DOI:10.1021/jf101472n

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Antioxidant Activity of Resveratrol in Several Fish Lipid Matrices: Effect of Acylation and Glucosylation

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The antioxidant activity of resveratrol (**1**) and several acylated and glycosylated derivatives on fish oil enriched systems has been studied. Two long-chain acylated derivatives, 3-stearoylresveratrol (**2**) and 4′-stearoylresveratrol (**3**), and three glucosyl derivatives, resveratrol-3-β-D-glucopyranoside (piceid, **4**), resveratrol-3,5-di-β-D-glucopyranoside (**5**), and resveratrol-3,4′-di-β-D-glucopyranoside (**6**), have been prepared and tested. The results have shown a notable antioxidant capacity of resveratrol and piceid in fish oil-in-water emulsions, similar to that of the potent antioxidant hydroxytyrosol. Lipophilization of resveratrol did not improve its antioxidant activity, either in emulsions or in bulk fish oil. Further glucosylation of piceid yielding compounds **5** and **6** did not improve either resveratrol or piceid antioxidant efficiency in emulsions or in bulk oil. In all of the examples, the hydroxyl group at the 4′-position seems to be relevant for the antioxidant efficiency of resveratrol, and it should be maintained to keep the antiradical activity. Finally, resveratrol has shown to be a very good antioxidant for fish muscle, as good as the potent antioxidant hydroxytyrosol.

KEYWORDS: Lipid oxidation; resveratrol; lipophilization; glucosylation; fish lipids; oil-in-water emulsions;

INTRODUCTION

Lipid oxidation is still today a problem in the food industry, especially for products that contain marine lipids. The high content of polyunsaturated fatty acids (PUFA) in fish oil, highly beneficial for human health (*1*−*3*), makes it very attractive to the rising market of functional products. However, at the same time, this high content of PUFA makes marine lipids highly susceptible to oxidation (*4*), consequently affecting the quality of fish oils during storage by deterioration of the flavor, odor, color, and texture and even producing toxic compounds. This phenomenon is aggravated in fish fillets due to their large surface area and is even worse in fish oil-in-water emulsions 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 (*5*, *6*).

Antioxidants are often added to food to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation steps, consequently delaying the oxidation process. New antioxidants capable of retarding oxidation in fish oil enriched foods would be desirable, even more so if these new antioxidants possessed relevant biological properties. Resveratrol, 3,4′,5-trihydroxystilbene, found in Japanese knotweed (*Polygonum cuspidatum*) and also in grapes and grape-derived products, such as red wine, is a remarkable phenolic compound. Resveratrol has shown cardiovascular protective properties (*7*) and antiinflammatory activity in different animal models (*8*, *9*), and especially important are its anticancer activity (*10*) and its capacity to extend lifespan in a variety of species through a mechanism of caloric restriction (*11*) and activation of various deacetylase enzymes (sirtuins). The antioxidant capacity of resveratrol has been demonstrated by inhibition of the lipid peroxidation induced by ADP and NADPH in rat liver microsomes (*12*), by the FTC (ferric-thiocyanate) method (*13*), as a radical scavenger using the DPPH test (*14*), and by other in vitro assays (*15*−*17*). Scarce examples of the use of resveratrol as a food antioxidant have been described so far. Wang et al. (*18*) showed that resveratrol was a better antioxidant than butylated hydroxytoluene (BHT) in pure lard (pork fat) as measured by the Rancimat method. In contrast, resveratrol behaved as a weak antioxidant against oxidation of sunflower and rapeseed oils, and also low efficiency was achieved in margarine (water-in-oil emulsion) (*19*).

The effectiveness of an antioxidant mainly relies on its chemical reactivity (as radical scavenger or as metal chelator), but it is also important how it interacts with other food components, their concentration, and especially their physical location in different homogeneous or heterogeneous food systems (for example, bulk oil or oil-in-water emulsion). For that reason, chemical modification of the antioxidants that change their physical characteristics, such as increasing their lipophilicity or making them amphiphilic, may be important for their antioxidant performance. A large number of resveratrol derivatives have been prepared, mainly to study their metabolic fate (*20*) and to try to improve their biological properties. For example, acetylated or fluoro derivatives of resveratrol have been assayed on a variety of cancer cell lines (*21*, *22*) or methylated derivatives as antithrombotic agents (*23*). Different in vitro radical scavenger activities have been reported in glycosylated resveratrol

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 Published on Web 08/05/2010

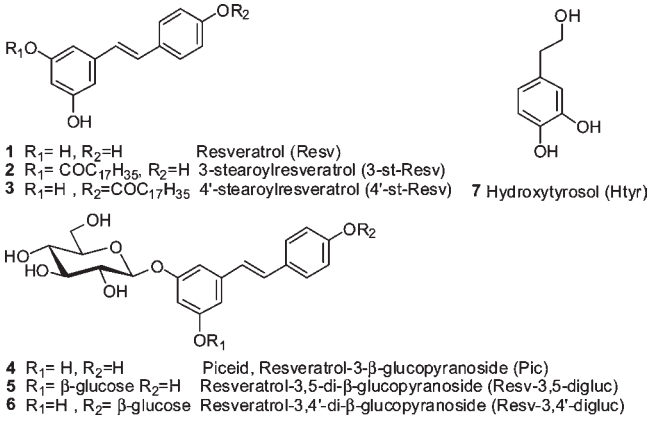

Figure 1. Chemical structures of resveratrol, 3-stearoylresveratrol, 4′-stearoylresveratrol, piceid, resveratrol-3,5-di-β-D-glucopyranoside, resveratrol-3,4′-di-β-D-glucopyranoside, and hydroxytyrosol.

compounds, such as piceid (resveratrol-3-β-D-glucopyranoside), resveratrol-4′-β-D-glucopyranoside, and resveratrol-3,5-di-β-D-glucopyranoside (*24*, *25*). Recently, Torres et al. (*26*) have described the enzymatic synthesis of 3-*O*-acyl and 4′-*O*-acyl-resveratrol derivatives and their radical scavenging activity as well.

In this work, resveratrol and several acylated and glycosylated derivatives have been examined as inhibitors of the oxidation of highly oxidative susceptible foodstuffs such as those containing fish lipids. Two long-chain acylated compounds, 3-stearoylresveratrol (**2**) and 4′-stearoylresveratrol (**3**), and three glucosyl derivatives, resveratrol-3-β-D-glucopyranoside (piceid, **4**), resveratrol-3,5-di-β-D-glucopyranoside (**5**), and resveratrol-3,4′-di-β-D-glucopyranoside (**6**), have been prepared and tested as food antioxidants (**Figure 1**). Stearic acid was selected because the C18 carbon chain is long enough to alter substantially the hydrophile−lipophile balance (HLB) of resveratrol, making it much more hydrophobic. Similarly, glucose moieties were selected because they could alter the HLB of resveratrol, making it much more hydrophilic. Hydroxytyrosol (**7**) has been used as a control. Three different food systems containing fish lipids have been studied: bulk fish oils, fish oil-in-water emulsions, and fish fillets. The rate of oxidation was monitored by the formation of lipid oxidation products during controlled sample storage. Chemico-physical properties such as radical scavenging capacity, reducing ability, or polarity of the resveratrol derivatives were measured to try to explain the antioxidant efficiency found and to predict the antioxidant behavior of the new compounds.

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 of either analytical or HPLC grade (Ridel-Haën, Seelze, Germany). Water was purified through a Millipore-Q plus (Millipore Corp., Bedford, MA). Immobilized lipases from *Candida antarctica* (Novozym 435) and from *Alcaligenes* sp. (lipase QLG) were kindly provided by Novozymes A/S and Meito Sangyo Co., respectively. Resveratrol was kindy provided by Prof. J. C. Espín (CEBAS, CSIC, Murcia, Spain). Piceid was purchased from Sigma Aldrich.

Preparation of Bulk Fish Oil. Cod liver oil samples (5 g) were placed in screw-capped 50 mL Erlenmeyer flasks. Before that, resveratrol and its derivatives 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 antioxidants. 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. Triplicate samples were prepared and oxidized. A first set of experiments was carried out to test the effect of antioxidant concentration. They were run during the period of time needed to identify the minimum concentration that would yield the maximum antioxidant capacity. A second set of experiments was performed to compare the effectiveness of different antioxidants. They were run during the period of 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. (*27*). Briefly, cod liver oil was emulsified in water using lecithin as emulsifier and sonicated 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 antioxidants. 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.

Preparation of Fish Minced Muscle. Ten kilograms of Atlantic horse mackerel (*Trauchurus trauchurus*) were debonned and eviscerated, and the white muscle was separated and minced to obtain a muscle homogenate. Streptomycin sulfate (200 ppm) was added for inhibiting microbial growth. The different antioxidant compounds were added at a concentration of 100 ppm (w/w). Portions of 10 g of minced muscle were placed into plastic bags. Control samples in the absence of antioxidants and samples with antioxidants were kept refrigerated at 4 °C during 11 days until samples showed microbial growth. Duplicate samples were taken at different sampling times. Inhibition of oxidation was calculated during the propagation period of controls according to the method of Frankel (*5*). Induction periods were calculated as the time (in days) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve. The experiment was done twice.

Synthesis of Resveratrol Derivatives. 3-Stearoylresveratrol and 4′-stearoylresveratrol were prepared by enzymatic acylation as described previously (*26*). Resveratrol-4′-β-D-glucopyranoside, resveratrol-3,5-di-β-D-glucopyranoside, and resveratrol-3,4-di-β-D-glucopyranoside were chemically synthesized through a modification of a previously described procedure (*28*). Briefly, resveratrol was randomly protected with *tert*-butyldimethylsilyl groups. After column chromatography separation, each silyl resveratrol derivative was glycosylated with the benzoyl-protected trichloroacetimidate glucopyranoside donor in dichloromethane using trimethylsilyl triflate as the promotor. Finally, protecting groups were cleaved with sodium methoxide in a methanol/THF mixture (5:1) in good yields.

Oxidation Analysis. *Sensory Analysis*. The attributes of fish oil, fish oil-in-water emulsions, and minced fish muscle 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 (*29*). A hedonic scale from 0 to 4 was used: 0, fresh seaweedy; 1, low odor; 2, slight paint; 3, moderate paint; and 4, strong paint.

Conjugated Diene and Triene Hydroperoxides. One hundred milligrams of fish oil samples and emulsion samples was dissolved in hexane and in ethanol, respectively. Absorbance was measured at 234 and 268 nm (UV−vis spectrophotometer, Perkin-Elmer) and calculated as millimoles of hydroperoxides per kilogram of oil as described previously (*30*). % 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. Synergism was calculated by comparing the induction periods (IP) of formation of hydroperoxides according to the method of Frankel (*5*).

$$\%\ \text{synergism} = \frac{100 \times [\text{IP}(\text{Antiox}_1 + \text{Antiox}_2) - (\text{IPAntiox}_1 + \text{IPAntiox}_2)]}{(\text{IPAntiox}_1 + \text{IPAntiox}_2)}$$

Lipid Extraction. Lipids were extracted from fish muscle according to the method of Bligh and Dyer (*31*) and quantified gravimetrically (*32*).

Peroxide Value. The peroxide value of fish muscle was determined on the lipid extract by using the ferric thiocyanate method (*33*) and was expressed as milliequivalents of oxygen per kilogram of lipid. Analyses were performed in duplicate.

TBARS. The thiobarbituric acid index (mg of malonaldehyde/kg of muscle) was determined on the muscle trichloroacetic extract according to the method of Vyncke (*34*). Analyses were performed in duplicate.

DPPH• Radical Scavenging Assay. Measurement of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity was performed according to reported recommendations (*35*). Conditions consist of an approximately 20 min reaction period and a molar ratio between DPPH• and antioxidant that permits 60−80% radical scavenging activity for the most potent antioxidant. Briefly, DPPH• 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• at 517 nm was measured. Ethanol was used as a blank solution. DPPH• ethanol solution (2 mL) served as the control. All tests were performed in triplicate. A dose−response curve was obtained for each 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•. The results are expressed as antiradical power (ARP), or $1/ED_{50}$.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The method is based on the capacity of a sample to scavenge the stable free radical cation 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) as compared to a standard antioxidant (Trolox). $ABTS^{\bullet+}$ was prepared as follows (*36*): 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 0.05, 0.10, 0.15, 0.2, and 0.3 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 using 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 micrograms per milliliter. The total antioxidant activity (TAA) of the fractions was expressed in millimoles of Trolox equivalents per gram. The TEAC value was defined as the concentration of an antioxidant that causes a decrease in the Abs_{734} equivalent to 1 μM Trolox.

Reducing Power of the Phenolic Compounds. The FRAP (ferric reducing/antioxidant power) method was used by adaptation of the procedure of Benzie and Strain (*37*). The FRAP reagent was prepared daily by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ), and 20 mM ferric chloride, in the ratio 10:1:1, respectively. TPTZ solution was prepared in 40 mM HCl, and 1.5 mL of FRAP reagent was 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. Dispersions of each compound (0.3 mM) were prepared in 1-octanol and were heated to 60 °C for 1 h to help to solubilize all of the antioxidant. UV spectra for each solution were performed, and the absorbance at the maximum was determined (A_0). Equal volumes of the organic solution (2 mL) and a sodium 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 **2** and **3** were calculated computationally using Crippen's fragmentation (*38*) in ChemBioDraw Ultra 11.0 software because the experimental measurements did not yield reliable results.

Statistical Analysis. Each sample type (antioxidant) was replicated in two independent storage experiments ($n = 2$) using different batches of bulk oils, emulsions, or mackerel muscle. Triplicate samples were prepared for the experiments carried out on bulk oils and fish oil-in-water emulsions, and duplicate samples were prepared for the experiments carried out on fish muscle. Each analysis was performed in duplicate. An average value of the replicate analyses was used in calculations of sample variation and significance testing. The data were compared by one-way analysis of variance, and the means were compared by a least-squares difference method (*39*). Significance was declared at $p < 0.01$. Correlations between the propagation rates of lipid oxidation products, and the physicochemical properties of phenolics were determined by Pearson coefficients. Statistical analyses were performed with the software Statistica.

RESULTS AND DISCUSSION

Radical Scavenging and Reducing Power Tests. The radical scavenging activity of resveratrol **1**, the stearoyl derivatives **2** and **3**, and the glycosylated derivatives **4**, **5**, and **6** was measured using two different methodologies, the DPPH and TEAC radical scavenging assays. Both experiments showed similar results in radical scavenging efficiency, except for the acylated derivatives, as follows: resveratrol (**1**) > piceid (**4**) > resveratrol-3,5-di-β-D-glucopyranoside (**5**) ∼ resveratrol-3,4′-di-β-D-glucopyranoside (**6**) ≫ 3-stearoylresveratrol (**2**) and 4′-stearoylresveratrol (**3**) (**Table 1**). This tendency does not change when the molar concentration of each antioxidant in the TEAC experiment is taken into account: resveratrol **1** (438 μM), piceid **4** (256 μM), and resveratrol diglucosides **5** and **6** (181 μM each). The DPPH test was carried out at the same molar concentration of each antioxidant. 4′-Stearoylresveratrol (**3**) is a better radical scavenger than its regioisomer **2** in the TEAC method and the opposite in the DPPH method. The main factor affecting radical scavenging activity seems to be the number of free phenolic OH groups, and then the glucosylated moieties seem to better assist in the stabilization of the radical than the stearoyl chains. Data obtained are in agreement with previous results that showed a high radical scavenging capacity for resveratrol **1**, both using the TEAC and DPPH methods (*14*, *16*, *17*), and also higher than that of piceid **4** (*14*, *24*). Recently, Torres et al. (*26*) reported a better radical scavenging efficiency for 4′-stearoylresveratrol (**3**) than for 3-stearoylresveratrol (**2**) using the TEAC assay in microplates, which is in agreement with the present results. Stivala et al. (*40*) reported a reverse scavenging efficiency for methylated derivatives of resveratrol using the DPPH method, similar to the results of this work with the stearoyl derivatives of resveratrol (major activity for compound **2** relative to **3**).

The results obtained for the reducing capacity measured using the FRAP assay were in general agreement with the scavenging capacity order of resveratrol and their derivatives. The ferric reducing ability decreased in the following order: resveratrol **1** > piceid **4** > resveratrol-3,5-di-β-D-glucopyranoside **5** ∼ resveratrol-3,4′-di-β-D-glucopyranoside **6** ≫ 4′-stearoylresveratrol **3** ∼ 3-stearoylresveratrol **2**.

Inhibition of Lipid Oxidation in Fish Oil-in-Water Emulsions. The antioxidant activity of resveratrol in oil-in-water emulsions was tested in preliminary thermal oxidation samples supplemented with different concentrations of resveratrol (**1**): 10, 25, 50, 100, 150, and 200 ppm. The oxidation experiments were run during 11 days, and results are shown in **Table 2**. All resveratrol concentrations added were significantly effective to inhibit the formation of conjugated diene and triene hydroperoxides. There was a significant effect of increasing concentrations of antioxidant up to concentrations around 50−100 ppm to inhibit extensively lipid oxidation of emulsions. These results were in accordance with the sensory scores corresponding to the different samples during the experiments. Control samples showed moderate paint odor by the 8th day of oxidation; samples supplemented with 10 ppm resveratrol showed this score by the 10th day and samples supplemented with 25 ppm by the 11th day. Others did not show paint odors during the whole experiment (**Table 3**). Filip et al. have reported a low activity of resveratrol supplemented at 12.5−50 ppm for inhibiting lipid peroxidation in water-in-oil emulsions (*19*). Such low effectiveness found for resveratrol in water-in-oil emulsion could be attributed to

Table 1. Radical Scavenging Activity Measured through TEAC and DPPH Tests, Electron-Donating Capacities Measured through FRAP Test, and Partition Coefficient Log P[a]

phenolic compound	TEAC	DPPH EC_{50}	FRAP	log P
Resv	0.1027 ± 0.02 a	3.1673 ± 0.05 a	0.8917 ± 0.00 f	1.85 ± 0.46
3-st-Resv	1.2818 ± 0.11 f	7.3755 ± 0.23 d	0.0274 ± 0.00 b	9.94[b]
4′-st-Resv	1.0741 ± 0.01 e	20.0081 ± 1.16 f	0.0515 ± 0.00 c	9.94[b]
Pic	0.2925 ± 0.02 b	3.3486 ± 0.19 b	0.8762 ± 0.01e	0.89 ± 0.06
Resv-3,5-digluc	0.5402 ± 0.05 c	5.1142 ± 0.08 c	0.7446 ± 0.1 d	−0.72 ± 0.02
Resv-3,4′-digluc	0.5162 ± 0.04 d	nd	0.0101 ± 0.00 a	−0.73 ± 0.12

[a] Values in each column followed by the same letter are not significantly different ($p < 0.01$). [b] Theoretical value.

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

phenolic antioxidant	conjugated dienes	conjugated trienes
control	0.0 ± 12.2 a	0.0 ± 17.7 a
Resv 10 ppm	51.0 ± 8.0 b	60.2 ± 1.3 b
Resv 25 ppm	66.6 ± 12.2 b	73.8 ± 13.3 bc
Resv 50 ppm	78.7 ± 4.8 bc	84.4 ± 5.0 c
Resv 100 ppm	80.7 ± 4.5 c	84.7 ± 6.9 c
Resv 150 ppm	80.9 ± 2.1 c	86.2 ± 2.6 c
Resv 200 ppm	80.6 ± 0.1 c	85.4 ± 1.1 c

[a] Values were taken on day 10 (mean ± SD). Values in each column followed by the same letter are not significantly different ($p < 0.01$). % 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 (*5*). Inhibition is calculated during propagation phase of controls.

Table 3. Sensory Attributes Corresponding to the Oxidation Experiments of Fish Oil-in Water Emulsions

phenolic antioxidant	day 2	day 4	day 8	day 10	day 12
control	fresh	low odor	moderate paint	strong paint	strong paint
Resv 10 ppm	fresh	low odor	slight paint	moderate paint	strong paint
Resv 25 ppm	fresh	low odor	low odor	slight paint	moderate paint
Resv 50 ppm	fresh	low odor	low odor	low odor	low odor
Resv 100 ppm	fresh	fresh	fresh	fresh	fresh
Resv 150 ppm	fresh	fresh	fresh	fresh	fresh
Resv 200 ppm	fresh	fresh	fresh	fresh	fresh

a low incorporation in the droplet interface. In fact, resveratrol, which shows a poor solubility in water (0.03 g/L) and higher solubility in organic solvents, is probably more dispersed in the oily droplets of the emulsion than in the interface.

The effect of acylation was studied by comparing the antioxidant capacity of resveratrol (**1**) and that of the acylated derivatives, 3-stearoylresveratrol (**2**) and 4′-stearoylresveratrol (**3**), in fish oil-in-water emulsions supplemented with 100 ppm of the antioxidants. Hydroxytyrosol (**7**), a highly potent phenolic antioxidant found in olives and olive oil, was used as a positive control. Results are shown in **Figure 2** and **Table 4**. Remarkably, resveratrol showed a very high antioxidant activity in fish oil-in-water emulsions, very similar to that of hydroxytyrosol. In contrast, the modification with the stearoyl groups in resveratrol decreased considerably its antioxidant efficiency. 3-Stearoylresveratrol and 4′-stearoylresveratrol were not able to inhibit the formation of lipid oxidation byproduct. Even more, 4′-stearoylresveratrol was found to be prooxidant with some values of lipid peroxidation products higher than control samples. The large differences in antioxidant activity found for the stearoyl resveratrol derivatives with respect to resveratrol cannot be simply justified by the difference in molar concentration of the antioxidants in this experiment (438 μM resveratrol, 202 μM 3-stearoylresveratrol, 202 μM 4′-stearoylresveratrol). It is important to comment that acylation of hydroxytyrosol with medium-size alkyl chains (octanoic acid) has shown higher antioxidant activity than hydroxytyrosol or hydroxytyrosol fatty acid esters with longer alkyl chains in fish oil-in-water emulsions (*41*). Similarly, chlorogenate esters and rosmarinate esters modified with medium-size alkyl chains (dodecyl and octyl, respectively) have shown higher antioxidant activity than chlorogenic acid and rosmarinic acid, respectively, or esters with shorter or longer alkyl chains in oil-in-water emulsions (*42*,*43*). Recently, we have proposed a potential explanation for this behavior (*44*). We have observed that the best antioxidant in a series of hydroxytyrosol fatty acid esters seems to be at the same time the most effective surfactant in aqueous media. Because the phenolic unit, responsible for most of the antioxidant activity, is common to all of these series of compounds, an effective surfactant would locate the antioxidant preferentially at the oil−water interface in the emulsions inhibiting lipid oxidation more efficiently.

Results of TEAC and FRAP (**Table 1**) showed better radical-scavenging and electron-donating properties for 4-stearoylresveratrol than for 3-stearoylresveratrol, but the opposite trend was found for DPPH values. DPPH measurements give an estimation of the number of hydrogen atoms donated for radical scavenging, and FRAP values are related to the number of donated electrons. The capacity to donate hydrogen atoms seems to be higher if the hydroxyl group is present in the 4′-position and decreases when this hydroxyl in the 4′-position is substituted by a fatty acid ester. That means that the hydroxyl group located at the 4′-position seems to be more acidic than that located at the 3-position. In fact, the bond dissociation energy of O−H is lower in para-substituted phenols than in meta-substituted ones and the donation of hydrogen atoms should be favored in the OH located at the 4′-position instead of the 3-position of resveratrol (**Figure 1**) (*45*). Considering these observations, it seems that the substitution of the hydroxyl group by a fatty acid ester did not improve the antioxidant capacity in vitro. Experimental results obtained in the emulsion experiments demonstrated that the changes in the polarity of the molecule due to the incorporation of the fatty acid ester did not improve their incorporation in the interface of the droplets of the emulsion. In fact, log P data (**Table 1**) indicate that the stearoyl resveratrol derivatives are highly apolar and, therefore, these compounds must be highly included in the oil droplets and much less incorporated at the water−oil interface compared to resveratrol.

The influence of adding a small amount of ethanol as carrier to help the antioxidants to incorporate into the emulsion was also analyzed. Results indicated increased antioxidant effectiveness of resveratrol and hydroxytyrosol when ethanol was used as antioxidant carrier. Ethanol is a protic solvent that can also contribute to scavenge lipid radicals. Also, the presence of an antioxidant in the location where oxidation is initiated or propagated could be essential for antioxidant efficacy (*46*). Such directionality seems to be defined by the antioxidant concentration and the polarity of the antioxidant carrier solvent (*47*). In emulsions, antioxidant efficiency is highly correlated with the facility of compounds to be located at the interface in which

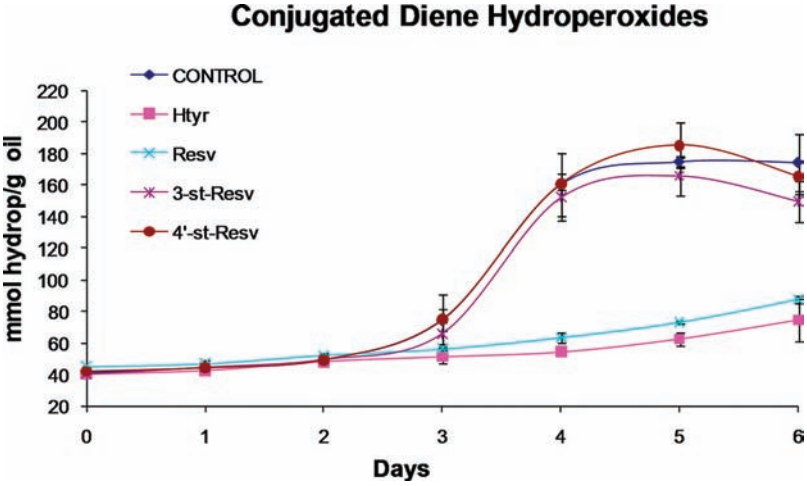

Figure 2. Time course of lipid oxidation of fish oil-in-water emulsions containing resveratrol and two stearoyl resveratrol derivatives measured by conjugated hydroperoxides (no carrier added).

Table 4. Inhibition by Resveratrol, 3-Stearoylresveratrol, 4′-Stearoylresveratrol, and Hydroxytyrosol on the Formation of Conjugated Diene and Triene Hydroperoxides in Fish Oil-in-Water Emulsions during Oxidation at 40 °C[a]

phenolic antioxidant	conjugated dienes		conjugated trienes	
	no carrier	with carrier	no carrier	with carrier
control	0.0 ± 13.3 a	0.0 ± 11.8 b	0.0 ± 8.0 a	0.0 ± 13.4 b
Htyr	74.5 ± 10.1 c	89.3 ± 3.6 d	80.1 ± 6.1 c	88.3 ± 3.7 d
Resv	64.8 ± 2.0 c	83.2 ± 2.5 d	75.4 ± 0.5 c	85.1 ± 3.8 d
3-st-Resv	18.4 ± 9.8 b	19.7 ± 14.7 bc	12.2 ± 9.5 ab	18.8 ± 10.7 bc
4′-st-Resv	6.7 ± 8.0 b	−21.0 ± 7.0 a	6.5 ± 10.0 ab	−15.5 ± 4.1 a

[a] Values were taken on day 6. Antioxidants were tested at the same concentration: 100 ppm (mean ± SD). Values in each column followed by the same letter are not significantly different ($p < 0.01$). Carrier used was ethanol. % 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 (*5*). Inhibition is calculated during propagation phase of controls.

Table 5. Inhibition by Resveratrol, Piceid, Resveratrol-3,5-di-β-D-glucopyranoside, and Resveratrol-3,4′-di-β-D-glucopyranoside on the Formation of Conjugated Diene and Triene Hydroperoxides in Fish Oil-in-Water Emulsions during Oxidation at 40 °C[a]

phenolic antioxidant	conjugated dienes	conjugated trienes
control	0.0 ± 7.4 b	0.0 ± 25.2 b
Resv	69.8 ± 5.7 d	73.0 ± 13.0 d
Pic	68.6 ± 10.4 cd	67.3 ± 2.0 d
Resv-3,5-digluc	53.9 ± 4.3 c	52.3 ± 3.2 c
Resv-3,4′-digluc	−27.3 ± 10.3 a	−23.1 ± 5.7 a

[a] Values were taken on day 10. Antioxidants were tested at the same concentration: 100 ppm (mean ± SD). Values in each column followed by the same letter are not significantly different ($p < 0.01$). % 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. Inhibition is calculated during propagation phase of controls.

oxidation takes place. The calculation of the synergism between ethanol and resveratrol was performed according to the method of Frankel (*5*). Considering the induction periods of diene hydroperoxide formation corresponding to the resveratrol (1.57), ethanol (2.56), and resveratrol + ethanol (5.0), the synergism calculated was around 20%. Therefore, these results indicate that a polar protic solvent such as ethanol seems to favor the location of resveratrol at the oxidative sensitive sites of the emulsion. Raghavan and Hultin (*47*) have also described a higher concentration of tocopherol incorporated into muscle membranes with ethanol as the carrier than when oil was used as the antioxidant carrier solvent. They suggested that the more hydrophilic character of ethanol favored the interaction with the polar surface of phospholipids.

Finally, the effect of glucosylation of resveratrol on its antioxidant activity in fish oil-in-water emulsions was studied. Resveratrol (**1**) was compared with three glucosyl derivatives, piceid (**4**), resveratrol-3,5-di-β-D-glucopyranoside (**5**), and resveratrol-3,4′-di-β-D-glucopyranoside (**6**), all of them at 100 ppm concentration. Results are indicated in **Table 5** and **Figure 3**. Piceid inhibited considerably the formation of conjugated hydroperoxides, very similarly to the data observed for resveratrol. If the molar concentration of each antioxidant in this experiment is taken into account, piceid (256 μM) turned out to be a better antioxidant in emulsions than resveratrol (438 μM). The differences found for the diglucosides of resveratrol in the oxidation inhibition were quite surprising. Whereas resveratrol-3,5-di-β-D-glucopyranoside exhibited only slightly lower antioxidant activity than piceid or resveratrol, resveratrol-3,4′-di-β-D-glucopyranoside showed a prooxidant profile. This result confirms the discussion above suggesting that the phenolic OH group at position 4′ is critical for the antioxidant capacity of resveratrol derivatives. In fact, the DPPH, FRAP, and TEAC values of resveratrol, piceid, and the diglucosylated resveratrol derivatives showed the same tendency (**Table 1**). When a second glycosidic group was introduced in the piceid molecule, the in vitro test showed a significant difference on antioxidant effectiveness if the substitution occurred at the 3- or 4′-position. Although both compounds showed a decrease of their antioxidant solution properties related to their parent compounds resveratrol and piceid, resveratrol-3,4′-di-β-D-glucopyranoside lost completely its activity against DPPH and FRAP methods.

With regard to the polarity of these compounds, the diglucosylated resveratrol derivatives **5** and **6** showed negative log *P* values (**Table 1**), indicating that they must be mainly dissolved in the aqueous phase, whereas piceid **4** and resveratrol **1** showed small positive values, probably indicating a higher incorporation in the water−oil interface and in the oil droplet. The lower antioxidant effectiveness of the diglucosylated resveratrol derivatives **5** and **6** with respect to piceid and resveratrol fits quite well with the polar paradox because an increment in the polarity of the antioxidant correlates with lower antioxidant effectiveness in emulsions. In contrast, it does not predict the dramatic differences in antioxidant activity between the two regioisomers **5** and **6**, which seems to be more related with the higher capacity to donate the hydrogen atom of the hydroxyl group at the 4′-position.

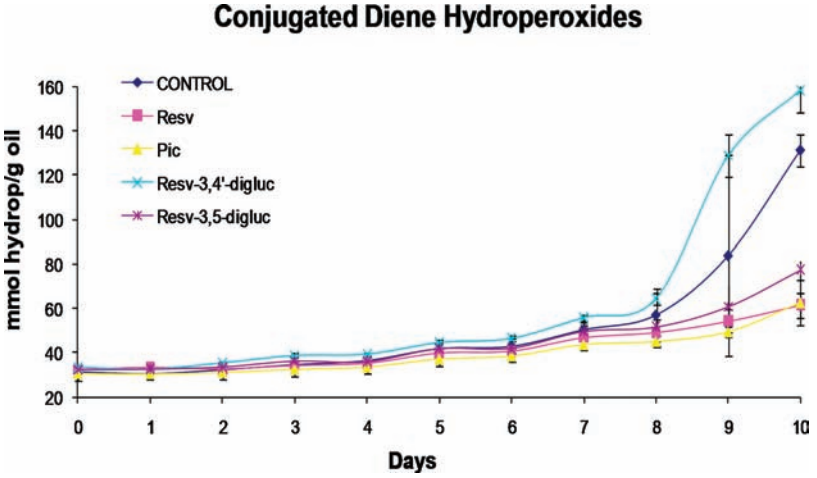

Figure 3. Time course of lipid oxidation of fish oil-in-water emulsions containing resveratrol, piceid, and two glucosylated resveratrol derivatives measured by conjugated diene hydroperoxides.

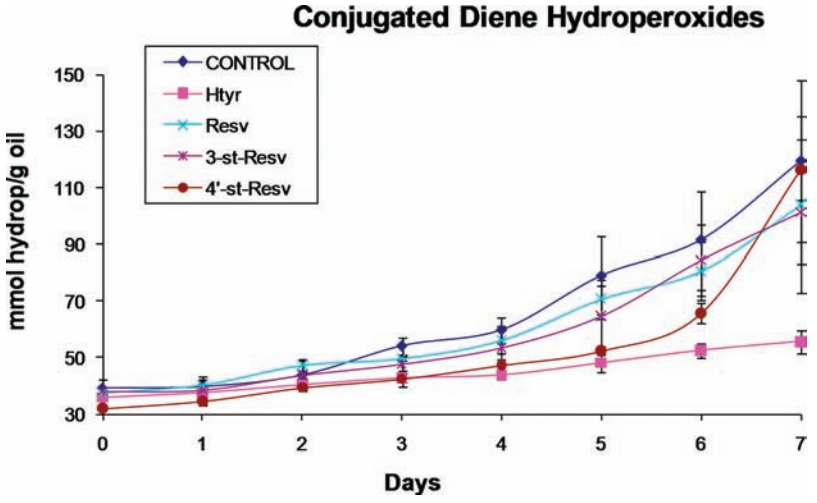

Figure 4. Time course of lipid oxidation of cod liver oil containing resveratrol and two stearoyl resveratrol derivatives measured by conjugated diene hydroperoxides.

Finally, it is important to mention that the antioxidant activity of phenolic derivatives in emulsions could vary with pH as has been observed in the case of hydroxytyrosol (*48*).

Inhibition of Lipid Oxidation in Bulk Fish Oils. The activity of resveratrol and two stearoyl isomers, 3-stearoylresveratrol and 4′-stearoylresveratrol, was tested in bulk fish oils subjected to thermal oxidation at 40 °C. Again, hydroxytyrosol was included as the antioxidant positive control. Antioxidants were assayed at 100 ppm. Results obtained are shown in **Figure 4**. Oxidation was quite effectively inhibited by hydroxytyrosol. However, 3-stearoylresveratrol, 4′-stearoylresveratrol, and resveratrol showed low activity for inhibiting the formation of lipid oxidation products (**Table 6**). Log *P* values of resveratrol (*15*) and the two stearoyl derivatives (**Table 1**) indicate that these compounds are quite hydrophobic and therefore quite soluble in the fish oil matrix and probably located far away from the air−oil interface. As predicted by the polar paradox, these apolar phenolic derivatives show low efficiency as antioxidants in an oil matrix. These data are in agreement with those reported by Filip et al. (*19*), who found low antioxidant activity for resveratrol in sunflower and rapeseed oils. Nevertheless, Wang et al. (*18*) described higher antioxidant activity for resveratrol than for BHT in retarding the induction periods of oxidation in pork fat measured through the Rancimat method. This activity was increased when one or two hydroxyl groups were introduced, increasing the polarity of the parent resveratrol.

Table 6. Inhibition by Resveratrol, 3-Stearoylresveratrol, 4′-Stearoylresveratrol, and Hydroxytyrosol on the Formation of Conjugated Diene and Triene Hydroperoxides in Bulk Fish Oil during Oxidation at 40 °C[a]

phenolic antioxidant	conjugated dienes	conjugated trienes
control	0.0 ± 1.2 a	0.0 ± 2.5 a
Htyr	79.4 ± 5.2 d	78.2 ± 7.7 e
Resv	19.2 ± 8.7 bc	35.0 ± 4.4 cd
3-st-Resv	22.8 ± 8.5 c	25.4 ± 7.3 c
4′-st-Resv	3.9 ± 6.5 b	5.9 ± 9.4 b

[a] Values were taken on day 7. Antioxidants were tested at the same concentration: 100 ppm (mean ± SD). Values in each column followed by the same letter are not significantly different ($p < 0.01$). % 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. Inhibition is calculated during propagation phase of controls.

When the glucosyl resveratrol derivatives were examined as antioxidants in bulk fish oils, they did not show important antioxidant effects (**Table 7** and **Figure 5**). Only piceid and resveratrol-3,5-di-β-D-glucopyranoside were able to inhibit oxidation to some extent (around 10−15%). In contrast, resveratrol-3,4′-di-β-D-glucopyranoside showed a prooxidant profile. It seems that increasing the

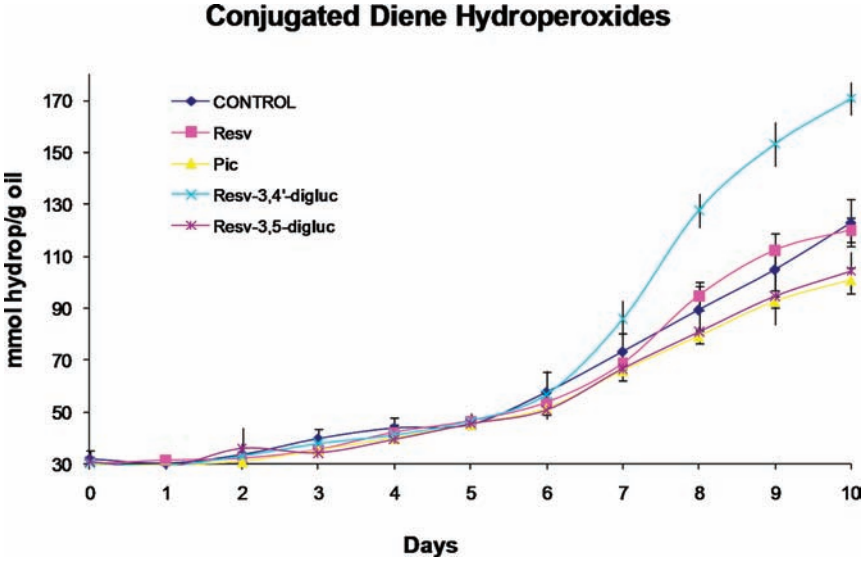

Figure 5. Time course of lipid oxidation of cod liver oil containing resveratrol, piceid, and two glucosylated resveratrol derivatives measured by conjugated diene hydroperoxides.

Table 7. Inhibition by Resveratrol, Piceid, Resveratrol-3,5-di-β-D-glucopyranoside, Resveratrol-3,4′-di-β-D-glucopyranoside, and Hydroxytyrosol on the Formation of Conjugated Diene and Triene Hydroperoxides in Bulk Fish Oil during Oxidation at 40 °C[a]

phenolic antioxidant	conjugated dienes	conjugated trienes
control	0.0 ± 11.3 b	0.0 ± 3.9 b
Resv	10.0 ± 3.4 bc	14.6 ± 3.9 c
Pic	14.2 ± 3.1 c	4.8 ± 5.1 bc
Resv-3,5-digluc	14.3 ± 15.1 b	12.0 ± 3.9 c
Resv-3,4′-digluc	−79.2 ± 11.7 a	−42.0 ± 6.3 a

[a] Values were taken on day 19. Antioxidants were tested at the same concentration: 100 ppm (mean ± SD). Values in each column followed by the same letter are not significantly different ($p < 0.01$). % 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 (*5*). Inhibition is calculated during propagation phase of controls.

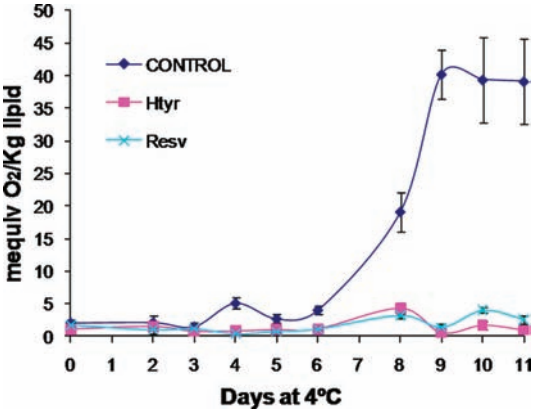

Figure 6. Time course of lipid oxidation of fish fillets containing resveratrol and hydroxytyrosol measured by peroxide content.

polarity of resveratrol by attaching one or two glucosyl units slightly improves its antioxidant activity in bulk fish oil with respect to resveratrol itself, but only if the phenolic hydroxyl group at the 4′-position of resveratrol is not conjugated. These results confirmed the above discussion stressing the importance of maintaining the hydroxyl group in the para-position (4′). The polar paradox would predict that the hydrophobicity of piceid (log $P = 0.84$, **Table 1** and ref *49*) will yield a better antioxidant activity in emulsions than in bulk fish oils, as is actually observed. In contrast, the polar paradox does not seem to explain why the highly polar resveratrol-3,5-di-β-D-glucopyranoside **5** is also more active in emulsions than in bulk fish oils.

When the antioxidant efficiency of resveratrol and its acylated and glucosylated derivatives in homogeneous and heterogeneous fish lipid matrices is compared with their radical scavenging and reducing power tests, no correlation could be found in fish oils, whereas some general trends can be drawn in oil-in-water emulsions. Resveratrol **1** is an efficient antioxidant in emulsions with similar activity as piceid **4**, the same tendency observed in the radical scavenging and reducing power tests. At the same time, **1** and **4** are better antioxidants than the diglucosylated resveratrol derivatives **5** and **6** in emulsions, but the differences found between **5** and **6** are not predicted by the TEAC, DPPH, or FRAP methods.

Inhibition of Lipid Oxidation in Fish Fillets. Considering the high antioxidant activity of resveratrol for inhibiting oxidation in fish oil-in-water emulsions, its capacity to inhibit rancidity in fish muscle was also determined. The antioxidant efficiency of resveratrol in minced fish muscle was examined in chilled experiments in which inhibition was achieved through the addition of 100 ppm of resveratrol. Once again, hydroxytyrosol was used as positive antioxidant control because it had previously proved its effectiveness in this type of food matrix (*41*, *50*). Oxidation was studied during 11 days. Sensory analysis demonstrated the detection of rancid off-flavors in controls by the seventh day. However, off-flavors were not detected in fish samples supplemented with resveratrol or hydroxytyrosol during the whole experiment. Data of the peroxide formation and TBARS corroborated sensory analysis and clearly showed that resveratrol is as good an antioxidant as hydroxytyrosol to protect frozen fish muscle from oxidation (**Figures 6** and **7**).

In conclusion, our results have shown a notable antioxidant activity of resveratrol and piceid in fish oil-in-water emulsions, similar to that of the potent antioxidant hydroxytyrosol. These findings open up the possibility of the use of these biologically relevant phenols also as antioxidants in this type of food matrix. Additional studies on antioxidant activity comparing resveratrol with even better antioxidants in emulsions such as α-tocopherol or hydroxytyrosol octanoate would be highly informative. Lipophilization of resveratrol with stearoyl moieties did not improve

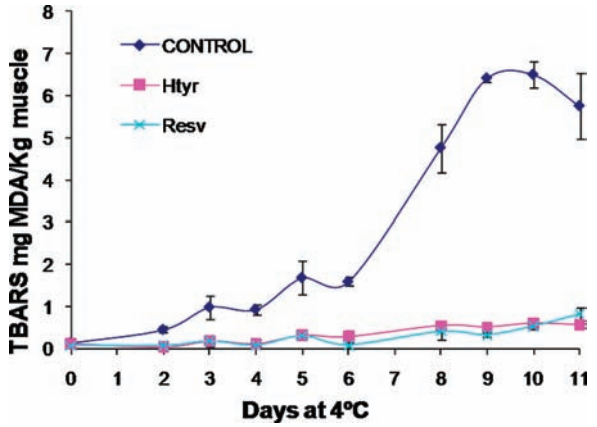

Figure 7. Time course of lipid oxidation of fish fillets containing resveratrol and hydroxytyrosol measured by TBARS.

its antioxidant activity, most probably because these highly hydrophobic compounds tend to be mainly located at the interior of the oil droplet and not in the oil−water interface. Further glucosylation of piceid yielding compounds **5** and **6** did not improve either resveratrol or piceid antioxidant efficiency in emulsions, possibly because these compounds are mainly located in the aqueous phase, due to their high polarity as shown by their log *P* values. Concerning fish oils, resveratrol showed low antioxidant activity and neither lipophilization nor glucosylation improved its efficiency. Furthermore, the hydroxyl group at the 4′-position is relevant for the antioxidant efficiency of resveratrol and should be maintained to keep the antiradical activity. Finally, it is important to remark that resveratrol is a very good antioxidant for fish muscle, as good as the potent antioxidant hydroxytyrosol.

ACKNOWLEDGMENT

We thank S. Lois for helpful technical assistance.

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Received for review April 19, 2010. Revised manuscript received June 23, 2010. Accepted July 1, 2010. We thank JAE-Doc program (R.L.) and Intramural Frontier Project (200680F0132) from CSIC and Comunidad de Madrid (P.T.) for financial support.