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Development of a Sensitive and Specific Solid Phase Extraction−Gas Chromatography−Tandem Mass Spectrometry Method for the Determination of Elenolic Acid, Hydroxytyrosol, and Tyrosol in Rat Urine

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A novel gas chromatography−tandem mass spectrometry (GC-MS/MS) method was developed, using an ion trap mass spectrometer, for the simultaneous determination of olive oil bioactive components, elenolic acid, hydroxytyrosol, and tyrosol, in rat urine. Samples were analyzed by GC-MS/MS prior to and after enzymatic treatment. A solid phase extraction sample pretreatment step with greater than 80% analytical recoveries for all compounds was performed followed by a derivatization reaction prior to GC-MS/MS analysis. The calibration curves were linear for all compounds studied for a dynamic range between 1 and 500 ng. The limit of detection was in the mid picogram level for tyrosol and elenolic acid (300 pg) and in the low picogram level for hydroxytyrosol (2.5 pg). The method was applied to the analysis of rat urine samples after sustained oral intake of oleuropein or extra virgin olive oil as a diet supplement.

KEYWORDS: Olive oil; oleuropein; hydroxytyrosol; tyrosol; elenolic acid; gas chromatography; tandem mass spectrometry

INTRODUCTION

The beneficial effects of the Mediterranean diet on human health, in terms of lowering the occurrence of chronic diseases, such as cancer and cardiovascular illnesses, have been well-established. Olive oil, extracted from the fruits of *Olea europaea* L., serves as the principal source of fat in this diet. The beneficial effects of olive oil could be associated with its content in bioactive substances, primarily polyphenols, which have been proven to possess antioxidant (*1*−*3*) antiinflammatory (*4*, *5*), antiatherogenic (*6*−*8*), antibacterial (*9*−*11*), and anticancer (*12*, *13*) properties. Moreover, these compounds possess antiviral activity and have been proven potent against HIV-1 infection and replication (*14*, *15*), while recent reports relate them with protective effects against diseases affecting the elderly, such as the Alzheimer's disease (*16*) and osteoporosis (*17*, *18*).

The major bioactive component of *O. europaea* has been shown to be oleuropein (OE), which is a glycosylated polyphenol. When OE is hydrolyzed (**Figure 1**), enzymatically or chemically, it produces structural subunits, i.e., OE aglycon, hydroxytyrosol (HT), and elenolic acid (EA). Another mild antioxidant phenol, called tyrosol (T), is also present in high amounts in olive oil (**Figure 1**). In vivo studies have shown that the olive oil phenolics are dose dependently absorbed in the body (*33*) and are excreted in urine (*19*) in either free form or conjugated as glucuronides, sulfates, methylated ethers, or others (*35*). Therefore, the determination as well as the bioavailability and metabolism (*19*, *20*) of the aforementioned substances are of vital importance for assessing their role on human health (*21*). In recent years, there has been a plethora of analytical methods developed for the separation and quantification of bioactive substances from *O. europaea* (*22*−*24*) and for in vitro or in vivo (*25*, *26*) studies thereof. The biological fate of olive oil-derived bioactive compounds has been studied using various methods (*27*−*36*) after the consumption of extra virgin olive oil (EVOO) (*19*, *31*, *32*, *34*, *36*), enriched EVOO (*33*), and single doses of pure HT (*20*, *30*) or OE (*29*, *36*, *37*). However, to our knowledge, in vivo studies incorporating sustained moderate daily doses of OE or EVOO for a long period of time, in accordance to Mediterranean dietary habits, have not been described previously.

The aim of the present study was to develop an analytical methodology based on gas chromatography coupled to tandem mass spectrometric detection (GC-MS/MS) for the specific and

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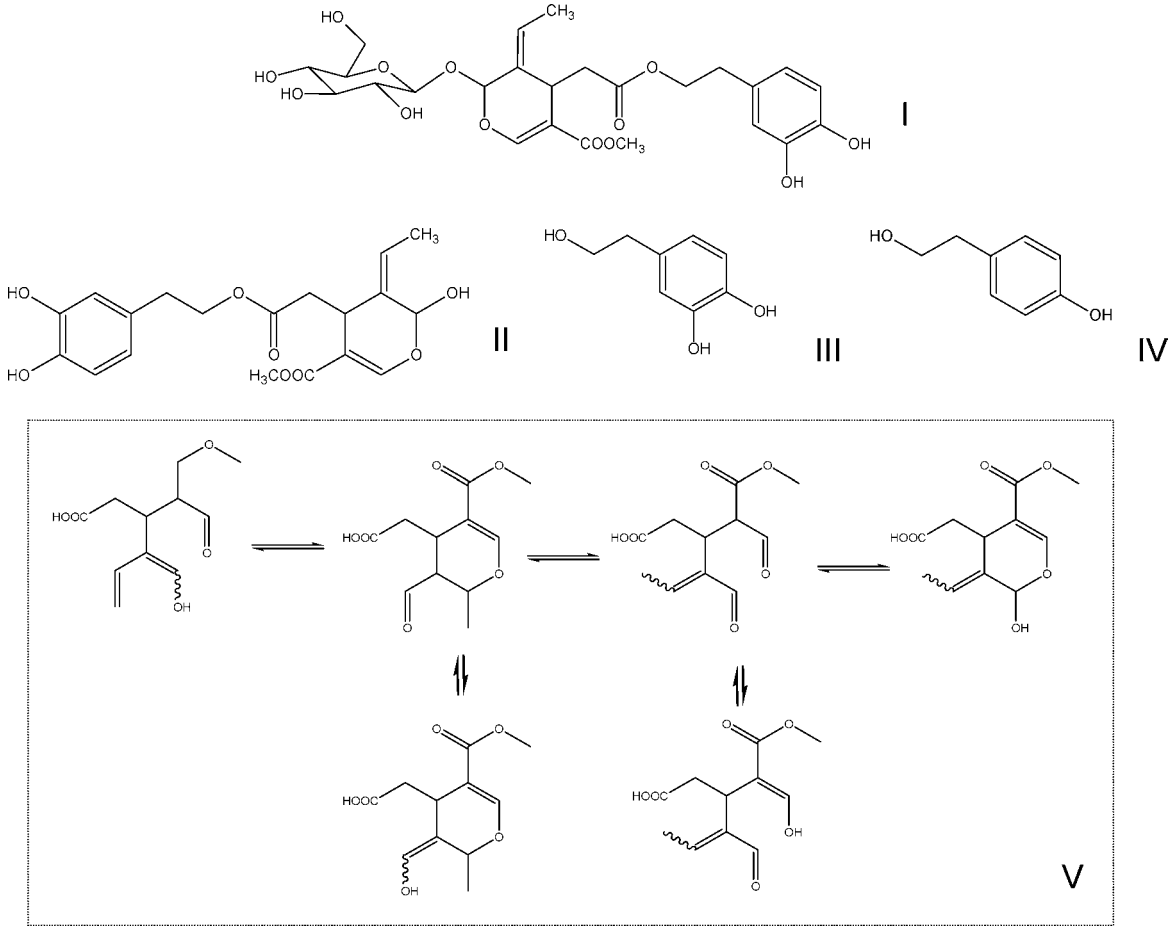

Figure 1. Molecular structures of OE (I) and its metabolites: OE's aglycon (II), HT (III), T (IV), and EA (V) (including its isomeric forms).

sensitive determination of the olive oil bioactive constituents, T, HT, and EA. To the best of our knowledge, this is the first time that a validated method for the qualitative and quantitative determination of EA, simultaneously with HT and T, is reported and has been applied to urine samples. Furthermore, this is the first GC-MS/MS method reported for the determination of OE bioactive metabolites, in contrast to several GC-selected ion monitoring (SIM)-MS methodologies developed so far. Another goal of the present work was to study whether sustained doses of OE or EVOO, in contrast to the single dose protocols that have been incorporated in the past, result in elevated levels of the aforementioned bioactive metabolites in the body, which could be related with beneficial effects on health.

MATERIALS AND METHODS

Reagents and Chemicals. 3-Hydroxyphenylethanol (3-HPE), used as an internal standard (IS), 2-hydroxyphenylethanol (2-HPE), T (4-HPE), and β-glucuronidase were purchased from Sigma-Aldrich (GmbH, Steinheim, Germany). Chemically pure HT (3,4-dihydroxyphenylethanol) was isolated from fresh olive fruits originating from *O. europaea* (var. Koroneiki), according to a previously described procedure (*28*). OE and EA were isolated from olive tree (*O. europaea* var. Koroneiki) leaves (*38*, *39*). Methanol, acetonitrile, and acetone were of analytical grade purity and were purchased from Merck (Darmstadt, Germany), whereas cyclohexane was provided by Lab-Scan (Dublin, Ireland). Ammonium acetate and acetic acid were obtained from Panreac (Barcelona, Spain). *N*-Methyl-*N*-trimethylsilyl-trifluoacetamide (MSTFA) was supplied by Macherey-Nagel (Düren, Germany), and trimethylsilyl-iodide (TMSI) was provided by Fluka (Buchs, Germany).

Solutions. Stock standard solutions of the analytes and the IS were prepared in methanol at a concentration level of 1 mg/mL. Working standard solutions of the analytes (T, HT, and EA) at concentration levels of 0.05, 0.5, and 10 μg/mL and of the IS at a concentration level of 10 μg/mL were prepared by dilution of the aforementioned stock solutions in methanol for the GC-MS/MS analysis. Accordingly, standard solutions of the analytes and 2-HPE as IS were prepared by dilution of the stock solutions in water for the high-performance liquid chromatography diode array detection (HPLC-DAD) analysis. Methanolic solutions of the analytes were stable for at least a 4 week period, when kept in dark-colored vials at -20 °C.

IS. 3-HPE was selected as the IS for the GC-MS/MS analysis, since it possesses structural similarity with T and HT. It is, therefore, expected to exhibit analogous solid phase extraction (SPE) recovery, chromatographic behavior, and fragmentation pattern with the aforementioned compounds. Similarly, 2-HPE was selected as the IS for the HPLC-DAD analysis.

Bioactive Compounds in EVOO. Briefly, T, HT, EA, and OE were extracted from an aliquot of 50 mL of EVOO (Puget, France) by shaking with 50 mL of cyclohexane and 50 mL of methanol. The two layers were separated, and the methanolic fraction was further extracted with cyclohexane (2×50 mL). The methanolic fraction was evaporated under vacuum, and the yellow oily residue obtained weighed 4.8 mg. T, HT, EA, and OE were quantified in EVOO by HPLC-DAD using a previously described method (*40*) with minor modifications. For the quantification of the analytes, calibration curves were constructed by the IS method (range 150−1500 ng). A 100 μg amount of the extracted residue was diluted in 1 mL of the initial mobile phase composition (AcN:0.05 M aqueous ammonium acetate, pH 5.0, 10:90), and 100 μL of sample was injected in the chromatograph and analyzed.

Animal Treatment. The animal treatment was conducted in accordance with the current legislation on animal experiments in France (*17*). Urine samples were provided by female Wistar rats given a control diet supplemented with 0.15 g OE/kg (OE rats) or with 50 g of EVOO/

kg (Puget, France) (OL rats) for 80 days. Blank urine samples were collected from rats given a diet free of EVOO or OE.

In Vivo Study. The basal levels of the metabolites under study found in the blank rat urine samples were taken into account for assessing the levels of these substances in urine samples of rats nourished with OE- or EVOO-enriched food (see Discussion).

Urine Sample Pretreatment. *β-Glucuronidase Hydrolysis Protocol.* To assess the amount of the conjugated forms of the phenolic compounds, urine samples were subjected to enzymatic hydrolysis with β-glucuronidase. A 50 μL amount of rat urine was added in 100 μL of 0.1 M acetate buffer (pH 5, adjusted with acetic acid). Subsequently, 5 μL of β-glucuronidase was added and the samples were incubated for 90 min at 37 °C. The reaction was quenched with 10 μL of TFA and deep freezing. Samples were then thawed at room temperature, 100 ng of IS was added, and they were subjected to SPE and derivatization according to the following described procedure. The conditions for the hydrolysis reaction were optimized in terms of time, pH, and enzymic units employed. Blank enzyme (addition of 50 μL of water in the buffer instead of urine) and blank urine (addition of 50 μL of urine obtained from untreated rats) control samples were subjected to the same hydrolytic procedure, to preclude possible interferences, as well as to establish basal background levels in the latter case. Analysis of urine samples without employing the enzymatic hydrolysis step provided the nonconjugated (free) fraction of phenolic compounds.

SPE. Purification of urine samples was performed using Oasis HLB Cartridges (60 mg, 3 cm^3) obtained from Waters (Milford, MA). Each cartridge was conditioned with 1 mL of MeOH and equilibrated with 1 mL of 2% aqueous AcOH. A 200 μL aliquot of urine sample was diluted in 800 μL of H_2O and acidified with 10 μL of TFA. Accordingly, the sample was centrifuged for 2 min at 10000 rpm and the supernatant was carefully loaded onto the cartridge along with 100 ng of the IS. A washing step with 1 mL of 2% (v/v) aqueous AcOH followed, and the SPE cartridge was dried under vacuum for 5 min. The analytes were eluted with 1 mL of acetone, and the eluate was evaporated to dryness under a gentle nitrogen stream at 25 °C. The residue was subjected to the derivatization procedure described below.

Derivatization Reaction. The samples were derivatized with 35 μL of MSTFA/TMSI 1000/2 (v/v) for 5 min at 60 °C. The reagent selectively reacts and derivatizes hydroxyl, phenolic, and carbonyl groups to their corresponding trimethyl-silyl ether derivatives. The mixture was then evaporated to dryness under a gentle nitrogen stream at 25 °C and was reconstituted with 10 μL of MSTFA/TMSI 1000/2 v/v. The injected sample volume for the GC-MS/MS analysis was 2 μL. This reconstitution step was added, to maximize the sensitivity of the method by injecting 3.5 more analyte than by injecting the original reaction mixture but also to avoid reproducibility problems from the partial evaporation of the derivatizing solution. It should be noted that the commonly used derivatization mixture MSTFA:TMSI:DTE (dithioerythritol) was tested and rejected because DTE interfered with HT during the chromatographic procedure.

Instrumentation. *GC-MS/MS Analysis.* GC-MS/MS analyses were performed on an ion trap mass analyzer (Finnigan GCQ Plus) coupled to a Trace GC 2000 (Thermo Finnigan, San Jose, CA) gas chromatograph. Separation of the substances was carried out on an Alltech AT5 (0.25 mm × 0.25 μm × 30 m) capillary column. An uncoated, deactivated silica capillary (0.25 mm × 2 m) was used as a precolumn. Helium was used as the carrier gas at a flow rate of 1 mL/min. The conditions for the chromatographic analysis were as follows. The initial temperature of 55 °C was maintained for 1 min, and then, it was raised to 170 °C at a rate of 10 °C/min, where it was maintained for 2 min. Then, the temperature was increased to 190 °C at a rate of 10 °C/min, where it was kept constant for 2 min. It was then raised to 225 °C at 10 °C/min, and finally to 325 °C at 25 °C/min, and was held there for 3 min. Samples were injected in the splitless mode using insert liners of silanized glass. The compounds were ionized by electron impact in the positive ion mode, using an electron energy of 70 eV. The source and injector temperatures were set to 250 °C, whereas the transfer line temperature was set at 300 °C. For the quantification of the compounds, an MS/MS approach was employed. The precursor ions, the applied excitation voltage, the scan range of the product ions, and the product ions selected for the quantification of each substance are listed in **Table 1**. The quantification of the molecules under study was carried out by extracting selected ions (extracted ion chromatograms, XIC) from the respective full scan MS/MS chromatograms. The Xcalibur 1.2 software was used for acquiring and handling the data.

Table 1. *m*/*z* Values of the Selected Precursor Ions of the Metabolites under Study and the IS Employed for the MS/MS Analysis, along with the Excitation Voltage Values Used for Their Subsequent Fragmentation[a]

	precursor ion	excitation voltage (V)	product ion range	product ions employed for quantification
IS	267	1.2	100–270	193, 233, 249
T	282	1.2	100–285	179, 193, 267
HT	267	1.0	100–270	149, 179
EA	305	1.0	100–310	273, 290

[a] The product ion scan range and the *m*/*z* values of the product ions employed for the quantification of the compounds are also shown.

HPLC-DAD Analysis. HPLC analyses were performed on a liquid chromatograph equipped with a Spectra system P4000 quaternary pump (Finnigan, Riviera Beach, FL), a 7725i injector (Rheodyne, Rohnert Park, CA) with a 100 μL loop, a Finnigan on-line degasser, and a Finnigan Spectra system UV6000LP diode array detector. Chromquest v.2.5.1 software controlled the equipment through the Finnigan SN4000 controller and carried out the data processing. The separation was performed on a C_8 reversed-phase column (250 × 4.6 mm, 5 μm particle size, Thermo) equipped with a C_8 precolumn. A gradient elution program using two solvents was employed for the separation of T, HT, EA, OE, and IS. Solvent A was 0.05 M ammonium acetate buffer (adjusted to pH 5.0 with glacial acetic acid), and solvent B was acetonitrile. The maxima of UV absorbance were determined to be 240 nm for OE and EA and 280 nm for T, HT, and 2-HPE. The time window for peak identification was set to 10% of t_R.

RESULTS

T, HT, EA, and OE in EVOO. The chromatographic separation of the four bioactive compounds extracted from EVOO by liquid−liquid extraction is shown in **Figure 2**. HT, T, and 2-HPE were detected at 280 nm (**Figure 2A**,**C**), while OE and EA were detected at 240 nm (**Figure 2B**,**D**). The quantity of T, HT, EA, and OE contained in 10 μg of the oily residue of the EVOO methanolic extract was found to be 314.9, 375.3, 1861.7, and 89.9 ng, respectively, as analyzed by HPLC-DAD. A 4.8 mg amount of oily residue resulted after the extraction of 50 mL of EVVO, and the mean amount of EVOO added per meal of an OL rat was 16.3 mL. On the other hand, the mean corresponding amount of OE consumed by the OE rats per meal was 45 mg.

GC-MS/MS Analysis. The total ion chromatograms (TICs), as well as the XICs, of a standard solution containing HT, T, EA, and IS and a blank rat urine sample spiked with the aforementioned compounds are depicted in **Figure 3**. Each XIC was obtained by selecting the product ions shown in **Table 1**. The chromatograms exhibited baseline resolution for all compounds, and the retention times for IS, T, HT, and EA were 13.30, 13.71, 16.85, and 22.15 min, respectively. Peaks eluting at 22.15 and 22.38 shown in **Figure 3B** both correspond to EA, since this compound possesses multiple isomeric forms (opened and closed ring) as shown in **Figure 1**. It should be noted that OE was not determined due to mass limitations imposed by the ion trap analyzer (M_r of the derivatized OE is greater than 1000 Da).

Mass Spectra. The parameters selected for the MS/MS experiments are shown in **Table 1**. The mass spectra of all

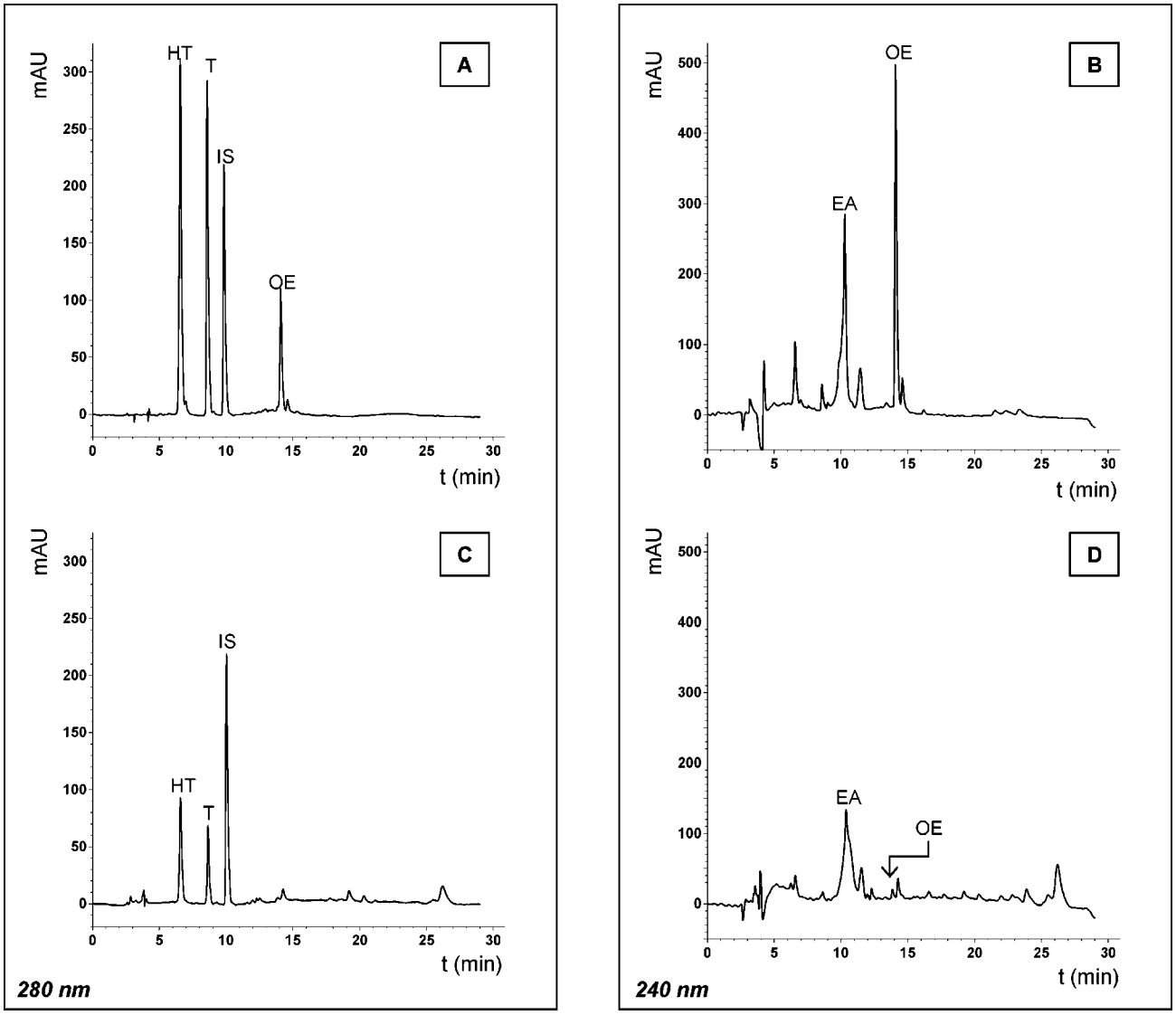

Figure 2. HPLC-DAD chromatographic separation of the four bioactive compounds HT, T, EA, and OE extracted from EVOO by liquid–liquid extraction at 280 (**A**,**C**) and 240 (**B**,**D**) nm. Panels **A** and **B** correspond to standard solution sample, whereas panels **B** and **D** correspond to the EVOO-extracted sample. IS (2-HPE) corresponds to the internal standard.

compounds under study (**Figure 4A**) include an ion at *m*/*z* 73, which is characteristic for the trimethylsilyl group, [$Si(CH_3)_3$] and peaks corresponding to the loss of CH_3- (15 Da), $Si(CH_3)_3O-$ (89 Da), $Si(CH_3)_3OCH_2-$ (103 Da), and $Si(CH_3)_3$-OH (90 Da) groups. The molecular ion or a characteristic major fragment ion from the mass spectrum of each compound was chosen as the precursor ion. Application of a suitable excitation voltage to the selected precursor ions resulted in the product ion spectra shown in **Figure 4B**. The MS/MS spectra (**Figure 4B**) reflect the efficiency of the ion trap to select ions specific for the analytes, thus increasing the signal-to-noise ratio and the specificity and sensitivity of the overall methodology, which is required for the analysis of complex biological samples.

Method Validation. The compounds under study were quantified comparing the peak area ratios of each analyte vs that of the IS ([analyte]/[IS]) with the corresponding ratios of the standards. The correlation coefficients R^2 of the calibration curves for each compound indicate good linearity, as shown in the equations included in **Table 2**, and were better than 0.99 for all compounds. The intraday precisions for T, HT, and EA (two different levels assayed, with five replicates per level) were 7.48, 5.82, and 1.71%, respectively, for the 10 ng level and 10.44, 10.89, and 4.37% for the 100 ng level, accordingly (**Table 3**). The interday precisions for T, HT, and EA (two different levels assayed, with five replicates per level at three different days) were found to be 13.26, 11.24, and 14.27%, respectively, for the 10 ng level and 5.91, 9.84, and 12.91% for the 100 ng level. These %relative standard deviation (RSD) values evaluated together along with the intraday precision results (**Table 3**) indicate that the proposed methodology is reproducible and suitable for bioavailability studies. The accuracy (%Er) of the method for T, HT, and EA was 7.12, 13.32, and 8.89, respectively, at the 40 ng level, whereas at the 400 ng level it was 12.37, 11.36, and 7.45 for T, HT, and EA, respectively (**Table 3**). The instrumental limit of detection (LOD) was determined to be 300, 2.5, and 300 pg for T, HT, and EA, respectively. By employing the IUPAC definition for assessing the limit of quantification (LOQ), it was found to be 1 ng for T and EA and as low as 8.5 pg for HT (**Table 2**).

SPE Recovery from Rat Urine. Recovery was calculated relative to the blank rat urine samples, which contain basal levels of the metabolites under study (see Discussion). The basal levels of the metabolites found in the blank samples were considered, and the recovery was thus determined by dividing the peak area

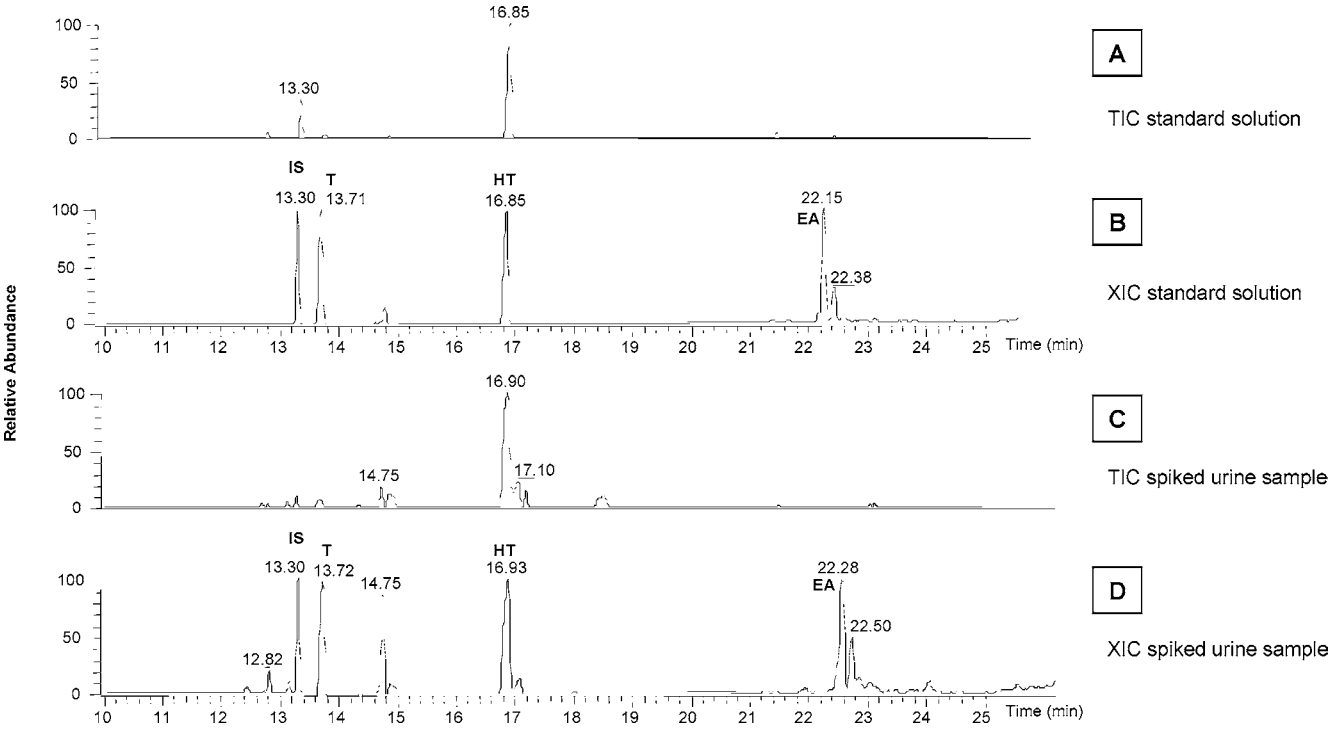

Figure 3. TIC and XIC of the compounds under study and the IS for a standard solution sample (**A**,**B**) and a spiked rat urine sample (**C**,**D**) after the GC-MS/MS analysis. The retention times (R_t) of IS, T, HT, and EA are 13.30, 13.71, 16.85, and 22.15 min, respectively.

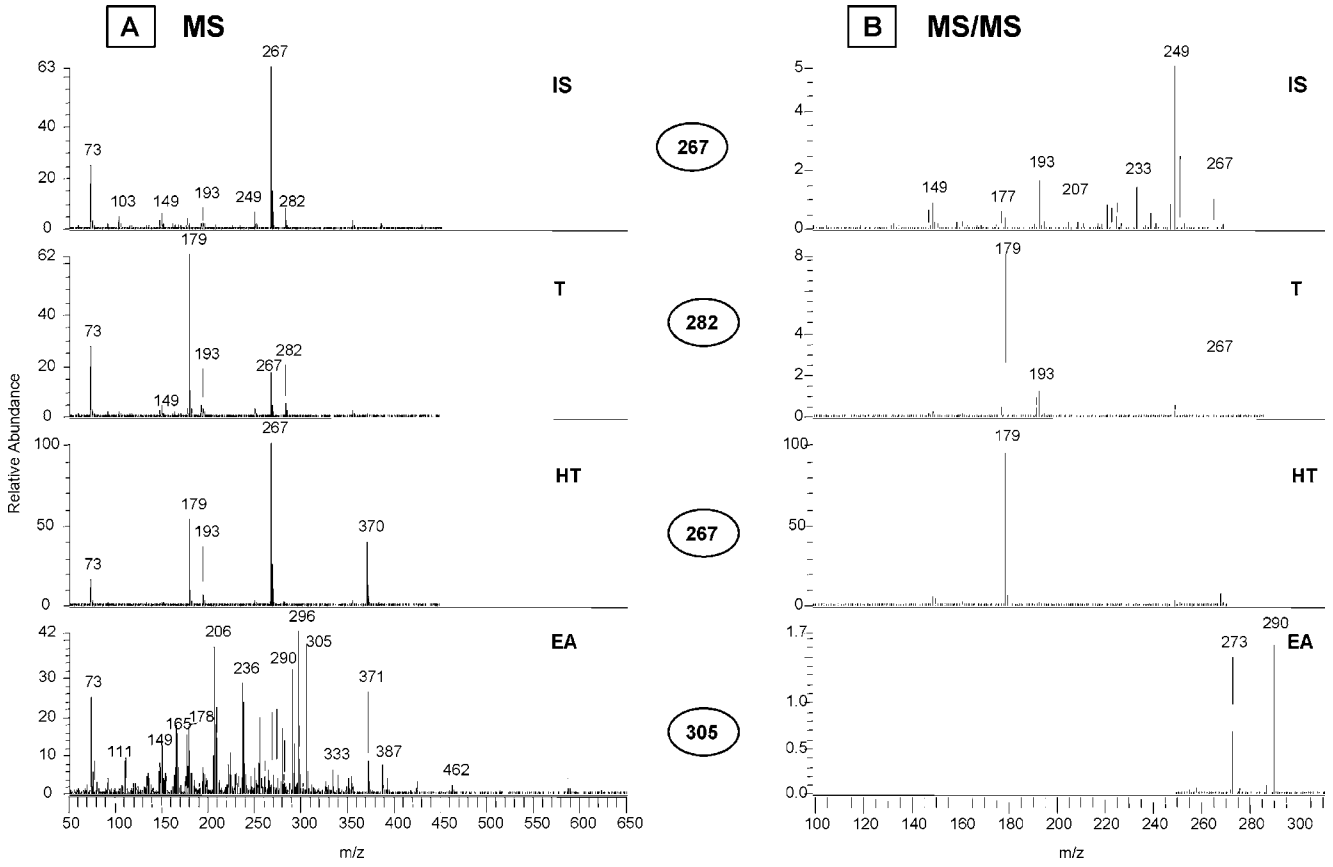

Figure 4. Mass spectra of IS, T, HT, and EA (**A**) and the respective full scan product ion (MS/MS) mass spectra (**B**) of the aforementioned compounds. The *m*/*z* values of the selected precursor ions for the MS/MS analysis are shown in circles (267, 282, 267, and 305 for IS, T, HT, and EA, respectively).

ratio of T, HT, and EA vs IS found in a blank urine sample spiked before SPE against that of a blank urine sample from the same batch spiked after SPE. This value was expressed as % percentage. The mean SPE recovery (%R, ± RSD, %) was found to be 76.2 (±7.5), 100.6 (±5.8), and 83.5 (±1.7)%, respectively, at the 10 ng level and 86.0 (±10.4), 82.8 (±10.9),

Table 2. Calibration Data Resulting from Linear Least Squares Regression Analysis for the Determination of T, HT, and EA by the Developed GC-MS/MS Methodology

	T[a]	HT[a]	EA[a]
linear fit	$y = 0.0048x + 0.0707$	$y = 0.0572x + 0.8304$	$y = 0.00811x + 0.00146$
slope (±SD[b])	0.0048 ± 0.0002	0.0572 ± 0.0026	0.00811 ± 0.00001
intercept (±SD[b])	0.0707 ± 0.0478	0.8304 ± 0.5512	0.00146 ± 0.00232
SEE[c]	0.099	1.144	0.005
correlation coefficient (R^2)	0.991	0.992	0.9993
linear range (ng)	1–500	1–500	1–500
LOD[d]	0.3 ng	2.5 pg	0.3 ng
LOQ[d]	1 ng	8.5 pg	1 ng

[a] The quantification of the compounds and the construction of the calibration curves were performed as a peak area ratio vs IS. [b] SD, standard deviation. [c] SEE, standard error of the estimate. [d] These values refer to the instrumental LOD and LOQ limits estimated in terms of the baseline noise.

Table 3. Precision (Intraday and Interday) and Accuracy Data, Expressed as Relative Standard Deviation (%RSD) and Relative Error (%Er), Respectively, and SPE Recovery (%R) for T, HT, and EA after Extraction from Spiked Rat Urine

	level (ng)	T/3-HPE	HT/3-HPE	EA/3-HPE
$\%RSD_{intraday}$	10	7.48	5.82	1.71
($n = 5$)	100	10.44	10.89	4.37
$\%RSD_{interday}$	10	13.26	11.24	14.27
($n = 15$, 3 days)	100	5.91	9.84	12.91
% Er	40	7.12	13.32	8.89
	400	12.37	11.36	7.45
%R (±%RSD)	10	76.2 (±7.5)	100.6 (±5.8)	83.5 (± 1.7)
($n = 5$)	100	86.0 (±10.4)	82.8 (±10.9)	71.6 (±4.4)

Table 4. Amounts (ng/mL of Rat Urine) of T and HT Detected Before (free) and After (Free and Conjugated) Hydrolysis with β-Glucuronidase in Urine Taken from Untreated, EVOO-Fed (OL Rat) and OE-Fed Rats (OE Rat)[a]

	untreated rats		EVOO-fed rats (OL rats)		OE-fed rats (OE rats)	
ng/mL of urine	T	HT	T	HT	T	HT
free	247.3	139.5	321.9	253.2	183.6	154.4
total	1630.1	330.1	1855.6	404.3	814.5	1036.7

[a] The difference between the free and the total amount of analytes corresponds to the amount of analytes found in their conjugated forms.

and 71.6 (±4.4)%, respectively, at the 100 ng level (**Table 3**). The mean SPE recovery of the IS at the 100 ng level ($n = 5$) was determined to be 106.6 (±1.8)%.

Application of the Method. The developed GC-MS/MS analytical methodology was applied for the quantification of the bioactive compounds under study, namely, T, HT, and EA, in urine samples obtained from rats treated with OE or EVOO, as described in the Animal Treatment section. The urinary levels of free, i.e., unconjugated metabolites (prior to enzymatic hydrolysis with β-glucuronidase), in a OL rat urine sample were determined to be 321.9 and 253.2 ng/mL for T and HT, respectively, and 183.6 and 154.4 ng/mL, respectively, in an OE rat urine sample (**Table 4**). In both cases, EA was not detected. When the urine samples were treated with β-glucuronidase (**Table 4**), the total amounts of the T and HT metabolites (free and conjugated) measured for the OL rat were 1855.6 and 404.3 ng/mL, respectively (**Figure 5B**), while the respective amounts for the OE rat were 814.5 and 1036.7 ng/mL, respectively (**Figure 5C**). EA was not detected after the enzymatic hydrolysis reaction for neither OL nor OE rat samples. These values of the total amount of compounds include the free forms of the metabolites (unconjugated), the glucuronidic form of the analytes, as well as other conjugated forms, like sulfate derivatives, because β-glucuronidase exhibits limited sulfatase activity. Thus, the respective amounts of T and HT metabolites found to be conjugated were 1533.7 and 151.1 ng/mL in the OL rat urine and 630.9 and 882.3 ng/mL in OE rat urine samples. It should be noted that for evaluating the amounts of free and total analytes (T and HT), the basal levels of these metabolites found in blank (untreated) rat urine samples should be taken into account (for more details, see the Discussion).

DISCUSSION

A novel GC-MS/MS-based analytical methodology has been developed for the simultaneous quantification of olive oil bioactive components, i.e., the antioxidants HT and T, and EA, which is a bioactive compound with antibacterial potency. To date, this is the first quantitative method for determining EA in biological samples. The developed GC-MS/MS methodology is more selective and sensitive over the respective GC-SIM methods reported previously (*33*, *34*), both for the quantification of the OE-derived metabolites and also for exploring their bioavailability and metabolism (*41*). The employment of the MS/MS analysis requires the selection of a specific precursor ion for each compound at its characteristic chromatographic retention time, thus ensuring a two-level specificity of the method. Furthermore, selection of specific product ions generated by the fragmentation of each precursor ion introduced a third level of specificity. The selectivity is attributed to the fact that the chromatographic analysis method allowed for baseline resolution of each compound and the absence of interfering substances from the matrix, which was confirmed by MS/MS spectra, at the characteristic retention times. Moreover, the use of full-scan product ion spectra enables the identification of the compounds by comparing the fragments of the substances with a custom-made tandem MS library constructed in our laboratory. Thus, the method could differentiate between structurally analogous substances, which is a common problem in the case of the metabolism of small molecules. Overall, the developed methodology includes an SPE cleanup, which is simple and exhibits sufficient extraction recovery for all of the analytes under study and a specific derivatization step prior to GC-MS/MS analysis.

Another novelty of the present study is the establishment of stable and moderate elevated levels of OE's metabolites in the body attributed to a sustained dose administration protocol applied to the nourishment of the rats, in contrast to the single dose protocols employed in the majority of the bioavailability studies previously reported. This simulates the Mediterranean diet habits in terms of nutritive intake of the bioactive compounds, absorption, and disposition thereof. An elevated single dose (attack dose) may result in different pharmaco-

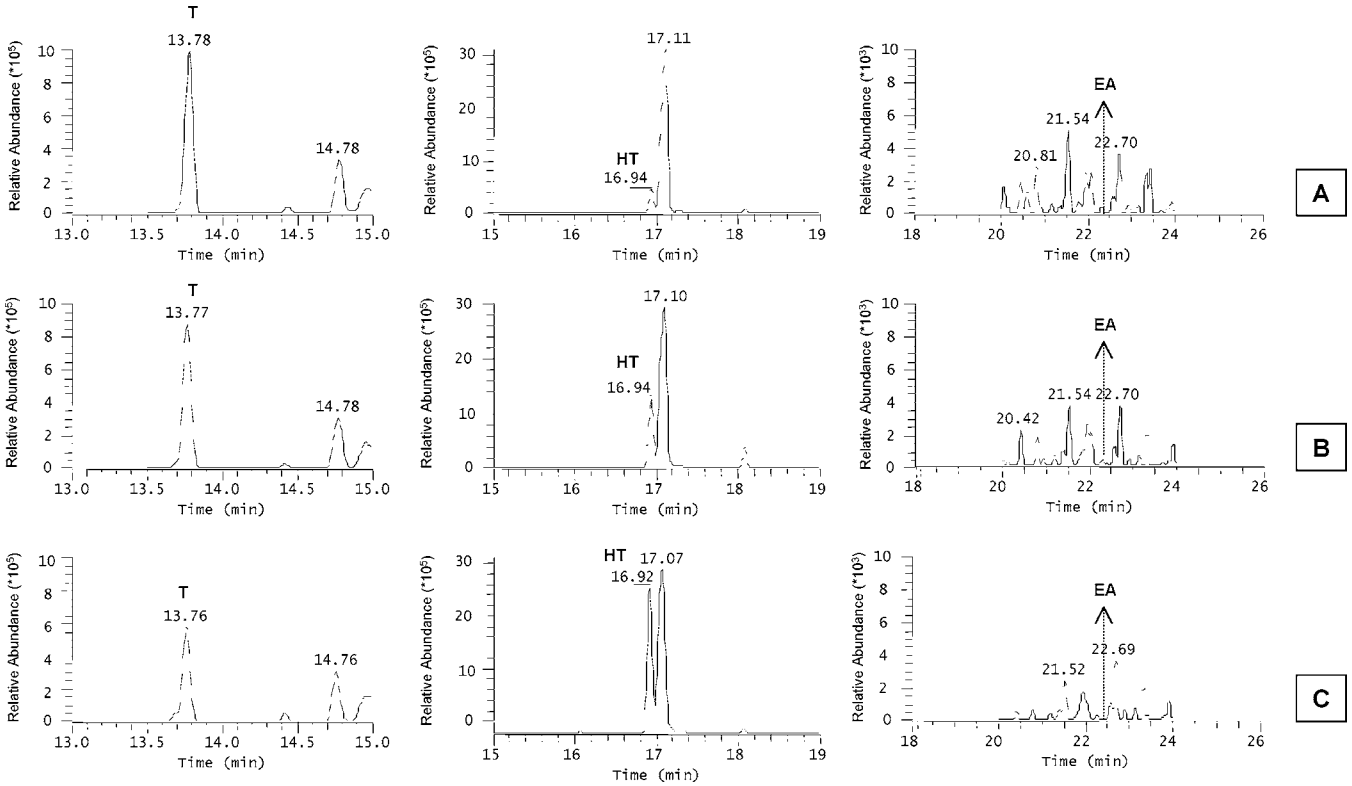

Figure 5. GC-MS/MS XICs for T, HT, and EA of a blank rat urine sample (**A**), a urine sample from a rat nourished with EVOO-enriched food (OL rats) (**B**), and a urine sample from a rat nourished with OE-enriched food (OE rats) (**C**). All of the samples were analyzed after enzymatic hydrolysis with β-glucuronidase. EA was not detected at the expected R_t (indicated by the arrow).

kinetics than those naturally occurring in the body. In the latter case, the initial blood concentration is elevated, resulting in a higher excretion rate of these substances in urine, which in turn may give misleading results regarding the bioavailability thereof.

It is worth mentioning that the development of the aforementioned methodology for the analysis of phenolic compounds had to overcome several difficulties. This is mainly because there are no biological fluids completely free (real blanks) of the phenolic substances under study. This is due to the presence of multiple forms of HT or T in other dietary sources and to the production of HT through dopamine's metabolism (*16*). Thus, we have decided to overcome the aforementioned difficulty of basal metabolite levels by spiking the blank urine samples before and after the SPE sample pretreatment, thus taking into account the background levels. The advantage of this approach is the consideration of the background metabolite levels, which is not accounted when synthetic urine is used (*31*, *34*).

To assess the percentage of the conjugated forms of the analytes and subsequently quantify the total levels of the aforementioned bioactive substances (free and conjugated as glucuronide metabolites), the samples were subjected to hydrolysis with β-glucuronidase in order to liberate the analytes from their conjugated forms (**Figure 5**). As mentioned above, the presence of HT and T in biological fluids originating from other dietary sources should be taken into account in their quantitative determination in biological samples. As it is shown in **Figure 5** and in **Table 4**, the amount of T detected in urine after enzymatic hydrolysis is derived mainly from the EVOO and other food sources and not from OE. However, the amount of HT detected after enzymatic hydrolysis increases for OL rats and is more elevated for OE rats (**Figure 5**). This indicates that OE is catabolized mainly to HT, which bares strong antioxidant properties and is then absorbed and excreted in urine as free HT but also as conjugated HT. In this case, the higher percentage of HT detected in conjugated forms indicates that a higher amount of this metabolite is absorbed after ingestion of pure OE, thus indicating a higher biological impact. **Table 4** shows that the metabolites are excreted after sustained moderate doses of OE or EVOO mainly conjugated as glucuronides, as reported previously (*31*, *36*), but also as free forms as a result of metabolic path saturation (*42*). It is noteworthy that the free vs total amount ratios remain constant for the blank and the OL rat urine samples, while it was significantly elevated for the OE rat urine sample. It's worth noting that EA was not detected in the samples analyzed following the sustained dose protocol. This may be due to its further metabolism to simpler molecules or to its conversion to other unknown isomeric forms during conjugation, taking into account that this molecule is sensitive to hydrolysis. On the other hand, EA may be excreted through another metabolic pathway. Nevertheless, the bioavailability of EA needs further study, since EA could prove rather useful as a biomarker for assessing the metabolism of OE, as it is not present in other food sources such as T and HT do. Thus, more in vivo experiments with animals treated with EA under different administration protocols are necessary for shedding light into the bioavailability and metabolism of this substance. In light of this, we are currently developing a liquid chromatography tandem mass spectrometry (LC-MS/MS)-based method, which allows for more sensitive metabolite determination.

In conclusion, the developed GC-MS/MS methodology allows the reliable, sensitive, and highly specific quantification of the metabolites under study in complex biological samples. The method demonstrates good precision, accuracy, and linearity for a wide dynamic range. The LOD for T and EA was in the mid picogram level, while that of HT was in the low picogram level.

This study corroborates the potential use of OE in the rapidly developing area of neutraceuticals. The fact that natural extracts and phytochemicals have a positive impact on human health may promote the development of nutraceuticals for reducing the risk of diseases. However, further bioavailability, pharmacokinetic, and epidemiological studies in animals and humans are necessary to confirm these results and recommend the intake of olive oil bioactive substances as prophylactic means to delay or even prevent the incidence of dysfunctions associated with reactive oxygen species.

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