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Targeted Lipidomics Profiling Reveals the Generation of Hydroxytyrosol-Fatty Acids in Hydroxytyrosol-Fortified Oily Matrices: New Analytical Methodology and Cytotoxicity Evaluation

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ABSTRACT: Lipophenols have been stressed as an emerging class of functional compounds. However, little is known about their diversity. Thus, this study is aimed at developing a new method for the extraction, cleanup, and ultrahigh-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UHPLC-ESI-QqQ-MS/MS)-based analysis of the lipophenols derived from hydroxytyrosol (HT): α -linolenic (HT-ALA), linoleic acid (HT-LA), and oleic acid (HT-OA). The method validated provides reliable analytical data and practical applications. It was applied to an array of oily (extra virgin olive oil, refined olive oil, flaxseed oil, grapeseed oil, and margarine) and aqueous (pineapple juice) matrices, nonfortified and fortified with HT. Also, the present work reported the formation of fatty acid esters of HT (HT-FAs) that seem to be closely dependent on the fatty acid profile of the food matrix, encouraging the further exploration of the theoretical basis for the generation of HT-FAs, as well as their contribution to the healthy attributions of plant-based foods.

KEYWORDS: *hydroxytyrosol, lipophenols, fatty acids, mass spectrometry, oily foods, cytotoxicity*

■ INTRODUCTION

Hydroxytyrosol (HT) is a naturally occurring phenolic compound with recognized health benefits. Based on cumulative evidence, upon diverse European regulations, it has been recommended the intake of 5 mg of HT per day to achieve such health benefits.¹

Alternatively, in the last few years, lipophenols (phenolic compounds esterified with fatty acids, FAs) have attracted considerable attention in the fields of food science, nutrition, and health.² From a biological point of view, lipophenols, either chemically synthesized or from natural sources, have shown several advantages compared to unesterified phenolics. In this aspect, it has been demonstrated that thanks to their fatty acid moiety, lipophenols can display improved physicochemical properties, as well as enhanced pharmacokinetic and bioavailability, since they present higher absorption at the small intestinal lumen, relative to unesterified phenolics. This allows lipophenols to possess chemical and biological features currently attributed to both fatty acids and polyphenols.^{3,4} However, such biological activities and the exact mechanisms responsible for them are still unclear. In this regard, to date, diverse studies focused on this type of compounds have reported their antioxidant, radical scavenging, anticarbonyl-stress, anti-inflammatory, and antimicrobial activities.^{2,5–8} Specifically related to the antioxidant activity of lipophenols, to date, in vitro models have demonstrated a parabolic behavior (“cutoff” effect) characterized by an initial enhancement of antioxidant efficiency in parallel to the increase of the alkyl chain length, until a critical chain length is reached, when the molecule experiences a slight drop of the

antioxidant activity.^{9,10} Nevertheless, nowadays, the effect of fatty acid chain length and the degree of unsaturation in the lipid part on the stability, bioavailability, and biological properties of lipophenols, in humans, remain poorly explored.

In addition to synthetic derivatives, natural lipophenols have been previously identified in plant-based foods.¹¹ Particularly, lipophenols of HT (hydroxytyrosol linked to α -linolenic acid (HT-ALA), hydroxytyrosol-linoleic acid (HT-LA), and hydroxytyrosol-oleic acid (HT-OA)) have been newly identified in olive oils.¹¹ This new description, jointly with the suggestion of bioactivity, has boosted the development of new analytical methods, which are essential for the complete characterization (chemical and functional) of these newly described compounds.

Previous studies have reported HT from natural sources as bioavailable for humans,¹² and its pharmacokinetics and bioavailability are dependent on the food matrix in humans.¹³ These works have also described biological activities of HT in vivo, related to anti-inflammatory and antioxidant activities, as well as to the inhibition of platelet aggregation.^{14,15} With respect to these issues, the bioavailability of HT seems to be susceptible to be enhanced, e.g., esterifying HT with fatty acids (FAs).^{4,11} Thus, the enhancement of HT lipophilicity may

Table 1. Multiple Reaction Monitoring (MRM) Parameters, Linearity, and the Limits of Detection and Quantification of the Separate Lipophenols of Hydroxytyrosol Obtained by UHPLC-QqQ-MS/MS

analyte ^a	retention time (min)	molecular weight	MRM	fragmentor (V)	collision energy (eV)	correlation coefficient (R ²)	calibration equation	LOD ^b (ng/mL)	LOQ ^c (ng/mL)
HT-ALA	3.810	414.28	413 > 277 ^d 413 > 260 ^e	90	9	0.995	$y = 2\,846\,922x + 38249$	0.166	0.373
HT-LA	4.026	416.29	415 > 279 415 > 262	90	9	0.998	$y = 1\,888\,104x + 15299$	0.166	0.375
HT-OA	4.350	418.31	417 > 281 417 > 264	90	9	0.997	$y = 841\,386x + 3937$	0.376	0.795

^aHT-ALA, hydroxytyrosol- α -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid. ^bLOD, limit of detection. ^cLOQ, limit of quantification. ^dQuantification transition. ^eConfirmation transition.

constitute an interesting strategy to augment the absorption and bioavailability of HT, providing additional value to those foods naturally containing free HT or containing HT as a part of oleuropein, thanks to the additional corresponding lipophenols like, e.g., olive oil or wine,^{16,17} and allowing the formulation of new products with valuable applications in health care.⁴ On the other hand, besides bioavailability, the determination of cytotoxicity of new natural drugs is a prerequisite for further assessing their pathophysiological significance in humans.

According to these antecedents, the present study uncovers the development and validation of a new ultrahigh-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UHPLC-ESI-QqQ-MS/MS)-based method that provides reliable analytical data and practical applications with optimized extraction of HT lipophenols (HT-ALA, HT-LA, and HT-OA) to obtain a rapid and simpler sample preparation, relative to the methodology previously described by Lee et al.¹¹ (4.350 min for our elution of all compounds vs 11.900 min, as well as a single liquid extraction for the newly developed methodology vs double liquid–liquid extraction), which does not lower the sensitivity and accuracy of the method. Also, the method developed was validated by its application on a range of oily (extra virgin olive oil, refined olive oil, flaxseed oil, grapeseed oil, and margarine) and aqueous (pineapple juice) matrices, fortified and nonfortified with HT, that allowed demonstrating the suitability and valuable contribution of the method developed for the assessment of plant-based foods on the presence and concentration of HT-fatty acids (HT-FAs).

MATERIALS AND METHODS

Chemicals and Reagents. The authentic standards of HT-ALA, HT-LA, and HT-OA (cited by elution order in Table 1) were synthesized and fully characterized (using NMR analysis) by the Institut des Biomolécules Max Mousseron (IBMM) (Montpellier, France), according to previously published procedures.¹¹ Acetone, butylated hydroxyanisole (BHA), diethyl ether, heptane, and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO), and all liquid chromatography–mass spectrometry (LC–MS)-grade solvents like H₂O, methanol, and acetonitrile were from J.T. Baker (Phillipsburg, NJ). A certified reference of FA standards from Sigma-Aldrich was used for their identification and quantification. Hydroxytyrosol with the specifications of purity 99.6%, moisture 3.7%, and pH 4.26 (1 M aqueous solution), used to fortify the food matrices, was provided by Seprox BIOTECH S.L. (Murcia, Spain). All

lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO) to obtain a stock solution (at mM concentration), and then successive dilutions were prepared in methanol/deionized Milli-Q water (50:50, v/v) to facilitate ionization in the mass spectrometer from 0.40 to 1000.00 nmol/L for calculating the range of linearity, precision, accuracy, and recovery, as well as to quantify these compounds in the food matrices analyzed. All stock solutions of lipophenols were stored at $-20\text{ }^{\circ}\text{C}$, in the dark.

The THP-1 cell line and the RPMI-1640 were purchased from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, U.K.). L-Glutamine, sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT), and phenazine methosulfate (PMS) were from Sigma-Aldrich (St. Louis, MO).

Food Matrices and Optimization of Lipophenol Extraction.

Food matrices extra virgin olive (*Olea europaea* L.) oil (Oleoestepa S.C.A., Sevilla, Spain), refined olive (*O. europaea* L.) oil (Bernal Romero, Aceites Manzano S.A., Murcia, Spain), flaxseed (*Linum usitatissimum* L.) oil (Laboratorios Almond, NaturGreen, Librilla, Spain), grapeseed (*Vitis vinifera* L.) oil (La Compagnie des Saveurs, Novers-Sur-Cher, France), margarine (Holland, Vandermoortele D. GmbH, Herford, Germany), and pineapple (*Ananas comosus*) juice (Juver Alimentacion S.L.U., Murcia, Spain) (as no oily matrix (control)) were selected because of their different concentration of HT, α -linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA). An exception was made for the extra virgin olive oil that constituted an example of natural source of HT at 5 mg/20 g; the remaining matrices were fortified by adding HT (novel food (EU) and generally recognized as safe (GRAS) food (USA)) to reach the final concentration of 5 mg/20 g for oily matrices and 5 mg/250 mL for pineapple juice, corresponding to the equivalent dose (per day) recognized as a promotor of human health according to European Food Safety Authority (EFSA).¹ The scientific opinion by EFSA in the regulation—Safety of HT as a novel food, according to Regulation (EC) No 258/97 (concerning novel foods and novel food ingredients)—showed the solubility of HT in all food matrices including pineapple juice (aqueous matrix).¹⁸ In addition, the stability of HT in pineapple juices was assessed (Table S1). All samples were analyzed within 7 days after the fortification of food matrices.

According to a previous study that presented a novel lipid extraction method using diverse solvents (acetone, chloroform/methanol, chloroform/methanol/H₂O, and dichloromethane/methanol), where acetone provided the highest extraction yield,¹⁹ in the present method, the optimization of lipophenol extraction was performed by carrying out two distinct extractions: extraction 1, single acetone extraction; and extraction 2, double acetone extraction and a deionized Milli-Q water intermediate cleanup step. The following extraction protocol was accomplished: 100 μL of each food matrix was weighed at 25 $^{\circ}\text{C}$ (an exception was made for portions of margarine that were preheated at 60 $^{\circ}\text{C}$ to melt them) and mixed with

1 mL of acetone/BHA (99.995:0.005 v/w). Samples were vortexed for 1 min, and an ultrasound-assisted extraction was performed at 40 kHz for 30 min (Branson 3510MT sonicator, Sigma-Aldrich, St. Louis, MO). Afterward, samples were centrifuged at 12 500 rpm for 5 min at 4 °C and dried at room temperature using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA). Then, the pellets were dissolved with 200 μ L of MeOH, vortexed for 1 min, and centrifuged at 12 500 rpm for 5 min at 4 °C. The supernatants were filtered through a 0.45 μ m PTFE filter (Millipore, MA) and subsequently analyzed by UHPLC-QqQ-MS/MS.

UHPLC-ESI-QqQ-MS/MS Analysis. Separation of lipophenols of HT was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), using the analytical column BEH C18 1.7 μ m (2.1 \times 50 mm) (Waters, Milford, MA.), according to the previous methodology with slight modifications.¹¹ The column temperature was 30 °C. The mobile phases consisted of 0.1% formic acid in deionized Milli-Q water (LC-MS grade) (A) and acetonitrile (B). The injection volume and flow rate were 15 μ L and 0.3 mL/min, respectively. The chromatographic separation of HT lipophenols was achieved upon the following linear-gradient (time (min), %B): (0.0, 30%); (3.0, 98%); (6.0, 100%), and (6.1, 30%). An additional post-run period of 1.5 min at 30% solvent B was considered for column equilibration. Analyses were performed by multiple reaction monitoring (MRM) in the negative mode, assigning preferential MRM transitions (quantification and confirmation) for the corresponding analytes (Table 1). The MS fragmentor parameters (ion optics: capillary exit voltage) and collision energy were optimized for each analyte (Table 1). The ionization and fragmentation conditions were as follows: gas temperature 325 °C, gas flow 8 L/min, nebulizer 30 psi, sheath gas temperature 350 °C, jetstream gas flow 12 L/min, capillary voltage 4000 V, and nozzle voltage 1000 V, according to the most abundant product ions. Data acquisition and processing were performed using Mass Hunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany).

Fatty Acid Extraction and Analysis by Gas Chromatography with a Flame Ionization Detector (GC-FID). Fatty acids were extracted according to methodology published by FAO (FAOLEX N° LEX-FAOC141241, <http://www.fao.org/faolex/results/details/es/c/LEX-FAOC141241/>) for the analysis of edible oils. Briefly, 1 g of sample was weighed and dried in a laboratory oven at 70 \pm 10 °C for 6 h. Fatty acid content was obtained by Soxhlet extraction with diethyl ether and heptane and by esterification with 1 M methanolic NaOH. Fatty acids were analyzed by gas chromatography with a flame ionization detector (GC-FID) (6890 GC Agilent Technologies, Waldbronn, Germany), using a GC column HP-88 (88%-cyanopropyl)aryl-polysiloxane (100 m \times 0.25 i.d., 0.2 μ m). The carrier gas was helium at 20 mL/min (column nominal pressure 54.8 psi), with injector at 250 °C in the splitless mode. The temperature program was set up as follows: initial temperature of 120 °C, a ramp of 6 °C/min to 230 °C, and constant temperature kept for 5 min. The equilibration time was 3 min, and 1 μ L of the extract was injected. The FID detector temperature was fixed at 280 °C with hydrogen and airflow rates at 40 and 450 mL/min in a constant makeup flow mode with helium as a makeup gas type at 45 mL/min. Fatty acids were analyzed and calculated for all fortified and nonfortified matrices in absolute (g/100 fw) by the following formula: "individual fatty acid (g/100 g fw) = individual fatty acid (% of fat)/100 \times total fat (g/100 g fw)", which is according to FAO/INFOODS guidelines for converting units, denominators, and expression, 2012.²⁰

Cell Culture and Drug Treatment. The THP-1 human monocytic cell line (ECACC General Cell Collection-88081201) was obtained from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, U.K.) (passages 17–18) and maintained in 75 cm² cell culture flasks to the logarithmic growth phase (0.6–1.4 \times 10⁶ cells/mL) in an incubator at 37 °C, with 5% CO₂ and 70% humidity. RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum was used to culture THP-1 cells. Once harvested at logarithmic growth, the cells were washed

with serum-free EMEM media, counted, and seeded in 96-well plates at 10⁴ cells per mL (100 μ L/well).

XTT-Based Viability Assay. The tetrazolium salt XTT-based cell viability assay was used to determine the cytotoxic effects of HT-OA, HT-LA, and HT-ALA on THP-1 cells. As mentioned before, THP-1 cells were seeded at 10000 cells/well into a 96-well microliter plate, in 100 μ L of a serum-free medium. After 24 h, THP-1 cells were treated with a medium containing 100.000, 50.000, 25.000, 5.000, 1.000, 0.200, 0.020, and 0.002 μ M of HT-OA, HT-LA, or HT-ALA. Negative control cells received either the medium alone or the medium + the solvent used to prepare the target compounds' dilutions. After incubation for 24 and 48 h, 1 mg/mL of the XTT labeling reagent in serum-free RPMI-1640 media without phenol red, containing 7.65 μ g of PMS, was added, and after 4 h, the formation of formazan was quantified by spectrophotometry at 450 nm (reference wavelength 690 nm), using a microtiter plate reader (Victor 3, PerkinElmer Las, Jügesheim, Germany).

Statistical Analyses. To determine the effect of the food matrix (fortified and nonfortified) on the quantification of the target compounds, one-way analysis of variance (ANOVA) and multiple range test of Tukey were carried out. Significant differences were set at $p < 0.05$. The data set was processed using the SPSS 25.0 software package (SPSS Inc., Chicago, IL).

Method Validation Parameters. The Food and Drug Administration (FDA) guidelines for the validation of chemical methods in food, feed, cosmetics, and veterinary products,²¹ and the International Conference on Harmonization (ICH)²² guidelines, were followed for the validation of the present method, to demonstrate the linearity, sensitivity, precision, accuracy, and recovery of the assay. Hydroxytyrosol is featured by a phenolic structure composed of hydroxyl groups on an aromatic ring. The application of the negative ESI mode in this method allowed detecting the low concentration of HT linked to FAs. The two most abundant fragment ions produced were considered as the product ion for multiple reaction monitoring (MRM) with quantification and confirmation purposes, to verify the identity of the target compounds detected (Table 1 and Figure S1). To optimize the resolution of the chromatographic peak definition, the fragmentor voltage and collision energy for each lipophenol were set up according to their maximum intensities (Table 1). The linearity was assessed in three replicates of eight concentrations, and the linearity range was established for each HT-FA in the range 0.19–418.31 ng/mL, using solutions of the three standards. Calibration curves were fitted by linear regression equations of the type "y = ax + b." Correlation coefficients (R^2) > 0.99 were considered acceptable for establishing the linearity of the analytical procedure. The sensitivity of the analytical method was described in terms of the limit of detection (LOD) and quantification (LOQ). The LOD is defined as the lowest concentration of analyte that can be detected but not necessarily quantified, while the LOQ is the lowest amount of analyte that could be determined quantitatively. The LOD and LOQ were established according to the signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.²² For the calculation of LOD and LOQ, the empirical method (no statistical method) was used, according to ICH and FDA, and also based on a previous report that compared empirical and statistical methods for the calculation of LOD and LOQ. In this work, the empirical method was demonstrated as providing much more realistic LOD/LOQ values than the statistical one.²³ The precision (relative standard deviation (RSD%)) and accuracy of the intra- and interday assays were evaluated in five replicates within the same day and throughout three consecutive days, respectively, for three concentrations (4.00, 62.50, and 1000.00 nmol/L) within the linearity range verified. The accuracy was determined from the percentage of the measured concentration relative to the nominal concentration. An RSD \leq 15% and accuracies ranging between 80.0 and 120.0% were considered satisfactory for the validation of the method.

The sample recovery (REC) describes the percentage of analyte recovered during the extraction and cleanup processes. However, when the analyte is spiked into samples that already contain the analyte, the "apparent recovery" was calculated as "REC (%) = (C_{spiked} - C_{unspiked}/C_{solvent}) \times 100", where C_{spiked} is the concentration obtained

for the spiked sample, C_{unspiked} is the concentration obtained for the unspiked sample, and C_{solvent} is the standard concentration in solvent (MeOH/deionized Milli-Q water (50:50, v/v)). In this respect, a critical constraint of the technique of spiking samples with a given compound already present is that recovery could be >100%. From a practical point of view, the selectivity of a method may be checked to study its capacity to measure the analyte of interest, when other specific interferences are present, thus enhancing or suppressing the sensitivity of the method. In this context, the matrix effect (ME) is considered as a measure of the percentage change in the signal caused by the matrix components and, in the present work, was calculated as “ME (%) = $(C_{\text{spiked post-extraction}}/C_{\text{solvent}}) \times 100$ ”, where $C_{\text{spiked post-extraction}}$ is the concentration obtained for the spiked sample after liquid extraction and C_{solvent} is the standard concentration in solvent (MeOH/deionized Milli-Q water (50:50, v/v)). An ME value of 100% indicates no effect, <100% indicates ionization suppression, and > 100% indicates ionization enhancement.²⁴ These parameters (REC% and ME%) were estimated in quintuplicate at three concentrations (4.00, 62.50, and 1000.00 nmol/L) according to the guidelines for the validation of chemical methods.^{21,22}

RESULTS AND DISCUSSION

Recently, organizations focused on human health promotion have discussed the benefits associated with the consumption of plant-based foods and the relative contribution of the diverse bioactive compounds, concluding that these bioactive nutrient and non-nutrient compounds would confer biological functions of different intensities.^{25,26} However, when a new type of compound emerges, the previous characterizations available need the development of appropriate and specific methods.²⁷ In this sense, the contribution of the new methodologies would allow understanding the actual diversity of the molecular forms and thus their relative input into the health-promoting capability of plant-based foods.

Based on these premises that evidence the necessity of developing a new method to describe the qualitative and quantitative presence of HT derivatives (in this case, regarding the fatty acid esters known as lipophenols), the current work describes a new method to evaluate the presence of HT-ALA, HT-LA, and HT-OA in plant-based foods. In this method, developed following the FDA guidelines,²¹ the term selectivity is referred to as the extent to which a method can determine particular analyte(s) in a mixture(s) or matrix(ces), overwhelming interferences from other components featured by similar chemical properties. In this regard, the separation of lipophenols of HT using the specific chromatography conditions detailed above was satisfactory both in solvent (MeOH/deionized Milli-Q water (50:50, v/v)) and in plant-food samples like extra virgin olive oil, displaying the following retention times: 3.810, 4.026, and 4.350 min for HT-ALA, HT-LA, and HT-OA, respectively (Figure 1). Indeed, the chromatographic separation obtained for the three target analytes evidences the appropriateness of the analytical approach.

Linearity, Sensitivity, Precision, and Accuracy of the Analytical Method. The linearity of an analytical method is referred to as its ability to provide an instrumental response proportional to the concentration of the analyte of interest in the sample. In the present method, linearity was tested in the concentration range 0.19–4.18 ng/mL, by evaluating the correlation coefficient (R^2) that was always higher than 0.995. This result indicated an adequate linearity according to the currently established rules for the validation of analytical methods (Table 1). The standard curves were assessed separately and in a pool with all lipophenol standards, with

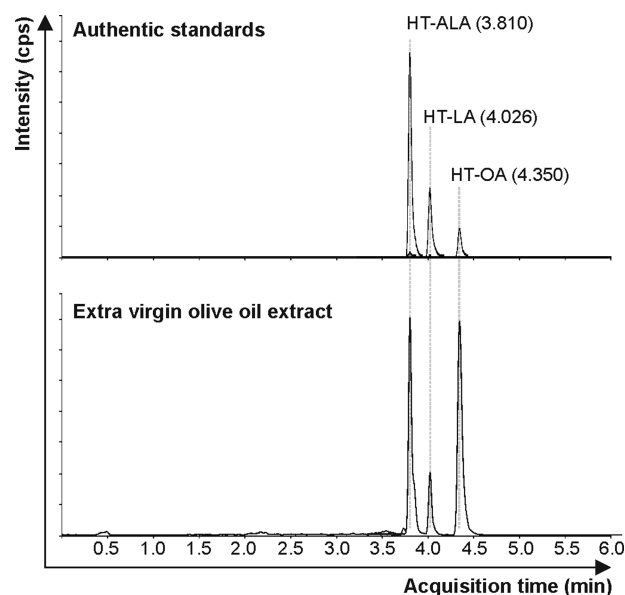


Figure 1. UHPLC-ESI-QqQ-MS/MS-extracted ion chromatograms corresponding to the authentic standards of lipophenols of hydroxytyrosol (HT-ALA, hydroxytyrosol- α -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid) and the compounds present in extra virgin olive oil using the quantification MRM transitions. CPS, charges per second.

identical satisfactory results. As shown in Table 1, the LODs were 0.166 ng/mL, on average, for HT-ALA and HT-LA and 0.376 ng/mL for HT-OA and the LOQs were 0.373, 0.375, and 0.795 ng/mL for HT-ALA, HT-LA, and HT-OA, respectively. The intraday precision (RSD%) for each compound ranged from 0.4 to 11.4%, and the interday RSD % values were found between 2.1 and 13.6% (Table 2). With respect to the accuracy of the analytical method, the intra- and interday accuracies ranged from 83.4 to 119.6% and from 80.5 to 119.9%, respectively (Table 2), thus being within the cutoff values approved by the FDA guidelines (precision values < 15% and accuracy between 80 and 120%).²¹

Sample Extraction Optimization, Recovery, and Matrix Effects. The optimization of the extraction procedure was performed based on a previous methodology for lipid extraction, which explored the use of three alternative solvents (acetone, chloroform/methanol/H₂O, and dichloromethane/methanol).¹⁹ Extraction 1 (single acetone extraction) provided the highest yields for HT-ALA, HT-LA, and HT-OA, surpassing the concentrations obtained when applying the conditions detailed for extraction 2 (double acetone extraction and deionized Milli-Q water intermediate addition step) by 92.9, 25.2, and 96.8%, respectively. The results obtained by extraction 1 allowed identifying an additional advantage, since this extraction needs a lower volume of extracting solvent and a reduced time for the extraction of lipophenols of HT. Nonetheless, it should be noticed that the intermediate addition of water could be of great relevance when analyzing additional plant-based foods including in their structure vegetal tissues organized by cellular units. In this case, the application of an intermediate water addition step would provide a hypotonic environment and the consequent osmotic shock would affect the cell membrane integrity, allowing the access of solvents to intracellular compartments and thus the lipid diffusion out of cells and its availability for chromatographic determinations.¹⁹

Table 2. Intraday and Interday Precision and Accuracy for the Determination of Lipophenols of Hydroxytyrosol

analyte ^a	nominal concentration (ng/mL)	intraday			interday		
		measured (ng/mL)	RSD (%)	accuracy (%)	measured (ng/mL)	RSD ^b (%)	accuracy (%)
HT-ALA	414.28	382.80 ± 1.68	0.4	92.4	387.03 ± 8.26	2.1	93.4
	25.89	30.41 ± 0.97	3.2	117.4	27.09 ± 3.43	12.7	104.6
	1.62	1.91 ± 0.11	5.7	118.2	1.94 ± 0.13	6.7	119.9
HT-LA	416.30	393.57 ± 2.62	0.7	94.5	374.18 ± 10.11	2.7	89.9
	26.02	31.11 ± 1.41	4.5	119.6	24.33 ± 3.30	13.6	93.5
	1.62	1.85 ± 0.09	4.6	113.8	1.58 ± 0.15	9.4	97.5
HT-OA	418.31	441.53 ± 7.15	1.6	105.6	345.78 ± 15.83	4.6	82.7
	26.14	21.81 ± 0.93	4.3	83.4	21.84 ± 0.68	3.1	83.5
	1.63	1.43 ± 0.16	11.4	87.9	1.31 ± 0.18	13.6	80.5

^aData are presented as mean ± standard deviation (SD) of five independent measurements ($n = 5$). HT-ALA: hydroxytyrosol- α -linolenic acid; HT-LA: hydroxytyrosol-linoleic acid; and HT-OA: hydroxytyrosol-oleic acid. ^bRSD, relative standard deviation.

Table 3. Recovery of Extraction and Matrix Effect of Lipophenols of Hydroxytyrosol in Extra Virgin Olive Oil

analyte ^a	nominal concentration (ng/mL)	recovery (%)	RSD (%)	matrix effect (%)	RSD ^b (%)
HT-ALA	414.28	99.4 ± 12.8	12.9	99.6 ± 13.4	13.5
	25.89	107.3 ± 13.0	12.1	108.0 ± 14.9	13.8
	1.62	96.3 ± 10.2	10.6	99.4 ± 12.2	12.3
HT-LA	416.30	102.6 ± 14.7	14.3	103.1 ± 12.3	12.0
	26.02	84.8 ± 5.2	6.1	86.4 ± 5.3	6.1
	1.62	85.8 ± 5.1	5.9	87.3 ± 7.9	9.0
HT-OA	418.31	86.7 ± 6.8	7.9	92.4 ± 7.1	7.7
	26.14	95.2 ± 9.7	10.2	104.4 ± 11.9	11.4
	1.63	86.3 ± 6.4	7.4	102.2 ± 9.0	8.8

^aData are presented as mean ± SD of five independent measurements ($n = 5$). HT-ALA, hydroxytyrosol- α -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid. All analyses were performed 5-fold. ^bRSD, relative standard deviation.

The sample recovery describes the percentage of lipophenols recovered after liquid or solid extraction.²⁴ This parameter may be influenced by several factors, including chromatographic conditions, ionization at the source, the extracting solvent, and the food matrix. In the present work, the recovery of HT lipophenols ranged between 84.8 and 107.3%. This parameter seems to be influenced by the standard concentration of lipophenols (Table 3). Typically, matrix effects are caused by coeluting additional components that can alter (reducing or increasing) the ionization of target analytes. In the current method, the matrix effect varied between 86.4 and 108.0%. These results indicate that the values recorded for recovery and matrix effect were in the appropriate range according to FDA guidelines (80.0–120.0%), thus ensuring the ruggedness of the analytical method developed. Moreover, all RSD% values were lower than 15.0%, which is good agreement with the requirement of analytical methods with quantification purposes.^{28–30}

Fatty Acid Composition. The fatty acid composition of the food matrices assessed consisted of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). During the characterization of the array of oily matrices assessed in the present work, a total of 18 FAs was detected (Table 4). Regarding the SFAs, lauric (C12:0), myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids showed significant statistical differences among samples ($p < 0.05$), highlighting margarine and HT-fortified margarine as the matrices with the highest contents of lauric (0.87 and 0.90 g/100 g fw, respectively) and myristic (0.47 and 0.46 g/100 g fw, respectively) acids, as well as refined olive and flaxseed oils that presented the greatest amount of palmitic acid (11.07 g/100 g fw, on average) and flaxseed oil that

provided the highest concentration of stearic acid (5.89 g/100 g fw). Two MUFAs (palmitoleic (C16:1 n7) and oleic (C18:1 n9) acids) were found at statistically different concentrations among the diverse fortified and nonfortified food matrices analyzed ($p < 0.05$), underlining oleic acid as the MUFAs present at the highest concentration among all food matrices considered (15.27–78.23 g/100 g fw). Extra virgin olive oil was the matrix featuring the highest value (78.23 g/100 g fw). With respect to the content of PUFAs, LA (C18:2 n6) and ALA (C18:3 n3) were found at significantly different concentrations among samples ($p < 0.05$), with grapeseed oil (nonfortified and fortified) being the matrix presenting the highest concentration of LA (74.23 g/100 g fw, on average) and flaxseed oil (nonfortified and fortified) being the matrix with the highest content of ALA (53.79 g/100 g fw, on average) (Table 4). These results concur with previous descriptions in studies comparing the fatty acid composition of various types of edible oils^{31,32} and evidenced that the fortification of oily plant-based foods with HT did not modify significantly the fatty acid profile and concentration.

Profile of Lipophenols of Hydroxytyrosol in Oily and Aqueous Matrices. The scientific opinion published by EFSA, regarding the biological properties of dietary HT and its contribution to health, especially regarding cardiovascular pathophysiology,¹ has caught the attention of the food industry on this phenolic compound that, in addition, is easily and cost-effectively recovered from olive oil extraction byproducts as “alperujo” (a combination of liquid and solid olive waste), “alpechin” (olive-mill wastewater), and “orujo” (olive oil cake).³³ Hence, in connection with the existence of appropriate natural sources and procedures to obtain HT, the development of applications for this bioactive phytochem-

Table 4. Concentration of Fatty Acids (g/100 g fw) in the Separate Food Matrices^b

matrix	fatty acid ^a																	
	C22:6 n3	C20:5 n3	C20:3	C24:1	C12:0	C14:0	C16:0	C16:1 n7	C17:0	C17:1 n8	C18:0	C18:1 n9	C18:2 n6	C18:3 n3	C20:0	C20:1	C22:0	C24:0
extra virgin olive oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	9.07b	0.53b	<0.10	<0.30	3.80b	78.23a	6.67f	0.50c	<0.10	<0.10	<0.10	<0.10
refined olive oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	11.00a	0.70a	<0.10	<0.10	3.40c	75.83c	7.80e	0.50c	<0.10	<0.10	<0.10	<0.10
fortified refined olive oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	11.13a	0.70a	<0.10	<0.10	3.37c	76.80b	7.80e	<0.10d	<0.10	<0.10	<0.10	<0.10
grapeseed oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	6.33d	<0.10c	<0.10	<0.10	3.80b	15.27f	74.27a	<0.30d	<0.10	<0.10	<0.10	<0.10
fortified grapeseed oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	6.30d	<0.10c	<0.10	<0.10	3.80b	15.33f	74.20a	<0.30d	<0.10	<0.10	<0.10	<0.10
flax oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	6.03e	<0.10c	<0.10	<0.10	5.90a	20.97e	12.83c	54.27a	<0.10	<0.10	<0.10	<0.10
fortified flax oil	<0.10	<0.10	<0.10	<0.10	<0.10b	<0.03b	5.97e	<0.10c	<0.10	<0.10	5.87a	20.77e	13.47b	53.70a	<0.10	<0.10	<0.10	<0.10
margarine	<0.06	<0.06	<0.06	<0.06	0.87a	0.47a	7.69c	<0.06c	<0.06	<0.06	1.59d	31.60d	10.00d	4.42b	<0.06	<0.06	<0.06	<0.06
fortified margarine	<0.06	<0.06	<0.06	<0.06	0.90a	0.46a	7.81c	<0.08c	<0.06	<0.06	1.61d	32.03d	10.18d	4.49b	<0.06	<0.06	<0.06	<0.06
pineapple juice	nd	nd	nd	nd	nd	nd	<0.04f	nd	nd	nd	<0.01	<0.10g	<0.03g	<0.01d	nd	nd	nd	nd
fortified pineapple juice	nd	nd	nd	nd	nd	nd	<0.04f	nd	nd	nd	<0.01	<0.10g	<0.03g	<0.01d	nd	nd	nd	nd
LSD ($p < 0.05$)	0.129	0.129	0.129	0.129	1.037	1.782	11.598	0.867	0.129	0.251	6.352	93.875	85.975	67.507	0.129	0.129	0.129	0.129

^aC22:6 n3, docosahexaenoic acid; C20:5 n3, eicosapentaenoic acid; C20:3, eicosatrienoic acid; C24:1, nervonic acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1 n7, palmitoleic acid; C17:0, heptadecanoic acid; C18:0, stearic acid; C18:1 n9, oleic acid; C18:2 n6, linoleic acid; C18:3 n3, α -linolenic acid; C20:0, arachidic acid; C20:1, eicosenoic acid; C22:0, behenic acid; C24:0, lignoceric acid. ^bMeans ($n = 3$) within a column with different letters are significantly different ($p < 0.05$ according to the ANOVA and Tukey's multiple range tests). Least significant difference, LSD.

ical is of special interest because of the tasteless feature of HT at low concentrations.³⁴ This characteristic allows the incorporation of HT in newly designed foods, thus contributing to the daily intake of the 5 mg per day recommended by EFSA.¹ In the current study, it was developed and validated, for the first time, an analytical method for the detection and quantification of HT esterified with FAs (lipophenols) in several fortified food matrices at the concentration recognized by EFSA as healthy (5 mg HT/20 g) thanks to Lee et al.,¹¹ who described, in 2016, the occurrence of HT-FAs in extra virgin olive oil. This approach allowed the assessment of fortified and nonfortified matrices and to provide their absolute concentration and their relative value as a source of the esterified forms of HT, which has never been taken into consideration previously. In this context, the method developed allowed assessing food matrices on three lipophenols of HT (HT-ALA, HT-LA, and HT-OA) (Figure 2). Regarding the nonfortified matrices, extra virgin olive oil was the food with the highest concentrations of HT-ALA, HT-LA, and HT-OA (82.97, 121.95, and 639.21 ng/g, respectively). So the concentration of HT-ALA in extra virgin olive oil surpassed significantly the content recorded in refined olive oil, the additional oily matrix being susceptible to containing this lipophenol, by 77.3%, while this lipophenol was detected in the level of the analytical technique background in the remaining matrices. With respect to HT-LA, the content in extra virgin olive oil was, on average, 19.9-fold higher than in grapeseed and refined olive oils and was not detected in flax oil, margarine, and pineapple juice. Finally, when considering HT-OA, the concentration found in extra virgin olive oil was 70.5% higher than the content observed in refined olive oil. This lipophenol was almost absent in flax oil, grapeseed oil, margarine, and pineapple juice (Figure 2). This result is consistent with the distribution of HT in the diverse plant matrices (olive drupes, olive oil, grapes, must, and wine).^{16,35} The absence of HT in flax oil, margarine, and pineapple juice does not allow the formation of HT-OA in such food matrices.

Interestingly, the fortification of the separate oily and aqueous matrices with HT gave rise to the formation of lipophenols of HT, whose relative abundance was dependent on the FA profile of the separate matrices (Figure 2). In this regard and concerning HT-ALA, when flax oil, the plant oil featured by the highest concentration of ALA, was fortified with 5 mg HT per 20 g of oil, it allowed the formation of the highest content of HT-ALA among the separate matrices under study (191.11 ng/g), followed by extra virgin and refined olive oils, which did not differ significantly between them (71.98 ng/g, on average) (Figure 2). The additional food matrices analyzed in the present work did not experience a significant increase in the HT-ALA concentration after fortification.

Related to HT-LA, extra virgin olive oil displayed the highest value also in comparison with fortified matrices that presented the following significantly decreasing concentrations: fortified refined olive oil (44.76 ng/g) > fortified grapeseed oil (24.10 ng/g) > fortified flax oil (8.41 ng/g) > fortified margarine (3.08 ng/g). Also, both fortified grapeseed oil and fortified refined olive oil displayed around 5-fold higher concentration than their respective nonfortified matrices.

Finally, the level of HT-OA in extra virgin olive oil (639.21 ng/g) again was higher than in those matrices, fortified, in which its concentration was equal to or higher than the limit of quantification of the method (flax (12.82 ng/g) and refined

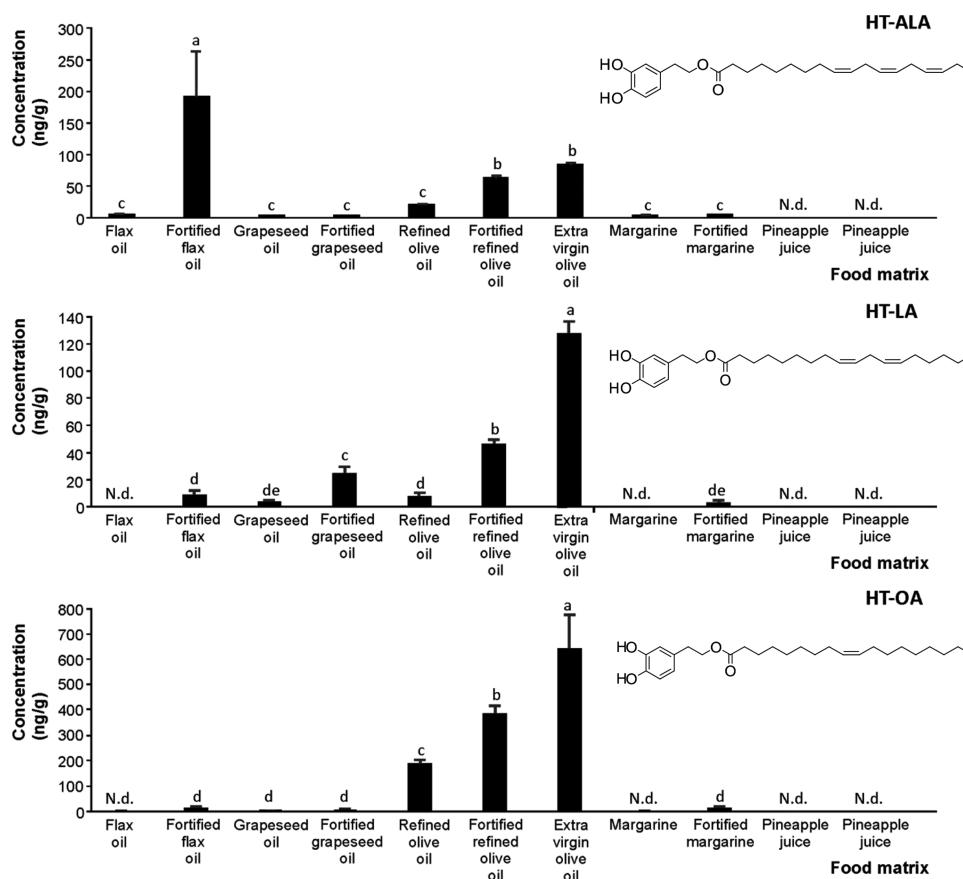


Figure 2. Structure and concentration (ng/g) of lipophenols of hydroxytyrosol HT-ALA, hydroxytyrosol- α -linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid in the array of fortified and nonfortified oily (extra virgin olive oil, refined olive oil, flaxseed oil, grapeseed oil, and margarine) and aqueous (pineapple juice) matrices evaluated. Bars with different lowercase letters are significantly different at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range tests.

olive (381.81 ng/g) oils). Besides, in these two matrices, fortification with 5 mg/20 g HT raised significantly the content of HT-OA relative to the nonfortified oils for flax oil and refined olive oil.

It is important to highlight that HT-LA and HT-OA were not detected in nonfortified matrices (flaxseed oil and margarine), while they were found in their respective fortified matrices. As expected, no lipophenols were detected in pineapple juice (fortified and nonfortified), which is in close agreement with the absence of fatty acids in this aqueous matrix.

The occurrence of HT lipophenols in plant-based foods is of high relevance because of their tentative contribution to the extra virgin olive oil profile in bioactive molecules of functional interest. Besides, those derivatives would be featured by increased lipophilicity, and thus a high cellular penetration and bioavailability across the intestinal barrier as compared to polar phenolics, while would constitute a form to reduce their biotransformation and oxidative degradation. Besides this biological advantage, the linkage of fatty acids with phenolic compounds would limit autoxidation of fatty acids, thus preserving the health benefits attributed to MUFAs and PUFAs.³ With respect to the functional advantages of lipophenols, the conjugation of phenolic compounds and FAs could be related to enhanced antioxidant activities by providing a synergy between the bioactivities of their constituents.

The synthesis of lipophenols by organic chemistry procedures is performed by chemical, enzymatic, or chemo-enzymatic procedures, most often through esterification/acylation of phenolic -OH with FAs.³ In this aspect, a practical approach to apply phenolic acids in a lipophilic formulation by lipase-catalyzed lipophilization to increase their hydrophobic affinity has been accomplished in recent years.³⁶ Enzymatic acylation of flavonoids may be performed either directly by esterification or by transesterification.³⁷ In nature, some acylated phenolic compounds have been described, and these lipophilic esters seem to exert antioxidant properties, thus protecting lipids from oxidation. On the other hand, the lipophenol derivatives would carry out the combined health beneficial properties of lipids and phenolic compounds.³ In this context, our study sheds more light on the lipophilization process, describing that it might occur both naturally and when the samples are fortified with phenolic compounds, as in the case of HT. To explain the generation of lipophenols after the addition of HT in the oily matrices, some possible scenarios need to be discussed. Lipase is an enzyme that catalyzes not only the hydrolysis of ester bonds but also their synthesis in nonaqueous media, which explains interesterification reactions during the oligomerization of triacylglycerols reported in virgin olive oils by an endogenous lipase.³⁸ Therefore, this mechanism could be responsible for the formation of lipophenols of HT in fortified food matrices, according to the findings described in the current study. The origin of lipase might be the microbial diversity of plant-based foods, mainly

composed of yeasts (lipase-positive yeasts) present in vegetable oils, affecting their final quality.³⁹ In this regard, recently, Plastina et al., 2019,⁴⁰ found HT-OA in olive oil byproducts, while this was not detectable in intact olives. A possible explanation for this fact hypothesized by the authors is that HT-OA could be formed in olive oil byproducts independently, as a result of the esterase activity coupling oleic acid and HT present in high amounts in those matrices. However, it should be noticed that nowadays the mechanism of formation of HT-OA, as well as the conditions needed for the successful development of these reactions, remains unclear.⁴⁰ On the other hand, the acidic features of edible oils could promote the transesterification reactions between phenolic alcohols and acylglycerols present in the oily matrices.⁴¹ However, the parameters of conversion like the yield of lipophenols, the effect of the extraction solvent on the recovery, the influence of carbon chain length or unsaturation degree of FAs, and the diversity and concentration of the reaction substrate should be further explored to achieve the reaction in satisfactory kinetics. Moreover, the oxidation of FAs could decrease the rate of the acylation reaction due to the formation of hydroperoxides. For this reason, all samples (fortified and nonfortified matrices) were extracted with acetone/BHA to avoid autoxidation. Particular works revealed that through introducing lipophilic molecules to the basic phenolic compound skeleton, not only physicochemical properties can be modified but also biological effects of the phenolic compound are improved.³⁷

Cytotoxicity of Lipophenols of Hydroxytyrosol. To evaluate the cytotoxic effect of HT lipophenols against the human-derived monocyte cell line THP-1, an XTT assay was carried out after 24 and 48 h in the presence of decreasing concentrations of HT-ALA, HT-LA, and HT-OA (100, 50, 25, 5, 1, 0.2, 0.02, and 0.002 μM) (Figure 3). This cell line has been widely used to investigate the function and regulation of monocytes and macrophages in the cardiovascular system. Also, the interactions between THP-1 cells and an array of vascular and immunological cells have allowed gaining an insight into the relevance of the monocyte–macrophage interconnection with other cell types during inflammation, particularly atherogenesis and obesity.⁴² Hence, although the THP-1 cell line may not express matching features relative to primary monocytes and macrophages, as well as their regulatory mechanisms when exposed to various inflammatory mediators, these cells are featured by physiological properties, already described in primary monocytes and derived macrophages in vivo.⁴³

The obtained results evidenced limited cytotoxicity of the HT-FAs evaluated. Indeed, the abovementioned range of concentrations of HT-LA did not significantly decrease viability on THP-1 cells, after neither 24 nor 48 h. Similarly, when applying HT-ALA and HT-OA to THP-1 cells, only the highest concentration caused a significant decrease of cell viability after 24 h (by 18.0 and 32.6%, respectively) and, for HT-OA, after 48 h (by 15.6%). On the other hand, interestingly, concentrations of HT-OA ranging between 1.000 and 0.002 μM significantly augmented the cell viability after 48 h (between 3.0 and 12.8%) (Figure 3).

According to these results, the limited cytotoxic effect observed concerning HT-ALA (at 24 h) and HT-OA (at 24 and 48 h) could be dependent on the nature of the lipid moiety introduced (Figure 3). On the other hand, the proliferative effect of the lowest concentration of HT-OA (1–0.002 μM) could indicate that these differential effects

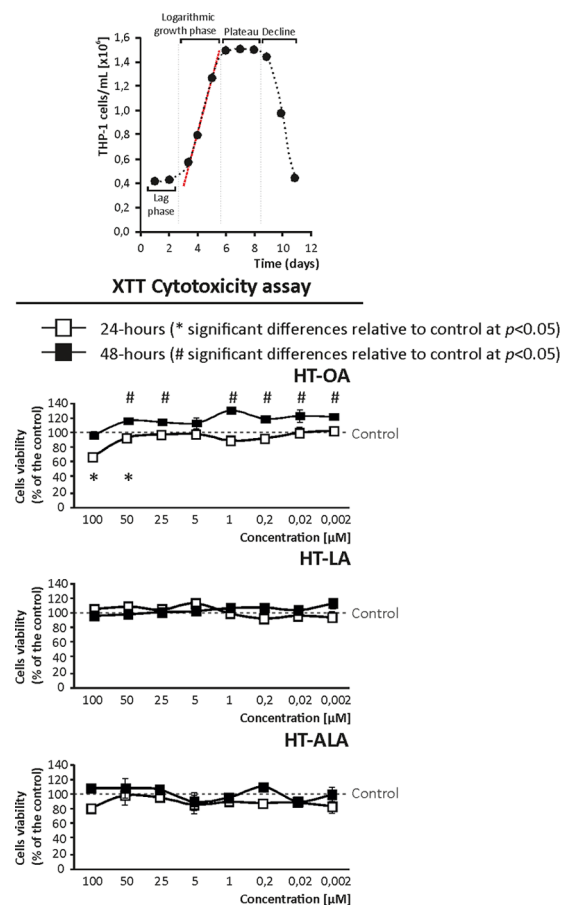


Figure 3. Cytotoxic activity of lipophenols of hydroxytyrosol (HT-OA, hydroxytyrosol-oleic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-ALA, hydroxytyrosol- α -linolenic acid). Cytotoxicity on THP-1 cells (monocyte cell line) with decreasing concentrations of lipophenols (100,000, 50,000, 25,000, 5,000, 1,000, 0.200, 0.020, and 0.002 μM) was measured by the XTT assay during the logarithmic growth phase of THP-1 cells. Cytotoxicity is expressed as mean \pm SD of the percentage of viability relative to the control at 24 and 48 h. Significant decreases of cell viability relative to control conditions after 24 h were set at $p < 0.05$ (*) and after 48 h at $p < 0.05$ (#).

could be due, first, to a result of the distinct cell penetration capacity of the HT lipophenols because of the association to the three different lipid moieties characterized in the present work, as well as a dose-dependent response of cells to these type of compounds, and especially concerning HT-OA. Moreover, the increase of cell viability could be due to the capacity of low concentrations of HT-OA (1.000–0.002 μM) to increase the cell membrane integrity⁴ and the induction of cell proliferation as a side effect of additional biochemical advantages (e.g., the reduction of oxidative stress), derived from the incorporation of these compounds, which would be in agreement with previous descriptions.⁴⁴ To date, the diversity in the cytotoxicity profile of HT-FAs has been hypothesized to be a consequence of their tridimensional conformation that may result in different stability in the cells.⁴

As concluding remarks, this work has evaluated the content of lipophenols of HT in oily matrices (nonfortified and fortified with HT) for the first time and in pineapple juice as a control matrix by a fast, accurate, and robust analytical method, detecting three HT-FAs (HT-ALA, HT-LA, and HT-OA) with a simple sample preparation. Additionally, the presence of HT-

FAs in both fortified and nonfortified oily matrices was observed. Hence, this study is the first indication that shows that the formation of HT-FAs rises when the matrices are fortified with the phenolic compound. Therefore, the results reported and discussed in the present work will encourage the need for additional research to unravel the theoretical basis for the generation of lipophenols (specifically HT-FAs), as a novel class of bioactive molecules, according to their tentative value to be used in the design and development of new nutraceutical and pharmaceutical applications or for providing added value to the current foods naturally containing free HT or as part of oleuropein. In this aspect, it is essential to investigate the parameters of the conversion reactions including the yield of lipophenols, as well as their stability, pharmacokinetics, and bioavailability, to initiate a study on their bioactivity on humans and their relative contributions to healthy features of the plethora of bioactive compounds contributing to human well-being already described in plant-based foods.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c01938>.

Analysis of the stability of hydroxytyrosol in fortified pineapple juices and the mass spectrum plot of lipophenols of hydroxytyrosol in extra virgin olive oil samples (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ALA, α -linolenic acid; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; FAs, fatty acids; GD-FID, gas chromatography with a flame ionization detector; GRAS, generally recognized as safe; HT, hydroxytyrosol; HT-ALA, hydroxytyrosol linked to α -linolenic acid; HT-LA, hydroxytyrosol linked to linoleic acid; HT-OA, hydroxytyrosol linked to oleic acid; LA, linoleic acid; LC-MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effect; MRM, multiple reaction monitoring; MUFAs, monounsaturated fatty acids; NMR, nuclear magnetic resonance; OA, oleic acid; PMS, phenazine methosulfate; PTFE, polytetrafluoroethylene; PUFAs, polyunsaturated fatty acids; REC, recovery; RSD, relative standard deviation; SFAs, saturated fatty acids; UHPLC-ESI-QqQ-MS/MS, ultrahigh-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry; XTT, sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate

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