Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil: understanding metabolism of polyphenols

Rocío García-Villalba[1], Alegría Carrasco-Pancorbo[1*], Ekaterina Nevedomskaya[2], Oleg A. Mayboroda[2], André M. Deelder[2], Antonio Segura-Carretero[1], Alberto Fernández-Gutiérrez[1*]

[1] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, c/.Fuentenueva s/n, E-18071 Granada, Spain.

[2] LUMC, Biomolecular Mass Spectrometry Unit, Department of Parasitology, Leiden, The Netherlands.

[*]Corresponding author:

Dr. A. Carrasco-Pancorbo or A. Fernández-Gutiérrez, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.

E-mail: alegriac@ugr.es or albertof@ugr.es

Fax: +34 958 249510

Abstract

Olive oil polyphenols have important biological properties which closely depend on their bioavailability, therefore it is essential to understand how polyphenols are absorbed, metabolized and eliminated from the body. An analytical methodology based on rapid resolution liquid chromatography (RRLC) coupled to mass spectrometry detection with a time of flight analyzer (RRLC-ESI-TOF MS) was developed for the determination of the main olive oil phenolic compounds and their metabolites in human urine. Urine samples from ten healthy volunteers were collected before and 2, 4 and 6 h after the intake of 50 mL of extra-virgin olive oil. The proposed method includes liquid-liquid extraction with ethyl acetate that provides extraction recoveries of the phenolic compounds studied between 35 and 75% from spiked urine samples. Good repeatability was obtained, since the relative standard deviations (RSDs) of peak areas in the intra- and inter-day studies were 4.3 and 6.5%, respectively.

Statistical studies allowed us to discriminate between the urine samples before and after the intake, and facilitated to find out the m/z values responsible of this discrimination. Based on the very accurate mass information and the isotopic pattern provided by TOF-MS analyzer, together with other available information, ten of these biomarkers and more than 50 metabolites, obtained through phase I and phase II biotransformation reactions, were tentatively identified. Additionally, kinetic studies of the metabolites identified as possible biomarkers were developed, obtaining maximal values in the first two hours for most compounds.

Keywords RRLC-ESI-TOF MS/ Olive oil / Phenolic compounds/ Urine sample / Metabolites

Introduction

Nowadays, a growing number of studies point to the important role that extra virgin olive oil (EVOO) plays as a crucial ingredient of the Mediterranean diet due to its beneficial effects on health, associated with a lower incidence of atherosclerosis, cardiovascular disease, and certain types of cancer [1-3]. These health protective effects of olive oil have been traditionally attributed to a high content of monounsaturated fatty acids (MUFAs) [4-6], mainly oleic acid. However, recently the importance of the antioxidants in the nonsaponificable fraction, especially phenolic compounds has been clearly demonstrated [7,8]. The phenolic content of olive oil depends on several agronomic and technological factors [9], but regardless the amount, the main families of phenols that have been described in olive oil are: simple phenols, lignans, flavonoids and secoiridoids [10,11]. The antioxidant properties of these compounds (strong radical-scavenging activity and the ability to inhibit prooxidation processes on human low density lipoproteins (LDL)) have been shown in numerous in vitro experiments [12-14]. Moreover, phenolic compounds may influence some fundamental physiological processes, for instance suppress platelet aggregation or reduce the proliferation of cancer cell [15,16].

One of the requirements to extend their protective effect and to evaluate their biological activity *in vivo* is the knowledge of their bioavailability and metabolism in humans. The study of recovery of polyphenols in plasma or urine after intake of olive oil is one of the ways to access their absorption and metabolism in human body [17].

Different analytical methods have been proposed to investigate the *in vivo* effect of the olive oil phenols in biological fluids so far, especially the effect of hydroxytyrosol, (Hyty) tyrosol (Ty) and oleuropein [18,19]. Several papers have described the absorption of Hyty and oleuropein after their administration as pure compounds, analyzing rat plasma by GC-MS [20] and HPLC with UV [21], fluorescence [22], mass spectrometry detection [23] and radioanalysis [24]. The oral or intravenous administration of these compounds to experimental animals and their detection in plasma has provided evidence of its absorption. However, studies in which phenolic ingestion is closer to typical dietary patterns may be more appropriate for estimating bioavailability than the administration of pure phenolic compounds. Besides, the rat and rodents in general are not the best model for the study of dietary problem of human

metabolism [25]. Miró-Casas et al. [26] proposed a GC-MS method to quantify Hyty and 3-O-methyl-Hyty in human plasma and urine after real-life doses of virgin olive oil. Both compounds appear rapidly in plasma (32 and 53 min respectively) as conjugated forms, mainly glucuronoconjugates. Because data on plasma phenol concentrations are scarce, an alternative is to look at olive oil phenols excreted in urine. The urinary levels of Ty and especially Hyty were determined mainly by GC-MS after olive oil consumption or olive oil enriched with phenolic extracts [27 - 31] and HPLC-radiometric detection after consumption of labeled Hyty. [32]. As a result of these studies it was postulated that Hyty and Ty were absorbed, metabolized and eliminated in urine in either free form or conjugated, mainly as glucuronides. Different metabolites of Hyty (sulfoconjugate, 3-O glucuronide conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenyl acetaldehyde (DOPAL), 3,4-dihydroxyphenylacetic acid (DOPAC)) were identified by analysis of radiolabelled metabolites after orally or intravenously dosing of the labelled Hyty [24,33]. Besides, Caruso et al. [34] described novel pathways of Hyty metabolism identifying for the first time homovanillic alcohol and homovanillic acid in human urine after ingestion of virgin olive oil.

Thus, literature data on the metabolism of olive oil phenolic compounds in humans are limited, and the majority of studies conducted in this area have been focused on Hyty, Ty and oleuropein. There are practically no reports about the bioavailability of other phenolic compounds described in olive oil, despite recent studies have demonstrated that individual phenolic fractions rich in lignans and especially in secoiridoids have a significantly stronger ability to decrease cell viability and the expression status of HER2 in different types of human breast carcinoma cells in comparison with simple phenols [11,35,36]. This lack of information is due to the absence of commercially available pure standards and the difficulty of developing sensitive method to detect the presumptively low concentrations of these compounds in biological systems.

Thus, the aim of the present study was to develop an analytical methodology based on RRLC-ESI-TOF MS to identify by first time, the urinary excretion of different families of phenolic compounds as free compounds or in conjugated form after ingestion of commercially available virgin olive oils, allowing to further elucidate the *in vivo* kinetics of the metabolites identified.

Experimental

Chemicals and samples

Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Fluka (Buchs, Switzerland) were used for preparing the mobile phases. For the extraction procedure, sodium acetate and methanol were purchased from Panreac (Barcelona, Spain), hydrochloric acid from Scharlau (Barcelona, Spain) and ethyl acetate from Lab-Scan (Dublin Ireland). Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). β-glucuronidase (type H-2 crude solution from *Helix pomatia*; 111000 U/mL of β-glucuronidase and 1079 U/mL of sulfatase) and taxifolin, used as internal standard (I.S.), were supplied by Sigma-Aldrich (St. Louis, MO, USA). A stock solution of taxifolin was prepared in methanol with a final concentration 100 µg/mL and stored at -20ºC until use. Synthetic urine, free of the tested compounds was purchased from Alltech Associates (Deerfield, IL, USA) and was prepared according to the manufacturer's instructions, dissolved in water.

Aiming to chose a very high-phenol olive oil for the intake, several olive oils were selected and analyzed and, finally a mixture of two extra virgin olive oils: 50% Arbequina Romanico, 50% Picual Señorío de Segura was used. The amount of phenolic compounds, for which we had available standards, was determined in this mixture in mg/kg: hydroxytyrosol: 8.31; tyrosol: 5.33; pinoresinol: 3.25; luteolin: 2.65; apigenin: 0.64; elenolic acid: 34.91; ligstroside aglycon: 40.58.

All chemical were analytical grade and used without further purification

Human experiments

Ten healthy volunteers (5 male and 5 female) with an age ranging between 24 and 35 years old participated in the intervention study. Subjects had an average weight of 71.8±11.5 kg (men, 81.2±7.3 kg, women 62.4±4.6 kg) and a body mass index of 24.4±2.9 (men, 26.2±2.7 women, 22.5±1.8). The study was approved by the ethical committee at the University of Granada. The subjects volunteered for the study and gave their written consent after receiving carefully information about the study. All volunteers followed a phenolic-free diet for 2 days (wash-out period), avoiding several foods and beverages from their diet (fruits, vegetables, cereals, nuts, honey, chocolate, coffee, tea, wine, beer, juices) and specifically excluding olive oil. On day 3 after the overnight fast, all subjects consumed 50 mL of olive oil with 30 g of bread (intake of

olive oil in the Mediterranean countries is estimated to be 30-50 mL/day [18]) between 8.00 and 8.30 a.m. at the laboratory. Subjects stayed at the laboratory the whole day and were allowed to drink only water (the same quantity for all volunteers) for 6 h in order to assure that olive oil was the only source of phenolic compounds. Urine samples were collected in special urine-collecting plastic bottles during the washout period (first void spot urine in the morning) and in three different time collection periods (at 0-2, 2-4, 4-6 h) after the ingestion. After the measurement of the urinary volume, the samples were immediately stored at -20ºC. A human urine sample obtained from fasted healthy individuals was prepared and used as a blank.

Extraction procedure

Urine is one of the easiest biological fluids to collect, but contains a high salt concentration that can easily interfere with the electrospray ionization process, so it is important to remove the electrolytes that are present prior to analysis. This is usually achieved in part by extraction into solvents, particularly those that are immiscible with water (ether or ethyl acetate).

2 mL of urine (pH~7) was acidified at pH 2.5 with 0.5 M HCl/MeOH (50/50, v/v) and a liquid-liquid extraction was carried out by addition to each sample of 3 mL ethyl acetate. After 10 min stirring in a vortex and 10 min centrifugation at 3500 r.p.m, the supernatant was evaporated to dryness. The dried sample was reconstituted in 100 µL of methanol. To assay the performance of the analytical determination (separation by liquid chromatography and detection by mass spectrometry) and to correct for variability, 10 µL of taxifolin (100 µg/mL), a flavanonol with similar structure to the compounds under study and that was not present in urine, was chosen as internal standard and added to the extract of the sample. Finally the extracts were stored at -20 ºC until analysis. The stability of the extracts was determined by injecting the same sample, stored at -20ºC, in consecutive days. Data proved to be rather consistent during at least 3 weeks.

Enzymatic hydrolysis

To verify the presence of the conjugated forms of polyphenols and their metabolites urine samples were subjected to enzymatic hydrolysis with deconjugating enzymes that hydrolyze glucuronides and sulphate ester.

2 mL of urine sample was adjusted to pH 5 by the addition of 800 µL 1M sodium acetate buffer. An aliquot of 45 µL of the crude enzymatic preparation (β-glucuronidase from *Helix Pomatia*), containing 5000 units of β-glucuronidase and 49 units of sulfatase, was added to the sample and incubated for 4 h at 37 ºC in a heating bath. After samples cooled to room temperature the pH was adjusted to pH 2.5 by the addition of 0.5 M HCl/MeOH (50/50, v/v) and the free aglycones were extracted with 3 mL of ethyl acetate, as described above.

The most suitable type of β-glucuronidase and the optimal incubation time for the enzymatic hydrolysis were investigated previously in literature [26].

Urines from the wash-out period were subjected to the same extraction procedures (with and without enzymatic hydrolysis) to establish basal background and to preclude possible interferences.

RRLC-ESI-TOF MS

Analyses were carried out operating on an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV-Vis (DAD) detector. The analytical column used for the separation of the compounds was a Zorbax C18 (4.6 x 150 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing, operating at 30 ºC and a flow rate of 0.8 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the separation was performed using the gradient elution program: 0 min, 95% A and 5% B; 10 min, 70% A and 30% B; 12 min, 67% A and 33% B; 17 min 62% A and 38% B; 20 min, 50% A and 50% B; 23 min, 5% A, 95% B; 25 min, 95% A and 5% B. Finally, the column was re-equilibrated for 10 min. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA) and degassed in an ultrasonic bath prior to HPLC analysis. A volume of 10 µL urine extracts was injected.

The RRLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). TOF analyzers provide greatly improved mass resolution (5,000–10,000 at 250 *m/z*) and significantly high sensitivity and accuracy. Due to the high flows of mobile phase used (0.8 mL/min) and in order to achieve a stable electrospray, a flow divisor 1:3 was used to reduce the flow

delivered into the mass spectrometer to 0.2 mL/min. According to this inflow, the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min, and dry gas temperature 190 ºC.

The transfer parameters of the mass spectrometer were similar to those previously optimized in recent works with the same families of phenolic compounds [11] acquiring spectra in the range of 50-800 *m/z* in negative mode. The negative ionization mode was found to be more sensitive than positive ionization mode as has been similarly described for other classes of phenolic compounds [37, 38]

SmartFormula^{TM} tool within DataAnalysis was used for the calculation of elemental composition of compounds; it lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulas, the TIP adds a second dimension to the analysis doing a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}). For routine screening, an error of 5 ppm and a threshold sigma value of 0.05 are generally considered appropriate.

The calibration of the MS was performed using sodium formate clusters by introducing externally a solution containing sodium hydroxide in the sheat liquid of some formic acid in water:isopropanol 1:1 v/v, for instance, with a pump at the beginning of the analysis (external calibration).

Statistic

All the datafiles were exported into mzXML format using Bruker Daltonics's DataAnalysis software. Only the chromatographic region from 1 to 29 min was used for further analysis.The alignment of chromatograms was performed using XCMS software (The Scripps Research Institute, La Jolla, USA) with default parameters [39]. The resulting tables included the detected ion features and their peak areas. As the primary interest was in metabolites of polyphenols, only those peaks were selected which were not present at 0 time point, but were present at later ones. These peaks were imported into Simca-P+ software package, version 12.0 (Umetrics, Umeå, Sweden) for further multivariate analysis. No normalization of the peak areas was performed firstly due to low systemic variance in the data (RSD of the total areas was within 30%) and secondly to avoid constrain effects of this type of normalization on data covariance structure [40].

Results and discussion

RRLC-ESI-TOF MS analysis

As a guideline for the optimization of the RRLC-ESI-TOF MS method for the determination of metabolites of olive oil polyphenols in human urine, we used conditions reported in previous studies [11]. Two different control samples were used for the method optimisation: synthetic urine and blank urine spiked with diluted olive oil extracts. Synthetic urine was used to ensure the absence of any endogenous compound that could interfere with the assay, and blank urines, obtained from volunteers after 2 days with free polyphenols diet and 12 h fasting, to make a closer approach to the real samples.

For the further optimisation and the evaluation of the extraction procedure the most representative components of the phenolic fraction of virgin olive oil (two analytes belonging to each family of phenolic compounds) were selected: simple phenols (Ty and Hyty), flavonoids (luteolin (Lut) and apigenin (Apig)), lignans (pinoresinol (Pin) and acetoxipinoresinol (Ac Pin)) and secoiridoids (Oleuropein aglycon (Ol Agl) and Ligstroside aglycon (Lig Agl)). Optimum separation with good response and peak shape were obtained for the eight compounds with both control samples. Even with the blank urine, the RRLC-ESI-TOF MS method provided a separation good enough among the eight polyphenols from interfering compounds present in the sample. Moreover, it is important to keep in mind that the application of mass spectrometry for the analysis of these analytes confers a high specificity and selectivity to the assay.

The extraction procedure was evaluated calculating recovery percentages of the main compounds by comparison between peak areas of every compound in the olive oil extract and those obtained from synthetic urine and blank urine spiked with the olive oil extract and extracted with ethyl acetate as explained above. Extraction efficiency ranged from 46.9 to 74.9% for the families of simple phenols, lignans and flavonoids, and were a bit lower (around 35%) for the family of secoiridoids (Table 1). The results were similar for both control samples (synthetic and blank urine), fact which indicates the specificity of the method with regard to the interference of endogenous compounds.

The precision of the assay was evaluated by three consecutive injections (n = 3) of the control samples in the same day (intra-day repeatability) and in three different days

(inter-day repeatability) obtaining values of relative standard deviation (RSD%) on the peak area ratio below 4.3 and 6.5% respectively. These RSD indicate that the proposed methodology is reproducible and suitable for carrying out these studies.

To evaluate the applicability of the method, all urine samples collected before (wash-out period) and after the olive oil consumption were analyzed. After each series of analysis, a quality control sample (synthetic urine spiked with an olive oil extract) was injected for assessing the performance of the chromatographic procedure in terms of retention time stability and signal sensitivity. Figure 1 shows the base peak chromatogram (BPC) of a human urine sample collected 2 hours after the oral administration with the optima RRLC-ESI-TOF MS conditions (a). It can be easily observed the complexity of this type of samples. In the same figure, it is possible to observe the extracted ion chromatograms (EIC´s) of some possible metabolites found in the human urine samples (b).

Discrimination of urine samples using statistical analysis

Due to the large number of samples in biological and medical research and their complexity (as shown in Fig. 1), full interpretation of the acquired data can be extremely difficult. For this purpose, chemometrics offers a variety of methods which allow investigation of the structure of all the data at once (e.g. Principal Component Analysis (PCA)) as well as discrimination and classification of the samples (e.g. Partial Least Squares (PLS), Artifitial Neural Networks (ANN) based methods).

There can be a number of processes that occur in the organism after the large intake of olive oil, which might be reflected in the composition of body fluids, but we have deliberately focused on metabolites of polyphenols. These metabolites should not be present at 0 time point, due to the low polyphenol diet that the subjects had before the experiment, and should appear at later time points. Thus among all the peaks picked by XCMS we selected those with no or low intensity at time point 0.

PCA was used to evaluate the quality of the data and observe its structure. A lot of the variation present in the data (75.4 %) is explained by the first two principal components as can be seen on the Fig. 2a. The samples before the intake are tightly clustered together which was expected with selection of peaks absent at this time point. Other samples are more scattered with 6 h time point tending to be closer to 0 time point,

which suggests that after 6 h the excretion of polyphenol metabolites declines and the organisms starts to return to its normal state. To justify this and to find the peaks that have most influence on the difference between samples at different time points batch PLS was used. The peaks, most influential for the model, can be selected on the basis of variable importance in the projection (VIP) scores. The list of these peaks with VIP>1 is given in Fig.2b.

Identification of the metabolites

In order to identify the compounds more responsible of the discrimination of the samples (biomarkers) and other possible metabolites, the urine profiles were studied in depth taking into account the polarity of the compounds and the knowledge about the possible organism's metabolism reactions. Furthermore, ESI-TOF MS analyzer provides information about accurate mass and isotopic pattern that allow obtaining a reduced number of possible molecular formula that then can be matched against available databases.

The chemical reactions of metabolism can be classified in *Phase I reactions* and *Phase II reactions*. Phase I reactions usually precede Phase II, though not necessarily, and the most important ones are oxidation, hydrogenation, hydration, decarboxilation, hydroxylation and methylation. Phase II reactions are usually known as conjugation reactions and include glucuronidation, sulfoconjugation, acetilation, glutamination.

To confirm the presence of the conjugated forms of polyphenols and their metabolites, analyses of the urine samples were conducted before and after enzymatic hydrolysis with deconjugating enzymes. It was possible to identify glucuronic form of the analytes, as well as other conjugated forms, like sulfated derivatives, because β-glucuronidase exhibits also limited sulfatase activity.

Using all the mentioned information, 60 metabolites could be tentatively identified in the urine profile. All metabolites are summarized in Tables 2 (phase I metabolites) and 3 (phase II metabolites) including retention time, *m/z* experimental, the mass error and sigma value (comparison of the theoretical and measured isotope pattern), molecular formula and a list of possible compounds. The compounds identified as biomarkers in the statistical analysis are indicated in bold. As can be seen all the compounds are phase I metabolites, specially those derived from deacetoxy oleuropein aglycon (DOA) and only one of them is a glucuronoconjugate.

Metabolites obtained through phase I reactions are shown in Table 2, classified by the type of metabolism reaction. Hydrogenation, hydroxylation, hydration and methylation were the reactions more frequently conducted, although the absorption, metabolism and excretion appeared to differ greatly between the various polyphenols. In Fig. 3, we can observe the extracted ion chromatograms (EIC´s) of some metabolites identified in table 2 during the wash-out period (I) and 2 hours after the intake of olive oil (II). Peaks corresponding to metabolites appeared at 0-2 h after the olive oil intake, whereas none of these compounds were detected during the wash-out period.

In general, the most abundant metabolites found in human urine came from compounds of the family of secoiridoids (Ol Agl, DOA, Lig Agl and deacetoxy ligstroside aglycon (D-Lig Agl)) that were the most likely absorbed and metabolized. There are no available information in literature about the absorption and metabolism of these compounds and, so far, it was thought that Ol Agl and Lig Agl may be hydrolyzed in the gastrointestinal tract and the resulting polar phenols, Hyty and Ty, absorbed and metabolized [30]. However, the results obtained in the present trial suggest that, at least, part of secoiridoids could be absorbed, metabolized and excreted in urine. One of the most probable pathways for the metabolism of these compounds was the hydrogenation. The elenolic acid (EA), compound which is part of the molecular structure of the secoiridoids, was also subjected to this hydrogenation reaction which could explain the presence of the hydrogenated metabolites of the secoiridoids. Some previous authors failed to detected EA metabolites in rat urine after olive oil ingestion [31]. In the present study the hydrogenated metabolite of EA appeared with high intensity and was identified in the statistical analysis as one of the possible biomarkers. Its determination results very interesting and it could be rather useful as a biomarker because it is not present in other food sources as, for instance, Hyty or Ty.

Hydroxylation and hydration were also common pathways for the secoiridoids, especially for Ol Agl and DOA (compounds containing in their structures a molecule of Hyty). Some doubts appeared about Lig Agl and D-Lig Agl (compounds containing in their structures a molecule of Ty) because the *m/z* values of their possible hydroxylated and hydrated metabolites matched up with those of other metabolites of DOA and Ol Agl. Taking into account the information available in literature about the limited diffusion into the cell of compounds with a lower degree of hydroxylation -such as Ty and their derivatives [41] - the most adequate way of interpretation it would be that these masses correspond to Hyty derivates. However with the techniques used in this

study it was not possible to confirm this information. Anyway, we have indicated in the Table 2 all the possibilities. Small amounts of hydroxylated and hydrated metabolites of hydroxytyrosol acetate (Hyty-Acet) were detected as well.

Methylation was the preferential pathway for Hyty and we could also find the methyl conjugate of DOA with high intensity. The results indicate that these compounds underwent the action of catechol-O-methyl transferase (COMT), enzyme involved in the catecholamine metabolism. Other phase I reactions, mainly hydroxylation and hydration, could precede or follow the action of COMT on compounds as Hyty, DOA and Ol Agl. In this way, metabolites as methyl Hyty + OH, methyl Ol Agl + OH, methyl DOA + OH, methyl Ol Agl + H_2O and methyl DOA + H_2O were found in the urine. Some compounds even suffered a double hydroxylation before or after the methylation (see Table 2). The presence, although in low quantities, of MOPAL (Methyl Hyty – H_2) and homovanillic alcohol (HVA) (methyl Hyty- H_2 + OH) previously identified in literature [24, 32, 34] implies a sequential oxidation of methyl-Hyty ethanol side chain catalyzed by alcohol and aldehyde dehydrogenase, respectively. In the same pathway of HVA, a compound with $[M-H]^-$ 347.1158 was tentatively identified as (Methyl DOA – H_2 + OH).

It is the first time that the presence of methylated derivatives, different from alcohol homovanillic, are studied and found in urine samples. No methyl conjugates of Ty and derivatives compounds (Lig agl and D-Lig Agl) were observed as could be expected, since methylation by COMT requires an ortho-diphenolic structure, absent in these compounds [41].

It is worth noting that the secoiridoid DOA participated in all the phase I reactions described in our study and all its metabolites showed up with high intensity. Besides, most of them (DOA + H_2, DOA- H_2, methyl DOA+ OH, DOA + H_2O, methyl DOA+ H_2O and DOA + CH_3) were identified as biomarkers of the olive oil intake.

The compounds in Table 3 are phase II metabolites, most of them identified as glucuronoconjugates. The identity of these compounds was confirmed with enzymatic hydrolysis, showing the disappearance of the peaks suspected of being glucuronoconjugates and the parallel increase of the corresponding unconjugated compounds (free polyphenols or phase I metabolites). Figure 4 shows, as an example, the extracted ion chromatograms (EIC´s) of three compounds identified as glucuronides

(on the left) and the corresponding unconjugated compounds obtained after the enzymatic hydrolysis (on the right).

As an example, the chromatographic peaks with $[M-H]^-$ 329.0868 disappeared after treatment of the urine samples with β-glucuronidase with the peak $[M-H]^-$ 153.0562 increasing in size, assuming that these peaks posses a glucuronide group. Both peaks showed the same $[M-H]^-$ ion, 329.0868 and were tentatively identified as monoglucuronidated metabolite of Hyty, although we could not distinguish between the structural isomers [41]. Many of the glucuronoconjugated compounds showed more than one peak with exactly the same $[M-H]^-$ (isomers). Similarly, peaks with $[M-H]^-$ 313.0929 and 555.1719 disappeared after hydrolysis with β-glucuronidase and were tentatively identified as glucuronides of Ty and Ol Agl + H_2 respectively. However, the position of the substituents on the aromatic ring was not possible to confirm with the technique used in this study and it would be necessary to use nuclear magnetic resonance spectroscopy (NMR) to get more structural information.

It is worth noting the presence of glucuronoconjugates of compounds belonging to the most representative phenolic families described in olive oil: simple phenols (Hyty, Ty and Hyty-Acet), secoiridoids (DOA, Ol Agl, D-Lig Agl and Lig Agl), and lignans (Pin, and Ac pin). To the best of our knowledge, the metabolism of lignans has not been reported in any detail and one of the few references has been developed recently [42]. In that study, pinoresinol-glucuronide was identified after incubation of pinoresinol using differentiated Caco-2/Tc7 cells monolayers as a model of the human intestinal epithelium.

As far as flavonoids are concerned, the product of both methylation and glucuronidation were observed and small peaks with *m/z* 459.0975 and 475.0868 were tentatively identified as methyl-monoglucuronides of apigenin and luteolin, respectively. The latter mentioned metabolite, methyl-luteolin glucuronide was also identified by Soler et al [42]. Although new metabolites were identified in the intestinal epithelial cells, they showed limited metabolism of olive oil phenolics, and it is most likely that glucuronides are products of hepatic metabolism [41, 42].

Besides, almost all the phase I metabolites identified in Table 2 were subsequently subjected to glucuronoconjugation reactions and new glucuronoconjugates also appeared, especially those related with the Ol Agl (Ol Agl – H_2 + glucuronide, Ol Agl + CH_3 + glucuronide, Mehtyl Ol Agl - H_2 + glucuronide).

Sulfated metabolites of the main olive oil phenols studied were not found in human urine (with the exception of Hyty- sulfoconjugate). This is in agreement with some previous studies in humans [27- 30] and *in vitro* studies with HepG2 cells [41] in which methylated and glucuronidated metabolites were the main conjugated observed.

Few metabolites in low amount of some compounds ingested (ty, luteolin, apigenin, pinoresinol, acetoxypinoresinol) were observed in urine samples. This could be because these compounds were poorly absorbed, excreted with the faeces, destroyed in the gut, accumulated in organs, or excreted through another metabolic pathway [17]

Excretion kinetics of polyphenols in human urine

The excretion kinetics of the compounds identified as biomarkers in human urine after the olive oil intake was followed along six hours. Figure 5 clearly illustrates the metabolic pattern of the compounds under study, showing the average results from the ten volunteers expressed as the amount excreted in μg-equivalents of taxifolin (I.S.). Due to the absence of pure standards, the metabolites were quantified on the basis of the response factors of I.S., which was also used to correct any possible deviations. The results indicated that the majority of the metabolites studied followed the same kinetic, reaching maximum concentrations in the human urine at 2 hours after the administration, except for the compound with $[M-H]^-$ 349.1283 (methyl DOA + OH) which appeared with higher intensity at 4 h. In the samples taken 6 h after the large intake of oil, a fast decrease of the intensity of the compounds was observed with a trend toward basal conditions. These results are in good agreement with previous studies about the kinetic of Hyty and Ty, where the maximum concentrations of the metabolites were reached in the first 2-4 h after the intake [28, 29].
Furthermore, during this period, the concentration of the compounds fluctuated widely with the individual, reflected as high SD value; but no significant difference in urinary recovery could be observed between men and women for any of the polyphenols analyzed. The basal levels of the studied metabolites found in the blank urine samples and in the wash-out period were practically zero.

Conclusions

A direct and reproducible RRLC-ESI-TOF MS method was developed to carry out the exploratory analysis of human urine after high intake of olive oil. Tentative identification of more than 60 metabolites of olive oil polyphenols was achieved by using the information obtained from the statistical analysis, the different metabolism reactions, polarity of the compounds, enzymatic hydrolysis and the valuable information from the time-of flight mass analyzer.

In other works previously developed specific information about the type of conjugates was not provided, and the authors were mainly focused on the determination of metabolites of Hyty, Ty and oleuropein. This is the first report in which metabolites of practically all the compounds described in olive oil have been found in human urine samples, suggesting that most of the compounds are absorbed to a greater or lesser extent. However, absorption and metabolism appeared to differ greatly between the various polyphenols, and the most abundant metabolites came from phenolic compounds containing a catechol group, such as Hyty and the secoiridoids Ol Agl and DOA. Phenolic compounds were subjected to different Phase I and Phase II reactions, but the most common metabolic reactions were methylation and glucuronidation.

Statistical analysis allowed us to discriminate between the urine samples and to identify at least ten possible metabolites (mainly derived from DOA) that could be used as biomarkers of olive oil intake. The developed method was also successfully applied to monitor the levels of these biomarkers in human urine after the intake of olive oil, and the results indicated that the highest level of most compounds was detected at 2h after administration.

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Figure Captions

Fig. 1 (a) Base peak chromatogram (BPC) of a human urine sample (2 h after the intake of olive oil) analyzed using the optima RRLC-ESI-TOF MS method. (b) A more detailed view of this BPC highlighting in grey all possible metabolites found in the human urine 2 h after the olive oil intake

Fig. 2 (a) Results of the multivariate statistical analysis of urine samples after the intake of olive oil. PCA scores plot, first two principal components cover 63.9% and 11.5% of the variation respectively. (b) m/z values and retention times of the possible biomarkers of the olive oil intake obtained by statistical analysis.

Fig. 3 Extracted ion chromatograms (EIC´s) of the main Phase I metabolites identified in human urine samples during the wash-out period (I) and 2 h after oral administration of 50 ml of olive oil (II). The numbering of the peaks in this figure refers to the numbering of the compounds in Table 2

Fig. 4 (a) Extracted ion chromatograms (EIC´s) of three compounds identified as glucuronides (on the left) and the corresponding unconjugated compounds obtained after the enzymatic hydrolysis (on the right). (I) samples in the was-out period (II) 2 h after oral administration (III) 2 h after oral administration and with the enzymatic hydrolysis (IV) samples in the washout period subjected to the same enzymatic hydrolysis. The numbering of the peaks in this figure refers to the numbering of the compounds in Table 3

Fig. 5 Excretion kinetics of the main biomarkers identified in the human urine after the olive oil intake. Bars represent the average of data obtained from ten individuals expressed as μg excreted of I.S during each time period and errors (SD) are expressed as line on the top of each bar. Concentrations at wash-out period (urine collected just before oil administration) were practically zero.

Table 1 Extraction recoveries of the main phenolic compounds identified in olive oil from synthetic and blank urine samples spiked with diluted extracts of olive oil. Samples were analyzed by RRLC-ESI-TOF MS and recoveries calculated as described in the experimental section. Values are means from three independent experiments.

Compounds	m/z	Recovery %	
		Blank urine	Synthetic urine
1. Hydroxytyrosol	153.0557	51.2±	46.9±
2. Tyrosol	137.0608	60.4±	56.1±
3. Luteolin	285.0405	74.9±	66.5±
4. Pinoresinol	357.1344	58.5±	64.1±
5. Acetoxipinoresinol	415.1398	69.3±	69.7±
6. Apigenin	269.0455	73.4±	63.7±
7. Oleuropein aglycon	377.1242	35.1±	37.3±
8. Ligstroside aglycon	361.1293	40.4±	46.5±

Table 2 Phase I metabolites, classified by the metabolism reaction, identified in human urine 2 h after the intake of olive oil

Phase I reactions		**Retention time (min)**	**m/z experimental**	**error ppm**	**sigma**	**Molecular Formula**	**Posible Compounds**
Hydrogenation (+ H_2)	**1**	**11.308**	**243.0868**	**2.3**	**0.004**	**$C_{11}H_{15}O_6$**	**EA + H_2**
	2	**14.084**	**321.1344**	**-2.3**	**0.014**	**$C_{17}H_{21}O_6$**	**DOA + H_2**
	3	15.355	305.1397	-0.9	0.107	$C_{17}H_{21}O_5$	D- Lig Agl + H_2 *
	4	14.686	379.1410	-3.1	0.029	$C_{19}H_{23}O_8$	Ol Agl + H_2 (isomer)
	5	**19.720**	**363.1455**	**-1.5**	**0.021**	**$C_{19}H_{23}O_7$**	**Lig Agl + H_2**
Dehydrogenation (-H_2)	**6**	**16.860**	**317.1027**	**1.0**	**0.019**	**$C_{17}H_{17}O_6$**	**DOA-H_2**
Hydroxylation (+ OH)	7	10.650	211.0615	-1.2	0.063	$C_{10}H_{11}O_5$	Hyty-Acet + OH *
	8	13.710	335.1132	1.2	0.052	$C_{17}H_{19}O_7$	DOA + OH
	9	16.493	319.1188	-0.3	0.008	$C_{17}H_{19}O_6$	D-Lig Agl + OH = DOA
	10	**16,660**	**393.1191**	**0.1**	**0.004**	**$C_{19}H_{21}O_9$**	**Ol Agl + OH**
	11	19.336	377.1248	-1.7	0.037	$C_{19}H_{21}O_8$	Lig Agl + OH = Ol Agl
Hydration (+ H_2O)	12	7.562	213.0768	4.8	0.063	$C_{10}H_{13}O_5$	Hyty- Acet + H_2O *
	13	**12.495**	**337.1282**	**3.3**	**0.007**	**$C_{17}H_{21}O_7$**	**DOA + H_2O**
	14	**14.084**	**321.1344**	**-2.3**	**0.014**	**$C_{17}H_{21}O_6$**	**D-Lig Agl + H_2O = DOA + H_2**
	15	14.686	379.1410	-3.1	0.029	$C_{19}H_{23}O_8$	Lig Agl + H_2O = Ol Agl + H_2
	16	16.626	395.1361	-3.4	0.080	$C_{19}H_{23}O_9$	Ol Agl + H_2O
Methylation (+ CH_3)	17	10.639	167.0716	-1.3	0.164	$C_9H_{11}O_3$	Hyty + CH_3 *
	18	**19.326**	**333.1345**	**-0.3**	**0.046**	**$C_{18}H_{21}O_6$**	**DOA + CH_3**
Methylation + others reactions	19	8.532	185.0817	1.5	0.057	$C_9H1_3O_4$	Methyl Hyty + OH
	20	10.121	165.0543	8.6	0.325	$C_9H_9O_3$	Methyl Hyty - H_2 (mopal) *
	21	11.342	181.0516	-5.4	0.228	$C_9H_9O_4$	Methyl Hyty- H_2+OH (HVA) *
	22	13.164	365.1242	0.1	0.013	$C_{18}H_{21}O_8$	Methyl DOA + 2 OH *
	23	**14.319**	**351.1444**	**1.5**	**0.015**	**$C_{18}H_{23}O_7$**	**Methyl DOA + H_2O**
	24	14.890	423.1315	-4.3	0.112	$C_{20}H_{23}O_{10}$	Methyl Ol Agl + 2 OH *
	25	14.904	409.1520	-3.8	0.022	$C_{20}H_{25}O_9$	Methyl Ol Agl + H_2O
	26	**14.921**	**349.1283**	**2.8**	**0.018**	**$C_{18}H_{21}O_7$**	**Methyl DOA + OH**
	27	14.971	347.1158	-6.2	0.149	$C_{18}H_{19}O_7$	Methyl DOA- H_2 + OH
	28	19.720	407.1355	-1.9	0.019	$C_{20}H_{23}O_9$	Methyl Ol Agl + OH

* means compounds present in small traces.
We represent in bold compounds found out in the statistical analysis responsible of the discrimination between urine samples before and after the intake of olive oil (biomarkers)

Table 3 Phase II metabolites, classified by the conjugation reaction, identified in human urine 2 h after the intake of olive oil.

Phase II reactions		Retention time (min)	m/z experimental	error ppm	sigma	Molecular Formula	Posible Compounds
Glucuronidation	1	5.672-6.107	329.0868	3.1	0.030	$C_{14}H_{17}O_9$	Hyty- glucuronide
	2	5.756	313.0912	5.3	0.012	$C_{14}H_{17}O_8$	Ty- glucuronide
	3	10.071,10.355	371.1071	-1.4	0.128	$C_{16}H_{19}O_{10}$	Hyty- Acet- glucuronide
	4	13.198	495.1497	2.2	0.130	$C_{23}H_{26}O_{12}$	DOA- glucuronide *
	5	11.709	533.1639	4.7	0.053	$C_{26}H_{30}O_{12}$	Pin- glucuronide
	6	12.04,12.362	591.1705	2.4	0.160	$C_{28}H_{31}O_{14}$	Ac Pin- glucuronide *
	7	12.914	479.1687	-14.2	0.093	$C_{23}H_{26}O_{11}$	D- Lig Agl- glucuronide
	8	13.014	553.1550	2.2	0.061	$C_{25}H_{29}O_{14}$	Ol Agl- glucuronide
	9	13.399	537.1626	-2.4	0.070	$C_{25}H_{29}O_{13}$	Lig Agl- glucuronide
Hydrogenation + Glucuronidation	10	10.907,11.592	497.1651	2.7	0.011	$C_{23}H_{29}O_{12}$	DOA + H_2- glucuronide
	11	12.830	481.1715	1.5	0.017	$C_{23}H_{29}O_{11}$	D-Lig Agl + H_2- glucuronide
	12	**13.064, 13.449**	**555.1709**	**1.8**	**0.006**	**$C_{25}H_{31}O_{14}$**	**Ol Agl+ H_2- glucuronide**
	13	13.551,14.723	539.1766	0.7	0.013	$C_{25}H_{31}O_{13}$	Lig Agl + H_2- glucuronide
Dehydrogenation + Glucuronidation	14	10.572,10.288	327.0713	2.5	0.019	$C_{14}H_{15}O_9$	Hyty- H_2- glucuronide
	15	14.904	551.1386	3.7	0.084	$C_{25}H_{27}O_{14}$	Ol Agl – H_2- glucuronide
Hydroxylation + glucuronidation	16	7.512	387.0918	3.8	0.041	$C_{16}H_{19}O_{11}$	Hyty-Acet + OH- glucuronide *
	17	13.750	569.1538	-4.6	0.077	$C_{25}H_{29}O_{15}$	Ol Agl + OH- glucuronide
Hydration + glucuronidation	18	10.723	513.1597	3.3	0.016	$C_{23}H_{29}O_{13}$	DOA + H_2O- glucuronide
	19	11.158	571.1649	3.4	0.037	$C_{25}H_{31}O_{15}$	Ol Agl + H_2O- glucuronide *
Methylation + glucuronidation	20	6.425-7.328	343.1022	3.6	0.023	$C_{15}H_{19}O_9$	Hyty + CH_3- glucuronide
	21	12.763	475.0868	3.0	0.124	$C_{22}H_{19}O_{12}$	Luteolin + CH_3- glucuronide *
	22	17.262	459.0975	3.6	0.044	$C_{22}H_{19}O_{11}$	Apigenin + CH_3- glucuronide
	23	13.315	567.1719	1.2	0.115	$C_{26}H_{31}O_{14}$	Ol Agl + CH_3- glucuronide *
Methylation + other reactions+ glucuronidation	24	6.040,7.261	357.0809	5.0	0.098	$C_{15}H_{17}O_{10}$	Methyl Hyty-H_2+OH-glucuronide*
	25	7.913, 8.465	341.0878	-1.6	0.095	$C_{15}H_{17}O_9$	Methyl Hyty- H_2- glucuronide*
	26	11.308, 11.776	527.1757	2.4	0.072	$C_{24}H_{31}O_{13}$	Methyl DOA + H_2O- glucuronide
	27	11.408	541.1553	1.9	0.070	$C_{14}H_{29}O_{14}$	Methyl DOA +2 OH- glucuronide
	28	11.709	525.1638	-4.6	0.173	$C_{24}H_{29}O_{13}$	Methyl DOA + OH- glucuronide*
	29	13.466, 14.250	569.1905	-5.1	0.007	$C_{26}H_{33}O_{14}$	Methyl Ol Agl + H_2- glucuronide
	30	14.268, 14.954	583.1642	4.6	0.037	$C_{26}H_{31}O_{15}$	Methyl Ol Agl + OH- glucuronide
	31	15.288	565.1563	7.3	0.147	$C_{26}H_{29}O_{14}$	Methyl Ol Agl- H_2- glucuronide *
Sulfoconjugation	32	6.559	233.0125	0.1	0.006	$C_8H_9O_6S$	Hyty -sulfate

* means compounds present in small traces.
We represent in bold compounds found out in the statistical analysis responsible of the discrimination between urine samples before and after the intake of olive oil (biomarkers)

Fig. 1 (a) Base peak chromatogram (BPC) obtained from a human urine sample (2 h after the intake of olive oil) analyzed using the optimum RRLC–ESITOF MS method. (b) A more detailed view of this BPC highlighting in grey all possible metabolites found in the human urine 2 h after olive oil intake

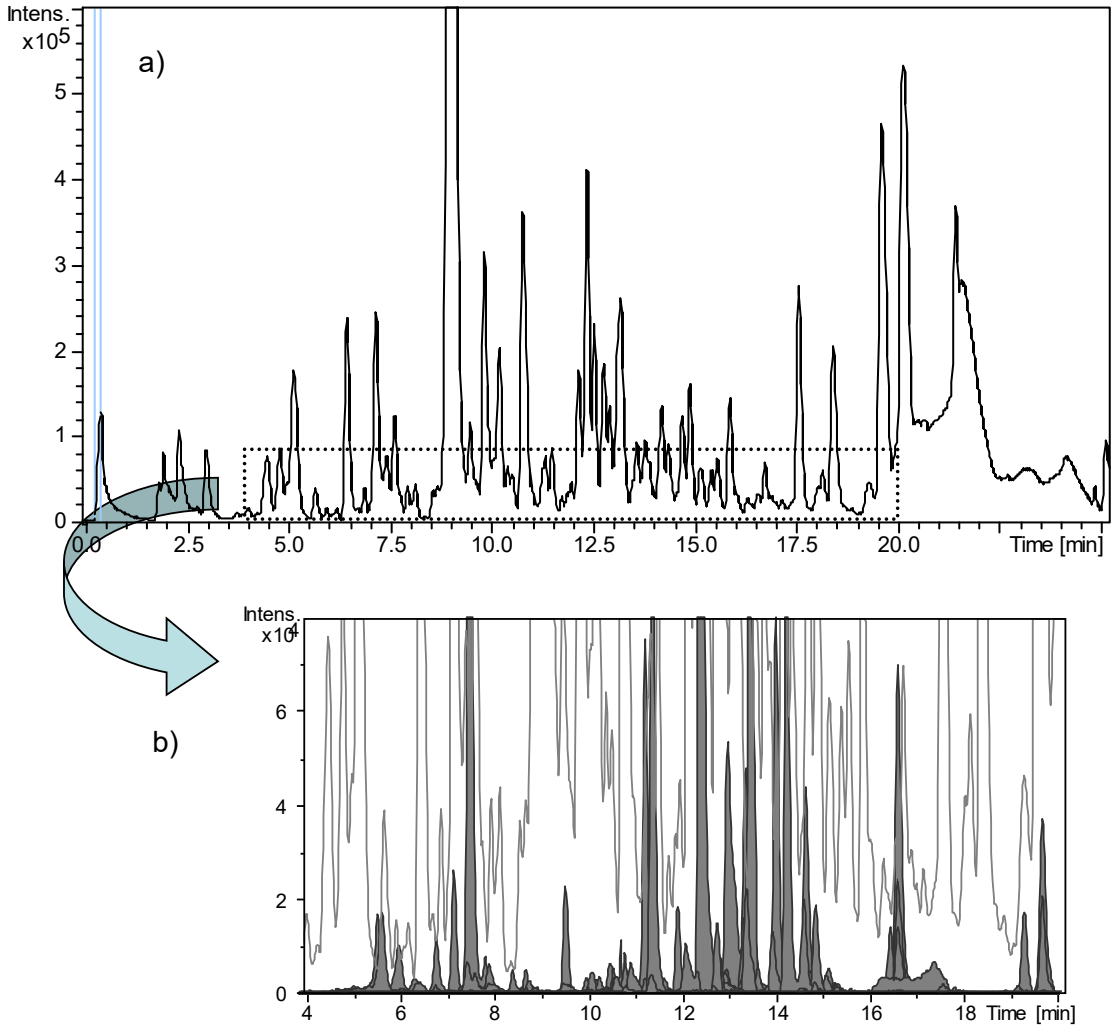
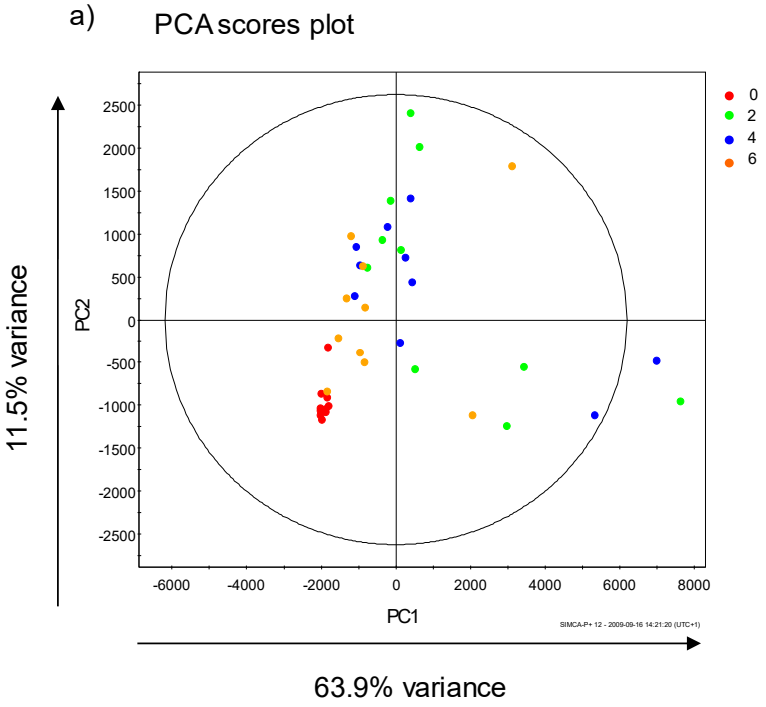


Fig. 2 Results from multivariate statistical analysis of urine samples after the intake of olive oil. (a) PCA scores plot, first two principal components cover 63.9% and 11.5% of the variation respectively. (b) Batch PLS scores plot of urine samples mapped across the time after intake. Dashed horizontal lines show two and three standard deviations for the dataset. (c) m/z values and retention times of the possible biomarkers of olive oil intake obtained by statistical analysis

a)



b)

m/z	Retention time, min
393.119	16.70
349.128	14.96
321.134	14.12
243.086	11.37
434.223	14.34
337.128	12.54
229.076	8.56
391.205	19.33
363.145	19.83
431.203	13.96
555.170	13.51
351.144	14.43
445.255	22.88
181.055	16.99
457.212	15.59
317.102	16.97
322.144	14.19
459.109	16.53
333.134	19.48
414.231	22.79

Fig. 3 Extracted ion chromatograms (EICs) of the main Phase I metabolites identified in human urine samples during the wash-out period (I) and 2 h after oral administration of 50 mL of olive oil (II). The numbering of the peaks in this figure refers to the numbering of the compounds in Table 2.

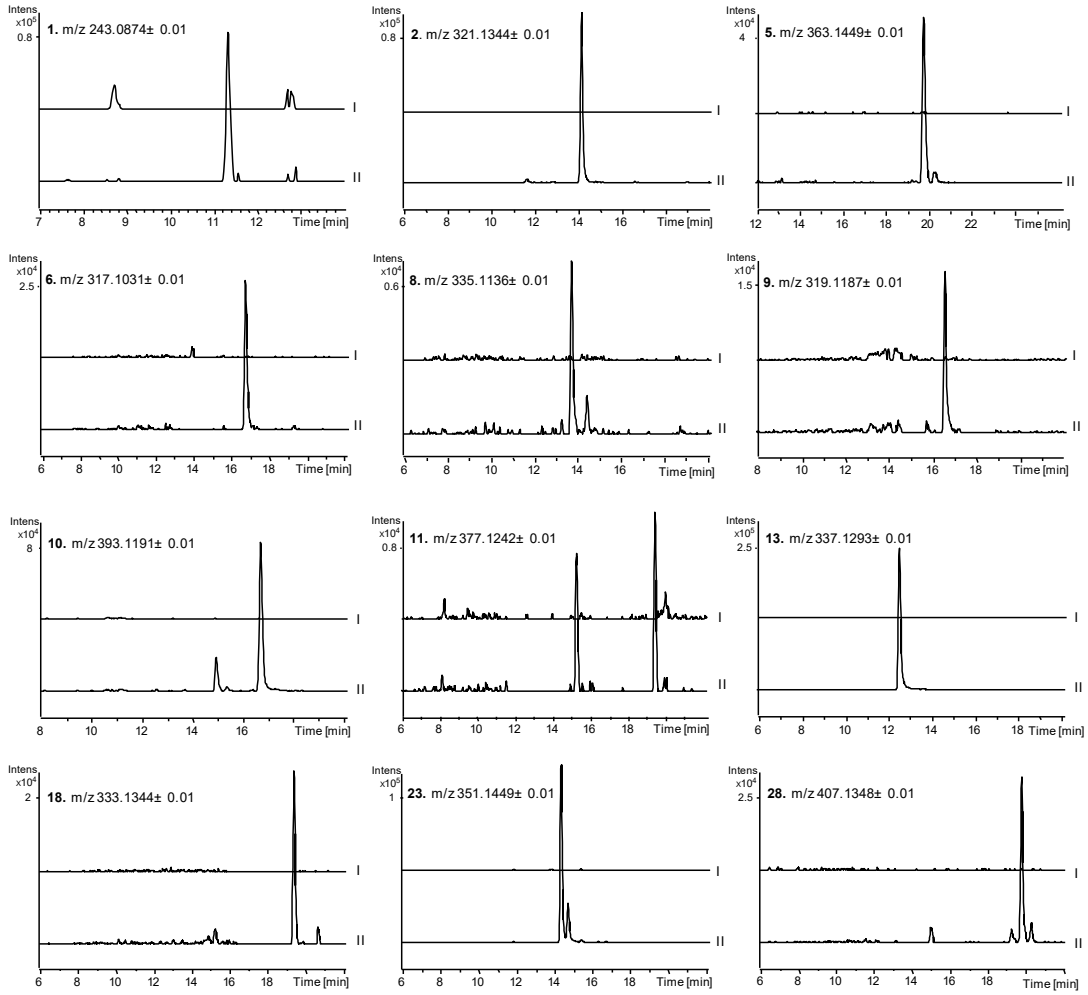


Fig. 4 (a) Extracted ion chromatograms (EICs) of three compounds identified as glucuronides (on the left) and the corresponding unconjugated compounds obtained after the enzymatic hydrolysis (on the right). (I) samples in the wash-out period, (II) 2 h after oral administration, (III) 2 h after oral administration and with the enzymatic hydrolysis, (IV) samples in the washout period subjected to the same enzymatic hydrolysis. The numbering of the peaks in this figure refers to the numbering of the compounds in Table 3

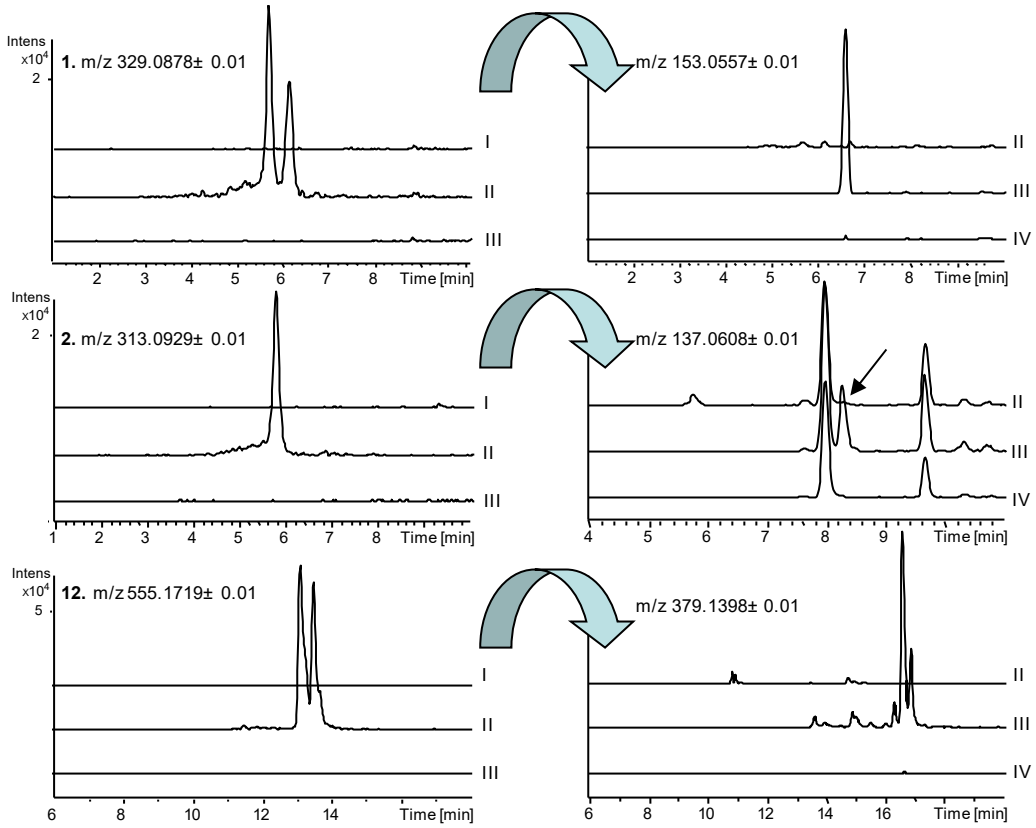


Fig. 5 Excretion kinetics of the main biomarkers identified in the human urine after the olive oil intake. Bars represent the average of data obtained from ten individuals expressed as µg excreted of I.S during each time period, and errors (SD) are expressed as the line on the top of each bar. Concentrations at wash-out period (urine collected just before oil administration) were practically zero