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Metabolism of the Olive Oil Phenols Hydroxytyrosol, Tyrosol, and Hydroxytyrosyl Acetate by Human Hepatoma HepG2 Cells

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To study the potential hepatic metabolism of olive oil phenols, human hepatoma HepG2 cells were incubated for 2 and 18 h with hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate, three phenolic constituents of olive oil. After incubation, culture media and cell lysates were hydrolyzed with β-glucuronidase and sulfatase and analyzed by LC-MS. In vitro methylation, glucuronidation, and sulfation of pure phenols were also performed. Methylated and glucuronidated forms of hydroxytyrosol were detected at 18 h of incubation, together with methylglucuronidated metabolites. Hydroxytyrosyl acetate was largely converted into free hydroxytyrosol and subsequently metabolized, yet small amounts of glucuronidated hydroxytyrosyl acetate were detected. Tyrosol was poorly metabolized, with <10% of the phenol glucuronidated after 18 h. Minor amounts of free or conjugated phenols were detected in cell lysates. No sulfated metabolites were found. In conclusion, olive oil phenols can be metabolized by the liver as suggested by the results obtained using HepG2 cells as a hepatic model system.

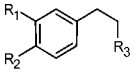
KEYWORDS: Phenols; virgin olive oil; metabolism; in vitro conjugation; HepG2 cells; LC-MS analysis

INTRODUCTION

Epidemiological studies have shown a relationship between the Mediterranean diet, rich in fruit, vegetables, and legumes, and a reduced incidence of pathologies such as coronary heart disease and cancer (*1*, *2*). A central hallmark of this diet is the high consumption of virgin olive oil as the main source of fat (*3*). Converging evidence suggests that the beneficial effects of olive oil are related not only to its high oleic acid content but also to the presence of antioxidants in the nonsaponifiable fraction, including phenolic compounds absent in seed oils (*4*−*6*).

The absolute concentration of phenols in olive oil is a result of complex interactions between several factors, including cultivar, degree of ripeness of the olives, climate, and extraction procedures (*7*). As a result of this, the total phenol content in olive oil has been reported to vary from 100 mg/kg to 1 g/kg (*8*). The main phenolic compounds in virgin olive oil are secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) and 2-(4-hydroxyphenyl)ethanol (tyrosol) that occur either as simple phenols or esterified with elenolic acid to form oleuropein and ligstroside aglycones (*9*). Recently, hydroxytyrosyl acetate [2-(3,4-dihydroxyphenyl)ethyl acetate] has also been identified in virgin olive oil (*9*) (**Figure 1**). These compounds, together with some flavonoids and lignans, constitute the phenolic fraction of olive oil (*7*, *9*, *10*).

The phenolic fraction of virgin olive oil has proved to have antioxidant activity in vitro, scavenging peroxyl, hydroxyl, and other free radicals, reactive nitrogen species, and superoxide anions and hypochlorous acid, breaking peroxidative chain reactions, and preventing metal ion catalyzed production of reactive oxygen species (for reviews, see refs *11* and *12*). Also, the inhibitory action of olive oil constituents on low-density lipoprotein (LDL) oxidation, platelet aggregation, activation of lipoxygenase or xanthine oxidase, etc. (*4*, *6*, *11*, *13*, *14*) contributes to the protective effect of olive oil against cardiovascular disease.



R_1=R_2=R_3=OH	Hydroxytyrosol (**1**)
R_1=H; R_2=R_3=OH	Tyrosol (**2**)
R_1=R_2=OH; R_3=$COOCH_3$	Hydroxytyrosyl Acetate (**3**)
R_1=R_3=OH; R_2=OCH_3	Homovanillic alcohol
R_1=R_3=OH; R_2=OGlucAc	Monoglucuronide conjugate of 1
R_1=H; R_2= OGlucAc; R_3=OH	Monoglucuronide conjugate of 2
R_1=OH; R_2= OGlucAc; R_3=$COOCH_3$	Monoglucuronide conjugate of 3
R_1=OCH_3; R_2=OGlucAc; R_3=OH	Methylglucuronide conjugate of 1

Figure 1. Chemical structures of olive oil phenols and metabolites.

Several human (*15*−*21*) and animal (*22*−*24*) studies have shown that olive oil phenols are bioavailable. In a human ileostomy study it was shown that up to 66% of the ingested olive oil phenols were absorbed in the small intestine (*18*). Urinary recoveries as high as 80% of the ingested amounts of hydroxytyrosol have been reported in humans (*20*, *21*), with considerably lower excretions of tyrosol (20−25% of the initial doses) (*19*). Over 90% of the urinary metabolites were conjugates (*15*, *17*−*21*), mainly glucuronidated metabolites, yet

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free phenols and methylconjugates, with or without glucuronidation, were also excreted in human urine. Sulfoconjugates of hydroxytyrosol, tyrosol, or their metabolites (methyl or glucuronide conjugates) have been observed only in animal experiments (*22*−*24*).

Using differentiated Caco-2 cell monolayers as a model system of the human intestinal epithelium, it has been shown that [^{14}C]hydroxytyrosol is transported via passive diffusion, bidirectionally (from the apical to the basolateral sides of this differentiated cells and vice versa), and in a dose-dependent manner. The only metabolite identified was homovanillic alcohol, the methylated derivative of hydroxytyrosol, which represented only 10% of the total radioactivity (*25*). Considering the limited metabolism of hydroxytyrosol by Caco-2 cells in contrast with the extensive conjugation of olive oil phenols observed in vivo, it follows that biotransformation of absorbed hydroxytyrosol should take place mostly in the liver.

The aim of this work was to study the metabolism of olive oil phenolics, hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate, using a human hepatoma cell line (HepG2) in culture as a model system of the human liver. Identification of metabolites was performed by HPLC-DAD and LC-MS analysis and by studying the behavior of the resulting metabolites after in vitro treatment with conjugating and deconjugating enzymes.

MATERIALS AND METHODS

Chemicals. Acetonitrile, methanol, sodium chloride, disodium hydrogen phosphate anhydrous, and potassium dihydrogen phosphate as well as 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). Tyrosol was from Janssen Chemical Co. (Beerse, Belgium). Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid by reduction with $LiAlH_4$ (*26*). Hydroxytyrosyl acetate was obtained from hydroxytyrosol in ethyl acetate after incubation with *p*-toluenesulfonic acid and purification by column chromatography following a patented procedure (*27*). All reagents were of analytical or chromatographic grade.

Cell Culture and Sample Preparation. Human hepatoma HepG2 cells were grown in DMEM F-12 medium supplemented with 2.5% FBS and 50 mg/L each of gentamicin, penicillin, and streptomycin. Cells were grown in 60 mm diameter plates, in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. The culture medium was changed every other day, and the cells were usually split 1:3 when they reached confluence.

The assay was carried out when cells reached ∼80% confluence [concentration of $\sim(2.5-3) \times 10^6$ per plate]. Because HepG2 cells grew well in serum-free medium, as tested previously in the laboratory, the incubation with olive oil phenols was carried out in the same medium deprived of serum but containing the antibiotic mixture in order to prevent any potential interference from serum components. On the day of the assay, cells were changed to the different experimental conditions (final volume = 3 mL). Control plates contained only FBS-free medium, whereas the experimental plates contained 100 μM concentrations of the different phenolic compounds, in triplicate. To all plates was added ascorbic acid (150 μM) to prevent potential oxidation of olive oil phenolics. 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 triplicate plates corresponding to a particular condition were collected in PBS and combined in an Eppendorf vial. After centrifugation at 1250 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 7 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.

Analysis of Olive Oil Phenols and Metabolites. *HPLC Procedure.* HPLC analyses were performed on an Agilent 1100 liquid chromatographic system equipped with a diode array UV−vis detector and a Rheodyne injection valve (20 μL loop). 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. Agilent Chemstation software system controlled the equipment and carried out the data processing. Elution was performed at a flow rate of 1.0 mL/min at room temperature, using as mobile phase a mixture of 1% (v/v) formic acid in deionized water (solvent A) and acetonitrile (solvent B). The solvent gradient changed according to the following conditions: from 100% A to 95% A in 10 min, to 85% A in 10 min, to 70% A in 5 min, to 65% A in 5 min, to 90% A in 5 min, and to 100% A in 5 min, followed by 5 min of maintenance. Chromatograms were acquired at 280 nm.

For quantification of hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate and their metabolites, standards of the three parent compounds were prepared in serum-free culture medium in a range of concentrations from 3.125 to 125 μM. A linear response was obtained for all of the standard curves, as checked by linear regression analysis. Percentage of recovery of standards added to the culture medium (varying from 91 to 100%), limits of detection (ranging from 0.5 to 0.75 μM), limits of quantification (1−2 μM), and precision of the assay (as the coefficient of variation, ranging from 1.9 to 10.2%) were considered to be acceptable and allowed quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecules).

LC-MS measurements were performed on an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies, Waldbronn, Germany). The liquid chromatographic system consisted of a quaternary pump (G1311A), an on-line vacuum degasser, an autosampler (G1313A), and a 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* 100−500) at a scan rate of 1.5 s. Selected ion monitoring was conducted at *m*/*z* 123, 153, 137, and 195. Diode array spectra were registered from 190 to 450 nm, with an interval of 2 nm.

In Vitro Conjugation of Olive Oil Phenols. Standard solutions of olive oil phenols were enzymatically conjugated in vitro using a pure enzyme or a rat liver microsomal fraction that contained both UDP-glucuronosyltransferase and sulfotransferase.

Preparation of Liver Microsomal Fraction. Livers from adult Wistar rats were frozen immediately after removal by immersion in liquid nitrogen. A piece of liver was 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. Liver microsomes were separated by successive centrifugation at 700*g* for 10 min and at 5000*g* for 10 min in a refrigerated centrifuge (4 °C) to remove nuclei and mitochondria. 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 (*28*). 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., München, Germany).

In Vitro Glucuronidation. Glucuronidation of olive oil phenols was performed using UDP-glucuronosyltransferase (UGT) from rat liver microsomes in the presence of UDP-glucuronic acid as the glucuronosyl

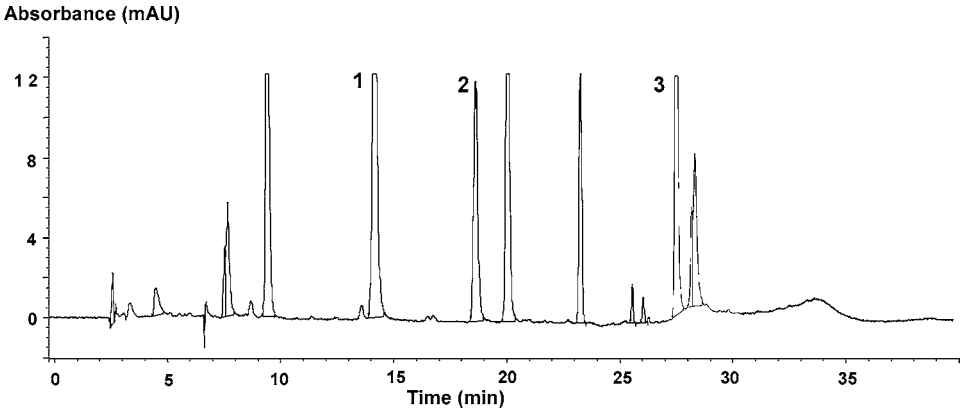

Figure 2. Chromatographic profile of serum-free culture medium spiked with 100 μM hydroxytyrosol (**1**), tyrosol (**2**), and hydroxytyrosyl acetate (**3**).

donor. The reaction mixture consisted of 390 μL of 50 mM Tris−chloride buffer, pH 7.4, containing 10 mM $MgCl_2$ and 1 mM DTT, 50 μL of UDP-glucuronic acid (5 mM final concentration), and 20 μL of liver microsomal suspension (100 μg of protein). The reaction was started by the addition of 40 μL of the phenolic compound solution (100 μM final concentration). Incubations were performed at 37 °C for 4 h (*29*). Aliquots of the reaction mixture were taken and analyzed by HPLC.

In Vitro Sulfation. Hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate were treated with cytosolic sulfotransferases from rat liver microsomes, using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfate donor. The assay mixture consisted of an 80 μM concentration of the polyphenol, 100 μM PAPS, 5 mM $MgCl_2$, and the enzyme protein (0.3 mg) in 50 mM Tris−HCl buffer, pH 7.4, in a total volume of 500 μL (*30*). The reaction was initiated by the addition of the liver microsomal fraction and incubated for 120 min at 37 °C, and then aliquots of the reaction mixture were taken and analyzed by HPLC.

In Vitro Methylation. Olive oil phenols were methylated in vitro using commercial catechol-*O*-methyltransferase (COMT) and *S*-adenosyl-L-methionine (SAM) as the methyl group donor. One hundred microliters of COMT (1000 units/mL) dissolved in 0.1 M phosphate buffer, pH 7.9, containing 10 mM $MgCl_2$ and 1 mM DTT, was kept at 37 °C for 10 min. After this time, SAM and the phenolic compound dissolved in the same buffer were added to final concentrations of 5 mM and 80 μM, respectively, in a total volume of 500 μL. The mixture was incubated at 37 °C for 4 h (*31*). The reaction was stopped by the addition of 25 μL of 4 mM perchloric acid, and the mixture was analyzed by HPLC.

Enzymatic Hydrolysis of Phenolic Conjugates from the Cell Culture. Metabolites obtained after incubation of HepG2 cells with olive oil phenols (100 μM) during 18 h were hydrolyzed enzymatically by incubating the culture media with β-glucuronidase (*32*) and sulfatase (*31*) and analyzing released compounds by HPLC to confirm conjugate identifications.

RESULTS AND DISCUSSION

Figure 2 shows the chromatographic profile of fresh culture medium spiked with 100 μM hydroxytyrosol (**1**), tyrosol (**2**), and hydroxytyrosyl acetate (**3**). Olive oil phenols, as well as their metabolites (**Figure 3**), were well resolved from other peaks corresponding to the culture medium, allowing quantitative and qualitative analysis of the studied phenols. No transformation of these compounds was observed when incubated in culture medium in the absence of HepG2 cells for up to 18 h. Conversely, two new peaks at 18.4 and 19.2 min appeared after 18 h of incubation of HepG2 cells in FBS-free culture medium with no added phenolic (data not shown). These new peaks, with absorption maxima at 273 and 258 nm, respectively, probably corresponded to basic metabolites formed by HepG2 cells in culture. None of these peaks interfered in the analysis of olive oil phenols or their metabolites.

Olive Oil Phenolic Metabolism by HepG2 Cells. Hepatic metabolism of hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate was monitored at short (2 h) and long (18 h) incubation times, analyzing the products formed as a consequence of cell metabolism in the extracellular culture medium as well as in cytoplasmatic contents after cell lysis. Cell uptake and metabolism of phenols was slow, with most hydroxytyrosol and tyrosol found as the original molecules in the culture medium after 2 h of incubation (data not shown). Only a new peak at 16.1 min (M2) was observed after incubation with hydroxytyrosol. Hydroxytyrosyl acetate showed an interesting behavior, with formation of deacetylated hydroxytyrosol after only 2 h, together with small amounts of peaks M1 + M2, M5, and M7 (data not shown). Because hydroxytyrosyl acetate was stable in the culture medium, the deacetylated hydroxytyrosol detected in the extracellular medium should be ascribed to the action of the hepatic cells.

At longer incubation times (18 h), up to five new peaks (M1−M5) were observed when cells were incubated with hydroxytyrosol, which could correspond to conjugated metabolites (**Figure 3A**). These same peaks, together with peak M7 and minor amounts of free hydroxytyrosyl acetate, were detected when cells were treated with this acetylated phenol (**Figure 3C**). Conversely, tyrosol appeared to be poorly metabolized, and only a minor peak at 15.7 min (M6) was formed after 18 h of incubation with HepG2 cells (**Figure 3B**). Most of this phenol appeared as the free molecule in the cell culture medium, pointing to a poor uptake and/or metabolism of tyrosol. On the other hand, only traces of free hydroxytyrosol, tyrosol, or hydroxytyrosyl acetate and some of their potential metabolites could be observed in the cell lysates after 2 and 18 h of incubation, suggesting no intracellular accumulation of these compounds or their metabolites (data not shown).

Identification of Olive Oil Phenolic Metabolites. To identify the different metabolites formed after incubation of olive oil phenols with human hepatoma cells, several steps were followed: in vitro conjugation of pure standards, enzymatic hydrolysis with β-glucuronidase and sulfatase of metabolites formed by HepG2 cells, and confirmation of structures by LC-MS.

In vitro conjugation of pure olive oil phenols was performed with COMT or rat liver microsomes in the presence of the corresponding methyl, sulfate, or glucuronic acid donors. No new peaks were found after in vitro sulfation (data not shown), and only a new peak derived from hydroxytyrosol was observed after incubation with COMT (data not shown). The spectroscopic characteristics of this peak (spectrum width and λ_{max} at 280 nm) were close to those of metabolite M5 and coincident

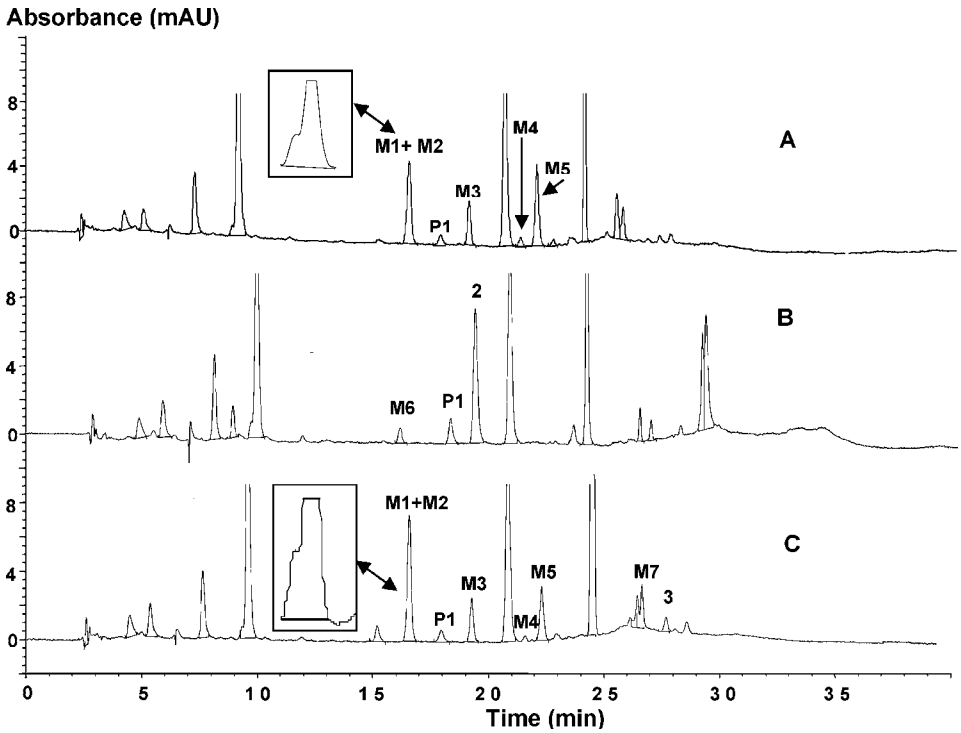

Figure 3. Typical profile of the chromatographic separation of different molecular species detectable in medium from HepG2 cells in culture after incubation for 18 h with 100 μM concentrations of the following compounds: (**A**) hydroxytyrosol (**1**); (**B**) tyrosol (**2**); and (**C**) hydroxytyrosyl acetate (**3**). Peaks M1–M7 correspond to different metabolites of the studied olive oil phenols formed after incubation with the hepatic cells; peak P1 is accumulated in the medium after 18 h of incubation in the absence of test compounds.

with homovanillic alcohol (4-hydroxy-3-methoxyphenylethanol). Spiking with homovanillic alcohol of this sample and that of the culture medium from cells incubated with hydroxytyrosol for 18 h confirmed the nature of this peak (M5) as the monomethylated derivative of hydroxytyrosol. No methyl conjugate of tyrosol was observed (data not shown) as was expected because methylation by COMT requires an ortho-diphenolic structure absent in tyrosol. When hydroxytyrosyl acetate was subjected to in vitro methylation, only free hydroxytyrosol and metabolite M5 could be detected, suggesting deacetylation of hydroxytyrosyl acetate in these in vitro conditions (data not shown).

Concerning in vitro glucuronidation, besides the peak corresponding to glucuronic acid (at 15.1 min, **Figure 4A**), major peaks of nonmetabolized hydroxytyrosol at 14.3 min (**Figure 4B**) and tyrosol at 18.9 min (**Figure 4C**) appeared after 4 h of treatment with liver microsomes. Of the new peaks observed when hydroxytyrosol was subjected to in vitro glucuronidation (**Figure 4B**), two of them (M1 and M2) showed retention times and spectroscopic characteristics similar to the peaks observed after incubation of hydroxytyrosol with HepG2 cells (**Figure 3A**). Similarly, the new peak found after in vitro glucuronidation of tyrosol, M6 (**Figure 4C**), was coincident with the metabolite formed after incubation of tyrosol with the human hepatoma cells (**Figure 3B**). A peak with a similar retention time was observed in the chromatogram corresponding to hydroxytyrosol glucuronidation, yet the spectroscopic characteristics of this peak ($\lambda_{max} = 259$ nm) were different from those of M6 ($\lambda_{max} = 272$ nm). When hydroxytyrosyl acetate was subjected to in vitro glucuronidation, the chromatographic profile of the samples was similar to that of free hydroxytyrosol (data not shown), suggesting that esterases in the rat liver microsomal fraction were capable of hydrolyzing the ester linkage to yield deacetylated hydroxytyrosol, which was then glucuronidated. No glucuronide derivative of hydroxytyrosyl acetate was detected in vitro. In all cases, the yield of in vitro conjugation was very low, as it occurred in the methylation assay.

Figure 5 shows the chromatograms obtained after treatment with β-glucuronidase of culture media after 18 h of incubation with hydroxytyrosol (**Figure 5A**), tyrosol (**Figure 5B**), or hydroxytyrosyl acetate (**Figure 5C**). Metabolites M1 + M2, M3, and M4 observed in the culture medium of cells incubated with hydroxytyrosol (**Figure 3A**) disappeared after treatment with β-glucuronidase. In turn, the area corresponding to peak M5 increased, and hydroxytyrosol, formerly absent in the medium, could be detected (**Figure 5A**). This indicates that the peaks that disappeared were glucuronides and methylglucuronides of hydroxytyrosol, because removal of the glucuronic acid moiety resulted in the formation of the parent hydroxytyrosol (**1**) and methylhydroxytyrosol (M5). Similarly, peak M6 in the culture medium of cells treated with tyrosol (**Figure 3B**) disappeared after hydrolysis with β-glucuronidase (**Figure 5B**), suggesting that this metabolite corresponded to the glucuronide derivative of tyrosol. Peaks M1 + M2, M3, M4, and M7 disappeared when culture media from cells incubated with hydroxytyrosyl acetate were hydrolyzed with β-glucuronidase, with a concomitant increase of free hydroxytyrosol and M5 peak areas and the appearance of free hydroxytyrosyl acetate (**Figure 5C**). As shown above, M1−M4 would correspond to glucuronides and methylglucuronides of hydroxytyrosol, whereas M7 would probably be a glucuronidated metabolite of hydroxytyrosyl acetate, accounting for the detection of free hydroxytyrosyl acetate after β-glucuronidase treatment.

Addition of sulfatase to serum-free culture medium resulted in a complicated chromatogram, with several new peaks appearing within the first 14 min of analysis. Fortunately, no further new peaks were observed at longer retention times, which allowed identification of metabolites in culture media from cells incubated with olive oil phenols after hydrolysis with sulfatase (**Figure 6**). The same metabolites as in nonhydrolyzed samples

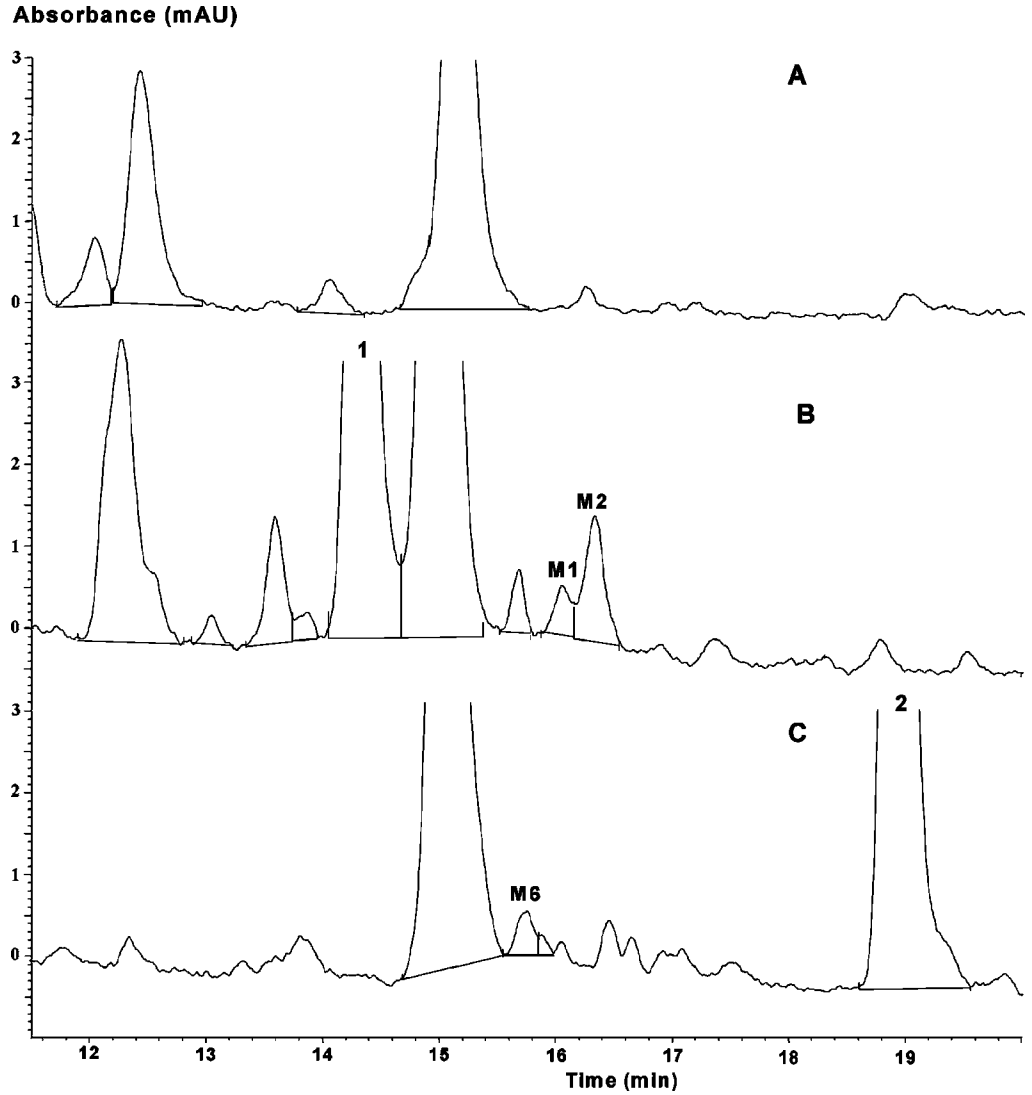

Figure 4. HPLC chromatograms of reaction media after in vitro glucuronidation with liver microsomes: (**A**) reaction medium with no added phenol (blank); medium after in vitro glucuronidation of (**B**) hydroxytyrosol (**1**) or (**C**) tyrosol (**2**). Peaks M1, M2, and M6 correspond to glucuronidated metabolites.

were observed after incubation with sulfatase (i.e., M1−M5 in media from hydroxytyrosol treatments, M1−M5 plus M7 in those from hydroxytyrosyl acetate, and M6 in media from cells treated with tyrosol), suggesting that no sulfate-conjugated metabolites of olive oil phenols are formed by HepG2 cells in culture. This agrees with the absence of sulfated metabolites after in vitro conjugation with liver microsomes mentioned above.

Culture media from incubations of HepG2 cells for 18 h with olive oil phenols were analyzed by LC-MS in an attempt to identify metabolites on the basis of the *m*/*z* values of the molecular ions and fragment ions. These results together with the UV−vis spectroscopic data are given in **Table 1**. Metabolites M1 and M2, which could not always be properly resolved due to their close retention times, showed similar UV spectra and a $[M - H]^-$ ion at *m*/*z* 329, characteristic of a monoglucuronidated metabolite of hydroxytyrosol. A fragment ion at *m*/*z* 153 corresponding to the deprotonated parent molecule (hydroxytyrosol) after loss of dehydrated glucuronic acid ($[M - H_2O]^-$ 176) was also observed. These data, along with those from the in vitro conjugation and enzymatic hydrolysis experiments, confirmed that these metabolites corresponded to the monoglucuronides of hydroxytyrosol, although we could not distinguish between structural isomers (4-hydroxy-3-glucuronide phenylethanol or 4-glucuronide-3-hydroxy phenylethanol).

Peaks M3 and M4, which were not found after in vitro conjugation yet disappeared from the culture medium when hydrolyzed with β-glucuronidase, were identified as methylglucuronides of hydroxytyrosol. The molecular ion $[M - H]^-$ and the fragment ions at *m*/*z* 343, 167, and 153 corresponded to the methylglucuronide, the monomethyl derivative (after the loss of dehydrated glucuronic acid), and the deprotonated parent molecule hydroxytyrosol (after a further loss of the methyl group), respectively. Again, we could not differentiate between isomers (4-methoxy-3-glucuronide phenylethanol or 4-glucuronide-3-methoxy phenylethanol).

Surprisingly, metabolite M5 did not give any signal in the MS detector after electrospray ionization in either the positive or negative modes. The same occurred when a pure standard of homovanillic alcohol was tested. However, as mentioned before, spiking of culture medium from cells incubated with hydroxytyrosol or of the reaction mixture after in vitro methylation of hydroxytyrosol confirmed this metabolite as homovanillic alcohol (4-hydroxy-3-methoxyphenylethanol). The corresponding isomer (4-methoxy-3-hydroxyphenylethanol) was not analyzed, although no further peaks suggestive of a second

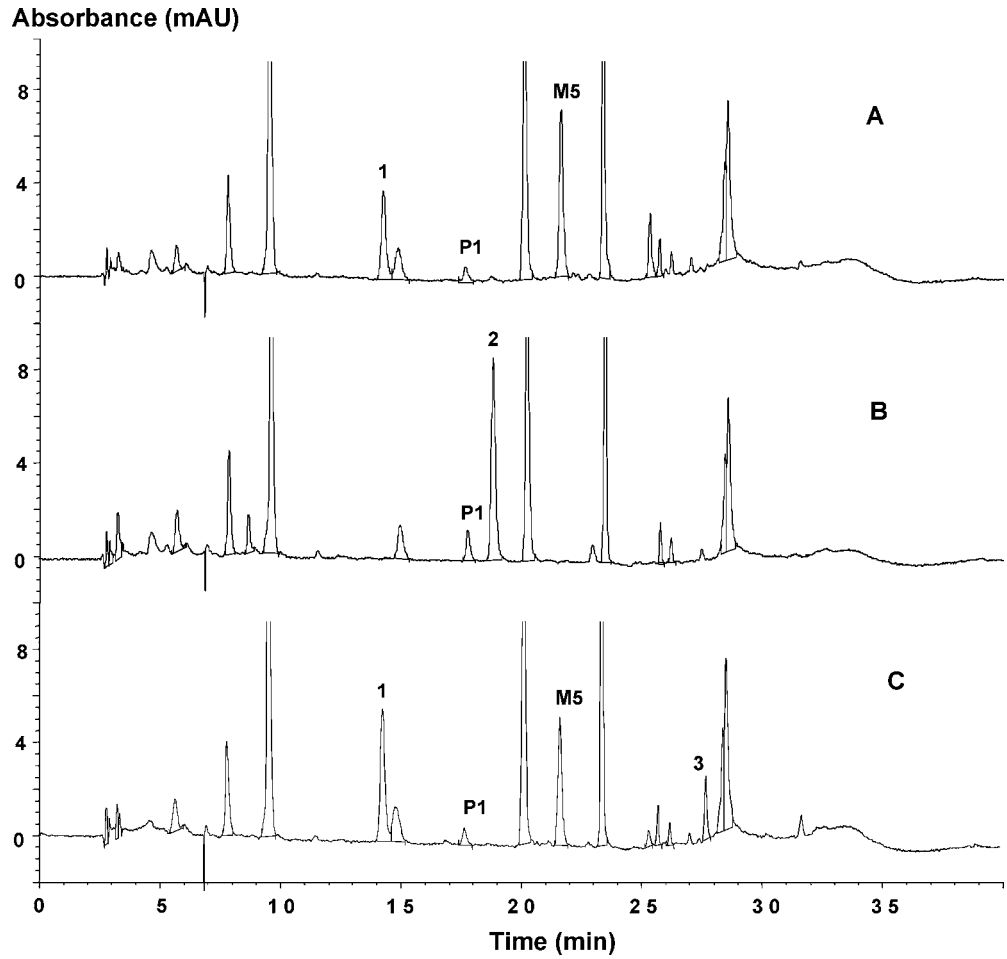

Figure 5. Chromatograms of culture media obtained after 18 h of incubation in the presence of 100 μM concentrations of the three olive oil phenols subjected to in vitro hydrolysis with β-glucuronidase. Culture medium of cells was treated with (**A**) hydroxytyrosol (**1**), (**B**) tyrosol (**2**), and (**C**) hydroxytyrosyl acetate (**3**). Peak M5 corresponds to a methylated metabolite of HTy; peak P1 is accumulated in the medium after 18 h of incubation in the absence of test compounds.

monomethylated conjugate of hydroxytyrosol were observed. It is noteworthy that this was the only metabolite that did not show any hypsochromic effect (**Table 1**), observed in the other hydroxytyrosol (and tyrosol) metabolites, with slightly lower UV-absorption maxima in comparison with the free phenolics due to the incorporation of the glucuronic acid moiety.

The tyrosol metabolite M6 was confirmed as the glucuronidated conjugate on the basis of its molecular ion at *m*/*z* 313 and fragment ion at *m*/*z* 137, corresponding to deprotonated tyrosol after the loss of dehydrated glucuronic acid (**Table 1**).

Finally, metabolite M7, which showed an absorbance maximum at 280 nm, presented a molecular ion $[M - H]^-$ at *m*/*z* 371. No fragment ions could be seen. However, this mass would correspond to the addition of a glucuronic acid moiety to the hydroxytyrosyl acetate molecule. This, together with the fact that this peak disappeared when hydrolyzed with β-glucuronidase, suggests that M7 could be a monoglucuronidated metabolite of hydroxytyrosyl acetate.

Quantification of Metabolites. Free and conjugated metabolites were quantified in the extracellular culture medium after 2 and 18 h of incubation with HepG2 cells. Differences occurred in the extent of metabolism of the three phenols studied, depending on their chemical structure and the length of contact with the hepatic cells. After 2 h, most hydroxytyrosol was present in the culture medium as the free molecule, with metabolites representing <15% of the total phenols in the extracellular medium. Half of these metabolites were methyl conjugates, the rest being monoglucuronides and methylglucuronides (**Figure 7**). At 18 h, however, hydroxytyrosol metabolites exceeded 75% of the analyzed phenols, with ~25% of free, nonmetabolized hydroxytyrosol being detected in the culture medium. The extent of glucuronidation was comparable to that of methylation (32 versus 26% of the total amount of phenols in the culture medium, respectively), with up to 18% of methylglucuronides. Conversely, tyrosol was poorly metabolized by HepG2 cells in culture. Virtually no tyrosol was metabolized after only 2 h of incubation, whereas <10% of this olive oil phenol was found as a glucuronidated metabolite after 18 h in culture with hepatocarcinoma cells (**Figure 7**).

As to hydroxytyrosyl acetate, this compound appeared to be more rapidly and extensively taken up and metabolized by HepG2 cells than hydroxytyrosol. Only 57 and 9% of free hydroxytyrosyl acetate were detected in the culture medium after 2 and 18 h, respectively. It is worth noting that nearly half of the hydroxytyrosyl acetate metabolites detected after 2 h of incubation corresponded to free hydroxytyrosol (22% of the total phenols in the culture medium). However, after 18 h no free hydroxytyrosol could be seen (**Figure 3C**), probably due to the efficient conjugation of deacetylated hydroxytyrosol. The extent of glucuronidation was higher in samples incubated with hydroxytyrosyl acetate, with 85 and 79% of the total amount of conjugated metabolites after 2 and 18 h, respectively (18 and 72% of total phenols, respectively), corresponding to glucuronides (with or without methylation) (**Figure 7**).

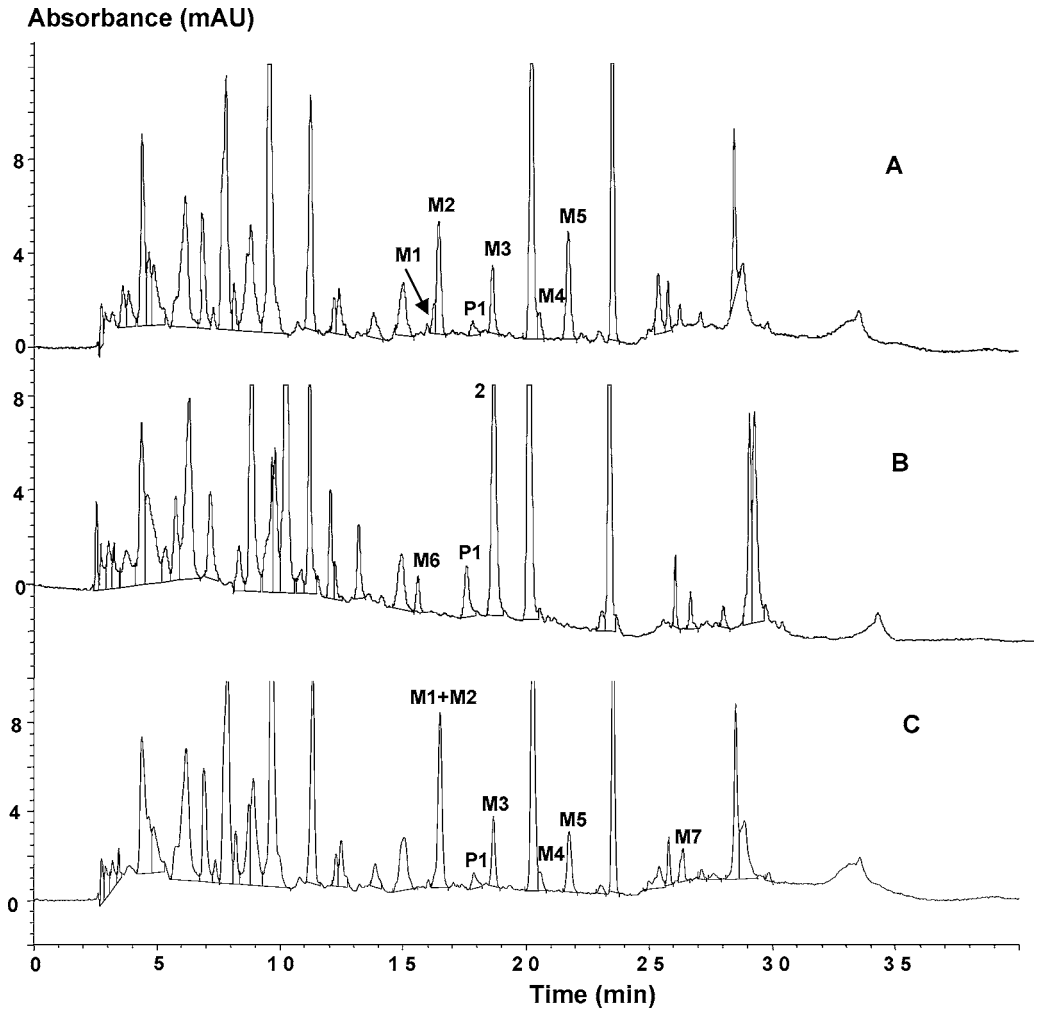

Figure 6. Chromatographic profile of culture media obtained after 18 h of incubation in the presence of 100 μM concentrations of the three olive oil phenols subjected to in vitro hydrolysis with sulfatase. Culture medium of cells was treated with (**A**) hydroxytyrosol (**1**), (**B**) tyrosol (**2**), and (**C**) hydroxytyrosyl acetate (**3**). Peaks M1–M7 correspond to metabolites of olive oil phenols.

Table 1. Chromatographic and Spectroscopic Characteristics of Hydroxytyrosol (**1**), Tyrosol (**2**), and Hydroxytyrosyl Acetate (**3**) and the Metabolites Formed after Incubation with HepG2 Cells

compound	MW	RT	λ_{max}	$[M-H]^-$	fragment ions	proposed structure
1	154	15.0	280	153.0	123.1	hydroxytyrosol
M1	330	16.2	277	329.0	153.0	monoglucuronide
M2	330	16.4	278	329.0	153.0	monoglucuronide
M3	344	18.7	276	343.1	166.9–153.0	methylglucuronide
M4	344	20.7	277	343.1	166.8–153.0	methylglucuronide
M5	168	21.8	280			homovanillic acid
2	138	18.9	276	137.1	119.1	tyrosol
M6	314	15.2	272	313.1	137.1	glucuronide
3	196	27.9	282	195.1	137.1–153.1–123.1	hydroxytyrosyl acetate
M7	372	26.5	280	371.1	no fragments	monoglucuronide

The results obtained here suggest that phase II metabolism of olive oil phenols takes place primarily in the liver, because extensive glucuronidation and methylation of olive oil phenols (except for tyrosol, which was only monoglucuronidated) took place after incubation with HepG2 cells. However, further studies either with Caco-2 cells or with perfused intestinal segments are needed to ascertain the lack of major metabolism of these phenolic compounds by the intestinal epithelium.

The main metabolites produced by HepG2 cells were glucuronides, methylglucuronides, and methyl conjugates, whereas no sulfate conjugates of any of the assayed phenols could be detected. It has been reported that this cell line has a low sulfotransferase activity (*33*). However, the absence of the formation of sulfated metabolites in vitro is in agreement with data from human studies, in which methylated and glucuronidated metabolites were the only conjugates observed in plasma and urine samples (*15*, *17*−*21*). Sulfate metabolites were detected in urine from animals only after intravenous administration of hydroxytyrosol (*22*−*24*), indicating that rat hepatocytes are capable of sulfation of this phenol. Nevertheless, when we attempted in vitro sulfation of olive oil phenolics using microsomes obtained from rat liver, no metabolites could be detected after 2 h of incubation. Perhaps longer incubation times would have been needed to achieve conjugation in vitro, although rat liver microsomes have been shown to be able to sulfate quercetin and isorhamnetin after 1 h of incubation (*30*).

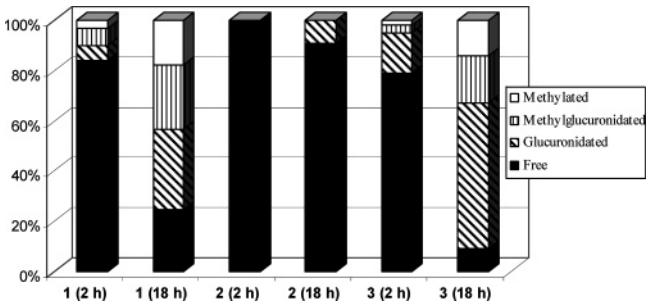

Figure 7. Relative percentages of parent compounds (black segments), glucuronides (slashed segments), methylglucuronides (striped segments), and methylated metabolites (open segments) accumulated in the culture media after 2 and 18 h of incubation of HepG2 cells in the presence of 100 μM hydroxytyrosol (**1**), tyrosol (**2**), and hydroxytyrosyl acetate (**3**). For hydroxytyrosyl acetate at 2 h, parent compounds comprise free plus deacetylated (**3**).

The extents of methylation and glucuronidation of hydroxytyrosol were similar after 2 h of incubation, although glucuronidation prevailed in the case of hydroxytyrosyl acetate. At longer incubation times (18 h), glucuronidation clearly predominated over methylation, especially for hydroxytyrosyl acetate, which might suggest induction of the activity of UGT isoforms by these compounds. Phase I and phase II enzymes are known to be inducible in hepatocytes (*34*), and specifically UGT1A1 has been shown to be inducible by the dietary flavonol quercetin both in Caco-2 cells and in human enterocytes (*35*).

Tyrosol, lacking an ortho-diphenolic structure, could not by methylated by COMT and the only metabolite formed was the glucuronide, although in this case the extent of biotransformation was surprisingly low, probably due to limited diffusion of tyrosol into the cell. Passive diffusion has been suggested as the mechanism of transport responsible for hydroxytyrosol uptake in Caco-2 cells (*25*). A lower degree of hydroxylation, such as in the case of tyrosol (**Figure 1**), should enhance diffusion through the lipid bilayer in cell membranes, minimizing interactions between the phenol hydroxyl groups and polar groups of membrane lipids (*36*). However, the results obtained here, with most tyrosol recovered unchanged in the culture medium, seem to indicate poor uptake of this phenol by HepG2 cells. Should similar reduced uptake occurred at the intestinal level, this would account for the low urinary recovery of tyrosol observed in humans (*19*).

Conversely, uptake of hydroxytyrosyl acetate appeared to be more effective than that of hydroxytyrosol and tyrosol. Chain length has been shown to increase uptake of alkyl gallates in Caco-2 cells and phospholipid vesicles (*36*), which might explain the lower amount of hydroxytyrosyl acetate (57% of the total phenols) in the culture medium after 2 h of incubation in comparison with cells treated with hydroxytyrosol (85%). Absorbed hydroxytyrosyl acetate can be glucuronidated by UGT as shown by the formation of a monoglucuronide (M7) (**Figure 3C**). Extensive deacetylation of hydroxytyrosyl acetate by carboxylesterases can also take place, generating free hydroxytyrosol. The hydrolysis of hydroxytyrosyl acetate can take place either within the cell upon absorption of the acetylated molecule or extracellularly by secreted esterases, accounting for the appreciable amount of free hydroxytyrosol detected in the culture medium after 2 h of incubation of HepG2 cells with hydroxytyrosyl acetate, which amounted to 22% of the total phenols. Either way, free hydroxytyrosol formed after hydrolysis was metabolized by phase II enzymes COMT and UGT as shown above.

In summary, extensive uptake and metabolism of the olive oil phenols hydroxytyrosol and hydroxytyrosyl acetate were observed using HepG2 cells as a model system of the human liver, with scarce metabolism of tyrosol. Glucuronidated and methylated conjugates were the main derivatives formed, resembling the metabolic profile of olive oil phenols observed in human plasma and urine.

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