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Uptake and Metabolism of New Synthetic Lipophilic Derivatives, Hydroxytyrosyl Ethers, by Human Hepatoma HepG2 Cells

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As a response to the increasing demand by the food industry for new synthetic lipophilic antioxidants, hydroxytyrosyl methyl, ethyl, propyl and butyl ethers have been synthesized from hydroxytyrosol, with similar or even higher antioxidant activity than free hydroxytyrosol. The uptake and metabolism of hydroxytyrosyl ethers with different alkyl side chain lengths (methyl, ethyl, propyl, and butyl) was studied after incubation for 2 and 18 h with HepG2 cells as a model of the human liver. LC-DAD and LC−MS were used for the identification of metabolites in culture media, cell lysates and samples hydrolyzed with β-glucuronidase and sulfatase. *In vitro* conjugation reactions of pure phenols were also performed. The results show an extensive uptake and metabolism by HepG2 cells after 18 h of incubation. A direct relationship between the lipophilic nature of the compound and the biotransformation yield was observed. Similar ratio of methyl and glucuronide forms were detected after 2 h of incubation while at 18 h high amounts of methylglucuronides and glucuronide metabolites were identified together with low amounts of methyl conjugates. In conclusion, alkyl hydroxytyrosyl ethers could be metabolized by the liver, their metabolic rate being higher for the more lipophilic compounds.

KEYWORDS: Alkyl hydroxytyrosyl ethers; HepG2 cells; uptake; metabolism; *in vitro* conjugation; LC−MS analysis

INTRODUCTION

Previous studies have suggested a relationship between virgin olive oil, the main source of fat in the Mediterranean diet, and a reduced risk of cancer and cardiovascular disease (*1*, *2*). These beneficial effects of virgin olive oil are attributed primarily to its minor components content such as phenolic compounds, the main group of natural antioxidants in olive oil, which play an important role due to their well-known biological activities and health effects (*3*−*5*). Particular attention has been focused on 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy) that occurs either as a simple phenol, esterified with elenolic acid to form oleuropein aglycone or with acetic acid to form hydroxytyrosyl acetate (2-(3,4-dihydroxyphenyl)ethyl acetate) in the phenolic fraction of virgin olive oil (*6*).

In vitro and *in vivo* studies with pure hydroxytyrosol have reported its ability for reducing the risk of coronary heart disease and atherosclerosis (*7*), and other biological effects such as antimicrobial (*8*), anti-inflammatory (*9*), inhibition of several lipoxygenases (*10*), or induction of apoptosis in HL-60 cells (*11*).

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Several human (*12*−*18*) and animal (*19*−*22*) studies have shown that olive oil phenols are absorbed, undergoing extensive intestinal and/or hepatic metabolism. HTy is mainly found as *O*-glucuronide conjugates, together with *O*-methyl forms like homovanillic alcohol or homovanillic acid, and monosulfate conjugates in plasma and urine after olive oil administration (*13*, *14*, *18*−*20*).

Previous studies by our group with HTy and hydroxytyrosyl acetate using a hepatic human cell line as a model of the human liver were carried out (*23*). Results showed an effective uptake and biotransformation of hydroxytyrosyl acetate, more extensive than that of HTy. A large conversion of hydroxytyrosyl acetate into free hydroxytyrosol and subsequent metabolism into methyl and glucuronides forms of hydroxytyrosol took place.

As a response to the food industry demand for new lipophilic antioxidants, a new group of antioxidants, hydroxytyrosyl ethers (**1**−**4**), have been synthesized from free hydroxytyrosol with potential application as antioxidants of lipophilic food. These compounds were shown to have a remarkable antioxidant activity as tested by different methods (i.e., Rancimat, DPPH, FRAP and ABTS assays), with even higher antioxidant activity than hydroxytyrosol or tocopherol (*24*). Their enhanced properties in comparison with hydroxytyrosol such as antioxidant activity and lipophilicity together with the higher stability that the ether form gives to their *o*-diphenolic structure make them

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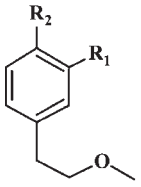
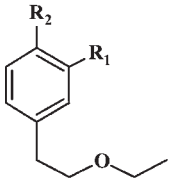
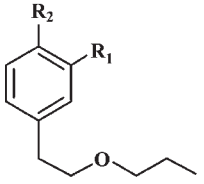


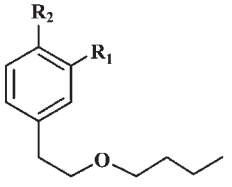
R_1	R_2	Name	Symbol
OH	OH	Hydroxytyrosyl methylether	1
OH	OGluA	Monoglucuronide conjugate of 1	M1/M2
OGluA	OH	Monoglucuronide conjugate of 1	M1/M2
OGluA	OCH_3	Methylglucuronide conjugate of 1	M3
OCH_3	OGluA	Methylglucuronide conjugate of 1	M3
OCH_3	OH	Monomethyl conjugate of 1	M4
OH	OCH_3	Monomethyl conjugate of 1	M4



R_1	R_2	Name	Symbol
OH	OH	Hydroxytyrosyl ethylether	2
OH	OGluA	Monoglucuronide conjugate of 2	M5/M6
OGluA	OH	Monoglucuronide conjugate of 2	M5/M6
OGluA	OCH_3	Methylglucuronide conjugate of 2	M7
OCH_3	OGluA	Methylglucuronide conjugate of 2	M7
OCH_3	OH	Monomethyl conjugate of 2	M8
OH	OCH_3	Monomethyl conjugate of 2	M8



R_1	R_2	Name	Symbol
OH	OH	Hydroxytyrosyl propylether	3
OH	OGluA	Monoglucuronide conjugate of 3	M9/M10
OGluA	OH	Monoglucuronide conjugate of 3	M9/M10
OGluA	OCH_3	Methylglucuronide conjugate of 3	M11
OCH_3	OGluA	Methylglucuronide conjugate of 3	M11
OCH_3	OH	Monomethyl conjugate of 3	M12
OH	OCH_3	Monomethyl conjugate of 3	M12



R_1	R_2	Name	Symbol
OH	OH	Hydroxytyrosyl butylether	4
OH	OGluA	Monoglucuronide conjugate of 4	M13/M14
OGluA	OH	Monoglucuronide conjugate of 4	M13/M14
OGluA	OCH_3	Methylglucuronide conjugate of 4	M15
OCH_3	OGluA	Methylglucuronide conjugate of 4	M15
OCH_3	OH	Monomethyl conjugate of 4	M16
OH	OCH_3	Monomethyl conjugate of 4	M16

Figure 1. Chemical structures of alkyl hydroxytyrosyl ethers (**1**−**4**) and their metabolites.

good candidates to be used by the food industry as bioantioxidants. The technological application in foods of these new types of lipophilic antioxidants constitutes one of our main objectives. However, the study of their absorption and metabolism in cellular or in animal models is a necessary step preliminary to its application in foods in order to evaluate their safety and potential biological activities. Therefore, the aim of the present study was to evaluate the uptake and metabolism of these new hydroxytyrosyl derivatives (hydroxytyrosyl methyl, ethyl, propyl and butyl ethers) (**1**−**4**), using a human hepatoma cell line (HepG2) in culture as a model system of the human liver. Identification of metabolites in cell culture media and cell lysates was performed by HPLC-DAD and LC−MS analysis, and compared with standards after *in vitro* treatment with conjugating enzymes or resulting metabolites after *in vitro* treatment with deconjugating enzymes.

MATERIALS AND METHODS

Chemicals. Methanol, sodium chloride, disodium hydrogen phosphate anhydrous, potassium dihydrogen phosphate, and formic acid were acquired from Panreac (Barcelona, Spain). Antibiotics (gentamicin, penicillin and streptomycin), enzymes (catechol-*O*-methyltransferase, β-glucuronidase from *Helix pomatia*, sulfatase), *S*-adenosyl-L-methionine chloride, UDP-glucuronic acid, and adenosine-3′-phospho-5′-phosphosulfate were purchased from Sigma Chemical Co. (Madrid, Spain). DMEM F-12 medium and fetal bovine serum (FBS) were from Biowhitaker (Innogenetics, Madrid, Spain). Lipophilic hydroxytyrosyl ethers (**1**−**4**) (**Figure 1**) were prepared by chemical synthesis from HTy (*25*). In order to identify the methyl conjugates that could be formed after incubation of HepG2 cells with hydroxytyrosyl ethers, standards of methyl derivatives were obtained by chemical synthesis from homovanillic alcohol (*26*). All reagents were of analytical or chromatographic grade. Standard solutions (10 mL) of hydroxytyrosyl methyl (**1**) (16.8 mg/L), ethyl (**2**) (18,2 mg/L) and propyl (**3**) (19.6 mg/L) ethers were prepared in 10% methanol while hydroxytyrosyl butyl ether (**4**) (21.0 mg/L) was dissolved in 20% methanol. Solutions of standard methylated conjugated (100 μM) were dissolved in 40% methanol. Diluted standards were prepared by dilution with distilled water.

Cell Culture. Human hepatoma HepG2 cells were cultured in 60 mm diameter plates in 8 mL of DMEM F-12 medium containing 2.5% FBS and 100 mg/L of each gentamicin, penicillin and streptomycin in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. The culture medium was changed every second day, and the cells were usually split 1:4 when they reached confluence.

Since HepG2 cells grew up well in serum-free medium and in order to prevent any potential interference from serum components, the incubation with the hydroxytyrosyl ethers (**1**−**4**) was carried out in the same medium containing the antibiotic mixture but deprived of serum. The experiments were carried out when cells reached about 80% confluence (concentration of $2.5-3 \times 10^6$ cells per plate approximately). Then, cells were changed to the different experimental conditions (final volume 6 mL): control plates contained only FBS-free medium, and experimental plates contained 100 μM of the different phenolic compounds (**1**−**4**), in duplicate. Ascorbic acid (200 μM) was added to prevent potential oxidation of the antioxidant compounds. After incubation for 2 h (short-term) or 18 h (long-term), the culture medium was separated and kept frozen at −20 °C until analysis. The cell layer was washed twice with PBS (0.01 M phosphate buffered saline solution, pH 7.4) and then collected by scraping. Cells from duplicate plates corresponding to a particular condition were collected in PBS and combined in an eppendorf vial. After centrifugation at 2000 rpm

for 5 min at 4 °C, the supernatant was removed and the cell pellet resuspended in 200 μL of PBS. Cells were sonicated for 10 min at room temperature to break down the cell membrane and to release the total amount of metabolites. After centrifugation at 5000 rpm for 10 min at 4 °C, the supernatant was transferred into an eppendorf vial and kept frozen at −20 °C.

HPLC Analysis. HPLC analyses were performed on an Agilent 1100 liquid chromatographic system equipped with a diode array UV/vis detector and with an autosampler Agilent 1200 (4 °C, 20 μL injection volume). A 250 mm × 4.6 mm i.d., 5 μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by a Tracer C-160K1 holder with an ODS precolumn was used. Elution was performed at a flow rate of 1.0 mL/min at room temperature, using as mobile phase a mixture of 0.1% (v/v) formic acid in deionized water (solvent A) and a mixture of 0.1% formic acid in methanol (solvent B). The solvent gradient changed according to the following conditions: from 100% A to 95% A in 5 min; 80% A in 10 min; 85% A in 2 min; 60% A in 5 min; 50% A in 8 min; 30% A in 5 min; 0% A in 5 min, followed by 10 min of maintenance; 100% A in 5 min, followed by 5 min of maintenance. Chromatograms were acquired at 280 nm.

For quantification of hydroxytyrosyl ethers (**1**−**4**) and their metabolites, standards of the four parent compounds were prepared in serum-free culture medium in a range of concentrations from 2.8 μM to 100 μM of hydroxytyrosyl methyl ether (**1**); 2.5 μM to 100 μM of hydroxytyrosyl ethyl ether (**2**); 3 μM to 100 μM of hydroxytyrosyl propyl ether (**3**) and 2.5 μM to 100 μM of hydroxytyrosyl butyl ether (**4**). A linear response was obtained for all the standard curves, as checked by linear regression analysis. Percentage of recovery of standards added to the culture medium (varying from 97 to 100%), limits of detection (ranging from 0.5 to 0.9 μM), limits of quantification (1.7−3.0 μM) and precision of the assay (as the coefficient of intra-assay variation, ranging from 2.8 to 5.0%) were considered acceptable and allowed quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecules).

LC−MS. LC−MS analyses were performed on an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies, Waldrom, Germany). The liquid chromatographic system consisted of a quaternary pump (G1311A), online vacuum degasser, autosampler (G1313A) (20 μL injection volume) and thermostatic column compartment, connected in line to a diode array detector (G1315B) before the mass spectrometer. Data acquisition and analysis were carried out with an Agilent ChemStation. Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above. Eluent flow (1 mL/min) was split 8:1 between the diode array detector and the mass spectrometer ion source. The mass spectrometer was fitted to an atmospheric pressure electrospray ionization (ESI) source, operated in negative ion mode. The electrospray capillary voltage was set to 3000 V, with a nebulizing gas (N_2) flow rate of 12 L/h and a drying gas temperature of 300 °C. Mass spectrometry data were acquired in the scan mode (mass range m/z 100−900) at a scan rate of 1.5 s. Selected ion monitoring was conducted at m/z 167.2, 357.2, 343.2, and 181.2 for hydroxytyrosyl methyl ether (**1**); at m/z 181.2, 357,2, 371,2 and 195.2 for hydroxytyrosyl ethyl ether (**2**); at m/z 195.2, 371.2, 386.2, and 209.2 for hydroxytyrosyl propyl ether (**3**); and at m/z 209.3, 385.3, 399.3, and 223.3 for hydroxytyrosyl butyl ether (**4**). Diode array spectra were registered from 190 to 450 nm, with an interval of 2 nm.

***In Vitro* Conjugation of Hydroxytyrosyl Derivatives (1−4).** Standard solutions of the assayed compounds were enzymatically conjugated *in vitro* using pure enzyme (catechol-*O*-methyltransferase, COMT) or a rat liver microsomal fraction that contained both UDP-glucuronosyltransferase and sulfotransferase.

Liver Microsomal Fraction. The preparation of the microsomal fraction from liver was according the procedure described by Graham et al. (*27*) and previously used by our group with some modifications (*23*). Briefly, from a fraction of rat liver homogenized in 10 volumes of ice-cold 50 mM Tris-chloride buffer (pH 7.4, containing 25 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose), the liver microsomes were separated by successive centrifugation. The nuclei and mitochondria were removed at 700*g* for 10 min and 5000*g* for 10 min in a refrigerated centrifuge (4 °C). The postmitochondrial supernatant fraction was centrifuged at 105000*g* for 60 min at 4 °C in a refrigerated ultracentrifuge. The final microsomal pellet was resuspended in 50 mM Tris-chloride buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 1 mM DTT and kept frozen at −80 °C until use. The final preparation was adjusted to a final protein concentration of 5 mg/mL, measured using a Bio-Rad protein assay kit (Bio-Rad Ltd., Munchen, Germany).

In Vitro Glucuronidation and Sulfatation of Hydroxytyrosyl Ethers (1−4). *In vitro* glucuronidation and sulfatation of hydroxytyrosyl derivatives (**1**−**4**) were performed using UDP-glucuronosyltransferase (UGT) and cytosolic sulfotransferase from rat liver microsomes in the presence of UDP-glucuronic acid as the glucuronosyl donor and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfate donor, respectively, as it has been described elsewhere (*23*, *28*).

Enzymatic Hydrolysis of Hydroxytyrosyl Ethers Conjugates from the Cell Culture. The culture medium from HepG2 cells incubated during 18 h with hydroxytyrosyl ethers (**1**−**4**) (100 μM) was hydrolyzed enzymatically using β-glucuronidase (*29*) and sulfatase (*30*) in order to confirm conjugated compounds by HPLC analysis.

RESULTS

In order to get a good quantitative and qualitative evaluation of hydroxytyrosyl ethers (**1**−**4**) as well as their metabolites, chromatographic conditions were optimized for the adequate elution of the evaluated compounds. No interference with other peaks corresponding to the culture medium was observed. Moreover, no transformation, degradation or derivatization of the tested compounds in the cell culture medium containing 200 μM ascorbic acid in absence of HepG2 cells were observed after 18 h of incubation.

Hepatic Metabolism of Hydroxytyrosyl Ethers (1−4). The metabolism of hydroxytyrosyl ethers (**1**−**4**) by HepG2 cells was monitored at two different incubation times (2 and 18 h). An active uptake and metabolism of hydroxytyrosyl ethers (**1**−**4**) at 18 h was observed. Thus, after 18 h incubation with HepG2 cells, only minor amounts of unmetabolized hydroxytyrosyl methyl ether (**1**), hydroxytyrosyl ethyl ether (**2**) and hydroxytyrosyl propyl ether (**3**) could be observed (**Figure 2a**−**c**), with 69.8%, 77.3% and 80.3% of transformation, respectively. Hydroxytyrosyl butyl ether (**4**) biotransformation was complete, and no traces of the original compound could be detected after 18 h (**Figure 2d**).

The chromatographic profile of the cell culture medium after 18 h incubation with hydroxytyrosyl methyl ether (**1**) (**Figure 2a**) was characterized by the formation of three different peaks. Peak at 21.6 min consisted of two unresolved conjugated metabolites (M1 and M2), and peaks at 24.4 and 30.6 min corresponded to metabolites M3 and M4, respectively. Hydroxytyrosyl ethyl ether (**2**) showed three new chromatographic peaks (M5−M8) after 18 h incubation in HepG2 cells together with nonmetabolized original molecule (**2**) (**Figure 2b**). Again, peak at 24.1 min consisted of two unresolved conjugated metabolites (M5 and M6), and peaks at 27.1 and 31.9 min corresponded to metabolites M7 and M8, respectively. When cells were incubated with hydroxytyrosyl propyl ether (**3**) during 18 h (**Figure 2c**), four peaks at retention times 29.6, 29.8, 30.5, and 34.2 min, labeled as M9−M12, were observed together with low amounts of nonmetabolized original molecule (**3**). The metabolism of hydroxytyrosyl butyl ether (**4**) generated the formation of three metabolites at retention times 30.3, 30.5, and 31.4 min, labeled as M13, M14 and M15 (**Figure 2d**). All detected metabolites showed a UV spectra maximum ranging from 278 to 282 nm, consistent with an *O*-diphenolic structure.

These results showed a direct relationship between the lipophilic nature of each compound and the higher level of metabolization.

After 2 h of incubation, most peaks found in the culture medium corresponded to the free original molecule, suggesting slow cellular uptake and metabolism of these compounds. The hydroxytyrosyl methyl ether (**1**) metabolism showed small

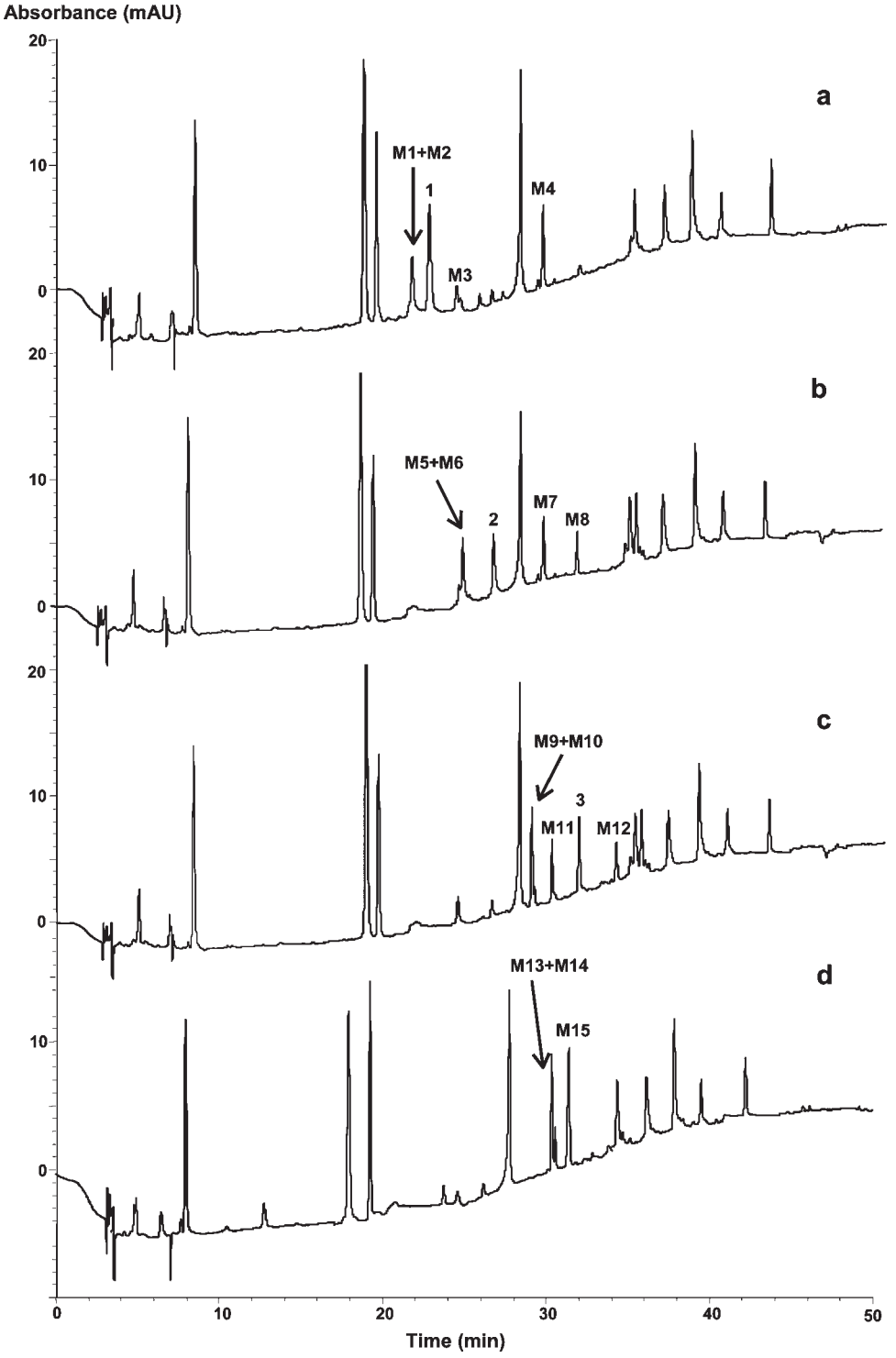


Figure 2. Chromatographic profile of different molecular species detected in culture medium from HepG2 cells after incubation for 18 h with 100 μM concentration of the following compounds: (**a**) hydroxytyrosyl methyl ether (**1**); (**b**) hydroxytyrosyl ethyl ether (**2**); (**c**) hydroxytyrosyl propyl ether (**3**); (**d**) hydroxytyrosyl butyl ether (**4**). Peaks M1 to M15 correspond to different metabolites of the studied synthetic compounds formed after incubation with the hepatic cells; peak 1, 2, and 3 are the parent compounds without transformation in the medium after 18 h incubation.

amounts of metabolites M1 and M2 at 21.6 min and metabolite M4 at 30.6 min. Similar behavior was observed for the other compounds (**2**−**4**), with the formation of two peaks at 24.0 and 24.1 min (M5 and M6) and 31.9 min (M8) for hydroxytyrosyl ethyl ether (**2**) and the chromatographic peaks at 29.6, 29.8, and 34.2 min corresponding to M9, M10 and M12 respectively for hydroxytyrosyl propyl ether (**3**). Hydroxytyrosyl butyl ether (**4**) metabolism at 2 h showed small amounts of metabolites M13 and M14 at 30.3 and 30.5 min, and a new chromatographic peak at 36.6 min labeled as M16 which was not observed at 18 h (data not shown).

On the other hand, only traces of free hydroxytyrosyl ethers (**1**−**4**) or their potential metabolites were observed in the cell lysates after 2 h or 18 h of incubation, suggesting no intracellular accumulation of these compounds or their metabolites (data not shown).

Moreover, HPLC chromatograms did not reveal the presence of free hydroxytyrosol resulting from the hydrolysis of

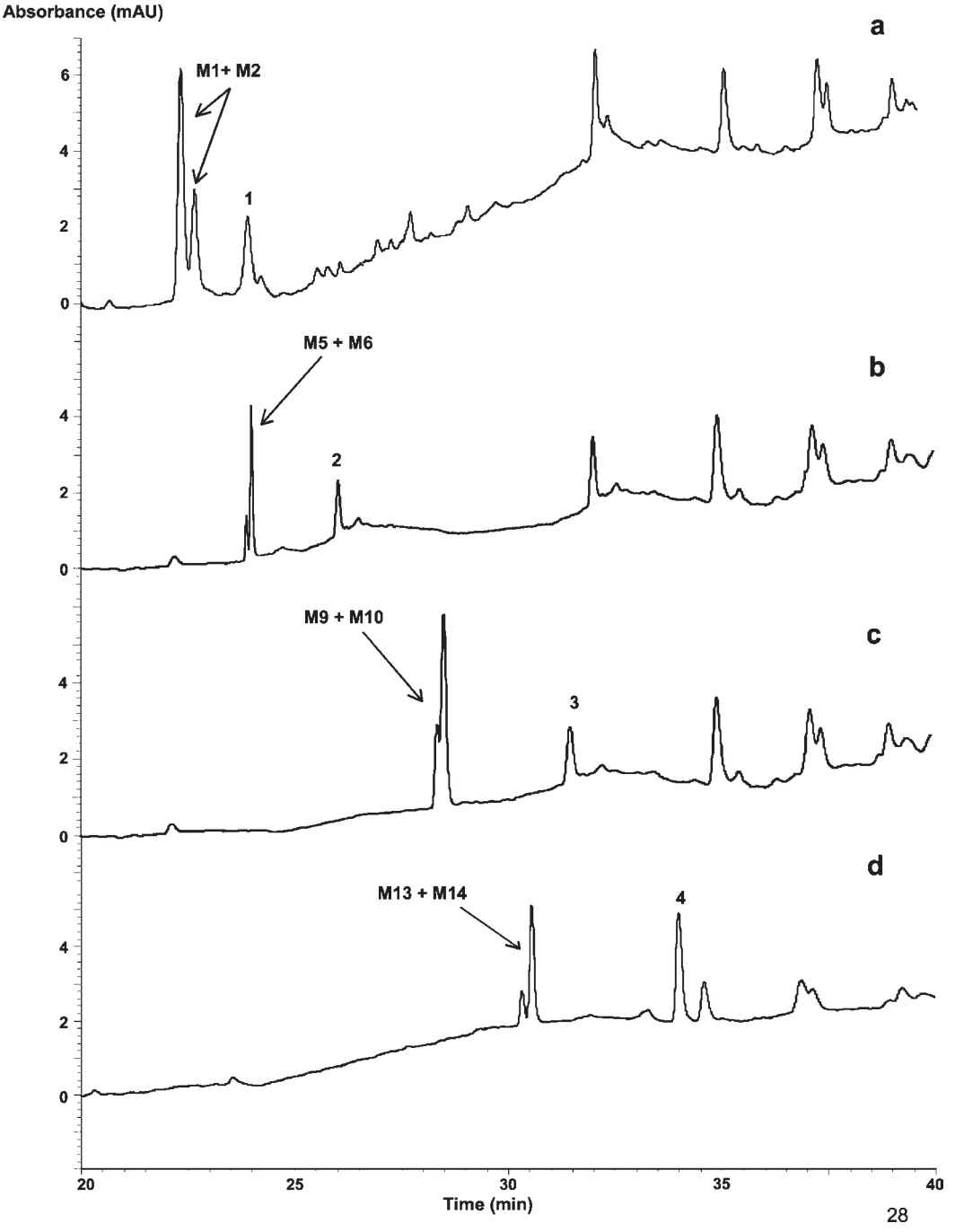


Figure 3. HPLC chromatograms of reaction media after *in vitro* glucuronidation with liver microsomes of hydroxytyrosyl methyl (**1**) (**a**), ethyl (**2**) (**b**), propyl (**3**) (**c**) and butyl (**4**) (**d**) ethers. Peaks M1+M2, M5+M6, M9+M10 and M13+M14 correspond to glucuronide metabolites, respectively.

hydroxytyrosyl ethers (**1**−**4**) by HepG2 cells neither in the cell culture medium nor cytoplasmatic content, after 2 or 18 h of incubation.

Identification of Hydroxytyrosyl Ether Metabolites. For the identification of the formed metabolites after incubation of hydroxytyrosyl derivatives (**1**−**4**) with human hepatoma cells, several steps were followed: *in vitro* glucuronidation and sulfatation of pure standards, comparison with synthetic alkyl methylated derivatives, enzymatic hydrolysis with β-glucuronidase and sulfatase of metabolites generated by HepG2, and confirmation of structures by LC−MS analysis.

After *in vitro* glucuronidation of pure hydroxytyrosyl derivatives (**1**−**4**), two metabolites of hydroxytyrosyl methyl ether (**1**) at 21.6 and 22.4 min (**Figure 3a**) were found. Their spectroscopic characteristics (spectrum width and λ_{max} at 278 nm) and retention times were coincident with the peak obtained in the culture medium from cells incubated with hydroxytyrosyl methyl ether (**1**) during 18 h, confirming the nature of peaks M1+M2 as monoglucuronide derivatives of hydroxytyrosyl methyl ether (**1**), without discerning between the two isomers. Hydroxytyrosyl ethyl ether (**2**) subjected to *in vitro* glucuronidation provided two chromatographic peaks at 24.0 and 24.2 min (**Figure 3b**). Their spectroscopic characteristics and retention times were coincident with those labeled as M5 and M6 present after incubation of hydroxytyrosyl ethyl ether (**2**) with HepG2 cells (**Figure 2b**), without discerning between the two isomers. Similarly, the metabolites present after incubation of hydroxytyrosyl propyl ether (**3**) with the HepG2 cells (M9 and M10) (**Figure 2c**) showed an

Table 1. Chromatographic and Spectroscopic Characteristics of Hydroxytyrosyl Methyl (**1**), Ethyl (**2**), Propyl (**3**) and Butyl (**4**) Ethers and the Metabolites Formed after Incubation with HepG2 Cells

				m/z		
compound	MW	RT	λ_{max}	$[M-H]^-$	fragment ions	proposed structure
1	168.2	22.3	280	167.2	122.0−153.9	hydroxytyrosyl methyl ether (**1**)
M1/M2	344.2	21.6	278	343.2	no fragments	monoglucuronide of **1**
M3	358.2	24.4	278	357.2	no fragments	methylglucuronide of **1**
M4	182.2	30.6	280			monomethyl of **1**
2	182.2	26.2	280	181.2	no fragments	hydroxytyrosyl ethyl ether (**2**)
M5/M6	358.2	24.0/24.1	278	357.2	no fragments	monoglucuronide of **2**
M7	371.2	27.1	276	370.2	no fragments	methylglucuronide of **2**
M8	196.2	31.9	280			monomethyl of **2**
3	196.2	32.5	282	195.1	no fragments	hydroxytyrosyl propyl ether (**3**)
M9/M10	372.2	29.6/29.8	280	371.1	195.1	monoglucuronide of **3**
M11	386.2	30.5	278	385.1	195.1	methylglucuronide of **3**
M12	210.2	34.2	282			monomethyl of **3**
4	210.1	34.7	280	209.1	no fragments	hydroxytyrosyl butyl ether (**4**)
M13/M14	386.1	30.3/30.5	278	385.1	209.0	monoglucuronide of **4**
M15	400.1	31.4	278	399.1	no fragments	methylglucuronide of **4**
M16	224.1	36.6	280			monomethyl of **4**

identical spectrum width and λ_{max} at 280 nm to the peak found after *in vitro* glucuronidation of this hydroxytyrosyl derivative, that involved two isomers (**Figure 3c**). Pure hydroxytyrosyl butyl ether (**4**) subjected to *in vitro* glucuronidation showed the same behavior as that described for the other compounds. Two peaks were detected at 30.3 and 30.5 min (**Figure 3d**), with spectrum width and λ_{max} at 278 nm matched up with those labeled as M13 and M14 present after incubation of hydroxytyrosyl butyl ether (**4**) with HepG2 cells (**Figure 2d**).

Concerning *in vitro* sulfatation, all hydroxytyrosyl ethers (**1**−**4**) provided sulfoderivatives in the presence of a sulfate acid donor. New chromatographic peaks were formed after *in vitro* sulfatation of hydroxytyrosyl methyl (**1**), ethyl (**2**), propyl (**3**) and butyl (**4**) ethers, respectively (data not shown). However, no similar peaks were detected in the culture medium after the incubation of hydroxytyrosyl ethers (**1**−**4**) by HepG2 cells during 2 and 18 h of incubation, suggesting that no conjugated sulfates were formed by the human hepatoma cells.

Pure synthetic methyl compounds were used as standards for the identification of the corresponding metabolites. The retention times and spectroscopic characteristics of synthetic methyl derivatives of hydroxytyrosyl ethers (**1**−**4**) permitted the identification of metabolite M4 at 30.6 min (**Figure 2a**) as homovanillic methyl ether, peak M8 at 31.9 min (**Figure 2b**) as homovanillic ethyl ether and peak M12 at 34.2 min (**Figure 2c**) as homovanillic propyl ether, present after the incubation with HepG2 cells for 18 h. No peak coincident with synthetic homovanillic butyl ether was found after the incubation of hydroxytyrosyl butyl ether (**4**) with HepG2 during 18 h. However, this methyl derivative of hydroxytyrosyl butyl ether was found at 36.6 min (metabolite M16) after 2 h of incubation (data not shown), suggesting it suffered further metabolism by cells to generate a methylglucuronide derivative at longer times of incubation.

After hydrolysis with β-glucuronidase of the cell culture media collected after incubation of HepG2 cells for 18 h with hydroxytyrosyl methyl ether (**1**), peaks M1+M2 and M3 disappeared, concomitant with an increase in the areas of peaks **1** and M4, corresponding to hydroxytyrosyl methyl ether (**1**) and homovanillic methyl ether, respectively. This confirmed the presence of a glucuronide moiety in the disappeared chromatographic peaks.

Peaks M5+M6 and M7 disappeared when the culture medium from cells incubated with hydroxytyrosyl ethyl ether (**2**) after 18 h was hydrolyzed with β-glucuronidase (data not shown). An increase in the areas of hydroxytyrosyl ethyl ether (**2**) and homovanillic ethyl ether (M8) confirmed the presence of glucuronides of hydroxytyrosyl ethyl ether (**2**) as M5 and M6 and glucuronide of homovanillic ethyl ether as M7.

Similarly, peaks M9, M10 and M11 detected in the culture medium from cells treated with hydroxytyrosyl propyl ether (**3**) during 18 h disappeared after hydrolysis with β-glucuronidase (data not shown), with the consequent increase of the areas of peak **3** and the peak corresponding to its methyl conjugate (M12). The results permitted the tentative identification of M9 and M10 as glucuronides of hydroxytyrosyl propyl ether (**3**) and M11 as the glucuronide of homovanillic propyl ether.

Peaks M13, M14 and M15 disappeared when culture media from cells incubated with hydroxytyrosyl butyl ether (**4**) after 18 h were hydrolyzed with β-glucuronidase (data not shown), while the area of the standard compound (**4**) was increased and a peak at 36.6 min formed. This peak was coincident in retention time and spectroscopic characteristic with the homovanillic butyl ether standard (M16). Therefore, metabolites M13 and M14 would correspond to glucuronides of hydroxytyrosyl butyl ether (**4**), and peak M15 to the methylglucuronide derivative.

In all cases, the yields of the *in vitro* conjugation were acceptable, allowing an easy identification of the formed metabolites in this study, although we were not able of differentiate among isomers.

Hydrolysis of the cell culture media with sulfatase did not result in significant changes in the chromatographic profiles, suggesting that no sulfate derivatives were formed after the incubation of HepG2 cells with the different alkyl hydroxytyrosyl ethers (**1**−**4**) (data not shown).

The metabolites obtained in the culture media from HepG2 cells after incubation for 2 and 18 h with hydroxytyrosyl derivatives (**1**−**4**) were analyzed by LC−MS for identity confirmation, based on the *m/z* values of the molecular and fragment ions. These results together with the UV/vis spectroscopic data are summarized in **Table 1**. As it was expected, the corresponding metabolites showed shorter retention times in the reverse phase column, in accordance with their increased polarity. Also, it is worth noting that, except for the methyl derivatives, all other conjugated forms showed a slight hypsochromic effect, with a reduction of 2−4 nm in the λ_{max} in comparison with the parent molecules, which is in agreement with previous results obtained with HTy and hydroxytyrosyl acetate incubated with HepG2 cells (*23*).

As for the LC−MS results, compound **1** showed a deprotonated molecular ion at *m/z* 167 plus a fragment ion at *m/z* 153.9,

consistent with the loss of a methyl group to yield hydroxytyrosol, and a second fragment at m/z 122. Metabolites M1 and M2 showed a $[M - H]^-$ ion at m/z 343.2, corresponding to glucuronide derivatives of the parent molecule (**1**). These data, along with those obtained from the *in vitro* conjugation and enzymatic hydrolysis experiments, confirmed the identity of peaks M1 and M2 as monoglucuronide conjugates of hydroxytyrosyl methyl ether (**1**). Peak M3, which disappeared from the culture medium after the *in vitro* hydrolysis with β-glucuronidase and presented a molecular ion at m/z 357, was identified as a methylglucuronide metabolite of hydroxytyrosyl methyl ether (**1**).

Regarding the hydroxytyrosyl ethyl ether metabolites M5 and M6, they were identified as monoglucuronides of hydroxytyrosyl ethyl ether (**2**) taking into account the resulting molecular ion at m/z 357.2. Metabolite M7 was identified as methylglucuronide derivative, considering the molecular ion at m/z 370.2. M5, M6 and M7 disappeared after the *in vitro* hydrolysis with β-glucuronidase.

Concerning the hydroxytyrosyl propyl ether metabolites M9 and M10, which disappeared from the cell culture medium after hydrolysis with β-glucuronidase, they were identified as monoglucuronides of hydroxytyrosyl propyl ether (**3**). These metabolites were confirmed on the basis of their molecular ion at m/z 371.0 also showing a fragment ion at m/z 195.1 corresponding to the deprotonated parent molecule (after the loss of dehydrated glucuronic acid). Metabolite M11 from hydroxytyrosyl propyl ether (**3**) metabolism after 18 h of incubation was identified as the methylglucuronide derivative, taking into account the resulting molecular ion at m/z 385.1 and fragment ion at m/z 195.1.

Metabolites M13 and M14, from hydroxytyrosyl butyl ether (**4**), which disappeared from the culture medium after hydrolysis with β-glucuronidase and were tentatively identified as monoglucuronides of hydroxytyrosyl butyl ether, were confirmed on the basis of their molecular ion at m/z 385.1 and fragment ions with m/z 209.0 (corresponding to the deprotonated parent compound after the loss of dehydrated glucuronic acid). Peak M15 had a molecular ion $[M - H]^-$ at m/z 399.0, and thus it was identified as the methylmonoglucuronide of hydroxytyrosyl butyl ether. LC−MS was not able to discern between isomers corresponding to glucuronide or methylglucuronide derivatives.

Finally, metabolites M4, M8, M12 and M16 did not give any signal in the single quadrupole mass detector after electrospray ionization either in the positive or the negative mode. However, the identification of these three metabolites was possible based on the comparative study with the synthetic standard methyl derivatives of hydroxytyrosyl ethers obtained by chemical synthesis from homovanillic alcohol. Thus, these chromatographic peaks M4, M8, M12 and M16 obtained after the incubation of hydroxytyrosyl ethers (**1**−**4**) with HepG2 cells were compared with the standard compounds by evaluating spectra characteristics and retention times obtained by HPLC-DAD analysis. Moreover, the increase of M4, M8, M12 and M16 after hydrolysis with β-glucuronidase confirmed their identity as methyl metabolites of hydroxytyrosyl methyl, ethyl, propyl and butyl ethers (**1**−**4**), respectively.

Quantification of Metabolites. The amounts of unmetabolized compounds and their metabolites in the extracellular culture medium were quantified after 2 and 18 h of incubation with HepG2 cells. Differences were observed considering the time of incubation as well as the chemical structure and the length of the alkyl chain of each assayed compound. Results are summarized in **Figure 4**.

The percentage of free, unmetabolized compounds detected in the culture medium after 2 h of incubation with hydroxytyrosyl ethers (**1**−**4**) represented 92.6, 90.5, 87.4 and 83.5% for hydroxytyrosyl methyl (**1**), ethyl (**2**), propyl (**3**) and butyl (**4**) ethers, respectively, showing a low uptake and metabolization of these compounds at short incubation times. While hydroxytyrosyl methyl ether (**1**) provided the same ratio of glucuronides and methyl conjugates (3.7%), the amounts of hydroxytyrosyl ethyl (**2**) propyl (**3**) and butyl (**4**) ether methyl conjugates (5.5, 7.9 and 9.3% respectively) were slightly and significantly higher than that of the glucuronide conjugates (4.0, 4.7 and 7.2, respectively). After 18 h of incubation, however, the metabolism was substantially higher in comparison with 2 h, with only 30.2, 22.7 and 19.7% of the parent hydroxytyrosyl methyl (**1**), ethyl (**2**) and propyl (**3**) ethers (**1**−**2**), respectively, detected unmetabolized in the culture medium (**Figure 4**). As for hydroxytyrosyl butyl ether (**4**), no free, unmetabolized compound was detected, being totally converted into the glucuronides and methylglucuronide metabolites. All compounds showed a similar metabolic behavior, detecting a higher amount of methylglucuronides, followed by glucuronides and finally methyl conjugates in the case of hydroxytyrosyl methyl (**1**), ethyl (**2**) and propyl (**3**) ether, since the cells spiked with butyl (**4**) ether did not show the presence of methyl compound after 18 h of incubation. Independently of the incubation time, a direct relationship between the lipophilic nature of the parent compound and the level of metabolization was observed, the hydroxytyrosyl butyl ether (**4**) being completely taken up and metabolized by HepG2 cells in culture, as it can be observed in **Figure 4**.

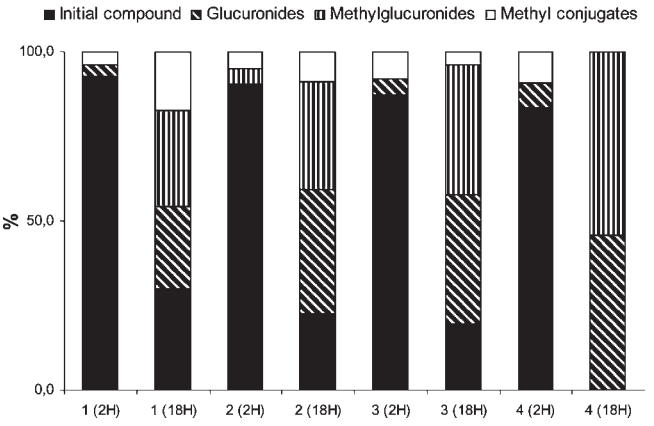


Figure 4. Relative percentages of parent compounds (black segments), glucuronides (slashed segments), methyglucuronides (striped segments), and methyl metabolites (open segments) accumulated in the culture media after 2 and 18 h of incubation of HepG2 cells in the presence of 100 μmol/L hydroxytyrosyl ethers (**1**−**4**).

DISCUSSION

Alkyl hydroxytyrosyl ethers (**1**−**4**), a new class of lipophilic antioxidants obtained from free hydroxytyrosol by chemical synthesis with different alkyl side chain lengths, have attracted our attention due to their similar or even higher antioxidant activity compared with HTy (*24*) and higher lipophilic nature. These compounds would answer the demands by the food industry of new lipophilic antioxidants to prevent oxidation of fatty foods and food ingredients. Preliminary results obtained by our group in enterocyte-like differentiated Caco-2 as a human model for the small intestinal epithelium showed the presence of high amounts of free hydroxytyrosyl ethers (**1**−**4**) in the basolateral side (unpublished observations). From this it follows that these compounds may undergo further metabolism in the liver.

In the present manuscript, we show the results on the uptake and metabolism of hydroxytyrosyl ethers (**1**−**4**) using a human hepatoma cell line (HepG2) in culture as a model system of the human liver. Differences occurred in the extent of metabolism of the four studied phenols (**1**−**4**), depending of their chemical

structure and the length of incubation with the hepatic cells. In general, the analyzed hydroxytyrosyl ethers (**1**−**4**) showed extensive uptake and metabolism by HepG2 cells after 18 h of incubation, being significantly higher than that observed after 2 h. On the other hand, the increasing lipophilic nature of these compounds as the length of the alkyl side chain increased determined the more extensive metabolism observed for hydroxytyrosyl butyl ether (**4**) in comparison to hydroxytyrosyl methyl ether (**1**). The quantitative evaluation of the results obtained after 2 and 18 h of incubation with HepG2 cells (**Figure 4**) confirmed the influence of increasing lipophilic nature in the metabolization yield, with a total percentage of biotransformation of 7.4, 9.5, 12.6 and 16.5% at 2 h for hydroxytyrosyl methyl (**1**), ethyl (**2**), propyl (**3**) and butyl (**4**) ethers, respectively, and as high as 69.8, 77.3, 80.3 and 100% at 18 h, respectively.

This is in agreement with previous results with hydroxytyrosol and hydroxytyrosyl acetate in HepG2 cells (*23*), as well as with results obtained with alkyl gallates in Caco-2 cells (*31*), where the addition of an acetate residue or increasing the side chain length, respectively, resulted in more extensive cellular uptake and metabolism. These results are in line with the passive diffusion suggested by Manna et al. (*22*) as the possible mechanism of transport responsible for hydroxytyrosol uptake in Caco-2 cells. In the present work, the high lipophilic nature of the studied compounds (**1**−**4**) might enable enhanced diffusion through the lipid bilayer in cell membranes.

Phase II enzymes, especially catechol-*O*-methyltransferase (COMT) and UDP-glucuronosyltransferases (UGT), play a crucial role in the metabolism of alkyl hydroxytyrosyl ethers (**1**−**4**), but apparently not sulfotransferases (SULT), since no sulfate metabolites could be detected. Methylation seems the primary metabolization step at short incubation times, together with glucuronidation although at a lower extent for hydroxytyrosyl ethyl (**2**) propyl (**3**) and butyl (**4**) ethers (**Figure 4**). The soluble, cytoplasmic form of COMT would be responsible for the rapid methylation of the uptaken compounds, followed by glucuronidation by microsomal UGT. At longer incubation times, the tested compounds would undergo further glucuronidation, especially the more lipophilic propyl and butyl ethers, until only glucuronides and methylglucuronide metabolites, but no methyl forms, are found after 18 h of incubation. This could respond to a strategy to increase polarity of these compounds in order to enhance their elimination.

It is worth noting that hydroxytyrosyl acetate metabolism by HepG2 cells comprised partial hydrolysis of the ester bond by cellular carboxylesterases, yielding deacetylated hydroxytyrosol (*23*). However, no free hydroxytyrosol was detected in the present study, indicating that the ether linkages were not hydrolyzed by the hepatic cells. Therefore, it could be hypothesized that the actual metabolites that could be expected to occur *in vivo* after absorption and metabolization of the alkyl hydroxytyrosyl ethers (**1**−**4**) would be the glucuronides and methylglucuronide derivatives identified in the present work. Considering the higher antioxidant activity of the alkyl hydroxytyrosyl ethers (**1**−**4**) in comparison with hydroxytyrosol (*24*), this could be considered as a positive feature, although it is necessary to assess the biological activity of the metabolites. Also, further *in vivo* studies are required to confirm the nature of circulating metabolites after alkyl hydroxytyrosyl ether (**1**−**4**) consumption and to study the pharmacokinetics of these compounds.

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