# High resolution mass spectrometric analysis of secoiridoids and metabolites as biomarkers of acute olive oil intake – an approach to study inter-individual variability in humans

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**Keywords:** bioavailability; metabolites; olive oil; phenolic compounds; secoiridoids.

**Abbreviations: CAE**, caffeic acid equivalents; **3,4-DHPEA – EA**, (3,4-dihydroxyphenyl)ethanol linked to elenolic acid; **3,4-DHPEA – EDA**, dialdehydic form of deacetoxy of oleuropein aglycon; **EVOO**, extra virgin olive oil; **HRMS**, High resolution mass spectrometry; **OO**, olive oil; ***p*-HPEA-EA**, *p*-hydroxyphenyl)ethanol linked to elenolic acid; ***p*-HPEA – EDA**, dialdehydic form of deacetoxy of ligstroside aglycon; **PLS-DA**, Partial Least Squares Discriminant Analysis; **OSC-PLS-DA**, Partial Least Squares Discriminant Analysis with Orthogonal Signal correction; **VOO**, virgin olive oil.

**Abstract**

Scope: Phenolic compounds are minor components of extra virgin olive oil (EVOO). Secoiridoids are the major components contributing to the phenolic content of EVOO. Information is lacking regarding their potential as biomarkers for EVOO intake.

Methods and results: Healthy volunteers (*n*=9) ingested 50 mL of EVOO in a single dose containing 322 mg/kg total phenolic content (caffeic acid equivalents) and 6 mg/20g hydroxytyrosol and its derivatives. Plasma was collected before (0h) and at 0.5, 1, 2, 4 and 6h after ingestion. Urine samples were collected prior to ingestion (0h) and at 0-4, 4-8, 8-15 and 15-24h. Samples were analysed by UPLC coupled with an Exactive Orbitrap MS. Partial Least Squares Discriminant Analysis with Orthogonal Signal Correction was applied to screen for metabolites that allowed samples discrimination. Plasma biomarkers and urine biomarkers were selected although individual variability was observed among volunteers. Results are in accordance with *in vitro* experiments performed (*in vitro* digestion and hepatic microsomal activity assays).

Conclusions: plasma (elenolic acid + $H_2$; *p*-HPEA-EA + $H_2$ + glucuronide) and urinary (3,4-DHPEA-EA, 3,4-DHPEA-EA + $H_2$+glucuronide, methyl 3,4-DHPEA-EA + $H_2$+glucuronide) secoiridoid compounds were selected as biomarkers to monitor EVOO intake showing good predictive ability according to multivariate analysis.

## Introduction

The major source of fat intake in the Mediterranean diet is olive oil (OO) and its consumption has been linked with increased longevity and a reduced frequency of chronic diseases, in particular coronary heart disease [1-5]. A European Food Safety Authority claim for OO reports that a daily consumption of 20g of OO containing at least 5 mg of hydroxytyrosol and its derivatives can protect low density lipoproteins particles from oxidative damage [6, 7]. These compounds are minor components of virgin olive oil (VOO) [8-10] and belong to the phenolic compounds family which main classes in VOO are: phenolic acids, phenolic alcohols, flavonoids, lignans and secoiridoids. The most abundant secoiridoids are the oleuropein and ligstroside aglycones - (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) and (*p*-hydroxyphenyl)ethanol (*p*-HPEA) linked to elenolic acid (EA), respectively. In addition, there are also dialdehydic forms of deacetoxy of oleuropein and ligstroside aglycons (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively) [8]. Phenolic content in VOO range from 40 to 900 mg/kg expressed as caffeic acid equivalents (CAE) [8, 11].

The digestion of VOO produces a micellar solution, with reported contradictory results on the stability of the major VOO phenolics (tyrosol, hydroxytyrosol and secoiridoids 3,4-DHPEA-EDA and *p*-HPEA-EDA) in the acidic environment of the stomach. While some identify a good stability in terms of hydrolysis [12, 13], others report that 3,4-DHPEA-EDA undergoes a rapid hydrolysis in a gastric acidic environment [14, 15] leading to an increase of hydroxytyrosol in the lumen, and consequently in plasma via passive diffusion [16, 17].

The *in vivo* effects of VOO phenolic compounds such as hydroxytyrosol are linked to their bioavailability [18] which depends on bioaccessibility, itself a function of the food matrix. A lipid matrix, contributes to increased hydroxytyrosol bioavailability compared to an aqueous matrix or a low fat yogurt [19-21], possibly via decreased rate of gastric emptying [20] and slow release of hydroxytyrosol from the lipid matrix [20, 22].

After absorption the phenolic compounds undergo phase I/II biotransformation [11, 18]. The majority of this metabolism occurs in the liver, namely in the smooth endoplasmic reticulum of the hepatocytes due to the enzymes contained in the microsomes. Phase II conjugation

processes of methylation, glucuronidation and sulfation have been described for VOO phenolic compounds [22-25].

VOO phenolic compounds are mainly absorbed in the small intestine [26] via passive diffusion [16], although absorption via intestinal membrane carriers might be also involved [16, 27]. Phenolic compounds that are not absorbed in the small intestine, such as secoiridoids, are degraded by the colonic microbiota [15].

Bioavailability of VOO phenolic compounds is affected by host factors, namely age and genomic profile, enzymatic activity or colonic microflora [16, 28]. In addition, in animal studies gender appeared as a factor conditioning bioavailability of hydroxytyrosol derivatives, related with enterohepatic circulation, and longer persistence of metabolites in organisms [27].

Although secoiridoids are the most abundant and complex family of phenolic compounds in VOO, their bioavailability has been poorly studied, in part because the main products of their metabolism (via hydrolysis) are hydroxytyrosol and tyrosol. However, hydroxytyrosol is not an ideal marker of compliance of VOO intake as it is present endogenously as a metabolite of the physiological neurotransmitter dopamine and can be produced after ingestion of red wine [29]. It is therefore difficult to reduce or control hydroxytyrosol concentration in biological fluids before interventions with VOO [18]. This warrants further research into the use of secoiridoids as biomarkers for VOO intake.

To obtain relevant information from metabolomics data, multivariate analysis is used [30] since, unlike univariate analysis, this approach can handle a large number of variables without requirement for the variables to be independent. Supervised methods, such as Partial Least Squares Discriminant Analysis (PLS-DA), are powerful tools in the search for markers that differ most between classes. The knowledge about the class to which a sample belongs to is used to select variables that are considerably different between groups of samples and which may be the candidate biomarkers. These approaches have been discussed in detail elsewhere [31, 32].

The primary aim of this study was to characterize, in human subjects, the bioavailability and the inter-individual variability of secoiridoids and their metabolites after an acute extra virgin olive oil (EVOO) intake. Other typical phenolic compounds in EVOO such as phenolic acids, phenolic alcohols, flavonoids, lignans, and their metabolites, were also monitored in the samples to ascertain whether any of these could also be used to study EVOO intake. The phenolic composition of EVOO was determined by LC-MS/MS. UPLC coupled with HRMS using the Orbitrap technology was used to overcome limitations of low resolution MS techniques as several isomeric forms of the oleuropein and ligstroside aglycones have been reported [33-36]. PLS-DA was applied for data analysis to search for markers that differ most between classes (e.g.: time collection point of sample) and allow validation of class spacing numerically [37]. Digestion and hepatic microsomal *in vitro* models were also used to evaluate the impact of digestion and microsomal enzymatic activity on EVOO secoiridoids. To the best of our knowledge this is the first study on HRMS simultaneous screening of EVOO phenolic compounds and metabolites in plasma and urine after an acute intake of EVOO.

## Materials and Methods

### *Materials*

Extra virgin olive oils used for human and *in vitro* assays were supplied by a Portuguese OO producer. Reference standards used were: apigenin, luteolin and tyrosol (≥99%), hydroxytyrosol (≥98%), and oleuropein (≥90%) purchased from Extrasynthese (Genay, France); enterolactone (≥90%), enterodiol and pinoresinol (≥95%) and caffeic acid (≥98%) purchased from Sigma-Aldrich (Sigma, USA). Bile salts, DMSO, pancreatin, pepsin and sodium bicarbonate were supplied from Sigma-Aldrich (Sigma, USA). HPLC grade, ACN, methanol, water, chloridric and formic acid *p.a*. were purchased from Carlo Erba (Italy). HPLC grade acetic acid and sodium carbonate were supplied from Panreac (Spain). Folin–Ciocalteu reagent was supplied

from Fluka (Switzerland). Rat pooled liver microsomes and NADPH-regenerating systems were supplied from BD Gentest (Discovery Labware Inc., Woburn, MA).

*Human study design*

Nine self-reported healthy volunteers (3 males and 6 females), non-smokers and not on any medication, participated in the study and gave their written consent. Participants were aged between 24 and 61 years old (mean 34.7, SD 12.7) and had a mean BMI of 23.5 (range 20.2-26.6, SD 2.4). Volunteers were required to follow a low phenolic diet (olives and any type of OO, as well as fruits, vegetables and plant products including tea and coffee were excluded) for two days and to fast overnight prior to intervention. On day 3, all fasted subjects consumed 50 mL of EVOO with 30 g of white bread. This dose was selected as it ensured compliance with EFSA health claim for phenolic compounds in OO [6, 7] in line with previous bioavailability studies [38].

Venous blood samples were taken before (baseline: 0h) and 0.5, 1, 2, 4 and 6h post the ingestion. Blood was collected in heparinized tubes at each time-point and immediately centrifuged at 657 ×*g* for 20 min at 4ºC. Urine was collected before (baseline: 0h) and in different collection periods (0-4, 4-8, 8-15 and 15-24h) following EVOO intake. The volume of urine collected over each time period was recorded. Plasma and urine samples were frozen at -80ºC prior to analysis. The study was approved by the Ethics Committee of the Faculty of Pharmacy, University of Lisbon (Permission number 02/CEECFFUL/2016). Protocols were according to the Declaration of Helsinki.

*Extraction of plasma*

Plasma samples were treated according to the method of Day *et al.* [39] with some modifications. ACN:formic acid (99.8:0.2, v/v) (1.25 mL) was added to 500 μL of plasma. Samples were vortexed over a 2 min period followed by 10 min ultrasound, and centrifuged at 16,000 g for 10 min at 4ºC. The supernatant was collected, frozen at -80ºC, lyophilized, and suspended in 50 μL methanol:water (80:20, v/v), before analysis. The extraction procedure was

evaluated, as described in **supplementary information (SI) section 1.1**, by spiking pool plasma with six standard phenolic compounds (apigenin, luteolin, enterolactone, enterodiol, pinoresinol and oleuropein) at known concentrations. These standards were selected as they represent different phenolic families and cover the mass range of the majority of metabolites detected in the samples.

*Urine*

Urine samples were defrosted centrifuged at 16,000 g for 10 min at 4ºC before analysis (one freeze-thaw cycle) without further processing.

*Upper gastro-intestinal in vitro digestion model*

An EVOO was subjected to an upper gastro-intestinal *in vitro* digestion model (*n*= 6 digestions) according to Soler A. *et al.* [13]. Oil and aqueous phases were separated and frozen at -20ºC prior to analysis by LC-MS/MS.

*In vitro microsomal assays*

An EVOO extract (**SI section 1.2**) was diluted to $10^{-5}$ M (CAE equivalents) in DMSO and used as described in **SI section 1.3** [40-42]. The supernatant fraction was analysed by LC-MS/MS.

*Total phenolic content determination*

The protocol described by Owen *et al.* [43] was used for the preparation of the phenolic compounds extract (**SI section 1.2**). The total phenolic content was determined [44] in triplicate (**SI section 1.2)**.

*LC-MS/MS assays*

Details of the analysis are presented in the **SI section 1.4**. Hydroxytyrosol and tyrosol standards were used to quantify these compounds and their secoiridoid derivatives in EVOO (results expressed as hydroxytyrosol and tyrosol equivalents).

*UPLC-Orbitrap MS assays*

Details of the analysis are presented in **SI section 1.5**. Identification was based on molecular ion and mass error tolerance (5 ppm).

*Data analysis*

The Thermo ToxID software was used to screen for EVOO phenolic compounds and corresponding metabolites in plasma and urine samples detected by UPLC-ESI-Orbitrap MS analysis. As our aim was to find discrimination patterns before and after EVOO intake, we screened for target compounds in plasma and urine samples in their free form and also as the corresponding Phase I (hydrogenation, dehydrogenation, hydration and hydroxylation) and Phase II metabolites (methylation, glucuronidation and sulfation), and mixed forms of Phase I and II metabolites (Phase I reaction + glucuronidation and/or methylation): secoiridoids and their metabolites as elenolic acid, hydroxy elenolic acid, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, methyl 3,4-DHPEA-EDA, methyl 3,4-DHPEA-EA, 10-hydroxy-3,4-DHPEA-EA, 10-hydroxy-3,4-DHPEA-EDA, *p*-HPEA-EA and *p*-HPEA-EDA. Other typical phenolics reported in VOO [45] were also screened: phenolic alcohols (tyrosol, hydroxytyrosol) and hydroxytyrosol acetate, phenolic acids (caffeic acid, *p*-coumaric acid, homovanillic acid, vanillic acid), phenolic aldehyde (vanillin), flavonoids (luteolin, apigenin) and lignans (1-acetoxypinoresinol, hydroxypinoresinol, pinoresinol, syringaresinol). All these compounds were tentatively identified by their exact mass. Criteria for variable inclusion in multivariate analysis were absence at 0h time point in plasma and urine [23] and presence in samples from at least two volunteers post EVOO ingestion. Participants were instructed to follow a low phenolic diet that excluded olives and any type of OO. Therefore by excluding variables present at baseline the variations in possible markers would be related to EVOO consumption minimizing interference due to other dietary components.

Alignment of retention time and *m/z* values was carried out across samples using a tolerance window of 5 ppm. Normalization of peak area of selected variables was performed using peak

area/total peak area for each chromatogram, and expressed as ppm (Peak area/total peak area X 1,000,000). Normalization allowed removal of systemic variation between spectra due to variations in sample or equipment. Partial Least Squares Discriminant Analysis with Orthogonal Signal correction (OSC-PLS-DA) was performed using SIMCA-P+ 13.0 software (**SI section 1.6**).

Determination of excretion of urinary biomarkers was done using hydroxytyrosol as standard. Normalized peak areas of the selected variables in urine were used for quantification. Values were corrected according to volume of urine collected per sample point.

## Results

*Phenolic composition of EVOO*

The EVOO ingested had a total phenolic content of 322 ± 6 mg/kg CAE and 6 mg/20g hydroxytyrosol and its derivatives and therefore meeting the EFSA recommendation for phenolic compounds in olive oil [6, 7]. Each volunteer ingested 14.7 mg of phenolics. Individual characterization of phenolic compounds in this EVOO is presented in **Table 1**.

*In vitro upper gastro-intestinal digestion and microsomal assays*

The *in vitro* digestion process led to a secoiridoids decrease (–0.49 fold change) due to chemical hydrolysis and an increase in phenolic alcohols (hydroxytyrosol: 1.66 fold change and tyrosol:0.64 fold change) in medium (**SI section 2.1 Table S1**). Secoiridoids were still detected in the oil phase, after digestion, showing that they were still available for absorption and metabolism. *In vitro* microsomal assays enabled to conclude that 3,4-DHPEA-EA and *p*-HPEA-EA decayed in the assay medium along with increases in the corresponding hydrogenated metabolites (**SI section 2.2 Figure S1**). Calculated half-life in rat liver microsomes was 14.4 h for 3,4-DHPEA-EA and 7.2 h for *p*-HPEA-EA.

*OSC-PLS-DA applied to plasma and urine results*

*Plasma samples*

Validation tests were performed for plasma extraction and results are presented in **SI section 2.3 (Tables S2 and S3)**. Eighteen compounds in plasma samples were selected as variables according to inclusion criteria (**Table 2**) and used for multivariate analysis. Possible isomeric forms were detected for several secoiridoid compounds, as reported before [36].

The parameters used to assess the OSC-PLS-DA modelling quality are summarized in **Table 3**. With the exception of the model with all plasma samples evaluated at the same time (data not shown), all OSC-PLS-DA models presented acceptable values for all quality and validation parameters, indicative that they were able to discriminate samples from baseline. These OSC-PLS-DA models (baseline *vs.* each time collection point) explained between 27.9 and 41.2% of the metabolite intensity variation ($R^2X$) and between 75.3 and 97.3% of the time point variation ($R^2Y$). In our work prediction values $Q^2$ were between 62.1 and 93.6% after cross-validation, indicating good predictive ability of the models.

Permutation tests allowed assessment of overfitting. Models were considered valid as the average of the $R^2$ values of the permuted models were less than half the $R^2$ value of the original model, indicating that more than half of the explained variability was not due to change [46]. p(corr*)* values were calculated for each of the models and are presented **SI section 2.4 (Table S4)**. Three variables (**P1A**, **P1B** and **P9B**) were discriminant for all time points and were selected as plasma biomarkers to monitor EVOO intake. Two other variables were discriminatory for one time point (**SI section 2.4 Table S4**) and nine variables were discriminant for two to four time points (**SI section 2.4 Table S4**).

*Urine samples*

Following the same approach, thirty-two compounds were selected in urine as variables for PLS analysis (**Table 4**). With the exception of the model with all samples evaluated at the same time (data not shown), all OSC-PLS-DA models presented acceptable values for all quality and validation parameters, indicative that they were able to discriminate samples when compared with baseline samples (**Table 5**). These OSC-PLS-DA models (baseline *vs.* each time collection point) explained between 48.0 and 61.0% of the metabolite intensity variation ($R^2X$) and

between 80.0 and 100% of the time point variation ($R^2Y$), with prediction values $Q^2$ between 77.6 and 99.4% after cross-validation. The obtained values of permutation tests were considered acceptable. p(corr*)* values were calculated for each of the models and are presented in **SI section 2.4 (Table S5)**. Nine variables were discriminant for all time points when compared with baseline samples (**U2**, **U9D**, **U9F**, **U9G**, **U9H**, **U11A**, **U11B**, **U11D** and **U11E**). These variables were selected as urinary biomarkers to monitor EVOO intake. Other four variables were discriminant for one time point (**SI section 2.4 Table S5)** and eighteen variables where discriminant for two or three time points (**SI section 2.4 Table S5**).

*Absorption and excretion profile of selected plasma and urinary biomarkers*

Absorption profiles of the discriminant plasma biomarkers presented maximum levels between 0.5 and 2h after EVOO intake, depending on each volunteer (**Figure 1**). In **Figure 2** are shown the excretion profiles of the nine discriminant urinary biomarkers, all hydroxytyrosol derivatives. Maximum concentrations in human urine were reached in the time period of 0-4h after oil intake and mean values for the nine volunteers are presented. The total amount excreted (24h) of each selected biomarker per volunteer is presented in **Figure 3**.

## Discussion

Within this work HRMS was used as a tool to identify, with a high selectivity and low error, the detected metabolites in plasma and urine samples after EVOO intake. ESI negative ionization mode was selected as it is more sensitive than the positive ionization mode [23, 47]. Moreover this approach overcomes complex sample preparation procedures related with GC-MS analysis [48].

Data obtained from UPLC-HRMS analysis of samples were treated by PLS-DA in order to maximize the separation of observations (plasma and urine samples) belonging to the different classes that have previously been defined (different time points in the study). This procedure allowed to establish a model to predict the class (variable Y) from the compounds measured in the UPLC-HRMS analysis (X variables). Orthogonal signal correction (OSC), a multivariate

pre-processing data filter, was used before proceeding with the PLS-DA to eliminate the signal information (X) that is unrelated (orthogonal) to the classes (Y) [49, 50]. Using this approach it was possible, to select biomarkers that were the best predictors of the class vector.

The OSC-PLS-DA models with all samples evaluated at the same time did not meet quality criteria. This could be due to the low number of variables and/or due to criteria used for variables inclusion (absence at baseline). Therefore, further models were performed at each time point to analyse the separation of samples of each time point compared with baseline samples. Using this approach three plasma secoiridoid compounds (elenolic acid + $H_2$ detected at two retention times; *p*-HPEA-EA + $H_2$ + glucuronide) and nine urinary (3,4-DHPEA-EA, 3,4-DHPEA-EA + $H_2$+glucuronide detected at four retention times, and methyl 3,4-DHPEA-EA + $H_2$ + glucuronide detected at four retention times) secoiridoid compounds were selected as biomarkers for monitoring EVOO intake in human intervention trials, as they were discriminant for all time collection points when compared to baseline. Moreover other secoiridoids were identified in plasma and urine as enabling discrimination of one or more time points. It is worth noting that although other phenolic compounds and metabolites were monitored in plasma and urine samples, the secoiridoids group clearly stood apart in terms of sample discrimination, results in agreement with others [23].

In our study the results from the *in vitro* experiments with microssomes showed that secoiridoids 3,4-DHPEA-EA and *p*-HPEA-EA were stable, when incubated with liver microsomes, with half-lives longer than 6h. A search for metabolites, allowed us to detect compounds, identified as the hydrogenated forms of the parent compounds. Although there are differences between rat and human microsomal activity, hydrogenation seems to be an important Phase I route of metabolism for these secoiridoids. This evidence was supported by results obtained in the human intervention as hydrogenated metabolites were detected in plasma for elenolic acid, a structural unit of phenolic secoiridoids. Therefore, the detection of elenolic acid in plasma (hydrogenated form) could be related with hydrolysis of oleuropein and ligstroside aglycones in the gastric or intestinal environment, as evidenced after *in vitro* digestion of EVOO. Discriminating metabolites resulting from Phase I metabolism, concerning

hydroxylation and hydration, were also detected in plasma such as *p*-HPEA-EDA + $H_2O$ and 3,4-DHPEA-EA + OH. These compounds discriminated samples for two to four time collection points. Our results are in accordance with others [23], that identified these metabolites as enabling discrimination in human urines before and after EVOO intake.

Absorption and metabolism of the secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA was studied before [12], by using perfused segments of *jejunum* and *ileum*, and hydrogenation followed by glucuronidation of these compounds was reported. Authors attributed the reduced metabolite forms to the action of NADPH-dependent aldo-keto reductases present in the small intestine. In our study, hydrogenated forms of *p*-HPEA-EDA and *p*-HPEA-EA glucuronides were also detected in plasma. Moreover, *p*-HPEA-EA + $H_2$ + glucuronide was selected within this work as a discriminating plasma metabolite.

In urine, 3,4-DHPEA-EA + $H_2$ + glucuronide and methyl 3,4-DHPEA-EA + $H_2$ + glucuronide were selected as biomarkers of EVOO intake for all time collection points. The higher polarity of 3,4-DHPEA-EA and methyl 3,4-DHPEA-EA hydrogenated glucuronides compared with *p*-HPEA-EDA and *p*-HPEA-EA corresponding metabolites, might explain their presence in urine and not in plasma. As the action site of NADPH-dependent aldo-keto reductases is in the elenolic acid unit [12] the same reactions can take place in oleuropein or ligstroside aglycones. Moreover hydrogenated metabolites of *p*-HPEA-EA and 3,4-DHPEA-EA glucuronides were selected before as human urinary biomarkers for EVOO intake [23].

It is worth noting the selection of 3,4-DHPEA-EA as discrimination marker in urine. This typical EVOO phenolic compound was also selected as marker in plasma, however not for all of the collection points. Other urinary discrimination markers included 3,4-DHPEA-EA + $H_2$ + glucuronide and methyl-3,4-DHPEA-EA + $H_2$ + glucuronide. The presence of methylated forms might be explained by the action of catechol-*O*-methyl-tranferase that catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to phenolic compounds having an *o*-diphenolic structure [51], although methyl 3,4-DHPEA-EA was also present in the EVOO.

In our study, glucuronide metabolites of *p*-HPEA-EDA, 3,4-DHPEA-EDA and methyl 3,4-DHPEA-EDA, were detected as major metabolites in urine, as were glucuronides of methyl 3,4-

DHPEA-EDA + OH, methyl 3,4-DHPEA-EA + $H_2$ and 3,4-DHPEA-EA + $H_2$/OH/$H_2O$. Glucuronides of *p*-HPEA-EDA, 3,4-DHPEA-EA + $H_2$/OH/ $H_2O$, and methyl 3,4-DHPEA-EDA + OH were previously identified in human urine samples after intake of EVOO [23]. Glucuronides of secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA derivatives were detected in human plasma samples after a bioavailability study with VOO [16]. In our study glucuronides of hydrogenated forms of *p*-HPEA-EDA and *p*-HPEA-EA were also detected in plasma as well as glucuronide of methyl 3,4-DHPEA-EDA+$H_2O$. UDP-glucuronosyltransferases are membrane-bounded enzymes that are located in the endoplasmic reticulum in many tissues and catalyze the transfer of a glucuronic acid from UDP-glucuronic acid to phenolic compounds or xenobiotics [51].

Metabolites of the secoiridoid group were detected with the same exact mass but at different retention times, suggesting the presence of isomers, which could be justified by the ring opening in the secoiridoid structure, due to keto-enolic tautomeric equilibrium [52]. The presence of two putative groups of isomers for secoiridoid derivatives as elenolic acid, oleuropein and ligstroside aglycones was previously reported [36].

Maximum absorption levels of the plasma biomarkers were detected between 0.5 and 2h and results are in accordance with others (maximum at 1h) [16]. Maximum excretion of urinary biomarkers occurred in the first 4h after EVOO intake and results are in accordance with others (maximum between 2 - 4h) [23]. Variability of results among volunteers was high for the selected biomarkers in plasma and urine which is reflected in the standard deviations obtained. Bioavailability of phenolic compounds not only depends on their concentration in the VOO but is also affected by the individual genomic profile with impact on enzymatic activity involved in the digestion and metabolism process. In fact polymorphism expression of conjugation enzymes [51] or individual variations in digestive enzymes [53] or bile salts [54] might justify the variations observed.

VOO phenolics undergo rapid absorption and fast renal elimination as evidenced in the absorption and excretion profiles of the selected biomarkers. Accumulation of metabolites in target tissues, studied before [55], could be considered when investigating the potential

bioactivity of VOO phenolic compounds. In this work, the lack of pure standards did not allow the calculation of a mass balance between the ingested EVOO phenolics, plasma and urinary metabolites and pharmacokinetic data.

The use of secoiridoids as biomarkers to monitor EVOO intake has the advantage of specificity. While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids are present exclusively in plants belonging to the family of *Oleaceae* including the olive tree *Olea europaea* L. [8]. Results of this work highlight the importance of secoiridoids as biomarkers of EVOO intake. These biomarkers would allow monitoring compliance of following the dose in interventions aimed to evaluate health benefits of EVOO intake in accordance with the EFSA claim [5]. Although secoiridoids are the major components in the phenolic fraction of EVOO [56], we have to take into account that several factors influence its content in EVOO. Olive tree cultivars, alternate bearing of the olive tree, environmental conditions, maturity of the fruits or technological factors are parameters that influence chemical composition of EVOO and therefore increase variability in the phenolic composition [1, 8, 57, 58]. The combination of digestion models with *in vitro* approaches of hepatic metabolism (e.g.: incubation with HepG2 cells or liver microsomes) can be useful in producing metabolites from EVOO. Recently it was demonstrated the use of Caco-2 cells as bioreactors for generation of phase II metabolites of hydroxytyrosol [59]. This approach would allow us to confirm the identity of biomarkers selected in this work. After identification it would be possible to produce biomarkers as authentic standard allowing absolute calibration of these compounds in samples. Moreover the use of HRMS/MS would enable structural clarification of the putatively identified isomers.

The secoiridoid metabolites detected in plasma and urine samples from volunteers were able to discriminate between different time points after EVOO intake. The most common metabolic reactions were Phase I hydrogenation and Phase II glucuronidation. The selected secoiridoid biomarkers can be added to the ones already described. Due to the variability in absorption, metabolism and elimination of compounds, future studies should include broader populations to

account for inter-individual variability. The role of the identified metabolites in beneficial health effects requires further investigation.

*Authors contributions: S.S., M.E.F, E.C., W.M. and M.R.B designed the research. S.S. conducted research, analyzed data and prepared the first draft of the manuscript. M.G.A performed statistical analysis. All authors: were responsible for the interpretation of data, critical review of the manuscript and approved the final manuscript.*

*This study was supported by QREN project Azeite+Global nº 12228, iNOVA4Health and National Programme for Scientific Re-equipment for the acquisition of the LC-MS/MS equipment that is part of the Portuguese National Mass Spectrometry Network (Contract RNEM- REDE/1518/REM/2005).*

*The authors have declared no conflict of interest.*

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Figure 1 - Plasma absorption-time profiles of variables P1A (elenolic acid + $H_2$), P1B (elenolic acid + $H_2$ detected at a different retention time) and P9B (*p*-HPEA-EA+$H_2$+glucuronide) for volunteers (1 to 9) and mean values. Data are expressed as normalized areas (ppm)

Figure 2 - Excretion profile of discriminatory biomarkers identified in urine after EVOO intake (μg excreted expressed as hydroxytyrosol equivalents). Data are expressed as mean ± SD

Figure 3 – Total excretion of discriminatory biomarkers identified in urine (0-24h) per volunteer (*n*=9) after EVOO intake (μg of hydroxytyrosol equivalents)

Table 1 – Phenolic compounds and elenolic acid in EVOO used in the human intervention identified by LC-MS/MS

| Retention time (min) | $[M-H]^-$ *m/z* | Ion Formula | MS/MS *m/z* | Compound | EVOO (mg/kg) |
|---|---|---|---|---|---|
| 3.6 | 153 | $C_8H_9O_3$ | 123, 95 | Hydroxytyrosol | 3.75 |
| 5.3 | 137 | $C_8H_9O_2$ | 119, 106 | Tyrosol | 2.09 |
| 19.3 | 377 | $C_{19}H_{21}O_8$ | 275, 307 | 3,4-DHPEA-EA* | 51.49 |
| 16.7 | 319 | $C_{17}H_{19}O_6$ | 195, 183 | 3,4-DHPEA-EDA* | 9.78 |
| 18.3 | 391 | $C_{20}H_{23}O_8$ | 377 | Methyl 3,4-DHPEA-EA* | 4.15 |
| 15.6 | 335 | $C_{17}H_{19}O_7$ | 199, 155 | OH - deacetoxy oleuropein aglycone* | 1.12 |
| 17.4 | 393 | $C_{19}H_{21}O_9$ | 317 | 10-OH oleuropein aglycone* | 5.67 |
| 21.0 | 361 | $C_{19}H_{21}O_7$ | 291, 259 | *p*-HPEA-EA** | 203.08 |
| 18.5 | 303 | $C_{17}H_{19}O_5$ | 285, 179 | *p*-HPEA-EDA** | 30.08 |
| 14.3 | 241 | $C_{11}H_{13}O_6$ | 139 | Elenolic acid* | 10.04 |
| 17.4 | 285 | $C_{15}H_9O_6$ | 133 | Luteolin | 3.19 |
| 18.7 | 269 | $C_{15}H_9O_5$ | 151 | Apigenin | 0.70 |
| 17.6 | 357 | $C_{20}H_{21}O_6$ | 151, 136 | Pinoresinol* | 0.49 |
| 17.6 | 415 | $C_{22}H_{23}O_8$ | 235, 151 | Acetoxipinoresinol* | 0.24 |
| 11.2 | 151 | $C_8H_7O_3$ | 136 | Vanillin | 0.14 |

*Quantified as hydroxytyrosol equivalents; **quantified as tyrosol equivalents.

Table 2 - Retention time, theoretical and experimental *m/z*, error and ion formula obtained in negative mode for compounds detected in plasma (P) and considered as variables for OSC-PLS-DA and corresponding abbreviations

| Variables included in OSC-PLS-DA | Retention time (min) | Theoretical *m/z* | Experimental *m/z*[a] | Error (ppm)[a] | Ion Formula | Abbreviation[b] |
|---|---|---|---|---|---|---|
| **Elenolic acid + $H_2$** | **10.35** | **243.0874** | **243.0862** | **-4.9** | **$C_{11}H_{15}O_6$** | **P1A** |
| **Elenolic acid + $H_2$** | **10.97** | **243.0874** | **243.0863** | **-4.5** | **$C_{11}H_{15}O_6$** | **P1B** |
| 3,4-DHPEA-EDA | 13.34 | 319.1187 | 319.1174 | -4.2 | $C_{17}H_{19}O_6$ | P2 |
| *p*-HPEA-EDA + $H_2O$ | 13.01 | 321.1344 | 321.1333 | -3.2 | $C_{17}H_{21}O_6$ | P3 |
| 3,4-DHPEA-EA | 9.70 | 377.1242 | 377.1250 | 2.2 | $C_{19}H_{21}O_8$ | P4 |
| 3,4-DHPEA-EA+OH | 13.68 | 393.1191 | 393.1175 | -4.0 | $C_{19}H_{21}O_9$ | P5A |
| 3,4-DHPEA-EA+OH | 13.94 | 393.1191 | 393.1173 | -4.5 | $C_{19}H_{21}O_9$ | P5B |
| *p*-HPEA-EDA + $H_2$ + glucuronide | 9.97 | 481.1715 | 481.1700 | -3.2 | $C_{23}H_{29}O_{11}$ | P6A |
| *p*-HPEA-EDA + $H_2$ + glucuronide | 10.24 | 481.1715 | 481.1701 | -2.9 | $C_{23}H_{29}O_{11}$ | P6B |
| *p*-HPEA-EDA + $H_2$ + glucuronide | 10.56 | 481.1715 | 481.1701 | -2.9 | $C_{23}H_{29}O_{11}$ | P6C |
| 3,4-DHPEA-EDA+OH+glucuronide | 11.14 | 511.1457 | 511.1480 | 4.4 | $C_{23}H_{27}O_{13}$ | P7 |
| Methyl 3,4-DHPEA-EDA + $H_2O$ + glucuronide | 10.12 | 527.1770 | 527.1753 | -3.3 | $C_{24}H_{31}O_{13}$ | P8A |
| Methyl 3,4-DHPEA-EDA + $H_2O$ + glucuronide | 10.35 | 527.1770 | 527.1756 | -2.7 | $C_{24}H_{31}O_{13}$ | P8B |
| Methyl 3,4-DHPEA-EDA + $H_2O$ + glucuronide | 10.71 | 527.1770 | 527.1755 | -2.9 | $C_{24}H_{31}O_{13}$ | P8C |
| *p*-HPEA-EA + $H_2$ +glucuronide | 11.73 | 539.1770 | 539.1757 | -2.4 | $C_{25}H_{31}O_{13}$ | P9A |
| ***p*-HPEA-EA + $H_2$ + glucuronide** | **11.94** | **539.1770** | **539.1758** | **-2.3** | **$C_{25}H_{31}O_{13}$** | **P9B** |
| *p*-HPEA-EA + $H_2$ + glucuronide | 12.15 | 539.1770 | 539.1759 | -2.1 | $C_{25}H_{31}O_{13}$ | P9C |
| Apigenin+$CH_3$+glucuronide | 11.33 | 459.0933 | 459.0949 | 3.5 | $C_{22}H_{19}O_{11}$ | P10 |

[a] mean value.

[b] letters account for putative isomers.

Compounds highlighted in bold enabled plasma samples discrimination after EVOO intake, for all collection points, as found by OSC-PLS-DA

Table 3 – Summary of parameters for assessing the OSC-PLS-DA modeling quality in plasma samples

| Model | OSC Filter | | | | OSC-PLS-DA | | | | | Permutation test | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Â | remaining SS (%) | eigen-value | N | $R^2X$ (cum) | $R^2Y$ (cum) | $Q^2$ (cum) | *p*-value | $R^2$ | $Q^2$ |
| t0h *vs*. t0,5h | 1 | 90.00 | 79.76 | 3.441 | 1 | 0.315 | 0.973 | 0.936 | $1.07x10^{-9}$ | 0.117 | -0.251 |
| t0h *vs*. t1h | 1 | 90.00 | 72.12 | 4.739 | 1 | 0.412 | 0.911 | 0.850 | $6.72x10^{-7}$ | 0.074 | -0.239 |
| t0h *vs*. t2h | 1 | 90.00 | 72.00 | 4.760 | 1 | 0.389 | 0.879 | 0.801 | $5.56x10^{-6}$ | 0.123 | -0.224 |
| t0h *vs*. t4h | 1 | 90.00 | 67.04 | 4.943 | 1 | 0.364 | 0.866 | 0.779 | $1.22x10^{-5}$ | 0.140 | -0.203 |
| t0h *vs*. t6h | 1 | 90.00 | 71.70 | 3.962 | 1 | 0.279 | 0.753 | 0.621 | $6.90x10^{-4}$ | 0.131 | -0.157 |

N, number of components in OSC and PLS-DA model, respectively; Â displays the angle between the component and the Y variable; SS, sum of squares, indicating the % of the original variance in the X-block that remains in the corrected X-matrix; $R^2X$(cum) and $R^2Y$(cum) are the cumulative modelled variation in the X and Y matrix, respectively; $Q^2$(cum) is the cumulative predicted variation in the Y matrix; the *p*-value from CV-ANOVA, based on the cross-validated predictive residuals, is listed for both models (significant result indicates a valid model).

Table 4 - Retention time, theoretical and experimental *m/z*, error and ion formula obtained in negative mode for compounds detected in urine (U) and considered as variables for OSC-PLS-DA and corresponding abbreviations

| Variables included in OSC-PLS-DA | Retention time (min) | Theoretical *m/z* | Experimental *m/z*[a] | Error (ppm)[a] | Ion Formula | Abbreviation[b] |
|---|---|---|---|---|---|---|
| *p*-HPEA-EA +$H_2$ | 23.06 | 363.1449 | 363.1443 | -1.7 | $C_{19}H_{23}O_7$ | U1 |
| **3,4-DHPEA-EA** | **22.86** | **377.1242** | **377.1233** | **-2.2** | **$C_{19}H_{21}O_8$** | **U2** |
| 3,4- DHPEA-EA + OH +Sulfate | 16.40 | 473.0759 | 473.754 | -1.2 | $C_{19}H_{21}O_{12}S$ | U3A |
| 3,4-DHPEA-EA + OH +Sulfate | 16.89 | 473.0759 | 473.0751 | -1.7 | $C_{19}H_{21}O_{12}S$ | U3B |
| Luteolin + $CH_3$+glucuronide | 12.80 | 475.0882 | 475.0905 | 4.8 | $C_{22}H_{19}O_{12}$ | U4 |
| *p*-HPEA-EDA +glucuronide | 15.48 | 479.1559 | 479.1548 | -2.2 | $C_{23}H_{27}O_{11}$ | U5A |
| *p*-HPEA-EDA +glucuronide | 16.39 | 479.1559 | 479.1549 | -2.1 | $C_{23}H_{27}O_{11}$ | U5B |
| 3,4-DHPEA-EDA+glucuronide | 15.18 | 495.1508 | 495.1498 | -2.0 | $C_{23}H_{27}O_{12}$ | U6 |
| Methyl 3,4-DHPEA-EDA +glucuronide | 16.88 | 509.1665 | 509.1653 | -2.2 | $C_{24}H_{29}O_{12}$ | U7A |
| Methyl 3,4-DHPEA-EDA +glucuronide | 18.08 | 509.1665 | 509.1653 | -2.3 | $C_{24}H_{29}O_{12}$ | U7B |
| Methyl 3,4-DHPEA-EDA + OH+glucuronide | 13.09 | 525.1614 | 525.1619 | 2.9 | $C_{24}H_{29}O_{13}$ | U8A |
| Methyl 3,4-DHPEA-EDA + OH+glucuronide | 13.15 | 525.1614 | 525.1623 | 1.8 | $C_{24}H_{29}O_{13}$ | U8B |
| Methyl 3,4-DHPEA-EDA + OH+glucuronide | 16.17 | 525.1614 | 525.1608 | -1.0 | $C_{24}H_{29}O_{13}$ | U8C |
| Methyl 3,4 DHPEA-EDA + OH+glucuronide | 16.50 | 525.1614 | 525.1607 | -1.3 | $C_{24}H_{29}O_{13}$ | U8D |
| 3,4-DHPEA-EA + $H_2$+glucuronide | 13.49 | 555.1719 | 555.1707 | -2.3 | $C_{25}H_{31}O_{14}$ | U9A |
| 3,4-DHPEA-EA + $H_2$+glucuronide | 13.71 | 555.1719 | 555.1706 | -2.3 | $C_{25}H_{31}O_{14}$ | U9B |
| 3,4-DHPEA-EA + $H_2$+glucuronide | 13.82 | 555.1719 | 555.1706 | -2.3 | $C_{25}H_{31}O_{14}$ | U9C |
| **3,4-DHPEA-EA + $H_2$+glucuronide** | **16.50** | **555.1719** | **555.1707** | **-2.3** | **$C_{25}H_{31}O_{14}$** | **U9D** |
| 3,4-DHPEA-EA + $H_2$+glucuronide | 16.61 | 555.1719 | 555.1706 | -2.3 | $C_{25}H_{31}O_{14}$ | U9E |
| **3,4-DHPEA-EA + $H_2$+glucuronide** | **16.87** | **555.1719** | **555.1706** | **-2.3** | **$C_{25}H_{31}O_{14}$** | **U9F** |
| **3,4-DHPEA-EA + $H_2$+glucuronide** | **17.76** | **555.1719** | **555.1706** | **-2.4** | **$C_{25}H_{31}O_{14}$** | **U9G** |
| **3,4-DHPEA-EA + $H_2$+glucuronide** | **18.09** | **555.1719** | **555.1706** | **-2.3** | **$C_{25}H_{31}O_{14}$** | **U9H** |
| 3,4-DHPEA-EA + OH+glucuronide | 14.33 | 569.1512 | 569.1533 | 3.7 | $C_{25}H_{29}O_{15}$ | U10A |
| 3,4-DHPEA-EA + OH+glucuronide | 14.68 | 569.1512 | 569.1518 | 1.0 | $C_{25}H_{29}O_{15}$ | U10B |
| 3,4-DHPEA-EA + OH+glucuronide | 14.80 | 569.1512 | 569.1539 | 4.8 | $C_{25}H_{29}O_{15}$ | U10C |
| **Methyl 3,4-DHPEA-EA + $H_2$+glucuronide** | **17.69** | **569.1876** | **569.1865** | **-1.9** | **$C_{26}H_{33}O_{14}$** | **U11A** |
| **Methyl 3,4-DHPEA-EA + $H_2$+-glucuronide** | **17.89** | **569.1876** | **569.1865** | **-1.9** | **$C_{26}H_{33}O_{14}$** | **U11B** |
| Methyl 3,4-DHPEA-EA + $H_2$+glucuronide | 17.97 | 569.1876 | 569.1864 | -2.0 | $C_{26}H_{33}O_{14}$ | U11C |
| **Methyl 3,4-DHPEA-EA + $H_2$+glucuronide** | **19.33** | **569.1876** | **569.1865** | **-1.9** | **$C_{26}H_{33}O_{14}$** | **U11D** |
| **Methyl 3,4-DHPEA-EA + $H_2$+glucuronide** | **19.66** | **569.1876** | **569.1868** | **-1.4** | **$C_{26}H_{33}O_{14}$** | **U11E** |
| 3,4-DHPEA-EA + $H_2O$+glucuronide | 12.99 | 571.1668 | 571.1660 | -1.5 | $C_{25}H_{31}O_{15}$ | U12A |
| 3,4-DHPEA-EA + $H_2O$+glucuronide | 13.52 | 571.1668 | 571.1658 | -1.9 | $C_{25}H_{31}O_{15}$ | U12B |

[a] mean value.

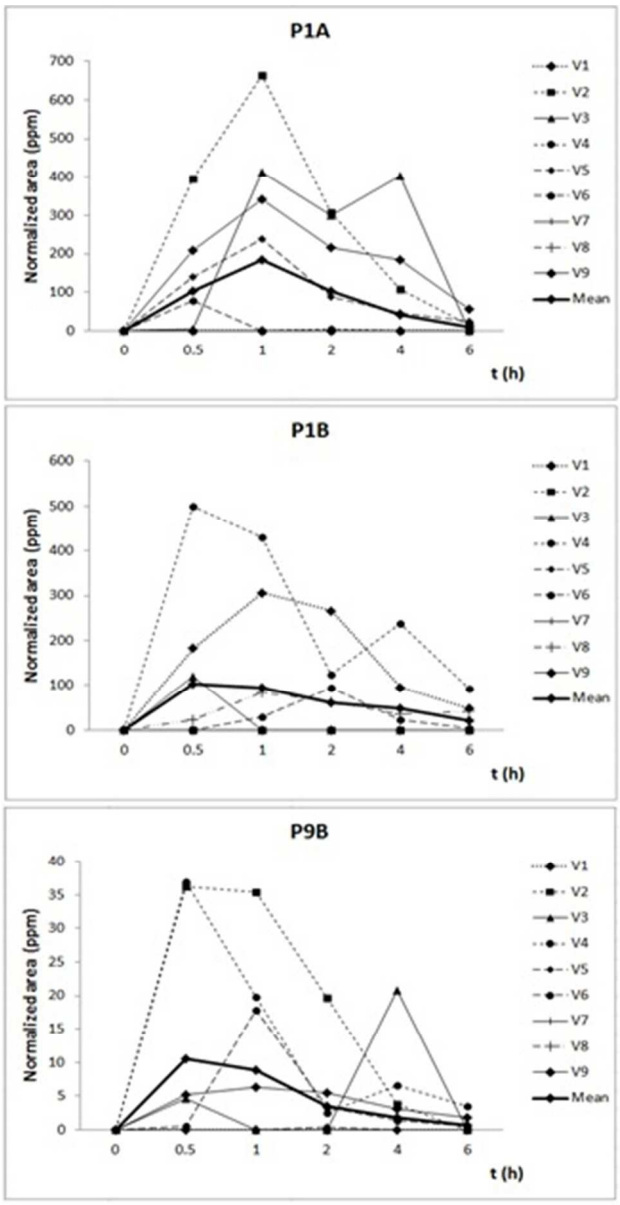
[b] letters account for putative isomers.

Compounds highlighted in bold enabled urine samples discrimination after EVOO intake, for all collection points, as found by OSC-PLS-DA.

Table 5 – Summary of parameters for assessing the OSC-PLS-DA modeling quality in urine samples

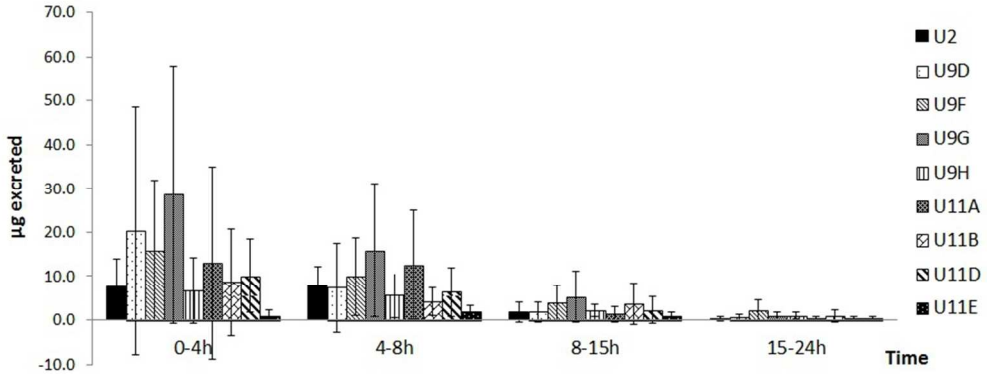
| Model | OSC Filter | | | | OSC-PLS-DA | | | | | Permutation test | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Â | remaining SS (%) | eigen-value | N | $R^2X$ (cum) | $R^2Y$ (cum) | $Q^2$ (cum) | *p*-value | $R^2$ | $Q^2$ |
| t0h *vs*. t0-4h | 1 | 89.99 | 80.35 | 3.538 | 1 | 0.573 | 0.800 | 0.776 | $1.36x10^{-05}$ | 0.096 | -0.216 |
| t0h *vs*. t4-8h | 1 | 90.00 | 75.18 | 4.219 | 1 | 0.610 | 1.000 | 0.994 | $3.02x10^{-16}$ | 0.032 | -0.260 |
| t0h *vs*. t8-15h | 1 | 90.00 | 68.95 | 5.589 | 1 | 0.480 | 0.998 | 0.988 | $5.07x10^{-15}$ | 0.029 | -0.277 |
| t0h *vs*. t15-24h | 1 | 90.00 | 68.40 | 4.740 | 1 | 0.560 | 0.997 | 0.986 | $8.84x10^{-14}$ | 0.008 | -0.266 |

N, number of components in OSC and PLS-DA model, respectively; Â displays the angle between the component and the Y variable; SS, sum of squares, indicating the % of the original variance in the X-block that remains in the corrected X-matrix; $R^2X$(cum) and $R^2Y$(cum) are the cumulative modelled variation in the X and Y matrix, respectively; $Q^2$(cum) is the cumulative predicted variation in the Y matrix; the p-value from CV-ANOVA, based on the cross-validated predictive residuals, is listed for both models (significant result indicates a valid model).



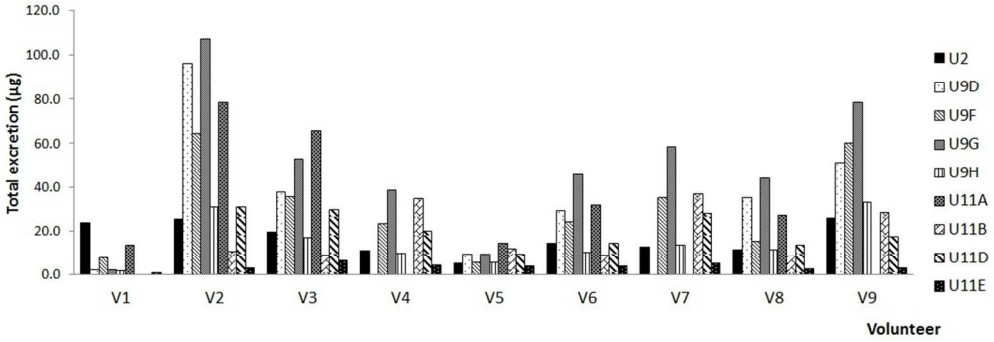

Plasma absorption-time profiles of variables P1A (elenolic acid + H2), P1B (elenolic acid + H2 detected at a different retention time) and P9B (p-HPEA-EA+H2+glucuronide) for volunteers (1 to 9) and mean values. Data are expressed as normalized areas (ppm)

86x166mm (96 x 96 DPI)



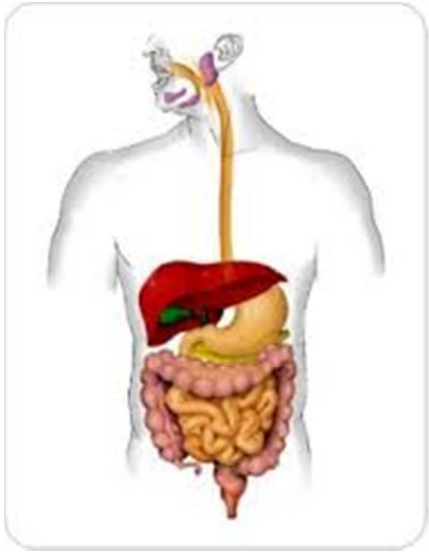

Excretion profile of discriminatory biomarkers identified in urine after EVOO intake (µg excreted expressed as hydroxytyrosol equivalents). Data are expressed as mean ± SD

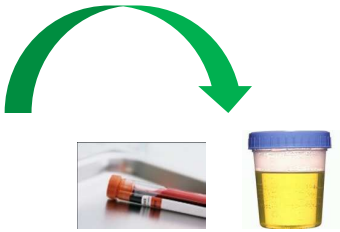
238x91mm (96 x 96 DPI)

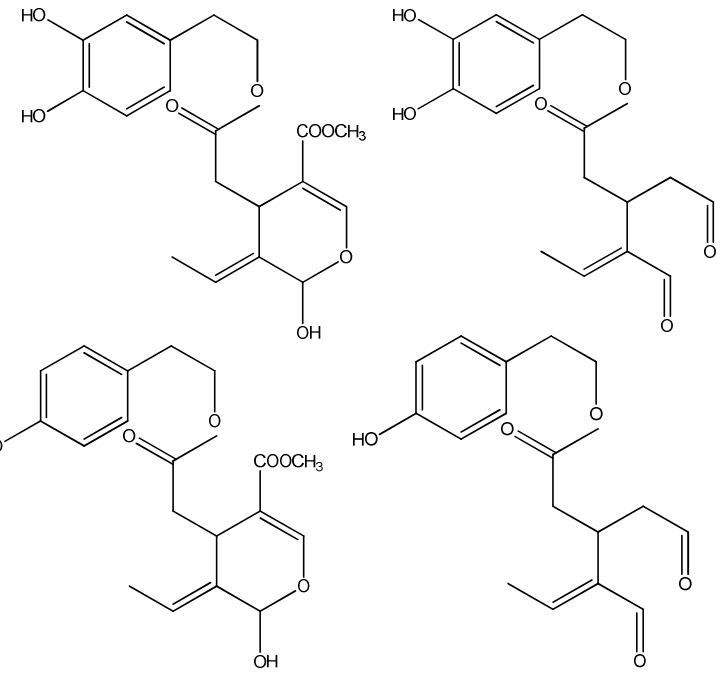



Total excretion of discriminatory biomarkers identified in urine (0-24h) per volunteer (n=9) after EVOO intake (µg of hydroxytyrosol equivalents)

282x98mm (96 x 96 DPI)






HO
HO
O
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COOCH3
O
OH
HO
HO
O
O
O
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HO
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COOCH3
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OH
HO
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O
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Secoiridoids are the major components contributing to the phenolic content of extra virgin olive oil (EVOO). Healthy volunteers (*n*=9) ingested 50 mL of EVOO in a single dose containing 6 mg/20 g of hydroxytyrosol and its derivatives. High resolution mass spectrometry and multivariate analysis enabled the identification of plasma and urinary secoiridoid compounds as biomarkers of EVOO intake.