JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Copper on the Antioxidant Activity of Olive Polyphenols in Bulk Oil and Oil-in-Water Emulsions

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The antioxidant activity and interactions with copper of four olive oil phenolic compounds, namely oleuropein, hydroxytyrosol, 3,4-dihydroxyphenylethanol-elenolic acid (**1**), and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (**2**), in olive oil and oil-in-water emulsions stored at 60 °C were studied. All four phenolic compounds significantly extended the induction time of lipid oxidation in olive oil with the order of activity being hydroxytyrosol > compound **1** > compound **2** > oleuropein > α-tocopherol; but in the presence of Cu(II), the stability of oil samples containing phenolic compounds decreased by at least 90%, and the antioxidant activity of hydroxytyrosol and compounds **1** and **2** became similar. In oil-in-water emulsions prepared from olive oil stripped of tocopherols, hydroxytyrosol enhanced the prooxidant effect of copper at pH 5.5 but not at pH 7.4. The stability of samples containing copper at pH 5.5 was not significantly different if oleuropein was present from that of the control. Oleuropein at pH 7.4, and compounds **1** and **2** at both pH values tested, reduced the prooxidant effect of copper. The lower stability and the higher reducing capacity of all compounds at pH 7.4 could not explain the higher stability of emulsions containing phenolic compounds at this pH value. However, mixtures containing hydroxytyrosol or oleuropein with copper showed higher 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity at pH 7.4 than at pH 5.5. Moreover, the compound **2**−copper complex showed higher radical scavenging activity then the uncomplexed compound at pH 5.5. It can be concluded that the formation of a copper complex with radical scavenging activity is a key step in the antioxidant action of the olive oil phenolic compounds in an emulsion containing copper ions.

KEYWORDS: Antioxidants; hydroxytyrosol; *Olea europaea*; oleuropein; copper; polyphenols; olive oil; 3,4-DHPEA-EA; 3,4-DHPEA-EDA; emulsions

INTRODUCTION

Lipid oxidation in virgin olive oil is of great concern to the consumer because it causes not only changes in the quality attributes of food, such as shelf life, appearance, and flavor, but also a strong decrease in the nutritional value and safety caused by the loss of antioxidants and formation of potentially toxic compounds. A large body of epidemiological studies shows that the incidence of coronary heart disease (CHD) and certain cancers in the Mediterranean countries is low, suggesting that this is largely due to the relatively safe and even protective diet of this southern area where virgin olive oil is the principal source of fat (*1*). The formulation of an antioxidant/atherosclerosis hypothesis led to experimental studies on the possible role of olive oil phenols in the protection against CHD observed in the Mediterranean area. Animal and in vitro studies suggest that the relatively high concentration of phenolic compounds in extra virgin olive oil may contribute to the healthy nature of this diet (*2*−*7*). Although many studies have investigated the antioxidant properties, the protective effects against cell injury, and the bioavalability of oleuropein and its aglycones, especially hydroxytyrosol, relatively few studies have investigated the stability and protective effect of these compounds during and after food processing. In fact, virgin olive oil is consumed mainly in food emulsions or in the presence of a water phase (soup, stews, and sauces), whereas most studies of its oxidative stability have been carried out in bulk oil (*8*−*18*). However, the behavior of antioxidants is more complex in emulsions than in bulk oil because more variables influence lipid oxidation, including the emulsifier and pH (*19*, *20*). The presence of the aqueous phase often decreases the activity of antioxidants because hydrogen-bonded complexes formed with water are ineffective in scavenging lipid radicals by hydrogen donation. Lipids in food emulsions exist as lipid dispersions in an aqueous

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10.1021/jf060033j CCC: $33.50
Published on Web 04/22/2006

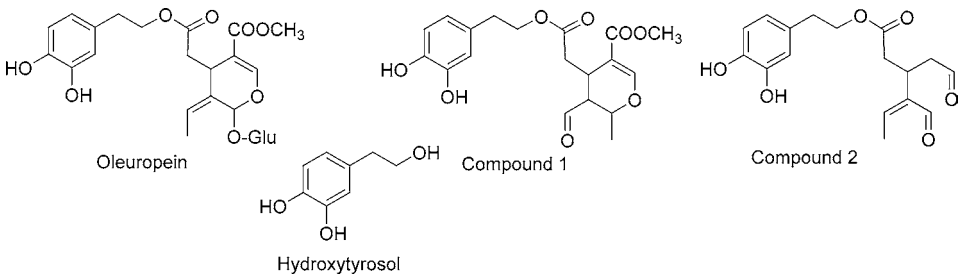

Figure 1. Structures of olive oil phenolics.

matrix that may contain a variety of water-soluble components including transition metals such as iron and copper. In the presence of these transition metal ions, both radical scavenging and metal chelation contribute to the antioxidative effects of the phenols. Phenols may chelate transition metal ions hence reducing metal-induced oxidative reactions, but they also reduce them. Since the reduced form is normally more active at catalyzing the decomposition of hydroperoxides into free radicals than the oxidized form, the metal−reducing properties of polyphenols can increase oxidative reactions (*21*).

Iron-catalyzed olive oil oxidation has been the subject of recent studies (*22*−*24*). However, iron is less effective as a catalyst of oxidation than copper at the same concentration, and most foods contain 3.1−31 μM Cu^{2+} (*21*). Therefore, more studies of copper-catalyzed lipid oxidation in emulsions systems are needed. Moreover, several important studies demonstrating the protective effect of olive oil phenolic compounds against chronic degenerative diseases were performed in the presence of copper ions (*2*−*4*, *25*−*30*), but the interactions and stability of these compounds in the presence of copper remains unclear. Therefore, the aim of this investigation was to study the ability of the most important olive oil phenolic compounds, namely hydroxytyrosol, 3,4-dihydroxyphenylethanol-elenolic acid (compound **1**), 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (compound **2**), and oleuropein (**Figure 1**), to inhibit lipid oxidation in bulk oil and emulsions in the presence of copper. To understand the antioxidant or prooxidant activity of phenolic compounds in emulsions containing this metal, the interactions of copper ions with olive oil phenolic compounds, namely their reducing capacity, stability in the presence of this metal, and the radical scavenging activity of complexes, were studied.

MATERIALS AND METHODS

Phenolic Compounds. Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A., Madrid, Spain) according to the procedure of Baraldi et al. (*31*). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi et al. (*32*). The aglycone **1** was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limirioli et al. (*33*). The olive oil component **2** was obtained from olive leaves according to the procedure of Paiva-Martins and Gordon (*34*).

Stripped Olive Oil. Olive oil stripped of natural tocopherols and phenols was prepared from commercial virgin olive oil by washing with 0.5 M NaOH (Merck) solution and passing twice through an aluminum oxide column (Merck). Complete removal of tocopherols was confirmed by HPLC, according to IUPAC Method 2.432.

Oxidation Experiments. Bulk refined olive oil samples and oil-in-water emulsions were oxidized in the dark at 60 °C. Each bulk oil sample was studied in triplicate, and each emulsion sample was studied in duplicate. Control samples containing no phenolic compounds were included in each experiment, both with (control 1) and without copper ions (control 2).

Emulsion Samples. The 30% oil-in-water emulsions (33 g) were prepared in 100 mL Erlenmeyer flasks. Olive oil (10 g), stripped of natural tocopherols and phenols, was mixed with each additive at the required concentration (0.4 and 1.2 mM, respectively). Tween 20 (0.66 g) was dissolved in the required buffer solution (22.3 g) (containing copper at 0.8 mg/kg when required), and the mixture was sonicated for 8 min in an ice bath. Isolation of oil from emulsions for analysis was by freezing, thawing, and centrifugation. Progress of oxidation was monitored by determination of the conjugated dienes (CD) (AOCS Official Method Ti 1a-64) and by determination of the *p*-anisidine value (AV) (AOCS Official Method Cd 18-90). Buffer solutions used were 0.05 M acetate buffer, pH 5.5, and 0.05 M 3*N*-morpholinopropanesulfonic acid (MOPS), pH 7.4.

Reducing Capacity. The reducing capacity at pH 5.5 and 7.4 was evaluated by measuring the formation of the complex between Cu(I) and bathocuproindissulfonic acid (BCDS) by visible spectrophotometry according to the procedure described by Mira et al. (*35*).

Stability of Phenolic Compounds at pH 5.5 and 7.4 in the Presence and Absence of Copper Chloride. The changes in each phenolic compound during storage were monitored by HPLC. Conditions were the same as described previously (*24*).

Determination of Radical Scavenging Activity of Complexes (36). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical was used as a stable radical. A 50% alcoholic solution at pH 5.5 or pH 7.4 of each phenolic compound (0.1 mL, 0.6 mM) in the presence of Cu(II) (0.3 mM) was added, after 15 min of complex reaction, to 3.5 mL of a 0.06 mM methanolic 1,1-diphenyl-2-picrylhydrazyl radical solution. The exact initial 1,1-diphenyl-2-picrylhydrazyl radical concentration (C_{DPPH}) in the reaction was calculated from a calibration curve with the equation

$$Y = 0.1105x + 0.0001 \qquad r^2 = 0.9999$$

as determined by linear regression. The change in absorbance with time was recorded, and the percentage of 1,1-diphenyl-2-picrylhydrazyl radical remaining at 60 min was determined. The test was performed in quadruplicate.

Statistical Analysis. Statistical analysis to determine significant differences in antioxidant activity involved plotting CD or AV against time to determine times to certain values, correcting for control samples 1 and 2, and then applying ANOVA one-way with Tukey's HSD multiple comparison to determine differences significant at the 5% level using SPSS 10.0 software.

Reducing capacity, radical scavenging activity of complexes, and stability of phenolic compounds at pH 5.5 and 7.4 were also analyzed by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$, using SPSS 10.0 software.

RESULTS AND DISCUSSION

The oxidative stability of olive oil samples stripped of natural phenolic compounds and tocopherols, containing added phenolic compounds (hydroxytyrosol, oleuropein, and compounds **1** and **2**) at 0.6 mmol/kg, in the absence and presence of Cu(II) at 0.8 mg kg^{-1}, was assessed by the Schaal oven test at 60 °C and compared to that of oil samples containing α-tocopherol. To verify if differences between samples were significant, the times for samples to reach the conjugated diene content of 0.4% were determined (**Figure 2**). In the absence of copper, hydroxytyrosol,

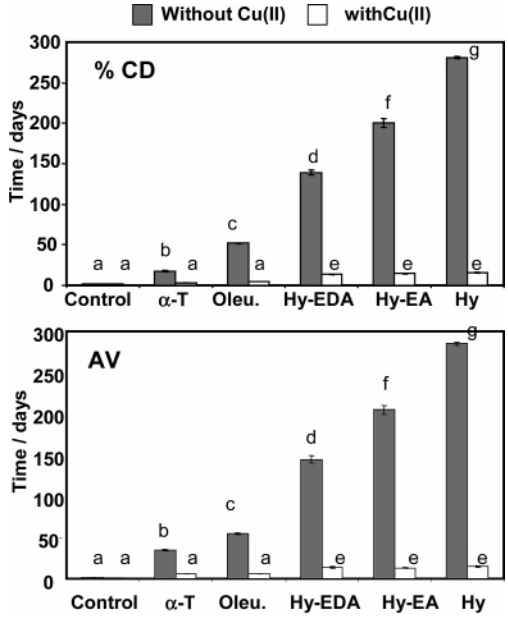

Figure 2. Time for oil samples to reach a conjugated diene content of 0.4% (% CD) and a *p*-anisidine value of 15 (AV) at 60 °C. Mean (error bars represent standard deviation) of triplicate stored samples. α-T, α-tocopherol; Hy, hydroxytyrosol; Oleu., oleuropein; Hy-EA, 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA); Hy-EDA 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA). Letters in each graph indicate samples that are significantly different ($p < 0.05$).

which is the most polar phenolic compound, showed the highest antioxidant capacity, which is in accordance with the polar paradox. However, compound **1** showed better activity than compound **2** despite having similar polarity, probably because of its better radical scavenging activity (*23*). As observed previously (*18*), oleuropein was less effective than predicted by the polar paradox. In the presence of copper, the antioxidant activity of these compounds was drastically affected (**Figure 2**), and the stability of samples containing phenols decreased by 90% but all olive oil polyphenols showed a much better antioxidant activity than tocopherol. The analysis of the time to AV = 15 confirmed the order of antioxidant activity (**Figure 2**, AV).

In emulsions which did not contain copper, the four compounds showed a marked increase in antioxidant activity with an increase in phenol concentration at both pH 5.5 and 7.4. However, the reduction in stability when Cu (II) was present was severe for all compounds at both pH values. In the presence of copper, hydroxytyrosol showed a prooxidant effect at pH 5.5 (**Figure 3**), with the effect increasing with phenol concentration. However at pH 7.4, an antioxidant effect could be observed (**Figure 4**). The stability of emulsion samples at pH 5.5 containing oleuropein and copper when assessed by the conjugated diene content was not statistically different from that of the control, but prooxidant effects could be observed when the stability of samples was assessed by the anisidine value (**Figure 3**). Oleuropein at pH 7.4 and compounds **1** and **2** at both pH values tested reduced the prooxidant effect of copper. The AV determinations confirmed these results for all compounds (**Figure 3**, AV; **Figure 4**, AV).

To understand the behavior of these compounds in the presence of copper, the stability of the compounds in the presence of copper (**Figure 5**), their reducing ability (**Table 1**), and their radical scavenging activity in the presence of copper(II) (**Figure 6**) were determined. **Table 1** shows the number of moles of copper(II) reduced by 1 mol of phenolic compound calculated using the calibration curves obtained with the appropriate buffer solution. All phenolic compounds showed a high Cu(II) reducing capacity, which was 5 times higher than the Fe(III) reducing capacity (*20*). The copper reducing capacity of hydroxytyrosol at pH 5.5 after 5 and 60 min was lower and statistically different from that of the other olive oil polyphenols tested. α-Tocopherol at pH 5.5 and after 60 min showed 50−55% of the reducing capacity of oleuropein, compound **1**, and compound **2** and 64% of the reducing capacity of hydroxytyrosol. At pH 7.4 in the presence of a Cu(II) chelating buffer (PBS buffer), all compounds showed a lower reducing capacity

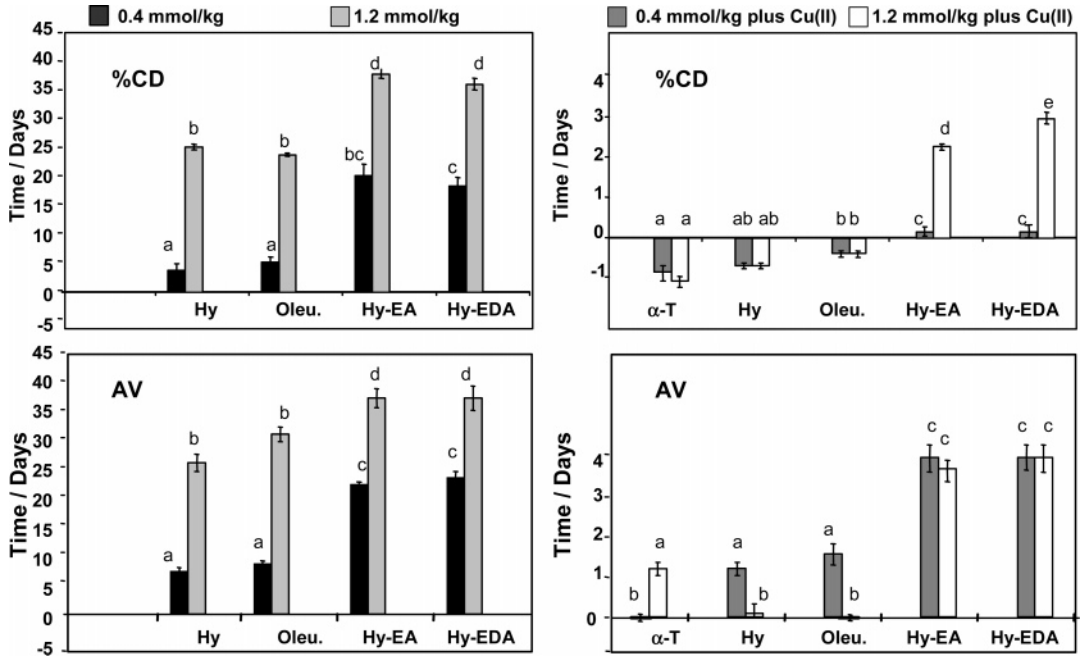

Figure 3. Time for olive oil-in-water emulsions samples at pH 5.5 to reach a conjugated diene content of 0.4% (% CD) and a *p*-anisidine value of 15 (AV) at 60 °C. Mean (error bars represent standard deviation) of duplicate stored samples. For abbreviations, see **Figure 2**. Letters in each graph indicate samples that are significantly different ($p < 0.05$).

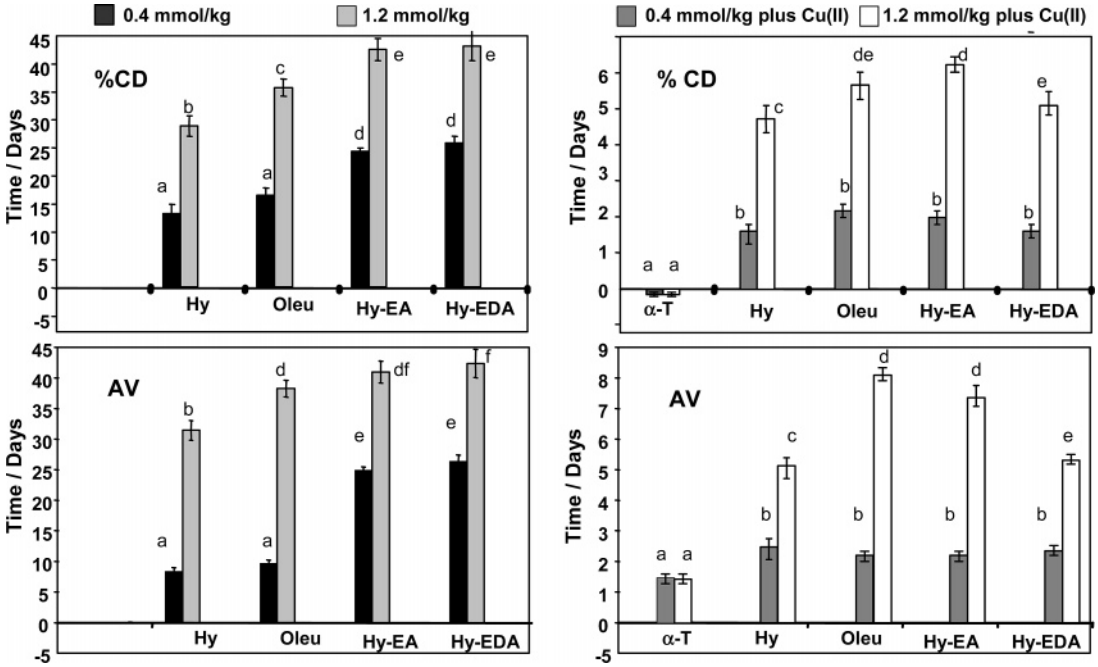

Figure 4. Time for olive oil-in-water emulsions samples at pH 7.4 to reach a conjugated diene content of 0.4% (% CD) and a *p*-anisidine value of 15 (AV) at 60 °C. Mean (error bars represent standard deviation) of duplicate stored samples. For abbreviations, see **Figure 2**. Letters in each graph indicate samples that are significantly different ($p < 0.05$).

Table 1. Number of Moles[a] of Cu(II) Reduced by 1 mol of Phenolic Compound after 5 min and 1 h

	mol Cu(I) formed/mol phenol					
	pH 5.5		pH 7.4			
	acetate buffer		PBS buffer		MOPS buffer	
compound	5 min	60 min	5 min	60 min	5 min	60 min
oleuropein	3.6 ± 0.2 a	4.6 ± 0.2 a	2.5 ± 0.1 a	3.5 ± 0.2 a	11.0 ± 0.7 a	10.9 ± 0.8 a
DHPEA-EDA	3.3 ± 0.1 ab	4.3 ± 0.2 a	2.0 ± 0.1 b	3.0 ± 0.2 b	9.3 ± 0.6 b	10.0 ± 0.5 a
DHPEA-EA	3.0 ± 0.2 b	4.2 ± 0.3 a	2.2 ± 0.1 b	3.5 ± 0.1 a	9.7 ± 0.6 bc	11.0 ± 0.6 a
hydroxytyrosol	2.5 ± 0.1 c	3.6 ± 0.1 b	2.4 ± 0.1 a	4.0 ± 0.2 c	10.0 ± 0.7 c	10.9 ± 0.8 a
tocopherol	2.0 ± 0.1 d	2.3 ± 0.1 c	1.0 ± 0.1 c	1.1 ± 0.1 d	8.4 ± 0.3 d	8.5 ± 0.1 b
ascorbic acid	3.1 ± 0.1 b	3.9 ± 0.2 d	1.6 ± 0.1 d	1.8 ± 0.1 e	7.3 ± 0.2 e	8.7 ± 0.3 b

[a] Mean of quadruplicate determinations. Letters in each column indicate samples that are significantly different ($p < 0.05$).

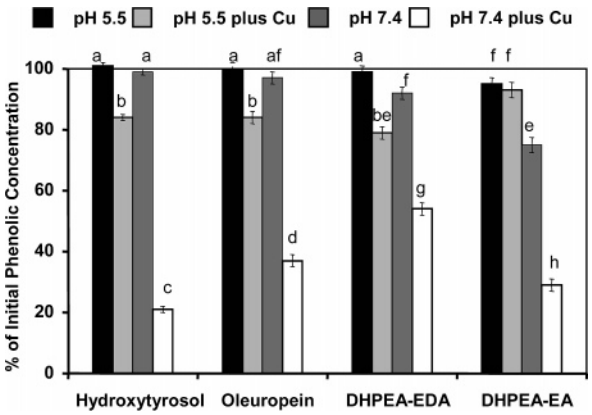

Figure 5. Changes in concentration of phenolic compounds after 15 min when stored as a mixture in aqueous ethanolic solutions at pH 5.5 and 7.4 at 37 °C in the presence and absence of copper(II). Mean (error bars represent range) of two determinations for each duplicate. Letters indicate samples that are significantly different ($p < 0.05$).

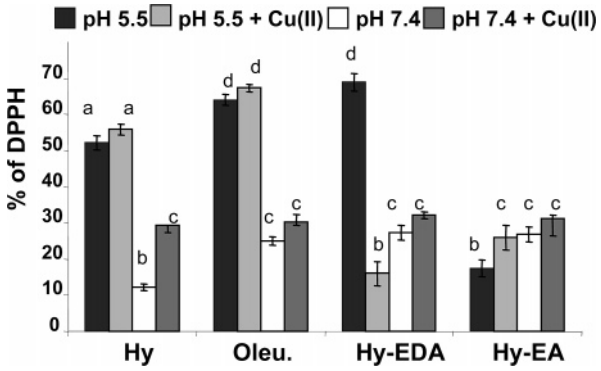

Figure 6. DPPH radical-scavenging effects of phenolic compounds after 60 min of reaction. Mean (error bars represent standard deviation) of quadruplicates. For abbreviations, see **Figure 2**. Letters indicate samples that are significantly different ($p < 0.05$).

than at pH 5.5 except for hydroxytyrosol; but in the absence of a chelating buffer (MOPS buffer), all compounds showed a similar high Cu(II) reducing capacity, which was higher than that of tocopherol and ascorbic acid. Knowing copper to be a very effective catalyst of oxidation, these results would help to explain the low and similar antioxidant activity of the compounds in the presence of Cu(II) in bulk oil. However, emulsions containing olive oil phenolics with Cu(II) were more stable at

pH 7.4 than at pH 5.5, and this could not be explained by the reducing capacity of the compounds. It was also observed that the compounds with the lower Cu(II) reducing capacity exhibited poorer antioxidant activity in the emulsions. Nevertheless, in the presence of a specific Cu(I) chelator (BCDS), the Cu(II) is rapidly reduced. In the absence of this Cu(I) chelator, the difference in the stability of the Cu(II)−phenol complexes at pH 7.4 may be significant, and this difference may explain the different antioxidant behavior observed for the different compounds in the emulsions. According to the literature, the monocatecholate complex of Cu(II) was observed to undergo a rate-limiting intramolecular electron transfer with $k = 1.9 \times 10^{-5}$/s (22 °C, $u = 0.125$ M $NaClO_4$) (*37*), and this indicates that reduction of Cu(II) should be a slower process than the one observed for all compounds. Moreover, since complex formation is normally required for reduction, the higher reducing capacity of hydroxytyrosol in PBS buffer may reflect not its higher reducing ability but the lower stability of its Cu(II) complex.

The stability of olive oil phenolic compounds was evaluated in the presence of copper at pH 5.5 and 7.4. At both pH values, hydroxytyrosol showed a remarkable stability in the absence of copper(II) when compared with other phenols with compound **1** being the most affected by the alkaline pH with a decrease to about 25% after 15 min (**Figure 5**). In the presence of copper, the stability of hydroxytyrosol was drastically reduced, and it had degraded even more than compound **1**. Compound **2** showed the best stability at pH 7.4 in the presence of Cu(II). These results would explain the lower capacity of hydroxytyrosol to inhibit the prooxidant activity of copper. Still, these effects did not explain the better stability at pH 7.4 of hydroxytyrosol and oleuropein.

Various low-molecular-weight superoxide dismutase (SOD)-like complexes of manganese, copper, and iron have been reported to exhibit SOD mimic activities (*38*, *39*). At a physiological pH, catechols readily form thermodynamically stable complexes with ferric ions (*40*). Catechols are also biosynthesized and used as iron-sequestering agents by microorganisms (*41*). These facts led us to investigate whether the copper chelates would have higher radical scavenging activity than the uncomplexed catechols. It was found that the compound **2**−copper complex obtained after 15 min of reaction exhibited a higher radical scavenging activity against the DPPH radical than the compound **2** at pH 5.5 (**Figure 6**). Moreover, all compound−copper complexes exhibited a similar and higher radical scavenging activity at pH 7.4 than at pH 5.5 (**Figure 6**). The higher radical-scavenging activity of copper complexes at pH 7.4 together with the higher stability of the olive oil-in-water emulsions at pH 7.4 would explain the better antioxidant activity of hydroxytyrosol and oleuropein at this pH.

Olive oil is used widely in the preparation of food emulsions in the form of soups and sauces in Mediterranean countries. Food may, together with the added water, contribute to a high concentration of free Cu(II) in prepared food, and copper-mediated oxidation is much faster when compared with oxidation catalyzed by other metals such as Fe(III). The addition of phenolic compounds to bulk oil significantly extended the induction time of lipid oxidation even in the presence of copper. In stripped olive oil-in-water emulsions, hydroxytyrosol enhanced the prooxidant effect of copper at pH 5.5 but not at pH 7.4. Oleuropein at pH 7.4 and compounds **1** and **2** at both pH values reduced the prooxidant effect of copper.

Since compound **2** is the most concentrated antioxidant compound normally found in the phenolic extract of virgin olive oil, it is interesting to note that this compound has good antioxidant behavior even in the presence of Cu(II). According to these results, when food is processed with olive oil in the presence of water, olive phenolic extracts with higher quantities of compounds **1** and **2** would be better than olive oil extracts with higher quantities of hydroxytyrosol, despite the higher antioxidant activity of hydroxytyrosol in bulk oil.

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Received for review January 4, 2006. Revised manuscript received March 21, 2006. Accepted March 21, 2006.

JF060033J