Different Tocopherols and the Relationship between Two Methods for Determination of Primary Oxidation Products in Fish Oil

Elin Kulås*,† and Robert G. Ackman‡

Norsk Hydro Research Centre, P.O. Box 2560, 3907 Porsgrunn, Norway and Dalhousie University, Halifax, Nova Scotia, B3J 2X4, Canada

The effectiveness of 2.32 mmol/kg (approximately 1000 ppm) of α-, γ-, or δ-tocopherol (TOH), as well as different levels of αTOH, on the formation of hydroperoxides in fish oil was studied by monitoring the peroxide value (POV) and the formation of conjugated dienes (CD) during storage at 30 °C. The same order of antioxidant activity was observed by both methods. Linear regression of POV on CD showed that these data were strongly correlated ($r^2 \geq 0.98$). The value of the slope of the regression lines, however, differed substantially and decreased with increasing hydrogen-donating ability of the different tocopherols and with increasing αTOH concentration. It is suggested that this is due, at least in part, to the contribution from hydroxy compounds to the CD measurements and a greater contribution from hydroperoxy epidioxides (two peroxide groups per conjugated diene unit) to the POV than to the CD value. The degrees of formation of both these groups of oxidation products are expected to be influenced by the rate of scavenging of lipid peroxyl and alkoxyl radicals by tocopherol (αTOH > γTOH > δTOH).

Keywords: *Autoxidation; conjugated dienes; fish oil; peroxide value; tocopherol*

INTRODUCTION

Fish oils, because of their content of the long-chain n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are increasingly being proposed for incorporation into foodstuffs. These polyunsaturated fatty acids are highly susceptible to oxidation, with simultaneous formation of adverse flavors, and adequate antioxidant protection is essential.

Peroxide value (POV) and conjugated diene (CD) measurements are both well-established methods for the determination of primary oxidation products in fats and oils. The widely used iodometric titration method (AOCS methods Cd 8-53 and Cd 8b-90; *1, 2*) for POV determination is based on the measurement of the iodine produced from potassium iodide by the peroxides present, and the result is expressed as milliequivalents of iodine per kg lipid (meq/kg). Peroxidation of unsaturated fatty acids is accompanied by a shift in the position of double bonds to form conjugated hydroperoxides, and the conjugated structure absorbs strongly at 232−234 nm. The result from this spectrophotometric CD analysis is reported either as % conjugated dienoic acid (AOCS method Ti 1a-64; *3*) or as the sample extinction coefficient $E^{1\%}_{1cm}$ (IUPAC method 2.505; *4*). For assessing primary oxidation, the CD method is faster and simpler than the POV method and requires very little sample. However, as the value obtained depends on the sample fatty acid composition, the CD method cannot be used for direct comparison of the oxidative state of different species of fats and oils (*5*). The UV absorbance at 268 nm is a measure of conjugated trienes and secondary oxidation products such as conjugated ketodienes and dienals (*4*).

* To whom correspondence should be addressed. Tel: +47 35924608. Fax: +47 35922722. E-mail: Elin.Kulas@hydro.com.
† Norsk Hydro Research Centre.
‡ Dalhousie University.

The CD values measured during oxidation of fats and oils have been found to correlate fairly well with POV (*6*, *7*). While POV is a direct measure of peroxides, it appears likely that oxidation products of other origins with conjugated diene structures, e.g. fatty acid hydroxy compounds, contribute to the CD value. It has been known for some time that hydrogen-donating antioxidants, particularly α-tocopherol (αTOH), affect the distribution of oxidation products formed during storage of unsaturated fatty acids (*8*, *9*). Hopia et al. (*10*) confirmed the formation of hydroxy fatty acids from methyl linoleate hydroperoxides, and the level increased in the presence of αTOH and its water-soluble carboxylic acid derivative, Trolox.

The purpose of this work was, when studying the antioxidant effectiveness of α-, γ-, and δTOH in fish oils, to verify that the order of antioxidant activity found was independent of the method used to monitor the formation of primary oxidation products. By studying the relationship between the POV and CD data, we also aimed at obtaining a relative measure of the contribution from oxidation products other than hydroperoxides to these tests.

MATERIALS AND METHODS

Materials. Anchovy oil (refined, deodorized, and winterized; POV < 1 meq/kg) was provided by Pronova Biocare (Sandefjord, Norway). The major fatty acids were C14:0 (6.9%), C16:0 (16.1%), C16:1 n-7 (9.2%), C18:1 n-9 (8.9%), C20:5 n-3 (EPA; 15.6%), and C22:6 n-3 (DHA; 9.8%), as determined by capillary gas chromatography (GC) with flame ionization detection of the methyl esters prepared by transesterification using BF_3-methanol (*11*). The initial level of αTOH was 0.12 mmol/kg oil (50 ppm), as determined by normal-phase high-performance liquid chromatography (HPLC). Menhaden oil, refined and bleached, was provided by Omega Protein (Reedville, VA). The major fatty acids were C14:0 (7.5%), C16:0 (19.0%), C16:1 n-7 (9.4%), C18:1 n-9 (8.6%), C20:5 n-3 (EPA; 10.5%), and C22:6

10.1021/jf0011541 CCC: $20.00
Published on Web 03/17/2001

n-3 (DHA; 13.1%). Immediately upon arrival, the oils were stored at −30 °C. The anchovy oil was used without further treatment to study the effectiveness of αTOH, but the menhaden oil triacylglycerols (TAG) were purified as described below. The *d*-α-tocopherol (purity ≥ 99%) and the *d*-*γ*-tocopherol (purity ≥ 95%) were supplied by Acros (Ghent, Belgium) and the *d*-*δ*-tocopherol was from Sigma (St. Louis, MO; purity ≥ 90%). The αTOH product contained no other tocopherols, but the *γ*TOH and *δ*TOH products contained 1.5% and 3.3% of other tocopherols, respectively, as determined by HPLC. All solvents were analytical grade.

Oil Purification. To study the effect of the different tocopherol homologues, menhaden oil was purified to remove the αTOH originally present, as well as other minor constituents such as free fatty acids, pigments, and preformed oxidation products. The multilayer column chromatographic purification procedure employed was based on that reported by Lampi et al. (*12*) using hexane as a solvent. The product oil solution was stripped of the eluent hexane by bubbling N_2 through it. This kept the solution cold and retarded the onset of autoxidation. The hexane concentration in the product oil was < 0.2 ppm, as determined by static headspace GC according to a method for the analysis of volatile secondary lipid oxidation products based on that reported by Frankel (*13*), but using an incubation temperature of 40 °C. The purified menhaden oil TAG was practically colorless and odorless, with POV < 0.5 meq/kg (AOCS method Cd 8-53; *1*) and *p*-anisidine value < 0.2 (AOCS method Cd 18-90; *14*). No αTOH could be detected (HPLC detection limit, 1 ppm).

Oxidation Tests. Purified menhaden oil TAG samples with 2.32 mmol αTOH/kg oil (1000 ppm), *γ*TOH (967 ppm), or *δ*TOH (934 ppm), and anchovy oil samples with 0.12 mmol αTOH/kg oil (50 ppm), 2.32 mmol αTOH/kg oil (1000 ppm), and 4.64 mmol αTOH/kg oil (2000 ppm) were prepared. Antioxidant was added immediately following the menhaden oil purification. The tocopherols were first dissolved in hexane, and the concentrations were determined spectrophotometrically as described in AOCS method Ce 8-89 (*15*). Accurate amounts were added to the oil samples by transferring known volumes of the tocopherol solutions to glass flasks and evaporating the solvent under a stream of N_2 before adding the desired amount of oil. The antioxidants were mixed with the oils by bubbling N_2 through the samples for 20 min. The tocopherol concentrations were then verified by normal-phase HPLC with fluorescence detection according to AOCS method Ce 8-89 (*15*), with the exception that a Partisil 5 *μ*m column (11 cm × 4.7 mm, Whatman, Clifton, NJ), equipped with a guard column, was used.

Fish oil samples (42 g each for the purified menhaden oil, 75 g each for the anchovy oil) were stored in triplicate in 100-mL uncapped brown glass bottles (9 × 4 cm i.d.) in the dark in an oven at 30 °C. The bottles were sampled at regular intervals for CD and POV analysis. POV was determined according to AOCS method Cd 8-53 (*1*), based on half of the weight of oil described in that procedure. CD values were determined by dissolving weighed oil samples (approximately 30 mg) in isooctane (50 mL) and reading the sample absorbance at 234 nm using a Hewlett-Packard HP 8453 UV−visible spectrophotometer. The $E^{1\%}_{1cm}$ was calculated from the absorbance reading and the sample oil concentration, and the result was reported as the increase from the measurement obtained at time zero of the particular oil studied.

Statistical Analysis. Linear regression of POV on CD for the different treatments was performed using StatMost (DataMost Corporation, Salt Lake City, UT). The slopes of the regression lines were compared using the Bonferroni *t*-test and were considered different when $P < 0.05$.

RESULTS AND DISCUSSION

The order of antioxidant activity of the tocopherols (2.32 mmol/kg oil; approximately 1000 ppm) in the purified menhaden oil TAG was *δ*TOH > *γ*TOH > αTOH on the basis of both POV and CD measurements

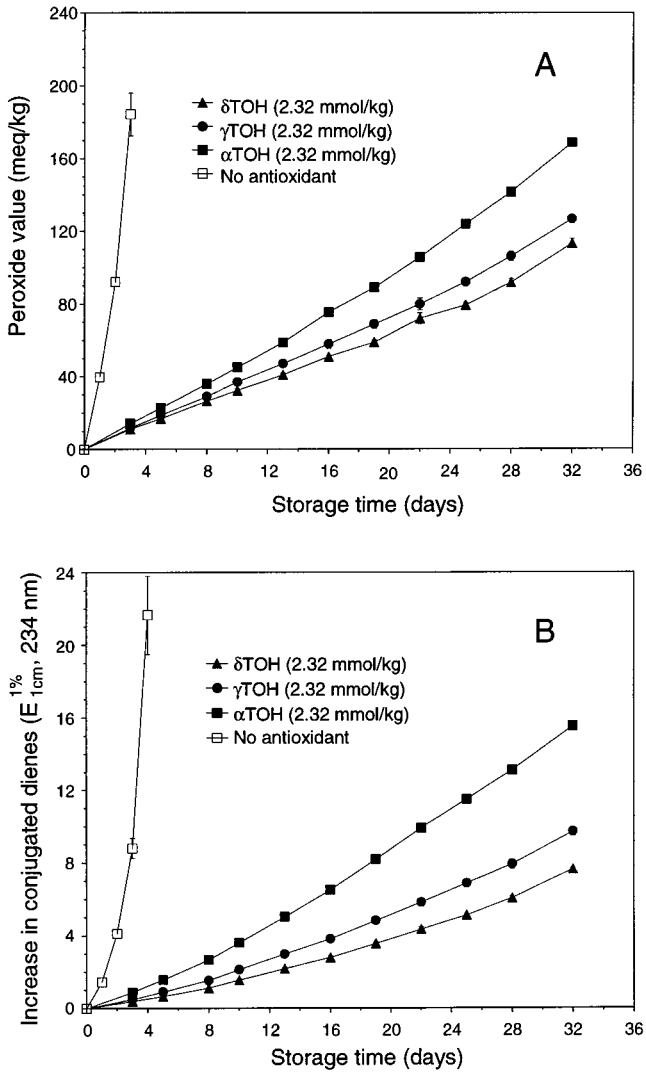


Figure 1. Effect of α-, *γ*-, and *δ*-tocopherol (TOH) (2.32 mmol/kg oil) on the formation of primary oxidation products in purified menhaden oil TAG during storage at 30 °C: (A) peroxide value measurements; (B) conjugated diene measurements. Data points are means ± standard deviation, $n = 3$.

(Figure 1). This is in agreement with the results obtained at similar levels of addition in several other oil substrates, including soybean oil (*16*) and corn oil (*17*, *18*). Relative tocopherol activity, on the basis of formation of hydroperoxides, has, however, been found to be concentration dependent (*18*), and the result obtained should be considered valid within this limited concentration range only.

A large further addition of αTOH to anchovy oil already containing 0.12 mmol/kg oil (50 ppm) of this antioxidant resulted in an increased rate of formation of primary oxidation products on the basis of both POV and CD measurements (Figure 2). A maximum antioxidant activity of αTOH at fairly low concentrations has also been observed in vegetable oils (*16*, *17*, *19*), and this inversion of activity has been suggested to be a result of the participation of αTOH and/or the α-tocopheroxyl radical in reactions other than those with fatty acid peroxyl radicals, as discussed by Huang et al. (*17*) and Kamal-Eldin and Appelqvist (*20*). Manufacturers of fish oils and n-3 concentrates for nutritional supplement use often add αTOH or tocopherol mixtures at levels of up to several thousand ppm. Results from this

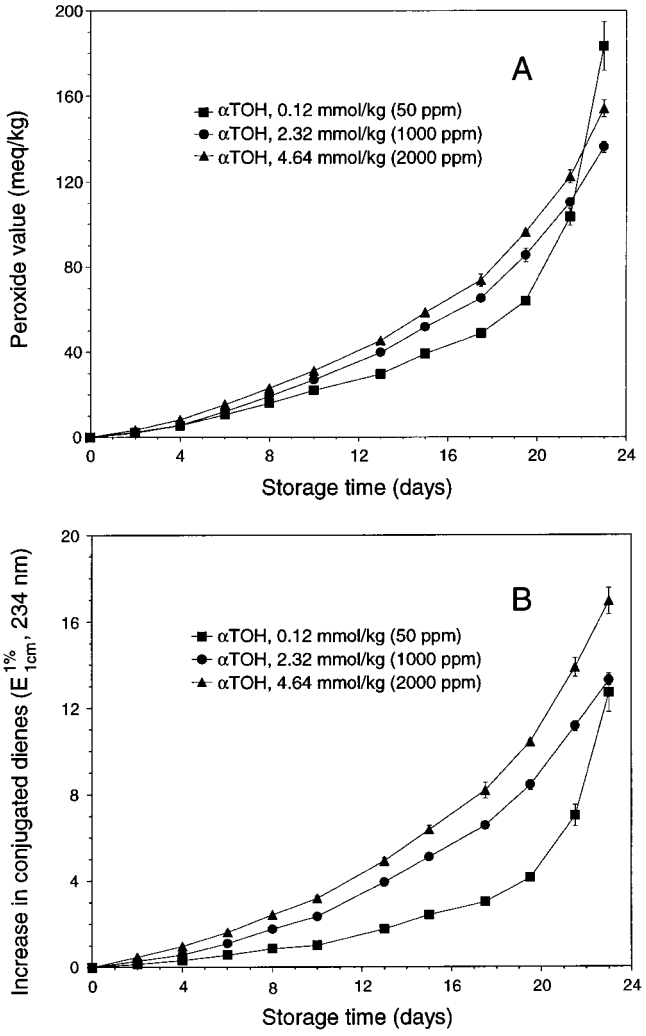


Figure 2. Effect of α-tocopherol (αTOH) on the formation of primary oxidation products in anchovy oil during storage at 30 °C: (A) peroxide value measurements; (B) conjugated diene measurements. Data points are means ± standard deviation, $n = 3$.

study suggest that such levels of αTOH are too high for optimum antioxidant activity on the basis of the rate of formation of primary oxidation products.

The POV and CD values measured during storage of the fish oils were strongly correlated (Figures 3 and 4, Table 1), and linear relationships between the two data sets were found for all the tocopherols tested, as well as for the levels of αTOH. The values of the regression line slopes, however, differed substantially and decreased in the following order: control > δTOH > γTOH > αTOH, as well as with increasing αTOH concentration. The slope of the regression line for the anchovy oil with 2.32 mmol αTOH/ kg oil and that of the purified menhaden oil TAG with the same amount of αTOH added were similar (Table 1), despite the difference in substrate composition and the fact that the two storage experiments reported were conducted at different times. Anchovy oil with 0.12 mmol αTOH/kg exhibited an induction period of 18−20 days (Figure 2), the induction period being the time required to reach a sudden increase in the rate of oxidation. Only POV and CD data points obtained before the end of the induction period were included in the linear regression analysis (Figure 4).

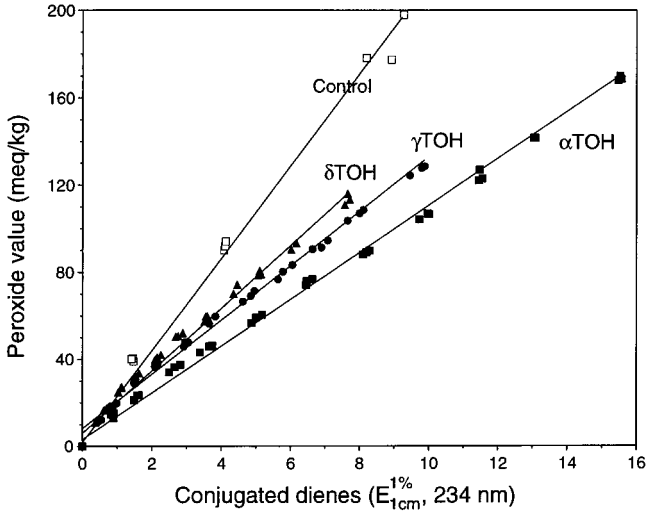


Figure 3. Relationships between the peroxide value and conjugated diene measurements obtained during storage of purified menhaden oil TAG with and without 2.32 mmol α-, γ-, or δ-tocopherol (TOH) per kg oil.

The tocopherols are chain-breaking antioxidants and act mainly by donating their phenolic hydrogen to lipid free radicals (*21*). The αTOH is structurally expected to be more potent as a hydrogen donor than γTOH, which in turn is expected to be more potent than δTOH, and their oxidation−reduction potentials support this order of hydrogen-donating power (*20*). In this study, the values of the slope of the regression lines presented in Figure 3 decreased in the order δTOH > γTOH > αTOH. From these results it appears that increasing the tocopherol hydrogen-donating power results in the formation of a higher proportion of oxidation products, other than fatty acid hydroperoxides, with a conjugated diene structure (or a lower proportion of oxidation products with more than one peroxide group per conjugated diene unit). The same phenomenon was observed when increasing the system hydrogen-donating ability by increasing the antioxidant concentration (Figure 4).

Although the product mixtures from the autoxidation of all polyunsaturated fatty acids, and EPA and DHA in particular, are extremely complex, it may be speculated that at least two groups of oxidation products contribute to the observed dependence of the relationship between POV and CD on the tocopherol homologue and concentration used, namely fatty acid hydroxy compounds and hydroperoxy epidioxides (Scheme 1). Homolytic cleavage of the hydroperoxide group, followed by β-scission of the alkoxyl radical intermediate and formation of volatile secondary oxidation products, is generally regarded as the most important pathway for decomposition of fatty acid hydroperoxides (*22*). In addition to reaction with fatty acid peroxyl radicals ($ROO^{\bullet}$) (reaction 1), hydrogen-donating antioxidants react with alkoxyl radical intermediates ($RO^{\bullet}$) in an analogous way (reaction 2), to form fatty acid hydroxy compounds (ROH) (*21*):

$$ROO^{\bullet} + TOH \rightarrow ROOH + TO^{\bullet} \quad (1)$$

$$RO^{\bullet} + TOH \rightarrow ROH + TO^{\bullet} \quad (2)$$

In accordance with this view, Hopia et al. (*10*) found that methyl linoleate hydroxy compounds were formed from methyl linoleate hydroperoxides in the presence

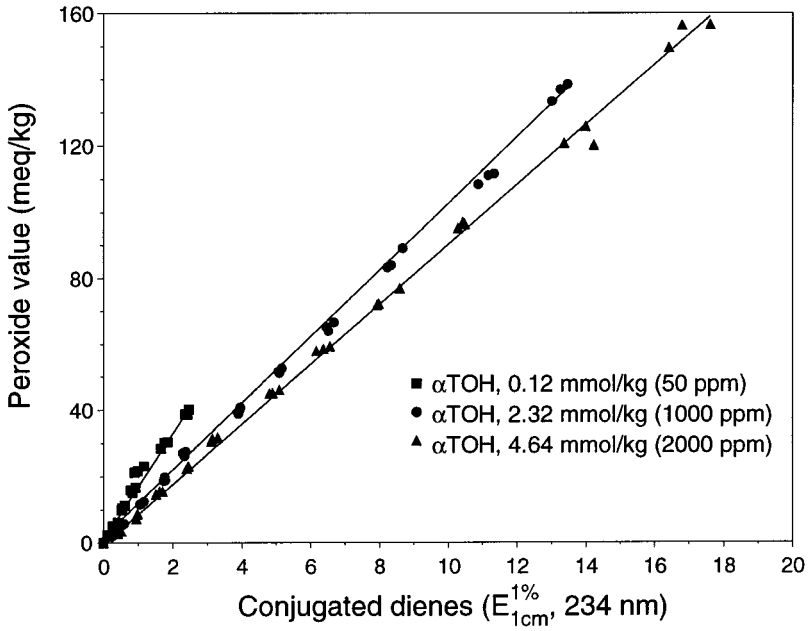


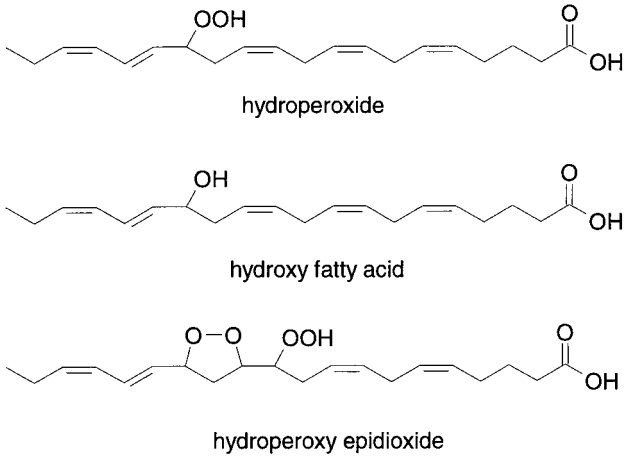
Figure 4. Relationships between the peroxide value (POV) and conjugated diene (CD) measurements obtained during storage of anchovy oil with different levels of α-tocopherol (αTOH). Only POV and CD data points obtained before the end of the induction period are included.

Table 1. Regression Coefficients (± Standard Error) and Coefficients of Determination (r^2) from the Linear Regression of POV on CD[a]

treatment (mmol TOH/kg oil)	slope	r^2	treatment (mmol TOH/kg oil)	slope	r^2
no antioxidant	20.1 (± 0.5)[a]	0.994			
δTOH (2.32)	13.8 (± 0.2)[b]	0.993	αTOH (0.12)	15.9 (± 0.4)[a]	0.98
γTOH (2.32)	12.3 (± 0.1)[c]	0.996	αTOH (2.32)	10.1 (± 0.1)[b]	0.998
αTOH (2.32)	10.3 (± 0.1)[d]	0.998	αTOH (4.64)	9.0 (± 0.1)[c]	0.998

[a] Values in each column followed by a different letter are significantly different ($P < 0.05$).

Scheme 1. Structures of a Typical Hydroperoxide, Hydroxy Fatty Acid, and Hydroperoxy Epidioxide Expected to Be Formed from the Autoxidation of Eicosapentaenoic Acid (EPA)



of αTOH and Trolox, with the corresponding inhibition of volatile decomposition products. The methyl linoleate hydroxy compounds were the only oxidation products, besides the hydroperoxides themselves, that appeared to absorb to any significant extent at 233 nm when analyzed by normal-phase HPLC. The hydroperoxides of the n-3 polyunsaturated fatty acids in fish oil decompose more readily than hydroperoxides of fatty acids with a lesser degree of unsaturation (*23*). In a study of the oxidative stability of lipids in frozen mackerel, Saeed and Howell (*24*) recently reported that the fatty acid hydroperoxides formed were rapidly converted to the more stable hydroxy compounds when preparing the extracted lipid samples for analysis. Fatty acid hydroxy compounds formed during lipid oxidation have the same UV absorption maxima as, and similar molar absorptivities to, their corresponding hydroperoxides (*25*), but do not contribute to the POV.

Hydroperoxy epidioxides are formed in a major pathway of the autoxidation of polyunsaturated fatty acids with three or more methylene-interrupted double bonds (e.g., linolenic acid, arachidonic acid, EPA, and DHA). In fact, the hydroperoxy epidioxides represented 25% of the total hydroperoxides found in autoxidized methyl linolenate (*26*). Rapid intramolecular peroxyl radical addition to a homoallylic double bond and subsequent reaction with a second oxygen molecule leads to the formation of these oxidation products, which have two peroxide groups per conjugated diene unit (*27*, *28*). Both peroxide groups are expected to contribute to the POV, the cyclic peroxide unit to a lesser extent than the hydroperoxide group because of a lower reactivity of dialkyl peroxides with iodide ion compared to that of hydroperoxides (*29*). The hydrogen-donating power of αTOH (5%) was found to completely inhibit 1,3-cyclization of methyl linolenate peroxyl radicals by trapping the peroxyl radicals as monohydroperoxides (*8*). On the basis of these results, an increase in the tocopherol hydrogen-donating power may be expected to reduce the formation of hydroperoxy epidioxides relative to that of hydroperoxides, and thereby the POV relative to the CD

value, which is in accordance with the observations in this study.

Fatty acid alkoxyl radicals are, in addition to being precursors of hydroxy compounds, also precursors of epoxy hydroperoxides and epoxy hydroxy compounds (*23*). Epoxy hydroperoxides, on the basis of their structure, are expected to contribute to the POV only, whereas epoxy hydroxy compounds, on the basis of their structure, are expected not to be detected by either the POV or CD measurements. One may expect a lower rate of epoxide formation with increasing tocopherol hydrogen-donating power due to alkoxyl radical scavenging by the antioxidant, and accordingly, a contribution from epoxy hydroperoxides to the observed POV−CD relationship in the same manner as that of the hydroperoxy epidioxides. Epoxy hydroperoxy compounds were, together with epoxy hydroxy compounds, considered to be minor secondary oxidation products compared to hydroperoxy epidioxides in oxidized methyl linolenate (*23*). Therefore, it seems likely that the hydroperoxy epidioxides are the most influential of these groups of oxidation products in fish oils as well. To verify the relative ability of the tocopherol homologues to affect the formation of the different groups of oxidation products discussed, a simpler model system such as methyl linolenate could be studied.

During autoxidation the fish oil fatty acids may cross-link through carbon−carbon, ether, or peroxide linkages, maintaining a conjugated diene unit with UV absorbance at 234 nm. The relative concentrations of these products depend on the temperature during oxidation and on the availability of oxygen (*23*), and it is difficult to predict whether the antioxidant hydrogen-donating power will affect the rate of formation of these compared to that of hydroperoxides. Hydroperoxides decompose rapidly when heated and a low POV is not necessarily indicative of a high quality oil. Fatty acid cross-linking to dimers and oligomers is important at high temperatures (*23*), and CD measurements may, contrary to POV, also be useful for the detection of oxidation products formed during heat treatments such as frying or deodorization.

In this study, we have demonstrated that CD measurements correlate very well with POV in fish oil exposed to air. The iodometric POV procedure is the most widely used method for the determination of primary oxidation products in fats and oils. It is, however, time-consuming and many sources of error have been identified (*7*). We found the CD method to be sensitive, simple, and very useful for stability tests with fish oils.

ACKNOWLEDGMENT

The authors thank Omega Protein (Reedville, VA) for providing the menhaden oil and Pronova Biocare (Sandefjord, Norway) for providing the anchovy oil.

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Received for review September 19, 2000. Revised manuscript received January 26, 2001. Accepted January 29, 2001. This work was financially supported by Pronova Biocare (Sandefjord, Norway) and the Norwegian Research Council.

JF0011541