DIFFERENTIAL ABSORPTION AND METABOLISM OF HYDROXYTYROSOL AND ITS PRECURSORS OLEUROPEIN AND SECOIRIDOIDS

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Abstract

This study investigated and compared the absorption, metabolism, and subsequently, the tissue distribution and excretion of hydroxytyrosol (HT) administered either in its free form or through its naturally occurring esterified precursors, namely oleuropein (OLE) and its aglycone forms known as secoiridoids (SEC). Here, rats were fed a diet supplemented with the equivalent of 5 mg phenol/kg/day for 21 days and the HT metabolites in the gastrointestinal digesta (stomach, small intestine and caecum), plasma, urine and metabolic tissues (liver and kidney) were analysed. Compared to HT and SEC, OLE showed greater stability during digestion, and, consequently, the bioavailability based on the urine excretion of HT metabolites was higher. OLE, as a glycoside molecule, reached the colon unaltered generating more diverse microbial metabolites. In terms of bioavailability, findings suggest that OLE might be the most suitable precursor of HT for incorporation into foods or nutraceutical formulations.

Keywords: Hydroxytyrosol; Microbial catabolism; Metabolic pathways; Olive oil, Phenolic compounds

1. INTRODUCTION

In recent years, numerous epidemiological and dietary intervention studies have demonstrated that plant-food consump-tion, attributed to bioactive chemical compounds, is directly associated with a lower risk of suffering chronic disease (Sikand, Kris-Etherton, & Boulos, 2015). The growing interest in these phyto-chemicals has enhanced the development and marketing of food supplements and nutraceuticals, which have become the fastest growing segments of the food industry. Over the last

decade, different engineering technologies (such as high pressure, supercritical fluid and molecular distillation) have been applied to extract components from raw materials or waste products and optimize their form and chemical structure to make them suitable for inclusion in new food products (Herrero, del Pilar Sánchez-Camargo, Cifuentes, & Ibáñez, 2015).

Specifically, an important expansion of the nutraceutical market has been observed with olive products due to the health benefits associated with their polyphenols (de Bock et al., 2013). The evidence of the protective effect of virgin olive oil (VOO) polyphenols for cardiovascular diseases (CVD) has been strengthened by the approved health claim based on the scientific report of the Panel on Dietetic Products, Nutrition and Allergies of EFSA (European Food Safety Authority) (COMMISSION REGULATION (EU) N° 432/2012 of 16 May 2012):

> Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil.

The most abundant polyphenols in olive fruit are oleuropein (OLE), demethyl-oleuropein, ligstroside and nüzhenide (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011). During mechanical extraction of VOO, hydrolysis reactions take place due to the activity of endogenous β-glucosidases, and aglycone derivatives known as secoiridoids (SEC) are released. From a chemical standpoint, SEC are characterized by the presence of elenolic acid (EA) or some of its derivatives, and hydroxytyrosol (HT) or tyrosol (TYR) in their molecule. The most abundant SEC in VOO are the dialdehydic form of decarboxymethyl EA linked to HT termed 3,4-DHPEA-EDA and the isomer of oleuropein aglycone (3,4-DHPEA-EA).

Based on the potential benefits of HT and its derivatives, different extracts or ingredients are available on the market. These are mainly obtained from the olive leaf, olive oil by-products (pomace) or waste-water, and the chemical nature of the phenolic compounds varies with the source. Generally, the extracts obtained from olive leaves are rich in OLE, the pomace yield extracts are rich in SEC and the waste-water gives extracts rich in HT. In this context, phenolic extracts derived from olive products can provide HT in different chemical structures, which could influence its uptake and metabolism. In the case of flavanols, it has been shown that their stereochemical configuration has a profound influence on their uptake and metabolism and their bioefficacy (Ottaviani et al., 2011). Similarly, the biological activity of the equol, a metabolite produced in vivo from the soy phytoestrogen daidzein by the action of gut microflora, depends on the enantiomers R-equol and S-equol (Muthyala et al., 2004).

Considering the wide range of phenolic extracts derived from olive products available on the market, this study was designed to study differences in the absorption and metabolism of HT either in its free form or through its natural occurring esterified precursors: oleuropein (OLE) and its aglycone forms known as secoiridoids (SEC). For

this proposal, rats were fed a diet supplemented with the equivalent of 5 mg phenol/kg rat weight for 21 days. The metabolism in the gastro-intestinal tract (GIT) was studied through the determination of the phenolic compounds and their metabolites in the contents (digesta) of the stomach, small intestine and caecal and in the faeces. Plasma, 24 h-urine and the metabolic tissues of liver and kidney were also analysed.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Hydroxytyrosol (HT) was provided by Seprox Biotech (Madrid, Spain), oleuropein by Extrasynthese (Genay, France), and homovanillic acid by Fluka Co. (Buchs, Switzerland). Catechol, p-hydroxyphenyl-acetic acid, 3,4-dihydroxyphenyl-acetic acid, 3-(4-hydroxyphenyl)propionic acid and hippuric acid were from Sigma-Aldrich (St. Louis, MO, USA). Ortophosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Hydroxytyrosol-3′-O-glucuronide (97.8% of purity), hydroxyl-tyrosol-4′-O-glucuronide (96.4% of purity), and homovanillic alcohol-4′-O-glucuronide (99.3% of purity) were synthe-sized according to the method reported by (Khymenets et al., 2006). Hydroxytyrosol-3-O-sulphate was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Methanol and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA).

2.2. Secoiridoid extract (SEC)

Secoiridoid extract was obtained from olive pomace by Pressurized Liquid Extraction (PLE), using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA, USA) based on our previously described method (Suárez, Romero, Ramo, Macià, & Motilva, 2009). Briefly, 10 g of freeze-dried olive-cake were mixed with 5 g of diatomaceous earth. The best extraction of phenolic compounds was obtained with these conditions: ethanol/water (80:20, v/v) solvent at 80 °C, 60% setting volume and two static cycles of 5 min in each extraction. Finally, the sample was purged with nitrogen (≥99.99% purity, Alphagaz, Madrid, Spain). After that, the ethanol
was rotary evaporated until its elimination (Buchi Rotavapor, New Castle, DE, USA). Aqueous extract was freeze-dried and stored at −80 °C in N2 atmosphere until use. The extract was mainly composed of 3,4-DHPEA-EDA (85%) and contained minor proportions of free HT and other secoiridoids providing HT such as 3,4-DHPEA-EA (**Table S1** Supplementary Material). To calculate the administered dose of 5 mg/kg weight of SEC, only 3,4-DHPEA-EDA was considered as it is the main secoiridoid derivative providing HT.

2.3. Animals and experimental procedure

Thirty-two male and female Wistar rats weighing between 300 and 350 g were obtained

from Charles River Laboratories (Barcelona, Spain). They were separated into four groups of 8 rats in each group (4 males, 4 females). Group A: Control, group B: hydroxytyrosol (HT), group C: secoiridoids (SEC) and group D: oleuropein (OLE) (**Fig. 1**). During the 21-day experiment, the animals were housed two per cage at a temperature- (21 ± 1 °C) and humidity controlled (55 ± 10%) room with a 12-h light/ dark cycle. Food and water were available ad libitum. The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 10-06/14, 31st July 2014).

For the supplemented diets, commercial feed pellets (Harlan Laboratories, Madison, WI, USA) were crushed in an industrial mill and mixed with Milli-Q

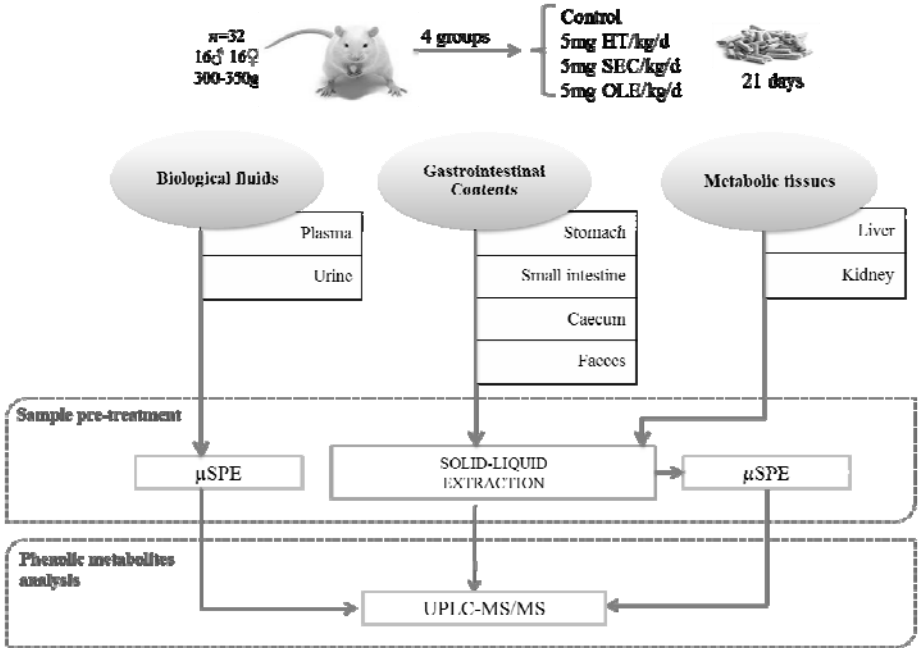

Fig. 1 – Experimental design of the study. µSPE, microelution solid-phase extraction; UPLC-MS/MS, Ultra-Performance liquid chromatography coupled to tandem MS.

water containing the equivalent of 5 mg of OLE, SEC or HT/kg rat weight in 16 g of crushed pellet (average daily consumption of each rat), respectively. New pellets were prepared and freeze-dried. Food and animals were weighed every 2 days to adjust the weekly dose of phenolic compound to 5 mg/kg rat weight/day. On the first day before treatment (day 0) and after treatment (day 21), the rats were caged in metabolic gavage for 24 hours to collect urine and faeces. The rats were sacrificed by intracardiac puncture after isoflurane anaesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). Blood was collected in EDTA tubes and plasma samples were obtained by centrifugation (3000 × g, 10 min at 4 °C) and stored at −80 °C until analysis. After blood collection, the rats were perfused with an isotonic solution of sodium chloride (NaCl) 0.9% to remove the remaining blood irrigating the tissues and their livers and kidneys were excised. Additionally, the digestive tract was excised and the contents of stomach, small intestine and caecum were obtained to analyse the phenolic metabolites,

expressed as nmols/g digesta. The small intestine content consisted of a pool of duodenum, jejunum and ileum contents. All samples were stored at −80 °C and freeze-dried for phenolic extraction and chromatographic analysis (**Fig. 1**).

2.4. Sample pre-treatment for phenolic metabolites analysis

Plasma and urine samples were processed using OASIS hydrophilic–lipophilic balance (HLB) μElution plates 30 μm (Waters, Milford, MA, USA). Pre-treatment of plasma was performed according to our method developed previously (Suárez et al., 2009). Briefly, 350 μL of plasma were mixed with 300 μL of phosphoric acid (4%) and 50 μL of catechol as the internal standard (IS). Then, the mixture was centrifuged and the supernatant was treated by microelution solid-phase extraction (μSPE). The cartridges were conditioned sequentially by using 250 μL of methanol and acidified Milli-Q water (Milli-Q water at pH 2 with diluted hydrochloric acid). After loading the plasma mixture, the plates were washed with 200 μL of Milli-Q water and 200 μL of 5% methanol. The retained phenolic compounds were then eluted with 2 × 50 μL of methanol and directly injected into the UPLC-MS/MS system. For the analysis of the urine samples, the cartridges were conditioned as described previously (Serra et al., 2013). Briefly, 100 μL of urine were mixed with 50 μL of phosphoric acid (4%) and 50 μL of catechol as IS, and this solution was loaded into the μSPE. The retained phenolic compounds were then eluted with 2 × 50 μL of methanol and injected into the UPLC-MS/MS system.

For the analysis of the GIT contents (stomach, small intestine and caecum) and faeces, 100 mg of freeze-dried sample was suspended in 1 mL of methanol/water (1:1, v/v). Each sample was shaken in a vortex (Multi vortex, VWR, Franklin, MA, USA) for 10 min. After that, the sample was centrifuged at 8784 × g for 10 min and supernatant was collected and centrifuged under the same conditions. Finally, the supernatant was filtered with 0.22 μm syringe filter and transferred into chromatographic vials until the chromatographic analysis.

The kidney and liver samples were sequentially pretreated with a combination of liquid–solid extraction (LSE) combined with μSPE (Serra et al., 2012). Briefly, for the analysis of LSE, 60 mg of freeze-dried sample was mixed with 50 μL of ascorbic acid (1%), 100 μL of phosphoric acid (4%), and 50 μL of catechol. The samples were treated four times with 400 mL of water/methanol/phosphoric acid 4% (94:4.5:1.5, v/v/ v). In each extraction, the sample was sonicated for 30 s maintaining the sample in ice to avoid heating and then centrifuged. The supernatants were collected, and an aliquot of 350 μL was treated with μSPE following the same method described above for the plasma samples.

2.5. Chromatographic analysis

The phenolic compounds were analysed by Acquity Ultra-Performance™ liquid chromatography coupled to tandem MS as the detector system (UPLC-MS/MS) from

Waters (Milford, MA, USA), as reported in our previous study (Serra et al., 2012). The column was an Acquity UPLC™ HSS T3 from Waters (100 mm, 2.1 mm i.d., 1.8 µm particle size). The mobile phase was 0.2% (v/v) acetic acid as solvent A and methanol as solvent B, with a flow rate of 0.4 mL/min. The gradient was performed as follows: 0–6 min, 3–15% B; 6–14 min, 15–70% B; 14–17 min, 70–00% B; 17–18 min, 100–3% B; 18–20 min, 3% B isocratic. The injection volume was 2.5 µL. The UPLC system was coupled to a PDA detector, AcQuity UPLC™ and a triple quadrupole detector (TQD™) mass spectrometer (Waters). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode, and the data were collected in the selected reaction monitoring (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h and desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as the nebulizing and collision gases, respectively. The SRM transitions and the individual cone voltages and collision energies were optimized for each analyte by injection of each standard compound into a mixture of methanol/water (1:1, v/v) at a concentration of 10 mg/L. Two SRM transitions were studied to find the most abundant product ions, selecting the

most sensitive transition for quantification and the second one for identification purposes. When standard phenolic compound were not available, the SRM parameters were selected by analysing the real sample in fullscan mode in MS and daughter-scan mode in tandem MS. Table S2 of Supplementary Material shows the tandem MS (MS/MS) transitions for quantification and confirmation, as well as cone voltage and collision energy values optimized for each phenolic compound. Table S2 also shows the commercial standards used to quantify each phenolic compound. When standard compounds were not available, the phenolic compound was tentatively quantified with its aglycone or with a phenolic compound with similar chemical structure.

2.6. Instrumental quality parameters

The instrumental quality parameters of the developed method, such as linearity, calibration curve, reproducibility, accuracy, detection limits (LODs) and quantification limits (LOQs) as well as extraction recovery and matrix effect were determined with a serial dilution of blank biological sample (plasma, urine, faeces, tissues and intestinal contents) with the standard phenolic compounds. The determination and calculation of these quality parameters are reported in our previous study (Suárez et al., 2009). The precision of the methods and accuracy were determined at three concentration levels, but only one concentration level is shown. The calibration curves (based on peak abundance) were plotted using $y = a + bx$, where y is the peak abundance ratio (analyte/internal standard) and x is the concentration ratio (analyte/internal standard). The internal standard (IS) used was catechol. For the other tissues (stomach, caecum, intestine and faeces), the calibration curves were plotted without IS, since catechol could be a metabolite.

2.7. Statistical analysis

Differences between diet groups were evaluated using a one-way ANOVA, followed by the LSD post-hoc test. Differences were considered statistically significant at $p < 0.05$. All analyses were performed with the Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1. Validation of the analytical procedure

Blank plasma, urine, faeces, intestinal contents and tissue samples with different phenolic concentration were analysed by sample pre-treatment and UPLC-MS/MS to determine the linearity, calibration curve, precision, accuracy, LOD and LOQ. The obtained results are shown in **Table S3** of the **Supplementary Material**.

The precision of the method for all the biological fluids and tissues was below 15%. The accuracy of the method for the different samples was between 95 and 105%. The extraction recovery (%R) and matrix effect (%ME) of the phenolic compounds in the different biological samples were higher than 75% and lower than 18%, respectively.

Once the instrumental quality parameters of the developed methods were studied, these were applied for the determination of hydroxytyrosol, oleuropein and their generated metabolites in the different biological samples

3.1. Phase I and II metabolism of HT, SEC and OLE in the gastrointestinal tract

Table 1 shows the phase I and II phenolic metabolite concentrations (nmols/g digesta) detected in the GIT contents (stomach, small intestine and caecum) and faeces after the diet supplementation (21 days) with 5 mg/kg weight rat/day of HT, SEC and OLE. The corresponding proposed pathways for phase I and II metabolism of the three HT precursors are shown in **Fig. 2**.

At the gastric level, the SEC group underwent a complete degradation of the main secoiridoid 3,4-DHPEA-EDA (Table 1), which was hydrolysed into HT and EA and further metabolized (**Fig. 2**). OLE underwent a similar process in the stomach but a remaining amount of the native form was detected, indicating that OLE molecule was more resistant to the gastric acidic hydrolysis than 3,4-DHPEA-EDA. Accordingly, previous studies have shown that OLE is stable in human gastric juice (Corona et al., 2006; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002) and 3,4-DHPEA-EDA has been shown to be more sensitive to temperature, pH and enzyme activity compared with the glycoside form of OLE (Briante et al., 2002). In stomach, both the administered HT and the resulting HT from the hydrolysis of OLE and SEC underwent direct sulphation (hydroxytyrosol sulphate, Sulf) or methylation (homovanillic alcohol, HVAlc) and a further oxidation process (homovanillic acid, HVAc) followed by sulphation (HVAc-Sulf and HVAlc- Sulf) with a remaining amount of free HT and observing very similar

metabolic profiles among the three HT precursors (**Table 1** and **Fig. 2**). Very few studies have analysed the in vivo metabolic capacity of the gastric mucosa to conjugate phenolic compounds, however, the presence of SULT1C2 isoform have been described in the stomach tissue (Nimmagadda, Cherala, & Ghatta, 2006). In a previous study by Zhao, Egashira, and Sanada (2004) they found that ferulic acid did not undergo any metabolic change in rat stomachs. In contrast, our results indicated that HT can suffer phase II metabolism at the gastric level of digestion, sulphation being the main conjugation process.

The detection of higher concentrations of phase I and II metabolites in the small intestine contents after the administration of the three precursors revealed an intense enzymatic activity in the intestinal epithelium with an efflux transport of the metabolites into the gut lumen, a phenomenon that has already been described for olive oil phenolic compounds (D'Angelo et al., 2001; Manna et al., 2000). It is worth noting that HVAc and its conjugated forms, HVAc-Sulf and HVAc-Glu, were the metabolites detected at the highest concentrations in the intestinal lumen in all groups including the control group (**Table 1**). HVAc is the major molecular species deriving from endogenous dopamine metabolism through the action of monoamine oxidase and catechol-O-methyltransferase in the central nervous system, which indicates that HVAc and its conjugated forms detected in the present study might be mainly of endogenous origin (D'Angelo et al., 2001). During digestion in the small intestine, part of the native form of OLE remained unaltered without being deglycosylated, resisting the action of lactase phlorizin hydrolase (LPH), an enzyme found in the lumen of the brush border of the small intestine with β-glucosidase activity towards glycoside compounds such as flavonoids (Del Rio et al., 2013). Compared to HT and SEC groups, OLE also presented in the small intestine significantly higher concentrations of some phase II metabolites such as HVAc-Sulf and hydroxytyrosol acetate sulphate (HTAc-Sulf) (Table 2). These results could indicate that OLE could suffer less

Table 1 – Phase I and II metabolites' concentration (nmols/g digesta) in the stomach and intestinal (gut and caecum) contents and faeces obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as the mean ± SEM.

Phenolic compound	Control	HT	SEC	OLE
Stomach content (nmols/g digesta)				
Hydroxytyrosol	n.d.	12.0 ± 2.69^{b}	8.75 ± 1.95^{b}	3.64 ± 0.79^{a}
Hydroxytyrosol sulphate	0.16 ± 0.13^{a}	3.28 ± 1.17^{b}	10.5 ± 10.2^{b}	3.45 ± 1.47^{b}
Homovanillic acid	1.67 ± 0.87^{a}	9.13 ± 1.74^{bc}	4.32 ± 1.95^{ab}	12.2 ± 4.12^{c}
Homovanillic acid sulphate	n.d.	0.17 ± 0.14^{a}	4.03 ± 2.66^{b}	0.55 ± 0.25^{a}
Homovanillic alcohol	n.d.	0.45 ± 0.29^{a}	4.74 ± 3.34^{b}	0.75 ± 0.22^{a}
Elenolic acid sulphate	–	–	6.99 ± 2.55^{b}	4.02 ± 1.25^{a}
Oleuropein	–	–	n.d.	4.14 ± 2.31
Small intestine (nmol/g digesta)				

Hydroxytyrosol sulphate	8.03 ± 1.53[a]	30.5 ± 5.75[b]	31.1 ± 12.6[b]	29.6 ± 6.22[b]
Hydroxytyrosol	n.d.	1.44 ± 0.91[a]	2.99 ± 0.53[b]	2.77 ± 0.58[b]
Hydroxytyrosol acetate	28.7 ± 8.63[a]	27.0 ± 7.36[a]	32.1 ± 6.5[a]	56.3 ± 13.0[b]
Homovanillic acid sulphate	48.3 ± 17.6[a]	36.6 ± 11.3[a]	50.8 ± 12.1[ab]	76.8 ± 14.4[b]
Homovanillic alcohol	16.0 ± 5.19[ab]	14.8 ± 2.67[a]	27.4 ± 7.74[bc]	32.9 ± 7.17[c]
Oleuropein	–	–	n.d.	6.95 ± 3.34
Caecum content (nmols/g digesta)				
Hydroxytyrosol	0.36 ± 0.24[a]	6.02 ± 1.32[b]	0.04 ± 0.00[a]	5.57 ± 0.65[b]
Hydroxytyrosol sulphate	n.d.	0.52 ± 0.52[a]	n.d.	3.12 ± 2.25[b]
Elenolic acid sulphate	–	–	0.02 ± 0.01[a]	9.38 ± 4.59[b]
Faeces (nmols/g)				
Hydroxytyrosol	n.d.	6.20 ± 1.35[c]	0.35 ± 0.01[a]	3.12 ± 0.35[b]
Hydroxytyrosol sulphate	n.d.	1.44 ± 0.78	0.01 ± 0.01	0.01 ± 0.01

n.d.: not detected.
[a–c] Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

degradation thanHT and SEC under alkaline intestinal conditions and therefore, it could have been more exposed to phase II metabolism. In faeces, the intact form of OLE was not detected, in contrast to a previous study in which OLE, HVAc, EA and OLE aglycone were detected in rat faeces collected over 24 h after an acute oral dose of OLE (Lin et al., 2013). This discrepancy could be explained by the differences in the administered dose, the dose administered in the present work being much lower (5 mg/kg vs 100 mg/kg). The free form of HT was detected in both the caecum content and the faeces, mainly in HT and OLE groups. In our previous work, free HT was also detected in human faeces after a sustained intake of an olive oil enriched with its own phenolic compounds (Mosele et al., 2014). Therefore, the present results confirm that the free form of HT is able to reach the large intestine, which could appear as a result of the deconjugation of the phase II metabolites by the action of the microbial enzymes, including β-glucuronidase and sulphatase (Selma, Espin, & Tomas-Barberan, 2009; Valdés et al., 2015).

3.2. Microbial metabolism of phenolics in the intestinal tract

The results showed that the microbial metabolism of HT, OLE and SEC started in the small intestine and was intensified in the lower part of the gut (caecum), detecting metabolites derived from oxidation, dehydroxylation, decarboxylation (α-oxidation) and carboxylation reactions along the GIT (**Table 2, Figs 3–5**). The microbial content of the GIT changes along its length, starting with a low number of microbes in the stomach, and increasing in the jejunum and the ileum, which are colonized by aerobe or facultative anaerobes. The highest concentrations are reached in the large intestine where the main microorganisms are anaerobes (Tiihonen, Ouwehand, & Rautonen, 2010; Zhao et al., 2015), which is in accordance with the amount of catabolites detected in caecum in the present study. Therefore, differences observed in the colonic metabolites can be attributed to the diverse microbiota populations that cause differences in the metabolite complexity in different sections of the digestive tract (Ouwehand & Vesterlund, 2003).

The results of this study demonstrated that each administered HT precursor underwent different catabolic transformations with more or less intensity depending on the molecular structure of the precursor. In the case of the free form of HT, we observed that the predominant microbial metabolic transformation was the oxidation, converting the primary alcohol into dihydroxyphenylacetic acid (diHPAAc), followed by rapid and subsequent dehydroxylation giving rise to hydroxyphenylacetic acid (HPAAc), which was the most abundant microbial metabolite after HT treatment (**Table 2** and **Fig. 3**). HPAAc could be further carboxylated leading to the

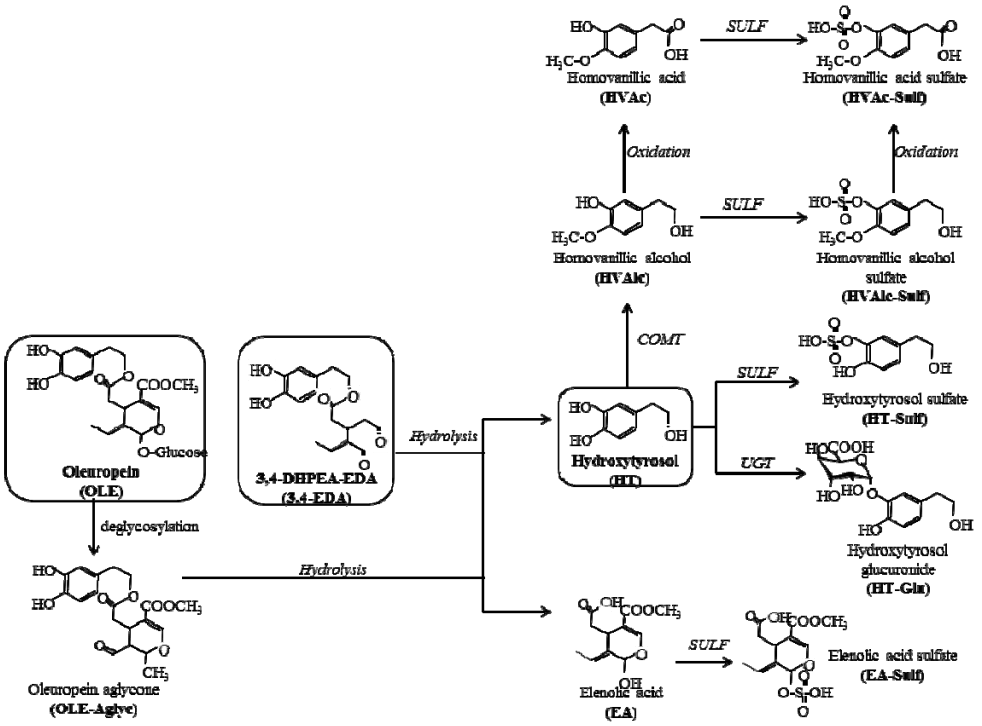

Fig. 2– Phase I and II metabolism pathways of HT, OLE and SEC. COMT: catechol-O-methyltransferase enzyme; SULF: sulphotransferase enzyme; UGT: glucuronosyl-transferase enzyme.

formation of hydroxyphenylpropionic acid (HPPAc) (**Fig. 3**), being the second most abundant microbial metabolite after HT intake (**Table 2**). The microbial origin and formation of these metabolites were demonstrated in our previous in vitro fermentation study with free HT (Mosele et al., 2014). When SEC was administered to rats, the microbial metabolic profile in GIT contents was similar to the HT group, lower concentrations (**Fig. 4**). On the other hand, OLE was characterized to undergo the same transformations as described in HT and SEC but in less intensity, and in addition, it suffered other kinds of microbial transformations. So, apart from the loss of a hydroxyl group, di- and mono-phenylacetic acids were also decarboxylated (α-oxidation) into phenolic compounds with lower molecular weights, including protocatechuic acid (PCAc) and

hydroxybenzoic acid (HBZAc) (**Fig. 5**). HBZAc was predominately transformed to its glycine conjugate termed hydroxyhippuric acid (HHiPAc), which could suffer a further

dehydroxylation and generate hippuric acid (HiPAc), a microbial transformation previously reported (Beyoglu & Idle, 2012). Consequently, of the three groups, OLE showed the most diverse microbial transformations and led to the formation of fermentation products with lower molecular weight (**Table 2**).

A significant higher concentration of diHPPAc was also observed after OLE compared to SEC and HT, which could be in great part due to the hydrolysis of OLE- aglycone into HTAc with a subsequent de-

Table 2 – Concentration (nmols/g digesta) of the microbial phenolic metabolites detected in the intestinal (gut and caecum) contents, and faeces obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as mean ± SEM.

Phenolic metabolite	Control	HTT	SEC	OLE
Small intestine (nmols/g digesta)				
Hydroxyphenylpropionic aci	9.29 ± 1.22^{a}	16.7 ± 1.22^{b}	11.8 ± 1.60^{a}	12.1 ± 1.33^{a}
Hydroxyphenylpropionic aci	2.57 ± 1.74^{a}	16.2 ± 6.15^{b}	15.4 ± 6.18^{b}	38.8 ± 9.27^{c}
Hydroxyphenylacetic acid sulphate	13.6 ± 3.83^{a}	12.4 ± 6.10^{a}	30.4 ± 9.06^{ab}	40.9 ± 6.22^{b}
Catechol	n.d.	2.73 ± 0.63^{b}	0.68 ± 0.21^{a}	4.32 ± 0.18^{c}
Hippuric acid	161 ± 49.9^{a}	186 ± 47.5^{a}	223 ± 50.1^{a}	367 ± 72.5^{b}
Caecum content (nmols/g digesta)				
Dihydroxyphenylpropionic	89.0 ± 60.3^{a}	195 ± 77.8^{ab}	147 ± 25.9^{ab}	403 ± 226^{b}
Hydroxyphenylpropionic aci	n.d.	592 ± 143^{b}	316 ± 70.4^{a}	252.09 ±
Dihydroxyphenylacetic acid	16.5 ± 5.26^{a}	50.4 ± 13.9^{b}	25.1 ± 7.40^{a}	31.9 ± 12.5^{ab}
Hydroxyphenylacetic acid*	65.1 ± 31.8^{a}	707 ± 31.1^{c}	493 ± 37.9^{b}	309 ± 99.08^{b}
Hydroxyphenylacetic acid	1.47 ± 0.97^{a}	n.d.	6.14 ± 5.16^{a}	34.9 ± 19.0^{b}
Phenylacetic acid	n.d.	26.52 ±	24.5 ± 2.18^{a}	n
Catechol	0.36 ± 0.28^{a}	1.62 ± 0.23^{b}	1.13 ± 0.17^{b}	1.67 ± 0.22^{b}
Faeces (nmols/g)				
Hydroxybenzoic acid	0.63 ± 0.32^{a}	0.46 ± 0.21^{a}	2.97 ± 1.59^{b}	5.82 ± 2.12^{c}
Hippuric acid	8.67 ± 0.44^{a}	6.65 ± 6.32^{a}	16.1 ± 4.34^{a}	48.84 ±
Phenylacetic acid	52.7 ± 13.0^{a}	70.2 ± 9.53^{ab}	268 ± 43.1^{b}	293 ± 50.0^{b}

n.d.: not detected.

* ∑ *p*- and *o*-hydroxyphenylacetic acid.

a–c Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

esterification, oxidation and carboxylation (**Fig. 5**). This metabolic pathway was described in our previous work, in which OLE was fermented in vitro with human faeces (Mosele et al., 2014).

Once in the portal bloodstream, microbial metabolites can rapidly reach the liver, where they can be subjected to further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion. This could explain the detection of metabolites such as HPPAc-Sulf, HPAAc- Sulf and HPAAc-Glu in the small intestine and caecum contents, mainly after the OLE administration.

The biological activities of the products of microbial fermentation of olive phenols have

not yet been systematically tested. However, a few reports have demonstrated that products of the colonic degradation of flavonoids, such as phenylacetic and phenylpropionic acids and their derivates, exhibit anti-inflammatory effects (Larrosa et al., 2009).

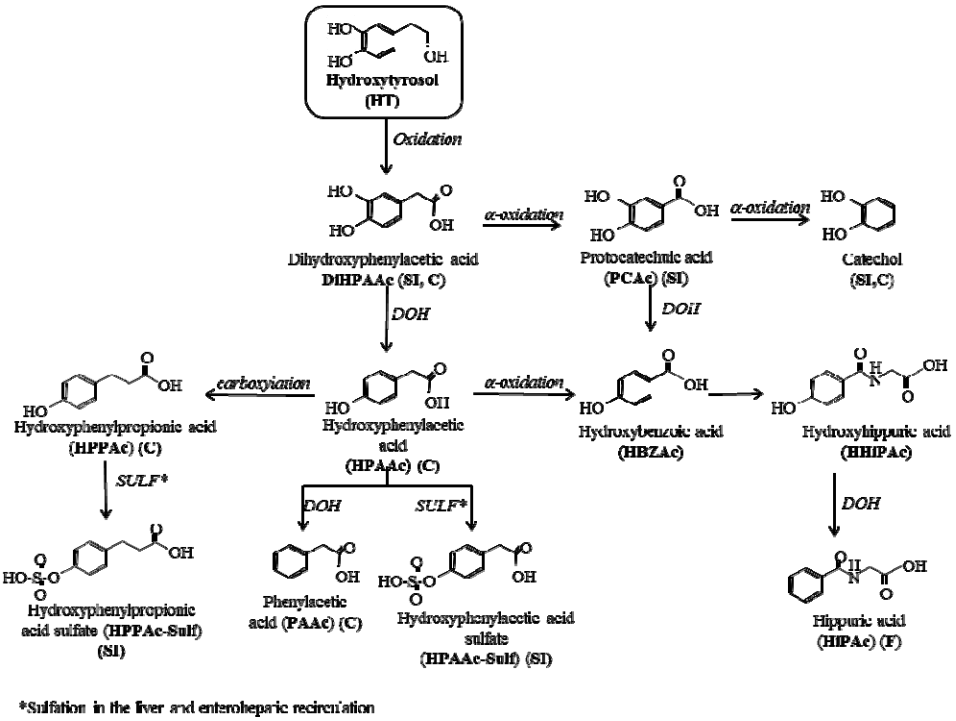

Fig. 3 – Proposed catabolism pathways of HT by the gut microbiota derived from the analysis of small intestine (SI) and caecum (C) contents and faeces (F). Compounds that were detected after HT treatment are highlighted in grey colour. DOH: dihydroxylation; SULF: sulphotransferase enzyme. *Phase II metabolism (sulphation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.

as well as antioxidant powers (Jaganath, Mullen, Lean, Edwards, & Crozier, 2009). It has also been pointed out that the local concentration of intact polyphenols and its metabolites that reach the intestine may have a significant redox effect (Scalbert et al., 2000). This might be the case of olive phenols, which in the present study, no matter the precursor (HT, SEC or OLE), have all been demonstrated to undergo extensive transforma tions in the GIT, resulting in the release of a large number of putative bioactive compounds, varying depending on the precursor compound. Metabolites could also be reabsorbed and exert their action in the colon as well as in other target tissues after absorption. Wang, Williams, Ferruzzi, and D'Arcy (2013) demonstrated the transference of some phenolic fermentation products across the Caco-2 cell monolayer. The results from the microbial metabolism in the present study are in close agreement with our previous in vitro fermentation experiments performed with human faecal inoculums (Mosele et al., 2014), which

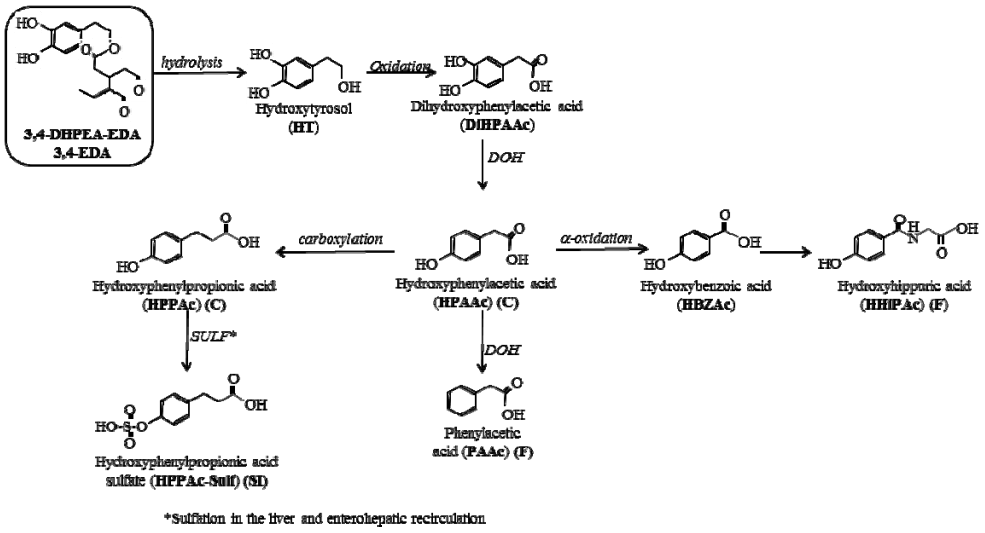

Fig. 4 – Proposed catabolism pathway of 3,4-DHPEA-EDA (main compound in the SEC extract) by the gut microbiota derived from the analysis of small intestine (SI), caecum contents (C) and faeces (F). DOH: dihydroxylation; SULF: sulphotransferase enzyme. *Phase II metabolism (sulphation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.

suggests that the rat model can be a good approach to performing a human approximation of the digestion and microbial metabolism of olive phenolic compounds. In fact, rodent animals are similar to humans at both the host geneticand the taxonomic levels of the microbiota, particularly at the phylum level. However, important differences may be appreciated at the lower taxonomic level (Kostic, Howitt, & Garrett, 2013). Moreover, it should remain clear that the amount and identity of the catabolites detected may significantly vary from individual to individual, as observed in the high deviation values in some determinations.

3.3. Plasma, urine excretion and tissue deposition

Table 3 shows the concentration of the phase I and II phenolic metabolites in plasma and urine after the sustained intake of free HT and its conjugated precursors OLE and SEC.

Phenolic compound	Control	HT	SEC	OLE
Plasma (μM)				
Hydroxytyrosol sulphate	n.d.	0.37 ± 0.03	0.36 ± 0.08	0.32 ± 0.07
Homovanillic alcohol	n.q	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Elenolic acid	n.q	–	0.09 ± 0.03[a]	0.22 ± 0.04[b]
Urine (μmol)				
Hydroxytyrosol	n.d.	0.03 ± 0.02[a]	5.38 ± 0.48[b]	4.01 ± 2.06[b]
Homovanillic acid	n.d.	4.26 ± 0.73[a]	9.11 ± 0.54[a]	169 ± 39.2[b]
Hydroxytyrosol sulphate	n.d.	57.4 ± 13.54[a]	72.6 ± 10.5[a]	169 ± 28.6[b]
Homovanillic acid	n.d.	85.6 ± 5.26[a]	92.6 ± 5.50[a]	557 ± 89.9[b]
Elenolic acid sulphate	n.d.	–	4.75 ± 0.90	6.95 ± 1.28
Hydroxytyrosol-4-*O*-	n.d.	1.56 ± 0.25[b]	0.46 ± 0.06[a]	1.80 ± 0.38[b]
Homovanillic acid	n.d.	n.d.	4.17 ± 0.30[a]	28.4 ± 5.35[b]
Elenolic acid	0.38 ± 0.09[a]	–	18.5 ± 3.13[b]	61.0 ± 9.14[c]
Methyloleuropein	n.d.	–	n.d.	1.63 ± 0.29
Oleuropein	n.d.	–	n.d.	2.13 ± 0.66
Oleuropein aglycone	n.d.	–	0.13 ± 0.02[a]	1.49 ± 0.22[b]
Oleuropein aglycone	n.d.	–	n.d.	2.03 ± 0.13

n.d.: not detected; n.q.: not quantified.

a–c Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

The results in plasma revealed that very low concentrations of metabolites were detected (nanomolar range), probably due to the low daily dose administered in this study and the distribution of the dose over the day. No differences were observed between the three groups, except for EA-Glu, which was only detected in the plasma of OLE group. A previous study performed in our lab (Serra et al., 2012) showed that after an acute intake by rats of an olive phenolic extract rich in SEC, the sulphate conjugates of HT and the native forms of OLE

and HT were detected in plasma at higher concentrations (between 21 and 89 μM) probably due to the higher administered dose (180 mg SEC/kg rat weight). We observed a similar metabolic fate after an acute intake of HT at different doses (1, 10, and 100 mg/kg rat weight), sulphation again being the most relevant conjugation pathway at the three administered doses (López de Las Hazas et al., 2015). The present study provides new data after a sustained intake of olive phenolic compounds, showing that when nutritionally relevant doses are administered in rats, a lower variety of metabolites is detected in the plasma (only HT-Sulf and HVAlc-Sulf) and at lower concentrations. In contrast to the plasma,

urine recovery of metabolites showed significant differences among the three HT precursors administered. Urine excretion of phase I and II metabolites was significantly higher when OLE was admi nistered in comparison with HT or SEC (**Table 3**). In some cases, such as for HVAc, the metabolite was 50-fold higher in the OLE than the other groups. Thus, it can be concluded from the urine excretion results that, among the three groups, the bioavailability of HT was more effective

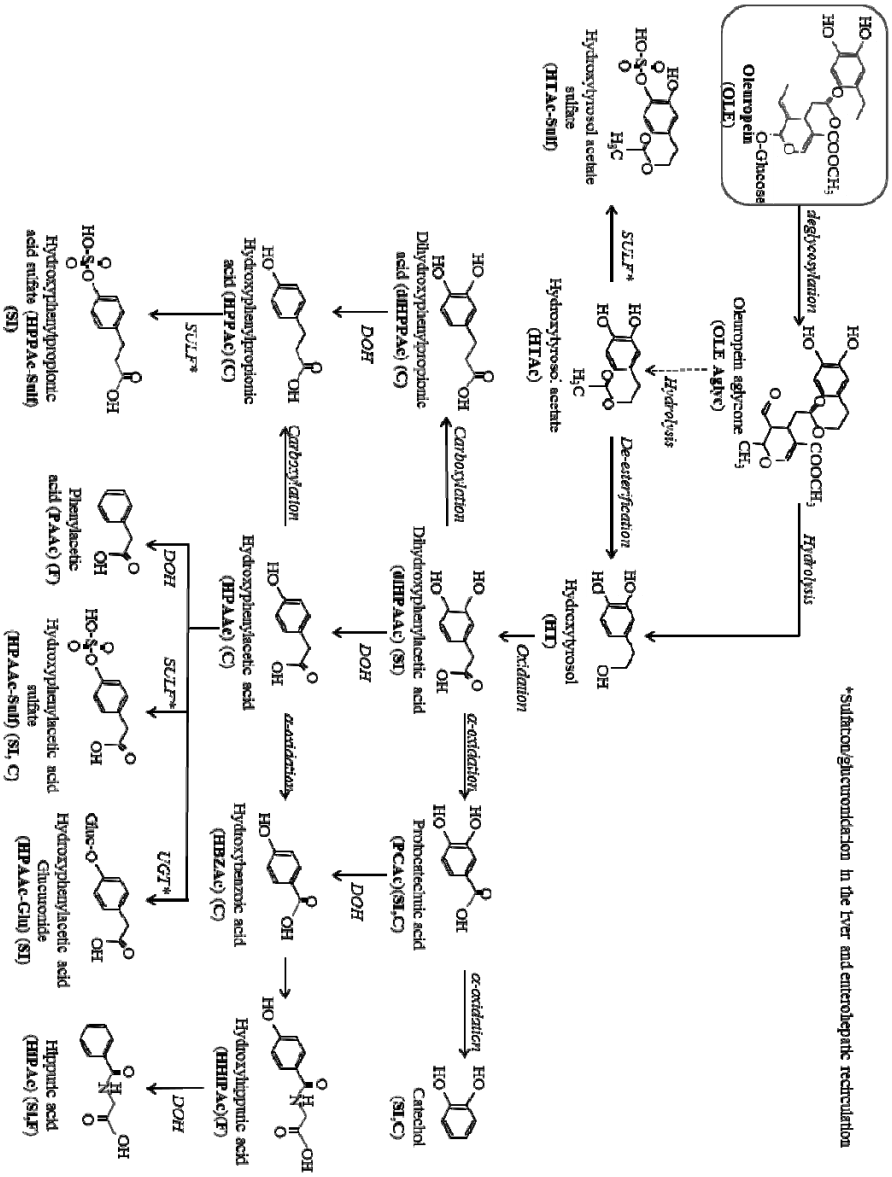

Fig. 5- Proposed catabolism pathway of OLE by the gut microbiota derived from the analysis of small intestine (SI), caecum contents (C) and faeces (F). DOH: dihydroxylation; SULF: sulphotransferase enzyme; UGT: glucuronosyl-transferase enzyme. *Phase II metabolism (sulphation and glucuronidation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.

after the diet supplementation with OLE. In fact, it has been established that 24 h- urine offers advantages over plasma measurements, mostly because they allow an accurate evaluation of the total polyphenols absorbed due to the very short plasma half-lives of the polyphenols (Medina - Remón, Tresserra - Rimbau, Arranz, Estruch, & Lamuela-Raventos, 2012). Regarding the colonic phenolic metabo-lites detected in the intestinal contents and faeces, a very low concentration of these metabolites was detected in the urine samples, and no significant differences were observed among the three groups or compared to the control (data not shown). These results could indicate rather that urine sampling of up to 24 h could be required to detect these HT microbial metabolites, or that they were not reabsorbed at colonic level.

So, results from plasma and urine showed that depending on the molecular structure of the HT precursors, the bioavailability could differ. In accordance, previous studies showed that the bioavailability of flavonoids also varied depending on their chemical structure. Results obtained from plasma and urinary levels of flavanol stereoisomers after their oral intake, showed that the ranking for the oral absorbability of flavanols was as follows: (−)-epicatechin > (+)-epicatechin = (+)-catechin > (−)-catechin, (−)-epicatechin (Ottaviani et al., 2011). Moreover, the sugar moiety of flavonoids has been suggested as an important determinant in their absorption in humans. Typically, absorption of flavonoid glycosides is associated with the release of the aglycone as a result of the action of LPH enzyme with β-glucosidase activity. However, a great part of the polyphenol conjugates with sugar moieties are resistant to the action of LPH enzymes and pass to the colon, where the colonic microbiota cleave conjugating moieties (Del Rio et al., 2013). This might be the case of the glycoside molecule of OLE, which could have been in part hydrolysed by LPH enzymes leading to HT in the upper part of the GIT, and another important amount of OLE could have passed to the colon.

Regarding the tissue distribution, in contrast to our previous study (Serra et al., 2012), in which a similar olive phenolic extract was administered to rats, in the present study, very few HT metabolites were detected in liver and kidney and at very low concentrations with no significant differences among the HT, SEC and OLE groups (**Table S4** of Supplementary Material). This fact could be due to the low daily dose administered (5 mg/kg rat weight/day) and the tissue perfusion with isotonic solution performed to remove the remaining blood irrigating tissues after the sacrifice of the rats. Accordinly with our previous study, HT-Sulf appeared again as the most deposited metabolite in both liver and kidney tissues.

4.CONCLUSIONS

The results from the present study show that, of the three HT precursors, the maximum efficiency in the bioavailability of HT and its metabolites was observed after diet supplementation with OLE. This could be due to the higher stability of OLE under digestion conditions observed along the GIT, which could have led to a major exposure of OLE to phase II metabolism. The higher bioavailability of HT through OLE was

mainly reflected in the urine excretion of phase I and II metabolites. Moreover, OLE as a glycoside molecule could have resisted in part the action of the β-glucosidase enzymes in the small intestine and passed unaltered to the colon with the resultant higher production of microbial fermentation catabolites with potential biological activity. The findings from this study revealed that the absorption and biotransformation of HT depends mainly on its precursor. Further studies are needed to find a possible correlation with biological efficacy, but in terms of bioavailability and microbial fermentation, the results indicate that OLE is the most suitable HT derivate for use in food or nutraceutical formulations.

Acknowledgments

This study was supported by the Spanish Ministry of Education and Science (AGL2012-40144-C03-03 and AGL2012-40144-C03-02 projects) and by the University of Lleida through the M.C. López de las Hazas grant.

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SUPPLEMENTARY MATERIAL

Table 1 of Supplementary Material. Phenolic composition of the secoiridoid extract (SEC) and nutritional composition of the standard fed pellets.

Secoiridoid extract	
Compound	**Concentration (mg/kg extract)**
HT	4176.82 ± 185.40
p-HPEA-EDA	936.37 ± 60.66
3,4-DHPEA-EDA	40995.97 ± 1085.46
p-HPEA-EA	378.82 ± 21.63
3,4- DHPEA-EA	1628.98 ± 274.01
OLE	480.00 ± 70.33
Standard fed pellets	
Ingredients: Wheat, corn, corn gluten, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, aminoacids mix, minerals and vitamins.	
Component	**% of diet**
Proteins	14.3 %
Fat	4 %
Carbohydrates	48 %
Fibers (∑ crude & neutral)	21 %
∑ minerals	2.7 %
∑ aminoacids	10.5 %
∑ vitamins (A, D, E, K, B_1, B_2, Niacin, B_6, Pantothenic Acid, B_{12}, Biotin, Folate, Choline)	

Values of extracts are means ± SD (n= 4). HT: Hydroxytyrosol; *p*-HPEA- EDA: Dialdehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EDA: Decarboxymethyl elenolic acid linked to hydroxytyrosol; *p*-HPEA-EA: Aldehydic form of elenolic acid linked to tyrosol; 3, 4- DHPEA-EA: Isomer of oleuropein aglycone. OLE: oleuropein.

Table 2 of Supplementary Material. SRM conditions for the analysis of phenolic metabolites by UPLC-MS/MS.

Phenolic compound	Quantification (SRM_1)			Identification (SRM_2)			Standard in which has been quantified
	SRM	Cone voltage (V)	Collision energy (eV)	SRM	Cone voltage (V)	Collision energy (eV)	
Hydroxytyrosol	153 > 123	35	10	153 > 95	35	25	Hydroxytyrosol
Hydroxytyrosol sulfate	233 > 153	40	15	233 > 123	40	25	Hydroxytyrosol-3-*O*-sulfate
Hydroxytyrosol disulfate	313 > 233	40	10	313 > 153	40	30	Hydroxytyrosol-3-*O*-sulfate
Hydroxytyrosol-3-*O*-glucuronide	329 > 153	40	20	329 > 123	40	25	Hydroxytyrosol
Hydroxytyrosol-4-*O*-glucuronide	329 > 153	40	20	329 > 123	40	25	Hydroxytyrosol
Homovanillic alcohol	167 > 152	35	15	167 > 122	35	25	Hydroxytyrosol
Homovanillic alcohol sulphate	247 > 167	40	15	247 > 152	40	25	Hydroxytyrosol
Homovanillic alcohol glucuronide	343 > 167	40	20	343 > 152	40	35	Hydroxytyrosol
Homovanillic acid	181 > 137	25	10	181 > 122	25	15	Hydroxytyrosol
Homovanillic acid sulphate	261 > 181	40	15	261 > 137	40	25	Hydroxytyrosol
Homovanillic acid glucuronide	357 > 181	40	20	357 > 137	40	30	Hydroxytyrosol
Hydroxytyrosol acetate sulphate	275 > 153	35	15	275 > 123	35	30	Hydroxytyrosol
Elenolic acid	241 > 139	30	15	241 > 127	30	20	Hydroxytyrosol
Elenolic acid sulphate	321 > 241	40	15	321 >	40	20	Oleuropein

Elenolic acid glucuronide	417 > 241	40	15	417 > 139	40	20	Oleuropein
Oleuropein	539 > 377	35	15	539 > 275	35	20	Oleuropein
Oleuropein glucuronide	715 > 539	55	15	715 > 377	55	25	Oleuropein
Oleuropein aglycone sulfate	457 > 377	40	15	457 > 275	40	20	Oleuropein
Oleuropein aglycone glucuronide	553 > 377	40	15	553 > 377	40	20	Oleuropein
Methyl oleuropein aglycone sulfate	471 > 391	40	15	391 > 275	40	20	Oleuropein
Oleuropein aglycone derivate 1	555 > 523	40	15	555 > 275	40	25	Oleuropein
Oleuropein aglycone derivate 2	571 > 539	40	15	571 > 377	40	25	Oleuropein
Catechol	108.9 > 90.9	40	15	-	-	-	Catechol
Phenylacetic acid	135 > 91	20	5	-	-	-	HPA
Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-	HPA
Hydroxyphenylacetic acid sulphate	231 > 151	20	20	231 > 107	20	25	HPA
Hydroxyphenylacetic acid glucuronide	327 > 151	20	15	327 > 107	20	25	HPA
Dihydroxyphenylacetic acid	167 > 123	20	10	-	-	-	DHPA
Hydroxybenzoic acid	137 > 93	30	15	-	-	-	HPA
Protocatechuic acid	153 > 109	45	15	-	-	-	Hydroxytyrosol
Hydroxyphenylpropionic acid	165 > 121	20	10	-	-	-	HPP
Hydroxyphenylpropionic acid sulphate	245 > 165	35	15	245 > 121	35	20	HPP

Dihydroxyphenylpropionic acid	181 > 137	20	15	-	-	-	HPP
Hippuric acid	178 > 134	40	10	-	-	-	Hippuric acid
Hydroxyhippuric acid	194 > 100	40	10	-	-	-	Hippuric acid

HPA*:* *p*-Hydroxyphenylacetic acid; HPP: 3-(4-Hydroxyphenyl)propionic; DHPA: 3,4-Dihydroxyphenylacetic

Table 3 of Supplementary Material. Linearity, calibration curves, reproducibility, accuracy, LOD and LOQs for the analysis of the commercial studied phenolic compounds by UPLC-MS/MS in spiked biological samples.

a) Plasma samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (0.5 µM)	Accuracy (*n*=3) (0.5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-2.77	y = 9.425 x + 0.013	11.2	98	0.002	0.005
Hydroxytyrosol-3-O-sulfate	0.03-277	y = 3.486 x + 0.024	9.8	102	0.005	0.015

b) Urine samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (25 µM)	Accuracy (*n*=3) (25 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-94.98	y = 5.412 x + 0.042	14.2	103	0.01	0.03
Oleuropein	0.07-78.67	y = 63.158 x – 0.107	13.4	99	0.02	0.07

c) Liver samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (2.5 µM)	Accuracy (*n*=3) (2.5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.009-179	y = 266.2 x + 0.019	9.8	100	0.003	0.009
Oleuropein	0.05-54.03	y = 108.85 x – 0.085	7.5	97	0.02	0.05

d) Kidney samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3)	Accuracy (*n*=3)	LOD (µM)	LOQ (µM)

			(2.5 µM)	(2.5 µM)		
Hydroxytyrosol	0.60-72.88	y = 7.811 x – 0.019	10.2	97	0.20	0.60
Oleuropein	0.08-21.95	y = 25.934 x – 0.019	9.5	96	0.03	0.08

e) Stomach content samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (5 µM)	Accuracy (*n*=3) (5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-636.36	y = 59.92 x + 65.54	7.9	96	0.01	0.03
Oleuropein	0.40-191.67	y = 305.74 x – 389.78	8.6	102	0.15	0.40

f) Small intestine content samples

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (10 µM)	Accuracy (*n*=3) (10 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.3-636.36	y = 108.02 x – 99.243	10.1	104	0.15	0.3
Oleuropein	0.19-191.67	y = 133.37 x + 529.84	12.6	103	0.03	0.19
p-Hydroxyphenylacetic acid	0.01-112.83	y = 520.91 x + 469.32	10.1	99	0.003	0.01
3,4-Dihydroxyphenylacetic acid	0.55-544.64	y = 63.319 x – 569.53	8.6	102	0.18	0.55
3-(4-Hydroxyphenyl)propioni acid	0.84-1084.34	y = 24.08 x + 159.63	9.9	97	0.25	0.84
Hippuric acid	5.44-543.58	y = 80.624 x – 1640.4	12.8	95	2.44	5.44
Catechol	0.3-745.75	y = 97.53 x – 124.56	10.5	105	0.15	0.3

g) Caecum content samples

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3)	Accuracy (*n*=3)	LOD (µM)	LOQ (µM)

			(5 µM)	(5 µM)		
Hydroxytyrosol	0.004-636.36	y = 110.12 x – 98.147	12.4	102	0.001	0.004
Oleuropein	0.004-592.67	y = 145.48x + 624.75	13.4	95	0.001	0.004
p-Hydroxyphenylacetic acid	0.002-728.3	y = 518.22 x + 396.24	12.4	96	0.001	0.003
3,4-Dihydroxyphenylacetic acid	0.45-544.64	y = 62.124 x – 475.04	11.5	99	0.15	0.45
3-(4-Hydroxyphenyl)propionic acid	0.84-1084.38	y = 20.084 x + 152.04	11.6	105	0.28	0.84
Catechol	0.75-745.75	y = 102.64 x – 113.29	12.2	102	0.001	0.004

h) Faeces

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (50 µM)	Accuracy (*n*=3) (5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.01-636.36	y = 96.116 x – 60.08	12.4	97	0.003	0.01
Hydroxytyrosol-3-*O*-sulfate	0.004-428.21	y = 450.49x + 142.58	11.9	99	0.001	0.004
p-Hydroxyphenylacetic acid	0.004-567.70	y = 559.73 x + 1009.6	12.7	101	0.001	0.004
Hippuric acid	0.54-543.58	y = 52.505 x – 120.03	13.0	103	0.2	0.54
Catechol	0.75-745.75	y = 103.57 x + 139.85	12.2	102	0.001	0.004

* Calibration curves (based on peak abundance) were plotted using y=a+bx, where *y* is the peak abundance ratio (analyte/IS) and *x* is the concentration ratio (analyte/IS). This was applied when catechol (IS) was not detected in samples.

Table 4 of Supplementary Material. Concentration of the metabolites detected in metabolic tissues (liver and kidney) obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as the mean ± SEM.

Phenolic compound	Control	HT	SEC	OLE
Liver (nmol/g tissue)				
Hydroxytyrosol sulfate	0.03 ± 0.00^{a}	2.74 ± 0.40^{b}	3.23 ± 0.30^{b}	2.05 ± 0.25^{b}
Homovanillic alcohol sulfate	n.d.	0.34 ± 0.10^{a}	0.60 ± 0.07^{a}	0.25 ± 0.02^{b}
Homovanillic acid glucuronide	0.01 ± 0.00^{a}	8.65 ± 0.86^{d}	2.70 ± 0.07^{b}	3.30 ± 0.06^{c}
Elenolic acid sulfate	n.d.	-	0.77 ± 0.49	0.55 ± 0.21
Elenolic acid glucuronide	n.d.	-	0.35 ± 0.02	0.38 ± 0.06
Methyloleuropein aglycone sulfate	n.d.	-	0.12 ± 0.01	0.12 ± 0.02
Oleuropein	n.d.	-	0.19 ± 0.02	0.22 ± 0.05
Kidney (nmol/g tissue)				
Hydroxytyrosol sulfate	1.82 ± 0.18^{a}	8.69 ± 0.32^{b}	6.65 ± 0.70^{b}	5.62 ± 0.95^{b}
Homovanillic alcohol sulfate	0.72 ± 0.12^{a}	2.12 ± 0.22^{b}	1.86 ± 0.19^{b}	1.79 ± 0.28^{b}
Homovanillic acid glucuronide	1.78 ± 0.08^{c}	0.79 ± 0.05^{a}	1.41 ± 0.11^{b}	1.02 ± 0.11^{a}
Elenolic acid sulfate	0.16 ± 0.03	-	0.16 ± 0.06	0.11 ± 0.03
Elenolic acid glucuronide	0.13 ± 0.04^{a}	-	0.55 ± 0.02^{b}	0.20 ± 0.02^{a}