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Model System for Testing the Efficacy of Antioxidants in Muscle Foods

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The objective of this research was to study the effect of the antioxidants, δ-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate in a model system of lean muscle and canola oil and to compare the effects with those in minced herring. Two carrier solvents with different dielectric constants (ϵ), ethanol (ϵ = 24) and oil (ϵ = 2), were used. Oxidation was measured using thiobarbituric acid reactive substances (TBARS) and sensory analysis. In both the lean muscle−canola oil model system and in herring muscle, the hydrophilic antioxidants, propyl gallate and TBHQ, were more effective in providing oxidative stability than the lipophilic antioxidants, δ-tocopherol and BHT. The oxidative stability of a cod muscle−canola oil system in the presence of propyl gallate, and δ-tocopherol was not affected by the dielectric constant of the carrier solvent, while BHA was more effective as an antioxidant when added in the polar solvent ethanol.

KEYWORDS: Model system; herring; δ-tocopherol; BHA; BHT; propyl gallate; TBHQ; dielectric constant

INTRODUCTION

Antioxidants are generally added to muscle foods to prevent or delay lipid oxidation (*1*). Numerous phenolic antioxidants, both natural and synthetic, have been shown to inhibit lipid oxidation (*2*, *3*). In an earlier paper (*4*), we had determined that the polarity of the antioxidant carrier solvent affected the distribution of a lipid-soluble antioxidant, δ-tocopherol, between the membrane lipid and triacylglycerol fractions of a cod−canola oil model system. We had tested carrier solvents with dielectric constants between 3 and 32 and found that a carrier solvent with a dielectric constant of 24−27 was able to incorporate the maximum amount of δ-tocopherol into the membrane lipid fraction of a cod muscle−canola oil mixture. However, it was observed that δ-tocopherol gave only moderate protection against lipid oxidation when preferentially incorporated into the membrane fraction of the minced washed cod−canola oil model system using the carrier solvent, ethanol.

The objective of this research was 3-fold. The first objective was to determine the effect of several phenolic antioxidants, δ-tocopherol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and propyl gallate dissolved in the carrier solvent ethanol (ϵ = 24) (*5*), on the oxidative stability of a cod−canola oil model system. The second objective was to determine the effect of a polar carrier solvent, ethanol (ϵ = 24), and a nonpolar carrier solvent, oil (ϵ = 2) (*6*), on the antioxidant effect of phenolic antioxidants in the lean fish−canola oil model system. The third objective was to compare the antioxidant effect of phenolic antioxidants in a lean fish−canola oil model system to that of a real system, minced herring. In a washed minced cod muscle system, Undeland et al. (*7*) had earlier shown that the development of thiobarbituric acid reactive substances and painty odor development was slow in the absence of trout hemoglobin addition, despite a high amount of hydroperoxides present in the muscle tissue. In our model system studies using minced, washed cod muscle and canola oil, we used the hemolysate prepared from cod to catalyze lipid oxidation.

MATERIALS AND METHODS

Materials. Whole herring (*Clupea harengus*) was a gift from Cape Sea Foods Inc., Gloucester, MA. Fillets of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) were purchased from local fish distributors in Gloucester, MA. Whole herring and fish fillets were transported to the laboratory on ice. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of ACS grade. Canola oil was purchased from a local supermarket.

Preparation of Muscle for Oxidative Stability Studies. Two different species of lean fish, cod and haddock, were used as model systems for oxidative stability studies. Haddock muscle was substituted for cod muscle in some of the experiments due to the unavailability of cod fillets in the local markets of Gloucester, MA. Minced, washed cod or haddock white muscle was chosen since they contain almost no triacylglycerols. Canola oil was added as a relatively stable exogenous triacylglycerol. Cod or haddock white muscle was minced twice with a KitchenAid grinder model KSM 90 (KitchenAid Inc., St. Joseph, MI) through a 3/16-in. diameter sieve. The minced muscle tissue was first washed with five parts of double distilled water. The second wash was made with five parts of 180 mM NaCl solution prepared in double distilled water. The washed muscle was adjusted to pH 7.0. The muscle

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was then blended for 0.5 min with 7% canola oil (based on the weight of the mince) in a Waring commercial blender model 51BL32 (Waring Commercial, Torrington, CT). The antioxidant solution (0.8−0.9% of the total weight) was added and blended for another 2.5 min. Cod hemoglobin was then mixed into a concentration of 6 μmol/(kg of sample). Streptomycin sulfate was added at 200 ppm on the basis of the muscle weight to prevent microbial spoilage.

The effects of antioxidants on lipid oxidation in herring muscle were studied by mincing the entire muscle (white and dark) as described above. Various antioxidants dissolved in ethanol were then added and chopped for 3 min in the Waring blender. After the addition of antioxidants to the muscle tissue, the cod−canola oil model system and the herring samples were transferred into 250-mL Erlenmeyer flasks (bottom diameter = 75 mm) and were evenly flattened out in the bottom of the flasks using an L-shaped stainless steel spatula. The sample thickness was around 1 cm. The capped sample bottles were stored on ice.

Preparation of Antioxidants in Carrier Solvents. Two different antioxidant carrier solvents and five different antioxidants were used. Ethanol with a dielectric constant (ϵ) of 24 and canola oil with $\epsilon = 2$ were used as antioxidant carrier solvents. δ-Tocopherol, BHT, BHA, propyl gallate, and TBHQ were used as antioxidants at the limit approved by FDA. δ-Tocopherol was added at a final concentration of 300 ppm, and the remaining antioxidants were added at a final concentration of 200 ppm on the basis of the total lipid content of the lean muscle−canola oil system or that of the herring muscle. The amount of carrier solvent added was around 0.8−0.9% of the muscle weight.

Determination of Lipid Content. The total lipid content of the minced muscle was determined according to the method of Sigfusson and Hultin (*8*). A 10-g amount of minced muscle was homogenized with 50 mL of a 1:1 (v/v) chloroform:methanol mixture for 60 s at moderate speed in a Waring commercial blender (Waring Products Division, Dynamics Corp. of America, CT). The homogenate was filtered through Whatman filter paper (No. 4) into a separatory funnel. A 20-mL aliquot of 0.5% NaCl solution was added to enhance phase separation, and the mixture was stored in the cold room for 3 h. The chloroform layer was used for total lipid determination. The total lipid content was determined gravimetrically by drying the chloroform layer on a hot plate at low setting to avoid overheating.

Preparation of Hemolysate. A modified method of Fyhn et al. (*9*) was used for preparing hemolysate from cod frames (which were obtained after filleting a whole cod fish). Cod frames were severed at the tail section. Blood was collected from the severed vein using a micropipet and transferred into heparin solution. Heparinized blood was washed with four volumes of ice cold 1.7% NaCl in 1 mM Tris buffer, pH 8.0. Centrifugation was done at 1000*g* for 10 min at 4 °C using a tabletop clinical centrifuge (IEC, Needham Heights, MA) to remove blood plasma. The red cells obtained were washed three times with 10 volumes of the above buffer and centrifuged at 1000*g*. Cells were then lysed in three volumes of ice cold 1 mM Tris buffer, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid stromal removal before centrifugation at 30000*g* for 15 min at 4 °C in a Beckmann ultracentrifuge model L5-65B (Beckmann Instruments Inc., Palo Alto, CA). The hemolysate obtained as the supernatant was stored at −80 °C.

Quantifying Hemoglobin Levels. The method of Brown (*10*) was used to quantify the hemoglobin level of the hemolysate. The hemolysate was diluted in ice cold 50 mM Tris buffer at pH 8.0. Around 1 g of sodium dithionate was added and mixed into the test tube. Carbon monoxide (Matheson Gas, MA) was then bubbled into the sample for 30 s. The sample was scanned between 440 and 360 nm against a blank that contained only the buffer using a model U-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The peak at 420 nm was recorded. Standard curves were plotted using bovine hemoglobin as a standard.

Measurement of Thiobarbituric Acid Reactive Substances. The method of Lemon (*11*) was modified according to the following procedure for measuring the thiobarbituric acid reactive substances (TBARS). TBARS was measured by extracting the samples with trichloroacetic acid (TCA) solution. A 1-g amount of the sample was extracted with 3 mL of 7.5% TCA solution by homogenization with a Bio homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 1 min. The samples were centrifuged at speed 6 in an IEC Clinical tabletop centrifuge (International Equipment Co., Needham Heights, MA) for 10 min. A 2-mL aliquot of the supernatant was mixed with 2 mL of 0.02 M TBA solution and heated in a boiling water bath for 40 min. The color developed was spectrophotometrically measured at 530 nm. A standard curve was plotted using tetraethoxypropane. Malonaldehyde concentration was calculated using an extinction coefficient of $1.6 \times 10^5\ M^{-1}\ cm^{-1}$. All the experiments were done in duplicate.

Determination of Lipid Hydroperoxides. A 1-g amount of minced cod muscle was taken in a disposable glass tube and homogenized for 1 min with 10 mL of chloroform:methanol (1:1) using a Bio homogenizer. The mixture was vortexed and then centrifuged at speed 6 for 10 min in an IEC Clinical tabletop centrifuge. The chloroform/methanol phase was removed and mixed with 3 mL of 0.5 M NaCl. The mixture was vortexed and centrifuged for 5 min at speed 6 in an IEC Clinical tabletop centrifuge to separate the sample into two phases. The chloroform phase was removed, and its volume was made to 10 mL using chloroform:methanol (1:1). Ammonium thiocyanate and ferrous chloride were prepared as in Shantha and Decker (*12*). A 25-μL aliquot of each reagent was added and vortexed for 10 s. The samples were incubated for 20 min at room temperature, and the absorbance was measured at 500 nm. A standard curve was prepared using cumene hydroperoxide

Sensory Analysis. A group of three to five trained panelists (*13*) did sensory analysis by sniffing the samples. All the panelists used the same samples for sensory evaluation. The samples were taken in a 250-mL Erlenmeyer bottles with caps. All samples were stored on ice. Panelists concentrated on detecting painty odors and rated it on a scale of 1−10, with 1 being least painty and 10 being most painty.

Statistical Analysis. All experiments were done at least twice. For TBARS measurements, TCA extracts were prepared from two samples taken out from each Erlenmeyer bottle and each of the samples was analyzed once. For sensory analysis, for each sample and at each time point, the average value of all the panelists was reported. Statistical analyses were done using the general linear model (GLM) procedure (Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

Effect of Ethanol and Canola Oil on the Oxidative Stability of Washed Cod. The effect of various levels of ethanol or canola oil on the oxidative stability of minced, washed cod muscle at pH 7.0 was studied. Cod hemolysate was used to promote oxidation. Ethanol was mixed at either 0.5, 1.0, or 1.5% of the cod muscle weight. Canola oil was mixed at either 3, 5, or 7% of the cod muscle weight. The results of TBARS measurements are given in **Figure 1**.

The addition of ethanol or canola oil significantly increased ($p < 0.05$) the oxidative stability of washed, minced cod muscle. However, there was no significant difference ($p > 0.05$) among the different levels of added ethanol or oil in improving this stability. The antioxidant effect of ethanol may be due to its free radical scavenging ability (*14*). When canola oil was analyzed for its tocopherol content using HPLC, it showed no detectable amounts of α-, β-, or δ-tocopherols. If rancid canola oil (peroxide value = 1.2 mmol equiv of cumene hydroperoxide/(kg of sample), with painty odor) was added to cod, it increased significantly ($p < 0.05$) the rate of oxidation of the washed, minced cod muscle, whereas the addition of canola oil (peroxide value = 0.12 mmol equiv of cumene hydroperoxide/(kg of sample), absence of painty odor) delayed the oxidation of cod muscle significantly. The antioxidative property of canola oil may be due to the presence of phenolic acids and carotenoids (*15*), which could have been retained in the canola oil after extraction and processing. Canola oil may also act as a free

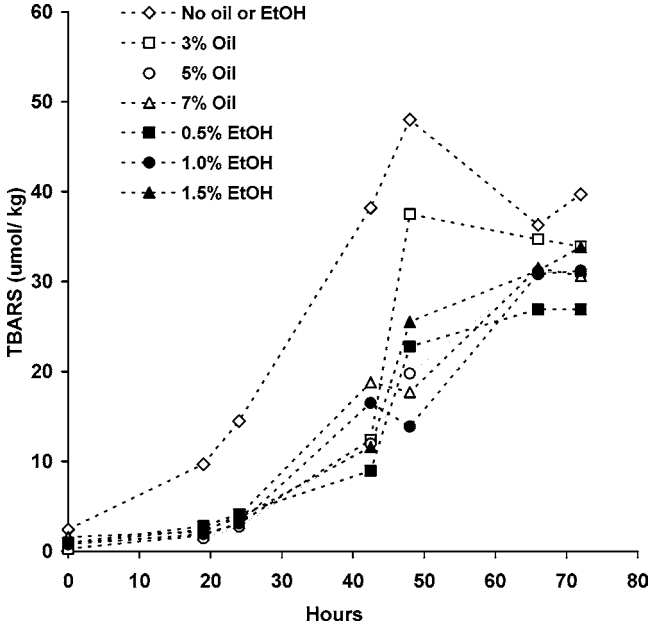

Figure 1. Effect of different levels of ethanol (EtOH) or canola oil on the oxidative stability of minced, washed cod at pH 7.0. Cod hemoglobin was added at 6 μmol/(kg of tissue). EtOH (■, 0.5%; ●, 1.0%; ▲, 1.5%); oil (□, 3%; ○, 5%; △, 7%); no oil or EtOH = cod muscle without oil or ethanol (◆).

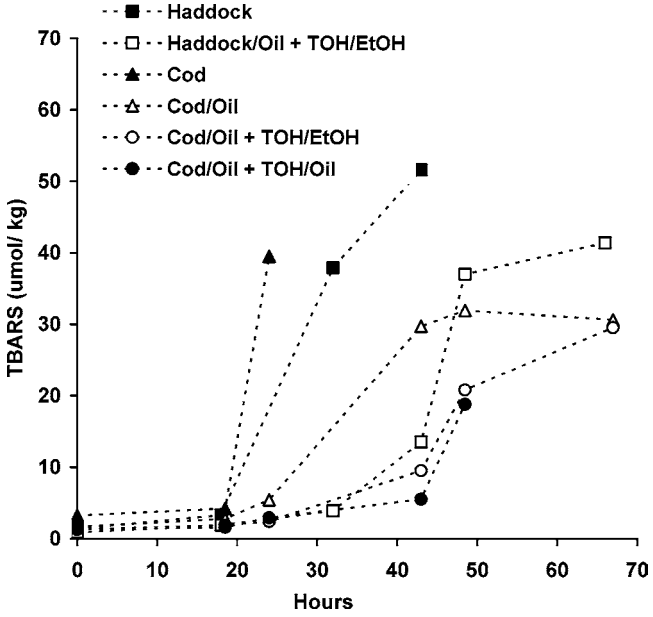

Figure 2. Effect of carrier solvent, ethanol or oil, on the antioxidative effectiveness of δ-tocopherol in a mixture of minced, washed cod (▲) or haddock muscle (■) and 7% canola oil at pH 7.06. Cod hemoglobin was added at 6 μmol/(kg of tissue). Cod/oil = mixture of cod and 7% oil (△); haddock/oil + TOH/EtOH = δ-tocopherol added at a concentration of 300 ppm in carrier solvent, ethanol, added to a mixture of haddock muscle and 7% oil (□); cod/oil + TOH/EtOH = δ-tocopherol added at a concentration of 300 ppm in carrier solvent, ethanol, added to a mixture of cod muscle and 7% oil (○); cod/oil + TOH/oil = δ-tocopherol added at a concentration of 300 ppm in the carrier solvent, canola oil, to a mixture of cod and 7% canola oil (●).

radical scavenger without producing the painty odor components and the components of TBARS associated with the oxidation of membrane lipids of fish.

Effect of the Polarity of Carrier Solvents on the Antioxidative Effect of δ-Tocopherol in a Lean Muscle−Canola Oil Model System. When δ-tocopherol was added at a concentration of 300 ppm on the basis of the total lipid content of the cod−canola oil model system using ethanol ($\epsilon = 24$) as the carrier, the tocopherol concentration in the membranes of the cod−canola oil model system was determined as 315 ppm (*4*). When oil ($\epsilon = 2$) was the carrier, the concentration of tocopherol in the membranes was around 130 ppm. The objective of this experiment was to determine whether the lipid-soluble antioxidant, δ-tocopherol, dissolved in the carrier, ethanol, would increase the oxidative stability of the cod−canola system compared to oil as carrier. To determine this, δ-tocopherol, dissolved in the carrier solvent ethanol or oil, was added to a cod−canola oil model system. The results of oxidation studies measured as TBARS are given in **Figure 2**. The addition of δ-tocopherol increased the oxidative stability of the cod−canola oil model system by around 24 h compared to the control. However, there was no significant difference among the two carrier solvents, ethanol and oil, in improving the oxidative stability.

In some of the model system studies, we had substituted cod with haddock muscle. In this experiment we also compared the oxidative stability of cod muscle with haddock muscle. Oxidative stability was judged by the measurement of TBARS and by the development of painty odor. For this purpose, δ-tocopherol, dissolved in ethanol was added to a mixture of haddock and 7% canola oil (haddock−canola oil model system) and oxidation was catalyzed using cod hemoglobin added at a concentration of 6 μmol/(kg of muscle tissue). In the absence of any added antioxidant, minced, washed cod muscle and haddock muscle exhibited a similar oxidative pattern (**Figure 2**). When δ-tocopherol was added to the lean muscle−canola oil model system, there was no significant ($p > 0.05$) difference between the oxidative stabilities of the cod−canola oil model system and that of the haddock−canola oil model system. These results show that cod and haddock muscle could be used interchangeably in our model system studies.

Effect of Various Antioxidants on the Oxidative Stability of the Lean Muscle−Canola Oil Model System. The effect of various phenolic antioxidants (with different hydrophile−lipophile balance values) dissolved in ethanol on the oxidative stability of minced, washed haddock muscle−canola oil mixtures was studied by the measurement of TBARS (**Figure 3a**) and by sensory analysis (**Figure 3b**). Cod hemoglobin was used to promote oxidation. Hydrophile−lipophile balance (HLB) is an empirical expression for the relationship between the hydrophilicity and lipophilicity of a molecule. The HLB value is affected by the number and the nature of polar groups, such as hydroxy groups, present in an antioxidant and could be calculated according to the formula of Davies and Rideal (*16*). The antioxidants tested for their effectiveness were δ-tocopherol (TOH), BHA, BHT, TBHQ, and propyl gallate. Among the synthetic antioxidants, the ascending order of lipophilicity is propyl gallate, TBHQ, BHA, and BHT (*17*). The order of effectiveness of various antioxidants in a haddock−canola oil model system measured as TBARS was TBHQ, propyl gallate > BHA ≫ BHT > TOH. The order of effectiveness on the development of painty odor was TBHQ > propyl gallate > BHA ≫ BHT > TOH. When propyl gallate or TBHQ was used, no oxidation in terms of increased TBARS or painty odor was observed for up to 12 days. The measurement of TBARS and painty odor development were discontinued after 12 days due to the development of microbial spoilage.

The effectiveness of antioxidants such as propyl gallate and TBHQ in inhibiting oxidative rancidity may be in part due to

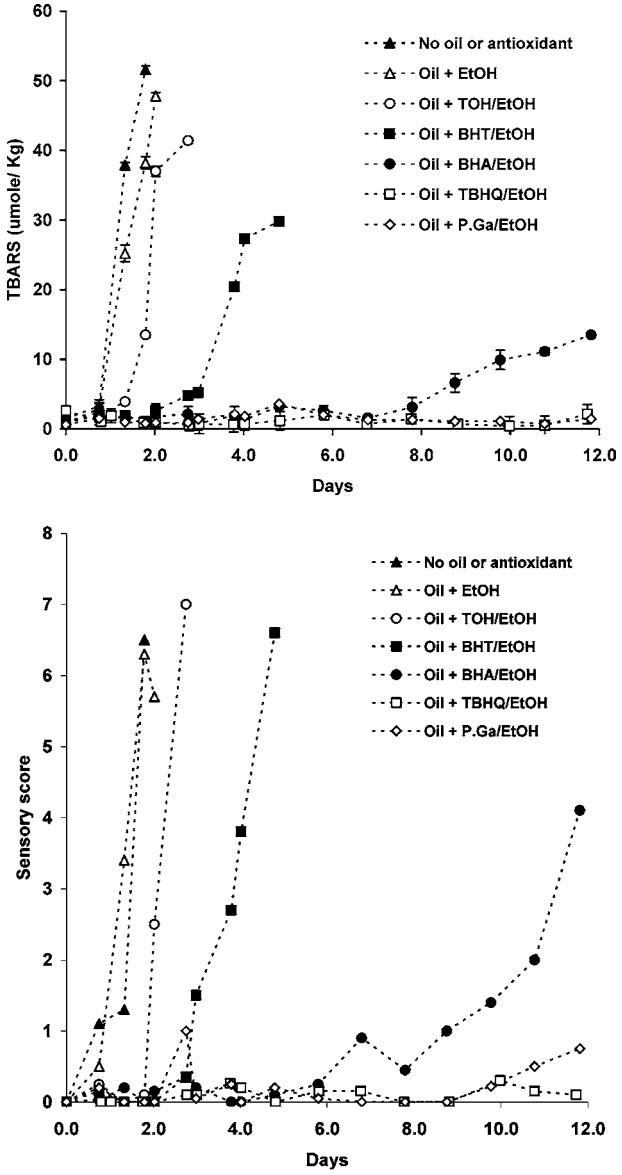

Figure 3. Effect of various antioxidants on TBARS measurement and painty odor in a mixture of minced, washed haddock muscle and 7% canola oil at pH 7.0. Cod hemoglobin was added at 6 μmol/(kg of tissue). No oil or antioxidant = haddock muscle without any added oil or antioxidant (▲); oil + EtOH = mixture of haddock muscle, 7% canola oil, and ethanol (△); oil + TOH/EtOH = haddock + 7% canola oil mixed with 300 ppm of δ-tocopherol in carrier ethanol (○); BHT/EtOH = butylated hydroxytoluene at a concentration of 200 ppm in carrier ethanol (■); BHA/EtOH = butylated hydroxyanisole at a concentration of 200 ppm in carrier ethanol (●); TBHQ/EtOH = tertiary butylhydroquinone at a concentration of 200 ppm in carrier ethanol (□); P.Ga/EtOH = propyl gallate at a concentration of 200 ppm in carrier ethanol (◇). Bars represent the standard error of each mean. (**a**, top) TBARS; (**b**, bottom) painty odor on a scale of 1–10.

the number of phenolic hydroxy groups available for free radical scavenging ability (*18*). Propyl gallate and TBHQ possess three and two hydroxy groups, respectively, compared to the single hydroxy group present in BHA, BHT, and δ-tocopherol. Ollila et al. (*19*) have shown a direct relationship between the number of hydroxyl groups present in an antioxidant and their interaction with the membranes. Due to the polar nature and the large surface area of the membranes (*20*), propyl gallate, with three hydroxyl groups, may interact more effectively with the membranes compared to BHA and BHT with one hydroxyl

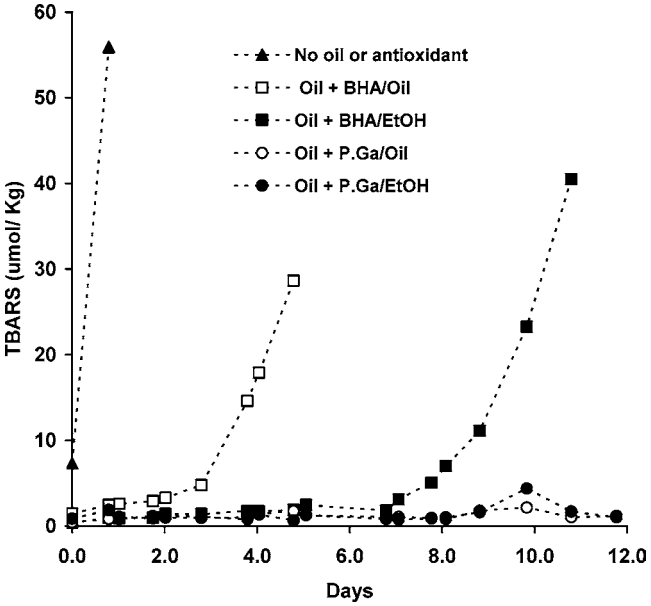

Figure 4. Effect of BHA and propyl gallate in carrier solvent ethanol or oil on the oxidative stability of the minced, washed haddock and 7% canola oil model system at pH 7.0. Oxidation catalyzed by cod hemoglobin added at 6 μmol/(kg of tissue). No oil or antioxidant = haddock muscle without any canola oil or antioxidant (▲); oil + BHA/EtOH = haddock muscle + 7% canola oil mixed with 200 ppm of butylated hydroxyanisole in carrier ethanol (■); P.Ga/EtOH = propyl gallate at a concentration of 200 ppm in carrier ethanol (●); BHA/oil = butylated hydroxyanisole at a concentration of 200 ppm in carrier canola oil (□); P.Ga/oil = propyl gallate at a concentration of 200 ppm in carrier canola oil (○). Bars represent the standard error of each mean.

group, respectively. The antioxidant efficiency of propyl gallate could also be attributed to its amphiphilic nature. The solubility of propyl gallate in water is 0.35 g/(100 mL of water) at 25 °C and in oil is 1.23 g/(100 g of cottonseed oil) at 30 °C (*21*), whereas antioxidants such as δ-tocopherol, BHT, and BHA are insoluble in water but soluble in the nonpolar oil fraction.

Effect of the Polarity of Antioxidant Carrier Solvents on the Oxidative Stability of the Lean Muscle−Canola Oil Model System. In our earlier experiment (**Figure 2**) we had determined that there was no effect on antioxidation when either ethanol or oil was used as the carrier for δ-tocopherol. The effect of the two antioxidant carriers, ethanol ($\epsilon = 24$) and canola oil ($\epsilon = 2$), on the effectiveness of propyl gallate and BHA in a haddock−7% canola oil model system was now tested. Propyl gallate was chosen as it was most effective among all the antioxidants tested in our system (**Figure 3a**,**b**). BHA was chosen as it showed moderate effectiveness in the lean muscle−canola oil model system. The results of oxidation studies in terms of TBARS measurement is given in **Figure 4**.

When propyl gallate was used as the antioxidant, no increase in TBARS measurement was observed using either carrier solvent ($p > 0.05$) for up to 12 days of storage. When BHA was used as the antioxidant, there was a significant difference ($p < 0.05$) between ethanol and oil in inhibiting oxidative rancidity. When BHA dissolved in ethanol was used, the model system was oxidatively stable up to 7 days of storage, while for BHA dissolved in oil, the model system was oxidatively stable up 3 days of storage.

Effect of Various Antioxidants on the Oxidative Stability of Minced Herring. In all our experiments we had used a model system of lean muscle and canola oil to study the antioxidant efficiency of various phenolic antioxidants. In this experiment,

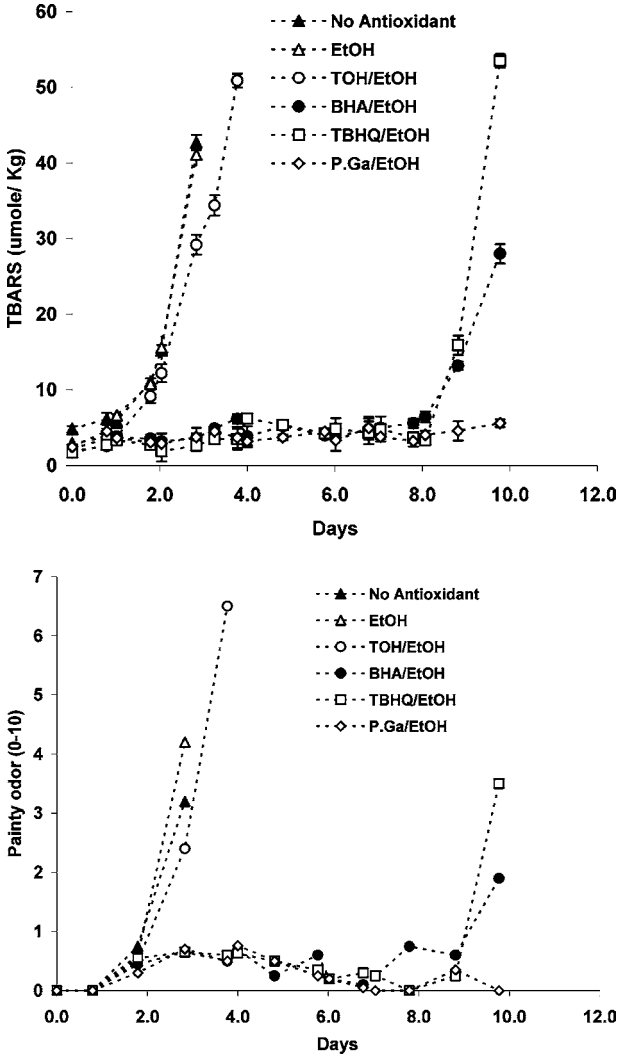

Figure 5. Effect of various antioxidants on the TBARS measurement and painty odor of minced herring (lipid content = 7.5%, pH = 6.69). No antioxidant = herring muscle without any antioxidant (▲); EtOH = herring muscle mixed with ethanol (△); TOH/EtOH = herring mixed with 300 ppm of δ-tocopherol in carrier ethanol (○); BHA/EtOH = butylated hydroxyanisole at a concentration of 200 ppm in carrier ethanol (●); P.Ga = propyl gallate at a concentration of 200 ppm in carrier ethanol (◇); TBHQ/EtOH = tertiary butylhydroquinone at a concentration of 200 ppm in carrier ethanol (□). Bars represent the standard error of each mean. (**a**, top) TBARS; (**b**, bottom) painty odor on a scale of 1–10.

we wanted to determine how an actual fish muscle would behave with these phenolic antioxidants. For this purpose, the effect of various antioxidants on the lipid oxidation of herring muscle was studied by mincing the entire muscle (white and dark muscle) and mixing it with antioxidants dissolved in the carrier solvent, ethanol. The total lipid content of the whole herring muscle was 7.5%, and the pH was 6.7. Two different controls were used. Minced herring muscle was used as one of the controls. A mixture of herring muscle and ethanol (added at 0.8% of the muscle weight) was used as the second control. No exogenous hemoglobin was added to any of the herring samples. The results of TBARS analysis are given in **Figure 5a** and the results of sensory analysis in **Figure 5b**. The order of effectiveness of various antioxidants in herring muscle measured as TBARS was propyl gallate > TBHQ, BHA ≫ δ-tocopherol. When propyl gallate was used as the antioxidant, no oxidative rancidity was observed for up to 10 days of storage, compared to δ-tocopherol where an increase in oxidative rancidity in terms of TBARS measurement was observed with 1 day of storage. The sensory scores for the different antioxidants followed a trend similar to the TBARS development. The order of effectiveness of various antioxidants in herring muscle was similar to that in the lean muscle−canola oil model system (**Figure 3a**,**b**), which indicates the effectiveness of using a lean muscle−canola oil model system for studying fatty muscle systems such as herring.

Conclusion. The data presented in this paper show the effect of various phenolic antioxidants in a model system comprised of lean fish and canola oil with oxidation catalyzed by cod hemolysate. This model system matches well with a real system, i.e., minced herring muscle. The polarity of the carrier solvents affected the efficiency of antioxidants with intermediate HLB (butylated hydroxyanisole) values and was less pronounced in antioxidants of high HLB (δ-tocopherol) and low HLB values (propyl gallate).

Abbreviations Used: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; HLB, hydrophilic−lipophilic balance; P.Ga, propyl gallate; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroquinone.

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