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Effect of Lipophilization of Hydroxytyrosol on Its Antioxidant Activity in Fish Oils and Fish Oil-in-Water Emulsions

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The effect of lipophilization of the antioxidant efficiency of hydroxytyrosol on fish oil enriched systems was studied. Hydroxytyrosol fatty acid esters with increasing size of the alkyl chain and different lipophilicity were tested in bulk fish oils and fish oil-in-water emulsions. Results showed a significant antioxidant activity of hydroxytyrosol esters in both systems especially in emulsions. The introduction of a lipophilic chain decreased the antioxidant effectiveness of hydroxytyrosol in homogeneous systems as fish oils. In emulsion systems, the presence of a short−medium lipophilic chain (acetate, butyrate or octanoate) improved the antioxidant efficiency of hydroxytyrosol favoring the physical location of the antioxidant in the interface, but longer alkyl chain (laurate) maintained or even decreased their antioxidant activity. A maximum of antioxidant efficiency seems to appear when the chain length of the hydroxytyrosol derivative is that of eight carbons which is probably associated with a preferential location of the diorthophenolic moiety in the right geometry. These results are of high importance for the optimum design of effective antioxidants for omega 3 enriched foods, which are very susceptible to suffer oxidation and, then, rancidity.

KEYWORDS: Lipid oxidation; antioxidants; hydroxytyrosol; lipophilization; fish oil; oil-in-water emulsions; free radical scavenging

INTRODUCTION

Fish oil is a source of polyunsaturated fatty acids (PUFA), which have clearly shown beneficial effects for human health (*1*−*3*). As a result, marine lipids have become very attractive as a food ingredient in the increasing market of functional foods. Nevertheless, the use of marine lipids is still limited in food applications mainly due to their high oxidative susceptibility (*4*, *5*). Lipid oxidation affects the quality of fish oils during storage by deterioration of the flavor, odor, color and texture, and the production of toxic compounds. This phenomenon is even more dramatic when fish oil-in-water emulsions are used in the preparation of processed food because the emulsification process leads to the formation of a large interfacial area and lipid oxidation has been suggested to be initiated at the emulsion droplet interface (*6*, *7*).

Antioxidants have been added to food for years to control rancidity (*6*, *8*, *9*) and are widely used today for better food preservation. Several factors affect the effectiveness of an antioxidant, such as its chemical reactivity (as radical scavenger or as metal chelator), the interaction with other food components, the environmental conditions (as pH or concentration) and the physical location of the antioxidant in different food systems (for example bulk oil or oil-in-water emulsion). Lipophilic or hydrophilic antioxidants in an edible form are needed to stabilize a wide variety of fish oil enriched foods. The "polar paradox" postulates that hydrophilic antioxidants are more effective in bulk oils whereas lipophilic antioxidants are more effective in oil-in-water emulsions (*9*). This hypothesis has been associated with the affinities of polar antioxidants toward the air−oil interface in bulk oils and of nonpolar antioxidants toward the oil−water interface in emulsions. The effectiveness of antioxidants in oil-in water emulsions is also dependent on their surface activity which influences the accumulation of the antioxidant at the oil−water interface (*10*). The surface activity of antioxidants can be modified by conjugation to alkyl chains, and the surfactants formed can then be incorporated to the oil−water interface in a more efficient mode. In addition, it has been suggested that the micelle size can be altered affecting the lipid oxidation rate (*10*). The significance of physical structures of bulk oils on lipid oxidation must be carefully considered to understand the mechanism involved in antioxidant effectiveness (*11*−*14*).

Phenolic derivatives are a family of antioxidants widely studied due to their antioxidant activity in food matrices and also due to their biological relevance (*15*, *16*). Hydroxytyrosol **1** (**Figure 1**) is the most potent antioxidant found in olives and olive oil. It has also been shown to be highly efficient to prevent oxidation in fish oils, fish oil-in-water emulsions and fish fillets (*17*). This is particularly interesting for stabilizing functional products containing ω-3 PUFA. Moreover, hydroxytyrosol has shown very interesting biological properties, such as inhibition of human low-density lipoprotein (LDL) oxidation (a critical step in atherosclerosis) (*18*) and inhibition of platelet aggregation (*19*), and also exhibits anti-inflammatory (*20*) and anticancer properties (*21*).

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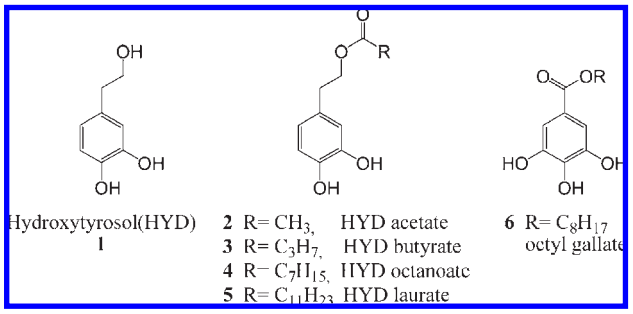

Figure 1. Chemical structures of hydroxytyrosol, hydroxytyrosol esters and octyl gallate.

Hydroxytyrosol is strongly hydrophilic, and this makes difficult its incorporation into fats and oils. Recently, lipophilic derivatives of hydroxytyrosol have been prepared in order to explore their antioxidant activity in different oil matrices. (*22*−*25*) In fact, these hydroxytyrosol fatty acid esters possess an amphiphilic nature due to the presence of the diorthophenolic moiety on one side of the molecule and the alkyl chain on the other side. The increase in the degree of lipophilicity of the antioxidant might locate it in a different area of the food matrix under study, and therefore its effect on lipid oxidation may result in being increased through a major accumulation at the oxidative sensitive interfaces.

The objective of this work has been to evaluate the capacity to inhibit lipid oxidation of a series of hydroxytyrosol fatty acid esters with increasing size of its alkyl chain and different lipophilicity (**Figure 1**). The antioxidants were tested in two different fish lipid enriched systems: bulk fish oils and fish oil-in-water emulsions. The rate of oxidation was monitored by the formation of lipid oxidation products (conjugated diene and triene hydroperoxides) during controlled sample storage. Chemical properties such as radical scavenging capacity and reducing ability of the series as well as their partition coefficients were measured in order to try to correlate them with the antioxidant efficiency obtained in the fish oil systems.

MATERIALS AND METHODS

Materials. High quality cod (*Gadus morhua*) liver oil composed by 40.6% of ω-3 PUFAs (3.7% of 18:3ω3; 1.3% of 20:4ω3; 14.9% of 20:5ω3; 2.8% of 22:5ω3 and 17.9% of 22:6ω3) was purchased from Fluka (New-Ulm, Switzerland). Soybean lecithin containing 40% L-α-phosphatidylcholine (Sigma, St. Louis, MO) was used as surfactant in oil-in-water emulsions. All chemicals and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany). Water was purified through a Millipore-Q plus (Millipore Corp., Bedford, MA).

Preparation of Bulk Fish Oil. Cod liver oil samples (5 g) were placed in screw-capped 50 mL Erlenmeyer flasks. Before that, hydroxytyrosol and its esters were incorporated in methanol solutions and methanol was then removed under a stream of nitrogen. Samples were subsequently sonicated for 5 min for a total dispersion of the phenolics. Control samples have no antioxidant added. The oxidative stability of fish oil was monitored during storage at 40 °C by sensory analysis and measuring the formation of conjugated diene and triene hydroperoxides. The experiments aimed to test the effect of antioxidant concentration were run during the period needed to identify the minimum concentration that would yield the maximum antioxidant capacity. The experiments aimed to compare the effectiveness of different antioxidants were run during the time needed to see clear differences in rancidity among antioxidant containing samples.

Preparation of Oil-in-Water Emulsions. Cod liver oil-in-water emulsions containing 1% lecithin and 10% fish oil were prepared in water, as previously described by Huang et al. (*26*). Briefly, cod liver oil was emulsified in water using lecithin as emulsifier, and sonicating at high power for 10 min in a cold glass container. Phenolics were added in methanol solutions into screw-capped 50 mL Erlenmeyer flasks, and then, methanol was removed under a stream of nitrogen before addition of oil-in-water emulsions (5 g). Samples were subsequently sonicated for 5 min for a total dispersion of phenolics. Control samples have no antioxidant added. The oxidative stability of emulsions was monitored during storage at 40 °C by sensory analysis and measuring the formation of conjugated diene and triene hydroperoxides and fluorescence compounds. Triplicate samples were prepared and oxidized. The experiments were run as described above for fish oils.

Synthesis of Hydroxytyrosol Fatty Acid Esters. Hydroxytyrosol was prepared from their corresponding carboxylic acid by reduction with lithium aluminum hydride (*27*). Preparation of lipophilic hydroxytyrosol derivatives **2**−**5** (**Figure 1**) was carried out by chemical acylation of the corresponding alcohols following the procedure described previously (*22*, *25*). General procedure: *Candida antarctica* lipase (Novozym 435) (180 mg) was added to a mixture of hydroxytyrosol (200 mg, 1.298 mmol, 1 equiv) and the acylating agent (20 equiv) in 45 mL of *tert*-butyl methyl ether using a dry round-bottom flask, and the mixture was stirred for 60 min at 40 °C. The enzyme was decanted and separated. The solvent was evaporated, and the product was purified by flash column chromatography.

Data for Hydroxytyrosol Octanoate (4). The pure compound was obtained using vinyl octanoate as the acylating agent and after column chromatography (hexane: ethyl acetate, 3:1) in 99% yield. ^{1}H NMR (300 mHz, $CDCl_3$): δ 6.80(d, 1H, J = 6 Hz); 6.75 (s, 1H); 6.63 (dd, 1H, J = 6 and 1.2 Hz); 4.26 (t, 2H, J = 5.4 Hz); 2.82 (t, 2H, J = 5.4 Hz); 2.39−2.29 (m, 4H); 1.67−1.59 (m, 4H); 1.29 (bs, 15H); 0.90 (m, 5H). ^{13}C NMR (75 mHz, $CDCl_3$): δ (ppm) 179.8; 174.9; 143.8; 142.5; 130.3; 121.1; 115.8; 115.4; 65.3; 34.4; 34.0; 31.6; 29.0; 28.8; 24.9; 24.7; 22.6; 14.1. ESIMS: found 303.1; $C_{16}H_{24}O_4Na$ $[M + Na]^+$ requires 303.1.

Oxidation Analysis. *Sensory Analysis*. The attributes of fish oil and fish oil-in-water emulsions were evaluated by an expert panel formed by four specialists trained in descriptive analysis of fishy off-flavors. Tasting was carried out in a room designed for the purpose. The raw samples were placed in separate sterile polystyrene Petri dishes immediately upon opening of the flask. Panelists concentrated on detecting rancidity/painty odors of the same raw samples that were used for chemical determinations.

Conjugated Diene and Triene Hydroperoxides. 100 mg of fish oil samples and emulsion samples were dissolved in hexane and in ethanol, respectively. Absorbance was measured at 234 and 268 nm (UV−vis spectrophotometer Perkin-Elmer) and calculated as mmol of hydroperoxides/kg of oil as described previously (*28*).

Measurement of Fluorescence Compounds. 100 mg of emulsion samples were dissolved in ethanol for measuring the formation of fluorescence compounds resulting of interaction among oxidation products and amino constituents present in the lecithin used as emulsifier. Fluorescence was measured at 345/416 nm and 393/463 nm (Perkin-Elmer LS 3B) and was standardized with a quinine sulfate solution (1 μg/mL in 0.05 M H_2SO_4) according to the procedure described by Medina et al.(*29*). The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the sample fluorescence at each excitation/emission maximum, and F_{st} is the corresponding fluorescence intensity of a quinine sulfate solution (1 μg/mL in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence shift (δF) was calculated as the ratio between both RF values: $\delta F = RF_{393/463nm}/RF_{345/416nm}$.

DPPH$^\bullet$ Radical Scavenging Assay. Measurement of DPPH$^\bullet$ radical scavenging activity was performed according to reported recommendations (*30*). Conditions consist of approximately 20 min reaction period and a molar ratio between DPPH$^\bullet$ and antioxidant that permits 60−80% radical scavenging activity for the most potent antioxidant. Briefly, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) in ethanol (250 μM, 2 mL) was added to 2 mL of the test compounds at different concentrations in ethanol. The final concentrations of the test compounds in the reaction mixtures were 0.5, 5, 10, 25, and 50 μM. Each mixture was then shaken vigorously and held for 30 min at room temperature in the dark. The decrease in absorbance of DPPH$^\bullet$ at 517 nM was measured. Ethanol was used as a blank solution. DPPH$^\bullet$ solution (2 mL) in ethanol (2 mL) served as the control. All tests were performed in triplicate. A dose response curve was obtained for every compound. ED_{50} corresponds to either micrograms or micromoles of product able to consume half the amount of free

radical divided by micromoles of initial DPPH$^{\bullet}$. The results are expressed as antiradical power (ARP), or $1/ED_{50}$.

TEAC Assay (*Trolox Equivalent Antioxidant Capacity*). The method is based on the capacity of a sample to scavenge the stable free radical cation, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) as compared to a standard antioxidant (Trolox). $ABTS^{\bullet+}$was prepared as follows (*31*): 3% H_2O_2 (45 μL) was added to a reaction mixture containing ABTS (54.9 mg, 1 mM) and horseradish peroxidase (HRP, 1.1 mg, 0.25 μM) in 50 mM gly HCl buffer, pH 4.5 (100 mL). The reaction mixture was left to stand at room temperature for 15 min in the dark. Then, the antioxidant solutions (50 μL) at concentrations of 0.3, 0.2, 0.15, 0.10, and 0.05 mg/mL in MeOH were added to the $ABTS^{\bullet+}$ solution (1950 μL). The decrease of absorbance at 734 nm with respect to the 1 mM solution of $ABTS^{\bullet+}$ was then measured used an UV−vis spectrophotometer Perkin-Elmer. The dose−response curves obtained with the antioxidant mixtures and Trolox were plotted as the percentage of absorbance decrease against the amount of antioxidants expressed as μg/mL. The total antioxidant activity (TAA) of the fractions was expressed in mmol Trolox equiv/g.

Reducing Power of the Phenolic Compounds. The FRAP (ferric reducing/antioxidant power) method was used by adaptation of the procedure of Benzie and Strain (*32*). The FRAP reagent was prepared daily by mixing acetate buffer 300 mM (pH 3.6), 2,4,6-tripyridyl-*s*-triazine (TPTZ) 10 mM and ferric chloride 20 mM, in the ratio 10:1:1, respectively. TPTZ solution was prepared in HCl 40 mM. 1.5 mL of FRAP reagent were incubated for 10 min at 37 °C. Then, 150 μL of water and 50 μL of phenolic solution (0.2−4 mg/L) were added and the absorbance was measured at 593 nm after 4 min. The standard curve was built with ferrous chloride. The number of donated electrons was calculated from the slopes of the lineal adjustments between the phenolic concentration and the FRAP activity.

Partitioning Coefficient Log *P* Solubility Measurement. 0.3 mM dispersions of each compound were prepared in 1-octanol and were heated up to 60 °C for 1 h to help to solubilize all the antioxidant. A UV spectrum for each solution was obtained, and its absorbance at the maximum was determined (A_0). Equal volumes of the organic solution (2 mL) and a phosphate buffer (0.1 M, pH = 7.4) were vigorously mixed using a vortex mixer for 1 min. The mixture was allowed to stabilize and separate for 30 min, and then the organic phase was obtained and its absorbance determined (A_x). Partition coefficient (log *P*) was determined from the fraction $P = A_x/(A_0 - A_x)$. All tests were performed in triplicate. Log *P* values for compounds **4** and **5** were calculated using Crippen's fragmentation(*33*) in the ChemBioDraw Ultra 11.0 software since the experimental measurements did not yield reliable results. The log *P* value for compound **6** was obtained from ref *34*.

RESULTS AND DISCUSSION

Inhibition of Lipid Oxidation in Fish Oils. Two preliminary experiments were performed in order to obtain the precise concentration of antioxidants to carry out the oxidation experiments in fish oil. First, the possible antioxidant effectiveness in fish oils of the simplest hydroxytyrosol ester, hydroxytyrosol acetate, was checked against hydroxytyrosol. Results obtained demonstrated a significant inhibition of the formation of lipid oxidation byproduct due to the addition of 100 ppm of hydroxytyrosol and hydroxytyrosol acetate during thermal oxidation (**Table 1**). The development of rancid off-flavors was totally inhibited in both samples related to controls. Scarce significant differences were found between the antioxidant efficiency of hydroxytyrosol and hydroxytyrosol acetate in fish oils according to results reported previously in olive oil (*11*). Hydroxytyrosol acetate, a substance easier to manipulate than hydroxytyrosol, was then used to test the effect of the addition of different concentrations (10, 25, 50, 100, 150, and 200 ppm) on the prevention of oxidation of bulk cod liver oils. Oxidation was studied during 10 days. Rancid off-flavors were detected in control samples by the sixth day of oxidation. Fish oils supplemented with 10 and 25 ppm delayed the apparition of rancidity for one day. Those samples supplemented with 50 ppm delayed rancidity for two days, and the other samples did not show rancid off-flavors until the ninth day. These data were in agreement with the detection of conjugated diene hydroperoxides the formation of which decreased with increasing amounts of hydroxytyrosol acetate present in the fish oil (**Table 2**). Concentrations of hydroxytyrosol acetate at 100 ppm were highly effective at inhibiting the lipid oxidation rate of bulk oil, and no improvement was observed when higher concentrations were used (150 and 200 ppm). Therefore, evaluation of the antioxidant activity was carried out at a concentration of 100 ppm of hydroxytyrosol and the corresponding same molar concentration (0.65 mmol/kg) of the lipophilic hydroxytyrosol derivatives.

Oxidation experiments were then carried out to study the effect of the ester moiety. The antioxidant activity of hydroxytyrosol and hydroxytyrosol fatty acid esters was checked using the same conditions described above (**Figure 2**). Octyl gallate, a commonly used food antioxidant, was included as a control antioxidant. It was selected due to its amphiphilic phenolic structure similar to that of hydroxytyrosol fatty acid esters. All ester derivatives inhibited the formation of conjugated hydroperoxides. The antioxidant efficiency order was found to be octyl gallate > hydroxytyrosol > hydroxytyrosol acetate > hydroxytyrosol butyrate > hydroxytyrosol octanoate > hydroxytyrosol laurate (**Table 3**). Sensory analysis corroborated chemical data. Rancidity was first detected in controls by the fourth day together with incipient rancid off-flavors in samples supplemented with laurate and

Table 1. Inhibition by 100 ppm Hydroxytyrosol and Hydroxytyrosol Acetate on the Formation of Conjugated Diene and Triene Hydroperoxides in Fish Oil and in Fish Oil-in-Water Emulsions during Oxidation at 40 °C (Mean ± sd)[a]

	fish oil (day 6)		fish oil-in-water emulsions (day 6)	
phenolic antioxidants	conjugates dienes	conjugated trienes	conjugates dienes	conjugated trienes
control	0.0 ± 33.2^{a}	0.0 ± 33.5^{a}	0.0 ± 13.3^{a}	0.0 ± 8.0^{a}
HYD	74.4 ± 5.2^{b}	69.2 ± 17.0^{bc}	89.3 ± 1.6^{b}	86.3 ± 3.7^{b}
HYD acetate	65.7 ± 5.2^{b}	47.5 ± 25.7^{b}	95.2 ± 2.3^{c}	93.5 ± 2.0^{c}

[a] % inhibition = $[(C - S)/C] \times 100$ where C = increment in the oxidation product formed in control from day 0 and S = increment in the oxidation product formed in sample from day 0 (*6*). Inhibition is calculated during propagation phase of controls. Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

Table 2. Inhibition by Hydroxytyrosol Acetate Supplemented at Different Concentrations on the Formation of Conjugated Diene and Triene Hydroperoxides in Fish Oil and in Fish Oil-in-Water Emulsions during Oxidation at 40 °C (Mean ± sd)[a]

	fish oil (day 8)		fish oil-in-water emulsions (day 10)	
phenolic antioxidants	conjugates dienes	conjugated trienes	conjugates dienes	conjugated trienes
control	0.0 ± 1.9^{a}	0.0 ± 4.9^{a}	0.0 ± 11.8^{a}	0.0 ± 18.4^{a}
HYD acetate 10	-5.3 ± 13.1^{ab}	-1.5 ± 3.2^{a}	-8.0 ± 32.1^{a}	8.6 ± 38.3^{ab}
HYD acetate 25	10.2 ± 3.9^{b}	34.1 ± 4.3^{b}	19.7 ± 2.3^{b}	46.4 ± 5.4^{b}
HYD acetate 50	38.5 ± 15.3^{c}	57.9 ± 6.6^{c}	56.7 ± 1.4^{c}	69.7 ± 2.0^{c}
HYD acetate 100	56.1 ± 12.1^{cd}	65.6 ± 9.9^{cd}	84.9 ± 3.2^{d}	92.6 ± 6.8^{d}
HYD acetate 150	69.5 ± 4.5^{d}	71.5 ± 2.2^{d}	78.5 ± 4.6^{d}	77.9 ± 3.0^{d}
HYD acetate 200	70.9 ± 1.9^{d}	66.3 ± 8.6^{cd}	94.1 ± 3.4^{de}	93.3 ± 8.1^{d}

[a] % inhibition = $[(C - S)/C] \times 100$ where C = increment in the oxidation product formed in control from day 0 and S = increment in the oxidation product formed in sample from day 0 (*6*). Inhibition is calculated during propagation phase of controls. Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

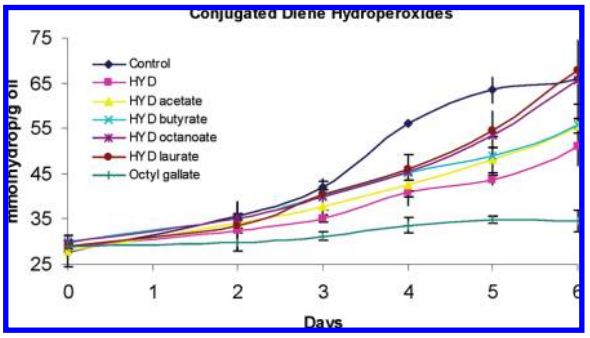

Figure 2. Time course of lipid oxidation of cod liver oil measured by conjugated hydroperoxides.

Table 3. Inhibition by Hydroxytyrosol and Several Hydroxytyrosol Esters on the Formation of Conjugated Diene and Triene Hydroperoxides in Fish Oil during Oxidation at 40 °C[a]

	conjugates dienes		conjugated trienes	
phenolic antioxidants	day 5	day 6	day 5	day 6
control	0.0 ± 7.8^{a}	0.0 ± 22.6^{a}	0.0 ± 3.3^{a}	0.0 ± 2.1^{a}
HYD	55.4 ± 2.9^{d}	38.5 ± 11.2^{bc}	48.5 ± 3.3^{c}	37.5 ± 6.5^{c}
HYD acetate	43.1 ± 7.5^{c}	26.8 ± 8.0^{b}	28.4 ± 7.6^{bc}	17.1 ± 5.6^{b}
HYD butyrate	40.4 ± 12.0^{bc}	26.1 ± 11.5^{b}	17.1 ± 7.4^{b}	0.8 ± 0.9^{a}
HYD octanoate	28.3 ± 14.9^{bc}	0.8 ± 5.4^{a}	14.8 ± 3.7^{b}	-11.4 ± 3.5^{a}
HYD laurate	25.2 ± 4.6^{b}	-5.1 ± 5.1^{a}	-8.6 ± 4.0^{a}	-12.4 ± 4.0^{a}
octyl gallate	79.8 ± 2.4^{e}	81.5 ± 6.3^{d}	72.9 ± 23.2^{d}	73.4 ± 7.8^{d}

[a] Antioxidants were tested at the same concentration: 0.65 mmol/kg (mean ± sd). % inhibition = $[(C - S)/C] \times 100$ where C = increment in the oxidation product formed in control from day 0 and S = increment in the oxidation product formed in sample from day 0 (*6*). Inhibition is calculated during propagation phase of controls. Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

octanoate esters. Incipient rancidity was developed in acetate and butyrate samples by the fifth day and, finally, in hydroxytyrosol supplemented samples. A decrease on antioxidant potency could be observed with the increment of the length chain and the subsequent increase of lipophilicity of the hydroxytyrosol derivatives (see **Table 4** for log *P* values). The presence of a third phenolic hydroxyl group in the structure of octyl gallate seems to be determinant for its antioxidant activity in comparison with that of the hydroxytyrosol esters in bulk oils. These results were in agreement with those reported by Gordon et al. (*11*) describing a better antioxidant activity for hydroxytyrosol than for hydroxytyrosol acetate in olive oil on the basis of the peroxide value and the *p*-anisidine value determined. The order of antioxidant efficiency found fits with the general hypothesis of the "polar paradox" where polar antioxidants are more efficient in fats and oils. Studies by Mateos et al. (*35*) and Trujillo et al. (*23*) have reported similar or scarce differences among antioxidant effectiveness of hydroxytyrosol and several hydroxytyrosol lipophilic esters in olive oil as measured by the Rancimat method. Such differences with the results obtained in this study and those reported by Gordon et al. (*11*) can be explained considering the differences on the oxidation experiments and the methods employed for determining the rate of oxidation. Oxidation experiments have been carried out at gentle oxidation conditions and measuring the formation of conjugates diene and triene hydroperoxides that yields information about the first stages of the oxidation process. The studies by Mateos et al. (*35*) and Trujillo et al. (*23*) were performed using much more severe oxidation conditions following the lipid oxidation by the Rancimat method that yields information about more advanced oxidation stages. These results indicated that no differences in oxidation capacity depending on polarity are observed when more advanced oxidation stages are studied, such as using the Rancimat assay.

A recent review on the enzymatic lipophilization of phenolic acids also discussed their antioxidant activity (*36*). The antioxidant capacity of ferulic acid, another important phenolic antioxidant, has been compared with several lipophilic ferulic acid esters. Nenadis et al. (*37*) observed no differences in antioxidant activity of ferulic acid and ethyl ferulate in triolein when peroxide values were measured. It is important to note that these compounds are quite apolar and possess very similar polarity (log *P* values of ferulic acid, 1.42, and ethyl ferulate, 2.02). Similarly, Kikuzaki et al. (*38*) reported comparable induction times for ferulic acid and several ferulic fatty acid esters in methyl linoleate as the oil matrix when measured by the Rancimat method. In contrast, the same authors, using the same conditions, observed a large variation when comparing the induction times also measured for gallic acid and several alkyl gallates. The relative order of decreasing antioxidant potency was methyl gallate $\gg$ propyl gallate $\sim$ lauryl gallate $>$ gallic acid $\sim$ stearyl gallate. The tendency found fits quite well with the polar paradox, since the less apolar compounds display better antioxidant activity, except for the case of gallic acid, the more polar of this series that is not the best antioxidant.

Inhibition of Lipid Oxidation in Fish Oil-in-Water Emulsions. As in the bulk oil case, a thermal oxidation study (40 °C) of hydroxytyrosol and hydroxytyrosol acetate at 100 ppm concentration in a fish oil-in-water emulsion was first performed. Both compounds were able to inhibit the formation of lipid oxidation byproduct, and the antioxidant effectiveness was slightly better for hydroxytyrosol acetate than for hydroxytyrosol (**Table 1**). Sensory scores confirmed these data. These results were in agreement with previous data by Gordon et al. (*11*). Hydroxytyrosol acetate is less polar than hydroxytyrosol which makes it more effective as an antioxidant in an emulsion due to the polar paradox. Then, different concentrations of hydroxytyrosol acetate (10, 25, 50, 100, 150, and 200 ppm) were tested in fish oil-in-water emulsions in order to select the optimum concentration of the ester derivatives. Oxidation was studied during 10 days. Rancid off-flavors were detected in controls and samples supplemented with 10 ppm of hydroxytyrosol acetate by the seventh day. Samples containing 25 and 50 ppm of the antioxidant retard the apparition of rancidity until the eighth and the ninth day, respectively. Finally, emulsions supplemented with concentrations ranged between 100 to 200 ppm of antioxidant inhibited completely the development of rancid off-flavors. Data of the formation of conjugated diene and triene hydroperoxides corroborated sensory analysis and decreased with increasing amounts of hydroxytyrosol acetate present in the samples (**Table 2**). As for fish oils, 100 ppm of hydroxytyrosol acetate was effective for inhibiting completely the lipid oxidation rate of fish oil-in-water emulsions, and no improvement was observed when higher concentrations were used (150 and 200 ppm). Consequently, evaluation of the antioxidant activity was performed at a molar concentration (0.65 mmol/kg) of hydroxytyrosol acetate and the corresponding hydroxytyrosol esters.

Oxidation experiments in emulsions containing hydroxytyrosol esters with increasing length chain were carried out in the same conditions described above. Again, octyl gallate was included as the antioxidant control. Oxidation was effectively inhibited by the addition of phenolic compounds as demonstrated by the kinetics of oxidation products (conjugated hydroperoxides) and fluorescent compounds (**Figure 3**). Rancidity was observed in controls by the fifth day and retarded in emulsions supplemented with antioxidants. Rancid off-flavors were detected in samples supplemented with hydroxytyrosol, acetate and butyrate derivatives by

Table 4. Log *P* Values, Radical Scavenging and Reducing Power of Hydroxytyrosol and Several Hydroxytyrosol Esters

phenolic antioxidants	DPPH EC_{50}	FRAP	TEAC	log *P*
HYD	0.7198 ± 0.0003	1.1440 ± 0,0956	0.3171 ± 0,0175	−0.08 ± 0.09
HYD acetate	0.5709 ± 0.0177	1.5071 ± 0,0793	0.2195 ± 0,0052	0.78 ± 0.21
HYD butyrate	0.5899 ± 0.0193	1.3692 ± 0,1005	0.2558 ± 0,0126	2.03 ± 0.44
HYD octanoate	0.6861 ± 0.0335	1.1279 ± 0,1203	0.3017 ± 0,0039	3.93
HYD laurate	0.9216 ± 0.0411	1.0350 ± 0,1048	0.4848 ± 0,0018	5.60
Octyl gallate	0.3444 ± 0.0101	1.7419 ± 0,1087	0.2324 ± 0,0021	2.11

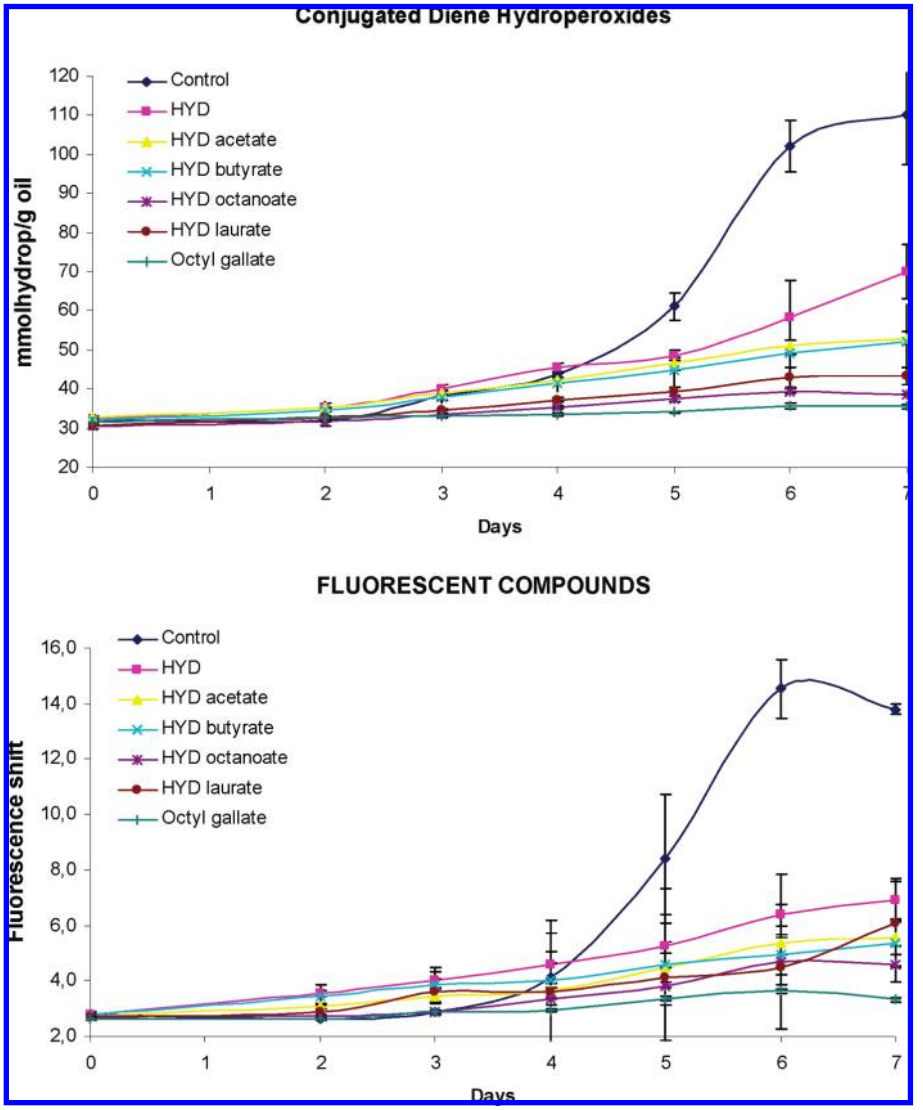

Figure 3. Time course of lipid oxidation of fish oil-in-water emulsions measured by conjugated diene hydroperoxides and fluorescent compounds.

the sixth day. Finally, incipient rancidity was observed in octanoate supplemented samples by the seventh day. **Table 5** shows the percent of inhibition on the formation of conjugated diene and triene hydroperoxides and fluorescent compounds. In contrast with the results in bulk fish oils, the antioxidant efficiency order was found to be octyl gallate ~ hydroxytyrosol octanoate > hydroxytyrosol laurate > hydroxytyrosol acetate ~ hydroxytyrosol butyrate ~ hydroxytyrosol. The more lipophilic antioxidants hydroxytyrosol octanoate and hydroxytyrosol laurate were more efficient in inhibiting oxidation than the less lipophilic antioxidants, hydroxytyrosol acetate and hydroxytyrosol butyrate. It is important to note that hydroxytyrosol octanoate and hydroxytyrosol laurate show very similar antioxidant potency in emulsions to that of octyl gallate, even when the hydroxytyrosol esters possess only two phenolic hydroxyl groups. Nenadis et al. (*37*) have also described higher antioxidant activity of the less polar ethyl ferulate than for ferulic acid when peroxide values were measured in an oil-in-water emulsion using triolein as the oil phase. This general trend seems to fit quite well with the "polar paradox". In contrast to these results, Yuji et al. (*39*) found that *p*-hydrophenylacetic acid (HPA) was a better antioxidant than more apolar compounds such as HPA butyrate and laurate in a Menhaden oil-in-water emulsion. These authors attributed the differences to a distinct physical location of the antioxidants into the emulsion. HPA esters seem to be better solubilized by the surfactant micelles, decreasing their effective concentration at the emulsion droplet interface.

Nevertheless, it is important to note that a maximum of antioxidant efficiency seems to appear in fish oil-in-water emulsions when the chain length of the hydroxytyrosol derivative is

Table 5. Inhibition by Hydroxytyrosol and Several Hydroxytyrosol Esters on the Formation of Conjugated Diene and Triene Hydroperoxides and Fluorescent Compounds in Fish Oil-in-Water Emulsions during Oxidation at 40 °C[a]

	conjugates dienes		conjugated trienes		fluorescent compounds	
phenolic antioxidants	day 6	day 7	day 6	day 7	day 6	day 7
control	0.0 ± 9.2^{a}	0.0 ± 29.2^{a}	0.0 ± 9.3^{a}	0.0 ± 24.6^{a}	0.0 ± 9.3^{a}	0.0 ± 24.6^{a}
HYD	66.7 ± 1.2^{b}	64.6 ± 5.4^{b}	70.8 ± 0.9^{b}	73.5 ± 1.5^{b}	69.6 ± 3.6^{b}	62.5 ± 3.5^{b}
HYD acetate	73.0 ± 2.3^{c}	73.2 ± 2.2^{bc}	78.9 ± 3.9^{c}	84.3 ± 2.9^{c}	77.6 ± 5.8^{c}	74.2 ± 5.9^{c}
HYD butyrate	75.4 ± 7.1^{cd}	74.2 ± 12.1^{bc}	74.9 ± 11.9^{bc}	76.7 ± 18.9^{bc}	81.2 ± 6.6^{cd}	88.7 ± 8.2^{d}
HYD octanoate	89.4 ± 0.9^{e}	91.7 ± 0.1^{e}	91.7 ± 1.5^{e}	96.8 ± 0.5^{f}	96.3 ± 3.9^{e}	95.1 ± 5.4^{d}
HYD laurate	84.3 ± 3.5^{de}	85.5 ± 3.0^{d}	79.7 ± 8.6^{bc}	87.3 ± 5.2^{cd}	96.9 ± 8.4^{de}	87.5 ± 2.8^{d}
octyl gallate	95.0 ± 1.0^{f}	95.6 ± 0.9^{f}	81.6 ± 1.5^{cd}	88.9 ± 1.2^{d}	91.4 ± 0.8^{e}	93.6 ± 0.8^{d}

[a] Antioxidants were tested at the same concentration: 0.65 mmol/kg (mean ± sd). % inhibition = $[(C - S)/C] \times 100$ where C = increment in the oxidation product formed in control from day 0 and S = increment in the oxidation product formed in sample from day 0 (*6*). Inhibition is calculated during propagation phase of controls. Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

that of eight carbons. The physical location of the antioxidants is dependent on their solubility in lipid and water. Therefore, more lipophilic hydroxytyrosol esters will increase the oxidation concentration at the droplet interface and consequently will help to protect oxidation of the fatty acids placed at the interior of the micelles. At the same time, the elongation of the alkyl chain may reduce the random rotation of the antioxidant in the interface of the emulsion, and this effect may preferentially locate the antioxidant in the interface with its orthophenolic moiety pointing to the aqueous phase. Finally, if the alkyl chain length of hydroxytyrosol esters is too long, this could place the antioxidant deeper in the interior of the micelle being less effective than when it is more preferentially positioned at the interface. This effect of the alkyl side chain variation has been also observed on ubiquinone derivatives by Yu et al. (*40*).

Radical Scavenging and Reducing Power Tests. The radical scavenging activity of hydroxytyrosol and several hydroxytyrosol fatty acid esters was measured using two different methodologies, the DPPH and the TEAC radical scavenging assays. The results obtained in both experiments were very similar showing the following radical scavenging efficiency: octyl gallate > hydroxytyrosol acetate > hydroxytyrosol butyrate > hydroxytyrosol octanoate ~ hydroxytyrosol > hydroxytyrosol laurate. The number of phenolic OH's is the first factor affecting radical scavenging activity. Moreover, the alkyl hydroxytyrosol esters of lower side chain length showed better radical scavenging activity than hydroxytyrosol. In contrast, hydroxytyrosol laurate, with longer side chain, showed worse activity than hydroxytyrosol.

The results obtained for the scavenging capacity were in agreement with the reducing capacity order of hydroxytyrosol and their fatty acid esters measured using the FRAP assay. The ferric reducing ability decreased in the following order: octyl gallate > hydroxytyrosol acetate > hydroxytyrosol > hydroxytyrosol butyrate > hydroxytyrosol octanoate > hydroxytyrosol laurate. The presence of the acetate chain improves slightly the reducing ability shown by free hydroxytyrosol. As it has been detected by the DPPH and TEAC assays, a reduction in the capacity for donating electrons was observed as the length chain increases (C4−C12).

Similar results have been recently published by Pereira-Caro et al. (*41*) for ether derivatives of hydroxytyrosol. These authors have found that short alkyl side chains improved the scavenger activity and the reducing ability of hydroxytyrosol but the presence of longer linear chains (C7−C9) decreased such antioxidant effects. Authors have related this behavior with the steric effect of the longer chain groups according to Lu et al. (*34*), who have indicated that the steric freedom is also important for the activity of antioxidants in solutions and liposomes. Gordon et al. (*11*) have suggested that intra- or intermolecular hydrogen bonding could hinder the scavenging effect of the hydroxyl groups.

The results of the radical scavenging and reducing power tests correlate somehow with the observed antioxidant capacity of hydroxytyrosol fatty acid esters in bulk fish oils since longer alkyl chains in the antioxidant yields worse antioxidant capacity. Nevertheless, hydroxytyrosol, which is the best antioxidant of the series in bulk fish oils, shows worse scavenging and reducing values than several of their fatty acid ester derivatives. In the case of the fish oil-in-water emulsions no correlation could be withdrawn with the scavenging or reducing data measured.

In conclusion, our results showed a significant antioxidant activity of hydroxytyrosol esters in both systems especially in emulsions. The introduction of a lipophilic chain decreased the antioxidant effectiveness of hydroxytyrosol in fish oils. This reduction seems to be dependent on the length of the chain and the subsequent increment of lipophilicity as predicted by the polar paradox and in contrast to previous results obtained in olive oil. In emulsion systems, the presence of a short−medium lipophilic chain improved the antioxidant efficiency of hydroxytyrosol favoring the physical location of the antioxidant in the interface, but a longer alkyl chain (laurate) maintained or even decreased their antioxidant activity. It is reasonable to consider that the adequate lipophilicity of the antioxidant places it at the best geometry and position within the oil−water interface leading to display its maximum antioxidant capacity. The results obtained support the "polar paradox" since the antioxidant activity of the lipophilic hydroxytyrosol esters was lower in bulk oils and higher in oil-in-water emulsions in comparison with hydroxytyrosol. The results also show the highly effective antioxidant effectiveness of hydroxytyrosol esters in fish oil systems especially in fish oil emulsions. These results are of high importance for the optimum design of effective antioxidant for omega 3 enriched foods, which are very susceptible to suffer rancidity.

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