Analysis of Free Hydroxytyrosol in Human Plasma Following the Administration of Olive Oil

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Abstract

Hydroxytyrosol (HT) from olive oil, a potent bioactive molecule with health benefits, has a poor bioavailability, its free form (free HT) being undetectable so far. This fact leads to the controversy whether attained HT concentrations after olive oil polyphenol ingestion are too low to explain the observed biological activities. Due to this, an analytical methodology to determine free HT in plasma is crucial for understanding HT biological activity. Plasma HT instability and low concentrations have been major limitations for its quantification in clinical studies. Here, we describe a method to detect and quantify free HT in human plasma by using liquid chromatography coupled to tandem mass spectrometry. The method encompasses different steps of sample preparation including plasma stabilization, protein precipitation, selective derivatization with benzylamine, and purification by solid-phase extraction. A high sensitivity (LOD, 0.3 ng/mL), specificity and stability of HT is achieved following these procedures. The method was validated and its applicability was demonstrated by analyzing human plasma samples after olive oil intake. A pharmacokinetic comparison was performed measuring free HT plasma concentrations following the intake of 25 mL of ordinary olive oil (nearly undetectable concentrations) versus an extra-virgin olive oil (C_{max} = 4.40 ng/mL). To our knowledge, this is the first time that an analytical procedure for quantifying free HT in plasma after olive oil dietary doses has been reported. The present methodology opens the door to a better understanding of the relationship between HT plasma concentrations and its beneficial health effects.

Keywords: Hydroxytyrosol; virgin olive oil; health; benzylamine derivatization; humans; LC-MS/MS.

1. Introduction

Virgin olive oil (VOO) is a vegetable oil, obtained directly from olives using only mechanical extraction. Major components of olive oil are triacylglycerols and small quantities of free fatty acids, being the main fatty acid in olive oil oleic acid (up to 83%). Minor components, constitute from 1 to 2% of the total oil weight and include more than 230 chemical compounds (aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile and phenolic compounds) [1]. Tyrosol, hydroxytyrosol (HT), and their secoiridoid derivatives (ligstroside and oleuropein) contribute for between the 60 to 80% of the total phenolic compounds content of virgin olive oil, depending on the olive cultivar and the geographical origin.

Among olive oil phenolic compounds, HT is the most potent as antioxidant, and its biological activities have stimulated a large research on its potential role in cardiovascular protection [2, 3]. The oxygen radical absorbance capacity (ORAC) of this phenol, an index of antioxidant activity, is twice that observed for oleuropein (the secoiridoid containing in its structure HT) and three and ten times more for than that for epicatechin (green tea polyphenol) and ascorbic acid, respectively. In *in vitro* and *ex vivo* models, HT shows antioxidant properties, higher than that of vitamin E, on lipids and DNA oxidation [4], as well as a broad spectra of bioactive effects [5]. In animal models, HT displays similar activities [6-8] and delays atherosclerosis progression [9, 10].

The previous biological activities of HT are relevant for the prevention of cardiovascular diseases (CVD) and some of them have been corroborated in humans after olive oil administration with varying concentrations of phenolic compounds [11-14]. In November 2011, the European Food Safety Authority (EFSA) released a health

claim concerning the benefits of the ingestion of 5 mg/day phenolic compounds from olive oil (including HT, tyrosol, and their secoiridoids) for protecting low density lipoprotein (LDL) from oxidation [15].

HT is well absorbed in the gastrointestinal tract in a dose-dependent manner [7, 13, 16] but its bioavailability is poor due to an important presystemic metabolism both in gut and liver, leading to the formation of sulfate and glucuronide conjugates [17-19], to the extent that concentrations in body fluids in its free form (free HT) were deemed undetectable so far [20]. It is not surprising that some authors caution that the attained concentrations after the ingestion of olive oil polyphenols are too low to explain the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations [21]. Several hypotheses have been postulated to explain discrepancies between nutritional intervention clinical trials where effects on secondary biomarkers of oxidation are dose-dependently related to the phenolic compounds content of virgin olive oil and their poor bioavailability. Among them, some authors postulate that glucuronide and/or sulfate conjugates of HT are biologically active as shown in endothelial cellular models [22] and endoplasmic reticulum stress models in HepG2 cells [23]. Several reports have provided evidence that phenolic compounds and their metabolites after virgin olive oil ingestion are incorporated in lipoproteins and this may explain their protecting antioxidant effects [25]. Also effects secondary to HT and its metabolites interaction with biological systems (nutrigenomics) must be considered [26].

The analysis of free HT, the chemical species assumed to be responsible for biological effects, has been limited by its instability in biological fluids as well as low concentrations. In our previous work we described the measurement of total HT (free + conjugated) in both human urine [27, 28] and plasma [29]. The novelty of the present

work is the measurement of free HT in human plasma. We report here an analytical method based on the selective derivatization of catechols with benzylamine in the presence of an oxidant to form fluorescent benzoxazoles [30]. Once derivatized, HT is highly ionizable and stable thus making it susceptible to be detected by LC-MS/MS at high sensitivity and specificity.

2. Materials and Methods

2.1. Chemicals and reagents

HT and HT acetate were supplied from Seprox Biotech (Madrid, Spain). HT-D_3 was obtained from Synfine Research Ltd. (Richmond Hill, ON, Canada). HT-D_4 and HT-3-*O*-sulphate were custom synthesized by Toronto Research Chemicals Inc. (Toronto, ON, Canada). Methanol, acetonitrile, ethyl acetate, acetic acid, hydrochloric acid, and formic acid were obtained from Merck (Darmstadt, Germany). Citric acid, ascorbic acid, taxifolin, oleuropein, $K_3Fe(CN)_6$ and benzylamine were supplied by Sigma-Aldrich (St Louis, MO, USA). HT glucuronide conjugates were synthesized according to a previously described method [17]. Oasis HLB 3cc Vac Cartridges (60 mg) (WAT094226) for solid-phase extraction were obtained from Waters Corporation (Milford, MA, USA). Ultra-pure water was supplied by a Milli-Q purification system. Blank plasma samples were obtained from the Hospital del Mar blood bank (Barcelona, Spain).

2.2. Standards

Chemical purity of HT-D_4 standard was 96% and isotopic purity was 96.8%. Chemical purity of HT was 99% (<0.3% HT acetate). Stock and working standards solutions were prepared in methanol and stored at -20ºC. Methanol was pre-treated with helium during 10 minutes to improve HT stability. Standard stock solutions were

prepared by dissolving 10 mg of each substance in 10 mL of methanol. Working solutions were prepared by further diluting the standard solutions and were kept in dark flasks at -20ºC. Different solutions were used for the calibration samples and for the quality control samples.

2.3. Olive oil samples

Two different olive oils (an extra-virgin olive oil with a high content on polyphenols, and an ordinary olive oil with a lower content) were assayed. The content of phenolic compounds in both olive oils is reported in the subheading 2.7.

2.4. Instrumentation: LC-MS/MS conditions

Identification and quantification analyses were performed using an Agilent 1200 series HPLC system (Agilent Technologies, Wilmington, DE) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface (EI).

The liquid chromatographic separation was performed using an Acquity UPLC® CSH C_{18} column (100 mm x 3.0 mm i.d., 1.7 μm particle size) from Waters Corporation (Milford, MA, USA) maintained at 40ºC with a column oven. The composition of mobile phase A was 0.01% (v/v) formic acid in water, and mobile phase B was acetonitrile. Initial conditions of the mobile phase were 40% B. After 2 min, it was linearly increased to 75% B over one min, then increased linearly to 100% B over 3 min, maintained at 100% for 4.5 additional min, to finally return to initial conditions in 5.5 min The total run time was 16 min at a flow rate of 0.35 mL/min. The injection volume was 5 μL and the selected reaction monitoring (SRM) mode was employed for quantification. The ion source was operated in positive EI mode. Nitrogen was employed as a drying and nebulizing gas, and a capillary voltage of 4 kV was used. The

pressure of the nebulizer was set at 30 psi. The source temperature was set to 350 ºC, and a gas flow rate of 10 L/min was used. Peak-response ratios of derivatized HT and the corresponding deuterated analog internal standard were used for the calculation of the concentrations.

2.5. Analysis procedure of HT in human plasma

Aliquots of 200 μL of plasma samples of the pharmacokinetic study were transferred to an Eppendorf tube containing citric acid (2M, 10% (v/v)). Calibration curves and quality control samples were prepared by adding to an Eppendorf tube the appropriate volume of working standard solutions, evaporating the methanol in a vacuum concentrator (SpeedVac®), and adding 200 μL of HT free plasma containing citric acid (2M, 10% (v/v)). All samples were spiked with the internal standard HT-D_4 (50 ng/mL; 20 μL) and plasma proteins were precipitated with 800 μL of cold (4ºC) methanol and then the samples were centrifuged (15,700 *g*; 10 min; 4ºC). The supernatants where then transferred to dark glass tubes. The derivatization reaction started by adding 200 μL of the reaction mix (($K_3[Fe(CN)_6]$ 50 mM in water : benzylamine, 1:1 (v/v)) to each sample. Samples were incubated at 40ºC for 1 hour. The reaction was stopped by the addition of 4 mL of water. Samples were submitted to a solid-phase procedure using Oasis HLB columns. Cartridges were conditioned with 2 mL of methanol and equilibrated with 2 mL of water. After loading the reaction mixture, interferences were washed away sequentially with 2 mL of water and 2 mL of a methanol : water mixture (20:80, v:v). The compounds of interest were then eluted with 2 mL of acetonitrile. After the evaporation of the solvent under nitrogen (30 min, 15 psi, 40 ºC), extracts were reconstituted with 100 μL of acetonitrile and analyzed by LC-MS/MS.

178 *2.6. Validation method*

179 The method was validated and the following characteristics were evaluated:
180 linearity of the calibration curves, precision, accuracy, matrix effect, and limits of
181 detection and quantification. The validation was performed following the European
182 Medicines Agency guidelines on bioanalytical method validation (URL:
183 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/
184 WC500109686.pdf Accessed 22.07.2015).

185 The linearity of the calibration curves was evaluated in a concentration range of
186 1.0-11.0 ng/mL. Calibration standards of HT were prepared for each analytical batch
187 and consisted of two replicates at 6 different concentrations (1.0, 3.0, 5.0, 7.0, 9.0 and
188 11.0 ng/mL). Calibration curves with their corresponding slope (s), intercept and
189 correlation coefficient (r) were calculated by weighting (1/x) least-squares linear
190 regression of the peak area ratio (analyte/IS) versus the concentration of the standards
191 using SPSS for Windows, version 18.0. The method was considered linear if the
192 coefficient of determination (r^2) was greater than 0.99.

193 Quality control (QC) samples were prepared in order to evaluate the precision
194 and accuracy of the method. To do so, known amounts of HT were spiked in HT-free
195 human plasma to achieve final concentrations of 1.0 (lower limit of quantification;
196 LLOQ), 2.0 (low QC), 8.0 (medium QC), and 10.0 (high QC) ng/mL. Five replicates of
197 each QC were analyzed against the calibration curve, and the obtained concentrations
198 were compared to their nominal value.

199 The precision was calculated measuring the coefficient of variation (CV) of the
200 estimated concentrations for five replicates of the QC samples that were analyzed in one
201 day (within-run precision) and also with the data obtained during three validation assays

that took place during three different days (between-run precision). Precision was considered acceptable if the CV was <15% for the QC with the exception of LLOQ, which was acceptable if the CV was within 20% of the nominal value.

The accuracy of the method was evaluated using five replicates of the QC samples by analyzing relative error (%) of the estimated concentrations. For the within-run accuracy, the values of the QC samples were analyzed in one day, whereas for the between-run accuracy the values of the QC from three different days were used. In all cases, the accuracy was acceptable if the mean concentration was within 15% of the nominal values, except for the LLOQ, which was acceptable if it was within 20% of the nominal value.

The matrix effect was evaluated using HT free plasma spiked at a low (2.0 ng/mL) and a high concentration (10 ng/mL). As this method consists in the detection of a derivatized compound and due to the lack of a pure derivatized-HT standard, the matrix effect was evaluated by measuring the precision and accuracy of the method in plasma obtained from six different donors.

The limit of detection (LOD) was estimated using the standard deviations (SD) of the obtained concentrations from six replicates of the LLOQ (1 ng/mL) and the slope (s) of the calibration curve, according to the following formula: LOD = 3.3 * (SD/s). Additionally, at the estimated LOD of the surrogated analytes, it was confirmed that the LOD was at least 3 times the signal to noise ratio of a blank plasma sample. The lower limit of quantification (LLOQ) was the lowest concentration calibrator experimentally tested that presented acceptable accuracy (80-120%) and precision (±20%).

2.7. Analysis of phenolic compounds in olive oil

225 Phenolic compounds were measured in olive oils as previously described [18]
226 with some modifications. Briefly, a 1 mL aliquot of olive oil was transferred into an
227 amber glass tube and the density of the olive oil was calculated by weighting the
228 aliquot. Aliquots were spiked with 10 μL of IS mix solution (containing 100 μg/mL of
229 HT-D_3 and taxifolin). Phenolic compounds were extracted by shaking for 60 min with
230 10 mL of a methanol/water solution (80:20, v/v) containing 1 mM ascorbic acid to
231 prevent catechol oxidation during the sample preparation procedure. After
232 centrifugation (1,700 *g*; 5 min), the organic phase was transferred to a new tube and
233 concentrated to a final volume of 2 mL by evaporating under a nitrogen stream at 29 ºC.
234 The samples were then acidified with HCl 0.5 N (cca. 75 μL) to p*H* 3.0 - 3.5, and
235 submitted to two consecutive extractions (30 min + 30 min) using 4 mL of ethyl
236 acetate/acetonitrile (4:1, v/v). After centrifugation (1,700 *g*; 5 min) the organic phases
237 were transferred to a new amber tube, evaporated under a nitrogen stream, reconstituted
238 in 100 μL of mobile phase, and analyzed by LC-MS/MS. All the analyses were run in
239 triplicate. The following phenolic compounds were monitored: tyrosol, HT, oleuropein,
240 oleuropein aglycon, and HT acetate. Total HT was defined as the sum of the equivalents
241 of HT, oleuropein, oleuropein aglycon, and HT acetate. The results were expressed in
242 nmol/g of oil. See Supplementary Material for a detailed description of the LC-MS/MS
243 settings and further details.

244 *2.8. Pharmacokinetics*

245 Once validated, the present method was applied to evaluate the
246 pharmacokinetics of free HT in human plasma following the ingestion (25 mL) of two
247 different olive oils (ordinary with 19.7 mmol HT/kg oil vs. extra-virgin with 398.2
248 mmol HT/kg oil) by a healthy male volunteer. Intervention days were separated by a
249 washout period of 4 days with a polyphenol-free diet. The study was conducted in

accordance with the Helsinki Declaration and took place in the Clinical Research Unit of Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain). Written informed consent was obtained prior to any study-related procedure. An indwelling catheter was inserted into a subcutaneous vein in the forearm and blood samples were collected at baseline and 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 hours after treatment administration. Blood was collected into 10 mL tubes containing EDTA and they were centrifuged (1,700 *g*, 10 min, 4ºC). The plasma aliquots were collected into Eppendorfs containing citric acid (2M, 10% (v/v)). They were immediately stored at -20 ºC and analyzed within a week.

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were determined from the curves constructed by plotting the plasma concentrations over time, whereas the area under the curve from 0 to 4 hours ($AUC_{0\text{-}4h}$) was calculated by the trapezoidal method. All parameters were calculated using GraphPad Prism (GraphPad Software, CA, USA, version 5.03 for Windows) and specific software from Microsoft (Excel PK Functions spreadsheet).

3. Results

3.1. Derivatization and MS/MS spectra of the derivatized HT, HT-D_3 and HT-D_4

The mass spectra of free-HT and the two internal standards (HT-D_3 and HT-D_4) were studied in order to evaluate characteristic ionization and fragmentations. Under the reaction conditions, all compounds underwent derivatization with 2 equivalents of benzylamine and, similarly to other nitrogen-containing compounds, they were easily ionized in positive mode producing an abundant $[M+H]^+$. The compounds formed in the derivatization reaction were the expected benzoxazoles, whose chemical structures and fragmentations are shown in **Figure 2**. The MS analysis of the products from the derivatization reaction of free HT, HT-D_3 and HT-D_4 revealed the expected molecular ions of 345, 347 and 349, respectively. The three benzoxazoles formed present a constant fragment at m/*z* 91, which is compatible with a loss of toluene ($C_6H_5CH_2$-) from the derivatized molecule. The loss of this toluene moiety generates the corresponding fragments at 254, 256 and 258, respectively. The fragment at m/z 223 (in the case of HT) and 225 (in the case of the internal standards) is compatible with the loss of toluene plus the methanol from the aliphatic chain.

It is noteworthy the different position of the deuterium atoms in HT-D_3 versus HT-D_4. In HT-D_3, the deuterated atoms are in the aromatic ring and, when the derivatization takes place, one deuterium is lost. Consequently, the final benzoxazole formed and its corresponding fragments present one deuterium less (**Figure 2 B**). In order to avoid this loss, HT-D_4 was evaluated as an alternative IS. The HT-D_4 has four deuterium atoms distributed in the aliphatic chain, which keeps intact during the derivatization reaction. Unlike HT-D_3, all the deuterium present HT-D_4 remained in the original molecule during the derivatization process and all the fragments formed are 4

290 Da higher than the corresponding non-deuterated analogue. Due to these observations,
291 HT-D_4 was selected as the preferred IS. The identification and quantification of HT and
292 HT-D_4 were performed following parameters described in **Table 1**.

293 Once the LC-MS/MS parameters were optimized, the absence of hydrolysis of
294 different HT metabolites (including sulfates, glucuronides of HT and HT acetate) under
295 the derivatization conditions was verified. Under the derivatization reaction conditions
296 no significant amounts of hydrolysis took place, indicating that HT metabolites do not
297 affect the selectivity when quantifying free HT in plasma using this procedure (data not
298 shown).

299 *3.2. Method validation*

300 The linearity of the calibration curve was evaluated from three different batches
301 that were prepared on three different days, as described above. Satisfactory
302 determination coefficients (r^2>0.99) were obtained for free HT in human plasma in the
303 selected concentrations range of 1.0-11.0 ng/mL. The individual linear regression
304 equations, the determination coefficients, and the interception and slope values are
305 shown in the **Supplementary Table 2**.

306 The within- and between-run precision presented acceptable values of CV, and
307 the intra- and inter-day accuracy values, measured as relative error, were always below
308 15% (**Table 2**).

309 The calculated LOD for free HT was 0.3 ng/mL, whereas the LLOQ was 1
310 ng/mL.

311 The matrix effect for derivatized-HT and its corresponding IS was evaluated by
312 duplicate in six different plasma batches at a low (2 ng/mL, QC-L) and a high (10

313 ng/mL, QC-H) concentration. As shown in **Table 3**, the fact of using different matrices
314 did not affect the precision or the accuracy of the method. The precision for QC-L was
315 12.4% and the accuracy was 14.0 %, for the QC-H the precision was 8.8 % and 7.9 %
316 for the accuracy.

317 *3.3. Analysis of total hydroxytyrosol present in olive oil*

318 Prior to the pharmacokinetics evaluation, the HT content of both olive oils was
319 measured. Taking into account that olive oil contains several esters of HT that can
320 release HT after the corresponding hydrolysis, *total HT* was defined as the sum of (1)
321 free HT, (2) HT acetate, (3) oleuropein aglycon, and (4) oleuropein. The results were
322 expressed in nmol/g of olive oil and are summarized in **Figure 3**. Total HT in extra-
323 virgin olive oil (398 nmol/g) was 20-fold higher than in ordinary olive oil (19.7
324 nmol/g). In both olive oils, oleuropein aglycon represented the major contribution to
325 total HT (70-80%), followed by free HT (18-24%) and HT acetate (3-4%). Oleuropein
326 (the precursor from oleuropein aglycon and the main secoiridoid in olive fruit [5]) was
327 not found in olive oil. These results are in agreement with the fact that extra-virgin olive
328 oil contains higher amount of phenolic compounds than ordinary olive oil, as during the
329 refining process an important part of the phenolic compounds are lost [31].

330

331 *3.4. Free hydroxytyrosol pharmacokinetics in human plasma*

332 Time-course plasma concentrations of free HT following the ingestion of 25 mL
333 of an ordinary olive oil and an extra-virgin olive oil are shown in **Figure 4**. The
334 pharmacokinetic parameters for free HT after extra-virgin olive oil intake were: AUC =
335 5.3 ng·h/mL, C_{max} = 4.4 ng/mL, T_{max} = 0.25 h, K_e = 0.25 h^{-1} and $T_{1/2}$ = 2.8 h. In the case
336 of ordinary olive oil, the C_{max} (0.2 ng/mL) was below the LOD (0.3 ng/mL) and,

consequently, the corresponding pharmacokinetic parameters could not be calculated. The results clearly show that, following the ingestion of an ordinary olive oil the concentrations of free HT are practically undetectable and, as expected, they are in contrast with the higher HT levels achieved after the extra-virgin olive oil intake.

4. Discussion

In the present work, we report for the first time the development and validation of an analytical method to detect with high sensitivity free HT in human plasma by LC-MS/MS after the intake of dietary doses of olive oil.

HT is the most potent phenolic antioxidant present in olive oil. It displays several health promoting properties including antioxidant, anti-inflammatory, antiproliferative, antimicrobial, among other cardio and neuroprotective activities [32]. To obtain a better understanding of HT biological activities in humans it is crucial to measure the levels of free HT that reach blood circulation. The extensive metabolism that this compound undergoes after absorption, transforms it into a large variety of metabolites (including sulfate and glucuronide conjugates, *o*-methylated forms, and *N*-acetylcysteine derivatives) [33]. As a result of this extensive metabolism, the amount of HT that remains unaltered in blood has been considered undetectable so far [20].

As previously mentioned, HT in particular and polyphenols in general are difficult to identify and quantify in biological samples because of their low concentrations and the large number of interferences (mainly proteins) that interact with the free forms [34]. There are different methods to determine the plasma or urinary concentrations of HT (and also tyrosol) in humans, but none of them describe the concentration of free HT following the intake of olive oil [35]. HT has been measured in plasma after the administration of pure HT to rats [36] and humans [37] using HPLC

and GC/MS, respectively. However, the sensitivity of the former methods, although suitable to measure the plasma concentrations achieved after a high dose HT supplementation (C_{max} in humans = 154 ng/mL), does not allow to detect the low concentrations of free HT after dietary olive oil intake (C_{max} = 4.4 ng/mL). The derivatization reaction presented in this method provides several advantages including a considerable increase of sensitivity, specificity, and stability of HT. As HT reacts with benzylamine, the resulting compound presents a greater molecular weight and becomes highly ionizable improving the corresponding capacity to be detected by positive ESI. This fact also increases the specificity by promoting the compound fragmentation into molecular ions with high mass. At the same time, the derivatization reaction protects the two hydroxyl groups of the catechol from autoxidation, generating a more stable compound. The derivatization reaction takes place in several steps (shown in **Figure 1**) and was developed inspired on a previous work of Pennington and co-workers [30]. It starts with the oxidation of the catechol group into an *o*-quinone in basic conditions. Then, a Michael addition followed by oxidation takes place. A condensation reaction between a second equivalent of benzylamine and most electrophilic carbonyl, followed by an isomerization of the imine forms a Schiff base. Finally, this Schiff base is in equilibrium with the dihydrobenzoxazole, which undergoes an irreversible oxidation to form the final product. An additional advantage of the present method lies in the use of deuterated HT. By using HT-D_4 (a deuterated compound that has all the deuterium atoms in the aliphatic chain) we managed to make free HT and the internal standard follow the same fragmentation patterns, thus guaranteeing a proper identification and quantification of free HT.

After having shown that the linearity, precision, accuracy, and matrix effect determinations were in accordance with the established values of the Guidelines on

bioanalytical method validation, a pharmacokinetic study of free HT in plasma was performed. The HT plasma values obtained after the ingestion of 25 mL of extra-virgin olive oil were much higher than the ones obtained after the intake of the same amount of an ordinary olive oil in which they were undetectable. According to our analyses, the 25 mL of extra-virgin olive oil contained 1.38 mg of HT equivalents. Taking into account that the AUC obtained was 5.3 ng·h/mL and assuming a plasma compartment of 3 L, the total amount of unaltered HT that reached the systemic circulation was 3.98 µg (which represents a 0.3% of the administered dose). This low bioavailability is not surprising if we take into account (i) that HT absorption is around 10% [37] and that HT is subject to an extensive metabolism. In previous urine analyses from metabolism studies, we found that free hydroxytyrosol represents 1-10% of total HT metabolites (Pérez-Mañá et al., 2015). Considering these facts, the expected % of free HT in plasma would range between 0.1 and 1%, which is consistent with our results.

According to *in vitro* studies, HT is one of the most potent antioxidants known [2, 3]. In the light of these results, the concentrations of the free HT that we found in plasma after olive oil intake cannot explain a direct *in vivo* antioxidant activity of HT. However, besides its direct chemical antioxidant activity it has been suggested that the biological effects of HT could be mediated through other secondary mechanisms such as transcriptomic effects [26] and the activity of metabolites [22, 23].

The present study has both strengths and limitations. The strength of this methodology is the high selectivity and sensitivity achieved after the derivatization procedure of HT and its subsequent LC-MS/MS analysis. A limitation of the present work is the small sample size of the pharmacokinetic study. More robust pharmacokinetic data with a larger sample size will be obtained from future studies.

In conclusion, we report here a method that allows for the first time the quantification of free HT in plasma after extra-virgin olive oil intake compatible with its real life dietary consumption. The methodology developed here is suitable to be used for administration studies of olive oil and nutritional supplements. The measurement of free HT in plasma will be a crucial tool for a better understanding of the dose-effect of this potent antioxidant and its beneficial health effects.

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Conflict of interest

The authors declare no potential conflicts of interest.

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562 **Figure captions**

563 **Figure 1**. Postulated mechanism of the derivatization reaction of hydroxytyrosol with
564 benzylamine

565 **Figure 2**. Mass spectra of benzoxazoles formed through the reaction of benzylamine
566 with hydroxytyrosol (A), trideuterated hydroxytyrosol (B), and tetradeuterated
567 hydroxytyrosol (C). Note that in (B) a loss of the aromatic deuterium of the molecular
568 ion [M+H] was observed after the derivatization reaction.

569 **Figure 3.** Olive oil content of hydroxytyrosol (HT), HT acetate, oleuropein aglycon,
570 oleuropein, and tyrosol. Total HT is expressed as the sum of the equivalents of HT
571 acetate, HT, oleuropein aglycon, and oleuropein, expressed in nmol/g. The results are
572 expressed as mean ± SD of three replicates.

573 **Figure 4.** Plasma concentrations over time curves of free hydroxytyrosol following the
574 ingestion of ordinary olive oil (25 mL) and extra-virgin olive oil (25 mL).