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Fatty Acid Hydroxytyrosyl Esters of Olive Oils Are Bioaccessible According to Simulated *In Vitro* Gastrointestinal Digestion: Unraveling the Role of Digestive Enzymes on Their Stability

Carolina Alemán-Jiménez,[#] Raúl Domínguez-Perles,[#] Juana I. Gallego-Gómez, Agustín Simonelli-Muñoz, Espérance Moine, Thierry Durand, Céline Crauste, Federico Ferreres, Ángel Gil-Izquierdo,^{*} and Sonia Medina^{*}



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ABSTRACT: Recently, new bioactive compounds were identified in olive oil, lipophenols, which are composed of a fatty acid (FA) and a phenolic core, such as HT (HT-FA). However, their bioaccessibility remains unknown. Thus, the present study uncovers the impact of the separate phases of gastrointestinal digestion on the release and stability of HT-FAs from oily matrices under *in vitro* simulated conditions. Accordingly, it was found that the bioaccessibility of HT derivatives is largely dependent on the type of FA that esterifies HT, as well as the food matrix. Also, the generation of HT-FAs during intestinal digestion was observed, with pancreatin being the enzyme responsible, to a higher extent, for the *de novo* formation of lipophenolic derivatives. These findings prompt us to identify new applications to oily matrices and their byproducts as potential functional ingredients for the promotion of health, where the possible formation of new lipophenols during digestion should be taken into consideration.

KEYWORDS: olive oil, hydroxytyrosol, lipophenols, *in vitro* gastrointestinal digestion, bioaccessibility, lipases

INTRODUCTION

Olive drupes and derived products are considered part of the Mediterranean diet and co-contribute to the healthy characteristics of this diet, especially because of their content of mono- and polyunsaturated fatty acids (FAs) and (poly)phenols such as hydroxytyrosol (HT).^{1,2} Moreover, oleic acid is the most abundant FA in olive oil, and HT is its principal phenolic compound.³ Also, the presence of lipophenol structures, composed of an FA and a phenolic core, has been reported in edible oils. These molecules result from the esterification of HT with fatty acids (e.g., α -linolenic, linoleic, and oleic acids (HT-ALA, HT-LA, and HT-OA, correspondingly)).^{4,5} To date, extra virgin and virgin olive oils (EVOO and VOO, respectively) are the oily matrices with the highest concentration of these compounds.⁵

Lipophenols offer functional advantages relative to their molecular constituents (HT and FAs). Specifically, these compounds are characterized by a higher lipophilicity, cell membrane affinity, and improved antioxidant activities.^{6–8} However, despite the suggestion of lipophenol's biological power,^{9,10} the actual functions remain almost unexplored. Nevertheless, regarding their antioxidant property, it may progressively be augmented along with the extension of the FA chain length up to a critical point, when additional extension could lower the radical scavenging power. Because of this phenomenon, the so-called cutoff effect, short- to medium-chain lipophilic esters of phenolic compounds (C8–C12) are more effective antioxidants relative to long-chain esters.¹¹ In

contrast, other studies have associated equal or higher activity with long-chain esters (C18–C20).^{12,13}

Aside from these considerations, the bioaccessibility of lipophenols remains to be assessed. In this regard, according to previous studies, intense hydrolysis of HT acetate after pancreatin and bile salt digestion has been reported,¹⁴ while tyrosol acyl esters are hydrolyzed by pancreatic lipase to produce free tyrosol, showing a certain sustained-release behavior.^{15,16} These results suggest that the time frame for retrieving biological activities from phenolic compounds could be extended due to lipophenol's bioactivity, thus allowing us to obtain additional health benefits.¹⁵ These authors reported that the stability of tyrosol esters under the physicochemical conditions associated with the *in vitro* simulation of gastrointestinal (GI) digestion was correlated both with the chain length and with the number of unsaturations.¹⁶ Nonetheless, the effect of the digestive process on the release of HT-FAs and their stability remains underexplored. Also, the experimental approach implemented thus far, based on the analysis of authentic standards instead of food matrices, is negligible because it ignores the effect of the matrix throughout the digestion.¹⁴ To overcome this limitation, a simulated GI

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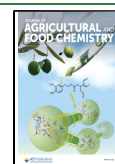


Table 1. Preparation of Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

simulated fluids	constituent ^a (mmol/L)					
	KCl	KH ₂ PO ₄	NaHCO ₃	NaCl	MgCl ₂ (H ₂ O) ₆	(NH ₄) ₂ CO ₃
simulated gastric fluid (SGF; pH 3)	6.90	0.90	25.00	47.20	0.10	0.50
simulated intestinal fluid (SIF; pH 8)	6.80	0.80	85.00	38.40	0.13	

^aKCl, potassium chloride; KH₂PO₄, potassium phosphate monobasic; NaHCO₃, sodium bicarbonate; NaCl, sodium chloride; MgCl₂(H₂O)₆, magnesium chloride hexahydrate; and (NH₄)₂CO₃, ammonium carbonate.

digestion by *in vitro* models could be a valuable, time-saving tool, as it requires fewer resources for obtaining preliminary but extremely important information for the evaluation of the structural and chemical changes under GI conditions.¹⁷

According to this background information and previous findings, the present work aims to gain further insight in the results of the GI digestion on HT-ALA, HT-LA, and HT-OA found in EVOO and VOO to identify the lipophenols released from the food matrix that remain stable during GI digestion and that are available for absorption. Also, the evaluation of the effect of pepsin, pancreatin, and pancreatic lipase allowed us to discover the influence of the digestive enzymes on the stability of lipophenols. The major outcomes retrieved contributed to setting up the bioaccessibility of HT-FAs in the small intestine compared with unesterified HT and to understanding the enzymatic mechanisms responsible for them, providing the theoretical basis of the transformation of lipophenols during the digestive process.

MATERIALS AND METHODS

Chemicals and Reagents. The authentic standards of high-purity HT-FAs (HT-ALA, HT-LA, and HT-OA) were synthesized and fully characterized by nuclear magnetic resonance (NMR)-based analysis by the Institut des Biomolécules Max Mousseron (IBMM) (Montpellier, France) according to previously published procedures.⁴ Acetone and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all LC-MS-grade solvents (deionized water, methanol, and acetonitrile) were from J.T. Baker (Phillipsburg, NJ, USA). A certified reference of FA standards from Sigma-Aldrich was used for their identification and quantification. Hydroxytyrosol, with the following specifications: purity 99.6%, moisture 3.7%, and pH 4.26 (1 M aqueous solution), was provided by Seprox BIOTECH S.L. (Murcia, Spain). All lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO, USA) to obtain a stock solution (at the mM range of concentration), and then successive dilutions were prepared in methanol/deionized Milli-Q water (50:50, v/v). All stock solutions of lipophenols were stored at -20 °C in the dark. Porcine pepsin (P6887), pancreatin from porcine pancreas (P7545, 8 × USP), and lipase from porcine pancreas (L3126) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Fatty Acid Composition Analysis of Edible Oils. Fatty acids of EVOO, VOO, and flaxseed oil (FO) were extracted according to the methodology employed previously⁵ and published by the FAO (FAOLEX No. LEX-FAOC141241, <http://www.fao.org/faolex/results/details/es/c/%20LEX-FAOC141241/>) for the analysis of vegetable oils. Briefly, samples (1 g) were weighed and dried at 70 ± 10 °C for 6 h. The fatty acid content was obtained by the Soxhlet extraction with diethyl ether and heptane and by esterification with methanolic NaOH (1 M). Fatty acids were determined by gas chromatography coupled to a flame ionization detector (GC-FID) (6890 GC Agilent Technologies, Waldronn, Germany). The absolute concentration (g/100 g fw) of fatty acids in the vegetable oils ($n = 3$) was calculated according to the formula individual fatty acid (g/100 g fw) = individual fatty acid (% of fat)/100 × total fat (g/100 g fw),

which was used according to FAO/INFOODS guidelines for converting units, denominators, and expression (2012).¹⁸

Vegetable Oil Samples and Preparation of Analytical Extracts of Hydroxytyrosol Fatty Acid Esters. Extra virgin olive oil (Picual monovarietal; Olimendros S.L. (Murcia, Spain)), VOO (Picual monovarietal; Salvador Gallego El Jota S.L. (Albacete, Spain)), and FO (Laboratorios Almond S.L. (Murcia, Spain)) were chosen due to their dissimilar concentration of HT esterified with ALA, LA, and OA, with FO used as the negative control sample due to its lack of HT-FA ester content.⁵ All oil samples were kept in dark glass bottles, closed with screw caps, and stored at 4 °C to avoid oil oxidation to the greatest possible extent. The HT-FAs were extracted from vegetable oils according to the methodology described previously.⁵

In Vitro Simulated Gastrointestinal Digestion. To reproduce the separate digestion phases (gastric, intestinal, and GI), the edible oils (EVOO, VOO, and FO) were processed based on the harmonized *in vitro* digestion protocol described in the literature,^{19,20} using the simulated gastric and intestinal fluids (SGF and SIF, respectively) stock electrolyte solution, developed according to the information provided in Table 1.

After digestion, the samples were centrifuged at 1600g for 5 min at 4 °C to separate the bioaccessible fraction (BF) and the upper oily phase or residual fraction (RF); the concentration of HT and HT-FAs in each fraction was analyzed. Blanks (negative controls) without enzymes were processed and analyzed in parallel under equal conditions. All samples were protected from light over the entire process. Both BF and RF fractions were frozen immediately at -80 °C and lyophilized. For the HT and HT-FA extraction, the lyophilized samples were then dissolved in 3 mL of an acetone/BHA (99.995:0.005, v/w) solution, vortexed for 1 min, sonicated for 30 min at 40 kHz, and centrifuged at 8750g for 5 min at 4 °C according to the procedure described.⁵ The samples were dried using a SpeedVac concentrator, and the dry extracts were reconstituted with 500 μL of MeOH, sonicated for 10 min, centrifuged at 8750g for 5 min, and filtered through a 0.45 μm filter (Millipore, Burlington, MA, USA).

UHPLC-ESI-QqQ-MS/MS Analysis. The separation and identification of HT and HT-FAs were performed using a UHPLC coupled with a triple quadrupole MS/MS (Agilent Technologies, Waldbronn, Germany) according to the methodology reported in the literature.⁵ The retention times recorded for unesterified HT, HT-ALA, HT-LA, and HT-OA were 0.6, 3.8, 4.0, and 4.4 min, respectively. The analyses were performed by multiple reaction monitoring (MRM) in the negative mode, and the quantification and confirmation MRM transitions were as follows: m/z 153 > 123 arbitrary mass units (amu) and m/z 153 > 95 amu, respectively, for HT; m/z 413 > 277 amu and m/z 413 > 260 amu, correspondingly, for HT-ALA; m/z 415 > 279 amu and m/z 415 > 262 amu, respectively, for HT-LA; and m/z 417 > 281 amu and m/z 417 > 264 amu, correspondingly, for HT-OA. Data acquisition and processing were done using the MassHunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany). Both HT and esters of HT were quantified using reference standards, and the concentrations were expressed as nanograms per gram of fresh weight (ng/g fw).

Statistical Analyses. All the analytical extractions of the oily matrices considered in the present work, as well as the products of the gastric, intestinal, and GI digestions, were analyzed in triplicate ($n = 3$), and the data were expressed as the mean ± SD. Statistical analyses were performed at 5% of the significance level using the SPSS 27.0

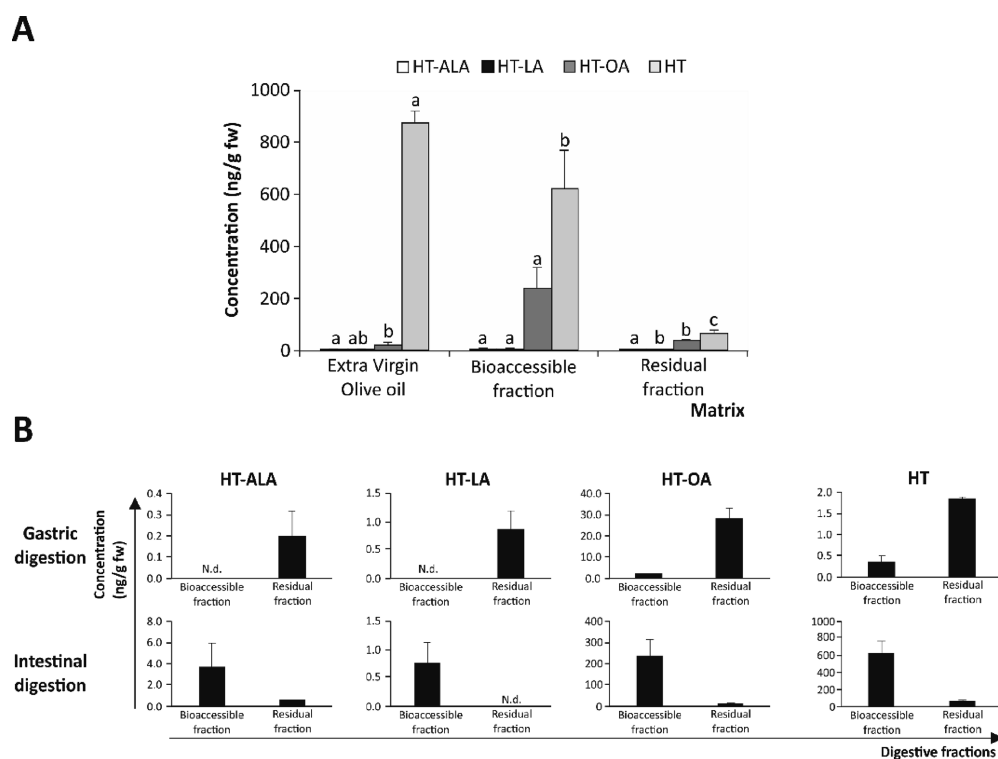


Figure 1. Concentration (ng/g fw) of HT and its lipophenolic derivatives in extra virgin olive oil (EVOO) and its bioaccessible and residual fractions (A) and bioaccessibility resulting from the individual gastric and intestinal digestions (B). N.d., not detected. Bars with a different lowercase letter indicate statistically significant differences among matrices at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

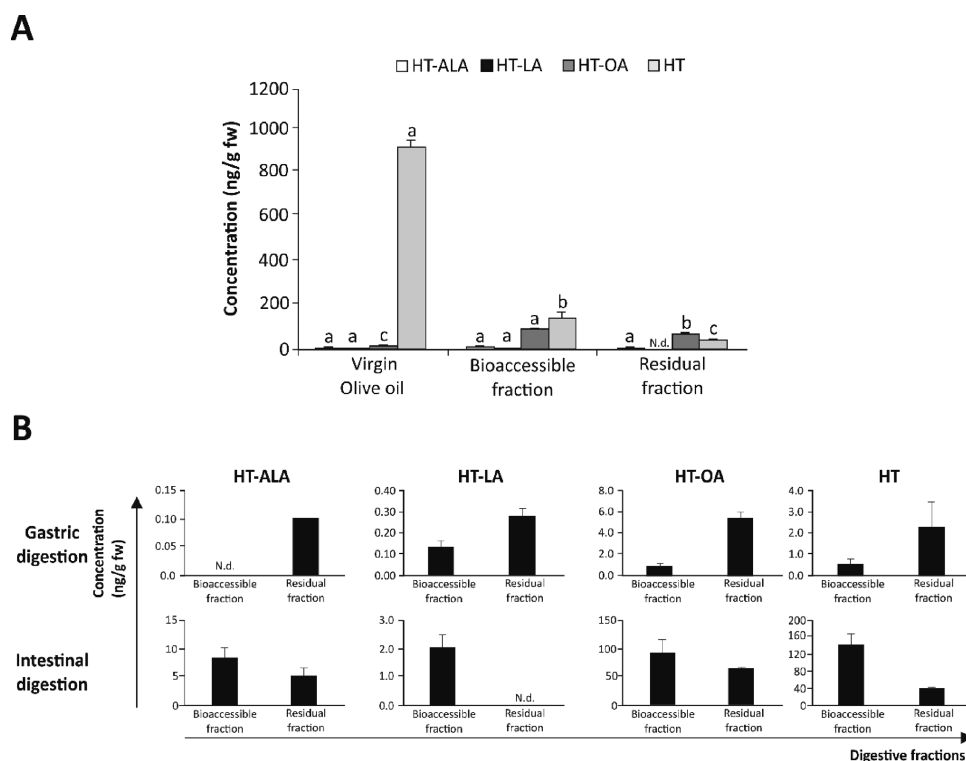


Figure 2. Concentration (ng/g fw) of HT and its lipophenolic derivatives in virgin olive oil (VOO) and its bioaccessible and residual fractions (A) and bioaccessibility resulting from the individual gastric and intestinal digestions (B). N.d., not detected. Bars with a different lowercase letter among matrices indicate statistically significant differences among matrices at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

software package (LEAD Technologies, Inc., Charlotte, NC, USA). Data were subjected to a one-way analysis of variance (ANOVA). The normal distribution of the residuals and the homogeneity of variance were tested with the Kolmogorov–Smirnov and Levene tests, respectively. When statistical differences were identified, the variables were compared using post hoc analysis with Tukey's multiple range test.

RESULTS AND DISCUSSION

Quantitative Profile of Hydroxytyrosol-Fatty Acids in Extra Virgin Olive Oil and Virgin Olive Oil. To understand the bioaccessibility of HT-FAs, first, the quantitative profile of unesterified and esterified HT in EVOO and VOO edible oils was set up. As expected, the retention time of HT-FAs displayed a reverse correlation with the degree of unsaturation of its lipid part. Thus, HT-ALA, HT-LA, and HT-OA were eluted at 3.8, 4.0, and 4.4 min, respectively, which are consistent with previous experimental results⁵ and other lipophenolic derivatives such as tyrosol esters.¹⁶ After this task, it was observed that the amount of unesterified and esterified HT of both olive oils was similar (Figures 1 and 2) but different from that corresponding to FO, which exhibited an absence of HT FAs (data not shown).

In this way, the unesterified HT was present as 874.74 and 934.09 ng/g fw in EVOO and VOO, respectively. These concentrations were in line with those available in the Phenol-Explorer database (<http://phenol-explorer.eu/>) on the HT content of EVOO (0.01–3.47 mg/100 g fw) and VOO (<0.01–7.43 mg/100 g fw). Regarding the HT-FAs, values of 2.80, 1.54, and 20.00 ng/g were recorded for HT-ALA, HT-LA, and HT-OA, respectively, in EVOO and 4.56, 2.59, and 11.90 ng/g in VOO (Figures 1 and 2, correspondingly), which are in agreement with previous reports on matching food matrices and analytes,⁵ although important factors such as variety, geographical origin, ripening stage, and production and extraction processes may play an important role in lipophenol content.²¹

Fatty Acid Composition of the Edible Oils Assessed.

As referred to before, it is worth noting that the bioaccessibility of specific bioactive compounds may be enhanced by the content of FAs in the food matrix through dispersing food components in the digestive tract or by promoting the secretion of pancreatic juice.²² In this scenario, as one of the objectives of the current study was to describe the bioaccessibility of the of HT-FAs, the quantitative profile of fatty acids of the edible oils under consideration (EVOO, VOO, and FO) was created, given its central importance to the formation of the target bioactive compounds, the HT-FAs. As expected and according to previous descriptions in the literature, the FA composition of the vegetable oils considered in the present work (EVOO, VOO, and FO) involved saturated, monounsaturated, and polyunsaturated fatty acids (SFAs, MUFAs, and PUFAs, correspondingly).²³ Specifically, the oily matrices studied contained a total of 13 individual FAs (Table 2).

The SFAs detected were palmitic (C16:0) and stearic (C18:0) acids, which showed statistically significant differences between samples ($p < 0.05$), with EVOO being the plant oil with the highest content of palmitic acid (12.07 g/100 g fw) in comparison with VOO and FO (10.97 and 5.77 g/100 g fw, respectively). On the other hand, FO provided the greatest concentration of stearic acid (4.40 g/100 g fw) that surpassed

Table 2. Concentration of Fatty Acids (g/100 g fw) in the Extra Virgin Olive Oil (EVOO), Virgin Olive Oil (VOO), and Flaxseed Oil (FO) Analyzed in the Present Work

fatty acids ^a	edible oils			LSD ($p < 0.05$)
	EVOO	VOO	FO	
C14:0	0.03a ^b	0.03a	0.03a	<0.01
C16:0	12.07a	10.97b	5.77c	0.17
C16:1 n7	0.93a	0.83a	0.10b	0.09
C17:0	0.10a	0.10a	0.10a	<0.01
C17:1 n8	0.10a	0.10a	0.10a	<0.01
C18:0	2.50c	2.63b	4.40a	0.06
C18:1 n9	77.53b	80.10a	19.97c	0.17
C18:2 n6	4.43b	4.10c	15.27a	0.09
C18:3 n3	0.63b	0.60b	53.53a	0.09
C20:0	0.37a	0.60a	0.33a	0.09
C20:1	0.30a	0.30a	0.10b	<0.01
C22:0	0.10a	0.10a	0.10a	<0.01
C24:0	0.47a	0.10b	0.10b	0.06

^aC14:0: myristic acid; C16:0: palmitic acid; C16:1 n7: palmitoleic acid; C17:0: heptadecanoic acid; C17:1 n8: heptadecenoic acid; C18:0: stearic acid; C18:1 n9: oleic acid; C18:2 n6: linoleic acid; C18:3 n3: alpha-linolenic acid; C20:0: arachidic acid; C20:1: eicosenoic acid; C22:0: behenic acid; and C24:0: lignoceric acid. ^bMeans ($n = 3$) within a row with different lowercase letters are significantly different at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

by 41.7%, on average, the contents recorded in EVOO and VOO, which remained in similar but lower levels.

Moreover, significant differences were recorded regarding the content of palmitoleic and oleic acids (C16:1 n7 and C18:1 n9, respectively) between the diverse edible oils analyzed ($p < 0.05$), with oleic acid underlined as having the most abundant MUFA in all vegetable oils considered (in the range of 19.97–80.10 g/100 g fw). The highest concentration of oleic acid was found in VOO (80.10 g/100 g fw) followed by EVOO (3.2% lower) and FO (75.1% lower) (Table 2).

Finally, when analyzing the content of PUFAs, linoleic (C18:2 n-6) and α -linolenic (C18:3 n-3) acids were found at the highest concentrations in FO (15.27 and 53.53 g/100 g fw, respectively), whereas EVOO and VOO displayed values in a similar lower level (4.27 and 0.62 g/100 g fw, on average, respectively) (Table 2).

The composition results regarding SFAs, MUFAs, and PUFAs are in accordance with information previously reported in the frame of studies comparing the FA composition of several types of edible oils.^{5,24} In this regard, the FA profile, together jointly with the presence of HT in the matrices referred to in the study of HT lipophenolic derivatives, would provide a very helpful choice for assessing the effect of GI digestion on the lipophenolic profile of vegetable oils, which is essential for discovering the biological interest of these compounds.

Bioaccessibility of Fatty Acid Esters of Hydroxytyrosol from Extra Virgin Olive Oil and Virgin Olive Oil. For this first description of the GI bioaccessibility of the HT lipophenols, an optimized and standardized static method was applied for the *in vitro* simulation of GI digestion, which allows mimicking the physicochemical conditions in the GI tract, *in vivo*, as closely as possible.^{19,20} The lipophenols of HT, HT-ALA, HT-LA, and HT-OA were analyzed in the three vegetable oils utilized in the present study. FO was selected

as a negative control as it does not contain either HT or its lipophenolic derivatives according to our previous work.⁵

These molecules were also assessed in the digestates obtained after *in vitro* gastric, intestinal, and GI digestion of the vegetable oils mentioned above. In this regard, both the upper oily or residual phase (RF) and the lower micellar or bioaccessible fraction (BF) resulting from digestion were collected and analyzed to retrieve accurate information on the theoretical and potential bioavailable fractions of these new lipophilic phenolic compounds, and also interconversions between unesterified HT and its esters derivatives, which would condition future *in vivo* research aimed at addressing the actual bioavailability of HT (free and esterified).

The *in vitro* GI digestion performed on EVOO (Figure 1A) and VOO (Figure 2A) revealed that ALA-, LA-, and OA-based lipophenols of esterified HT were present in the BF. The concentrations of HT-FAs corresponding to EVOO were 3.68, 4.04, and 239.41 ng/g fw, while for the VOO samples, these were 8.22, 1.80, and 92.00 ng/g fw for HT-ALA, HT-LA, and HT-OA, respectively, evidencing that the esterified forms of HT were bioaccessible. However, the rate of bioaccessibility depended on both the specific FA-based esterification and the compositional features of the food matrix, as already reported for free HT pharmacokinetics and bioavailability, *in vivo*, after ingestion of different oily matrices.²⁵

These results are in good agreement with previous reports on the bioaccessibility of other esters of HT, namely, phosphatidyl-hydroxytyrosol (PHT, phospholipid derivatives of HT with phosphatidylcholine).¹⁷ In their work, Martin et al. described that the portion of HT within the BF, regarding the esterified form (PHT), was significantly higher relative to unesterified HT.¹⁷ In addition, although at lower concentrations than in BF, both unesterified HT and esterified HT were detected in RF (Figures 1A and 2A). Just as with PHT, the amphiphilic properties of HT esterified with FAs could cause its dispersion in the aqueous media, which in turn indirectly enabled the dispersion of the vehiculated HT.¹⁷

When analyzing the bioaccessibility rate of HT and its lipophenol derivatives, it was found that the unesterified form presented a bioaccessibility of 70.9 and 15.4% for EVOO and VOO, respectively. These results are in line with previous descriptions concerning an important loss of HT in the duodenal compartment (~50%, on average) retrieved from alperujo (olive-mill waste) digestions.²⁶ These authors described that when digesting only the target bioactive compounds in analytical solvents (without the presence of additional food constituents, such as starch, casein, and fiber), the bioaccessibility of HT was enhanced by ~20%. This effect could be due to the presence of the referred macromolecules, which can bind polyphenols and retain them within the food matrix. Hence, several authors have emphasized the significance and complexity of phenolic compound interactions within the food matrices associated to their bioaccessibility.^{26,27}

In relation to the concentrations of lipophenols in EVOO, the concentration of HT-FAs in BF increased 1.3-, 3.0-, and 11.9-fold for HT-ALA, HT-LA, and HT-OA, respectively (Figure 1A). This fact may be due to the interactions between free HT and FAs, where HT could act as a nucleophilic compound able to trap ALA, LA, and OA present in the oily matrix to form the corresponding esters via enzymatic or chemical means under GI conditions,²⁸ therefore exerting "negative" effects on free HT bioaccessibility but ameliorating

the concentration of the esterified forms of HT in the digestate. A similar behavior was observed for VOO as a result of the GI digestion, which resulted in a 1.8- and 7.7-fold rise in concentration for HT-ALA and HT-OA, respectively, in the BF when compared to those found in VOO, with the exception of HT-LA, whose bioaccessibility decreased up to ~70%, on average (Figure 2A). In addition, it should also be stressed that the most abundant HT-FA in the RF was HT-OA, which increased 2.0- and 5.6-fold in comparison with the amounts found in EVOO and VOO, respectively (Figures 1A and 2 and Table 2). This differential occurrence of HT-FAs from EVOO and VOO after GI digestion could be owed to the interactions of these compounds with the food matrix components such as amino acids and proteins, in spite of their low content (0.07–2.4 mg/kg). The presence of these components depends on the ripening stage or olive cultivar, among other factors,^{29,30} and may generate fatty acid-binding protein reactions^{31,32} that can hamper the bioaccessibility of these compounds.

Conventionally, the RF has not been considered for the evaluation of the bioaccessibility of phenolic compounds after the digestions. However, this fraction is of high relevance because of its high concentration of polyphenols that are not absorbed in the small intestine, which can be metabolized by the gut microbiota,³³ giving rise to additional bioactive derivatives that could also contribute to the healthy attributes of foods. Thus, both fractions (BF and RF) resulting from GI digestion may be responsible for the positive health effects attributed to plant-based foods, including edible oils.³⁴ In this matter, the capacity of the intestinal microbiota to modify the quantitative phytochemical profile of the digestion products and thereby the bioavailability/absorption capacity of bioactive compounds present in olive and olive oils that have not been absorbed in the small intestine is remarkable, and as a result, the substrate for these metabolic reactions (nonabsorbed phenolics) should not be disregarded. In fact, the gut microbiota plays a prominent role in the biotransformation of both esterified and aglycone forms of phenolic compounds, but the metabolism of lipophenols by the gut microbiota is still unclear. To date, there is only one study that evaluated the fecal microbial metabolism of lipophenols utilizing an *in vitro* fermentation model as well as the *in vivo* absorption and plasma pharmacokinetics of tyrosol esters in rats, where standards of tyrosol and tyrosol esters were administered in the drinking water.³⁵ The authors concluded that tyrosol ester derivatives acted longer *in vivo* than native tyrosol by observing a second absorption peak in pharmacokinetic profiles, possibly due to microbiota degradation, with the esterified molecules exhibiting an improved bioavailability as compared to that of free tyrosol. The incubation of tyrosol esters in the fermentation solution liberated free tyrosol in a time-dependent manner, indicating the occurrence of hydrolysis.³⁵ However, this finding should be tested with rats fed with natural sources of these lipophenols, not with standards, and in the natural concentrations in which they are found.

Contribution of the Gastric and Intestinal Digestion Phases to the Bioaccessibility of HT-FAs. To understand the impact of the digestion process on the bioaccessibility of HT-FAs, the gastric digestates from EVOO and VOO were also analyzed (Figures 1B and 2), which provided valuable information on the release of the target lipophenols on each digestion stage. Upon this analysis, it was observed that unesterified HT and its lipophenol derivatives were present at

Table 3. Concentration (ng/g fw) of the Bioaccessible and Residual Fractions (BF and RF, Respectively) of HT and Its Lipophenolic Derivatives as a Result of the Only Intestinal Digestion on the Different Substrates (Extra Virgin Olive Oil, Virgin Olive Oil, and Their Gastric Digestates)

oil matrix	analyte ^a	concentration (ng/g fw)				
		olive oil	substrate of the intestinal digestion			
			gastric digestate		olive oil	
			BF	RF	BF	RF
EVOO ^a	HT	874.47 ± 41.88a ^b	619.88 ± 144.42b	64.46 ± 12.70d	279.05 ± 63.95c	542.00 ± 34.15b
	HT-ALA	2.80 ± 0.95b	3.68 ± 2.30b	0.61 ± 0.06b	235.96 ± 23.77a	0.64 ± 0.02b
	HT-LA	1.54 ± 0.49bc	4.04 ± 1.97b	N.d.	34.19 ± 1.61a	N.d.
	HT-OA	20.00 ± 8.11c	237.64 ± 76.94b	12.68 ± 2.72c	3599.94 ± 107.20a	102.35 ± 0.41bc
VOO	HT	934.09 ± 29.53a	143.76 ± 25.08bc	39.58 ± 1.27c	207.75 ± 131.67b	251.66 ± 3.38b
	HT-ALA	4.56 ± 1.52b	8.22 ± 1.79b	5.12 ± 1.25b	32.44 ± 5.17a	4.59 ± 0.54b
	HT-LA	2.59 ± 0.46b	1.98 ± 0.44b	N.d.	5.87 ± 1.51a	N.d.
	HT-OA	11.90 ± 4.90b	91.18 ± 2.97b	63.90 ± 4.21b	1205.44 ± 160.57a	85.81 ± 7.79b

^aHT, hydroxytyrosol; HT-ALA, hydroxytyrosol esterified with α -linolenic acid; HT-LA, hydroxytyrosol esterified with linoleic acid; and HT-OA, hydroxytyrosol esterified with oleic acid. ^bData are shown as means \pm SD ($n = 3$). Data within a single row followed by the same lowercase letter are not significantly different at $p < 0.001$ according to the analysis of variance (ANOVA) and Tukey's multiple range test. N.d., not detected.

high concentrations in the RF obtained from the gastric digestion for both EVOO and VOO.

When evaluating the impact of gastric digestion on the EVOO lipophenols, low concentrations of HT and HT-FAs were observed in the BF for all individual analytes (not detected, not detected, and 1.77 and 0.33 ng/g for HT-ALA, HT-LA, HT-OA, and HT) (Figure 1B). Similar results were obtained regarding VOO (not detected and 0.13, 0.82, and 0.52 ng/g for HT-ALA, HT-LA, HT-OA, and HT) (Figure 2B).

The low concentrations recorded could be due to the lability of lipophenols against the physicochemical conditions and the enzymatic activity proper of the gastric digestion, which would be associated with a low release or stability of the target analytes. This would be consistent with previous reports on the bioaccessibility of HT in olive leaves,³⁶ further supporting the influence of the food matrix, the gastric enzymes, or acidic conditions previously described.³⁷ Nonetheless, the development of intestinal digestion on the chyme provided a high concentration of free HT in the BF. This could be due to the hydrolysis of oleuropein and its aglycone during intestinal digestion that depends on the lipase activity, thus leading to the appearance of HT, as previously stated by Rocchetti et al.³⁸ Interestingly, as a result of intestinal digestion, the concentration of HT-FAs significantly increased in the BF over the entire GI process, tentatively due to the putative enzymatic activity of pancreatic lipase that may catalyze both the hydrolysis or synthesis of esters.³⁹

As far as we know, to date, most research on the bioaccessibility of lipophenols has been performed on solutions of authentic standards exposed to the GI physicochemical conditions. Therefore, despite the referred attributes of the synthesis of lipophenols as a result of the digestive enzymatic activity, additional works have evidenced that HT derivatives, such as hydroxytyrosol acetate, undergo intense hydrolysis during intestinal digestion mainly due to the enzymatic activity of pancreatin.¹⁴ Similarly, it has been reported that tyrosol acyl esters are hydrolyzed by pancreatic lipase to produce free tyrosol.^{15,16} Moreover, the stability of resveratrol esters with caprylic acid during digestion is negatively correlated with the degree of substitution of the FA moiety, given that after 120 min of incubation at 37 °C, ~54 and ~11% of monoesters and

diesters are hydrolyzed, respectively, while no hydrolysis of the triesters has been noticed.⁴⁰ However, these results have been associated with an "artificially" obtained (only with chemically or enzymatically synthesized standards) higher bioaccessibility relative to the effects that occur during the GI digestion of foods because these models do not allow monitoring the extractive capacity of the process, the interconversion between molecules, or the *de novo* synthesis of the target analytes (from the complex pool of molecules present in the food matrix). This gap in knowledge has been overwhelmed by a study on the bioaccessibility of HT with alperujo (solid byproduct of olive oil extraction). This study described a lower bioaccessibility of HT when applying GI digestion on a vegetable matrix due to the presence of fibers and sugars that could interact with HT and the digestive enzymes, modulating the bioaccessibility rate.²⁶ Accordingly, the assessment of the bioaccessibility of HT or HT-FAs needs to include a food matrix to obtain robust and nonspeculative conclusions.

The relevance of the intestinal digestion phase for the presence of HT lipophenols in the product of the GI digestion prompted us to explore the effect of the intestinal digestion considered individually on the intact food matrix, avoiding the uncertainly detrimental effect that could be occurring during the gastric stage (Table 3). Thus, when the intestinal digestion was performed on both EVOO and VOO (without the previous gastric phase), the concentration of HT lipophenols observed in the BF was higher relative to that obtained following the physiological workflow (Table 3). The content of HT-ALA, HT-LA, and HT-OA in BF of the intestinally digested EVOO was ~64-, ~8-, and ~15-fold higher, respectively, in comparison with the BF obtained after a complete GI digestion (gastric plus intestinal digestions). This trend was similar to VOO, where the content of lipophenols in the BF increased ~4-, ~3-, and ~13-fold for HT-ALA, HT-LA, and HT-OA, respectively, relative to BF obtained from the sequential gastric and intestinal digestions (Table 3).

On the contrary, the highest concentration of unesterified HT was found in the BF from intestinal digestions developed on gastric digestates of EVOO (619.88 ng/g fw) in comparison with the direct intestinal digestion of the intact food matrix (55.0% lower, $p < 0.001$) (Table 3), while regarding VOO, no statistically significant differences were found, reinforcing the

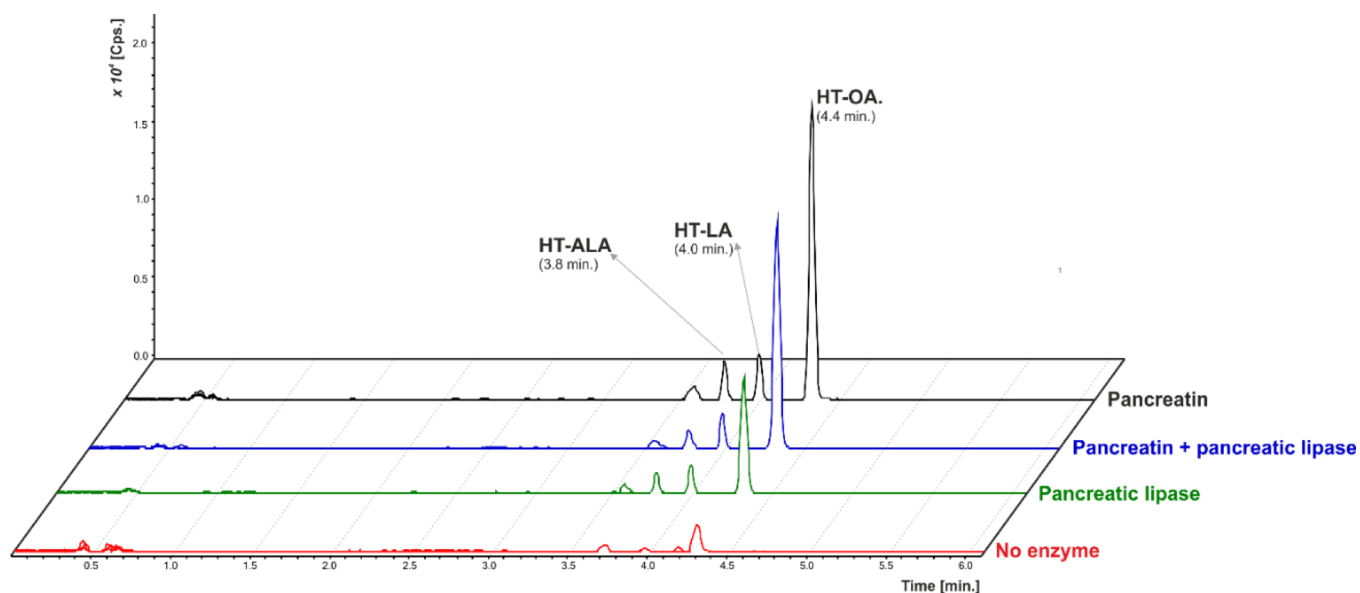


Figure 3. Representative UHPLC-ESI-QqQ-MS/MS-extracted ion chromatograms corresponding to the bioaccessible lipophenols of hydroxytyrosol (HT-ALA, hydroxytyrosol- α -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid) in digestates obtained after applying different enzyme combinations on extra virgin olive oil during simulated *in vitro* intestinal digestion using the quantification MRM transitions. CPS, charges per second.

Table 4. Concentration (ng/g fw) of Hydroxytyrosol Esterified with Fatty Acids after Intestinal Digestion of Extra Virgin Olive Oil with Different Combinations of Digestive Enzymes

analyte ^a	control (no enzyme)	pancreatin	pancreatic lipase	pancreatin + pancreatic lipase
HT-ALA	4.58 \pm 1.44 ^b	228.85 \pm 14.87a	193.94 \pm 27.88a	173.40 \pm 4.55 ^a
HT-LA	0.68 \pm 0.17 ^c	25.77 \pm 2.25a	21.33 \pm 7.50ab	14.54 \pm 0.42b
HT-OA	31.83 \pm 2.02 ^c	4853.34 \pm 69.57a	2235.37 \pm 244.55b	2876.17 \pm 315.30b

^aHT-ALA: hydroxytyrosol esterified with α -linolenic acid; HT-LA: hydroxytyrosol esterified with linoleic acid; and HT-OA: hydroxytyrosol esterified with oleic acid. ^bMean \pm standard deviation ($n = 3$) followed by the same lowercase letter are not statistically significant at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

relevance of the food matrix composition for the final bioaccessibility results. The distinct behavior in the digestive hallmark of HT and its lipophenol derivatives could be due to the sparing solubility of HT in olive oil and its consequent location in the aqueous phase (>99%), while, boosted by the amphiphilic nature, HT-FAs are distributed between both the oily and aqueous phases.⁴¹

Thereby, as expected, HT-OA was the most abundant lipophenol in the food matrix and the GI digestates (Table 3). This lipophenol should be formed through the enzymatic esterification of HT with OA, which is the most abundant FA in the oily matrices considered in the present work (EVOO and VOO) (Table 2). Based on the findings from the formation of HT-FAs under intestinal digestive conditions, the use of protectors of HT against the gastric conditions might be of interest to take advantage of the bioactivities of HT-FAs from biological or technological points of view.

Relative Influence of the Intestinal Enzymes on the Generation of Hydroxytyrosol Lipophenols. Given the relevance of the intestinal stage for the bioaccessibility of HT and its lipophenolic derivatives, the separate and joint effects of intestinal enzymes (pancreatin and pancreatic lipase) on these compounds were further investigated to gain further understanding of their role on the release of HT-FAs into the intestinal lumen and their stability. With this objective, EVOO was digested using SIF containing individual and combined intestinal enzymes at 37 °C for 120 min. The incubation of

EVOO in the SIF without any digestive enzyme was considered as a negative control. This approach allowed retrieving critical information on their relative influence on the bioaccessibility of HT-FAs.

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3.) are biocatalysts with a high selectivity and stability that catalyze hydrolysis, esterification, transesterification, and alcoholysis reactions.⁴² To date, lipases have been applied to the production of FA derivatives by the agro-food and nutraceutical industries because of their potential uses as flavoring esters, fatty acid esters of antioxidants, and structured lipids with high regio- and stereo-selectivities.⁴³ Up to now, the biosynthesis of HT-FAs has been done using commercial enzymes on pure standard compounds.³⁹ These reactions have been reported as being closely dependent on the acyl chain length and the degree of unsaturation of FAs that affect the conversion yield of the esterification process due to differences in their spatial configuration.^{44,45}

In the current work, the generation of HT esters with an equivalent carbon alkyl chain (C18) but with different degrees of unsaturation, C18:1, C18:2, and C18:3 corresponding to HT esterified with OA, LA, and ALA, respectively, was monitored, for the first time, after the intestinal digestion of EVOO with individual and combined intestinal enzymes (pancreatin and pancreatic lipase). The conversion yield of HT-FAs was more pronounced when the reaction was catalyzed only by pancreatin than when pancreatic lipase was

present (alone or in combination with pancreatin) (Figure 3). HT-OA was the lipophenol with the highest concentration in the intestinal digestate, independently of the enzymes or their combination applied (4853.34, 2235.37, and 2876.17 ng/g with pancreatin, pancreatic lipase, and combinations of the two enzymes, respectively), and showed no statistical differences between pancreatic lipase and the combination of both enzymes ($p > 0.05$) (Table 4). Regarding HT-LA and HT-ALA, the final concentrations achieved in the intestinal digestate, as well as the differences between enzymatic conditions, varied to a lesser extent relative to HT-OA (Figure 3 and Table 4). This may be due to the degree of unsaturation of the FAs involved in the esterification of HT, which may affect their conformation and thereby the reaction time, as stated by previous research.⁴⁴

The generation of HT-FAs has already been described in oily food matrices after fortifying food matrices with HT,⁵ as well as in olive oil byproducts during processing and storage, while this was not detectable in intact olives.⁴⁶ A hypothesis supporting these findings suggests that HT esters could be formed independently as a result of the esterase activity from lipase-positive yeasts, which are present in plant-based foods. However, the molecular mechanisms responsible for the *de novo* formation of lipophenols, as well as the optimal conditions needed for the successful development of such reactions, remain unclear. Likewise, recently, the formation of tyrosol esters, particularly with OA, has been described, identifying crushing and kneading processes when enzymes are activated and the reactions triggered.⁴⁷ Within this frame, the present study contributes to the understanding of the generation of lipophilic hydroxytyrosyl esters during GI digestion in oily matrices, with a discussion of some possible scenarios being necessary.

The different efficiency regarding the formation of the separate lipophenols (HT-ALA, HT-LA, and HT-OA) may be associated with the degree of unsaturation, which in turn modifies the FA spatial configuration and the lipase selectivity. Moreover, the concentration of the precursors in the food matrix (both unesterified HT and FAs) seems to be critical for the synthesis of HT-FAs during digestion as well since the content of free OA in EVOO and VOO was, on average, ~99 and ~95% in comparison with the ALA and LA concentration, respectively (Table 2). This is in agreement with recent research, which utilized nanoparticles with immobilized lipase from *Thermomyces lanuginosus* and employed olive leaf aqueous extract for the synthesis of bioactive hydroxytyrosyl FA esters. The study reported that the conversion yields (%) for OA (C18:1), LA (C18:2), and myristic acid (C14:0) were 55.0, 50.4, and 64.3%, respectively, establishing that the number of double bonds had a direct correlation with the molecular structure of the FA, which, as a result, would affect the advance of the acylation reactions.⁴⁵ On the contrary, additional studies working with the pure standard of long-chain unsaturated FAs indicated that there was no relationship between chain length and conversion, as the highest conversion was obtained with eicosapentaenoic acid (EPA) (68.0%) relative to OA (46.0%). The variation in these findings may be caused by the selectivity of lipase for the FAs, with the degree of unsaturation being a more relevant factor rather than FA chain length.³⁹ In addition, lipase performs heterogeneous reactions, and its catalytic activity is maximum when the enzyme is adsorbed at an oil–water interface. Generally, long-chain esters are mainly dispersed in the oil droplet, while short-chain esters are

found mostly in the water droplet.⁴⁸ For lipophenols, a cutoff effect that involves a too-short or a too-long hydrophobic chain does not ensure a proper interfacial location, which in turn does not result to an optimal lipase activity,⁴⁹ and the cutoff effect is simply a natural consequence of the differential solubility of antioxidants in the aqueous, interfacial, and oil regions of an emulsion.⁵⁰

Furthermore, other phenolic acyl esters, such as tyrosol-FAs, have shown a sustained-release behavior to free tyrosol molecules during the time-consuming digestion process¹⁶ that could improve the bioactivity scope of polyphenols because of extended actions. In this sense, the current work brings new knowledge and strategies for the design of slow-release formulations of HT-FAs that could enhance the phenolic loading cycle time by prolonging the terminal half-life and thereby lengthening their bioactivity. This is particularly important, as several biological properties have been recently suggested for these compounds, namely, antidiabetic, proliferative, and antioxidant capacities.^{47,51} In addition, it has been demonstrated that the esterification of phenolic compounds did not compromise the bioactivity of the native molecule but instead may enhance its bioavailability and expand its application domains.⁵²

As concluding remarks, the present study provides new evidence on the bioaccessibility of lipophenolic derivatives of HT from diverse olive oils (EVOO and VOO), showing that they are bioaccessible after performing a simulation of GI digestion under *in vitro* conditions, depending largely on the type of FA esterifying HT as well as the food matrix. More importantly, the generation of HT-FAs during intestinal digestion was reported in the present work, as well as the identification of the enzymes responsible for most of the synthesis identified (pancreatin). These findings are of critical relevance from a clinical and technological point of view because of the potential biological effects that could be expected from *de novo* synthesized lipophenols, which could have a critical impact on both the half-life of plant-based foods and the biological benefits already described on those foods and foodstuffs containing the components of lipophenols (*e.g.*, HT and FAs). Indeed, the major outcomes retrieved suggest that the use of oily matrices and their byproducts as potential functional ingredients, foods, supplements, or cosmetics, for health promotion and disease risk reduction, should take into consideration the formation of new lipophenols during digestion and, consequently, the transformation of the final bioactive scope and the effects within the frame of specific pathophysiological processes. Ultimately, further assays concerning the bioavailability of HT-FAs are still needed, with a high importance placed on validating whether lipophenol molecules would migrate across the epithelial barrier, their metabolization circulating reaction, the transporters for these compounds, and the metabolic events that affect their final shelf-life in the organism and bioactivity in the separate tissues and cell types.

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Notes

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ABBREVIATIONS

BF, bioaccessible fraction; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; EVOO, extra virgin olive oil; FAs, fatty acids; FO, flaxseed oil; HT, hydroxytyrosol; HT-ALA, hydroxytyrosol esterified with α -linolenic acid; HT-FA, hydroxytyrosol esterified with fatty acid; HT-LA, hydroxytyrosol esterified with linoleic acid; HT-OA, hydroxytyrosol esterified with oleic acid; MRM, multiple reaction monitoring; MUFAs, monounsaturated fatty acids; NMR, nuclear magnetic

resonance; PUFAs, polyunsaturated fatty acids; RF, residual fraction; SFAs, saturated fatty acids; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; VOO, virgin olive oil

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