Antioxidant Activity of Hydroxytyrosol Acetate Compared with That of Other Olive Oil Polyphenols

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Hydroxytyrosol acetate was synthesized, and the antioxidant activity of this olive oil component was assessed in comparison with that of other olive oil components, namely hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and α-tocopherol in bulk oil and oil-in-water emulsions. The activity of the compounds was also assessed by scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Hydroxytyrosol acetate had a weaker DPPH radical scavenging activity than hydroxytyrosol, oleuropein, or 3,4-DHPEA-EA but it had a radical scavenging activity similar to that of α-tocopherol. In oil, the antioxidant activity of hydroxytyrosol acetate was much higher than that of α-tocopherol or oleuropein, but in an emulsion 3,4-DHPEA-EA and α-tocopherol were more effective as antioxidants than hydroxytyrosol acetate. The antioxidant activity of hydroxytyrosol acetate was rather similar to that of hydroxytyrosol in oil and emulsions despite the difference in DPPH radical scavenging activity.

Keywords: *Olive oil; hydroxytyrosol; 3,4-DHPEA-EA; oleuropein; hydroxytyrosol acetate; α-tocopherol; emulsion; antioxidants*

INTRODUCTION

Virgin and extra virgin olive oils are among the very few oils consumed without refining, and which consequently contain phenolic compounds besides tocopherols, which may play a role in the health benefits of the oil. Epidemiological evidence shows that the Mediterranean diet is associated with lower incidence of both coronary heart disease (CHD) and certain tumors (*1, 2*). Olive oil, which is rich in monounsaturated, and low in saturated, fatty acids is the major fat component of this diet. The oxidative stability of low-density lipoproteins (LDL) isolated from animals fed virgin olive oil is increased in comparison with a control comprising a triglyceride preparation with similar fatty acid composition, and this increased stability is attributable to the minor components in the oil (*3, 4*). In recent years there has been much interest in the effects of antioxidants in retarding oxidative modification of LDL, which is believed to be a key step in the initiation of atherosclerosis (*5*).

Olive oil hydrophilic extracts contain a large number of phenolic compounds including phenyl-alcohols, such as 3,4-dihydroxyphenylethanol (3,4-DHPEA or hydroxytyrosol) and *p*-hydroxyphenylethanol (*p*-HPEA or tyrosol), as well as phenyl-acids. Oleosidic forms of 3,4-DHPEA, in particular the dialdehydic form of elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA), an isomer of oleuropein aglycon (3,4-DHPEA-EA), and the dialdehydic form of elenolic acid linked to p-HPEA (p-HPEA-EDA) have been identified as the major secoiridoid compounds of virgin olive oil (*6*).

From comparisons with a limited number of phenolic constituents, it has been claimed that hydroxytyrosol is the most active antioxidant compound in virgin olive oil, although the concentration of free hydroxytyrosol is sometimes low (*7*−*10*). Oleuropein, a hydroxytyrosol derivative, which occurs in large amounts in olive leaves and olive fruits (up to 14% of the dry weight in unripe olives), has shown a weak antioxidant activity in olive oil (*7, 9 ,10*). Both hydroxytyrosol and oleuropein have been shown to be scavengers of superoxide anions, and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals, but hydroxytyrosol was more effective than oleuropein (*1*). Both compounds also scavenged hydroxyl radicals, but in this case oleuropein showed greater activity (*11*).

Studies with the Rancimat method at 120 °C showed that the oxidative stability of olive oil at elevated temperatures mainly correlated with the concentration of hydroxytyrosol and its oleosidic forms. 3,4-DHPEA-EA and 3,4-DHPEA-EDA showed similar antioxidant activity to hydroxytyrosol under these conditions (*12, 13*). These compounds also inhibited copper sulfate-induced LDL oxidation (*14*).

Recently another hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-dihydroxyphenylethyl acetate) has been found in olive oil (*15*) (Figure 1). The aim of this research has been to investigate the antioxidant activity of this compound in comparison with that of the phenolic extract from olive oil and the pure components hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and α-tocopherol in bulk oil and oil-in-water emulsions. The activity of the single compounds and the phenolic extract were also assessed by scavenging of diphenylpicrylhydrazyl (DPPH) radicals.

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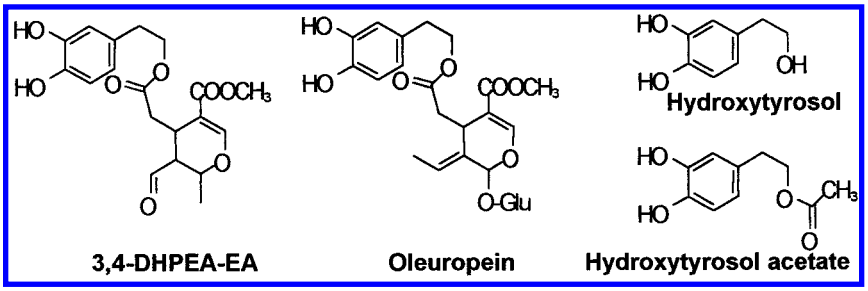

Figure 1. Structures of olive oil phenolics.

MATERIALS AND METHODS

Materials. Phenolic extract was obtained from commercial virgin olive oil following the procedure described by Montedoro et al. (*6*) and quantified by the Folin-Ciocalteu reaction, expressed as caffeic acid equivalent. All calculations were made by assuming a mean relative molecular mass of the phenolic extract of 300. Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S. A., Madrid, Spain) according to the procedure of Baraldi et al. (*16*). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Garibaldi et al. (*17*). 3,4 -DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limirioli et al. (*18*). α-Tocopherol, trolox, tween 20, and soybean phosphatidylcholine were obtained from Sigma. Sodium acetate, acetic acid, sodium hydrogen phosphate, sodium phosphate, and silica gel (130−230 mesh) were supplied by Merck (Merck Farma e Quimica, S. A., Lisbon, Portugal). $LiAlH_4$, benzyl bromide, dicyclohexylcarbodiimide (DCC), *p*-toluenesulfonic acid (TSA), Pd−C 10%, 1,4-cyclohexadiene, 1-octanol, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were supplied by Sigma-Aldrich. All other reagents were analytical grade or purer.

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.

Preparation of Hydroxytyrosol Acetate. The phenolic groups of hydroxytyrosol were protected by the reaction with benzyl bromide according to the method of Kanai et al. (*19*). The 2-[(3,4-dibenzyloxyphenyl]ethanol was purified by eluting through a silica gel column with chloroform/ethyl acetate (7:3) (v/v). The yield of this reaction was 80%. This compound (0.8 g) and acetic acid (0.14 mL) were dissolved in pyridine (3 mL); DCC (0.6 g) and TSA (0.02 g) were then added. After the mixture was stirred at room temperature for 72 h, acetic acid was added and the mixture was filtered. Chloroform was added and the organic layer was washed with hydrochloric acid (5 M), water, sodium carbonate solution (0.5 M), and water. The chloroform phase was evaporated in a vacuum rotary evaporator at 35−40 °C and the mixture was purified by eluting through a silica gel column with dichloromethane/light petroleum 40−60 °C (1:1) (v/v). 2-[(3,4-Dibenzyloxyphenyl]ethyl acetate was obtained, and the yield of this reaction was 40%. The benzyloxy groups from this compound were removed by catalytic hydrogenation with a palladium-on-carbon catalyst (10%) and 1,4-cyclohexadiene, according to the method of Felix et al. (*20*). The hydroxytyrosol acetate obtained was purified by eluting through a silica gel column with ethyl acetate/light petroleum 40−60 °C (1:1) (v/v). The yield of this reaction was 75%. NMR (methanol-*d*) δH 6.69−6.49 (m, 3H, Ar*H*), 4.91 (br s, 2H, O*H*), 4.16 (t, J = 7.1 Hz, 2H, CH_2−O), 2.74 (t, J = 7.1 Hz, CH_2−Ph), 1.99 (s, 3H, CH_3); δC 173.0 (*C*O), 146.2 and 144.9 (Ar*C*-OH), 130.7 (Ar*C*-CH_2), 121.1, 117.0, and 116.3 (Ar*C*-H), 66.6 (CH_2−O), 35.4 (Ph-CH_2), 20.8 (CH_3).

Determination of Radical Scavenging Activity. 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical was used as a stable radical (*21*). For each phenolic compound, several concentrations were tested. Phenolic compound solution (0.1 mL) was added to 3.5 mL of a 0.06 mM methanol DPPH radical solution. The decrease in absorbance was determined at 515 nm at 0, 5, and every 10 min until 250 min. The exact initial DPPH radical concentration (C_{DPPH}) in the reaction was calculated from a calibration curve with the equation

$$Y = 0.1108x + 0.0001 \quad (r^2 = 0.999)$$

as determined by linear regression. For each compound tested, the reaction kinetics were plotted, and from these graphs the percentage of DPPH radical remaining at several times was determined. The values were transferred onto another graph showing the percentage of residual DPPH radical as a function of the molar ratio of phenolic compound to DPPH radical. Antiradical activity was defined as the relative concentration of antioxidant required to lower the initial DPPH concentration by 50% [EC_{50} (mol/L phenolic compound per unit DPPH concentration)].

Measurement of Partition Coefficient (log *P*). A solution (0.3 mM) of each compound in 1-octanol was kept at 60 °C for an hour. An UV spectrum was then run, and the value of absorbance at the maximum was measured (A_0). Equal volumes of organic solution and acetate buffer (0.1 M, pH 5.5) or phosphate buffer (0.1M, pH 7.4) were vortexed (2500 rpm) for 1 min. The UV spectrum of the organic layer was determined after 30 min (A_x). The partition coefficient (log *P*) was calculated according to the relationship, $P = A_x/(A_0 - A_x)$. A solution of *n*-octanol saturated with water was used as the blank.

Bulk Oil Samples. Olive oil (10 g), stripped of natural tocopherols and phenols, with each additive at a final concentration of 0.3 mmol/kg or 0.6 mmol/kg was stored in a 10-cm Petri dish.

Emulsion Samples. 30% Oil-in-water emulsions (33 g) were prepared in 100-mL Erlenmeyer flasks. The emulsions were made by mixing 10 g of olive oil, stripped of natural tocopherols and phenols, containing each additive at a final concentration of 0.3 mmol·kg^{-1}, 0.66 g of tween 20 as emulsifier, and 22.3 g of acetate buffer (0.1 M, pH 5.5), 3*N*-morpholinopropanesulfonic acid (MOPS) or phosphate buffer (0.1 M, pH 7.4). The mixture was sonicated for 10 min in an ice bath.

Oxidation Experiments. *Bulk Oil and Emulsions.* Samples were oxidized in the dark at 60 °C. Each sample was studied in triplicate. Isolation of oil from emulsions for analysis was by freezing, thawing, and centrifugation. Progress of oxidation was monitored by determination of the peroxide value (PV) (AOCS Official Method Cd 8-53) or conjugated dienes (CD) (AOCS Official Method Ti 1a-64) and *p*-anisidine value (AV) (AOCS Official Method Cd 18-90).

Statistical Analysis. Statistical analysis to determine significant differences in antioxidant activity involved plotting PV or CD against time to determine times to certain values and then applying ANOVA one-way with Tukey's HSD multiple comparison to determine differences significant at the 5% level.

RESULTS AND DISCUSSION

DPPH Scavenging Test. The radical scavenging activity, assessed by the antioxidant concentration

Table 1. DPPH Radical-Scavenging Effects of Phenolic Compounds after Various Reaction Times (15 min, 60 min, and 250 min)[a]

	time 15 min[b]		time 60 min[b]		time 250 min[b]	
compound	EC_{50}[c]	no. of reduced DPPH	EC_{50}[c]	no. of reduced DPPH	EC_{50}[c]	no. of reduced DPPH
phenolic extract	0.40[a] (± 0.01)	1.3	0.36[a] (± 0.01)	1.4	0.26[a] (± 0.01)	1.5
hydroxytyrosol acetate	0.26[b] (± 0.01)	2.0	0.22[b] (± 0.01)	2.2	0.14[b] (± 0.01)	3.6
α-tocopherol	0.25[b] (± 0.01)	2.0	0.25[c] (± 0.01)	2.0	0.24[a,c] (± 0.01)	2.1
trolox	0.24[b] (± 0.01)	2.1	0.24[c] (± 0.02)	2.1	0.24[c,a] (± 0.02)	2.1
oleuropein	0.22[c] (± 0.01)	2.2	0.18[d] (± 0.01)	2.6	0.12[b] (± 0.01)	4.0
hydroxytyrosol	0.19[d] (± 0.01)	2.7	0.13[e] (± 0.01)	3.6	0.099[d] (± 0.004)	5.1
3,4-DHPEA-EA	0.12[e] (± 0.0)	4.3	0.10[f] (± 0.01)	4.7	0.062[e] (± 0.002)	8.0

[a] Superscripts within a row indicate samples that were significantly different ($p < 0.05$). nd, not determined. [b] Mean (standard deviation in parentheses) of four determinations. [c] EC_{50} expressed as mol of antioxidant/mol of DPPH radical.

Table 2. Time for Stripped Olive Oil Samples Containing Additives (0.3 mmol·kg^{-1}) to Reach a Peroxide Value of 50 Meq·kg^{-1} at 60 °C

compound	time (days)[a]
control	2.36 (± 0.37)[a]
oleuropein	8.51 (± 0.33)[b]
α-tocopherol	20.24 (± 0.18)[c]
3,4-DHPEA-EA	64.38 (± 1.45)[d]
phenolic extract	66.03 (± 0.79)[d,e]
hydroxytyrosol acetate	66.54 (± 0.78)[e,d]
hydroxytyrosol	70.45 (± 0.13)[f]

[a] Mean (standard deviation in parentheses) of triplicate stored samples. Superscripts within a column indicate samples that were significantly different ($p < 0.05$).

required for 50% reduction in DPPH radical concentration in 15 min (EC_{50}), decreased in the following order: 3,4-DHPEA-EA ≫ hydroxytyrosol > oleuropein > hydroxytyrosol acetate, α-tocopherol, trolox > phenolic extract (Table 1). After 15 min, the reaction of DPPH radical with α-tocopherol and trolox (the water-soluble analogue of α-tocopherol) was nearly at the steady state. Hydroxytyrosol acetate, the other pure phenolics, and the phenolic extract were similar to tocopherol and trolox in reacting rapidly with the DPPH radical, but they also showed slower reactions with the radical, with hydroxytyrosol acetate showing a 46% reduction in EC_{50} between 15 and 250 min. For an antioxidant to be effective in interrupting the propagation step of lipid oxidation, it must react much faster with lipid radicals than linoleic acid does, despite being present at much lower concentrations than the polyunsaturated fatty acid in an oil, and hence the EC_{50} value at 15 min, which is a measure of more rapid reactions with DPPH radicals, is probably more relevant than the slower reactions. The EC_{50} value at 15 min for hydroxytyrosol acetate (0.26 mol AH/mol DPPH) was significantly greater than for hydroxytyrosol and oleuropein (0.19 and 0.22 mol AH/mol DPPH). The acetate group of the ester may hinder the scavenging effect of the hydroxyl groups by intra- or intermolecular hydrogen bonding.

Oxidative Stability of Bulk Olive Oil Containing Olive Oil Phenolics. The antioxidant effects of pure olive oil phenolic compounds in olive oil, stripped of natural phenolic compounds and tocopherols, were assessed by the peroxide value (PV) to determine primary oxidation products and by the *p*-anisidine value determination (AV) to determine secondary oxidation products. On the basis of the time to a PV of 50 meq·kg^{-1}, antioxidant efficiency for 0.3 mmol·kg^{-1} addition decreased in the following order: hydroxytyrosol > hydroxytyrosol acetate, phenolic extract, 3,4-DHPEA-EA ≫ α-tocopherol > oleuropein > control (Figure 2a, Table 2). The AV determinations confirmed the order

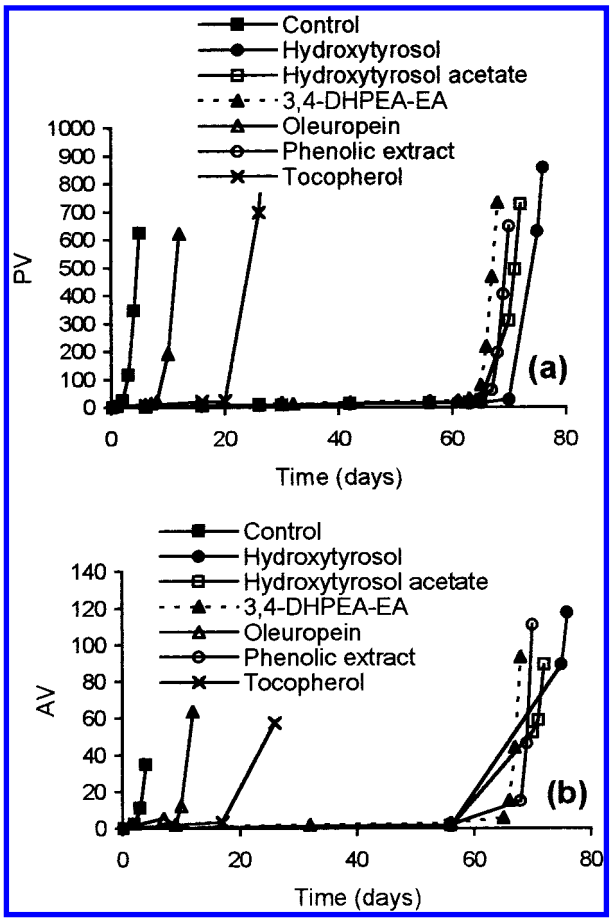

Figure 2. Effect of pure olive oil phenolics and olive oil phenolic extract at 0.3 mmol·kg^{-1} on the oxidation of olive oil triacylglycerols stored at 60 °C, assessed by (a) the peroxide value and (b) the anisidine value.

Table 3. Partition Coefficient of Phenolic Compounds

	Log P[a]	
compound	pH 5.5	pH 7.4
hydroxytyrosol	0.03 (± 0.01)	0.02 (± 0.01)
DHPEA-EA	1.13 (± 0.08)	1.16 (± 0.08)
hydroxytyrosol acetate	0.82 (± 0.01)	0.82 (± 0.05)
oleuropein	0.17 (± 0.01)	0.13 (± 0)
phenolic extract	0.78 (± 0.03)	nd
α-tocopherol	1.37 (± 0.04)	1.38 (± 0.01)

[a] Mean (standard deviation in parentheses) of triplicate determinations.

of antioxidant activity (Figure 2b). The polarity of antioxidants was assessed by the partitioning between octanol and water (Table 3). Comparing the order of activity as antioxidants in oil with the DPPH radical

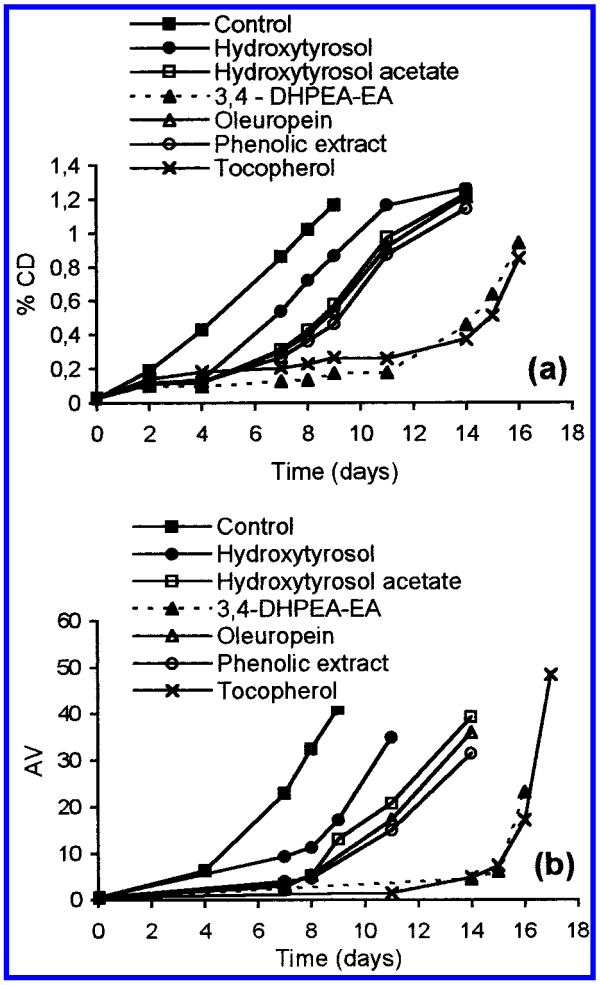

Figure 3. Effect of pure olive oil phenolics and olive oil phenolic extract at 0.3 mmol·kg^{-1} on the oxidation of stripped olive oil triacylglycerols-in-water emulsions at pH 5.5, stored at 60 °C, assessed by (a) the conjugated diene content and (b) the anisidine value.

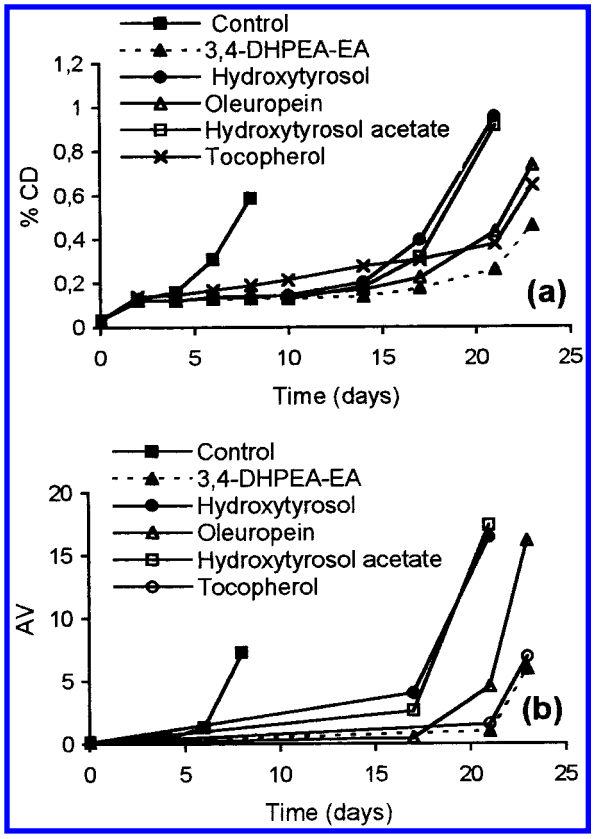

Figure 4. Effect of pure olive oil phenolics at 0.3 mmol·kg^{-1} on the oxidation of stripped olive oil triacylglycerols-in-water emulsions made with MOPS buffer at pH 7.4, stored at 60 °C, assessed by (a) the conjugated diene content and (b) the anisidine value.

scavenging activity, it is clear that hydroxytyrosol acetate is only slightly less effective as an antioxidant in oil than the more polar antioxidant hydroxytyrosol despite being considerably less polar and having reduced radical scavenging activity assessed by the DPPH scavenging test. According to the polar paradox, more polar antioxidants are more effective in less polar media (*22*, *23*). The good antioxidant activity of hydroxytyrosol acetate in oil suggests that any inter- or intramolecular hydrogen bonding between the ester group of this molecule and the active hydroxyl groups, which contributes to reduced radical scavenging activity in the DPPH test, does not occur in oil, presumably because of van der Waals interactions between triglyceride molecules and the ester side chain. However, even if hydroxytyrosol acetate and hydroxytyrosol had similar radical scavenging activity, the polar paradox would suggest that there would be a marked difference in antioxidant activity in oil but the effect is rather small. Oleuropein, which is a glycoside, is less effective than predicted by the polar paradox in less polar media as previously observed for other glycosides (*24*).

Oxidative Stability of Olive-Oil-in-Water Emulsions containing Olive Oil Phenolics. Antioxidant effects were studied at 60 °C in oil-in-water emulsions prepared from olive oil stripped of natural phenolic compounds and tocopherols. The emulsions were physically stable during all the experiments. Oxidation was more rapid in emulsions (Figures 3 and 4) than in bulk oil (Figure 2). The oxidative stability of the emulsions

Table 4. Time for Stripped Olive Oil-in-Water Emulsion Samples to Reach a Conjugated Diene Content of 0.4 %

	time (days) to $CD = 0.4\%$[a])		
	pH 5.5	pH 7.4	
compound	acetate buffer	phosphate buffer	MOPS buffer
control	3.79 (±0.08)[a]	3.03 (±0)[a]	6.80 (±0.50)[a]
hydroxytyrosol	5.99 (±0.25)[b]	6.57 (±0.04)[b]	16.97 (±0.52)[b]
hydroxytyrosol acetate	7.78 (±0.29)[c]	7.84 (±0.28)[c]	17.65 (±0.50)[b]
oleuropein	7.94 (±0.17)[c]	7.02 (±0)[b]	20.48 (±0.99)[c]
phenolic extract	8.38 (±0.45)[c]	nd	nd
DHPEA-EA	13.40 (±0.59)[d]	8.13 (±0.17)[c]	22.58 (±0.81)[d]
α-tocopherol	14.14 (±0.27)[d]	12.04 (±0.33)[d]	21.21 (±0.24)[c,d]

[a] Mean (standard deviation in parentheses) of triplicate stored samples. Superscripts within a row indicate samples that were significantly different ($p < 0.05$). nd, not determined.

containing added phenolic compounds was tested at pH 5.5 (acetate buffer, 0.1 M) and at pH 7.4 (MOPS buffer, 0.1M).

As shown in Figures 3 and 4, the order of antioxidant activity in emulsions was different from that in bulk oil. On the basis of the time to a conjugated diene content of 0.4%, the order of antioxidant activity at pH 5.5 (Table 4) was α-tocopherol, 3,4-DHPEA-EA > phenolic extract, oleuropein, hydroxytyrosol acetate > hydroxytyrosol > control; whereas at pH 7.4 in MOPS buffer, the order of antioxidant activity was 3,4-DHPEA-EA, α-tocopherol, oleuropein > hydroxytyrosol acetate, hydroxytyrosol ≫ control. The *p*-anisidine value determinations confirmed the order of activity (Figures 3 and 4). Hydroxytyrosol acetate was more effective than hydroxytyrosol in an emulsion at pH 5.5 (Figure 3) but there was no significant difference between the two antioxidants in MOPS buffer at pH 7.4 (Figure 4). The effects of the additives at pH 7.4 were confirmed by storing a set of samples with the additives in emulsions buffered with a phosphate buffer (Table 4). Oxidation in phosphate buffer was faster than in MOPS buffer, and differences in the order of activity were apparent. Hydroxytyrosol acetate was significantly more active than oleuropein and hydroxytyrosol in phosphate buffer. The differences in the rates of oxidation between the two buffers suggests that MOPS may have an antioxidant action. The order of antioxidant activity at pH 5.5 and at pH 7.4 in phosphate buffer was similar, although the differences between DHPEA-EA and α-tocopherol, and between hydroxytyrosol acetate and oleuropein were only significant at pH 7.4. There is no change in partition coefficient with a change in pH (Table 3), and consequently the explanation for the change in relative activity with pH must lie elsewhere. Hydroxytyrosol acetate is less effective than hydroxytyrosol as a radical scavenger (Table 1), but it is less polar and this helps to make it more effective as an antioxidant in an emulsion. The antioxidant activity in an emulsion depends on partitioning of components between hydrophilic and hydrophobic phases, the complex interfacial effects at the oil−water interfaces, the radical-scavenging properties, and stability of the antioxidants. Oleuropein has a greater radical-scavenging activity than hydroxytyrosol acetate, and consequently would be a better antioxidant in the absence of other effects. However, oleuropein is the more polar of the two molecules and this reduces its activity in an emulsion due to the polar paradox. The net effect is that the two compounds are similar in antioxidant activity in an emulsion.

In conclusion, this study has demonstrated that hydroxytyrosol acetate has a weaker DPPH radical scavenging activity than hydroxytyrosol, but the two compounds are similar in antioxidant activity with hydroxytyrosol acetate being slightly less effective in oil, but slightly more effective in an emulsion at pH 5.5 and pH 7.4. DHPEA-EA is slightly less effective than hydroxytyrosol acetate in oil but is the most effective hydroxytyrosol derivative in an emulsion.

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