ABSTRACT:

Hydroxytyrosol (HOTYR) and tyrosol (TYR), main phenolic compounds of olive oil, have been reported to contribute to the prevention of cardiovascular diseases due to their antioxidant activities, e.g., protection of low-density lipoprotein (LDL) oxidation. Their bioavailability in humans is poor, and they are found in biological fluids mainly as conjugated metabolites. Low concentrations of free phenols are unlikely to explain the biological activities seen in humans after olive oil intake. In this context, antioxidant activities of conjugated metabolites, in a range of concentrations compatible with their dietary consumption, were evaluated. Concentrations of metabolites and their core compounds were estimated in an intervention study of 11 healthy volunteers whose diet was supplemented with 50 ml of virgin olive oil, using high-performance liquid chromatography coupled to mass spectrometry for the simultaneous analysis of 3′-O- and 4′-O-HOTYR-glucuronides and 4′-O-glucuronides of TYR and homovanillil alcohol in human urine. Glucuronides and core compounds were tested for their chemical (hydrogen donation by 1,1-diphenyl-2-picrylhydrazyl free radical test) and in vitro biological (inhibition of Cu-mediated LDL oxidation) antioxidant activities at the concentration ranges observed in human biological fluids (range, 0.01–10 μM) after dietary olive oil consumption. None of the glucuronides displayed significant antioxidant activities at the concentrations tested.

Introduction

Recent intervention clinical trials have provided evidence that the phenolic content of an olive oil contributes to the protection against lipid oxidative damage in humans in a dose-dependent manner (Covas et al., 2006a,b). Some of the most representative phenolic compounds in olive oil are hydroxytyrosol (HOTYR) and tyrosol (TYR) and their secoiridoid derivatives oleuropein and ligstroside, respectively. One of the first steps in relating biological activities of dietary phenol compounds to health benefits in humans is to demonstrate their bioavailability from diet. Several intervention studies in human and animal models have reported that olive oil phenolic compounds are rapidly absorbed and extensively metabolized (Visioli et al., 2000; Weinbrenner et al., 2004). As a result, olive oil phenols appear in biological fluids mainly as phase II metabolites (e.g., glucuronides and sulfates) of HOTYR, TYR, and O-methylconjugate of HOTYR [homovanillil alcohol (HVAlc)], whereas their free forms are present only at very low concentrations (Miró-Casas et al., 2001, 2003a). The transformation of HOTYR and TYR into their phase II metabolites was predicted to negatively influence their activities as antioxidants (Nenadis et al., 2005). However, in a study of rats (Tuck et al., 2002), it was reported that urinary excretion of 3′-O-HOTYR-glucuronide, but not its 3′-O-sulfate conjugate, was a more potent antioxidant [by 1,1-diphenyl-2-picrylhydrazyl (DPPH) test] than its parent compound HOTYR. Therefore, the proper evaluation of the bioactivity of olive oil phenol metabolites is crucial to understanding the impact of metabolism on the beneficial health effects promoted by dietary consumption of phenol-rich olive oils (Weinbrenner et al., 2004; Covas et al., 2006b). In addition, properly estimated concentrations for both phenols and their main metabolites in humans after olive oil consumption are required to establish the concentration range for their in vitro and in vivo bioactivities evaluation.

The aim of the present study was to assess the antioxidant capacity of the olive oil phenol glucuronides, within a range of biologically relevant concentrations. The range of concentrations tested was derived from those detected in human urine after a dietary dose of virgin olive oil (VOO).

Materials and Methods

Reagents and Chemicals. Hydroxytyrosol [2-(3,4-dihydroxyphenylethanol] and tyrosol [4-hydroxyphenylethanol] were purchased from...
Extrásynthèse (Lyon, France). Homovanillyl alcohol, 3-(4-hydroxyphenyl)propanol (HOPhPr), DPPH free radical, and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were supplied by Sigma-Aldrich (St. Louis, MO). Methanol (MeOH) and acetonitrile were of analytical grade and were obtained from Schaarlau Chemie (Barcelona, Spain). The mobile phase was filtered through a 0.22-μm nylon membrane (Whatman, Maidstone, UK). Sodium bisulfite, acetic acid, ammonium hydroxide, hypochloric acid, and phosphoric acid were supplied by Merck (Lichrosolv; Barcelona, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). 4’-O-hydroxytyrosol glucuronide (‘O-Gluc-HOTYR), 3’-O-hydroxytyrosol glucuronide (‘O-Gluc-HYR), 4’-O-tyrosol glucuronide (‘O-Gluc-TYR), 4’-O-homovanillyl alcohol glucuronide (‘O-Gluc-HVAalc), and 4’-O-hydroxyphenylpropanol glucuronide (‘O-Gluc-HOPhPr) were synthesized according to a previously described method (Khymenets et al., 2006) (Fig. 1). Standard stock solutions of all analytes (olive oil phenols and corresponding glucuronides) and corresponding internal standards (I.S.; HOPhPr and ‘O-Gluc-HOPhPr as I.S.2 and I.S.1, respectively) were prepared in MeOH and kept at –20°C.

Study Design and Sample Collection. Eleven healthy volunteers (six males and five females, aged 22–44 years) were recruited. The institutional ethics committee Comité Étic de Investigació Clínica-Institut Municipal d’Assistència Sanitària approved the protocol, and the participants signed an informed consent document. Before the dietary intervention, volunteers followed a 1-week washout period during which they followed low-phenolic antioxidant diet, and the last 3 days they were also asked to exclude any phenol-rich food from their diet. Sunflower oil was provided as a source of fat for all purposes. On the intervention day, at fasting state, 50 ml (44 g) of VOO were administered in a single dose with bread (200 g).

The amount of total HOTYR, HVAalc, and TYR in VOO was determined as described previously (Miró-Casas et al., 2003b).

A spot urine sample was collected at 8:00 AM at fasting state. Urine was collected from 0 to 6 and 6 to 24 h after VOO consumption on the intervention day, preserved with sodium bisulfite (1 mM final concentration) at acidic conditions (0.24 M HCl final concentration), and stored at –20°C before use.

Analysis of Free and Glucuronon conjugated HOTYR, TYR, and HVAalc in Urine Samples by Solid-Phase Extraction-Ultraperformance Liquid Chromatography-Multiple Reaction Mode. Samples were spiked with 500 ng/ml I.S.1 and 1000 ng/ml I.S.2, diluted 1:1 with 4% H3PO4, and applied to preconditioned columns with 2 ml of MeOH and equilibrated with 2 ml of water (Oasis HLB 3 cc, 60-mg cartridges; Waters Corporation, Dublin, Ireland). They were then washed with 2 ml of water, extracted with 3 ml of MeOH, evaporated under nitrogen (25°C, 10–15 psi), reconstituted in 200 μl of solvent A of mobile phase, filtered using Spin-X Centrifuge 2-ml polypropylene tubes with a 0.22-μm Corning nylon filter (Corning Life Sciences, Lowell, MA), and analyzed by ultraperformance liquid chromatography-mass spectrometry as described below.

Analysis was performed using a Waters Acquity UPLC system (Waters, Milford, MA) coupled to a triple quadrupole (Quattro Premier XE; Waters) mass spectrometer with an electrospray interface (Waters). Gradient chromatographic separation of HOTYR, TYR, HVAalc, and their glucuronides was performed on an Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7-μm particle size) (Waters Corporation) at 40°C using 1 mM ammonium acetate at pH 5 (mobile phase A) and 100% acetonitrile (mobile phase B). The flow rate was 0.4 ml/min. All compounds were monitored in negative ionization using the multiple reaction mode (for details, see Supplemental Table 1).

HOTYR, TYR, and HVAalc were quantified by comparing their peak area ratios with HOPhPr (as I.S.2) and their glucuronides with ‘O-Gluc-HOPhPr (as I.S.1). The correlation coefficient was >0.99 in all cases, and their linear range was 20 to 2000 ng/ml. Both limits of detection and quantification in all cases were not higher than the lower calibrator (20 ng/ml), except for HOTYR, and recovery was within 89 to 97% for all tested compounds.

Low-Density Lipoprotein Resistance to Oxidation Assay. Low-density lipoprotein (LDL) extraction from blood samples and conjugated dienes formation, after Cu-mediated LDL oxidation were performed as described previously (Fitó et al., 2000), with some modifications for testing in small sample volumes. In brief, 160 μl of LDL was diazylated against phosphatase-buffered saline (final concentration, 0.05 g of apolipoprotein-B/liter) and incubated with 10 μl of MeOH in the presence (samples) or absence (control) of the tested compounds. Next, 10 μl of a 100 μM cupric sulfate solution was added to a 96-well, half-area, flat-bottom UV-transparent microplate (Corning Life Sciences). In addition, 10 μl of mineral oil (Sigma-Aldrich) was layered over the reaction mixture, and the plate was covered with an adhesive optical transparent film. Absorbance at 234 nm was continuously monitored at 15-min intervals for 24 h at 36°C using an Infinite M200 Reader (Tecan Iberica, Minndorf, Switzerland). Controls and samples at 0.01, 0.1, 1, 5, and 10 μM (for all tested compounds) were evaluated in run in duplicate at the same time, and each experiment was repeated three times. The length of the lag phase was determined by the intercept of the propagation phase tangent and the extrapolated line for the slow reaction. Lag time was calculated as the ratio between values observed for each compound and those corresponding to the control LDL oxidation reaction. The interseries CV(%) was 9.4.

DPPH Assay. The antiradical capacity of all tested compounds was evaluated by the DPPH stable radical method, as described previously (Touriño et al., 2005), with some modifications for testing in small reaction volumes. In brief, compounds (5 μl) were added to aliquots (250 μl) of a 60 μM DPPH solution, both made up in degassed MeOH, in a 96-well transparent flat-bottom microwell (Greiner Bio-One GmbH, Frickenhausen, Germany), covered by an adhesive ViewSeal transparent film (Greiner Bio One GmbH) and analyzed for absorbance at 517 nm in an Infinite M200 Reader every 2 min up to 1 h and subsequently every 30 min over the next 23 h at 25°C in the dark. Each calibration curve and the five different concentrations of the tested compounds (0.002, 0.125, 0.25, 0.5, and 1 mM) were acquired in duplicate, and each experiment was repeated three times. The percentage of remaining DPPH was calculated according to the calibration curves made separately for each experiment and read within the same plate and at the same time points as the analyzed samples. The within plate and between plate CV(%) of slopes for calibration curves, as well as intraday and interday performance, did not exceed 10% for the 24-h period of kinetic measurements. Results were expressed as ED50 (the concentration able to consume half the initial amount of free radical, expressed as the molar ratio compound to radical) and inversely to its antiradical capacity. The total stoichiometry of the reaction was evaluated at three points (30 min, 4 h, and 24 h) and expressed as amount of hydrogen atoms transferred from the analyte to the DPPH radical according to the following formula: nH = 1/(ED50 × 2).

Data Evaluation, Quantification, and Statistical Analysis. Normality of continuous variables was assessed by the Kolmogorov-Levene test. A least-squares (1/Ed) regression analysis was used to obtain correlation coefficients and slopes. For data comparison, a paired Student’s t test was used. Data from LDL resistance to oxidation test were evaluated using one-way analysis of variance. Statistical analyses were performed with...
SPSS for Windows (version 12.0; SPSS Inc., Chicago, IL), and significance was defined as $P < 0.05$. Data are expressed as mean ± S.D.

Results and Discussion

The total amounts of HOTYR and TYR consumed with 50 ml of VOO, estimated as a fraction combining free forms of HOTYR and TYR and those resulting from the acidic hydrolysis of their secoiridoid derivatives present in olive oil, were 22.0 ± 1.4 (3.38 ± 0.22 mg) and 15.2 ± 0.9 μM (2.10 ± 0.12 mg), respectively. Despite a washout period, almost all basal urines (0 h) contained some traces (under quantification limit of the method applied) of HOTYR, TYR, and HVAlc and their glucuronides (4′-O-Gluc-TYR, 4′-O-Gluc-HVAlc, 4′-O-Gluc-HOTYR, and 3′-O-Gluc-HOTYR) (data not shown). Concentrations of phenols and their metabolites were calculated in urine samples collected within 0 to 6 and 6 to 24 h after VOO acute intake. TYR and HOTYR, as well as the methylated metabolite of the latter, HVAlc, in their free form were detected at significant but relatively low concentrations (2.38 ± 1.14, 1.83 ± 1.12, and 1.64 ± 0.92 μM, respectively) in all urine samples collected 6 h after VOO acute intake. Lower concentrations were observed in urine samples collected from 6 to 24 h; however, they never exceeded 0.1 μM (data not shown). Concentrations of O-glucuronide metabolites were substantially higher in 6-h postprandial urine samples compared to their parent compounds: 6.65 ± 2.91, 2.66 ± 1.18, 3.89 ± 1.69, and 2.32 ± 1.31 μM for 4′-O-Gluc-TYR, 4′-O-Gluc-HVAlc, 4′-O-Gluc-HOTYR, and 3′-O-Gluc-HOTYR, respectively. Glucuronides were also detected in 6- to 24-h urine samples in a 0.1 to 0.6 μM concentration range (data not shown). Results show that the fraction of olive oil phenols absorbed is rapidly and extensively metabolized, mainly to glucuronides. On the whole, urinary concentrations of free phenols and their glucuronides do not exceed 10 μM, which might represent their highest concentration attained after olive oil dietary intake in human biological fluids.

The impact of glucuronidation on the antioxidant activity of olive oil phenols was evaluated by determining their protective effect against Cu-induced oxidation of LDL. The LDL oxidation test was performed at concentrations that covered all biologically relevant ranges: 0.01 to 0.1 μM (mainly referring to plasmatic postprandial phenols concentrations reported earlier by Miró-Casas et al. (2003a) and 1 to 5 10 μM (the postprandial urinary phenols concentrations estimated within this study). No data could be generated for HVAlc at concentrations of 5 and 10 μM due to a regular deterioration in the monitored diene conjugate formation, the cause of which was unknown to us. Both HOTYR and HVAlc expressed much stronger protective effects than Trolox against LDL oxidation by extending the lag time of dienes formation to almost twice the concentration of 1 μM (Fig. 2A), which was in agreement with previously reported data (Turner et al., 2005; Rietjens et al., 2007). In contrast, TYR appeared to have very weak activity in this model even at the highest concentration (10 μM) (Fig. 2A), in agreement with an earlier report (Briante et al., 2003). Both glucuronides of HOTYR appeared to maintain some residual activity in protecting LDL from Cu-mediated oxidation (Fig. 2A),

![Graph A](https://example.com/grapha.png)

**Fig. 2.** Antioxidant activities of olive oil phenols HOTYR and TYR and their metabolites: A, changes in lag time ratio of in vitro Cu-induced LDL oxidation in the presence of different concentrations of HOTYR, TYR, HVAlc, and their glucuronides compared with Trolox as a reference compound (see Material and Methods for explanation). *, significance at $P < 0.001$ level for linear trend of response. B, the 24- and 1-h kinetics of remaining DPPH (%) of reaction between DPPH radical and tested compounds (see Materials and Methods for explanations).
particularly at the highest concentration tested (5 and 10 μM). In contrast, the glucuronidation of HVAlc entails the loss of activity against lipid oxidation. Not surprisingly, TYR glucuronide was totally inactive. Overall, glucuronidation of phenols rapidly decreases the protective activities of olive oil phenols against Cu-mediated LDL oxidation.

Because free radical activities play a central role in LDL oxidation, olive oil phenols and their glucuronides were assessed for their radical-scavenging capacities (hydrogen donation abilities) using the DPPH assay. The 24-h kinetics of DPPH radical scavenging (percentage of remaining DPPH) of parent compounds and glucuronides are shown in Fig. 2B. After the first few minutes of reaction, HOTYR appeared to be the strongest antiradical scavenger (Fig. 2B). The Trolox reaction reached its plateau within the first 5 min of incubation, whereas HOTYR induced the first decay in absorbance within the first 3 min, followed by a slower slope (up to 60 min), and only afterward did the reaction reach its plateau. This biphasic kinetic behavior is well known for HOTYR as a catechol antioxidant (Gordon et al., 2001; Goupy et al., 2003) and can be explained by the degradation products of HOTYR that are involved in the reaction until equilibrium is reached. Compared with HOTYR, the reaction of HVAlc with DPPH was slower and the equilibrium could be reached until close to the end of the 24-h incubation period. Nevertheless, at 2 h a more reduced DPPH could be detected in the reaction with HVAlc than with Trolox, and at 24 h it was close to the amount reduced by HOTYR (Fig. 2B).

Some long-term residual activities in DPPH radical scavenging were observed for HOTYR glucuronides but not for HVAlc and TYR glucuronides. Due to the variation in the kinetic behavior of the tested compounds, their total stoichiometry of reaction was estimated at three time points: 30 min, 4 h, and 24 h (see data shown in Supplemental Table 1). Among the parent olive oil phenols, HOTYR displayed excellent radical-scavenging activities, in agreement with previous reports (Briante et al., 2003; Roche et al., 2005), whereas TYR was found to be practically inactive. HVAlc shows some scavenging activity, but it is lower than that for HOTYR with respect to kinetics and stoichiometry of reaction. Overall results demonstrate that HOTYR and HVAlc glucuronides do not display the antiradical activities present in their parent compounds. Previous observations suggesting that radical scavenging activity of glucuronides was higher than that of HOTYR do not agree with the present results (Tuck et al., 2002). The present study made use of well characterized pure compounds (Khymenets et al., 2006), instead of compounds extracted from urine. We believe that this result explains the discrepancies found in a previous report (Tuck et al., 2002). In addition, our results confirm previous theoretical studies in which the bond dissociation enthalpy of phenolic hydroxyl groups used to calculate the H-atom-donating capacity of HOTYR and its phase II metabolites glucuronides and sulfates already predicted the loss of activity of metabolites (Nenadis et al., 2005).

Results of this study show that after a single dose of VOO (50 ml), two glucuronides of HOTYR, one glucuronide of TYR, and one glucuronide of HVAlc are the main metabolites detected in humans. Although olive oil polyphenols with different degrees display radical scavenging (DPPH test) and capacity to protect LDL against oxidation, their glucuronides do not. To the best of our knowledge, this is the first report showing their antioxidant capacity at concentrations relevant to real-life doses of olive oil. Overall, the lack of antioxidant activity of glucuronides, compared with that of the parent compounds, suggests that they are not chief contributors that explain the antioxidant effects provided by olive oils rich in phenolic compounds seen in humans. Additional studies are needed to solve this enigma.

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References

Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Hospital del Mar Research Institute, Barcelona, Spain (O.K., M.P., M.Fa., R.d.l.T.); CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Santiago de Compostela, Spain (O.K., M.Fi., M.P., M.-I.C., R.d.l.T.); Cardiovascular Risk and Nutrition Research Group, IMIM-Hospital del Mar Research Institute, Barcelona, Spain (M.Fi., D.M.-A., M.-I.C.); Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona, Spain (S.T., J.L.T., J.J.); Universitat Autònoma de Barcelona (UAB), Barcelona, Spain (M.Fa.); and Universitat Pompeu Fabra (CEX-UPF), Barcelona, Spain (O.K., R.d.l.T.)

Olha Khymenets; Montserrat Fito; Sonia Tourino; Daniel Munoz-Aguayo; Mitona Pujadas; Josep Luis Torres; Jesus Joglar; Magi Farre; Maria-Isabel Covas; Rafael de la Torre


Address correspondence to: Rafael de la Torre, PharmD, PhD, Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Hospital del Mar Research Institute, C/Dr.Aiguader 88, Barcelona 08003, Spain. E-mail: rtorre@imim.es.