

Silva, S., Garcia-Aloy, M., Eduardo Figueira, M., Combet, E. , Mullen, W. and Rosário Bronze, M. (2018) 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. *Molecular Nutrition and Food Research*, 62(2), 1700065. (doi: [10.1002/mnfr.201700065](https://doi.org/10.1002/mnfr.201700065))

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

This is the peer-reviewed version of the following article: Silva, S., Garcia-Aloy, M., Eduardo Figueira, M., Combet, E. , Mullen, W. and Rosário Bronze, M. (2018) 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. *Molecular Nutrition and Food Research*, 62(2), 1700065, which has been published in final form at [10.1002/mnfr.201700065](https://doi.org/10.1002/mnfr.201700065). This article may be used for non-commercial purposes in accordance with [Wiley Terms and Conditions for Self-Archiving](#).

<http://eprints.gla.ac.uk/148481/>

Deposited on 20 September 2017

1
2
3 1 **High resolution mass spectrometric analysis of secoiridoids and metabolites as biomarkers**
4
5 2 **of acute olive oil intake – an approach to study inter-individual variability in humans**

6
7 3 Sandra Silva^{1,2}, Mar Garcia-Aloy³, Maria Eduardo Figueira², Emilie Combet^{4*}, William
8
9 4 Mullen^{5*} and Maria Rosário Bronze^{1,2*}

10
11
12
13 6 ¹ iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras,
14
15 7 Portugal

16
17 8 ² Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de
18
19 9 Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

20
21 10 ³ Biomarkers & Nutrimental Lab, Department of Nutrition and Food Science, XaRTA,
22
23 11 INSA, Campus Torribera, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n,
24
25 12 08028 Barcelona, Spain

26
27 13 ⁴ Human Nutrition, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary
28
29 14 and Life Sciences, University of Glasgow, Glasgow G31 2ER, UK

30
31 15 ⁵ Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow G12 8QQ,
32
33 16 UK

34
35 17 *These authors contributed equally to this work.

36
37 18 Correspondence: Maria Rosário Bronze (Professor)/ Faculty of Pharmacy, Universidade de
38
39 19 Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal/mrbronze@ff.ulisboa.pt/ Fax number
40
41 20 + 351 21 794 64 70

42
43
44 21
45 22 **Keywords:** bioavailability; metabolites; olive oil; phenolic compounds; secoiridoids.

46
47 23 **Abbreviations:** CAE, caffeic acid equivalents; **3,4-DHPEA – EA**, (3,4-dihydroxyphenyl)ethanol linked
48
49 24 to elenolic acid; **3,4-DHPEA – EDA**, dialdehydic form of deacetoxy of oleuropein aglycon; **EVOO**,
50
51 25 extra virgin olive oil; **HRMS**, High resolution mass spectrometry; **OO**, olive oil; **p-HPEA-EA**, *p*-
52
53 26 hydroxyphenyl)ethanol linked to elenolic acid; **p-HPEA – EDA**, dialdehydic form of deacetoxy of
54
55 27 ligstroside aglycon; **PLS-DA**, Partial Least Squares Discriminant Analysis; **OSC-PLS-DA**, Partial Least
56
57 28 Squares Discriminant Analysis with Orthogonal Signal correction; **VOO**, virgin olive oil.

29 **Abstract**

30 Scope: Phenolic compounds are minor components of extra virgin olive oil (EVOO).
31 Secoiridoids are the major components contributing to the phenolic content of EVOO.
32 Information is lacking regarding their potential as biomarkers for EVOO intake.
33 Methods and results: Healthy volunteers ($n=9$) ingested 50 mL of EVOO in a single dose
34 containing 322 mg/kg total phenolic content (caffeic acid equivalents) and 6 mg/20g
35 hydroxytyrosol and its derivatives. Plasma was collected before (0h) and at 0.5, 1, 2, 4 and 6h
36 after ingestion. Urine samples were collected prior to ingestion (0h) and at 0-4, 4-8, 8-15 and
37 15-24h. Samples were analysed by UPLC coupled with an Exactive Orbitrap MS. Partial Least
38 Squares Discriminant Analysis with Orthogonal Signal Correction was applied to screen for
39 metabolites that allowed samples discrimination. Plasma biomarkers and urine biomarkers were
40 selected although individual variability was observed among volunteers. Results are in
41 accordance with *in vitro* experiments performed (*in vitro* digestion and hepatic microsomal
42 activity assays).
43 Conclusions: plasma (elenolic acid + H₂; *p*-HPEA-EA + H₂ + glucuronide) and urinary (3,4-
44 DHPEA-EA, 3,4-DHPEA-EA + H₂+glucuronide, methyl 3,4-DHPEA-EA + H₂+glucuronide)
45 secoiridoid compounds were selected as biomarkers to monitor EVOO intake showing good
46 predictive ability according to multivariate analysis.

47 Introduction

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

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

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

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

1
2
3 75 processes of methylation, glucuronidation and sulfation have been described for VOO phenolic
4
5 76 compounds [22-25].
6
7 77 VOO phenolic compounds are mainly absorbed in the small intestine [26] via passive diffusion
8
9 78 [16], although absorption via intestinal membrane carriers might be also involved [16, 27].
10
11 79 Phenolic compounds that are not absorbed in the small intestine, such as secoiridoids, are
12
13 80 degraded by the colonic microbiota [15].
14
15 81 Bioavailability of VOO phenolic compounds is affected by host factors, namely age and
16
17 82 genomic profile, enzymatic activity or colonic microflora [16, 28]. In addition, in animal studies
18
19 83 gender appeared as a factor conditioning bioavailability of hydroxytyrosol derivatives, related
20
21 84 with enterohepatic circulation, and longer persistence of metabolites in organisms [27].
22
23 85 Although secoiridoids are the most abundant and complex family of phenolic compounds in
24
25 86 VOO, their bioavailability has been poorly studied, in part because the main products of their
26
27 87 metabolism (via hydrolysis) are hydroxytyrosol and tyrosol. However, hydroxytyrosol is not an
28
29 88 ideal marker of compliance of VOO intake as it is present endogenously as a metabolite of the
30
31 89 physiological neurotransmitter dopamine and can be produced after ingestion of red wine [29].
32
33 90 It is therefore difficult to reduce or control hydroxytyrosol concentration in biological fluids
34
35 91 before interventions with VOO [18]. This warrants further research into the use of secoiridoids
36
37 92 as biomarkers for VOO intake.
38
39 93 To obtain relevant information from metabolomics data, multivariate analysis is used [30] since,
40
41 94 unlike univariate analysis, this approach can handle a large number of variables without
42
43 95 requirement for the variables to be independent. Supervised methods, such as Partial Least
44
45 96 Squares Discriminant Analysis (PLS-DA), are powerful tools in the search for markers that
46
47 97 differ most between classes. The knowledge about the class to which a sample belongs to is
48
49 98 used to select variables that are considerably different between groups of samples and which
50
51 99 may be the candidate biomarkers. These approaches have been discussed in detail elsewhere
52
53 100 [31, 32].
54
55
56
57
58
59
60

1
2
3 101 The primary aim of this study was to characterize, in human subjects, the bioavailability and the
4
5 102 inter-individual variability of secoiridoids and their metabolites after an acute extra virgin olive
6
7 103 oil (EVOO) intake. Other typical phenolic compounds in EVOO such as phenolic acids,
8
9 104 phenolic alcohols, flavonoids, lignans, and their metabolites, were also monitored in the
10
11 105 samples to ascertain whether any of these could also be used to study EVOO intake. The
12
13 106 phenolic composition of EVOO was determined by LC-MS/MS. UPLC coupled with HRMS
14
15 107 using the Orbitrap technology was used to overcome limitations of low resolution MS
16
17 108 techniques as several isomeric forms of the oleuropein and ligstroside aglycones have been
18
19 109 reported [33-36]. PLS-DA was applied for data analysis to search for markers that differ most
20
21 110 between classes (e.g.: time collection point of sample) and allow validation of class spacing
22
23 111 numerically [37]. Digestion and hepatic microsomal *in vitro* models were also used to evaluate
24
25 112 the impact of digestion and microsomal enzymatic activity on EVOO secoiridoids. To the best
26
27 113 of our knowledge this is the first study on HRMS simultaneous screening of EVOO phenolic
28
29 114 compounds and metabolites in plasma and urine after an acute intake of EVOO.
30
31
32 115

33 116 **Materials and Methods**

34 117 35 36 118 *Materials*

37
38 119 Extra virgin olive oils used for human and *in vitro* assays were supplied by a Portuguese OO
39
40 120 producer. Reference standards used were: apigenin, luteolin and tyrosol ($\geq 99\%$), hydroxytyrosol
41
42 121 ($\geq 98\%$), and oleuropein ($\geq 90\%$) purchased from Extrasynthese (Genay, France); enterolactone
43
44 122 ($\geq 90\%$), enterodiol and pinoresinol ($\geq 95\%$) and caffeic acid ($\geq 98\%$) purchased from Sigma-
45
46 123 Aldrich (Sigma, USA). Bile salts, DMSO, pancreatin, pepsin and sodium bicarbonate were
47
48 124 supplied from Sigma-Aldrich (Sigma, USA). HPLC grade, ACN, methanol, water, chloridric
49
50 125 and formic acid *p.a.* were purchased from Carlo Erba (Italy). HPLC grade acetic acid and
51
52 126 sodium carbonate were supplied from Panreac (Spain). Folin–Ciocalteu reagent was supplied
53
54
55
56
57
58
59
60

1
2
3 127 from Fluka (Switzerland). Rat pooled liver microsomes and NADPH-regenerating systems were
4
5 128 supplied from BD Gentest (Discovery Labware Inc., Woburn, MA).

6
7 129

8
9 130 *Human study design*

10
11 131 Nine self-reported healthy volunteers (3 males and 6 females), non-smokers and not on any
12
13 132 medication, participated in the study and gave their written consent. Participants were aged
14
15 133 between 24 and 61 years old (mean 34.7, SD 12.7) and had a mean BMI of 23.5 (range 20.2-
16
17 134 26.6, SD 2.4). Volunteers were required to follow a low phenolic diet (olives and any type of
18
19 135 OO, as well as fruits, vegetables and plant products including tea and coffee were excluded) for
20
21 136 two days and to fast overnight prior to intervention. On day 3, all fasted subjects consumed 50
22
23 137 mL of EVOO with 30 g of white bread. This dose was selected as it ensured compliance with
24
25 138 EFSA health claim for phenolic compounds in OO [6, 7] in line with previous bioavailability
26
27 139 studies [38].

28
29 140 Venous blood samples were taken before (baseline: 0h) and 0.5, 1, 2, 4 and 6h post the
30
31 141 ingestion. Blood was collected in heparinized tubes at each time-point and immediately
32
33 142 centrifuged at 657 ×g for 20 min at 4°C. Urine was collected before (baseline: 0h) and in
34
35 143 different collection periods (0-4, 4-8, 8-15 and 15-24h) following EVOO intake. The volume of
36
37 144 urine collected over each time period was recorded. Plasma and urine samples were frozen at -
38
39 145 80°C prior to analysis. The study was approved by the Ethics Committee of the Faculty of
40
41 146 Pharmacy, University of Lisbon (Permission number 02/CEECFFUL/2016). Protocols were
42
43 147 according to the Declaration of Helsinki.

44
45 148

46
47 149 *Extraction of plasma*

48
49 150 Plasma samples were treated according to the method of Day *et al.* [39] with some
50
51 151 modifications. ACN:formic acid (99.8:0.2, v/v) (1.25 mL) was added to 500 µL of plasma.
52
53 152 Samples were vortexed over a 2 min period followed by 10 min ultrasound, and centrifuged at
54
55 153 16,000 g for 10 min at 4°C. The supernatant was collected, frozen at -80°C, lyophilized, and
56
57 154 suspended in 50 µL methanol:water (80:20, v/v), before analysis. The extraction procedure was
58
59
60

1
2
3 155 evaluated, as described in **supplementary information (SI) section 1.1**, by spiking pool plasma
4
5 156 with six standard phenolic compounds (apigenin, luteolin, enterolactone, enterodiol, pinoresinol
6
7 157 and oleuropein) at known concentrations. These standards were selected as they represent
8
9 158 different phenolic families and cover the mass range of the majority of metabolites detected in
10
11 159 the samples.

12
13 160

14 15 161 *Urine*

16
17 162 Urine samples were defrosted centrifuged at 16,000 g for 10 min at 4°C before analysis (one
18
19 163 freeze-thaw cycle) without further processing.

20
21 164

22 23 165 *Upper gastro-intestinal in vitro digestion model*

24
25 166 An EVOO was subjected to an upper gastro-intestinal *in vitro* digestion model ($n=6$ digestions)
26
27 167 according to Soler A. *et al.* [13]. Oil and aqueous phases were separated and frozen at -20°C
28
29 168 prior to analysis by LC-MS/MS.

30
31 169

32 33 170 *In vitro microsomal assays*

34
35 171 An EVOO extract (**SI section 1.2**) was diluted to 10^{-5} M (CAE equivalents) in DMSO and used
36
37 172 as described in **SI section 1.3** [40-42]. The supernatant fraction was analysed by LC-MS/MS.

38
39 173

40 41 174 *Total phenolic content determination*

42
43 175 The protocol described by Owen *et al.* [43] was used for the preparation of the phenolic
44
45 176 compounds extract (**SI section 1.2**). The total phenolic content was determined [44] in triplicate
46
47 177 (**SI section 1.2**).

48
49 178

50 51 179 *LC-MS/MS assays*

52
53 180 Details of the analysis are presented in the **SI section 1.4**. Hydroxytyrosol and tyrosol standards
54
55 181 were used to quantify these compounds and their secoiridoid derivatives in EVOO (results
56
57 182 expressed as hydroxytyrosol and tyrosol equivalents).

1
2
3 183 *UPLC-Orbitrap MS assays*

4 184 Details of the analysis are presented in **SI section 1.5**. Identification was based on molecular ion
5
6 185 and mass error tolerance (5 ppm).
7
8

9 186

10
11 187 *Data analysis*

12
13 188 The Thermo ToxID software was used to screen for EVOO phenolic compounds and
14
15 189 corresponding metabolites in plasma and urine samples detected by UPLC-ESI-Orbitrap MS
16
17 190 analysis. As our aim was to find discrimination patterns before and after EVOO intake, we
18
19 191 screened for target compounds in plasma and urine samples in their free form and also as the
20
21 192 corresponding Phase I (hydrogenation, dehydrogenation, hydration and hydroxylation) and
22
23 193 Phase II metabolites (methylation, glucuronidation and sulfation), and mixed forms of Phase I
24
25 194 and II metabolites (Phase I reaction + glucuronidation and/or methylation): secoiridoids and
26
27 195 their metabolites as elenolic acid, hydroxy elenolic acid, 3,4-DHPEA-EA, 3,4-DHPEA-EDA,
28
29 196 methyl 3,4-DHPEA-EDA, methyl 3,4-DHPEA-EA, 10-hydroxy-3,4-DHPEA-EA, 10-hydroxy-
30
31 197 3,4-DHPEA-EDA, *p*-HPEA-EA and *p*-HPEA-EDA. Other typical phenolics reported in VOO
32
33 198 [45] were also screened: phenolic alcohols (tyrosol, hydroxytyrosol) and hydroxytyrosol
34
35 199 acetate, phenolic acids (caffeic acid, *p*-coumaric acid, homovanillic acid, vanillic acid), phenolic
36
37 200 aldehyde (vanillin), flavonoids (luteolin, apigenin) and lignans (1-acetoxypinoresinol,
38
39 201 hydroxypinoresinol, pinoresinol, syringaresinol). All these compounds were tentatively
40
41 202 identified by their exact mass. Criteria for variable inclusion in multivariate analysis were
42
43 203 absence at 0h time point in plasma and urine [23] and presence in samples from at least two
44
45 204 volunteers post EVOO ingestion. Participants were instructed to follow a low phenolic diet
46
47 205 that excluded olives and any type of OO. Therefore by excluding variables present at baseline
48
49 206 the variations in possible markers would be related to EVOO consumption minimizing
50
51 207 interference due to other dietary components.
52

53
54 208 Alignment of retention time and *m/z* values was carried out across samples using a tolerance
55
56 209 window of 5 ppm. Normalization of peak area of selected variables was performed using peak
57
58
59
60

1
2
3 210 area/total peak area for each chromatogram, and expressed as ppm (Peak area/total peak area X
4
5 211 1,000,000). Normalization allowed removal of systemic variation between spectra due to
6
7 212 variations in sample or equipment. Partial Least Squares Discriminant Analysis with Orthogonal
8
9 213 Signal correction (OSC-PLS-DA) was performed using SIMCA-P+ 13.0 software (**SI section**
10
11 214 **1.6**).

12
13 215 Determination of excretion of urinary biomarkers was done using hydroxytyrosol as standard.
14
15 216 Normalized peak areas of the selected variables in urine were used for quantification. Values
16
17 217 were corrected according to volume of urine collected per sample point.
18
19 218

219 **Results**

220 *Phenolic composition of EVOO*

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

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

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

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

237 *Plasma samples*

1
2
3 238 Validation tests were performed for plasma extraction and results are presented in **SI section 2.3**
4
5 239 (**Tables S2 and S3**). Eighteen compounds in plasma samples were selected as variables
6
7 240 according to inclusion criteria (**Table 2**) and used for multivariate analysis. Possible isomeric
8
9 241 forms were detected for several secoiridoid compounds, as reported before [36].
10
11 242 The parameters used to assess the OSC-PLS-DA modelling quality are summarized in **Table 3**.
12
13 243 With the exception of the model with all plasma samples evaluated at the same time (data not
14
15 244 shown), all OSC-PLS-DA models presented acceptable values for all quality and validation
16
17 245 parameters, indicative that they were able to discriminate samples from baseline. These OSC-
18
19 246 PLS-DA models (baseline vs. each time collection point) explained between 27.9 and 41.2% of
20
21 247 the metabolite intensity variation (R^2X) and between 75.3 and 97.3% of the time point variation
22
23 248 (R^2Y). In our work prediction values Q^2 were between 62.1 and 93.6% after cross-validation,
24
25 249 indicating good predictive ability of the models.
26
27 250 Permutation tests allowed assessment of overfitting. Models were considered valid as the
28
29 251 average of the R^2 values of the permuted models were less than half the R^2 value of the original
30
31 252 model, indicating that more than half of the explained variability was not due to change [46].
32
33 253 $p(\text{corr})$ values were calculated for each of the models and are presented **SI section 2.4 (Table**
34
35 254 **S4)**. Three variables (**P1A**, **P1B** and **P9B**) were discriminant for all time points and were
36
37 255 selected as plasma biomarkers to monitor EVOO intake. Two other variables were
38
39 256 discriminatory for one time point (**SI section 2.4 Table S4**) and nine variables were
40
41 257 discriminant for two to four time points (**SI section 2.4 Table S4**).
42
43
44 258

259 *Urine samples*

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

1
2
3 266 between 80.0 and 100% of the time point variation (R^2Y), with prediction values Q^2 between
4
5 267 77.6 and 99.4% after cross-validation. The obtained values of permutation tests were considered
6
7 268 acceptable. $p(\text{corr})$ values were calculated for each of the models and are presented in **SI section**
8
9 269 **2.4 (Table S5)**. Nine variables were discriminant for all time points when compared with
10
11 270 baseline samples (**U2, U9D, U9F, U9G, U9H, U11A, U11B, U11D and U11E**). These variables
12
13 271 were selected as urinary biomarkers to monitor EVOO intake. Other four variables were
14
15 272 discriminant for one time point (**SI section 2.4 Table S5**) and eighteen variables where
16
17 273 discriminant for two or three time points (**SI section 2.4 Table S5**).

274

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

276 Absorption profiles of the discriminant plasma biomarkers presented maximum levels between
277 0.5 and 2h after EVOO intake, depending on each volunteer (**Figure 1**). In **Figure 2** are shown
278 the excretion profiles of the nine discriminant urinary biomarkers, all hydroxytyrosol
279 derivatives. Maximum concentrations in human urine were reached in the time period of 0-4h
280 after oil intake and mean values for the nine volunteers are presented. The total amount excreted
281 (24h) of each selected biomarker per volunteer is presented in **Figure 3**.

282

283 **Discussion**

284 Within this work HRMS was used as a tool to identify, with a high selectivity and low error, the
285 detected metabolites in plasma and urine samples after EVOO intake. ESI negative ionization
286 mode was selected as it is more sensitive than the positive ionization mode [23, 47]. Moreover
287 this approach overcomes complex sample preparation procedures related with GC-MS analysis
288 [48].

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

1
2
3 294 pre-processing data filter, was used before proceeding with the PLS-DA to eliminate the signal
4
5 295 information (X) that is unrelated (orthogonal) to the classes (Y) [49, 50]. Using this approach it
6
7 296 was possible, to select biomarkers that were the best predictors of the class vector.
8
9 297 The OSC-PLS-DA models with all samples evaluated at the same time did not meet quality
10
11 298 criteria. This could be due to the low number of variables and/or due to criteria used for
12
13 299 variables inclusion (absence at baseline). Therefore, further models were performed at each time
14
15 300 point to analyse the separation of samples of each time point compared with baseline samples.
16
17 301 Using this approach three plasma secoiridoid compounds (elenolic acid + H₂ detected at two
18
19 302 retention times; *p*-HPEA-EA + H₂ + glucuronide) and nine urinary (3,4-DHPEA-EA, 3,4-
20
21 303 DHPEA-EA + H₂+glucuronide detected at four retention times, and methyl 3,4-DHPEA-EA +
22
23 304 H₂ + glucuronide detected at four retention times) secoiridoid compounds were selected as
24
25 305 biomarkers for monitoring EVOO intake in human intervention trials, as they were discriminant
26
27 306 for all time collection points when compared to baseline. Moreover other secoiridoids were
28
29 307 identified in plasma and urine as enabling discrimination of one or more time points. It is worth
30
31 308 noting that although other phenolic compounds and metabolites were monitored in plasma and
32
33 309 urine samples, the secoiridoids group clearly stood apart in terms of sample discrimination,
34
35 310 results in agreement with others [23].
36
37 311 In our study the results from the *in vitro* experiments with microsomes showed that
38
39 312 secoiridoids 3,4-DHPEA-EA and *p*-HPEA-EA were stable, when incubated with liver
40
41 313 microsomes, with half-lives longer than 6h. A search for metabolites, allowed us to detect
42
43 314 compounds, identified as the hydrogenated forms of the parent compounds. Although there are
44
45 315 differences between rat and human microsomal activity, hydrogenation seems to be an
46
47 316 important Phase I route of metabolism for these secoiridoids. This evidence was supported by
48
49 317 results obtained in the human intervention as hydrogenated metabolites were detected in plasma
50
51 318 for elenolic acid, a structural unit of phenolic secoiridoids. Therefore, the detection of elenolic
52
53 319 acid in plasma (hydrogenated form) could be related with hydrolysis of oleuropein and
54
55 320 ligstroside aglycones in the gastric or intestinal environment, as evidenced after *in vitro*
56
57 321 digestion of EVOO. Discriminating metabolites resulting from Phase I metabolism, concerning
58
59
60

1
2
3 322 hydroxylation and hydration, were also detected in plasma such as *p*-HPEA-EDA + H₂O and
4
5 323 3,4-DHPEA-EA + OH. These compounds discriminated samples for two to four time collection
6
7 324 points. Our results are in accordance with others [23], that identified these metabolites as
8
9 325 enabling discrimination in human urines before and after EVOO intake.
10
11 326 Absorption and metabolism of the secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA was
12
13 327 studied before [12], by using perfused segments of *jejunum* and *ileum*, and hydrogenation
14
15 328 followed by glucuronidation of these compounds was reported. Authors attributed the reduced
16
17 329 metabolite forms to the action of NADPH-dependent aldo-keto reductases present in the small
18
19 330 intestine. In our study, hydrogenated forms of *p*-HPEA-EDA and *p*-HPEA-EA glucuronides
20
21 331 were also detected in plasma. Moreover, *p*-HPEA-EA + H₂ + glucuronide was selected within
22
23 332 this work as a discriminating plasma metabolite.
24
25 333 In urine, 3,4-DHPEA-EA + H₂ + glucuronide and methyl 3,4-DHPEA-EA + H₂ + glucuronide
26
27 334 were selected as biomarkers of EVOO intake for all time collection points. The higher polarity
28
29 335 of 3,4-DHPEA-EA and methyl 3,4-DHPEA-EA hydrogenated glucuronides compared with *p*-
30
31 336 HPEA-EDA and *p*-HPEA-EA corresponding metabolites, might explain their presence in urine
32
33 337 and not in plasma. As the action site of NADPH-dependent aldo-keto reductases is in the
34
35 338 elenolic acid unit [12] the same reactions can take place in oleuropein or ligstroside aglycones.
36
37 339 Moreover hydrogenated metabolites of *p*-HPEA-EA and 3,4-DHPEA-EA glucuronides were
38
39 340 selected before as human urinary biomarkers for EVOO intake [23].
40
41 341 It is worth noting the selection of 3,4-DHPEA-EA as discrimination marker in urine. This
42
43 342 typical EVOO phenolic compound was also selected as marker in plasma, however not for all of
44
45 343 the collection points. Other urinary discrimination markers included 3,4-DHPEA-EA + H₂ +
46
47 344 glucuronide and methyl-3,4-DHPEA-EA + H₂ + glucuronide. The presence of methylated forms
48
49 345 might be explained by the action of catechol-*O*-methyl-transferase that catalyzes the transfer of a
50
51 346 methyl group from S-adenosyl-L-methionine to phenolic compounds having an *o*-diphenolic
52
53 347 structure [51], although methyl 3,4-DHPEA-EA was also present in the EVOO.
54
55
56 348 In our study, glucuronide metabolites of *p*-HPEA-EDA, 3,4-DHPEA-EDA and methyl 3,4-
57
58 349 DHPEA-EDA, were detected as major metabolites in urine, as were glucuronides of methyl 3,4-

1
2
3 350 DHPEA-EDA + OH, methyl 3,4-DHPEA-EA + H₂ and 3,4-DHPEA-EA + H₂/OH/H₂O.
4
5 351 Glucuronides of *p*-HPEA-EDA, 3,4-DHPEA-EA + H₂/OH/ H₂O, and methyl 3,4-DHPEA-EDA
6
7 352 + OH were previously identified in human urine samples after intake of EVOO [23].
8
9 353 Glucuronides of secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA derivatives were detected
10
11 354 in human plasma samples after a bioavailability study with VOO [16]. In our study glucuronides
12
13 355 of hydrogenated forms of *p*-HPEA-EDA and *p*-HPEA-EA were also detected in plasma as well
14
15 356 as glucuronide of methyl 3,4-DHPEA-EDA+H₂O. UDP-glucuronosyltransferases are
16
17 357 membrane-bounded enzymes that are located in the endoplasmic reticulum in many tissues and
18
19 358 catalyze the transfer of a glucuronic acid from UDP-glucuronic acid to phenolic compounds or
20
21 359 xenobiotics [51].
22
23 360 Metabolites of the secoiridoid group were detected with the same exact mass but at different
24
25 361 retention times, suggesting the presence of isomers, which could be justified by the ring opening
26
27 362 in the secoiridoid structure, due to keto-enolic tautomeric equilibrium [52]. The presence of two
28
29 363 putative groups of isomers for secoiridoid derivatives as elenolic acid, oleuropein and
30
31 364 ligstroside aglycones was previously reported [36].
32
33 365 Maximum absorption levels of the plasma biomarkers were detected between 0.5 and 2h and
34
35 366 results are in accordance with others (maximum at 1h) [16]. Maximum excretion of urinary
36
37 367 biomarkers occurred in the first 4h after EVOO intake and results are in accordance with others
38
39 368 (maximum between 2 - 4h) [23]. Variability of results among volunteers was high for the
40
41 369 selected biomarkers in plasma and urine which is reflected in the standard deviations obtained.
42
43 370 Bioavailability of phenolic compounds not only depends on their concentration in the VOO but
44
45 371 is also affected by the individual genomic profile with impact on enzymatic activity involved in
46
47 372 the digestion and metabolism process. In fact polymorphism expression of conjugation enzymes
48
49 373 [51] or individual variations in digestive enzymes [53] or bile salts [54] might justify the
50
51 374 variations observed.
52
53 375 VOO phenolics undergo rapid absorption and fast renal elimination as evidenced in the
54
55 376 absorption and excretion profiles of the selected biomarkers. Accumulation of metabolites in
56
57 377 target tissues, studied before [55], could be considered when investigating the potential

1
2
3 378 bioactivity of VOO phenolic compounds. In this work, the lack of pure standards did not allow
4
5 379 the calculation of a mass balance between the ingested EVOO phenolics, plasma and urinary
6
7 380 metabolites and pharmacokinetic data.
8
9 381 The use of secoiridoids as biomarkers to monitor EVOO intake has the advantage of specificity.
10
11 382 While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables
12
13 383 belonging to various botanical families, secoiridoids are present exclusively in plants belonging
14
15 384 to the family of *Oleaceae* including the olive tree *Olea europaea* L. [8]. Results of this work
16
17 385 highlight the importance of secoiridoids as biomarkers of EVOO intake. These biomarkers
18
19 386 would allow monitoring compliance of following the dose in interventions aimed to evaluate
20
21 387 health benefits of EVOO intake in accordance with the EFSA claim [5]. Although secoiridoids
22
23 388 are the major components in the phenolic fraction of EVOO [56], we have to take into account
24
25 389 that several factors influence its content in EVOO. Olive tree cultivars, alternate bearing of the
26
27 390 olive tree, environmental conditions, maturity of the fruits or technological factors are
28
29 391 parameters that influence chemical composition of EVOO and therefore increase variability in
30
31 392 the phenolic composition [1, 8, 57, 58]. The combination of digestion models with *in vitro*
32
33 393 approaches of hepatic metabolism (e.g.: incubation with HepG2 cells or liver microsomes) can
34
35 394 be useful in producing metabolites from EVOO. Recently it was demonstrated the use of Caco-2
36
37 395 cells as bioreactors for generation of phase II metabolites of hydroxytyrosol [59]. This approach
38
39 396 would allow us to confirm the identity of biomarkers selected in this work. After identification
40
41 397 it would be possible to produce biomarkers as authentic standard allowing absolute calibration
42
43 398 of these compounds in samples. Moreover the use of HRMS/MS would enable structural
44
45 399 clarification of the putatively identified isomers.
46
47 400 The secoiridoid metabolites detected in plasma and urine samples from volunteers were able to
48
49 401 discriminate between different time points after EVOO intake. The most common metabolic
50
51 402 reactions were Phase I hydrogenation and Phase II glucuronidation. The selected secoiridoid
52
53 403 biomarkers can be added to the ones already described. Due to the variability in absorption,
54
55 404 metabolism and elimination of compounds, future studies should include broader populations to
56
57
58
59
60

1
2
3 405 account for inter-individual variability. The role of the identified metabolites in beneficial health
4
5 406 effects requires further investigation.
6

7 407

8
9 408 *Authors contributions: S.S., M.E.F, E.C., W.M. and M.R.B designed the research. S.S.*

10
11 409 *conducted research, analyzed data and prepared the first draft of the manuscript. M.G.A*

12
13 410 *performed statistical analysis. All authors: were responsible for the interpretation of data,*

14
15 411 *critical review of the manuscript and approved the final manuscript.*
16

17 412

18
19 413 *This study was supported by QREN project Azeite+Global n° 12228, iNOVA4Health and*

20
21 414 *National Programme for Scientific Re-equipment for the acquisition of the LC-MS/MS*

22
23 415 *equipment that is part of the Portuguese National Mass Spectrometry Network (Contract*

24
25 416 *RNEM- REDE/1518/REM/2005).*
26

27 417

28
29 418 *The authors have declared no conflict of interest.*
30

31 419

32
33 420

References

34 421

35
36 422 [1] Tripoli, E., Giammanco, M., Tabacchi, G., Di Majo, D., *et al.*, The phenolic compounds of
37 423 olive oil: structure, biological activity and beneficial effects on human health. *Nutrition*
38 424 *research reviews* 2005, 18, 98-112.

39 425 [2] Covas, M. I., Olive oil and the cardiovascular system. *Pharmacological research : the official*
40 426 *journal of the Italian Pharmacological Society* 2007, 55, 175-186.

41 427 [3] Lopez-Miranda, J., Perez-Jimenez, F., Ros, E., De Caterina, R., *et al.*, Olive oil and health:
42 428 summary of the II international conference on olive oil and health consensus report, Jaen and
43 429 Cordoba (Spain) 2008. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2010, 20,
44 430 284-294.

45 431 [4] Urpi-Sarda, M., Casas, R., Chiva-Blanch, G., Romero-Mamani, E. S., *et al.*, Virgin olive oil and
46 432 nuts as key foods of the Mediterranean diet effects on inflammatory biomarkers related to
47 433 atherosclerosis. *Pharmacological research : the official journal of the Italian Pharmacological*
48 434 *Society* 2012, 65, 577-583.

49 435 [5] Silva, S., Bronze, M. R., Figueira, M. E., Siwy, J., *et al.*, Impact of a 6-wk olive oil
50 436 supplementation in healthy adults on urinary proteomic biomarkers of coronary artery
51 437 disease, chronic kidney disease, and diabetes (types 1 and 2): a randomized, parallel,
52 438 controlled, double-blind study. *The American journal of clinical nutrition* 2015, 101, 44-54.

53 439 [6] Scientific Opinion on the substantiation of health claims related to polyphenols in olive and
54 440 protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865),
55 441 maintenance of normal blood HDL-cholesterol concentrations (ID 1639), maintenance of
56 442 normal blood pressure (ID 3781), "anti-inflammatory properties" (ID 1882), "contributes to the
57
58
59
60

- 1
2
3 443 upper respiratory tract health" (ID 3468), "can help to maintain a normal function of
4 444 gastrointestinal tract" (3779), and "contributes to body defences against external agents" (ID
5 445 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal* 2011, 9 (4).
6 446 [7] Scientific Opinion on the substantiation of health claims related to olive oil and
7 447 maintenance of normal blood LDL-cholesterol concentrations (ID 1316, 1332), maintenance of
8 448 normal (fasting) blood concentrations of triglycerides (ID 1316, 1332), maintenance of normal
9 449 blood HDL-cholesterol concentrations (ID 1316, 1332) and maintenance of normal blood
10 450 glucose concentrations (ID 4244) pursuant to Article 13(1) of Regulation (EC) No 1924/2006.
11 451 *EFSA Journal* 2011 9 (4).
12 452 [8] Servili, M., Montedoro, G., Contribution of phenolic compounds to virgin olive oil quality.
13 453 *European Journal of Lipid Science and Technology* 2002, 104, 602-613.
14 454 [9] Perez-Jimenez, F., Ruano, J., Perez-Martinez, P., Lopez-Segura, F., Lopez-Miranda, J., The
15 455 influence of olive oil on human health: not a question of fat alone. *Molecular nutrition & food*
16 456 *research* 2007, 51, 1199-1208.
17 457 [10] Harwood J., A. R., *Handbook of olive oil analysis and properties* 2000.
18 458 [11] Preedy, V. R., Watson, R. R., *Olives and olive oil in health and disease prevention*, Elsevier,
19 459 USA 2010.
20 460 [12] Pinto, J., Paiva-Martins, F., Corona, G., Debnam, E. S., et al., Absorption and metabolism of
21 461 olive oil secoiridoids in the small intestine. *The British journal of nutrition* 2011, 105, 1607-
22 462 1618.
23 463 [13] Aranzazu Soler, M. P. R., Alba Macià, Shikha Saha, Caroline S.M. Furniss, Paul A. Kroon,
24 464 Maria J. Motilva, Digestion stability and evaluation of the metabolism and transport of olive oil
25 465 phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. *Food chemistry* 2010,
26 466 119, 703-714.
27 467 [14] Rubio, L., Macia, A., Valls, R. M., Pedret, A., et al., A new hydroxytyrosol metabolite
28 468 identified in human plasma: hydroxytyrosol acetate sulphate. *Food Chem* 2012, 134, 1132-
29 469 1136.
30 470 [15] Corona, G., Tzounis, X., Assunta Dessi, M., Deiana, M., et al., The fate of olive oil
31 471 polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-
32 472 dependent biotransformation. *Free radical research* 2006, 40, 647-658.
33 473 [16] Suarez, M., Valls, R. M., Romero, M. P., Macia, A., et al., Bioavailability of phenols from a
34 474 phenol-enriched olive oil. *The British journal of nutrition* 2011, 106, 1691-1701.
35 475 [17] Manna, C., Galletti, P., Maisto, G., Cucciolla, V., et al., Transport mechanism and
36 476 metabolism of olive oil hydroxytyrosol in Caco-2 cells. *FEBS letters* 2000, 470, 341-344.
37 477 [18] de la Torre, R., Bioavailability of olive oil phenolic compounds in humans.
38 478 *Inflammopharmacology* 2008, 16, 245-247.
39 479 [19] Tuck, K. L., Freeman, M. P., Hayball, P. J., Stretch, G. L., Stupans, I., The in vivo fate of
40 480 hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and
41 481 oral dosing of labeled compounds to rats. *The Journal of nutrition* 2001, 131, 1993-1996.
42 482 [20] Gonzalez-Santiago, M., Fonolla, J., Lopez-Huertas, E., Human absorption of a supplement
43 483 containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its
44 484 transient association with low-density lipoproteins. *Pharmacological research* 2010, 61, 364-
45 485 370.
46 486 [21] Visioli, F., Galli, C., Grande, S., Colonnelli, K., et al., Hydroxytyrosol excretion differs
47 487 between rats and humans and depends on the vehicle of administration. *The Journal of*
48 488 *nutrition* 2003, 133, 2612-2615.
49 489 [22] Miro-Casas, E., Covas, M. I., Farre, M., Fito, M., et al., Hydroxytyrosol disposition in
50 490 humans. *Clinical chemistry* 2003, 49, 945-952.
51 491 [23] Garcia-Villalba, R., Carrasco-Pancorbo, A., Nevedomskaya, E., Mayboroda, O. A., et al.,
52 492 Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil:
53 493 understanding the metabolism of polyphenols. *Analytical and bioanalytical chemistry* 2010,
54 494 398, 463-475.

- 1
2
3 495 [24] Mateos, R., Goya, L., Bravo, L., Metabolism of the olive oil phenols hydroxytyrosol, tyrosol,
4 496 and hydroxytyrosyl acetate by human hepatoma HepG2 cells. *Journal of agricultural and food*
5 497 *chemistry* 2005, *53*, 9897-9905.
- 6 498 [25] Miro Casas, E., Farre Albadalejo, M., Covas Planells, M. I., Fito Colomer, M., *et al.*, Tyrosol
7 499 bioavailability in humans after ingestion of virgin olive oil. *Clinical chemistry* 2001, *47*, 341-343.
- 8 500 [26] Vissers, M. N., Zock, P. L., Roodenburg, A. J., Leenen, R., Katan, M. B., Olive oil phenols are
9 501 absorbed in humans. *The Journal of nutrition* 2002, *132*, 409-417.
- 10 502 [27] Dominguez-Perles, R., Aunon, D., Ferreres, F., Gil-Izquierdo, A., Gender differences in
11 503 plasma and urine metabolites from Sprague-Dawley rats after oral administration of normal
12 504 and high doses of hydroxytyrosol, hydroxytyrosol acetate, and DOPAC. *European journal of*
13 505 *nutrition* 2015.
- 14 506 [28] D'Archivio, M., Filesi, C., Vari, R., Scaccocchio, B., Masella, R., Bioavailability of the
15 507 polyphenols: status and controversies. *International journal of molecular sciences* 2010, *11*,
16 508 1321-1342.
- 17 509 [29] de la Torre, R., Covas, M. I., Pujadas, M. A., Fito, M., Farre, M., Is dopamine behind the
18 510 health benefits of red wine? *European journal of nutrition* 2006, *45*, 307-310.
- 19 511 [30] Saccenti, E., Hoefsloot, H. C. J., Smilde, A. K., Westerhuis, J. A., & Hendriks, M. M. W. B. ,
20 512 Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics* 2013,
21 513 *10*.
- 22 514 [31] Gromski, P. S., Muhamadali, H., Ellis, D. I., Xu, Y., *et al.*, A tutorial review: Metabolomics
23 515 and partial least squares-discriminant analysis--a marriage of convenience or a shotgun
24 516 wedding. *Analytica chimica acta* 2015, *879*, 10-23.
- 25 517 [32] Madsen, R., Lundstedt, T., Trygg, J., Chemometrics in metabolomics--a review in human
26 518 disease diagnosis. *Analytica chimica acta* 2010, *659*, 23-33.
- 27 519 [33] Caruso, D., Colombo, R., Patelli, R., Giavarini, F., Galli, G., Rapid evaluation of phenolic
28 520 component profile and analysis of oleuropein aglycon in olive oil by atmospheric pressure
29 521 chemical ionization-mass spectrometry (APCI-MS). *Journal of agricultural and food chemistry*
30 522 2000, *48*, 1182-1185.
- 31 523 [34] Obied, H. K., Bedgood, D. R., Jr., Prenzler, P. D., Robards, K., Chemical screening of olive
32 524 biophenol extracts by hyphenated liquid chromatography. *Analytica chimica acta* 2007, *603*,
33 525 176-189.
- 34 526 [35] Fu, S., Arraez-Roman, D., Menendez, J. A., Segura-Carretero, A., Fernandez-Gutierrez, A.,
35 527 Characterization of isomers of oleuropein aglycon in olive oils by rapid-resolution liquid
36 528 chromatography coupled to electrospray time-of-flight and ion trap tandem mass
37 529 spectrometry. *Rapid communications in mass spectrometry : RCM* 2009, *23*, 51-59.
- 38 530 [36] Vichi, S., Cortes-Francisco, N., Caixach, J., Insight into virgin olive oil secoiridoids
39 531 characterization by high-resolution mass spectrometry and accurate mass measurements.
40 532 *Journal of chromatography. A* 2013, *1301*, 48-59.
- 41 533 [37] Garcia-Aloy, M., Llorach, R., Urpi-Sarda, M., Jauregui, O., *et al.*, A metabolomics-driven
42 534 approach to predict cocoa product consumption by designing a multimetabolite biomarker
43 535 model in free-living subjects from the PREDIMED study. *Molecular nutrition & food research*
44 536 2015, *59*, 212-220.
- 45 537 [38] Vissers, M. N., Zock, P. L., Katan, M. B., Bioavailability and antioxidant effects of olive oil
46 538 phenols in humans: a review. *European journal of clinical nutrition* 2004, *58*, 955-965.
- 47 539 [39] Day, A. J., Mellon, F., Barron, D., Sarrazin, G., *et al.*, Human metabolism of dietary
48 540 flavonoids: identification of plasma metabolites of quercetin. *Free radical research* 2001, *35*,
49 541 941-952.
- 50 542 [40] Guantai, E. M., Ncokazi, K., Egan, T. J., Gut, J., *et al.*, Enone- and chalcone-chloroquinoline
51 543 hybrid analogues: in silico guided design, synthesis, antiplasmodial activity, in vitro
52 544 metabolism, and mechanistic studies. *Journal of medicinal chemistry* 2011, *54*, 3637-3649.
- 53 545 [41] Margout, D., Gattacceca, F., Moarbess, G., Wein, S., *et al.*, Pharmacokinetic properties and
54 546 metabolism of a new potent antimalarial N-alkylamidine compound, M64, and its

- 1
2
3 547 corresponding bioprecursors. *European journal of pharmaceutical sciences : official journal of*
4 548 *the European Federation for Pharmaceutical Sciences* 2011, 42, 81-90.
- 5 549 [42] Obach, R. S., Prediction of human clearance of twenty-nine drugs from hepatic
6 550 microsomal intrinsic clearance data: An examination of in vitro half-life approach and
7 551 nonspecific binding to microsomes. *Drug metabolism and disposition: the biological fate of*
8 552 *chemicals* 1999, 27, 1350-1359.
- 9 553 [43] Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., *et al.*, The antioxidant/anticancer
10 554 potential of phenolic compounds isolated from olive oil. *European journal of cancer* 2000, 36,
11 555 1235-1247.
- 12 556 [44] Stamatakis, G., Tsantila, N., Samiotaki, M., Panayotou, G. N., *et al.*, Detection and isolation
13 557 of antiatherogenic and antioxidant substances present in olive mill wastes by a novel filtration
14 558 system. *J Agric Food Chem* 2009, 57, 10554-10564.
- 15 559 [45] Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gomez-Caravaca, A. M., *et al.*, Phenolic
16 560 molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant
17 561 activity and analytical methods. An overview of the last decade. *Molecules* 2007, 12, 1679-
18 562 1719.
- 19 563 [46] Borel, P., Desmarchelier, C., Nowicki, M., Bott, R., Morange, S., & Lesavre, N. ,
20 564 Interindividual variability of lutein bioavailability in healthy men: characterization, genetic
21 565 variants involved, and relation with fasting plasma lutein concentration. *The American journal*
22 566 *of clinical nutrition* 2014, 100, 168-175.
- 23 567 [47] Garcia-Villalba, R., Larrosa, M., Possemiers, S., Tomas-Barberan, F. A., Espin, J. C.,
24 568 Bioavailability of phenolics from an oleuropein-rich olive (*Olea europaea*) leaf extract and its
25 569 acute effect on plasma antioxidant status: comparison between pre- and postmenopausal
26 570 women. *European journal of nutrition* 2014, 53, 1015-1027.
- 27 571 [48] Suarez, M., Macia, A., Romero, M. P., Motilva, M. J., Improved liquid chromatography
28 572 tandem mass spectrometry method for the determination of phenolic compounds in virgin
29 573 olive oil. *Journal of chromatography. A* 2008, 1214, 90-99.
- 30 574 [49] Pujos-Guillot, E., Hubert, J., Martin, J.-F., Lyan, B., Quintana, M., ... Manach, C. , Mass
31 575 spectrometry-based metabolomics for the discovery of biomarkers of fruit and vegetable
32 576 intake: citrus fruit as a case study. *Journal of Proteome Research* 2013, 12, 1645–1659.
- 33 577 [50] Trygg, J., Holmes, E., & Lundstedt, T. , Chemometrics in metabolomics. *Journal of*
34 578 *Proteome Research* 2007, 6, 469-479.
- 35 579 [51] Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., Polyphenols: food sources
36 580 and bioavailability. *The American journal of clinical nutrition* 2004, 79, 727-747.
- 37 581 [52] Fu, S., Arraez-Roman, D., Segura-Carretero, A., Menendez, J. A., *et al.*, Qualitative
38 582 screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography
39 583 and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Analytical*
40 584 *and bioanalytical chemistry* 2010, 397, 643-654.
- 41 585 [53] Lindahl, A., Ungell, A. L., Knutson, L., Lennernas, H., Characterization of fluids from the
42 586 stomach and proximal jejunum in men and women. *Pharmaceutical research* 1997, 14, 497-
43 587 502.
- 44 588 [54] Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., *et al.*, Characterization of the
45 589 human upper gastrointestinal contents under conditions simulating
46 590 bioavailability/bioequivalence studies. *Pharmaceutical research* 2006, 23, 165-176.
- 47 591 [55] Lopez de Las Hazas, M. C., Rubio, L., Krotonoulas, A., de la Torre, R., *et al.*, Dose effect on
48 592 the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats.
49 593 *Molecular nutrition & food research* 2015.
- 50 594 [56] Sanchez de Medina, V., Priego-Capote, F., de Castro, M. D., Characterization of
51 595 monovarietal virgin olive oils by phenols profiling. *Talanta* 2015, 132, 424-432.
- 52 596 [57] Council, I. O., *World Olive Encyclopaedia*, International Olive Oil Council, Barcelona, Spain
53 597 1996.
- 54
55
56
57
58
59
60

1
2
3 598 [58] Vinha A. F., F. F., Silva B.M., Valentão P., Gonçalves A., Pereira J., Oliveira M. B., Seabra
4 599 R.M, Andrade P.B. , Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): Influences
5 600 of cultivar and geographical origin. *Food Chemistry* 2005, 89, 561-568.

6 601 [59] Catalan, U., Lopez de Las Hazas, M. C., Rubio, L., Fernandez-Castillejo, S., *et al.*, Protective
7 602 effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial
8 603 dysfunction in human aortic endothelial cells. *Molecular nutrition & food research* 2015, 59,
9 604 2523-2536.

10
11 605

12
13 606

14
15 607

16
17 608

18
19 609

20
21 610

22
23 611

24
25 612

26
27 613

28
29 614

30
31 615

32
33 616

34
35 617

36
37 618

38
39 619

40
41 620

42
43 621

44
45 622

46
47 623

48
49 624

50
51 625

52
53 626

54
55
56
57
58
59
60

1
2
3 627 Figure 1 - Plasma absorption-time profiles of variables P1A (elenolic acid + H₂), P1B (elenolic
4
5 628 acid + H₂ detected at a different retention time) and P9B (*p*-HPEA-EA+H₂+glucuronide) for
6
7 629 volunteers (1 to 9) and mean values. Data are expressed as normalized areas (ppm)

8
9 630

10
11
12 631 Figure 2 - Excretion profile of discriminatory biomarkers identified in urine after EVOO intake
13
14 632 (μg excreted expressed as hydroxytyrosol equivalents). Data are expressed as mean \pm SD

15
16 633

17
18
19 634 Figure 3 – Total excretion of discriminatory biomarkers identified in urine (0-24h) per volunteer
20
21 635 ($n=9$) after EVOO intake (μg of hydroxytyrosol equivalents)

22
23 636

24
25 637

26
27 638

28
29 639

30
31 640

32
33 641

34
35 642

36
37 643

38
39 644

40
41 645

42
43 646

44
45 647

46
47 648

48
49 649

50
51 650

52
53 651

54
55 652

56
57 653

58
59
60

654

655

656 Table 1 – Phenolic compounds and elenolic acid in EVOO used in the human intervention

657 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 ₈ H ₉ O ₃	123, 95	Hydroxytyrosol	3.75
5.3	137	C ₈ H ₉ O 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	<i>p</i> -HPEA-EA**	203.08
18.5	303	C ₁₇ H ₁₉ O ₅	285, 179	<i>p</i> -HPEA-EDA**	30.08
14.3	241	C ₁₁ H ₁₃ O ₆	139	Elenolic acid*	10.04
17.4	285	C ₁₅ H ₉ O ₆	133	Luteolin	3.19
18.7	269	C ₁₅ H ₉ O 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 ₈ H ₇ O ₃	136	Vanillin	0.14

658

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

659

660

661

662

663

664

665 Table 2 - Retention time, theoretical and experimental m/z , error and ion formula obtained in
 666 negative mode for compounds detected in plasma (P) and considered as variables for OSC-PLS-
 667 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 + H₂	10.35	243.0874	243.0862	-4.9	C₁₁H₁₅O₆	P1A
Elenolic acid + H₂	10.97	243.0874	243.0863	-4.5	C₁₁H₁₅O₆	P1B
3,4-DHPEA-EDA	13.34	319.1187	319.1174	-4.2	C ₁₇ H ₁₉ O ₆	P2
<i>p</i> -HPEA-EDA + H ₂ O	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
<i>p</i> -HPEA-EDA + H ₂ + glucuronide	9.97	481.1715	481.1700	-3.2	C ₂₃ H ₂₉ O ₁₁	P6A
<i>p</i> -HPEA-EDA + H ₂ + glucuronide	10.24	481.1715	481.1701	-2.9	C ₂₃ H ₂₉ O ₁₁	P6B
<i>p</i> -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 ₂ O + glucuronide	10.12	527.1770	527.1753	-3.3	C ₂₄ H ₃₁ O ₁₃	P8A
Methyl 3,4-DHPEA-EDA + H ₂ O + glucuronide	10.35	527.1770	527.1756	-2.7	C ₂₄ H ₃₁ O ₁₃	P8B
Methyl 3,4-DHPEA-EDA + H ₂ O + glucuronide	10.71	527.1770	527.1755	-2.9	C ₂₄ H ₃₁ O ₁₃	P8C
<i>p</i> -HPEA-EA + H ₂ + glucuronide	11.73	539.1770	539.1757	-2.4	C ₂₅ H ₃₁ O ₁₃	P9A
<i>p</i>-HPEA-EA + H₂ + glucuronide	11.94	539.1770	539.1758	-2.3	C₂₅H₃₁O₁₃	P9B
<i>p</i> -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

668 ^a mean value.

669 ^b letters account for putative isomers.

670 Compounds highlighted in bold enabled plasma samples discrimination after EVOO intake, for

671 all collection points, as found by OSC-PLS-DA

672

673

674

675

676

677

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

Model	OSC Filter		OSC-PLS-DA							Permutation test	
	N	\hat{A}	remaining SS (%)	eigen-value	N	R ² X (cum)	R ² Y (cum)	Q ² (cum)	<i>p</i> -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

680 N, number of components in OSC and PLS-DA model, respectively; \hat{A} displays the angle
681 between the component and the Y variable; SS, sum of squares, indicating the % of the original
682 variance in the X-block that remains in the corrected X-matrix; R²X(cum) and R²Y(cum) are the
683 cumulative modelled variation in the X and Y matrix, respectively; Q²(cum) is the cumulative
684 predicted variation in the Y matrix; the *p*-value from CV-ANOVA, based on the cross-validated
685 predictive residuals, is listed for both models (significant result indicates a valid model).

686

687

688

689

690

691

692 Table 4 - Retention time, theoretical and experimental m/z , error and ion formula obtained in
 693 negative mode for compounds detected in urine (U) and considered as variables for OSC-PLS-
 694 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
<i>p</i> -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	C₁₉H₂₁O₈	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
<i>p</i> -HPEA-EDA +glucuronide	15.48	479.1559	479.1548	-2.2	C ₂₃ H ₂₇ O ₁₁	U5A
<i>p</i> -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 + H₂+glucuronide	16.50	555.1719	555.1707	-2.3	C₂₅H₃₁O₁₄	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 + H₂+glucuronide	16.87	555.1719	555.1706	-2.3	C₂₅H₃₁O₁₄	U9F
3,4-DHPEA-EA + H₂+glucuronide	17.76	555.1719	555.1706	-2.4	C₂₅H₃₁O₁₄	U9G
3,4-DHPEA-EA + H₂+glucuronide	18.09	555.1719	555.1706	-2.3	C₂₅H₃₁O₁₄	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 + H₂+glucuronide	17.69	569.1876	569.1865	-1.9	C₂₆H₃₃O₁₄	U11A
Methyl 3,4-DHPEA-EA + H₂+glucuronide	17.89	569.1876	569.1865	-1.9	C₂₆H₃₃O₁₄	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 + H₂+glucuronide	19.33	569.1876	569.1865	-1.9	C₂₆H₃₃O₁₄	U11D
Methyl 3,4-DHPEA-EA + H₂+glucuronide	19.66	569.1876	569.1868	-1.4	C₂₆H₃₃O₁₄	U11E
3,4-DHPEA-EA + H ₂ O+glucuronide	12.99	571.1668	571.1660	-1.5	C ₂₅ H ₃₁ O ₁₅	U12A
3,4-DHPEA-EA + H ₂ O+glucuronide	13.52	571.1668	571.1658	-1.9	C ₂₅ H ₃₁ O ₁₅	U12B

695 ^a mean value.

696 ^b letters account for putative isomers.

697 Compounds highlighted in bold enabled urine samples discrimination after EVOO intake, for all

698 collection points, as found by OSC-PLS-DA.

699

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

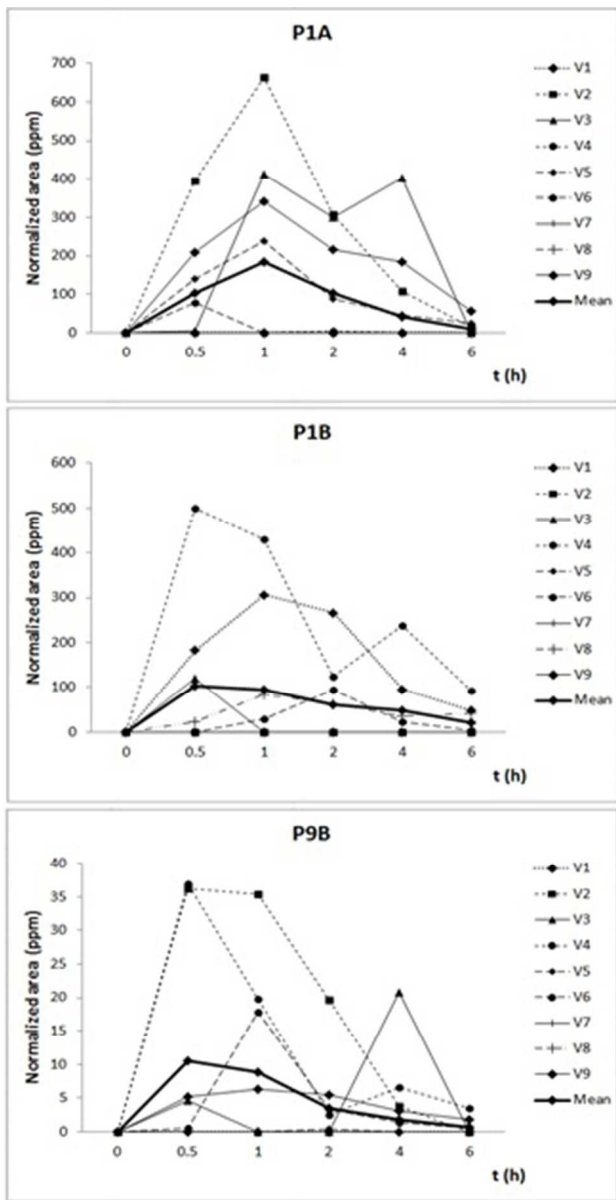
Model	OSC Filter		OSC-PLS-DA						Permutation test		
	N	\hat{A}	remaining SS (%)	eigen- value	N	R ² X (cum)	R ² Y (cum)	Q ² (cum)	p-value	R ²	Q ²
t0h vs. t0-4h	1	89.99	80.35	3.538	1	0.573	0.800	0.776	1.36x10 ⁻⁰⁵	0.096	-0.216
t0h vs. t4-8h	1	90.00	75.18	4.219	1	0.610	1.000	0.994	3.02x10 ⁻¹⁶	0.032	-0.260
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

702 N, number of components in OSC and PLS-DA model, respectively; \hat{A} displays the angle
 703 between the component and the Y variable; SS, sum of squares, indicating the % of the original
 704 variance in the X-block that remains in the corrected X-matrix; R²X(cum) and R²Y(cum) are the
 705 cumulative modelled variation in the X and Y matrix, respectively; Q²(cum) is the cumulative
 706 predicted variation in the Y matrix; the p-value from CV-ANOVA, based on the cross-validated
 707 predictive residuals, is listed for both models (significant result indicates a valid model).

708

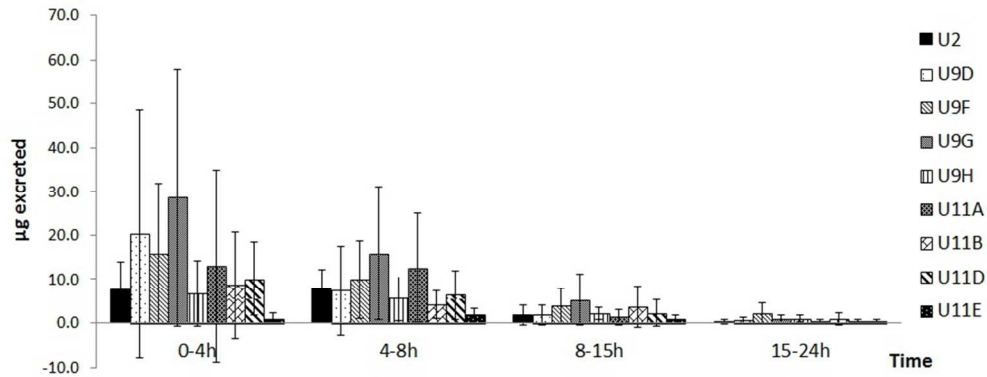
709

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



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)

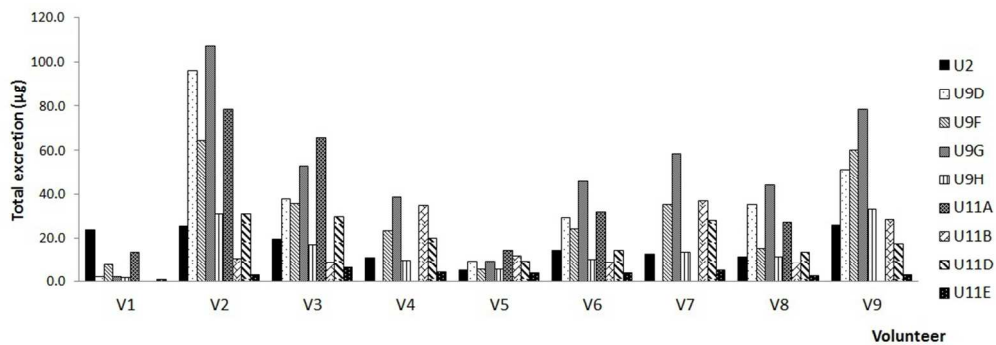
86x166mm (96 x 96 DPI)



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)

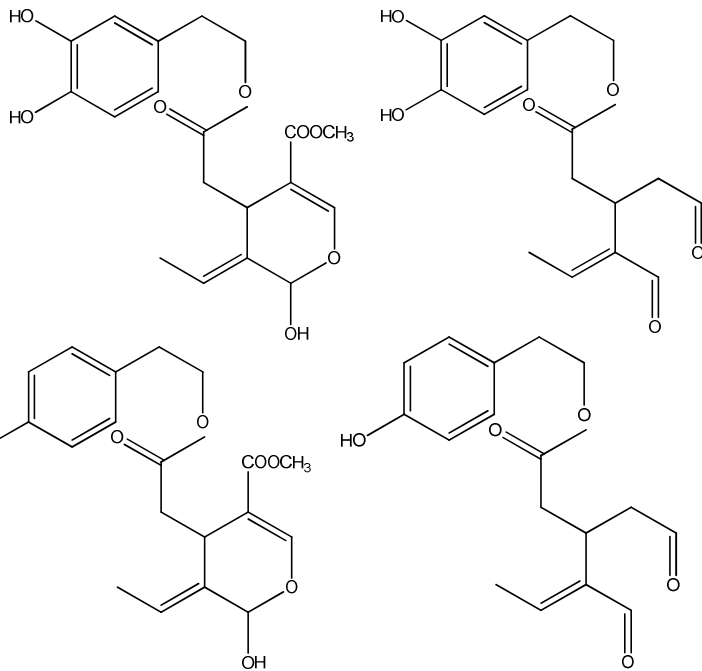
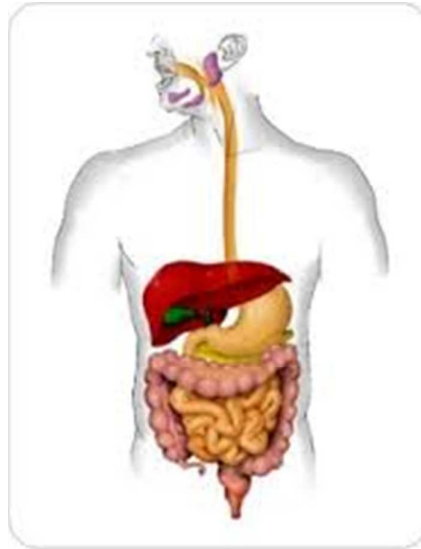
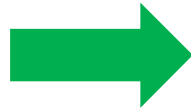
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



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)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

1
2
3
4
5 Secoiridoids are the major components contributing to the phenolic content of extra
6 virgin olive oil (EVOO). Healthy volunteers ($n=9$) ingested 50 mL of EVOO in a single
7 dose containing 6 mg/20 g of hydroxytyrosol and its derivatives. High resolution mass
8 spectrometry and multivariate analysis enabled the identification of plasma and urinary
9 secoiridoid compounds as biomarkers of EVOO intake.
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60