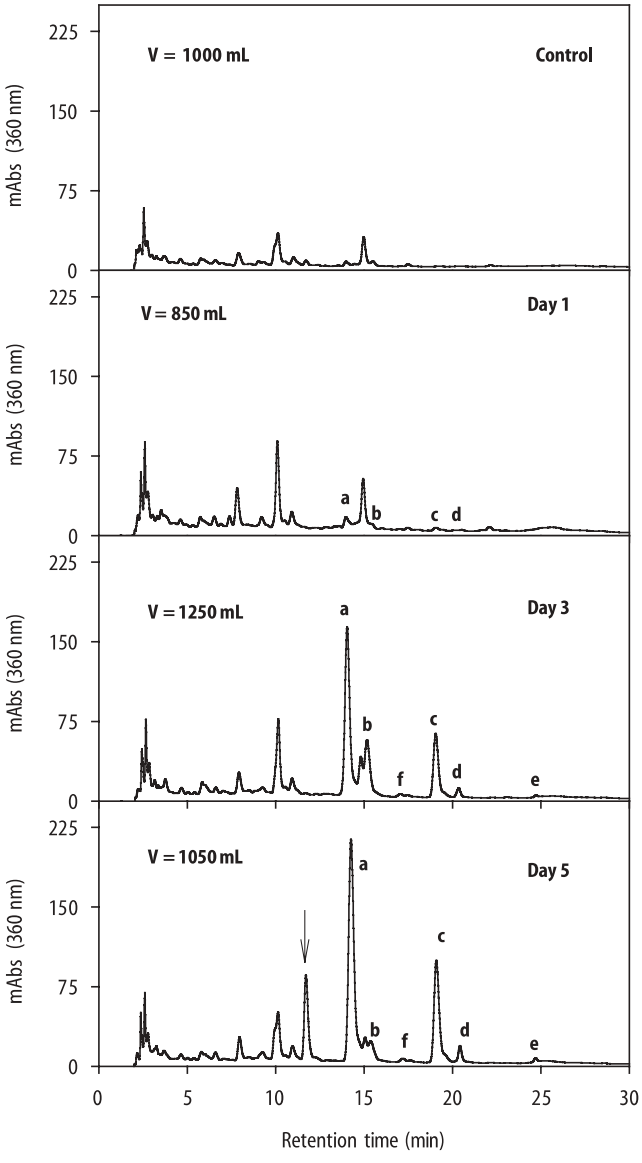

Fig. 6 HPLC analysis, at different days, of the urine excretion of colonic microflora metabolites after pomegranate juice supplementation. **a** 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide (MS = 406); **b** unidentified aglycone metabolite, tentatively, trihydroxy-6H-dibenzo[*b,d*]pyran-6-one derivative (MS = 244); **c** hydroxy-6H-dibenzo[*b,d*]pyran-6-one glucuronide (MS = 390); **d** 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (MS = 228); **e** hydroxy-6H-dibenzo[*b,d*]pyran-6-one (MS = 212); **f** unidentified aglycone metabolite, tentatively, trihydroxy-6H-dibenzo[*b,d*]pyran-6-one derivative (MS = 244), similar to metabolite **b**, but with different hydroxyl substitution. **V** 24-h urine volume collected. The arrow at day 5, in this volunteer, indicates one dietary unidentified compound non-related with pomegranate juice intake

Isolation and identification of urine metabolites

Compound *d*, the aglycone of the main metabolite in plasma and urine, was isolated as reported in Materials and Methods by a combination of liquid-liquid extraction, solid-phase extraction, column chromatography and thin layer chromatography. The purity of the isolated compound was checked by HPLC and analysed by direct inlet MS in the HPLC-MS equipment, and ^{1}H NMR. The mass analysis showed a molecular ion at m/z$^+$ 229 consistent with a $C_{13}O_4H_8$ molecule. MS-MS analyses of the m/z$^+$ 229 ion isolated in the ion trap showed

Table 2 Daily recovery of individual ellagitannin-derived metabolites in urine of the different healthy volunteers

Volunteers (V) and metabolites	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Metabolite **a** (MS = 406)						
V1	0.044	0.415	1.063	0.900	0.853	0.293
V2	0.265	0.348	1.215	1.829	1.093	0.601
V3	0.016	0.066	0.327	0.348	0.388	0.147
V4	n. d.	0.021	0.023	0.056	0.022	0.002
V5	n. d.	0.041	0.073	0.016	0.010	0.0007
V6	0.006	0.151	0.292	0.224	0.153	0.067
Metabolite **d** (MS = 228)						
V1	n. d.	0.022	0.073	0.042	0.045	0.013
V2	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V3	0.003	0.006	0.014	0.019	0.018	0.001
V4	n. d.	n. d.	0.001	0.003	0.002	n. d.
V5	n. d.	0.003	0.006	0.004	0.005	n. d.
V6	n. d.	0.015	0.070	0.032	0.043	0.001
Metabolite **c** (MS = 390)						
V1	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V2	0.088	0.051	0.865	1.604	1.037	0.547
V3	0.004	0.054	0.128	0.166	0.176	0.046
V4	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
Metabolite **e** (MS = 212)						
V1	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V2	n. d.	n. d.	n. d.	0.078	0.027	0.025
V3	n. d.	n. d.	0.002	0.004	0.004	0.0003
V4	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V5	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
V6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
Total						
V1	0.044	0.437	1.137	0.904	0.898	0.305
V2	0.353	0.399	2.081	3.512	2.157	1.173
V3	0.020	0.072	0.346	0.454	0.443	0.174
V4	n. d.	0.021	0.024	0.059	0.025	0.002
V5	n. d.	0.044	0.080	0.020	0.015	0.001
V6	0.006	0.165	0.362	0.257	0.196	0.069
Mean ± SD	0.07 ± 0.14	0.19 ± 0.18	0.67 ± 0.80	0.87 ± 1.33	0.62 ± 0.82	0.29 ± 0.45

Values are expressed as grams of excreted metabolites; *n. d.* not detected

fragments at m/z$^+$ 201 (M-CO), 185 (M-CO-O), 172 and 157. Its UV spectrum in methanol showed maxima at 348, 304, 295, 278 and a shoulder at 265 nm (Fig. 5, metabolite *a*). The ^{1}H NMR analysis showed a characteristic spectrum of this type of compounds [22], with a singlet integrating two protons at 8.4 ppm corresponding to the hydrogen signal of both phenolic hydroxyls, a doublet at 7.9 ppm (J 8.9 Hz) corresponding to one proton at H10, another doublet at 7.7 ppm (J = 8.9 Hz) corresponding to one proton at H1, a doublet at 7.4 ppm (J = 2.5 Hz) corresponding to one proton at H7, one double-doublet at 7.1 ppm (J = 8.9 and 2.5 Hz) corresponding to one proton at H9, one double-doublet at 6.7 ppm (J = 8.9 and 2.5 Hz) corresponding to one proton at H2 and one doublet at 6.6 ppm (J = 2.5 Hz) corresponding to one proton at H4 (for proton numbering see Fig. 7).

Other metabolites were also isolated by TLC and analysed by HPLC-MS-MS. The aglycone with M+H at m/z$^+$213 was also isolated and with a UV spectrum consistent with this type of compound (Fig. 5, metabolite *c*). The metabolite *b* was also purified and both MS data (Fig. 4b) and UV spectrum (Fig. 5, metabolite *b*) were confirmed. Another compound with UV spectrum like ellagic acid was also isolated, although this was not detected in the urine as it was present in amounts below the detection limit. The MS-MS analysis was consistent with an ellagic acid methyl ester (m/z$^-$ 315). This type of compound was also previously reported in rat urine after intake of pomegranate husk ellagitannins.

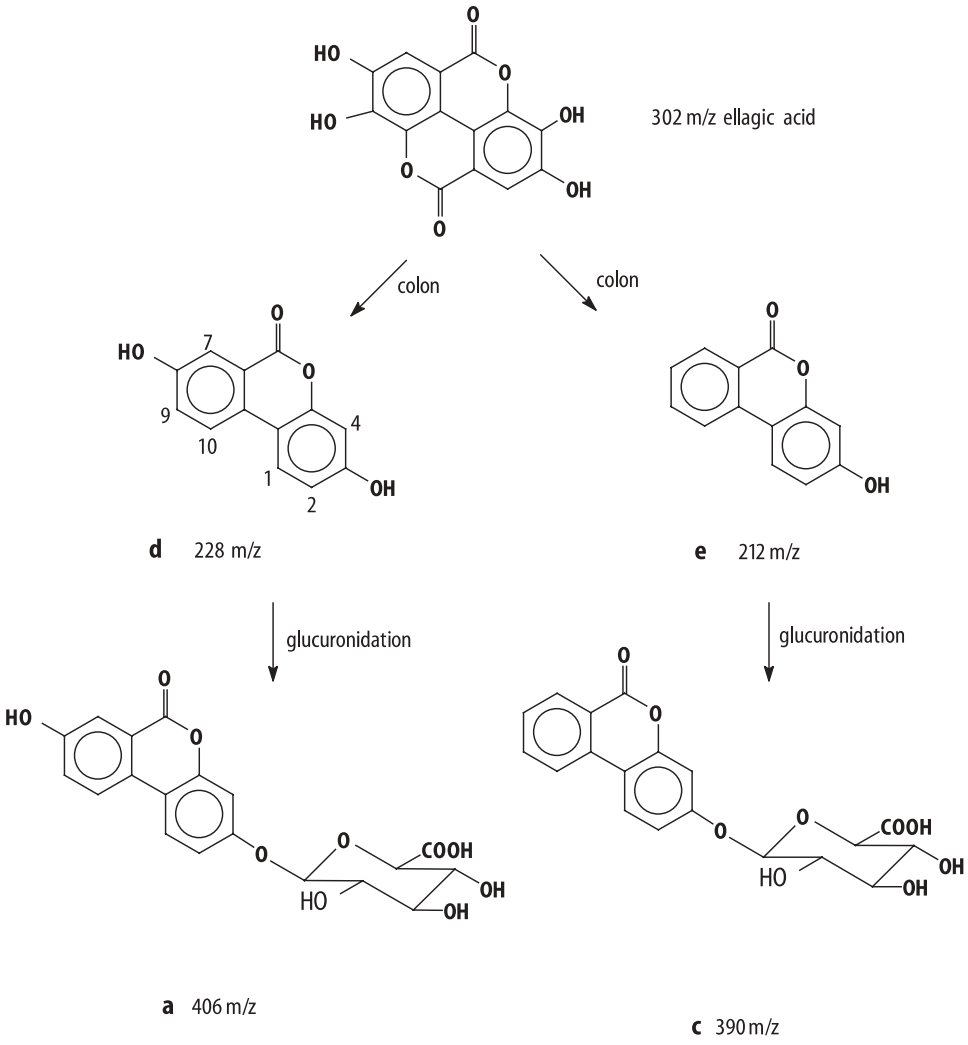

Fig. 7 Proposed metabolism of ellagic acid by colonic microflora

Excretion of pomegranate phenolic metabolites

In this supplementation experiment (intake of 1 litre of pomegranate juice daily for 5 days), every volunteer ingested a total of 24.3 g ellagic acid equivalents (as punicalagin, ellagic acid and ellagic acid glycosides). A large variability in the transformation of ellagic acid metabolites in the corresponding hydroxy-6H-dibenzopyran-6-one derivatives was observed in the healthy volunteers. One of them metabolised and excreted in urine more than 50 % of the ingested ellagic acid, while others excreted below 1 % of the ingested ellagic acid as this type of compound or any other compounds that could be detected in the HPLC analysis either with DAD-UV or MS-MS detection (Table 3). Regarding the urinary excretion kinetics, the metabolites were detected in urine after 24 h of the first pomegranate juice intake to reach a maximum excretion between day 3 and 4 (results not shown).

Table 3 Urinary excretion of ellagitannin-derived metabolites in healthy volunteers

Volunteers	Total excretion (g)*	Total excretion (mmoles)	Excreted percentage (%)
V1	3.76	16.50	20.50
V2	9.67	42.40	52.70
V3	1.51	6.60	8.20
V4	0.13	0.60	0.70
V5	0.16	0.70	0.90
V6	1.05	4.60	5.80
Total Mean ± SD	2.71 ± 3.66	11.90 ± 16.00	14.80 ± 19.90

* Values are expressed as grams of the purified metabolite **e** (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one)

Biological effects

None of the analysed haematological parameters listed in Materials and Methods was affected after the pomegranate juice supplementation in all volunteers (results not shown). When the serobiochemical parameters were evaluated, only total proteins, albumin and calcium concentrations showed a slight, although statistically significant, decrease after juice supplementation (Table 4). In addition, there were also slight, but statistically significant, differences in the serum lipid fraction showing an increase in the pre-β-lipoprotein, VLDL-cholesterol, LDL-triglycerides and VLDL-triglycerides, while there was a decrease in β-lipoprotein and LDL-cholesterol (Table 4).

Antioxidant activity

No differences were observed between samples from "control days" and samples from "study days" in both urine and plasma with the *in vitro* assays used in this study (results not shown). Antioxidant activity of punicalagin (referred to 1 μM) showed a TEAC value of 8.4 μM with DPPH assay. However, in the same assay, the metabolite 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one (1 μM) showed a TEAC value of 0.2 μM which meant 42-fold lower activity than punicalagin. The differences between punicalagin and this metabolite were much more evident with ABTS assay because 1 μM punicalagin showed a TEAC value of 15 μM, whereas the metabolite showed a TEAC value of 4.2 nM which meant 3500-fold lower activity than punicalagin. No activity was detected with the rest of the metabolites.

Discussion

After the dietary supplementation with 1 litre of pomegranate juice (more than 5 g of polyphenols per day including ellagitannins, ellagic acid derivatives and anthocyanins), none of the initially ingested compounds or metabolites of hydrolysis as well as conjugated with glucuronic acid or sulphate were detected in human plasma

Table 4 Serobiochemical parameters of healthy subjects before (CD) and after (PD) pomegranate juice supplementation

Parameters	Mean (CD)	Mean (PD)	NVR
Glucose (mg/dL)	84.16±5.56	85.53±4.71	75–110
Urea (mg/dL)	29.16±4.60	24.76±3.85	10–50
Creatinine (mg/dL)	1.03±0.09	1.06±0.13	0.6–1.1
Uric acid (mg/dL)	4.63±1.11	4.23±1.07	2.4–5.7
Total proteins (g/dL)	7.40±0.16	7.13±0.18*	6.6–8.7
Albumin (g/dL)	4.63±0.12	4.49±0.11*	3.5–5.3
Calcium (mg/dL)	10.03±0.25	9.95±0.24*	8.1–10.4
Phosphorus (mg/dL)	3.47±0.52	3.64±0.42	2.7–4.5
Total bilirubin (mg/dL)	1.20±0.59	0.88±0.40	0.1–1.1
Cholesterol (mg/dL)	156.41±19.23	153.13±27.45	50–230
Triglycerides (mg/dL)	71.83±8.00	92.3±36.85	50–200
HDL (mg/dL)	63.25±8.78	57.81±11.16	45–75
LDL (mg/dL)	78.41±12.65	77.15±17.07	< 130
AST (U/L)	21.41±5.60	21.60±5.05	5–37
ALT (U/L)	21.25±11.74	20.00±8.27	5–37
ALP (U/L)	80.08±38.27	79.50±36.64	35–130
GGT (U/L)	18.66±6.34	17.33±6.81	5–39
Cholinesterase (U/L)	10468±1085.05	9818.17±939.7	4300–11500
Ferritin (ng/mL)	83.50±66.94	75.40±51.43	10–160
Homocysteine (μM)	9.50±2.55	9.93±2.85	5–15
Chylomicrons (%)[a]	0.75±0.22	1.35±0.50	0–2
α-lipoprotein (%)[a]	45.81±1.77	42.97±1.38	40.7–71.9
Pre-β-lipoprotein (%)[a]	12.70±1.41	17.97±3.8*	0–29.6
β-lipoprotein (%)[a]	40.72±2.50	37.75±4.47*	9.8–46.2
LDL-cholesterol (mg/dL)[b]	97.66±22.32	82.65±26.29*	41.9–152.3
LDL-triglycerides (mg/dL)[b]	24.40±4.19	31.89±4.00*	0.0–43.5
HDL-cholesterol (mg/dL)[b]	58.27±6.98	57.39±6.59	31.5–88.8
HDL-triglycerides (mg/dL)[b]	21.27±3.45	22.66±2.70	12.4–31.0
VLDL-cholesterol (mg/dL)[b]	13.87±2.48	27.01±8.87*	10.9–56.6
VLDL-triglycerides (mg/dL)[b]	24.28±7.65	37.36±15.08*	0.0–102.3

CD control days; *PD* study days (pomegranate juice supplementation); *NVR* normal values range; [a] Values from the lipidogram; [b] Values from the ultracentrifugation; Values are shown as the mean ± SD (n = 6); * $P \leq 0.05$

or urine. The anthocyanins ingested, although pomegranate juice is rich in these pigments, were in smaller amounts than those previously reported for the detection of untransformed anthocyanins in plasma of human volunteers [23, 24]. No intact punicalagin, punicalin or ellagic acid conjugates were detected in human plasma or urine, although they have been detected in a previous study on the ellagitannin absorption and metabolism in rats [10]. This could be due to the much higher intake of ellagitannins in the experiment with rats, in which a huge amount of ellagitannins were ingested daily (the equivalent of 200 litres of pomegranate juice [10]).

In general, the metabolites associated to pomegranate juice intake coincide with those previously reported to be produced in rats after pomegranate husk intake. These metabolites (6H-dibenzo[*b,d*]pyran-6-one derivatives) were reported to be produced by intestine bacteria in rats after consumption of ellagic acid [7] and ellagitannins [10]. In the study achieved with rats, these metabolites were detected in urine, in significant amounts, after 4 days [10], suggesting that the rat needed some time to adapt the microflora to metabolise the pomegranate tannins to produce these metabolites. A previous work concerning the disposition of ellagic acid in mice indicated that 22% of the ingested ellagic acid was excreted in urine after 24h and only one 6H-dibenzo[*b,d*]pyran-6-one derivative was detected in faeces. Perhaps, the 24-h study was not enough to detect 6H-dibenzo[*b,d*]pyran-6-one derivatives in both plasma and urine [9].

In humans, these metabolites were detected in urine and plasma after 24h of juice intake, suggesting that the metabolites were produced in the distal part of the intestine by the action of the colon bacteria, as it was reported in rats. There were, however, large differences in the metabolite production between the different volunteers, and in some of them the metabolites were only produced in very small amounts. This could be due to differences in the colon microflora, that could be either unable to metabolise tannins into the 6H-dibenzo [*b,d*]pyran-6-one derivatives or metabolised them into other compounds, such as aliphatic acids (acetic, propionic and butyric) that were not detected under these analytical conditions. In addition, other dietary constituents such as proteins, calcium, etc. could precipitate tannins and render them less accessible to the action of microbes, thus excreting them with faeces.

It should be stressed that the maximum total concentration of these metabolites in plasma, depending on the subject (Table 1) could reach very high values (18.6 μM). In fact, some metabolites could reach in plasma the highest values reported in the literature so far (6.7 μM for metabolite *a*, and 10.8 μM for metabolite *c*) [25].

These 6H-dibenzo [*b,d*]pyran-6-one metabolites have also been reported in both the faeces of bats [22] and the beaver hair secretions (castor fibre) [26], since these animals also ingest large amounts of ellagitannins from berries and wood, respectively. In addition, these compounds have also been found as constituents of 'clover stone', a type of renal calculus found in sheep which can also ingest large amounts of ellagitannins [26]. In fact, the metabolite *d* (3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one) has been previously named as 'urolithin B' [22, 26].

The presence of these 6H-dibenzo[*b,d*]pyran-6-one derivatives has also been detected in both the plasma and urine of Iberian pigs, which, when raised extensively, ingest large amounts of ellagitannins with the acorns that constitute the main ingredient in their diet (own observations, unpublished results).

Despite the high *in vitro* antioxidant activity reported for pomegranate juice [2], it should be stressed that the daily intake of 1 L of pomegranate juice by healthy humans (total supplementation with 5.6 g of antioxidant phenolics per day) did not have a significant effect in the antioxidant capacity of both plasma ($ABTS^{\cdot+}$ and FRAP methods) and urine ($ABTS^{\cdot+}$ and $DPPH^{\cdot}$ methods). Therefore, our results do not agree with a previous report that described the increase of antioxidant capacity in plasma ($ABTS^{\cdot+}$ assay) upon pomegranate juice supplementation [4]. This previous study claimed that the polyphenols were responsible for such effect; however, no data were provided concerning the bioavailability and metabolism of polyphenols to justify this effect. Therefore, the reported claim about the antiatherogenic and antiatherosclerotic properties of pomegranate juice attributable to its antioxidant properties should be taken with caution.

In general, the lack of effect observed in the present study could be expected as none of the potent *in vitro* antioxidant metabolites present in the pomegranate juice were detected in plasma as such in detectable amounts, and the antioxidant activity of the microflora metabolites was very low. In fact, the antioxidant activity of the metabolite 3,8-dihydroxy-6H-dibenzo [*b,d*]pyran-6-one was 42-fold and 3570-fold lower than that of punicalagin with DPPH and ABTS methods, respectively. Moreover, the main metabolites in plasma were detected as glucuronide derivatives which showed undetectable antioxidant activity (results not shown). Additional evidence to support the lack of antioxidant activity of the *in vivo* generated metabolites is that the remarkable differences in the presence of these metabolites among different volunteers in the present study did not correlate with differences in the plasma or urine antioxidant activity, thus corroborating that these metabolites had no effect on the direct control of oxidation in plasma.

Moreover, no effect was observed on the activity of the antioxidant enzyme glutathione peroxidase (results not shown) which was in agreement with a recent study

on the effect of the administration of high dietary doses of pomegranate ellagitannins to rats [27].

Concerning the haematological and serobiochemical parameters, only a few of them were affected by the pomegranate juice supplementation. There was a small, but statistically significant, reduction in the seric proteins, albumin and calcium, but the decrease was always within normal (non-pathological) values (Table 4). This behaviour could be expected for a supplementation with tannins that precipitate proteins and it is well established that tannins decrease the digestibility of proteins in animals [28]. As the main carrier of calcium in plasma is albumin, it was also logic to detect the corresponding decrease in calcium associated to that of albumin.

In addition, pomegranate juice ingestion also slightly affected the levels of some plasmatic lipids. HDL and LDL were not affected by pomegranate juice intake, in agreement with another report [4]. A decrease in both LDL-cholesterol and β-lipoprotein (both related to the atherosclerosis development) was detected. However, other lipid fractions, i. e. VLDL-cholesterol, pre-β-lipoprotein, LDL-triglycerides and VLDL-triglycerides slightly increased during this polyphenol supplementation experiment which made evident that no conclusive health-beneficial effects can be inferred from the intake of 1 litre of pomegranate juice during 5 days by healthy volunteers. Perhaps, more conclusive effects should be expected in subjects with hypercholesterolemia, hypertriglyceridemia, etc. or other pathologies and also with longer juice supplementation. In fact, approximately the same effects were observed in all volunteers, despite the large differences in the plasma levels and urine excretion of the ellagic-derived metabolites. This suggested that, in this case, the effect observed could be associated to the decrease in the absorption of proteins and/or lipids in the intestine by combination with the large amount of ellagitannins ingested with pomegranate juice.

Finally, according to the results presented above, it should be stressed that the potential systemic biological effects of pomegranate juice ingestion should be attributed to the colonic microflora metabolites rather than to the polyphenols present in the juice. In this context, the present study also calls for caution concerning the huge output of data regarding the *in vitro* antioxidant capacity of foodstuffs and their claimed potential *in vivo* extrapolation.

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