Properties of α-, γ-, and δ-Tocopherol in Purified Fish Oil Triacylglycerols[1]

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ABSTRACT: The effect of α-tocopherol (αTOH) (50–2000 ppm), γ-tocopherol (γTOH) (100–2000 ppm), and δ-tocopherol (δTOH) (100–2000 ppm) on the formation and decomposition of hydroperoxides in purified fish oil triacylglycerols (TAG) was studied. The tests were conducted at 30°C in the dark. Purified fish oil TAG oxidized very rapidly with no apparent induction period. The relative ability of the tocopherols to retard the formation of hydroperoxides decreased in the order αTOH > γTOH > δTOH at a low level of addition (100 ppm), but a reverse order of activity was found when the initial tocopherol concentration was 1000 ppm. This dependence of relative antioxidant activity on tocopherol concentration was caused by the existence of concentrations for maximal antioxidant activity for αTOH and for γTOH. An inversion of activity, on the basis of hydroperoxide formation, was observed for αTOH at 100 ppm and for γTOH at 500 ppm, whereas the antioxidant activity of δTOH increased with level of addition up to 1500–2000 ppm. None of the tocopherols displayed any prooxidant activity. All three tocopherols strongly retarded the formation of volatile secondary oxidation products in a concentration-dependent manner. At concentrations above about 250 ppm there appeared to be a linear relationship between rate of consumption of αTOH and initial αTOH concentration, in accordance with the linear relationship observed between the initial rate of formation of hydroperoxides and the initial αTOH concentration. The rate of consumption of γTOH also increased with initial concentration, but to a lesser extent than for αTOH. At high levels of addition the rate of consumption of δTOH was independent of initial concentration, appearing to reflect the greater stability of this tocopherol homolog and participation in reactions with lipid peroxyl radicals only.

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Long-chain n-3 fatty acids play an important role in human development and in the prevention and treatment of disease (1). The content of such fatty acids renders fish oils highly susceptible to oxidation. Fishy off-flavors develop rapidly during storage, and adequate antioxidant protection is essential for the successful utilization of these oils in food products. Unlike most vegetable oils, α-tocopherol (αTOH) is generally the only tocopherol homolog naturally present in oils from wild fish (2,3). In the case of phospholipid-rich fish membranes, αTOH may be regarded as essential because it fits better than the other tocopherols into the bilayers of such animal tissues (4).

The antioxidant activities of the tocopherols in vegetable oil models have been thoroughly studied. Although the effectiveness and relative antioxidant activity of the tocopherols in oils are generally regarded as being dependent on both the characteristics of the lipid substrate and the experimental conditions (5,6), γ-tocopherol (γTOH) and δ-tocopherol (δTOH) have frequently been found to be superior to αTOH (7–9). An inversion of activity may take place at certain tocopherol concentrations, above which an increase in the rate of lipid autoxidation occurs, and at sufficiently high levels of addition prooxidant effects have been observed, particularly with αTOH (10–12). Tocopherol efficiency and relative order of antioxidant activity have been demonstrated to vary with the degree of unsaturation of the substrate (13,14). Inversion of activity of αTOH was observed at a somewhat lower concentration in butter oil triacylglycerols (TAG) (15) than in the more oxidizable rapeseed oil TAG (16), suggesting an increased tocopherol requirement for optimal activity with degree of substrate unsaturation. The level of αTOH in fish oils is reduced by at least half by commercial refining and deodorization, generally to below 100 ppm, and for food use relatively high levels of natural antioxidants, often several thousand ppm αTOH or mixed tocopherols, are added in order to protect the product oil. The findings in vegetable oils have suggested that a reduction in tocopherol concentration may be beneficial.

The majority of studies on properties of antioxidants in fish oils have been accelerated by elevated temperatures (17–19), and/or activities have been compared at one antioxidant concentration only (13,17,20). The reaction mechanisms for oxidation and hydroperoxide decomposition are known to change with temperature (21), and results obtained at high temperatures may thus not be directly transferable to ambient conditions. Also, results from vegetable oils suggest that antioxidant activities of the tocopherols in fish oil should be evaluated over a wide concentration range. Ternary blends of tocopherols, lecithin, and ascorbyl palmitate have been found to retard autoxidation in fish oils efficiently (19,22). Information on the properties of the individual tocopherols is also important when developing antioxidant systems containing other antioxidants in addition to tocopherols. The cost is an important consideration when deciding what antioxidant(s) to use in fish oils and n-3 concentrates as well as in aquaculture feeds containing fish oil; commercial tocopherol

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blends generally are less expensive than the natural *d*-αTOH products.

Few autoxidation studies have measured the effect of antioxidants on the decomposition of hydroperoxides, yet the volatile products formed are very relevant to the development of rancidity in an oil. The objectives of this work were to study, in a purified fish oil TAG fraction, the effect of *d*-αTOH, *d*-γTOH, and *d*-δTOH, individually, over a wide concentration range on the rate of formation of both hydroperoxides and volatile secondary oxidation products. Minor natural antioxidant constituents in an oil, and catalysts such as metal ions and preformed oxidation products, are known factors in lipid autoxidation, and catalysts have also been suggested to induce the sometimes observed prooxidant effect of αTOH (6). Purification of the fish oil by column chromatography thus allows better control over factors that influence the autoxidation process.

EXPERIMENTAL PROCEDURES

Materials. Anchovy oil (refined, deodorized, and winterized) was from Pronova Biocare (Sandefjord, Norway). The *d*-αTOH (purity ≥99%) and the *d*-γTOH (purity ≥95%) were supplied by Acros (Ghent, Belgium), and the *d*-δTOH was from Sigma (St. Louis, MO; purity ≥90%). The αTOH product contained no other tocopherols, whereas the γTOH and the δTOH contained 1.5 and 3.3% other tocopherols, respectively, as determined by high-performance liquid chromatography (HPLC). Silica gel was purchased from Aldrich (Milwaukee, WI), Celite 545 from Supelco (Bellefonte, PA), and activated carbon from Fisher Scientific (Fair Lawn, NJ). All solvents were analytical grade.

Oil purification. The refined fish oil was further purified using a modified version of a previously reported multilayer chromatographic method (23). A chromatographic column (60 × 4 cm i.d.) was packed sequentially with four adsorbents, all suspended in hexane. The bottom layer was 40 g activated silica gel (60–200 mesh), which had been washed with distilled water and with methanol before activation. The next two layers were 40 g of a 1:2 mixture of Celite 545/activated carbon (50–200 mesh) and 40 g of a 1:2 mixture of Celite 545/granulated sugar. The top layer was 40 g of activated silica gel. The oil (230 g) was dissolved in hexane (230 mL) and applied to the column. Eluent was collected under N_2 until all the oil solution had been drawn down into the column. The product solution was stripped of hexane by bubbling N_2 through it. This kept the solution cold and retarded onset of autoxidation of the purified TAG. The hexane concentrations in the oil samples after stripping ranged from 0.02 to 0.2 ppm, as determined by static headspace gas chromatography (GC) as later described for volatile oxidation products, but with hexane as external standard. The chromatographic column and flasks used for collecting product and removal of hexane were covered with aluminum foil to prevent light-induced oxidation during the purification process. The purified oil was colorless and odorless, and components more polar than triacylglycerols, such as mono- and diacylglycerols and preformed oxidation products, were removed. The level of hydroperoxides was <0.5 meq/kg as determined by the peroxide value (POV), and the *p*-anisidine value (*p*AV) was 0.2. The iron content was 15 ng/mL, while the copper content was <10 ng/mL. No αTOH could be detected in the purified oil (limit of detection 1 ppm). The major fatty acids were 14:0 (6.9%), 16:0 (16.1%), 16:1n-7 (9.2%), 18:1n-9 (8.9%), 20:5n-3 [eicosapentaenoic acid (EPA), 15.6%], and 22:6n-3 [docosahexaenoic acid (DHA), 9.8%].

Oxidation tests. Purified fish oil TAG samples containing αTOH (50–2000 ppm), γTOH (100–2000 ppm), or δTOH (100–2000 ppm) were prepared immediately following oil purification. An oil sample with 100 ppm αTOH was included in the storage experiments with γTOH and δTOH for direct comparison of their activities. The tocopherols were first dissolved in hexane and the concentrations determined spectrophotometrically as described in AOCS method Ce 8-89 (24). Accurate amounts of the tocopherols 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 oil by bubbling N_2 through the samples for 20 min, and tocopherol concentrations were verified by HPLC analysis. Portions (1.5 g) of the different oil treatments were stored in 20-mL uncapped glass vials (40 × 25 mm i.d.) in the dark in a thermostatted oven at 30°C. Triplicate portions of each oil treatment were removed from the oven at regular intervals for analysis of hydroperoxides (by determination of conjugated dienes), volatile secondary oxidation products (as C3-aldehydes), and residual tocopherol content.

Analyses. Fatty acid composition was determined by capillary GC with flame-ionization detection of the methyl esters prepared by transesterification using BF_3/methanol (25). The POV was measured according to AOCS method Cd 8b-90 (24) using a 1:10 dilution of the $Na_2S_2O_3$-solution, and the *p*AV was measured according to AOCS method Cd 18-90 (24). Percentage of TAG in the oil was determined by Iatroscan analysis according to the method of Sigurgisladottir *et al*. (25). The iron and copper contents were determined by electrothermal atomic absorption spectrophotometry equipment with Zeeman background correction. The oil samples were dissolved in methyl isobutyl ketone and diluted with 2-propanol. Ammonium hydrogen phosphate and magnesium nitrate dissolved in 2-propanol were used as the matrix modifiers in the determination of iron. Acidified palladium nitrate and magnesium nitrate dissolved in 2-propanol were used as the matrix modifiers in the determination of copper.

Conjugated dienes 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 (Palo Alto, CA). The $E^{1\%}_{1\,cm}$ was calculated from the absorbance reading and the sample oil concentration, and the result is reported for each study as the increase from the measurement obtained at time zero. Tocopherol concentrations were determined by normal-phase HPLC with fluorescence detection according to

AOCS method Ce 8-89 (24), with the exception that a Partisil 5 μm column (11 cm × 4.7 mm; Whatman, Clifton, NJ), equipped with a guard column, was used.

Propanal is one of the major volatile secondary oxidation products of n-3 fatty acids, and may be analyzed by static headspace GC as a marker of the formation of this complex group of oxidation products (26). Oil samples (0.50 g) in 10-mL crimp-sealed glass vials were equilibrated for 20 min at 40°C in an oven. A headspace sample (1.0 mL) was manually injected, using a heated gas-tight syringe, into a PerkinElmer Sigma 3B GC (Norwalk, CT) equipped with a capillary SPB-1701 column (14% cyanopropylphenyl, 86% dimethylsiloxane, 30 m × 0.32 mm i.d., 1 μm phase; Supelco, Bellefonte, PA) and a flame-ionization detector. The analysis was isothermal at 70°C. Propanal was identified by comparison of the retention time with that of an authentic reference sample on two GC columns of different polarity; however, it was discovered that 2-propenal, another volatile oxidation product from n-3 fatty acids, coeluted with propanal on the SPB-1701 column. C3-aldehydes (propanal + 2-propenal) are therefore reported as a marker of volatile secondary oxidation products and quantified by comparison of the peak area with that of an external standard of propanal in sunflower oil. The repeatability of the method was considered good, as the coefficient of variation from the analyses of the standard, in triplicate, ranged from 0.7 to 2.7%.

The data for hydroperoxide formation at the different sampling times were evaluated using a *t*-test. Initial hydroperoxide formation refers to the results obtained at day 2 of storage. The data for tocopherol consumption were evaluated by analysis of variance, followed by Tukey's test for multiple comparisons, using StatMost™ (DataMost Corporation, Salt Lake City, UT). Differences in treatment means were considered significant when $P < 0.05$. When studying the effect of αTOH, the induction periods were estimated as the storage time when an abrupt increase in oxidation rate was observed. For the samples that remained in the induction period throughout the experiment, the induction period was estimated by extrapolating the curve for tocopherol consumption to zero level.

RESULTS AND DISCUSSION

Effect of α-, γ-, and δTOH on formation of hydroperoxides and C3-aldehydes. Formation of hydroperoxides in purified fish oil TAG was very rapid with no apparent induction period (Fig. 1A). The αTOH retarded the formation of both hydroperoxides and volatile secondary oxidation products at all levels of addition. In the presence of 50 ppm αTOH, the rate of formation of hydroperoxides increased rapidly after an induction period of about 4 d (Fig. 1A). Further addition of αTOH to 100 ppm had no significant effect on the amount of hydroperoxides initially formed (day 2 of storage), but the induction period was extended by about 2 d. In the presence of >100 ppm αTOH, there was a significant increase in the initial formation of hydroperoxides, and the difference in oxidative status between the samples increased with storage time. Hence, of the concentrations tested, αTOH displayed an inversion of activity at 100 ppm. Contrary to the effect on the formation of hydroperoxides, the amount of C3-aldehydes formed decreased with increasing αTOH concentration (Fig. 1B). The formation of C3-aldehydes in purified fish oil TAG with no antioxidant was very rapid (26 ppm after 2 d of storage) and is not included in Figure 1B. The induction periods were in accordance with those observed for the formation of hydroperoxides.

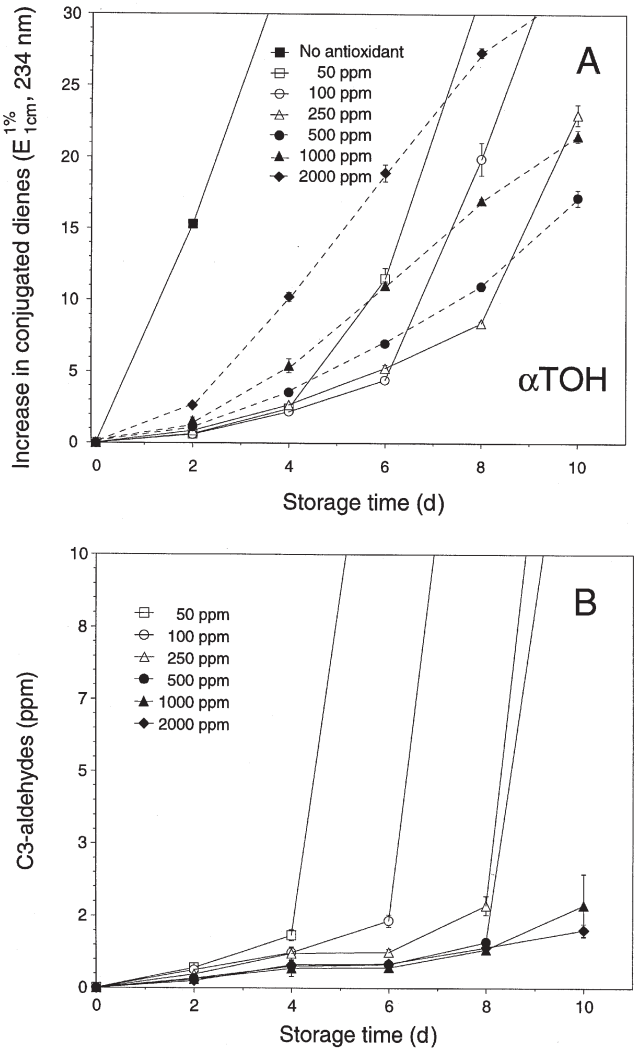

FIG. 1. Storage of purified fish oil triacylglycerols with different levels of α-tocopherol (αTOH) at 30°C in the dark: (A) formation of conjugated dienes, (B) formation of C3-aldehydes. Data points are means ± standard deviation ($n = 3$).

The γTOH retarded both hydroperoxide and C3-aldehyde formation at all levels of addition (Fig. 2). The levels of hydroperoxides formed at 500 ppm γTOH were significantly lower than those of the other oil samples containing γTOH after 4 d of storage and up to the end of the induction period (Fig. 2A), and an inversion of activity of γTOH occurred at this concentration. The initial hydroperoxide formation (day 2 of storage) was lower in the sample with 100 ppm αTOH than at either of the γTOH concentrations tested. As was

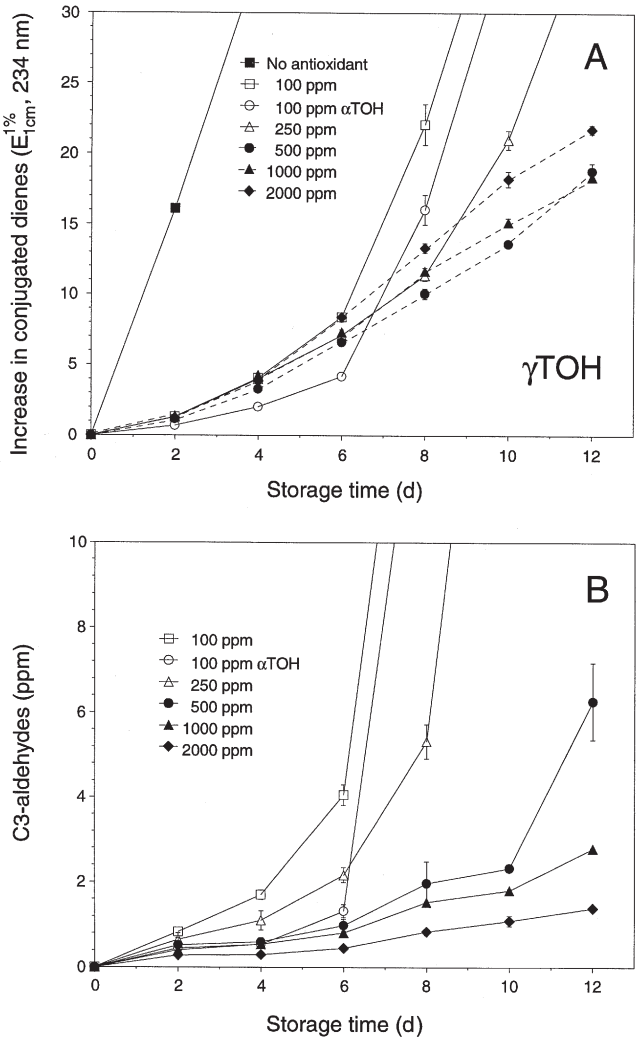

FIG. 2. Storage of purified fish oil triacylglycerols with different levels of γ-tocopherol (γTOH) at 30°C in the dark: (A) formation of conjugated dienes, (B) formation of C3-aldehydes. Data points are means ± standard deviation (n = 3).

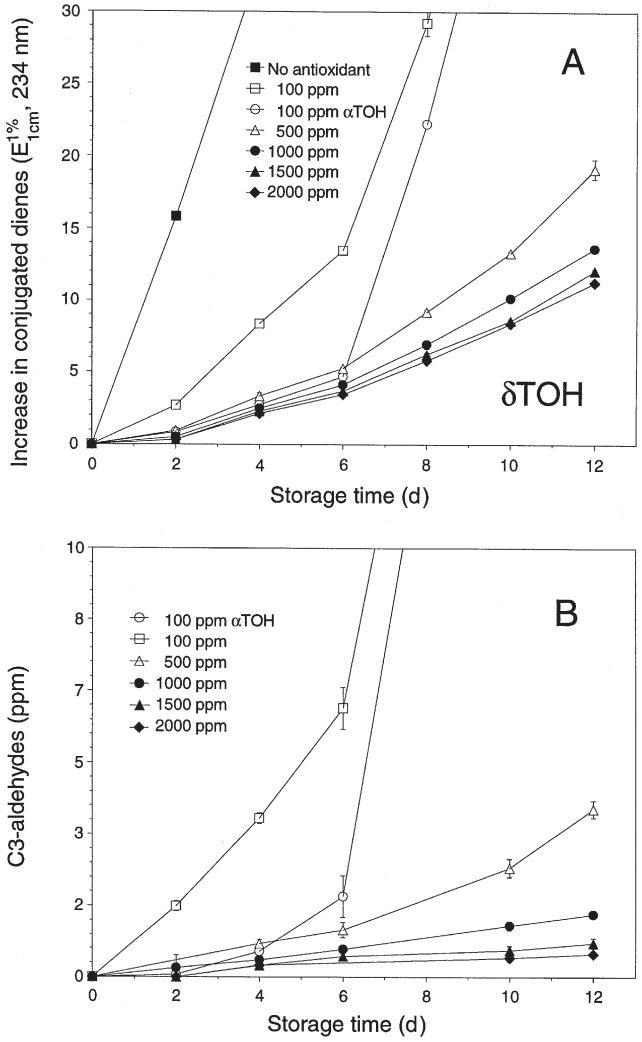

FIG. 3. Storage of purified fish oil triacylglycerols with different levels of δ-tocopherol (δTOH) at 30°C in the dark: (A) formation of conjugated dienes, (B) formation of C3-aldehydes. Data points are means ± standard deviation (n = 3).

found for αTOH, γTOH inhibited the formation of C3-aldehydes in a concentration-dependent manner (Fig. 2B).

As was found for αTOH and γTOH, δTOH retarded the formation of hydroperoxides and C3-aldehydes at all levels of addition (Fig. 3). The antioxidant activity increased with δTOH concentration up to 1500 ppm, when no further improvement of oil stability was obtained. At 100 ppm δTOH, accumulation of both hydroperoxides and C3-aldehydes was rapid while the samples were still in the induction period, which ended after 6 d of storage. The difference in the induction period between the samples with 100 ppm αTOH and 100 ppm δTOH was small, but αTOH at this concentration was a much better inhibitor of both hydroperoxide and C3-aldehyde formation than δTOH.

As in purified fish oil TAG, an inversion of activity of αTOH, on the basis of hydroperoxide formation, was observed at 100 ppm in corn oil (10), soybean oil (12), purified rapeseed oil TAG (16), and olive oil (27). A limited number of antioxidant concentrations were tested in all these studies, as well as in the present study, and the true optimal concentrations may thus vary somewhat among the different vegetable oils and between vegetable oils and the highly oxidizable fish oil. The concentration for maximum antioxidant activity of γTOH found in this study (500 ppm) was somewhat higher than what was observed in other fats and oils (10,12, 15,16). In addition to the degree of unsaturation, the substrates previously studied are likely to have differed with regard to concentration of minor constituents expected to influence the autoxidation process, as well as storage conditions. Taking this into account, the influence of substrate composition on the concentrations for maximum tocopherol activity appears to be small. The antioxidant effectiveness of δTOH in corn oil increased with concentration (11), in accordance with the results in purified fish oil TAG.

In this study, all three tocopherols retarded the formation of C3-aldehydes, and the antioxidant activity on the basis of these volatile secondary oxidation products increased with tocopherol concentration. A discrepancy between the effect of

tocopherols on hydroperoxide formation and hexanal formation was previously found in corn oil (10,11), and, recently, the same phenomenon was observed in purified rapeseed oil TAG (16). Hopia *et al*. (28) found that αTOH markedly inhibited the decomposition of methyl linoleate hydroperoxides and the formation of hexanal in a concentration-dependent manner. In addition to stabilizing the hydroperoxides, αTOH was suggested to be an effective inhibitor of β-scission of alkoxyl radicals by hydrogen donation to form stable methyl linoleate hydroxy compounds. It is likely that the formation of propanal, also a product of β-scission of an alkoxyl radical, is inhibited according to the same mechanism.

The flavor of fish oil has been found to deteriorate rapidly during storage, although the levels of hydroperoxides, as POV, remain low (22). Propanal and 2-propenal are two of the major volatile secondary oxidation products from n-3 fatty acids and can easily be analyzed by static headspace GC as markers of hydroperoxide decomposition in fish oils. However, secondary oxidation products with higher molecular weight, such as 4-hexenal and the 2,4,7-decatrienals, are thought to be more influential with regard to the development of rancid flavor in fish oils, even though only present at trace levels (29). In order to study the effect of the individual tocopherols on the formation of these longer-chain oxidation products, a more sophisticated dynamic headspace method is required.

Tocopherol consumption. The consumption of the tocopherols during storage appeared to be characterized by an initial low rate of consumption followed by a more rapid loss of αTOH after about 2 d of storage, after 4 d of storage for γTOH, and after 6 d of storage for δTOH (results not shown). Complete consumption of the antioxidant was reached at the end of the induction periods. An additional sample with 100 ppm αTOH was included when studying the properties of γTOH and δTOH. The rates of consumption of αTOH in the three storage experiments were not significantly different, and the tocopherol data were therefore directly compared. The overall rates of consumption of αTOH and γTOH (ppm/d) were strongly correlated with the amount of tocopherol added (Fig. 4). Above a certain initial concentration (about 250 ppm for αTOH and 500 ppm for γTOH) the relationships appear linear, the value of the slope being highest for αTOH. The rate of consumption of δTOH reached a constant value of about 50 ppm/d (δTOH ≥ 1000 ppm) and became independent of the initial tocopherol concentration.

Relative order of antioxidant activity. At 100 ppm, the order of antioxidant activity of the tocopherols was αTOH > γTOH > δTOH on the basis of the formation of both hydroperoxides and C3-aldehydes, corresponding to the reverse of the order of the rate of consumption of the antioxidant (Fig. 4; $P < 0.05$). When added at a level of 1000 ppm, the tocopherols retarded hydroperoxide formation in the order δTOH > γTOH > αTOH, again corresponding to the reverse of the order of the rate of tocopherol consumption (Fig. 4). These findings were confirmed in a separate storage experiment (Kulås, E., and R.G. Ackman, unpublished results).

The tocopherols retard lipid autoxidation mainly by donating a hydrogen atom to a lipid peroxyl radical, forming hydroperoxides and tocopheroxyl radicals (TO•), and subsequent reaction of TO• with a second lipid peroxyl radical to peroxy tocopherones (6). The αTOH structurally is expected to be more potent as a hydrogen donor than γTOH, which in turn is expected to be more potent than δTOH. Their oxidation-reduction potentials support this order of hydrogen-donating power (6), and αTOH is, in fact, regarded as one of the best chain-breaking phenolic antioxidants known (30,31). At low concentrations in the purified fish oil TAG, its hydrogen-donating ability made αTOH a strong antioxidant, while higher levels of γTOH and δTOH were required to inhibit hydroperoxide formation adequately. An αTOH-mediated accumulation of hydroperoxides, in accordance with the findings in this study, was explained by the participation of αTOH or αTO• in reactions other than with lipid peroxyl radicals (6,10,32). The mechanisms and relative importance of these side reactions are not fully understood but may involve chain-transfer reactions with intact lipid molecules and preformed hydroperoxides, direct reaction with oxygen, and interactions with transition metal ions. The assumption that αTOH/αTO• participates in side reactions is in accordance with the high rate of tocopherol consumption observed at high initial αTOH concentrations in this study. The γTOH, being a less active hydrogen-donor than αTOH, is to a lesser extent consumed in side reactions. The even more stable δTOH does not appear to participate in side reactions, as the rate of tocopherol consumption became independent of the level of addition. The tocopherols were found to effectively retard the formation of volatile secondary oxidation products in the purified fish oil TAG. Based on these findings, it is considered likely that, in addition to the participation of TOH/TO• in certain side reactions, protection of the already formed hydroperoxides also contributed to the observed accumulation of these at high concentrations of αTOH and of γTOH.

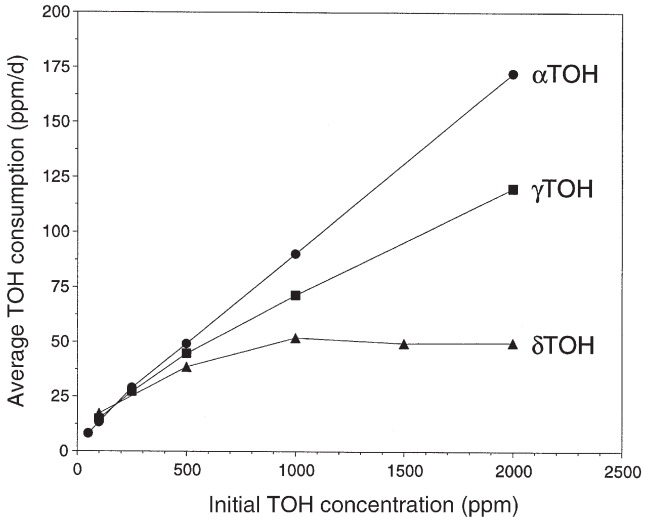

FIG. 4. Relationships between average rate of consumption of α-, γ-, and δ-tocopherol (TOH) and initial TOH concentration in purified fish oil triacylglycerols.

The αTOH, at concentrations tested in this study, was previously found to exert a prooxidant effect in various lipid systems (10,12), including fish oils (20,22). No prooxidant effect was observed for either tocopherol at any of the levels of addition to purified fish oil TAG in the present study. These results support the findings in purified rapeseed oil TAG (16) and purified sunflower oil TAG (32), where αTOH and γTOH were antioxidants at all levels tested (≤2000 ppm). The main reason for these contradictory findings is likely to be differences in the stability of the control oil, as even minor amounts of tocopherol have been shown to retard oxidation of TAG (33). Purification by column chromatography leaves the control oil devoid of any tocopherol, and the autoxidation of fish oil TAG is very rapid and with no apparent induction period.

The influence of the method used for evaluation of antioxidant efficiency on the results that may be obtained is illustrated in Figure 5, where the induction periods, as well as initial rates of formation of conjugated dienes and C3-aldehydes, are plotted vs. initial αTOH concentration. In a previous study we found that the level of hydroperoxides in purified fish oil TAG with tocopherol increased in a linear manner with storage time early in the storage period (≤3 d). Initial rates of hydroperoxide formation in the present study were therefore estimated from the data obtained after 2 d of storage. Above about 250 ppm αTOH there appears to be a linear relationship between the initial rate of hydroperoxide formation and the αTOH initial concentration, in accordance with the linear relationship observed between the rate of αTOH consumption and the amount of αTOH added (Fig. 4). The induction period, on the other hand, increased in a curvilinear fashion with αTOH concentration and was not greatly reduced by additional αTOH (above 500 ppm). This can be explained by the high rate of αTOH consumption in this concentration range (Fig. 4). The stabilization of hydroperoxides by αTOH and retardation of C3-aldehyde formation is also apparent from Figure 5. The C3-aldehyde curve appears to be a mirror image of the induction period curve, and we suggest that, at relatively high rates of hydroperoxide formation (αTOH > 500 ppm), inhibition of hydroperoxide decomposition is somewhat reduced. This trend was also evident later in the storage period when the C3-aldehyde concentrations were higher. From these observations it appears that, when αTOH is the only antioxidant added, an αTOH concentration of 500 ppm is a good compromise between the formation of hydroperoxides and the formation of volatile secondary oxidation products.

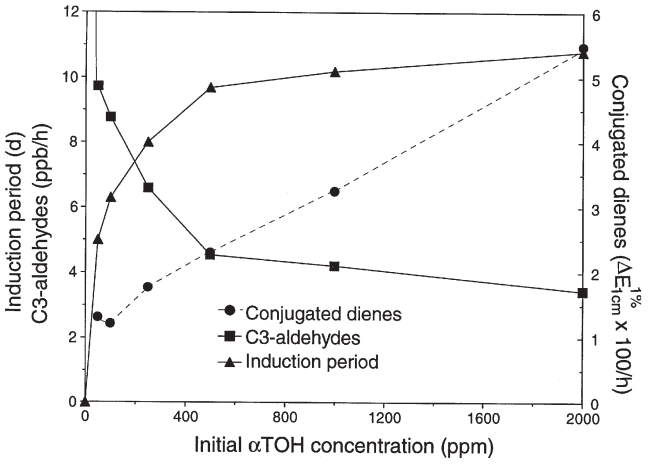

FIG. 5. Comparison of the induction period and initial rates of formation of conjugated dienes and C3-aldehydes in purified fish oil triacylglycerols with different levels of αTOH. Initial rates of formation of oxidation products were estimated from the data obtained after 2 d of storage. See Figure 1 for abbreviation.

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