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Antioxidant Properties of Tocopherols in Fish Oils

by

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List of Abbreviations

αTOH	α-Tocopherol
βTOH	β-Tocopherol
γTOH	γ-Tocopherol
δTOH	δ-Tocopherol
AOCS	American Oil Chemists' Society
AP	Ascorbyl palmitate
Ax	Astaxanthin
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Conjugated diene
CV	Coefficient of variation
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ESR	Electron spin resonance
FID	Flame ionization detection
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IP	Induction period
n-3	Omega-3
n-6	Omega-6
MS	Mass spectrometry
*p*AV	*para*-Anisidine value
PCA	Principal component analysis
PG	Propyl gallate
POV	Peroxide value
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
TAG	Triacylglycerol
TBA	Thiobarbituric acid

TBHQ	*tert*-Butylhydroquinone
TV	Total volatiles
UV	Ultraviolet

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Abstract

α-Tocopherol (vitamin E) is generally the only tocopherol naturally present in oils from marine fish, and the tocopherol concentrations in refined fish oils are substantially lower than in most vegetable oils. To reduce the susceptibility of fish oils to oxidation and adverse flavour formation, further antioxidant protection is needed. In this work, the properties of α-tocopherol (αTOH), γ-tocopherol (γTOH) and δ-tocopherol (δTOH) in purified fish oil triacylglycerols (TAG) were studied over a wide concentration range and at an oil temperature close to ambient and in the dark. Purification of the substrate oils (menhaden oil and anchovy oil) by column chromatography removed the majority of the antioxidant and prooxidant molecules present and accordingly allowed better control over factors known to influence the autoxidation process.

The work presented demonstrates that the tocopherols affect many stages of the autoxidation process in purified fish oils, including the formation of hydroperoxides, the decomposition of hydroperoxides, the formation of nonvolatile secondary oxidation products, as well as the composition of volatile secondary oxidation products. In all the oxidation tests addressing these different aspects of lipid autoxidation, as well as in a study of the effect of the tocopherols on the oxidative degradation of astaxanthin, the results obtained could be related to the relative hydrogen-donating ability of the tocopherols, which decreases in the order of αTOH > γTOH > δTOH.

The number of methyl groups on the chroman ring of the tocopherols had a remarkable effect on their antioxidant properties in fish oil. The relative ability of the tocopherols to retard the formation of hydroperoxides in purified fish oil TAG decreased in the order of αTOH > γTOH > δTOH at a low initial level of addition (100 ppm), but a reverse order of activity was found when the initial tocopherol concentration was 500 ppm and above. This dependence of relative antioxidant activity on tocopherol concentration was caused by the existence of concentrations for maximum antioxidant activity for αTOH, and for γTOH. The removal of minor oil components reduced the rate of TAG autoxidation, but did not appear to affect the αTOH concentration for inversion of activity. Contrary to their effect on primary oxidation, all three tocopherols retarded the formation of volatile secondary oxidation products in a concentration-dependent manner. Principal component analysis suggested that high tocopherol hydrogen-donating power directs the formation of the more flavour-potent carbonyls, and, moreover, that the C3-aldehydes together (propanal + 2-propenal) are a more suitable marker of secondary oxidation of n-3 fatty acids than propanal alone. An important finding in this work was that the addition of ascorbyl palmitate to fish oil with αTOH reduced the initial rate of autoxidation, thereby eliminating the αTOH-mediated accumulation of hydroperoxides, only when the αTOH level was above that for maximum activity. Ascorbyl palmitate is known to regenerate αTOH from its tocopheroxyl radical. This emphasizes the importance of the αTO•, as opposed to αTOH itself, in side-reactions inducing hydroperoxide formation in fish oil.

1 Introduction

The consensus of evidence over the last two decades is that increasing the dietary intake of omega-3 (n-3) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), is beneficial in various human disorders including cardiovascular and inflammatory conditions. A related issue is the requirements of DHA for normal neural development in the human foetus and premature infants. Fatty fish and fish oils are rich sources of EPA and DHA.

Unfortunately, their degree of unsaturation also renders the PUFA in fish oil highly susceptible to oxidation, and lipid oxidation is the most critical parameter affecting the shelf life of fish oils and food products where marine lipids have been incorporated. Lipid autoxidation, a free radical chain reaction, leads to the formation of fatty acid hydroperoxides, which then decompose to a wide range of secondary oxidation products. Many of these are low molecular weight compounds with strong flavour attributes, imparting the characteristic and disagreeable sensation of rancid fish oil. Encapsulation in gelatin capsules is frequently used to make fish oils palatable. This may, however, give a false sense of security, as even encapsulated oils have been found to contain high levels of fatty acid oxidation products (Sagredos, 1991 and 1992; Shukla and Perkins, 1998).

The total world-wide consumption of fish oil is about 1.2 million metric tons (Barlow, 1997). Up until a few years ago, most of the oil was hydrogenated for use in margarines and shortenings. This use of fish oils has decreased, mainly due to the findings of possible adverse health effects from the intake of *trans* fatty acids. As many people do not eat fish regularly, dietary supplements of fish oil/n-3 concentrates have become popular. Pharmaceutical products based on n-3 fatty acids from fish oil have also been developed. However, n-3 retail products are estimated to account for < 1% of the world-wide fish oil consumption in 2001 (Barlow, 1997). The next step in the effort to increase the popular intake of n-3 PUFA is to substitute other fats and oils in different

food products with fish oils. This requires more effective control methods against lipid oxidation.

Several studies have shown harmful effects in animals from some of the most common synthetic food antioxidants (Fitch Haumann, 1990). Moreover, the general trend toward the use of all-natural ingredients in foods has caused renewed interest in naturally occurring antioxidants such as the tocopherols and ascorbic acid (vitamin C). Another aspect of lipid oxidation is *in vivo* peroxidation, which has been implicated in DNA and protein modification, and the deposition of arterial plaque associated with low-density lipoprotein modification (Porter *et al.*, 1995). An increased intake of PUFA increases the physiological requirements for antioxidants that are also effective *in vivo* (Muggli, 1994).

Fish oil is an important ingredient in aquaculture feeds. Due to the rapid growth in the aquaculture industry, this now constitutes a major area of application of fish oil. Recent years have seen the introduction of so-called "high energy" diets in salmon aquaculture. Such diets contain 30%-40% of the feed as oil and result in enhanced growth rates of the fish. This increases the susceptibility of the components of the feed, including the red pigment astaxanthin, to oxidative degradation, as well as the oxidative stress to the fish, and better antioxidant protection is necessary.

Vitamin E was discovered in the early 1920's by Evans and Bishop (1922) as a dietary factor in animal nutrition essential for normal reproduction. The name tocopherol was deduced from the Greek words "tokos" (childbirth) and "phorein" (to bring forth), and the suffix "-ol" was added to indicate the phenolic nature. The tocopherols are the most important natural antioxidants in fats and oils. They are radical chain-breaking antioxidants, i.e. they act as antioxidants mainly by scavenging lipid free radicals. The four tocopherols, α- (αTOH), β- (βTOH), γ- (γTOH) and δ-tocopherol (δTOH), differ only by the number and patterns of substitution of methyl groups on the phenolic ring. This small difference in molecular structure has, however, great impact on their

effectiveness as antioxidants both *in vivo* and *in vitro*. α-Tocopherol is the major lipid-soluble chain-breaking antioxidant in mammalian membranes (Burton and Ingold, 1989), and is according to the Merck Index (Budavari *et al.*, 1996) synonymous with vitamin E. The term vitamin E is, however, frequently used to include all the tocopherols, as well as the tocotrienols (e.g. Kamal-Eldin and Appelqvist, 1996; Scott, 1997; Azzi and Stocker, 2000). It is generally agreed that the relative order of biochemical antioxidant effect *in vivo* is αTOH > βTOH > γTOH > δTOH (Kamal-Eldin and Appelqvist, 1996), while no simple relationship appears to exist between the tocopherol content and substrate stability *in vitro*. The antioxidant effectiveness of αTOH *in vitro* has in many studies been found to be inferior to that of the other tocopherols, and sometimes a prooxidant effect has been observed (e.g. Cillard and Cillard, 1980; Terao and Matsushita, 1986; Jung and Min, 1990; Huang *et al.*, 1994 and 1995). This may be due to the participation of αTOH or its tocopheroxyl radical (αTO•) in reactions other than with lipid peroxyl radicals (Marinova and Yanishlieva, 1992a; Kamal-Eldin and Appelqvist, 1996; Fuster *et al.*, 1998), but the relative importance of these side-reactions remains to be assessed.

The effectiveness of antioxidants in retarding autoxidation in fish oils is of relative recent interest compared to the wide range of reports on the properties of antioxidants in vegetable oils, lard and tallow. Unlike in most vegetable oils, αTOH is generally the only tocopherol naturally present in oils from wild fish (Ackman and Cormier, 1967). The level of αTOH in fish oils is substantially reduced by commercial refining and deodorization, generally to below 100 ppm, and for food and health-supplement use relatively high levels of natural antioxidants, often several thousand ppm αTOH or mixed tocopherols, are added in order to protect the product oil. However, the properties of the different tocopherol homologues in fish oil have not been adequately studied, and findings in vegetable oils (Huang *et al.*, 1994 and 1995; Fuster *et al.*, 1998; Lampi *et al.*, 1999) have indicated that the amount of tocopherol added to commercial fish oil products may be too high for optimum antioxidant protection.

1.1 Thesis Objectives

The general objectives of this research project were to:

1. Study the effect of αTOH on the rate of formation of hydroperoxides in fish oil, and to determine if minor fish oil components interact with αTOH to change its effectiveness as an antioxidant.

2. Determine the concentrations for maximum antioxidant activity of αTOH, γTOH and δTOH in fish oil on the basis of formation of both hydroperoxides and volatile secondary oxidation products.

3. Test the hypothesis that the difference in hydrogen-donating power of the tocopherols affects the composition of volatile secondary oxidation products in oxidizing fish oil.

4. Study the effects of astaxanthin on the rate of autoxidation in fish oil, as well as to examine the ability of the tocopherols to protect astaxanthin from oxidative degradation, aiming at increasing the stability of both PUFA and pigment in aquaculture feeds and in salmon oil.

2 Literature Review

2.1 Fish Oils

Marine oils originate from the bodies of fatty fish, the livers of lean fish and the blubber layers of marine mammals such as seal and whale. Commercial fish oils are mainly produced from sardine/pilchard, anchovy, menhaden, horse mackerel, sand launce, capelin, herring and cod liver. Production of fish oil involves heating of the fish to denature proteins and liberate water and oil, pressing to separate the liquid part of the fish mass from the protein, and centrifugation to separate the oil from the so-called stickwater (Ackman, 1994). If intended for human consumption, the crude fish oil is generally, as are most vegetable oils, refined, bleached and steam-deodorized (Bimbo, 1990). Alkali refining removes phospholipids, free fatty acids, oil insolubles and some coloured bodies. A bleaching step with an activated clay filter is for removal of pigment and oxidation products. Deodorization involves vacuum steam-stripping at a high temperature to remove free fatty acids, aroma compounds and other volatiles. By employing proper conditions, an oil with very little odour and taste can be obtained by this process.

A refined and deodorized fish oil consists mainly of triacylglycerols. These have a very complex fatty acid composition, but 8 fatty acids usually dominate. The principal fatty acids of some commercial fish oils are shown in Table 2.1 (Bimbo, 1990). The ethylenic bonds of unsaturated fatty acids normally have the *cis*-configuration. The concentrations of EPA and DHA (molecular structures shown in Fig. 2.1) in fish oils differ significantly among species. Within species variations also occur, reflecting geographical factors, season of catch, nutritional status and so forth. Minor refined fish oil components may include cholesterol/cholesterol esters, αTOH, free fatty acids, mono- and diacylglycerols, phospholipids, hydrocarbons, wax esters, alcohols, as well as oxidation products not removed in the refining and deodorization processes.

Table 2.1. Principal fatty acids of some commercial fish oils (Adapted from Bimbo, 1990).

Fatty acid[a]	Menhaden (% w/w)	Sardine (% w/w)	Anchovy (% w/w)	Cod liver (% w/w)	Capelin (% w/w)	Herring (% w/w)
C14:0	9	8	9	3	7	7
C16:0	20	18	19	13	10	16
C16:1 n-7	12	10	9	10	10	6
C18:1[b]	11	13	13	23	14	13
C20:1 n-9	1	4	5	0	17	13
C22:1 n-11[c]	Trace	3	2	6	14	20
C20:5 n-3	14	18	17	11	8	5
C22:6 n-3	8	9	9	12	6	6

[a]The shorthand notation refers to the carbon chain length, the number of double bonds and the position of the first double bond from the hydrocarbon end of the molecule
[b]25% C18:1 n-7, 75% C18:1 n-9
[c]Also accompanied by other isomers of C22:1

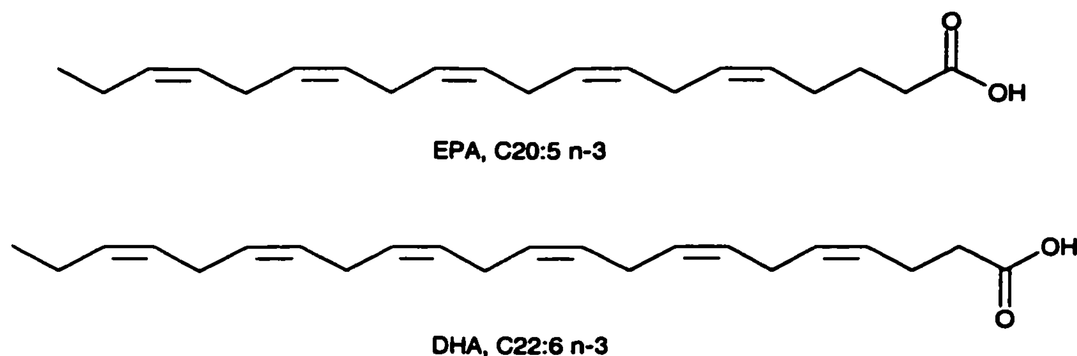


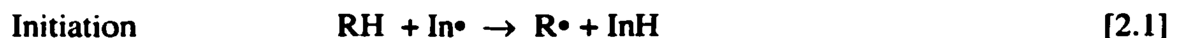
Figure 2.1. Structures of EPA and DHA.

2.2 Lipid Autoxidation

Formation of hydroperoxides in systems of unsaturated fatty acids may take place according to three different oxidation processes: autoxidation, photooxidation and enzymatic oxidation. Autoxidation is the spontaneous free radical reaction of organic compounds with oxygen. Lipid photooxidation is an oxidation process resulting from exposure of unsaturated fatty acids to light in the presence of a photosensitizer (e.g. chlorophyll, haem compounds). The singlet oxygen generated is able to add directly to a double bond and form a hydroperoxide without the involvement of free radicals. Enzymatic oxidation is important in animal and plant systems. In this process, enzymes like lipoxygenase catalyse the formation of hydroperoxides from unsaturated fatty acids. As enzymes are generally deactivated by heat during refining of fish oils, only autoxidation is considered important in this work.

2.2.1 General Mechanism of Lipid Autoxidation

The mechanism of lipid autoxidation has been extensively reviewed (Chan, 1987; Porter *et al.*, 1995; Frankel, 1998). It is well established that autoxidation is a free radical chain process and like other radical chain processes it can be described in terms of the initiation, propagation, and termination steps [2.1 - 2.4]. RH refers to a fatty acid or fatty acid ester and In• is an initiator of autoxidation.



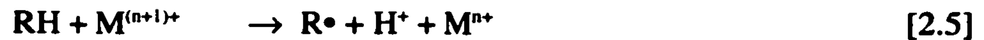
Initiation $RH + In\bullet \rightarrow R\bullet + InH$ [2.1]

Propagation $R\bullet + O_2 \rightarrow ROO\bullet$ [2.2]

$ROO\bullet + RH \rightarrow ROOH + R\bullet$ [2.3]

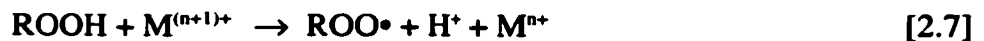
Termination $2ROO\bullet \rightarrow [ROOOOR] \rightarrow \text{nonradical products} + O_2$ [2.4]

Initiation is the process that is the most difficult to define, because of the very small concentration of radicals involved and the likelihood of there being more than one process. For example, In• may illustrate a transition metal ion, it may be a radical generated by photolysis or high-energy irradiation, or a radical, e.g. RO•, obtained by decomposition of a hydroperoxide. According to Chan (1987) the most probable initiation processes in autoxidation of unsaturated lipids are the metal catalysed reactions [2.5], [2.6] and [2.7], where M represents a transition metal. Transition metals possessing two or more valence states with a suitable oxidation-reduction potential between them (e.g. Cu, Fe, Mn and Co) are considered strong prooxidants (Gordon, 1990). Reaction [2.6] is generally much faster than reaction [2.7] (Chan, 1987).



$$RH + M^{(n+1)+} \rightarrow R\bullet + H^+ + M^{n+} \quad [2.5]$$

$$ROOH + M^{n+} \rightarrow RO\bullet + OH^- + M^{(n+1)+} \quad [2.6]$$



$$ROOH + M^{(n+1)+} \rightarrow ROO\bullet + H^+ + M^{n+} \quad [2.7]$$

Once a lipid radical is formed, it reacts very quickly with molecular oxygen to form the peroxyl radical ROO• [2.2]. The second, and rate-limiting, step of propagation is abstraction of a hydrogen atom from another fatty acid by ROO• to generate a hydroperoxide and a new fatty acid radical [2.3]. However, propagation steps may be more complicated than [2.2] and [2.3], and reactions like fragmentation, rearrangement and cyclization have also been observed in the autoxidation process (Porter *et al.*, 1995). At the later stages of oxidation the peroxyl radicals react with each other to form non-radical products via an unstable tetroxide intermediate [2.4].

The rate of fatty acid autoxidation is influenced by a number of factors, including the amount of oxygen present, the degree of unsaturation of the substrate, the presence of antioxidants and prooxidants (especially copper and iron), the storage temperature and exposure to light. Molecular oxygen reacts with the carbon-centered free radical R• at or near the diffusion controlled rate of 10^9 $M^{-1}s^{-1}$ (Porter *et al.*, 1995). At oxygen pressures

above 100 torr, the reaction of alkyl radicals R• with oxygen is thus very rapid and the concentration of ROO• is much greater than R•. The rate of autoxidation becomes independent of oxygen pressure and is given by equation [2.8] (Porter *et al.*, 1995; Frankel, 1998). The k_p and k_t are the propagation and termination rate constants, respectively, and R_i is the rate of radical initiation. Equation [2.8] is considered valid at early stages of oxidation only, at lower temperatures, and for fatty acids with less than three double bonds (Frankel, 1998). Under these conditions the decomposition of hydroperoxides, which greatly complicates the kinetics of lipid oxidation, is considered insignificant.

$$d[ROOH]/dt = k_p\,(R_i/2k_t)^{1/2}[RH] \qquad [2.8]$$

The rate constant, k_p, for the rate-limiting step of propagation [2.3] depends primarily on the strength of the C-H bond being broken. The bond dissociation energy for an allylic hydrogen is approximately 10 kcal/mol greater than that of a bisallylic hydrogen (Porter *et al.*, 1995). As a result, reasonable rates for the autoxidation of monounsaturated fatty acids, such as oleic acid, can only be achieved at elevated temperatures, while the oxidation of PUFA occurs readily at room temperature. The rate of oxidation of PUFA increases approximately two-fold for each active bisallylic methylene group, and the reactivity of C22:6 is thus about 5 times greater than that of C18:2 (Frankel, 1998).

2.2.2 Primary Oxidation Products

The autoxidation of polyunsaturated fatty acids results in the formation of a complex mixture of hydroperoxides, called primary oxidation products, which differ both in the position of the HOO-group and the geometrical isomerism of the double bonds. The autoxidation of linoleic acid (C18:2 n-6) and its esters has been extensively studied (Porter *et al.*, 1980; Porter *et al.*, 1981; Chan and Coxon, 1987) and may serve as a simple model for the autoxidation of unsaturated fatty acids containing more than two methylene-interrupted double bonds. Abstraction of a bisallylic hydrogen at C-11 results in the

formation of a pentadienyl radical and subsequent oxygenation gives rise to four major products (Fig. 2.2). These conjugated diene hydroperoxides result from oxygen addition at either the 9 or 13 position. Two of the products have *cis, trans* geometry in the conjugated diene unit while the other two display *trans, trans* geometry. Accordingly, autoxidation of linolenic acid (C18:3 n-3), where the double bond system may be considered as two separate 1,4-diene systems, gives rise to 8 isomers (*cis, trans* and *trans, trans* of 4 positional isomers). Based on this, a complex system of 16 monohydroperoxide isomers may be expected from the autoxidation of EPA (C20:5 n-3).

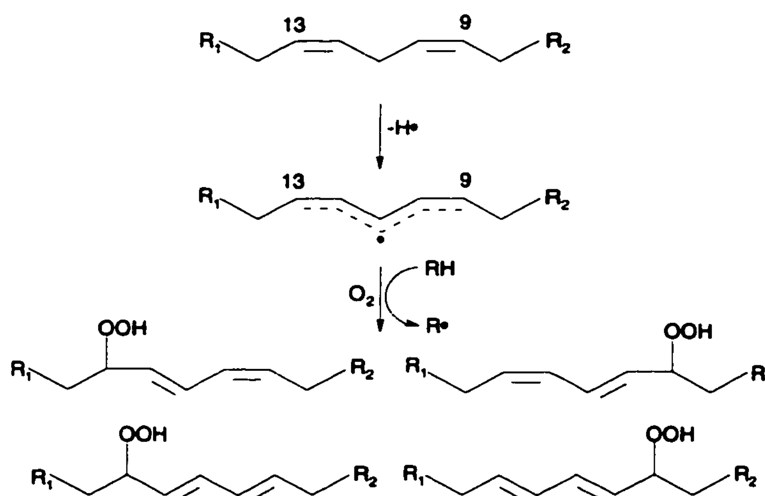
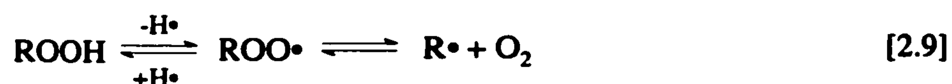


Figure 2.2. Formation of linoleic acid hydroperoxides.
$R_1 = (CH_2)_3CH_3$, $R_2 = (CH_2)_6COOH$, RH = fatty acid.

A hydroperoxide can reversibly lose a hydrogen radical and eliminate oxygen from the peroxyl radical intermediate (β-fragmentation) (Porter *et al.*, 1981; Chan *et al.*, 1982):



$$ROOH \underset{+H\bullet}{\overset{-H\bullet}{\rightleftharpoons}} ROO\bullet \rightleftharpoons R\bullet + O_2 \quad [2.9]$$

This β-fragmentation mechanism explains the formation of hydroperoxides with *trans, trans* geometry. Oxygen addition to a pentadienyl radical in the propagation step [2.3] generates a *cis, trans* peroxyl radical, which may abstract a hydrogen from a donor to generate the *cis, trans* hydroperoxide. Bond rotation and β-fragmentation of the *cis, trans* peroxyl radical yields a new pentadienyl radical, and subsequent oxygen addition gives the *trans, trans* hydroperoxide. The presence of reactive hydrogen donors such as αTOH favours the formation of the *cis, trans* isomer (Chan *et al.*, 1982).

2.2.3 Secondary Oxidation Products

Hydroperoxides of unsaturated lipids are unstable, particularly at high temperatures and in the presence of transition metal ions, and decompose to a wide variety of volatile flavour compounds, and oxygenated compounds of the same chain length, as well as oxidation products of higher molecular weight (Fig. 2.3). The formation of secondary oxidation products proceeds via alkoxyl radical (RO•) and peroxyl radical (ROO•) intermediates (Grosch, 1987; Frankel, 1998). Thermal decomposition of hydroperoxides involves homolytic cleavage of the hydroperoxide group to yield an alkoxyl radical and a hydroxyl radical (Grosch, 1987). At moderate temperatures (< 40°C), the presence of reagents that can attack the HOO-group, like transition metal ions and haem compounds, becomes important. Reactions [2.6] and [2.7] show how transition metal ions catalyse the formation of alkoxyl and peroxyl radicals by the degradation of hydroperoxides. The formation of alkoxyl radicals from hydroperoxides is an irreversible reaction, while the peroxyl radicals may revert to hydroperoxides [2.9] (Gardner, 1987).

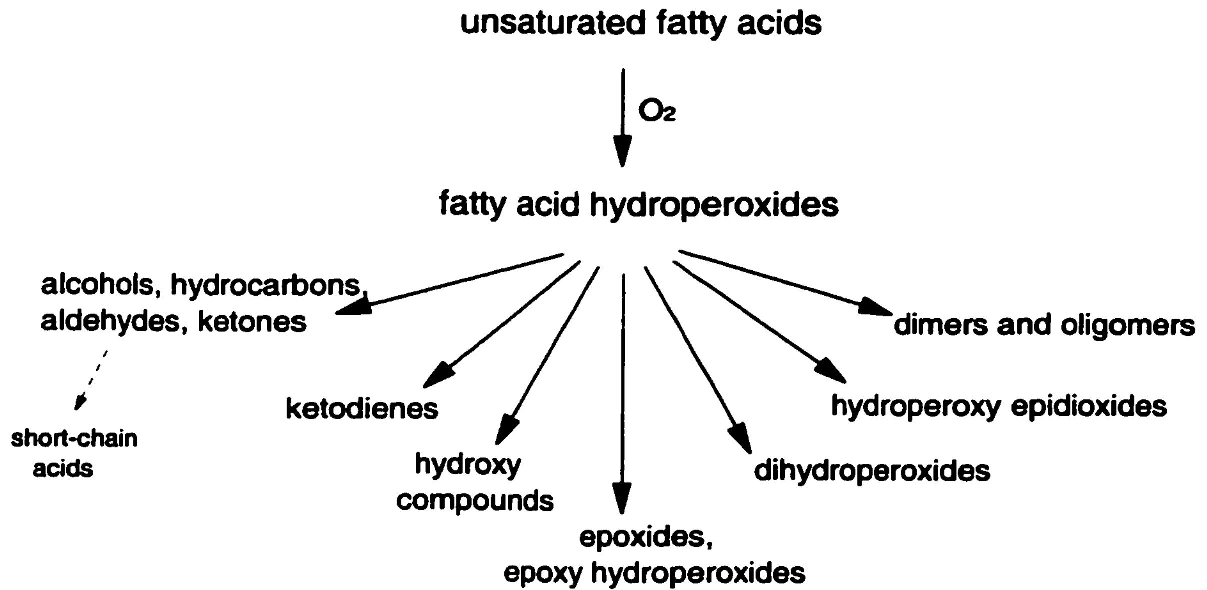


Figure 2.3. Major classes of secondary lipid oxidation products.

Volatile secondary oxidation products from lipid hydroperoxides include aldehydes (saturated, mono-, di- and triunsaturated), hydrocarbons, alcohols, ketones and furans. Lipid hydroperoxides are themselves essentially without any odour and taste, but some of the volatile oxidation products, particularly the carbonyls, are powerful flavour compounds with very low flavour threshold values. Common flavour characteristics of individual carbonyls formed from autoxidation of lipids include green, cucumber-like, sharp, painty, beany, rancid and fishy (Grosch, 1987). It is generally accepted that the volatile secondary oxidation products are generated via fatty acid alkoxyl radicals according to the mechanism presented in Figure 2.4 (Grosch, 1987; Frankel, 1998). The alkoxyl radical is cleaved by homolytic β-scission of the carbon-carbon bond on either side of the carbon carrying the oxygen. Scission A results in the formation of an unsaturated aldehyde and an alkyl radical. The alkyl radical can either react with a hydroxyl radical to form an alcohol or abstract a hydrogen from an unsaturated fatty acid. A new and shorter chain primary hydroperoxide may also form. By scission of the

carbon-carbon bond between the oxygen bearing carbon and the double bond (scission B), a vinyl radical and an aldehyde are formed. The vinyl radical can either react with a hydroxyl radical and form a 1-enol, which tautomerizes to the corresponding aldehyde, or abstract a hydrogen from an unsaturated fatty acid. Short-chain fatty acids can be produced by further oxidation of aldehydes.

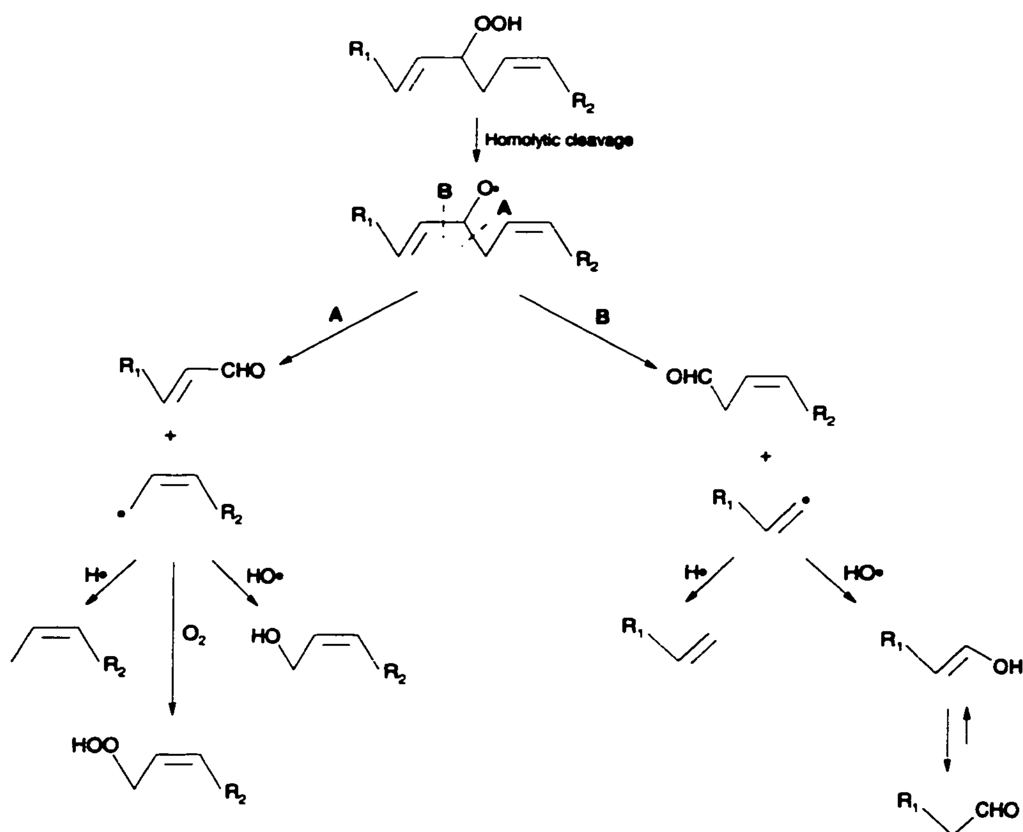


Figure 2.4. Reaction scheme for the homolytic cleavage of monohydroperoxides and the formation of volatile secondary oxidation products (Adapted from Frankel, 1998).

Many studies have been carried out on the formation of volatile oxidation products from specific fatty acids and their methyl esters (Badings, 1970; Frankel, 1982; Grosch, 1987; Rørbæk, 1994; Frankel, 1998). Many of the volatiles identified in these studies can be

formed either by fragmentation of monohydroperoxides according to Fig. 2.4 or by further oxidation of secondary oxidation products. Reaction pathways for individual volatiles may thus be difficult to elucidate.

Alkoxyl radicals undergo several competing reactions to the β-scission pathway illustrated in Figure 2.4, and the short-chain cleavage products generally account for less than 10% of the total end products (Gardner, 1987). Alkoxyl radicals may abstract a hydrogen atom from an unsaturated fatty acid to continue the radical chain reaction or from an antioxidant. In both cases, a stable alcohol is formed. Epoxy hydroxy and epoxy hydroperoxy compounds, formed by intramolecular alkoxyl radical addition to a neighbouring double bond, have been identified as secondary oxidation products of methyl linoleate and methyl linolenate (Frankel, 1998). The conversion of lipid hydroperoxides to the corresponding lipid ketones usually accounts for a relatively large proportion of end products (Gardner, 1987). Several mechanisms for the formation of ketodienes have been proposed, involving both alkoxyl and peroxyl radical intermediates.

Peroxyl radical cyclization is an important process in the autoxidation of fatty acids or esters having three or more methylene-interrupted double bonds (Neff *et al.*, 1981; Porter *et al.*, 1981; Chan *et al.*, 1982). Intramolecular peroxyl radical addition to a *cis* homoallylic double bond and subsequent reaction with oxygen yields 5-membered hydroperoxy epidioxides as oxidation products. Peroxyl, alkoxyl and alkyl radicals may also combine to form peroxide linked, ether linked or carbon-carbon linked dimers and oligomers.

The nature of the lipid substrate (e.g. degree of unsaturation, presence of antioxidants/prooxidants) and the reaction conditions (e.g. temperature and availability of oxygen) has a great impact not only on the overall rate of autoxidation, but also the relative rates of formation of the different classes of oxidation products. As already mentioned, peroxyl radical cyclization is a competing reaction to hydrogen abstraction

only in the autoxidation of fatty acids with three or more double bonds, and this pathway explains the uneven distribution of positional isomers of hydroperoxides of linolenate (Neff *et al.*, 1981). The presence of a strong hydrogen donor was found to substantially reduce the formation of hydroperoxy epidioxides of methyl linolenate relative to that of monohydroperoxides, as well as the ratio of *trans*, *trans* to *cis*, *trans* hydroperoxides, due to rapid scavenging of the initially formed *cis*, *trans* peroxyl radical (Peers *et al.*, 1981; Chan *et al.*, 1982).

The temperature influences the decomposition routes of the hydroperoxides, and the homolytic cleavage to alkoxyl and hydroxyl radicals becomes relatively more important at elevated temperatures (Grosch, 1987). Porter *et al.* (1980) found that the ratio of *cis*, *trans* to *trans*, *trans* hydroperoxides of linoleic acid decreased when the temperature was increased from 10°C to 50°C. The temperature also affects the radical termination reactions forming dimers and oligomers. Due to a reduction in the solubility of oxygen, as well as an increased rate of peroxide decomposition, carbon-carbon linked and ether linked dimers are favoured at elevated temperatures, while peroxide linked dimers predominate at ambient temperatures (Frankel, 1998). The formation of dimers and oligomers is of particular significance in vegetable oils at the elevated temperatures of frying and deodorization, when the hydroperoxides are rapidly decomposed.

2.3 Methods to Assess Quality and Stability of Fats and Oils

2.3.1 Assessment of Lipid Oxidation

The measurement of lipid oxidation is complicated by the fact that it involves different reaction stages with different reaction products, and that it is a dynamic process where the composition of oxidation products is continuously changing. Sensory evaluation is very important in the assessment of oil quality, however, it is time consuming, non-specific and it is difficult to set quality standards. Also, the primary oxidation products and the higher molecular weight secondary oxidation products are not themselves flavour compounds, but they are very important for oil stability. A number of different chemical

and physical methods have been developed for the quality evaluation of fats and oils. The most commonly used are briefly described in this section. The methods are limited to the detection of a certain class of oxidation products only. For the assessment of the oxidative state of an oil, more than one analytical method should therefore be employed to at least determine one class of secondary oxidation products in addition to the level of hydroperoxides.

The determination of the peroxide value (POV) is the most common method to evaluate the oxidative state of an oil. The POV is the quantity of peroxide oxygen present in an oil sample, expressed in milliequivalents per kg sample (Hahm and Min, 1995). The widely used iodometric titration method for the determination of POV is based on the measurement of the iodine produced from potassium iodide by the hydroperoxides present in the oil. Although widely accepted, the results of POV determinations vary according to the procedures used. The two principal sources of error are the absorption of iodine at fatty acid double bonds and the liberation of iodine from potassium iodide by oxygen present in the sample solution (Hahm and Min, 1995).

Formation of lipid hydroperoxides leads to double bond displacement to form conjugated diene (CD) hydroperoxides. This conjugated structure absorbs strongly at a 232-234 nm (White, 1995). A CD value is reported as the percentage of conjugated dienoic acid in the oil or as the sample extinction coefficient ($E^{1\%}_{1cm}$). The CD method offers several advantages over the POV method in that it is faster, simpler, requires no chemical reagents, does not depend on a chemical reaction or colour development, and can be conducted on small (< 100 mg) sample sizes. As the magnitude of the CD value depends on the fatty acid composition of an oil, the CD values cannot be easily compared from one type of oil to another. Individual hydroperoxides may also be separated and quantified by high-performance liquid chromatography (HPLC). This method is mainly used when studying model systems limited to one or two fatty acids.

The *p*-anisidine value (*p*AV) is a common method for the determination of secondary oxidation products in an oil. The *p*AV is mainly a measure of 2-alkenals, which under acidic conditions form Schiff base compounds creating a yellowish colour measurable at 350 nm (White, 1995). The *p*AV procedure is quick and simple, but lacks sensitivity. The *p*AV is often used in conjunction with the POV to calculate the so-called total oxidation value, or totox value, equal to twice the POV plus the *p*AV.

The thiobarbituric acid (TBA) test is another method that relates to the level of aldehydes present in an oil. The TBA reacts with malondialdehyde to give a red chromogen, which may then be determined spectrophotometrically (Rossel, 1991). The test has the advantage that it can be carried out on whole foods, and it is commonly used despite of wide criticism for lack of accuracy. Other secondary oxidation products like hydroxy compounds, ketodienes and hydroperoxy epidioxides may be analysed by HPLC. As for the HPLC determination of individual hydroperoxides, such analyses requires simple lipid model systems. Dimers and oligomers can be separated from smaller lipid components by size-exclusion chromatography.

The compounds responsible for the flavour of oxidized lipids have in common that they are more or less volatile, and the analysis of volatile components often provides the most useful information related to consumer acceptance. The most widely used methods for determination of volatiles are static and dynamic headspace analyses. Static headspace analysis is a very simple method based on transferring a certain volume of gas from the headspace above a lipid sample and injecting this gaseous sample directly into a gas chromatograph (GC). Proper sampling can only be performed when equilibrium is reached, and the equilibration time depends on the temperature, the viscosity of the sample and the solubility of the volatiles in the oil (Przybylski and Eskin, 1995). At relatively low temperatures (40°C) compounds like pentane, hexane, propanal, pentenal and hexanal in an oil can be analysed by static headspace (Frankel, 1993a). Equilibration at higher temperatures improves the liberation of the higher molecular weight volatiles

but also implies increased degradation of the hydroperoxides and the labile secondary oxidation products. Dynamic headspace analysis involves a concentration step for improved sensitivity. A continuous stream of inert gas is passed above the surface (purging) or bubbled through the sample (stripping) and the volatiles are collected in a trap, based on either adsorption or cold-trapping. The trapped volatiles are liberated by solvent or thermal desorption and analysed by GC. Dynamic headspace analysis allows for the determination of less volatile secondary oxidation products.

2.3.2 Estimation of Oil Stability

The rate of lipid oxidation can be estimated by measuring the loss of substrate (fatty acid or O_2) or the formation of oxidation products. The stability, or susceptibility to oxidation, of different oil samples are then often evaluated by comparison of their induction periods (IP), which is the time required to reach a sudden increase in the rate of oxidation, or by the use of another suitable end-point of oxidation.

The measurement of the loss of the lipid substrate is fairly straightforward, particularly when studying model systems of fatty acid methyl or ethyl esters. It is not commonly used for bulk oils as it requires a transesterification step before the GC analysis and also lacks sensitivity. The weight-gain method, based on an increase in the weight of an oil sample due to oxygen absorption, is, although not very sensitive, widely accepted (Frankel, 1998). Measurements of the depletion of headspace oxygen over a lipid sample by GC analysis is also common (Hahm and Min, 1995). Stability tests may also be carried out by periodically determining the concentration of oxidation products by one, or preferably several, of the methods described in the previous section or by sensory analysis.

To estimate the susceptibility of an oil to oxidation, it is often subjected to an accelerated test where commonly temperature and/or oxygen pressure is elevated to increase the rate of oxidation. Common high temperature tests include oxygen absorption tests, the Schaal

oven test, the Active Oxygen Method and the Rancimat™/Oil Stability Index metods (Shahidi and Wanasundara, 1997). These are all rapid and simple but may be unreliable as the mechanism of lipid oxidation is known to change at elevated temperatures (Frankel, 1993b). The order of activity of different antioxidants has also been found to depend on whether they were tested at high or low temperatures (Frankel, 1993b), and antioxidants should therefore be evaluated at temperatures close to those of the real storage conditions. As fish oils oxidize rapidly, stability tests may be performed within a reasonable time period (days to weeks) at temperatures close to ambient.

2.4 Protection of Lipids by Antioxidants

Antioxidants are regarded as compounds capable of retarding lipid oxidation processes. They may be classified into two groups, namely primary or chain-breaking antioxidants, which react with lipid radicals to convert them to more stable products, and secondary or preventive antioxidants which retard the rate of oxidation by processes other than interruption of the autoxidation propagation step. A molecule may act as a primary antioxidant if it is able to donate a hydrogen atom rapidly to a lipid radical and if the radical derived from the antioxidant is more stable than the lipid radical, or is converted to other stable products. Primary antioxidants (AH) thus interfere with lipid autoxidation chiefly by competing with the propagation reaction [2.3]. By reaction with a lipid peroxyl radical (ROO•), a lipid hydroperoxide (ROOH) and a less active antioxidant radical (A•) are formed:

$$ROO\bullet + AH \rightarrow ROOH + A\bullet \qquad [2.10]$$

Alkoxyl radicals can participate in an analogous way:

$$RO\bullet + AH \rightarrow ROH + A\bullet \qquad [2.11]$$

Most chain-breaking antioxidants are phenolic compounds. The radical formed in the reaction of a phenol with a lipid radical is stabilized by delocalization of the unpaired electron around the aromatic ring.

The tocopherols are the most important natural antioxidants in fats and oils. They occur as homologues (α, β, γ and δ) with varying extent and patterns of methylation of a chroman ring having a saturated phytyl side chain (Fig. 2.5). The phytyl tail has three chiral centers (position 2, 4′ and 8′), making a total of 8 stereoisomeric forms possible. All naturally occurring tocopherols have the same molecular configuration: RRR or *d*-tocopherols. The tocotrienols have the same chroman heads as the corresponding tocopherols but contain three isolated double bonds in their phytyl tails.

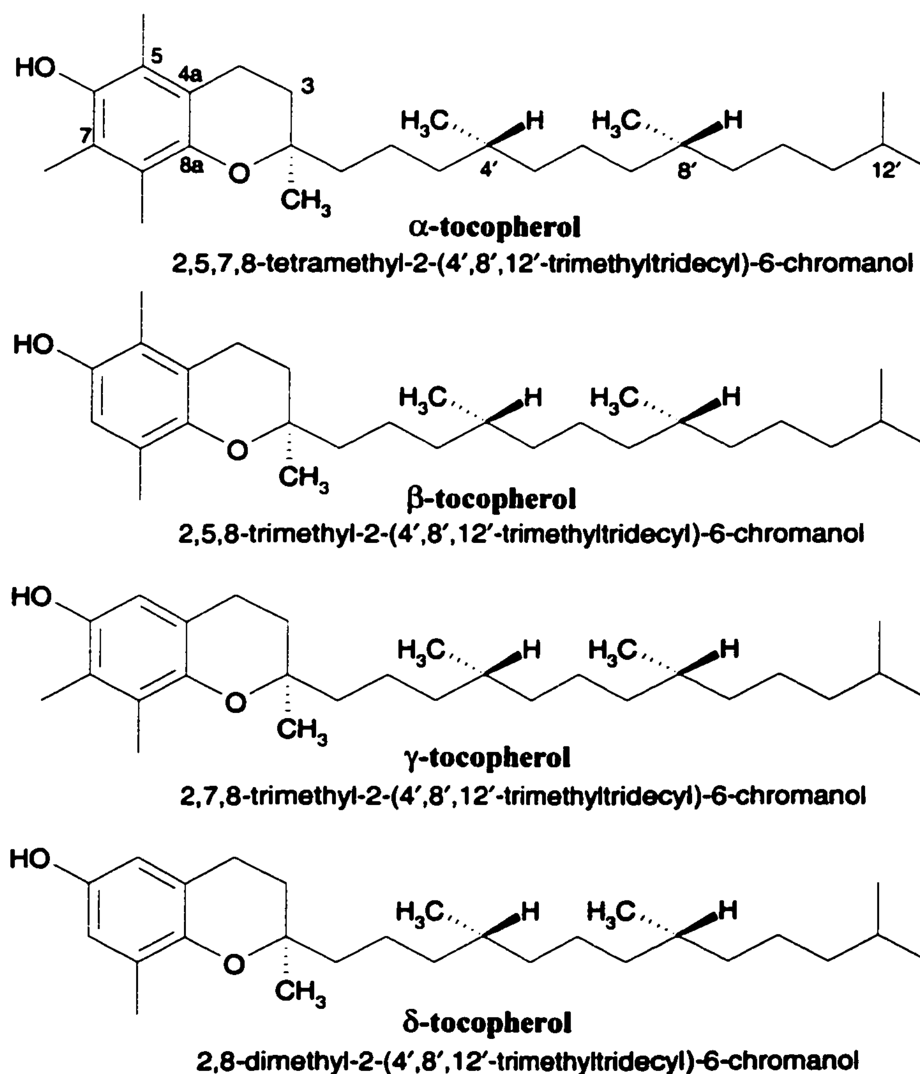


Figure 2.5. Structures of α-, β-, γ- and δTOH.

The tocopherols are present in oilseeds, leaves, and other green parts of higher plants (Kamal-Eldin and Appelqvist, 1996), and vegetable oils therefore contain considerable amounts of these antioxidants (Table 2.2). With the exception of palm oil, rice bran oil and wheat germ oil, tocotrienols are much less abundant (Schuler, 1990), and the antioxidant activity of the tocotrienols has not been fully established. Some of the tocopherol present in unrefined vegetable oils decomposes during processing, and the loss of α-tocopherol in soybean oil was found to be 4.3% after refining, 15% after bleaching and 20-51% after deodorization (Frankel, 1996). The βTOH is often either not found or present at very low levels in vegetable oils.

Table 2.2. Approximate content of tocopherol in some vegetable oils (Adapted from Schuler, 1990).

Oil	**Tocopherol (mg/kg)**			
	αTOH	βTOH	γTOH	δTOH
Olive	1-240	0	0	0
Palm	180-260	Trace	320	70
Rapeseed/canola	180-280	-	380-590	10-20
Soybean	30-120	0-20	250-930	50-450
Sunflower	350-700	20-40	10-50	1-10

Tocopherol concentrations in animal fats are generally much lower than in vegetable oils. Since fish do not synthesize tocopherol, the levels in fish oil are directly related to the fish's diet and vary with geographical location, season and sexual maturity (Ackman and Cormier, 1967). α-Tocopherol is generally the only tocopherol homologue naturally present in marine fish (Ackman and Cormier, 1967; Aminullah Bhuiyan *et al.*, 1993; Hemre *et al.*, 1997), and thus also in commercial fish oils. Its concentration is substantially reduced by commercial refining and deodorization. The concentration of αTOH determined in samples of five commercial fish oils are given in Table 2.3 (Kinsella, 1987). As oilseed meals are now commonly added to commercial aquaculture

feeds, farmed fish may have somewhat higher levels of tocopherols other than αTOH. In a study of the distribution of tocopherols in Atlantic salmon, Parazo *et al.* (1998) found that αTOH accumulated to a greater extent in the critical phospholipid-rich tissues compared to γTOH, which was preferentially incorporated into the adipose tissues.

Table 2.3. α-Tocopherol found in some commercial fish oils (Adapted from Kinsella, 1987).

Fish oil	αTOH (mg/kg)
Anchovy	291
Capelin	140
Cod liver	220
Menhaden	75
Herring	92

Other naturally occurring phenolic compounds that have antioxidant properties include flavonoids, phenolic acids (e.g. coumaric acid, ferulic acid, caffeic acid), sesamol (from sesame oil) and catechins (from tea leaves). Spices have been studied as a source of antioxidants by many authors, and rosemary and sage extracts are particularly potent (Shahidi and Wanasundara, 1992). Antioxidant extracts from rosemary and sage are commercially available. The phenolic antioxidants are the most active antioxidants from natural sources and as a consequence, most synthetic antioxidants are substituted phenols. Common synthetic antioxidants are propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ). The U.S. Food and Drug Administration generally allow PG, BHA, BHT and TBHQ or a combination to be used at a level not above 200 ppm (Fitch Haumann, 1990).

An important group of secondary antioxidants are the chelating agents which include ethylenediaminetetraacetic acid (EDTA), citric acid and phosphoric acid. Metal chelators act as antioxidants by complexing metal ions and thus retarding free radical formation and

hydroperoxide decomposition. Ascorbic acid (vitamin C) and its lipophilic derivative ascorbyl palmitate are commonly used in stabilizing fatty foods. They are known to act synergistically with αTOH by reducing the tocopheroxyl radical intermediate and thereby regenerate the tocopherol (Lambelet *et al.*, 1985; Frankel, 1996). The multiple effects of ascorbic acid also include metal inactivation, hydroperoxide reduction to produce stable alcohols, and oxygen scavenging in aqueous systems (Frankel, 1996). Secondary antioxidant effects of phospholipids have been ascribed to their metal chelating character (Gordon, 1990). Phospholipids may also act by releasing protons and bring about rapid decomposition of hydroperoxides without the formation of free radicals (Gordon, 1990). An alternative mechanism may involve the regeneration of primary antioxidants by hydrogen donation (Totani, 1997).

2.4.1 Antioxidant Properties of Tocopherols

General chemistry of the tocopherols

The tocopherols react with lipid radicals mainly according to reactions [2.12] and [2.13]. The resonance-stabilized tocopheroxyl radical formed upon transfer of the phenolic hydrogen to a peroxyl radical combines with a second peroxyl radical, thus yielding an antioxidant stoichiometric factor of two (Burton and Ingold, 1981; Liebler and Burr, 1995).

$$ROO\bullet + TOH \rightarrow ROOH + TO\bullet \quad [2.12]$$

$$ROO\bullet + TO\bullet \rightarrow \text{nonradical products} \quad [2.13]$$

The tocopherols are all excellent hydrogen donors. Their reactivity with peroxyl radicals is controlled by the fused 6-membered heterocyclic ring, which confers additional stabilization to the radical formed compared to that of a simpler phenoxyl radical through an interaction between the p-orbitals on the two *para*-oxygen atoms (Burton *et al.*, 1983; Burton *et al.*, 1985). Differences in the relative activities of the tocopherols are related to the number and position of the methyl groups on the phenolic ring. The presence of

electron-releasing substituents in positions *ortho* and/or *para* to the hydroxyl group of a phenol facilitates rapid hydrogen donation to peroxyl radicals and increases the stability of the phenoxyl radical (Pokorný, 1987). The fully methylated αTOH is therefore structurally expected to be the most potent tocopherol homologue. Accordingly, the reactivity of the tocopherols with peroxyl radicals was found to decrease in the order αTOH > βTOH ≈ γTOH > δTOH (Table 2.4; Burton and Ingold, 1981; Niki *et al.*, 1986).

Table 2.4. Relative tocopherol activity.

Tocopherol homologue	Relative reactivity with peroxyl radicals[a] (%)	Relative biological activity[b] (%)
αTOH	100	100
βTOH	71	50
γTOH	68	10
δTOH	28	3

[a]Determined by Burton and Ingold (1981) for the oxidation of styrene in chlorobenzene solution
[b]Kamal-Eldin and Appelqvist (1996)

αTOH is one of the most reactive phenolic antioxidants known (Burton and Ingold, 1981; Burton *et al.*, 1983), and its rate constant for the reaction with peroxyl radicals is about 250 times that of the well-known synthetic antioxidant BHT (Burton and Ingold, 1989). Not surprisingly, considering its peroxyl radical scavenging ability, αTOH is the major lipid-soluble chain-breaking antioxidant in mammalian membranes (Burton and Ingold, 1989; Kamal-Eldin and Appelqvist, 1996). The vitamin E activity of the tocopherols (Table 2.4) is governed not only by their chemical reactivities but also by their bioavailability, and there is a selective distribution of the natural RRR-αTOH to tissues and membranes. This transfer mechanism has been found to be mediated by the αTOH transfer protein (α-TTP) expressed in the liver. Although not important for the rate of reaction with peroxyl radicals *in vitro*, the phytyl tail is essential for retention of the

tocopherols in cell membranes (Burton and Ingold, 1989). The tocopherols also inhibit photosensitized oxidation, predominately by physical quenching of singlet oxygen (Kamal-Eldin and Appelqvist, 1996). Their relative quenching efficiency is αTOH > βTOH > γTOH > δTOH.

Tocopherol oxidation products

Figure 2.6 shows the main oxidation pathways for αTOH during fatty acid autoxidation. In the presence of sufficient amounts of peroxyl radicals in nonpolar systems, αTOH is primarily oxidized to 8a-peroxy-substituted tocopherones (Yamauchi *et al.*, 1993; Liebler and Burr, 1995; Kamal-Eldin and Appelqvist, 1996), which may hydrolyse to α-tocopherolquinone. Another important group of oxidation products is the epoxytocopherones and their hydrolysis products 2,3-epoxy-α-tocopherolquinone and 5,6-epoxy-α-tocopherolquinone. The mechanism of epoxide formation is not known, but data obtained by Liebler and Burr (1995) suggest that epoxide formation, like the formation of 8a-peroxy-α-tocopherones, results in the net consumption of two peroxyl radicals, and that the αTOH antioxidant stoichiometry is thus not affected by the relative formation of epoxides.

Dimers of αTOH are formed by bimolecular self-reaction of the α-tocopheroxyl radical, and upon further oxidation spirodimers and trimers may be produced. According to Yamauchi (1999) the rate constant for the reaction of αTO• with a peroxyl radical is 5 orders of magnitude greater than that for dimer formation, and dimerization has been found to mainly occur in reaction mixtures with excess tocopherol (Yamauchi *et al.*, 1995; Yamauchi, 1999). The dimers with free phenolic hydrogen can still act as antioxidants. Reaction products of fatty acid alkyl radicals and αTO•, the 6-*O*-alkyl-tocopherols, are formed under air-insufficient conditions only (Yamauchi *et al.*, 1993 and 1995). The oxidation products of γTOH and δTOH are less well studied, but their oxidation appears to follow the same main pathways as for αTOH. Unlike αTOH,

the γ- and δ-homologues have a free *ortho*-position 5, which makes peroxyl radical addition to their tocopheroxyl radical possible at this position (Kamal-Eldin and Appelqvist, 1996).

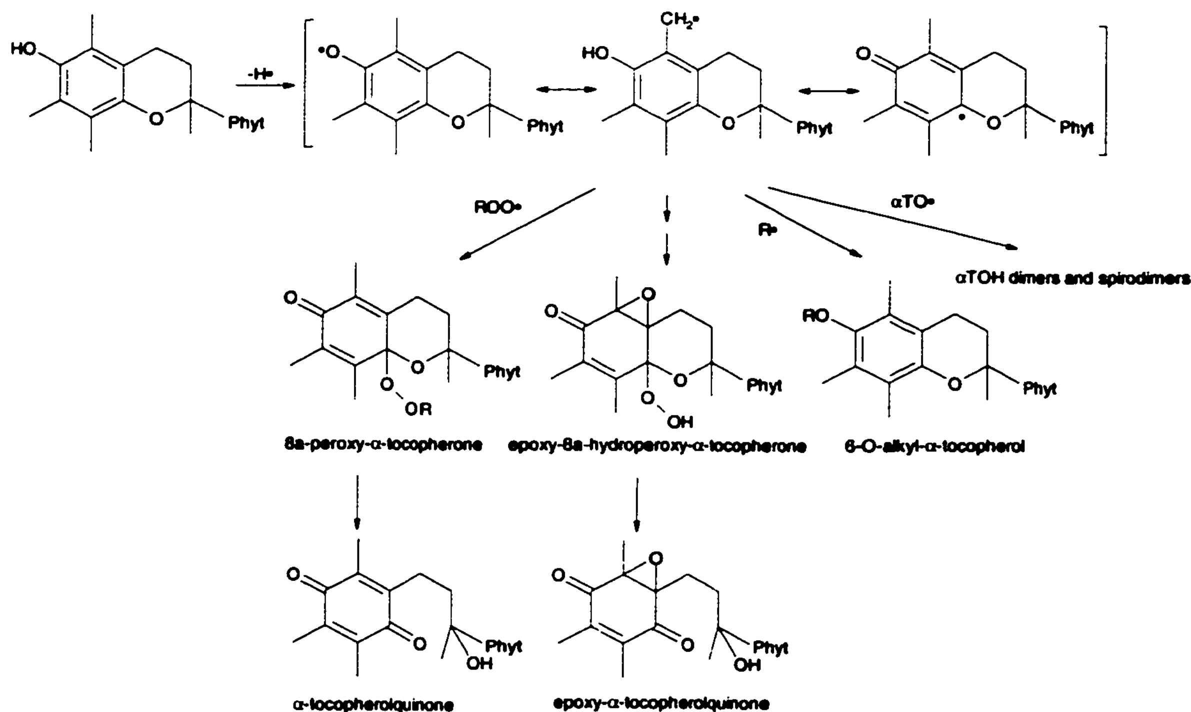


Figure 2.6. Reaction products of αTOH formed during fatty acid autoxidation. Adapted from Yamauchi (1999).

Effects of tocopherols on the autoxidation of lipids

Extensive research on the antioxidant properties of the tocopherols *in vitro* has been carried out, particularly in vegetable oils and in solutions of methyl linoleate or linoleic acid, and the many reports available reflects the widespread confusion regarding their relative and absolute antioxidant potency. Although it is generally agreed that the relative antioxidant activity of the tocopherols *in vivo* is in the order αTOH > βTOH > γTOH > δTOH (Kamal-Eldin and Appelqvist, 1996; Azzi and Stocker, 2000), which is in

accordance with the relative hydrogen-donating power expected from their chemical structures, many studies have concluded that αTOH was the least effective of the tocopherols in improving the stability of fats and oils (Lea and Ward, 1959; Olcott and van der Veen, 1968; Chow and Draper, 1974; Cort, 1974; Pongracz, 1984; Gottstein and Grosch, 1990; Pongracz *et al.*, 1995; Elmadfa and Wagner, 1997). Burton and Ingold (1981) found that their relative rate of reaction with peroxyl radicals of styrene in chlorobenzene (αTOH > βTOH ≈ γTOH > δTOH) did, in fact, decrease in the same order as their vitamin E activities. The tocopherol antioxidant activity *in vitro* therefore appears to be influenced by other factors in addition to their peroxyl radical scavenging abilities.

The activity of the tocopherols has been found to depend on the composition of the lipid substrate (Lea, 1960; Chow and Draper, 1974), the concentration used (Huang *et al.*, 1994; Fuster *et al.*, 1998; Lampi *et al.*, 1999), the experimental conditions of the storage tests (Lea and Ward, 1959; Lea, 1960; Marinova and Yanishlieva, 1992a), the method used to follow oxidation (Huang *et al.*, 1994 and 1995), as well as the endpoint of the oxidation test (Lea, 1960). This makes a direct comparison of the published data on tocopherol activity difficult. Moreover, in some reports the concentration of tocopherols naturally present in the substrate used is either not specified or the substrate is considered "essentially free of natural tocopherols". A study by Lampi *et al.* (1997) showed that the presence of even minor amounts of tocopherol (< 5 ppm) substantially retarded autoxidation.

In-depth studies by Huang and co-workers using tocopherol-stripped corn oil (Huang *et al.*, 1994 and 1995) and by Lampi and co-workers using purified rapeseed and sunflower oil (Lampi *et al.*, 1997; Fuster *et al.*, 1998; Lampi *et al.*, 1999) have provided important information about the *in vitro* properties of the tocopherols. An inversion of activity of αTOH at 100 ppm was observed in corn oil at 60°C, as addition of αTOH to higher concentrations increased the rate of formation of hydroperoxides (Huang *et al.*, 1994). Maximum antioxidant activity of γTOH was observed at 250-500 ppm. In the initial stage

of oxidation, αTOH promoted hydroperoxide formation at 250 ppm and higher when compared to the original tocopherol-stripped corn oil. In contrast to hydroperoxide formation, the ability of αTOH and γTOH to retard the formation of hexanal, a decomposition product of hydroperoxides of n-6 fatty acids, was improved with increasing tocopherol concentration. The antioxidant activity of δTOH increased with the level of addition when added at 100-2000 ppm on the basis of both hydroperoxide formation and decomposition (Huang *et al.*, 1995). Whether tocopherol mixtures acted as antioxidants or prooxidants in corn oil depended on the concentration of αTOH in the mixtures (Huang *et al.*, 1995). Both αTOH and γTOH were efficient antioxidants in purified sunflower oils at concentrations as high as 2000 ppm (Fuster *et al.*, 1998) and in purified rapeseed oil (5-500 ppm) (Lampi *et al.*, 1999). A relative increase in hydroperoxide formation at αTOH concentrations above 100 ppm in purified rapeseed oil paralleled an increased rate of consumption of the antioxidant (Lampi *et al.*, 1999). In purified sunflower oil, αTOH was a better antioxidant than γTOH at low concentrations ($\leq$ 40 ppm), but a reverse order of activity was obtained at concentrations above 200 ppm (Fuster *et al.*, 1998). Maximum antioxidant activity of α-, γ- and δTOH in soybean oil was found at 100, 250 and 500 ppm, respectively, above which the tocopherols promoted hydroperoxide formation (Jung and Min, 1990). Prooxidant effects of αTOH have also been claimed in sunflower oil (Pongracz *et al.*, 1995), olive oil (Satue *et al.*, 1995), methyl linoleate (Terao and Matsushita, 1986), aqueous dispersions of linoleic acid (Cillard and Cillard, 1980; Koskas *et al.*, 1984), as well as in fish oils (Bragadóttir *et al.*, 1995; Burkow *et al.*, 1995; Hamilton *et al.*, 1998).

The inversion of activity of tocopherol, and αTOH in particular, above a certain level of addition, explains some of the earlier contradictory findings in the literature regarding the relative antioxidant activity of the tocopherols, as well as their activity compared to other phenolic antioxidants. The mechanism responsible for the inversion of tocopherol activity, which has sometimes been found to induce a prooxidant effect, has not been

clarified. It is assumed that it is caused by the participation of αTOH and/or αTO• in reactions other than with peroxyl radicals (Marinova and Yanishlieva, 1992a; Kamal-Eldin and Appelqvist, 1996; Fuster *et al.*, 1998). These so-called side-reactions may include the interaction with minor oil constituents such as metal ions and preformed hydroperoxides, the generation of new radicals during tocopherol oxidation to quinone/epoxyquinones, direct reaction with oxygen, as well as chain-transfer with intact fatty acids or hydroperoxides (reviewed by Kamal-Eldin and Appelqvist, 1996). The relative importance of these side-reactions will be discussed in Section 3.

2.4.2 Antioxidant Protection of Fish Oils

The effect of added antioxidants to fats and oils is dependent on the concentration and type of antioxidants naturally present. Addition of tocopherols to refined vegetable oils often does not improve their oxidative stability, as these oils already contain relatively high levels of tocopherols (Hudson and Ghavami, 1984; Pokorný, 1987). Fish oils, on the other hand, require additional stabilization by antioxidants if intended for human consumption.

Burkow *et al.* (1995) evaluated different commercial antioxidant systems for their ability to stabilize cod liver oil at 1500 ppm using the Rancimat™ method. Of the two individual tocopherols tested, δTOH gave the longest induction period, and αTOH was the least effective, while the induction period values for the tocopherol mixtures tested (Tenox GT-2: α:γ:δ 12:65:23, Covi-ox T-70: α:β:γ:δ 12:2:56:30 and Pristine 180: > 80% γ +δ) were intermediate. Rosemary and sage extracts were weak antioxidants when tested using the Rancimat™ at 80°C, however, when tested at 5°C a rosemary extract was very efficient. The efficiency of rosemary extracts has also been studied by Wada and Fang (1992), who found that a αTOH-rosemary mixture delayed the onset of oxidation in sardine oil longer than either of the antioxidants alone. Two rosemary extracts and their major active components, carnosol and carnosic acid, effectively inhibited hydroperoxide formation in fish oil (Frankel *et al.*, 1996). Several flavonoids have also shown promise

as stabilizers for fish oils (Nieto *et al.*, 1993; Wanasundara and Shahidi, 1998). Another natural antioxidant, sesamol, was a better antioxidant than αTOH at 2000 ppm in pollock oil (Saito and Nakamura, 1990). The addition of nitrogen-containing phospholipids alone to fish oil had little antioxidant activity compared to a tocopherol mixture (Totani, 1997). However, phosphatidylethanolamine showed a remarkable synergistic effect with tocopherols, and this was ascribed to its ability to regenerate tocopherols from tocopheroxyl radicals.

An early paper by Olcott and van der Veen (1968) reports on the effects of α-, γ- and δ-tocopherols individually at concentrations up to 15 000 ppm on the oxidative stability of menhaden oil. The antioxidants were evaluated using the weight-gain method at 30°C. The induction period values obtained decreased in the order δTOH > γTOH > αTOH. Inversion of activity or prooxidant effects was not apparent for either of the tocopherols, possibly due to the low sensitivity of the method used to follow the oxidation.

Ternary antioxidant systems containing tocopherol, ascorbic acid/ascorbyl palmitate and lecithin are promising as stabilizers of fish oils, due to the synergistic action of these antioxidants. Yi *et al.* (1991) and Han *et al.* (1991) evaluated the antioxidant effects of δTOH and ascorbic acid in a sardine oil/lecithin/water system using the Rancimat™. Both lecithin and ascorbic acid were found to act as synergists of δTOH in the water-in-oil emulsion. In a recent study by Hamilton *et al.* (1998), refined fish oil with 2% δTOH, 0.1% ascorbyl palmitate and 0.5% lecithin showed no significant oxidation, as determined by the peroxide value, over a period of 6 months when stored in air at 20°C. Off-flavours, on the other hand, developed in the oil despite the low POV.

TBHQ, an efficient antioxidant in vegetable oils (Pokorný, 1987), has been shown to be the most effective of the common synthetic antioxidants in retarding oxidation in fish oils. Chahine (1978) showed that TBHQ was very active in herring oil, followed by PG, BHT and BHA. Similarly, TBHQ was more active in protecting unsaturated mackerel-skin

lipids against autoxidation than αTOH, BHA or BHT at a level of 0.02% (Ke *et al.*, 1977). By measuring oxygen uptake in capelin oil, Bragadóttir *et al.* (1995) found that 100 ppm synthetic antioxidant showed decreasing antioxidant activity in the following order: TBHQ > PG > BHT > BHA. However, although cleared in the U.S. since the early 1970's and recently in Canada, TBHQ is not approved for food use in Europe.

3 Protection of α-Tocopherol in Nonpurified and Purified Fish Oil

3.1 Introduction

The order of antioxidant activity of the tocopherols in many lipid systems *in vitro* has been found to be δTOH > γTOH > αTOH (e.g Cillard and Cillard, 1980; Lambelet and Löliger, 1984), and αTOH is generally considered a weak antioxidant when added alone to oils rich in polyunsaturated fatty acids. However, αTOH is the tocopherol homologue displaying the highest activity *in vivo* (Kamal-Eldin and Appelqvist, 1996; Azzi and Stocker, 2000). As the physiological tocopherol requirement is a function of the amount and type of fatty acids ingested (Muggli, 1994), the intake of fish oil and other PUFA supplements requires a high intake of vitamin E to protect the body lipids from *in vivo* peroxidation. It is therefore likely that αTOH will remain important as an antioxidant additive to fish oils for human consumption. Means to increase the *in vitro* antioxidant activity of αTOH are thus highly relevant in the effort to improve the stability of fish oils.

The antioxidant activity of the tocopherols does not increase linearly with concentration, and an inversion of activity may take place. This phenomenon is of particular importance for αTOH, and at sufficiently high levels of addition prooxidant effects of this antioxidant have been claimed (e.g. Jung and Min, 1990; Huang *et al.*, 1994). The accumulation of hydroperoxides at high αTOH concentrations observed in many different lipid systems has been suggested to be the result of the participation of αTOH and/or αTO• in reactions other than with fatty acid peroxyl radicals (Pokorný, 1987; Marinova and Yanishlieva, 1992a; Kamal-Eldin and Appelqvist, 1996). The relative importance of these side-reactions has not been clarified. Lampi *et al.* (1999) observed a relative increase in hydroperoxide formation in purified rapeseed oil triacylglycerols (TAG) at αTOH concentrations higher than 100 ppm, however, contrary to the findings of Jung and Min (1990) and Huang *et al.* (1994), αTOH did not display prooxidant activity in the purified TAG when compared to the purified control oil with no antioxidant. This is in

accordance with a study by Fuster *et al.* (1998) using purified sunflower TAG, where αTOH was an antioxidant at concentrations as high as 2000 ppm. These authors suggested that αTOH is not a prooxidant *per se* but may act as a prooxidant synergist (or cooxidant) when present at high concentrations together with known prooxidants such as transition metal ions or lipid hydroperoxides.

The objectives of this work were to study the influence of αTOH on the rate of formation of hydroperoxides in nonpurified and purified menhaden oil, and to determine the extent to which the presence of minor constituents in the oil, such as preformed lipid oxidation products, as well as the food-acceptable antioxidants citric acid and ascorbyl palmitate, affect its activity. Purification by column chromatography removes the majority of antioxidant and prooxidant molecules normally present in an oil, and the use of the purified TAG fraction as the substrate thus allows better control over factors known to influence the autoxidation process.

3.2 Materials and Methods

This work was carried out at Dalhousie University.

Materials

Menhaden oil, refined and bleached, was provided by Omega Protein (Reedville, VA). Immediately upon arrival, the oil was stored at -30°C, and before use it was held at room temperature overnight. The *d*-α-tocopherol (purity ≥ 99%) was supplied by Acros (Ghent, Belgium), citric acid (purity ≥ 99.9%) by Fisher (Fair Lawn, NJ) and ascorbyl palmitate (purity ≥ 95%) by Sigma (St. Louis, MO). The αTOH product contained no other tocopherols as verified by HPLC. Silica gel was purchased from Aldrich (Milwaukee, WI), Celite 545 from Supelco (Bellefonte, PA) and activated carbon from Fisher. All solvents were analytical grade.

3.2.1 Oil Purification

Menhaden oil was purified using a modified version of a previously reported multilayer column chromatographic method (Lampi *et al.*, 1992). A chromatographic column (60 cm x 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 (w/w) of Celite 545:activated carbon (50-200 mesh) and 40 g of a 1:2 mixture (w/w) of Celite 545:granulated sucrose. 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. The collection of eluent under N_2 started when the oil-front had passed through the column and continued until all the oil solution had been drawn down into the column. The product oil solution was stripped of hexane by bubbling N_2 through it. This kept the solution cold and retarded the onset of autoxidation of the purified TAG. The hexane concentration in the stripped oil samples used in this study ranged from 0.02 to 0.2 ppm, as determined by static headspace GC according to the method for analysis of C3-aldehydes described in Section 4.2, using hexane as an external standard in sunflower oil. 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 oil recovery was 30-35%.

3.2.2 Analytical Methods

The peroxide value (POV) was measured according to the American Oil Chemists' Society (AOCS) method Cd 8b-90 using a 1:10 (v/v) dilution of the $Na_2S_2O_3$-solution. The sensitivity of this method is reported to be about 0.5 meq/kg (Frankel, 1998), and this value was considered the limit of detection. The *p*-anisidine value (*p*AV) was determined according to AOCS method Cd 18-90. Colour intensity was measured by reading the oil absorbance against isooctane at 460 nm using a Hewlett Packard HP 8453 UV-Visible spectrophotometer.

Determination of fatty acid composition

Fatty acid compositions were determined by capillary GC with flame-ionization detection (FID) of the methyl esters prepared by transesterification using BF_3-methanol following the method of Morrison and Smith (1964). To the lipids dissolved in hexane (1 mL), an 8% BF_3-methanol reagent (1 mL) was added, and the samples were kept at 100°C for 60 min in a heating block. The reaction mixture was then cooled and diluted with distilled water (2 mL), and the fatty acid methyl esters were extracted with hexane (1 mL x 3). The combined hexane extract was washed with distilled water (2 mL) and dried with anhydrous sodium sulphate. The solvent was evaporated, and the lipid was redissolved in hexane (1 mL). The fatty acid methyl esters were analysed using a Perkin Elmer model 8420 GC fitted with an Omegawax 320 column (30 m x 0.32 mm i.d., 0.25 µm phase; Supelco). The oven temperature was 180°C for 8 min, followed by an increase to 220°C at 3°C/min, and a final hold at 220°C for 10 min. Fatty acid peaks were identified by comparison of their retention times with those of a standard mixture of known fatty acid methyl esters. Quantification was based on theoretical response factors.

Analysis of lipid composition by Iatroscan

Lipid compositions of fish oils and purified fish oil TAG fractions were determined by Iatroscan thin-layer chromatography-FID using an Iatroscan TH-10 Mark III analyser attached to a SP4200 computing integrator. The silica gel coated quartz rods (Chromarods-SIII) were scanned prior to spotting the oil samples dissolved in chloroform using 1µL Drummond Microcap pipettes. The rods were kept in a humidity chamber for 5 min before development in a solvent system consisting of hexane:chloroform:isopropanol:formic acid (80:14:1:0.2 v/v/v/v). They were then dried at 100°C for 3 min prior to scanning. The peak retention times were compared to those of standards of triacylglycerol (tripalmitin), free fatty acid (palmitic acid), diacylglycerol (1,2-dipalmitin) and monoacylglycerol (1-monopalmitin). The result is reported as area% triacylglycerols.

Metal analysis

The iron and copper contents were determined by Else Austad at the Norsk Hydro Research Centre, Norway, using electrothermal atomic absorption spectrophotometry with Zeeman background correction (SIMAA 6000). The oil samples (1 mL) were dissolved in methyl isobutyl ketone (0.5 mL) and diluted with 2-propanol (4 mL). 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. Conostan S-21 metalorganic standard was employed, dissolved in methyl isobutyl ketone and diluted with 2-propanol.

Determination of conjugated dienes

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. It was critical to the analysis that the quartz cuvette used was properly cleaned, as oxidized polar lipid material tended to adsorb onto the walls of the cuvette. The cuvette was therefore washed in a solution of KOH in methanol each day of analysis and rinsed with acetone between each sample UV reading. The $E^{1\%}_{1cm}$ was calculated from the absorbance reading and the sample oil concentration, and the result is reported as the increase from the measurement obtained at time zero of that particular experiment. The $E^{1\%}_{1cm}$ was independent of the sample oil concentration. This was verified by plotting the results from the analysis of 8 replicates of an oil sample against the sample weight.

Tocopherol analysis

Normal phase HPLC with fluorescence detection, according to AOCS method Ce 8-89, was used to measure αTOH in this study, as well as γTOH and δTOH in the work described in Sections 4, 5, 6 and 7. The HPLC system consisted of a Partisil 5 μm

column (11 cm x 4.7 mm; Whatman, Clifton, NJ), equipped with a guard column, and connected to a Waters UK6 injector, a Waters 6000A solvent delivery system, a Waters 420 AC fluorescence detector with 292 nm excitation and 330 nm emission filters, and a Perkin Elmer LCI-100 laboratory computing integrator. The isocratic mobile phase was hexane:2-propanol (99.5:0.5, v/v) set at 1.5 mL/min flow rate and sonicated just prior to use. The concentrations of standard solutions of the tocopherols in methanol were determined by measuring the UV absorbance, using a Hewlett Packard HP 8453 UV-Visible spectrophotometer, at the following wavelengths and using the corresponding divisor factors: αTOH: 292 nm, 0.0076; γTOH: 298 nm, 0.0091; δTOH: 298 nm, 0.0087. There was a linear relationship between the tocopherol HPLC peak response and the tocopherol standard concentration in the range covering the expected concentrations of tocopherol in the lipid samples (αTOH: $r^2 = 0.9998$; γTOH: $r^2 = 0.99996$; δTOH: $r^2 = 0.9997$). For quantification of the tocopherols in the lipid samples, oil was weighed and dissolved in hexane before injection. The peaks were identified by comparison of the their retention times with those of the tocopherol standards. Quantification was achieved by comparison of the sample tocopherol peak response with that of an external standard with similar peak response. Whenever necessary, the HPLC column was cleaned by successively passing solvents (about 50 mL of each) with increasing concentrations of isopropanol in hexane (2, 5, 15, 25 and 50%) through the column in the reverse flow direction. The column was then reversed to the proper flow direction and gradually re-equilibrated to the solvent system used in the tocopherol analysis.

3.2.3 Oxidation Tests

Antioxidant was added immediately following oil purification. The αTOH was dissolved in hexane and the concentration determined spectrophotometrically as described in AOCS method Ce 8-89. Accurate amounts of αTOH were added to the oils by transferring known volumes of the αTOH solution to glass flasks and evaporating the solvent under a stream of N_2, before adding the desired amount of oil. The αTOH was then mixed with the oil by bubbling N_2 through the samples for 20 min. The αTOH concentrations were

verified by HPLC as already described. Citric acid and ascorbyl palmitate were added to the purified oils as freshly prepared solutions in acetone, the solvent being immediately removed by N_2 stripping. This allowed antioxidant addition without having to heat the oil samples. The dissolution of citric acid and ascorbyl palmitate in the oil was visually verified. Portions (1.5 g) of the different oil treatments were stored in 20 mL uncapped glass vials (40 mm x 25 mm i.d.) at 30°C in the dark in an oven. Three vials of each oil treatment were removed from the oven at regular intervals for the analysis of conjugated dienes, and, when evaluating the effect of ascorbyl palmitate, for analysis of residual αTOH as well. One analytical determination was performed for each oil portion.

The oxidative stabilities of samples of menhaden oil and purified menhaden oil TAG with 50, 100, 250 and 500 ppm αTOH were compared. Purified menhaden oil TAG was then used as the substrate in the following experiments: i) a study of the effect of the addition of citric acid (50 ppm) on the stability of the substrate with 0, 50, 100, 250 and 500 ppm αTOH, ii) a study of the effect of the addition of ascorbyl palmitate (250 ppm) on the stability of the substrate with 0, 100, 250 and 500 ppm αTOH.

Statistical analysis

The initial rates of hydroperoxide formation, R_h, were estimated by linear regression of the hydroperoxide levels on storage time (storage time ≤ 3 days) using StatMost32™ (DataMost Corporation, Salt Lake City, UT). The slope estimates were compared using a *t*-test. The rates of hydroperoxide formation were considered different when $P < 0.05$.

3.3 Results

3.3.1 Purification of Fish Oil

Menhaden oil and a typical batch of purified menhaden oil TAG were characterized with regard to the content of oxidation products, lipid composition, colour, αTOH concentration, and iron and copper concentration (Table 3.1, p. 40). The fatty acid compositions determined in the menhaden oil and the purified menhaden oil TAG are

given in Appendix A. An anchovy oil was also purified according to the same procedure, and the results from the chemical characterization of the nonpurified and purified oils are shown in Table 3.1. The purified fish oil TAG fractions were practically colourless and odourless, and lipid components more polar than TAG, e.g. mono- and diacylglycerols and preformed oxidation products, had been removed. Cholesterol esters and free fatty acids were not detected in either of the nonpurified and purified oils by Iatroscan analysis. Typical Iatroscan chromatograms of a nonpurified and a purified oil are shown in Appendix B. Purification did not alter the amount of iron in the oils, while copper was below the limit of detection in both the nonpurified and purified fractions. The concentration of water in a typical batch of purified menhaden oil TAG was very low, 20 ppm, as determined by the Karl Fischer titration method.

No αTOH was detected by HPLC analysis of the purified TAG fractions. The αTOH HPLC limit of detection was estimated to 3 ppm, based on linear regression analysis of the standard deviation (SD) of the peak response, from triplicate injections of tocopherol standards, on the tocopherol standard concentration. The limit of detection was then calculated from three times the value of the intercept. A lower limit of detection, 1 ppm, was determined when performing this analysis at the Norsk Hydro Research Centre. As no αTOH was detected in the oils used as substrates in the work carried out in Norway, the purification procedure was considered to reduce the αTOH concentration in the oils to below 1 ppm.

The formation of hydroperoxides in the purified menhaden oil TAG was very rapid and without an apparent induction period, whereas the nonpurified menhaden oil exhibited an induction period of about 4 days (Fig. 3.1). This demonstrates the contribution of αTOH or other antioxidants naturally present to the oxidative stability of commercial fish oils. Hopia *et al.* (1996) determined the molar absorptivity for hydroperoxides of methyl linoleate to be 24 000. When using this value to estimate the amount of hydroperoxides

(as EPA-OOH) formed in fish oil, an increase in $E^{1\%}_{1cm}$ of 10 corresponds to a hydroperoxide content of approximately 1.4%.

The UV-spectra from the conjugated diene analysis of a freshly purified menhaden oil TAG batch and of an oxidized menhaden oil TAG sample (no antioxidant, kept 24 hours at 30°C) is shown in Appendix C. There was a large difference in the concentration of hydroperoxides with a conjugated diene structure (absorbance at 234 nm) between these samples. Conjugated trienes, as well as certain secondary oxidation products such as ketodienes, have an absorption band at about 268 nm (IUPAC, 1987). There was only a small increase in the sample absorbance in this region of the UV-spectrum upon fatty acid autoxidation.

Table 3.1. Characterization of menhaden oil and processed anchovy oil and typical batches of the respective purified TAG fractions.

Analysis[a]	**Menhaden oil**[e]	**Purified menhaden oil**	**Anchovy oil (processed)**[f]	**Purified anchovy oil**
1. POV (meq/kg)	6.0 ± 0.6	< 0.5	1.0 ± 0.2	< 0.5
2. *p*AV	30.0 ± 0.2	0.4 ± < 0.1	22.1 ± 0.1	0.2 ± < 0.1
3. TAG (area%)	98.7	100	99.2	100
4. Colour (A460 nm)[b]	0.41	0.05	0.24	0.02
5. αTOH (ppm)[c]	47.9 ± 0.4	ND	42 ± 2	ND
6. Iron (ng/mL)	35 ± 6	38 ± 6	20 ± < 1	15 ± 6
7. Copper (ng/mL)[d]	ND	ND	ND	ND

[a]Analysis 1 and 2: means ± SD, n = 2; 3: mean of 10 Iatroscan rods; 5-7: means ± SD, n = 3
[b]Absorbance at 460 nm
[c]Limit of detection 3 ppm
[d]Limit of detection 6 ng/mL
[e]Refined and bleached
[f]Refined, deodorized and winterized (EPAX3000TG)

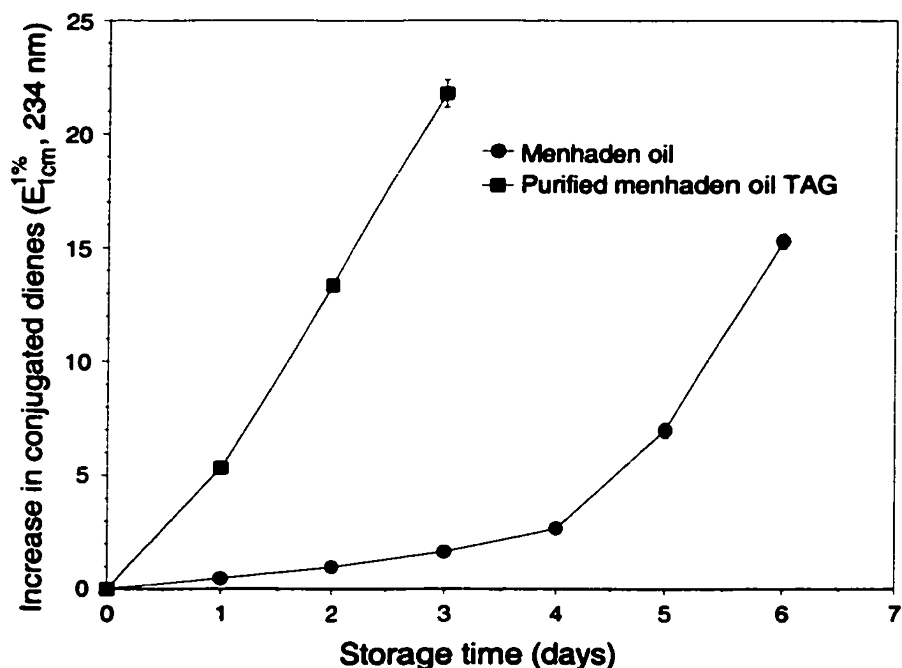


Figure 3.1. Effect of menhaden oil purification on the formation of hydroperoxides during storage at 30°C. Data points are means ± SD (n = 3).

3.3.2 Oxidation Tests

Comparison of αTOH properties in menhaden oil and purified menhaden oil TAG

Addition of αTOH up to a concentration of 100 ppm in nonpurified menhaden oil did not have any effect on the initial rate of hydroperoxide formation (Fig. 3.2A). The induction period of 4 days at 50 ppm αTOH, however, was prolonged by about one day at 100 ppm αTOH. Further increases in the αTOH concentrations resulted in an increased initial rate of hydroperoxide formation, but these oil samples remained in the induction period throughout the experiment. Hence, of the concentrations tested in this experiment, an inversion of antioxidant activity of αTOH was observed at 100 ppm.

In the purified menhaden oil TAG (Fig. 3.2B), the pattern of hydroperoxide formation was similar to that of the menhaden oil, with an inversion of activity taking place at 100 ppm αTOH. The αTOH inhibited hydroperoxide formation at all levels of addition.

Figure 3.3A shows a plot of the initial rate of hydroperoxide formation, R_h, versus αTOH concentration in both the nonpurified and purified oils. The rate of formation of hydroperoxides was higher in the menhaden oil than in the purified menhaden oil TAG at all αTOH levels tested. The effect of purification appeared to be influenced only to a limited extent by αTOH concentration, purification being slightly more beneficial to oil stability at the higher αTOH levels.

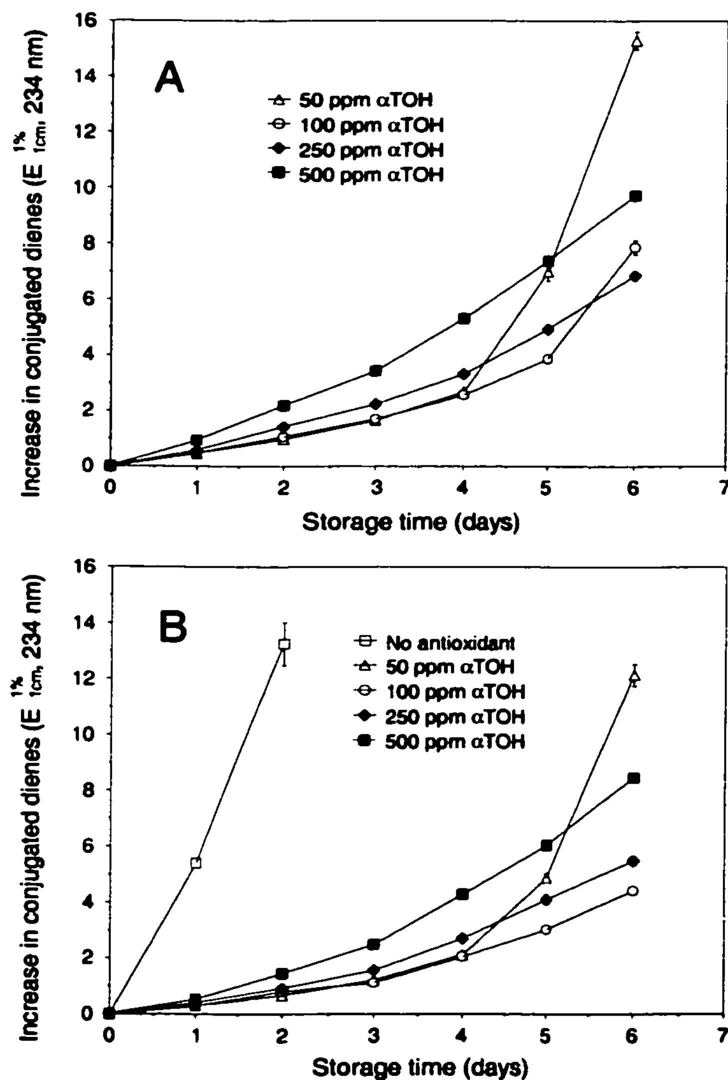


Figure 3.2. Formation of hydroperoxides in menhaden oil (A) and in purified menhaden oil TAG (B) during storage at 30°C in the dark. Data points are means ± SD (n = 3).

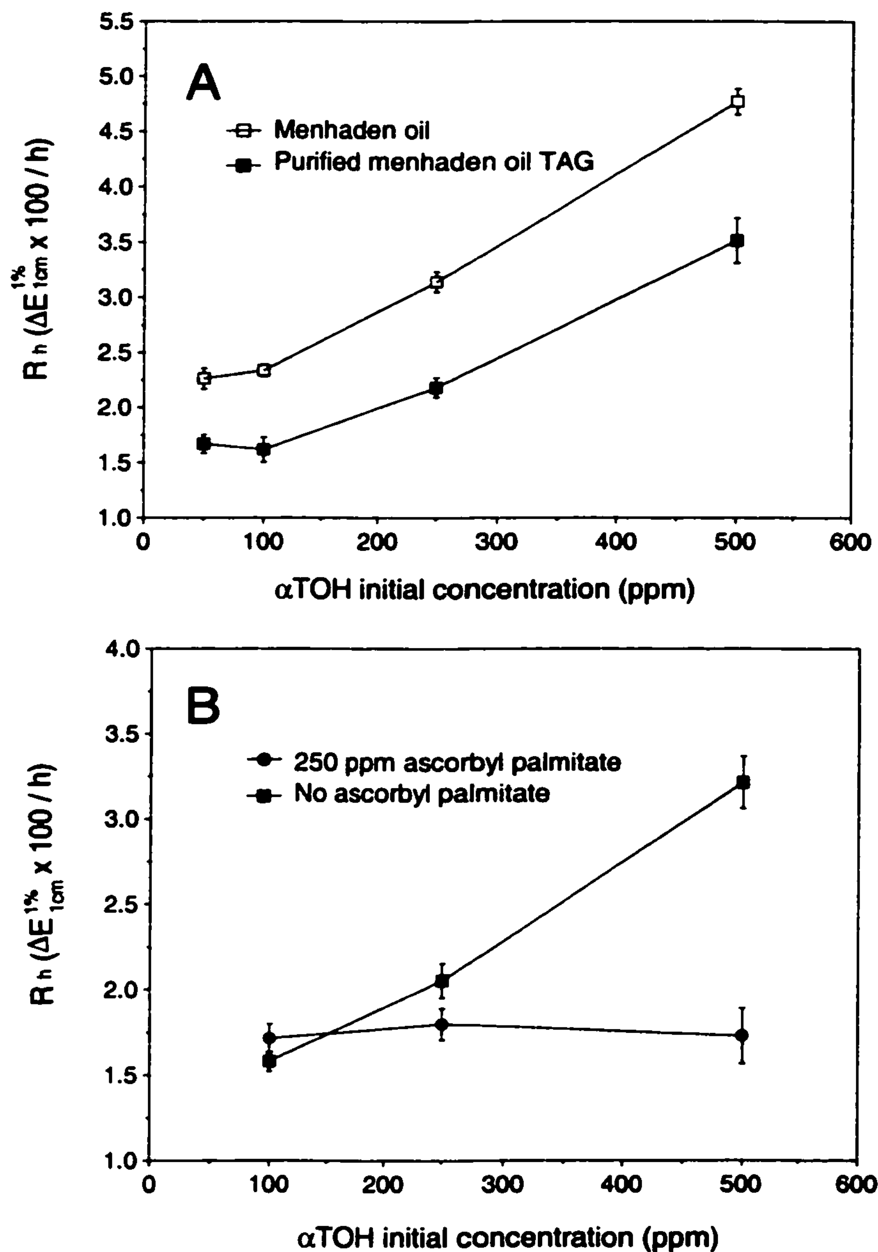


Figure 3.3. Relationships between initial rate of hydroperoxide formation, R_h, and initial αTOH concentration: (A) Effect of menhaden oil purification, (B) Effect of ascorbyl palmitate in purified menhaden oil TAG. Data points are slopes ± standard error.

Effect of other antioxidants

The concentration of iron in the original menhaden oil was very low, but was not reduced by purification (Table 3.1). Citric acid (Fig. 3.4) was therefore added in an attempt to eliminate a possible influence of this metal on the antioxidant activity of αTOH. Citric acid is considered a very effective metal chelator and is widely used during processing of vegetable oils (Frankel, 1998). Citric acid addition (50 ppm) did not have any effect on the rate of oxidation of purified menhaden oil TAG without any tocopherol or at either of the four αTOH levels tested, and thus did not alter the concentration for inversion of activity of the tocopherol (results not shown). The citric acid was also added to another commercial fish oil with a higher iron concentration, which resulted in a substantial reduction in the rate of hydroperoxide formation. This experiment was used as a positive control for the properties of the specific citric acid used. These oxidation curves are shown in Appendix D.

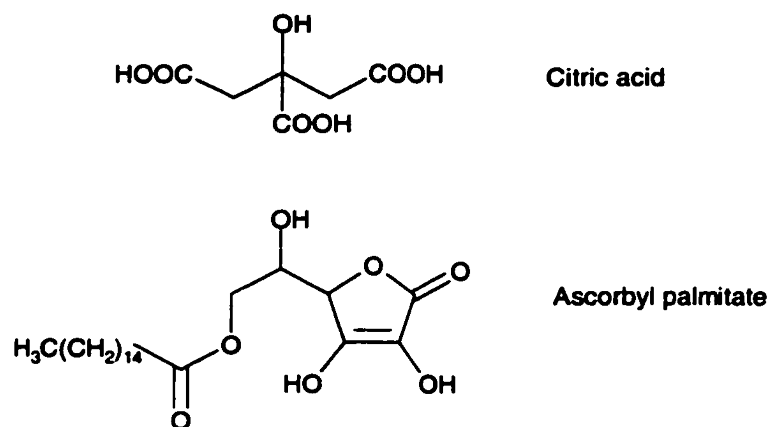


Figure 3.4. Structures of citric acid and ascorbyl palmitate.

It is well known that ascorbic acid, as well as its oil-soluble derivative ascorbyl palmitate (Fig. 3.4), can act as antioxidant synergists to αTOH (Schuler, 1990; Frankel, 1998). They readily reduce αTO•, thereby regenerating αTOH and increasing the effectiveness of

the tocopherol. Figure 3.3B shows a plot of the initial rate of formation of hydroperoxides versus αTOH starting concentration in the purified menhaden oil TAG with and without ascorbyl palmitate (250 ppm). The samples remained in the induction period throughout the experiment. At the αTOH concentration for inversion of activity, 100 ppm, ascorbyl palmitate had no effect on the rate of oxidation. At higher αTOH concentrations, the presence of ascorbyl palmitate reduced the initial rate of hydroperoxide formation to approximately that in purified menhaden oil TAG with 100 ppm αTOH. The addition of ascorbyl palmitate to purified menhaden oil TAG with no αTOH reduced the rate of formation of hydroperoxides slightly (Fig. 3.5), but the autoxidation still proceeded with no apparent induction period. Further addition of ascorbyl palmitate to menhaden oil TAG already containing 500 ppm αTOH and 250 ppm ascorbyl palmitate had an additional stabilizing effect on the oil (Fig. 3.5). Ascorbyl palmitate protected αTOH from being consumed when the initial αTOH concentration was 500 ppm, and had also an initial sparing effect on αTOH when the tocopherol start concentration was 100 ppm (Fig. 3.6). The data presented in Fig. 3.6 also demonstrates that the addition of more ascorbyl palmitate to purified menhaden TAG already containing 500 ppm αTOH and 250 ppm ascorbyl palmitate, further reduced the consumption of αTOH.

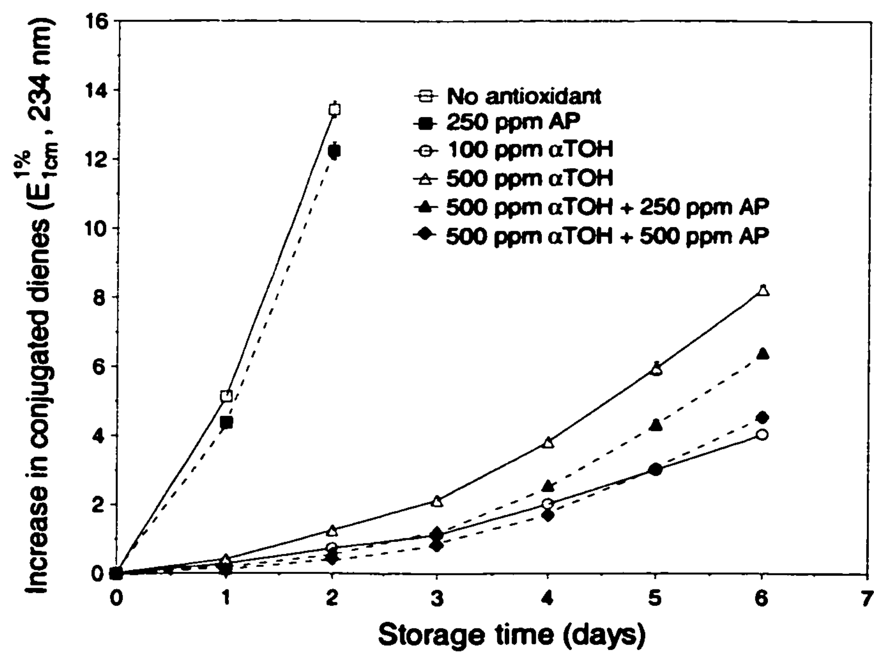


Figure 3.5. Effect of ascorbyl palmitate (AP) on the formation of hydroperoxides in purified menhaden oil TAG with and without αTOH (500 ppm) at 30°C. Data points are means ± SD (n = 3).

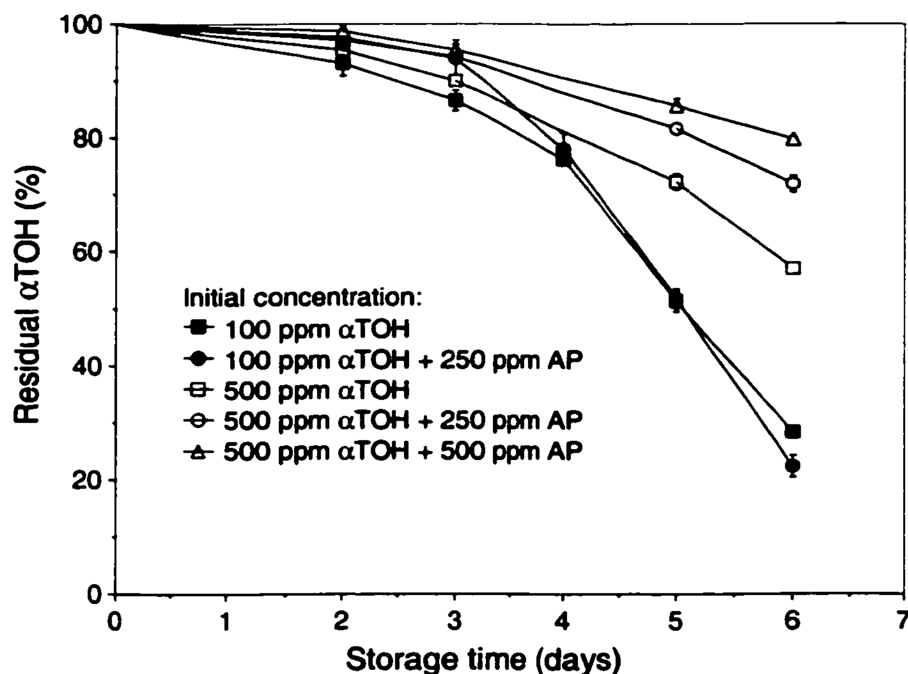


Figure 3.6. Effect of ascorbyl palmitate (AP) on the consumption of αTOH during storage of purified menhaden oil TAG at 30°C. Data points are means ± SD (n = 3).

3.4 Discussion

The presence of minor constituents in menhaden oil did have an effect on the rate of autoxidation of its fatty acids, but appeared only to a limited extent to influence the relative antioxidant activity of αTOH. An inversion of activity of αTOH on the basis of formation of hydroperoxides was observed at 100 ppm in both menhaden oil and purified menhaden oil TAG. An increase in the rate of oxidation at levels of addition of αTOH above 100 ppm has also been observed by several investigators in vegetable oils (Jung and Min, 1990; Huang *et al.*, 1994; Blekas *et al.*, 1995; Lampi *et al.*, 1999), while a somewhat lower concentration for maximum antioxidant activity was determined in a more saturated substrate, purified butter oil TAG (Lampi and Piironen, 1998). A limited number of tocopherol concentrations was tested in these studies, as well as in the present

study, and the true optimum level may thus vary slightly among different vegetable oils and between vegetable oils and fish oil, reflecting the different degrees of susceptibility toward oxidation. However, for practical purposes, the influence of substrate composition on the antioxidant behaviour of αTOH in bulk oils, with regard to concentration for inversion of activity, appears to be of minor importance.

An initial prooxidant effect of αTOH in the menhaden oil at 250 ppm and higher, on the basis of hydroperoxide formation, was observed, but αTOH inhibited hydroperoxide formation at all levels of addition in the purified menhaden oil TAG. The classification of αTOH as a prooxidant or antioxidant clearly depends on the composition of the oil to which a comparison is made, and these results illustrate the importance of properly defining the composition of the control oil. In this study, αTOH concentrations above 100 ppm promoted hydroperoxide formation, but the time the samples remained in the induction period was prolonged. When evaluating antioxidants by the duration of the induction period only, valuable information about the oxidation status of the oil while in the induction period may be lost.

αTOH side-reactions

The tocopherols inhibit lipid autoxidation by scavenging lipid peroxyl radicals (ROO•) according to reactions [3.1] and [3.2] (Burton and Ingold, 1981; Liebler and Burr, 1995).

ROO• + TOH → ROOH + TO• [3.1]

TO• + ROO• → nonradical products [3.2]

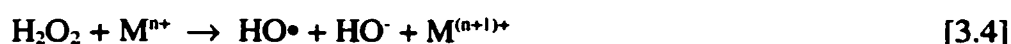
The αTOH-mediated promotion of oxidation frequently observed in fats and oils, as well as in the present work, appears to be related to the excellent hydrogen-donating power of this tocopherol compared to that of other phenolic antioxidants. The proposed mechanisms for the decrease in antioxidant strength of αTOH at high concentrations,

involving the participation at αTOH and αTO• in certain side-reactions, are reviewed below and discussed in relation to the findings of the present study.

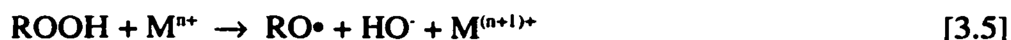
The αTOH-mediated accumulation of hydroperoxides has been suggested to be enhanced by the presence of high initial amounts of hydroperoxides (reviewed by Kamal-Eldin and Appelqvist, 1996). At low temperatures favouring hydrogen-bonding, αTOH may donate hydrogen atoms to lipid hydroperoxides decomposing them to alkoxyl radicals, thereby generating radicals that may act as chain-carriers [3.3]. A related mechanism is the αTOH-catalysed decomposition of α-tocopheroxy-peroxyl radical adducts (8a-peroxy-α-tocopherones) (Kamal-Eldin and Appelqvist, 1996). In contrast, Koskas *et al.* (1984) and Frankel and Gardner (1989) showed that the addition of tocopherols increased the stability of linoleic acid hydroperoxides. Similarly, Hopia *et al.* (1996) found that αTOH markedly inhibited the decomposition of methyl linoleate hydroperoxides in a concentration-dependent manner.

$$\alpha TOH + ROOH \rightarrow RO\bullet + H_2O + \alpha TO\bullet \qquad [3.3]$$

Transition metal ions (M) can generate radicals and act as prooxidants by the reactions:



$$H_2O_2 + M^{n+} \rightarrow HO\bullet + HO^- + M^{(n+1)+} \qquad [3.4]$$



$$ROOH + M^{n+} \rightarrow RO\bullet + HO^- + M^{(n+1)+} \qquad [3.5]$$

$$ROOH + M^{(n+1)+} \rightarrow ROO\bullet + H^+ + M^{n+} \qquad [3.6]$$

The metal-catalysed decomposition of preformed hydroperoxides, reaction [3.5], is considered the most important reaction in the initiation step of lipid autoxidation (Frankel, 1998). It has been demonstrated in a model system that both αTOH and γTOH are easily degraded by Fe^{3+} and Cu^{2+} (Cort *et al.*, 1978). At high metal concentrations, αTOH and αTO• may act as prooxidants (or prooxidant synergists) by reducing transition

metal ions to their lower state valences (reactions [3.7] and [3.8]), which are the most active forms of these metals in lipid autoxidation (reactions [3.4] and [3.5]).

$$\alpha TOH + M^{(n+1)+} \rightarrow \alpha TO\bullet + H^{+} + M^{n+} \quad [3.7]$$

$$\alpha TO\bullet + M^{(n+1)+} \rightarrow \alpha TO^{+} + M^{n+} \quad [3.8]$$

Cillard and Cillard (1980) suggested that the generation of the radical HOO• according to reaction [3.9] was responsible for an observed prooxidant effect of αTOH in linoleic acid. The more reactive αTOH was thought to be more easily oxidized by air than γTOH and δTOH. However, direct oxidation of αTOH by triplet O_2 is spin-forbidden, but in the presence of transition metal ions the oxidation of αTOH may be enhanced, due to the generation of reactive oxygen species from $^3O_2$ (Kamal-Eldin and Appelqvist, 1996).

$$\alpha TOH + O_2 \rightarrow \alpha TO\bullet + HOO\bullet \quad [3.9]$$

Accumulation of hydroperoxides at high concentrations of αTOH has also been related to undesirable side-reactions of the tocopheroxyl radical. The αTO• may reinitiate the chain reaction by abstraction of a hydrogen atom from a fatty acid(RH) [3.10]. The rate constant for this reaction is very low, but it has been suggested to be important at high αTOH concentrations (Mukai *et al.*, 1993).

$$\alpha TO\bullet + RH \rightarrow \alpha TOH + R\bullet \quad [3.10]$$

Further reactions of αTO• during lipid autoxidation yield two main groups of nonradical products (Fig. 2.6; Liebler and Burr, 1995; Yamauchi *et al.*, 1995; Murkovic *et al.*, 1997). The first consists of 8a-substituted tocopherones, which may rearrange to form α-tocopherolquinone, while epoxyhydroperoxy-α-tocopherones and their hydrolysis products epoxy-α-tocopherolquinones make up the second group of oxidation products.

Suggested pathways for the formation of α-tocopherolquinone (Gottstein and Grosch, 1990) and epoxyhydroperoxy-α-tocopherones (Liebler *et al.*, 1990) both involve abstraction of hydrogen from intact unsaturated fatty acids. The alkyl radicals generated are expected to react very rapidly with oxygen and hydroperoxide accumulation may be induced.

In this study, the rate of formation of hydroperoxides was greater in the menhaden oil than in the purified menhaden oil TAG at all αTOH levels tested. The decomposition of hydroperoxides generates radicals which then propagate the autoxidation chain reaction, and a higher concentration of preformed hydroperoxides (initial POV 6.0 meq/kg), as well as secondary oxidation products, in the original menhaden oil is most likely the main factor responsible for the observed difference in the oxidation rate. A low initial POV has been demonstrated to increase the storage stability of olive oil (Satue *et al.*, 1995) and methyl linoleate (Terao and Matsushita, 1986). El-Magoli *et al.* (1979) found that volatile secondary oxidation products also accelerated lipid autoxidation. In the present study, the removal of the preformed oxidation products by menhaden oil purification appeared to be only slightly more beneficial to oil stability at the highest αTOH level (500 ppm) compared to the effect at a tocopherol concentration of 100 ppm. It appears likely that other factors contribute more to the peroxidizing effect of αTOH than tocopherol-hydroperoxide interactions, particularly at the hydroperoxide levels commonly found in refined fish oils.

Even traces of metal ions, particularly iron and copper, may influence the rate of oxidation of unsaturated fatty acids, mainly by catalysing the decomposition of hydroperoxides. The prooxidant effect of trace metal ions has been shown to depend on the type of their associated ligands (Odumosu and Hudson, 1978), and it is likely that the efficiency of metal chelators is affected by the form of metal ligands as well. The form in which the metals occurred in the menhaden oil is not known, and an influence of metal ions on the properties of αTOH thus cannot be excluded on the basis of lack of effect of

citric acid in the purified menhaden oil TAG. However, it is suggested that when the metal concentration is very low, other side-reactions of αTOH/αTO• may be of equal or greater importance than reactions [3.7] and [3.8]. This is considered to be in agreement with several investigators who have observed an αTOH-mediated promotion of oxidation in bulk oils even though the metal concentrations were below the limits of detection (< 10 ppb iron, < 1 ppb copper in Fuster *et al.*, 1998; < 10 ppb iron, < 10 ppb copper in Lampi *et al.*, 1999). Addition of 50 ppm iron, as $FeSO_4$, to purified sunflower oil TAG did, however, lead to a stronger positive correlation between the amount of αTOH destroyed during oxidation and the initial tocopherol concentration (Fuster *et al.*, 1998). These findings were, at least in part, attributed to direct tocopherol-transition metal ion interactions. A more rapid loss of tocopherol at high metal concentrations may, on the other hand, also be caused by rapid generation of radicals from an increased rate of metal-catalysed decomposition of the hydroperoxides.

An important finding in the present study was the dependence of the antioxidant activity of ascorbyl palmitate on the concentration of αTOH in the oil. Ascorbyl palmitate has previously been found to suppress an observed prooxidant effect of αTOH in an aqueous dispersion of linoleic acid (Cillard and Cillard, 1986) and in methyl linoleate (Terao and Matsushita, 1986) when tested at one αTOH concentration only (1.25×10^{-4} M and 1000 ppm, respectively). In this study, the addition of ascorbyl palmitate to purified menhaden oil TAG reduced the rate of autoxidation, thereby eliminating the initial αTOH-mediated accumulation of hydroperoxides, when the tocopherol level was above that for maximum antioxidant activity only. It is also noteworthy that ascorbyl palmitate exerted a strong influence on the rate of hydroperoxide formation in the initial phase of the storage period (≤ 3 days), when very little of the added αTOH had been consumed (Fig. 3.6). At 100 ppm αTOH, the presence of ascorbyl palmitate did not reduce the initial rate of hydroperoxide formation, despite having an initial sparing effect on the αTOH (Fig. 3.6). A subsequent increase in the rate of αTOH consumption to approximately that of αTOH

in the samples without ascorbyl palmitate, is thought to mainly reflect depletion of the ascorbyl palmitate.

The multiple antioxidant effects of ascorbic acid include hydrogen donation to regenerate tocopherol, metal inactivation, reduction of hydroperoxides to stable alcohols, and scavenging of oxygen in aqueous systems (Frankel, 1998). In addition, ascorbic acid has been found to act as a prooxidant at low concentrations in aqueous systems by reducing trace metal ions to their more active lower valence states (Schuler, 1990; Frankel, 1998). The properties of ascorbyl palmitate in bulk oils are less well studied. Chemical evidence for the regeneration of αTOH from αTO• by ascorbyl palmitate was presented by Lambelet *et al.* (1985). Subsequently, most reports on an antioxidant effect of ascorbyl palmitate in oils have attributed this to the ability of ascorbyl palmitate to regenerate αTOH. In a study of the effects of ascorbyl palmitate in sunflower oil and lard at 500 ppm αTOH, Marinova and Yanishlieva (1992b) concluded that ascorbyl palmitate was a synergist to αTOH, by regenerating αTOH from its radical, but that ascorbyl palmitate itself also acted as a radical type inhibitor (or primary antioxidant). Hydroxyl free radical scavenging properties of ascorbyl palmitate was recently demonstrated by Perricone *et al.* (1999). Direct peroxyl radical scavenging and/or regeneration of trace amounts of αTOH not removed by purification may explain the slight antioxidant effect of ascorbyl palmitate observed when added alone to purified menhaden oil TAG (Fig. 3.5). The importance of oxygen scavenging by ascorbyl palmitate on autoxidation in bulk oils remains to be assessed.

Maintaining a relatively high αTOH concentration in the purified menhaden oil TAG during storage, by the action of ascorbyl palmitate, had a stabilizing effect on the oil. This suggests that the αTOH itself is not responsible for the increased rate of hydroperoxide formation observed above the concentration for inversion of activity. Lambelet and Löliger (1984) used electron spin resonance (ESR) spectroscopy to study the behaviour of tocopheroxyl radicals generated in oxidized chicken fat. The intensity of

the ESR signal of αTO• increased with increasing αTOH starting concentration. These results suggest that there is an increase in the rate of generation of αTO• at αTOH concentrations above 100 ppm in menhaden oil and purified menhaden oil TAG. This is in accordance with the observed higher overall rate of consumption of αTOH when the level of addition was 500 ppm (38.8 ppm/day) than when the level of addition was 100 ppm (11.9 ppm/day). It is suggested that ascorbyl palmitate regenerates αTOH from αTO•, thus reducing the amount of αTO• available for further oxidation and participation in side-reactions. The effect of ascorbyl palmitate therefore increases with increasing rate of αTO• generation. These results emphasize the participation of αTO•, as opposed to αTOH itself, in the hydroperoxide forming side-reactions in unsaturated fatty acid substrates with relatively high initial αTOH concentrations.

4 Properties of α-, γ- and δ-Tocopherols in Autoxidizing Purified Fish Oil Triacylglycerols

4.1 Introduction

Unlike most vegetable oils, αTOH is generally the only tocopherol homologue naturally present in oils from wild fish (Ackman and Cormier, 1967), and the concentration after refining and deodorization is low. For food and supplement 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 concentrations used are often somewhat arbitrary, and have frequently been determined based on the length of induction periods from accelerated oil stability tests. The results presented in Section 3.3 demonstrated that the length of an induction period is not necessarily related to the oxidative quality of an oil while in the induction period. Contrary to observations in vegetable oils, there is limited information available about the properties of the different tocopherols in fish oils. The inversion of activity found for αTOH and γTOH in certain vegetable oils (Jung and Min, 1990; Huang *et al.*, 1994) even suggests that a reduction in the tocopherol concentration in commercial fish oil products may be beneficial.

The majority of the studies reported on the properties of antioxidants in fish oils have been accelerated by elevated temperatures (Han *et al.*, 1991; Yi *et al.*, 1991; Kaitaranta, 1992; Koschinski and Mcfarlane, 1993; Burkow *et al.*, 1995), and sometimes activities have been compared at one antioxidant concentration only (Lea, 1960; Kaitaranta, 1992; Bragadóttir *et al.*, 1995; Burkow *et al.*, 1995). The reaction mechanisms for oxidation and hydroperoxide decomposition are known to change with temperature (Frankel, 1998), and results obtained at high temperatures may thus not be directly transferable to ambient conditions. Also, the non-linear tocopherol concentration-activity relationships often obtained in vegetable oils suggests 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 effectively retard autoxidation in fish oils (Koschinski and Mcfarlane, 1993; Hamilton *et al.*, 1998). Detailed information about the properties of the individual tocopherols is also valuable when developing antioxidant systems containing other antioxidants in addition to tocopherol. The cost is an important consideration when deciding which antioxidant(s) to use in fish oils and n-3 concentrates, as well as in aquaculture feeds containing fish oil, and commercial tocopherol blends from soybean oil are generally cheaper than the natural *d*-αTOH products but more expensive than synthetic *dl*-αTOH.

Few autoxidation studies measure the effect of antioxidants on the decomposition of hydroperoxides, yet, the volatile products formed are very relevant to the development of off-flavours in an oil. The objectives of this work were to study, in a purified fish oil TAG fraction, the effect of αTOH, γTOH and δTOH, individually and in combination, over a wide range of concentrations, on both the rate of formation of hydroperoxides and of volatile secondary oxidation products.

4.2 Materials and Methods

Materials

Freshly processed South American Pacific fish oil (refined, deodorized and winterized; EPAX3000TG), with a composition similar to that of anchovy oil, was provided by Pronova Biocare (Sandefjord, Norway). The refined fish oil was further purified by column chromatography as described in Section 3.2.1. The purified oil was colourless and odourless, and components more polar than triacylglycerols, such as mono- and diacylglycerols and preformed oxidation products, had been removed. In a typical purified TAG batch, the level of hydroperoxides was < 0.5 meq/kg as determined by the POV, and the *p*AV was 0.2 (Table 3.1, p. 40). 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 in a typical purified fish oil TAG batch 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%). The concentrations of the minor fatty acids are given in Appendix A (purified anchovy TAG-1). The analytical methods employed when characterizing the purified oil are described in Section 3.2.2. The residual hexane concentration in the oil samples was not determined, but they are likely to have been within the same concentration ranges as in the purified menhaden oil TAG samples studied in Section 3 (0.02-0.2 ppm).

The *d*-α-tocopherol (purity ≥ 99%) and the *d*-γ-tocopherol (purity ≥ 95%) were supplied by Acros (Ghent, Belgium), and the *d*-δ-tocopherol (purity ≥ 90%) was from Sigma (St. Louis, MO). The αTOH product contained no other tocopherols, whereas the γTOH and the δTOH contained 1.5% and 3.3% of other tocopherols, respectively, as determined by HPLC analysis of solutions of the tocopherol products in hexane.

Oxidation tests

The investigation of the effects of the individual tocopherols on the rate of formation of primary and secondary oxidation products in purified fish oil TAG was carried out at Dalhousie University. 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 according to the procedure described in Section 3.2.3. An oil sample with 100 ppm αTOH was included in the storage experiments with γTOH and δTOH for direct comparison of their activities. The tocopherol concentrations were verified by HPLC analyses. Portions (1.5 g) of the different oil treatments were stored in 20 mL (40 mm x 25 mm i.d.) uncapped glass vials in the dark in an oven at 30°C. Three vials of each oil treatment were removed from the oven at regular intervals for the analysis of hydroperoxides (by the determination of conjugated dienes), volatile secondary oxidation products (as C3-aldehydes), and the tocopherol concentrations. One analytical determination was performed for each oil portion.

The investigation of the effects of tocopherol mixtures on the formation of primary and secondary oxidation products in purified fish oil TAG was carried out at the Norsk Hydro Research Centre, Norway. Fish oil samples with 1500 ppm total initial tocopherol concentration were prepared according to the procedure described in Section 3.2.3. The initial concentration of αTOH was either 1500 ppm, 1000 ppm, 500 ppm or 200 ppm. The remaining tocopherol was made up of equal amounts (by weight) of γTOH and δTOH. A fish oil with 500 ppm αTOH as the only antioxidant was also prepared, as well as an oil with 500 ppm each of α-, γ- and δTOH and 500 ppm ascorbyl palmitate. The ascorbyl palmitate was added as a freshly prepared solution of known concentration in acetone, and the oil was stripped of solvent by bubbling N_2 through it. Portions (1.5 g) of the different antioxidant treatments were stored in 17 mL (40 mm x 23 mm i.d.) uncapped glass vials in the dark at 30°C in an oven. Triplicate portions of each oil treatment were removed from the oven at regular intervals for the analysis of hydroperoxides (by the determination of conjugated dienes), volatile secondary oxidation products (as C3-aldehydes), and the tocopherol concentrations.

Analyses

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 at Dalhousie University and a Shimadzu (Duisburg, Germany) UV-1200 spectrophotometer at the Norsk Hydro Research Centre. The $E^{1\%}_{1cm}$ 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, as described in Section 3.2.2 for the experimental work carried out at Dalhousie University. At the Norsk Hydro Research Centre, the HPLC system consisted of a Partisil 5 μm column (11 cm x 4.7 mm;

Whatman, Clifton, NJ), equipped with a guard column, and connected to a Waters Ultra Wisp sample processor, a Waters 600E system controller, and a Waters 470 scanning fluorescence detector. The experimental conditions are described in Section 3.2.2. Linear regression curves for each of the tocopherols were plotted from the peak responses from the analysis of three standard solutions of different concentrations, and the sample tocopherol concentrations were calculated using an Atlas chromatography data collection and processing system (LabSystems, Cheshire, UK).

Propanal is one of the major volatile secondary oxidation products of n-3 fatty acids, and may be analysed by static headspace GC as a marker of the formation of this complex group of oxidation products (Frankel, 1993a). Oil samples (0.50 g) in 10 mL crimp-sealed glass vials (Supelco, Bellefonte, PA) 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 Perkin Elmer Sigma 3B GC equipped with a capillary SPB-1701 column (14% cyanopropylphenyl, 86% dimethylsiloxane, 30 m x 0.32 mm i.d., 1μm phase; Supelco) and a flame-ionization detector. The analysis was isothermal at 70°C. When developing the method, standard samples of propanal (Sigma, St. Louis, MO) in sunflower oil were heated for 0-30 min at 40°C and analysed as just described. The relationship between the GC peak area and the heating time is shown in Appendix E, and at a heating time of 20 min, there was an equilibrium between the propanal in the oil and in the headspace above the oil samples. Propanal was identified by comparison of its GC retention time with that of an authentic reference sample. However, when carrying out the dynamic headspace analysis of the volatile secondary oxidation products on a more polar column (Section 6), it was discovered that 2-propenal (acrolein), another volatile oxidation product from n-3 fatty acids, coeluted with propanal on the SPB-1701 column. The C3-aldehydes (propanal + 2-propenal) is therefore reported as a marker of volatile secondary oxidation products. The concentrations of C3-aldehydes in the oil samples were estimated by comparing the FID response with that of a standard with a known concentration of propanal in sunflower oil. Despite the manual injection technique, the

repeatability of the method was considered good, as the coefficient of variation from the analysis of the standards, in triplicate, ranged from 0.7 to 2.7% in this study. The relevance of propanal and 2-propenal as markers of volatile secondary oxidation products is discussed in Section 6.3.3.

For the investigation of the effects of the tocopherol mixtures on fish oil TAG autoxidation at the Norsk Hydro Research Centre, the static headspace GC method was slightly modified to accommodate the equipment used. The samples were equilibrated at 45°C for 15 min in a Dani 3950 autosampler (Monza, Italy), and 1mL headspace was automatically injected into a Varian 3700 GC (Palo Alto, CA) via a transfer line held at 75°C. The GC column was the SPB-1701. The GC oven was programmed with an initial hold time of 3 min at 40°C, a raise to 70°C at a rate of 10°C/min and a final hold of 2 min. There was a linear relationship ($r^2 = 0.999$) between the concentration of a propanal standard in sunflower oil and the GC FID response within the concentration range of interest. The presence of an equilibrium for propanal between the oil and gas phases after 15 min heating at 45°C was verified by analysing standard samples of propanal in sunflower oil held at different lengths of time in the autosampler silicone oil bath. In the freshly prepared fish oil TAG samples, residual hexane from the purification of the oil dominated the chromatogram in the region of elution of propanal/2-propenal. The curves for the formation of C3-aldehydes were therefore extrapolated to zero concentration at the start of the storage tests. After two days of exposure to air, the hexane had evaporated from the oil samples. Typical static headspace GC chromatograms obtained from the analysis of C3-aldehydes in purified fish oil TAG are shown in Appendix F.

The results presented in Section 3.3 showed that the level of hydroperoxides in the purified menhaden oil TAG appeared to increase in a linear manner with storage time early in the storage period (≤ 3 days). Initial rates of hydroperoxide formation in this study were therefore estimated from the data obtained after two days of storage. 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 αTOH-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.

Statistical analysis

The data for the formation of hydroperoxides were compared using a *t*-test (StatMost™, DataMost Corporation, Salt Lake City, UT). The data for tocopherol consumption, in the samples with 100 ppm tocopherol, were also evaluated by analysis of variance using StatMost™. Differences in treatment means were considered significant when $P < 0.05$.

4.3 Results and Discussion

4.3.1 Individual Tocopherols

Effect of α-, γ- and δTOH on the formation of hydroperoxides and C3-aldehydes

The formation of hydroperoxides in purified fish oil TAG with no antioxidant was very rapid and with no apparent induction period (Fig. 4.1A). The hydroperoxides were determined as the conjugated dienes accompanying hydroperoxide formation. 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 days (Fig. 4.1A). Further addition of αTOH to 100 ppm had no effect on the initial rate of hydroperoxide formation, but the induction period was extended by about 2 days. In the presence of > 100 ppm αTOH, there was a significant increase in the initial rate of formation of hydroperoxides, and the difference in sample oxidative status between the treatments increased with storage time. Hence, at 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. 4.1B). The induction periods were in accordance with those observed for the formation of hydroperoxides.

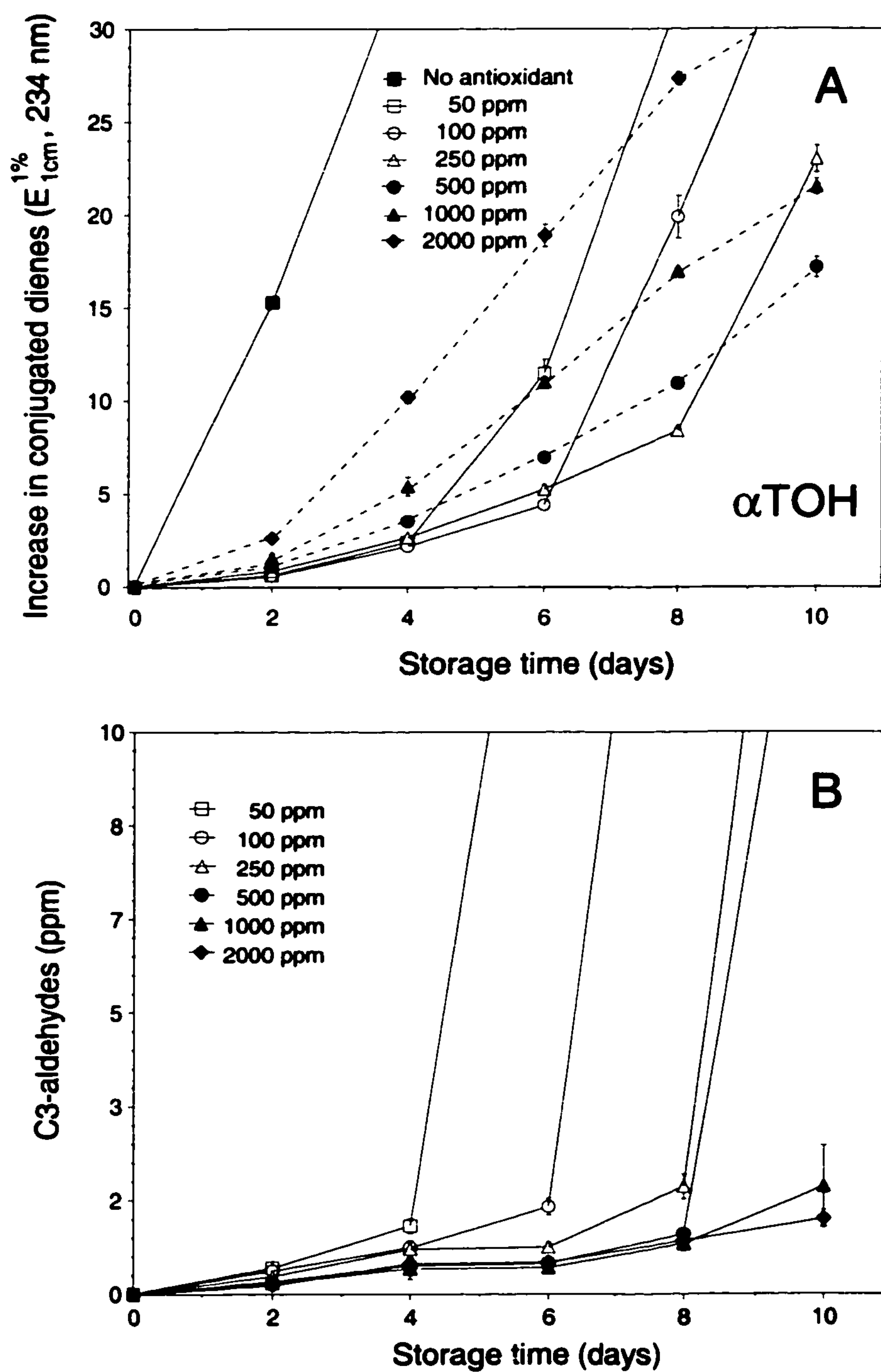


Figure 4.1. Formation of hydroperoxides (A) and C3-aldehydes (B) during storage of purified fish oil TAG with and without αTOH at 30°C in the dark. Data points are means ± SD (n = 3).

The γTOH retarded both hydroperoxide and C3-aldehyde formation at all levels of addition (Fig. 4.2). The levels of hydroperoxides formed at 500 ppm γTOH were significantly lower than those of the other oil samples containing γTOH after 4 days of storage and up to the end of the induction period (Fig. 4.2A). The initial rate of hydroperoxide formation was lower in the sample with 100 ppm αTOH than at either of the γTOH concentrations tested. As was found for αTOH, γTOH inhibited the formation of C3-aldehydes in a concentration-dependent manner (Fig. 4.2B).

As was found for αTOH and γTOH, δTOH retarded the formation of hydroperoxides and C3-aldehydes at all levels of addition (Fig. 4.3). 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 days of storage. The difference in the length of 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.

Figures 4.1B, 4.2B and 4.3B are expansions of the lower concentration range. The full plots for the C3-aldehyde concentrations in the oil samples after the end of the induction period, as well as in the purified oil without any added tocopherol, are shown in Appendix G.

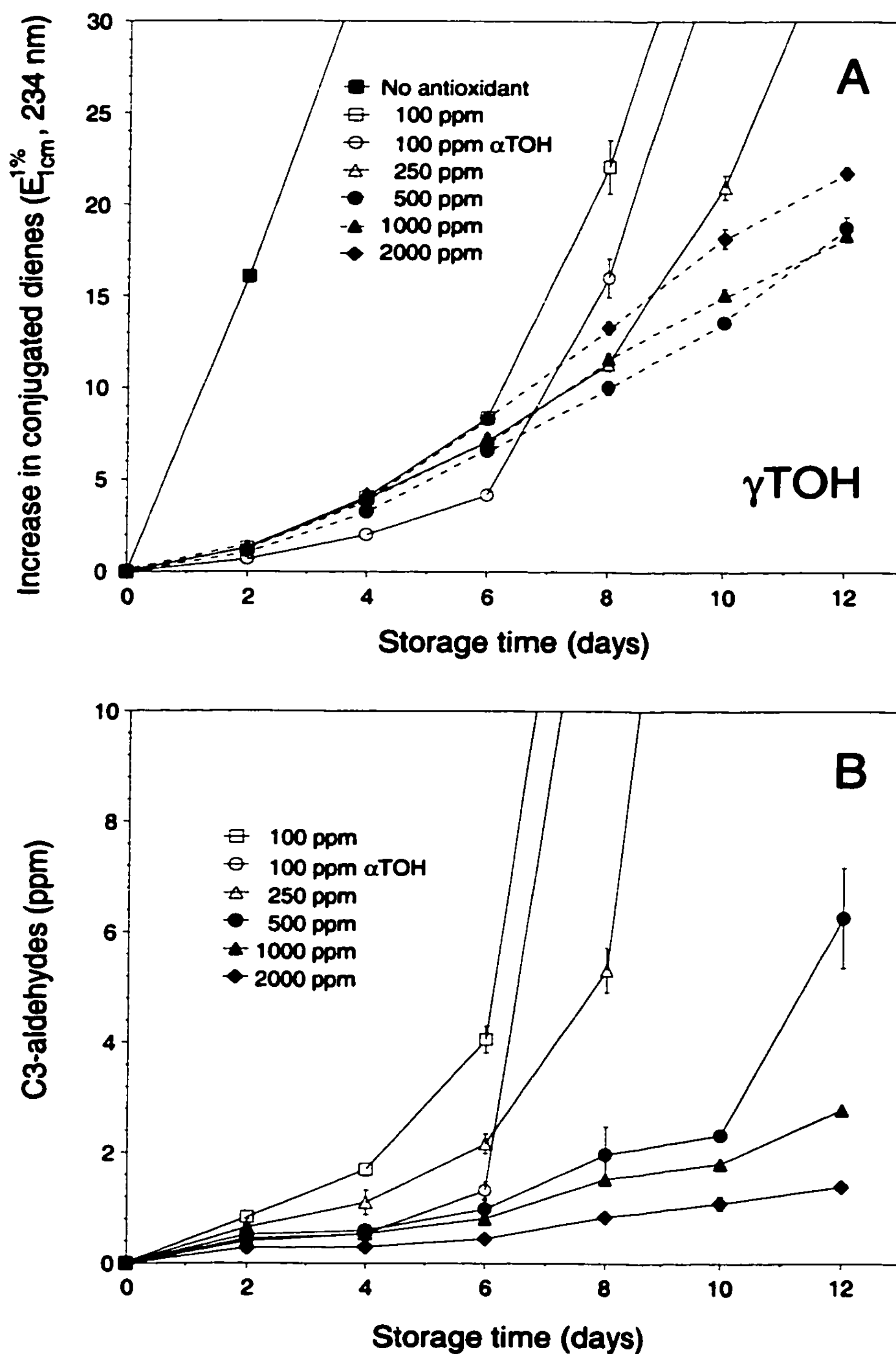


Figure 4.2. Formation of hydroperoxides (A) and C3-aldehydes (B) during storage of purified fish oil TAG with and without γTOH at 30°C in the dark. Data points are means ± SD (n = 3).

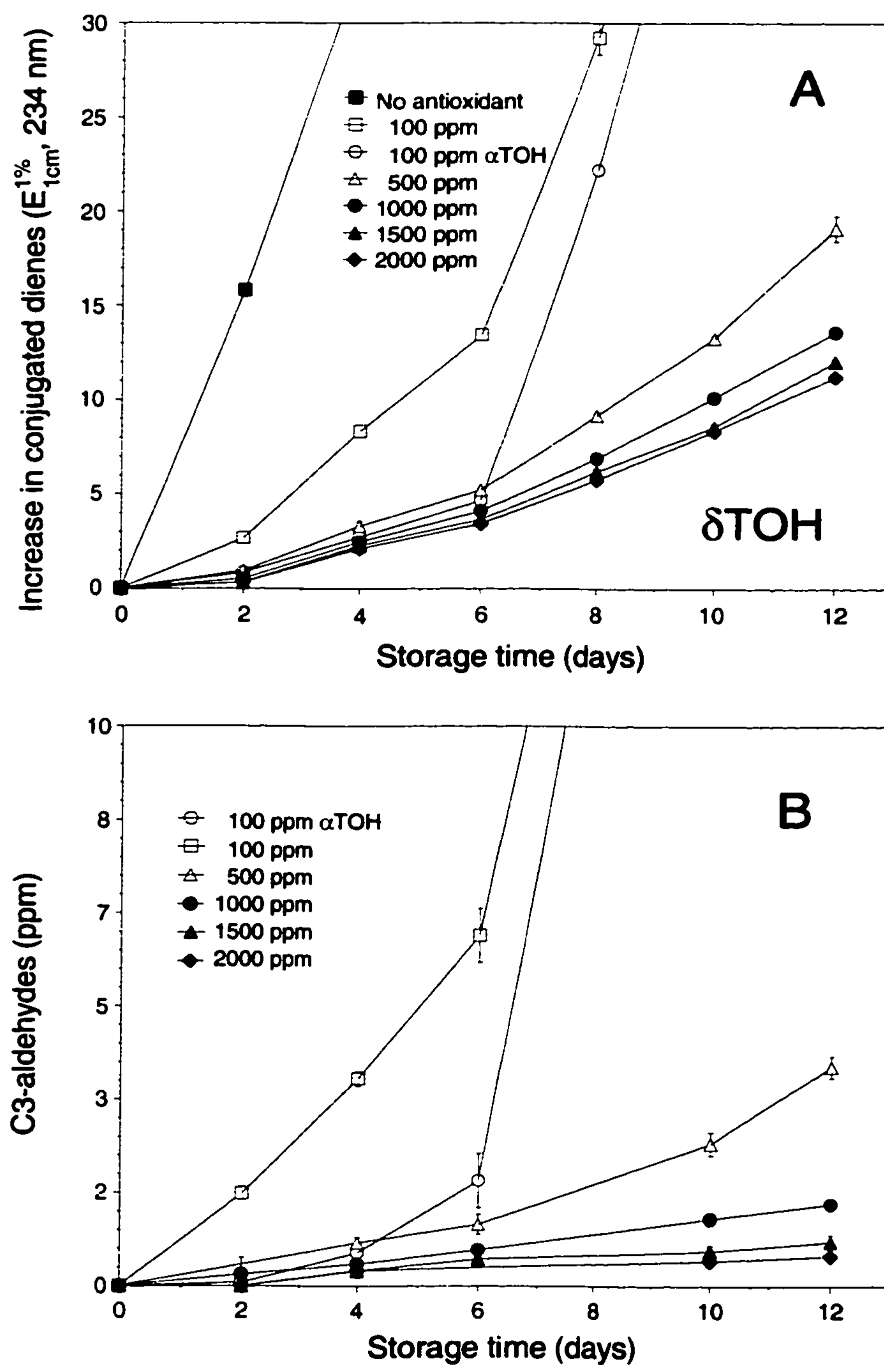


Figure 4.3. Formation of hydroperoxides (A) and C3-aldehydes (B) during storage of purified fish oil TAG with and without δTOH at 30°C in the dark. Data points are means ± SD (n = 3).

In accordance with the results in this study, as well as those presented in Section 3.3, an inversion of activity of αTOH, on the basis of hydroperoxide formation, was observed at 100 ppm in corn oil (Huang *et al.*, 1994), soybean oil (Jung and Min, 1990), purified rapeseed oil TAG (Lampi *et al.*, 1999) and olive oil (Blekas *et al.*, 1995). The tocopherols originally present had been removed from these oils either by molecular distillation or by column chromatography. A limited number of antioxidant concentrations were tested in all these studies, as well as in the present study, and the true optimum 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 has been observed in other fats and oils (Jung and Min, 1990; Huang *et al.*, 1994; Lampi and Piironen, 1998), indicating a higher requirement for γTOH for optimum activity with TAG susceptibility to oxidation. In addition to the degree of unsaturation, the substrates previously studied are likely to have differed with regard to the 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 and of little practical significance. The antioxidant effectiveness of δTOH in tocopherol-stripped corn oil increased with concentration (Huang *et al.*, 1995), in accordance with the result in purified fish oil TAG.

According to the findings in this study, all of the three tocopherols retard the formation of C3-aldehydes in fish oil, and the antioxidant activity increases with tocopherol concentration. A discrepancy between the effect of tocopherols subject to inversion of activity on hydroperoxide formation and on hexanal formation has previously been found in corn oil (Huang *et al.*, 1994 and 1995). Recently, Lampi *et al.* (1999) observed the same phenomenon in purified rapeseed oil TAG. Hopia *et al.* (1996) 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 formation of C3-aldehydes, also products of β-scission of alkoxyl radicals, is inhibited according to the same mechanism.

Hamilton *et al.* (1998) recently observed that the flavour of fish oil deteriorated rapidly during storage although the levels of hydroperoxides, as POV, remained low. Propanal and 2-propenal are two of the major volatile secondary oxidation products from n-3 fatty acids, and can easily be analysed by static headspace GC as markers of hydroperoxide decomposition in fish oils. However, secondary oxidation products of higher molecular weights, such as 4-hexenal and the 2,4,7-decatrienals, are thought to be more influential with regard to the development of off-flavours in fish oils, even though only present at trace levels (Karahadian and Lindsay, 1989a). 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. The findings from an evaluation of the relative ability of the tocopherols to direct the formation of the different groups of volatile secondary oxidation products formed in autoxidizing fish oil are presented in Section 6.

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 days of storage (Fig. 4.4A), after about 4 days of storage for γTOH (Fig. 4.4B), and after about 6 days of storage for δTOH (Fig. 4.4C). Complete consumption of the antioxidant was reached at the end of the induction period. 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. Attempts to linearize the data presented in Figure 4.4 were not successful, and instead average rates of tocopherol

consumption for the different treatments were calculated. The average rates of consumption of αTOH and γTOH (ppm/day) were strongly correlated with the amount of tocopherol added (Fig. 4.5). Above a certain initial concentration (about 250 ppm for αTOH and 500 ppm for γTOH) the relationships appeared linear, the value of the slope being highest for αTOH. The rate of consumption of δTOH reached a constant value of about 50 ppm/day (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 both formation of hydroperoxides and C3-aldehydes, corresponding to the reverse of the order of the rate of consumption of the antioxidant (Fig. 4.5). When added at a level of 1000 ppm, the tocopherols appeared to retard hydroperoxide formation in the order δTOH > γTOH > αTOH, again corresponding to the reverse of the order of the rate of tocopherol consumption (Fig. 4.5). These findings were confirmed in the study presented in Section 6. Contrary to what might have been expected, considering that antioxidants retard oxidation by being preferentially oxidized, high tocopherol antioxidant activity was related to low rate of consumption of the antioxidant.

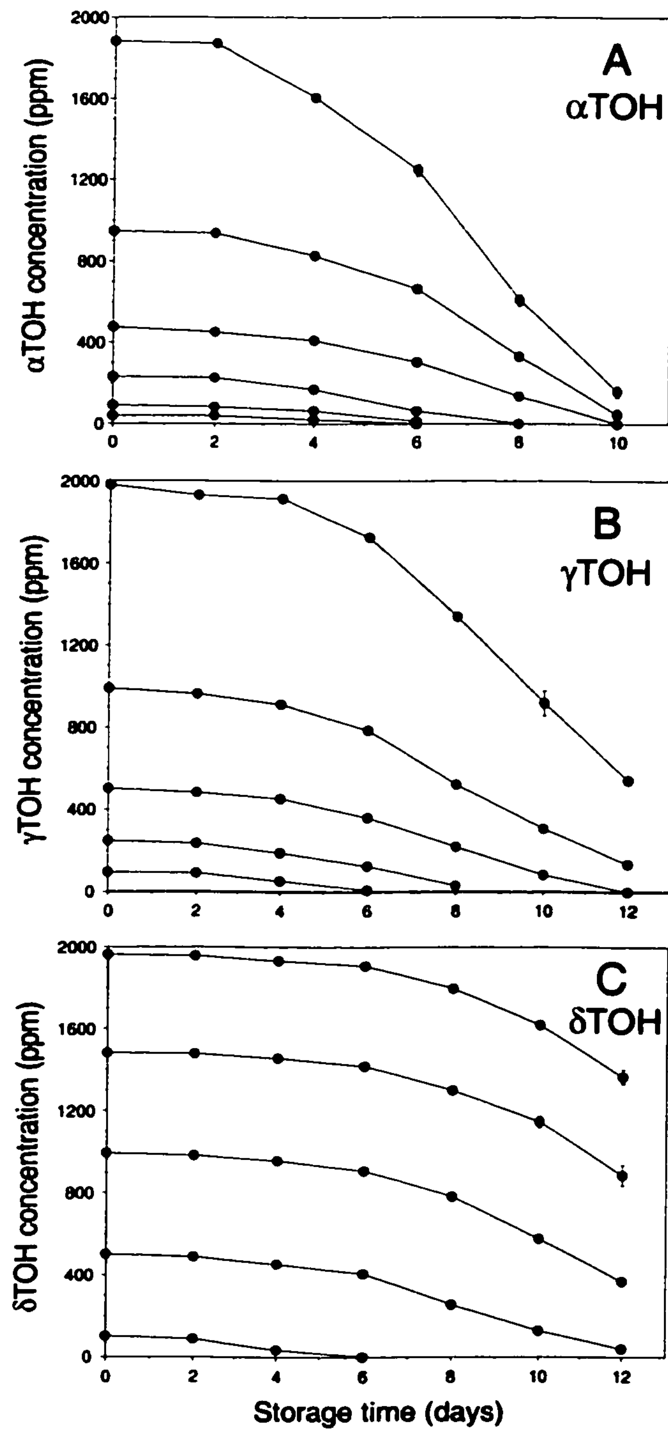


Figure 4.4. Consumption of antioxidant during storage of purified fish oil TAG at 30°C in the dark: (A) αTOH, (B) γTOH, (C) δTOH. Data points are means ± SD (n = 3).

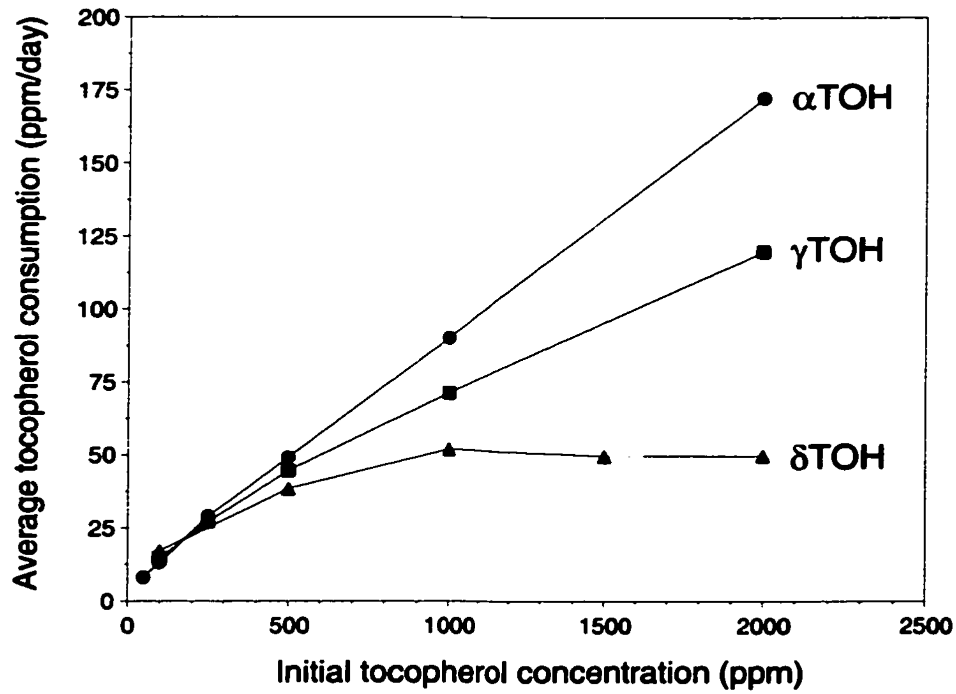


Figure 4.5. Relationships between the average rate of tocopherol consumption and the initial tocopherol concentration in purified fish oil TAG with αTOH, γTOH or δTOH.

The general chemistry of the tocopherols is presented in Section 2.4.1. In summary, the tocopherols retard lipid autoxidation mainly by donating a hydrogen atom to a lipid peroxyl radical, forming hydroperoxides and tocopheroxyl radicals, and subsequent reaction of the TO• with a second lipid peroxyl radical. 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. At low concentrations in the purified fish oil TAG, its high rate of reaction with lipid peroxyl radicals made αTOH a strong antioxidant, while higher levels of γTOH and δTOH were required to adequately inhibit hydroperoxide formation. An αTOH-mediated accumulation of hydroperoxides has previously been explained by the participation of αTOH/αTO• in reactions other than with peroxyl radicals (Marinova and Yanishlieva, 1992a; Kamal-Eldin and Appelqvist, 1996; Fuster *et al.*, 1998). The relative importance of these reactions was discussed in Section 3.3 in relation to the results obtained from the investigation of the properties of αTOH in purified menhaden oil TAG. The assumption that 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. It is considered likely that, in addition to the participation of TO• in side-reactions, a stabilization by αTOH and γTOH of hydroperoxides already formed has also contributed to the observed accumulation of hydroperoxides at high concentrations of these antioxidants.

The αTOH, at concentrations tested in the present work, has previously been found to exert a prooxidant effect in various lipid systems (Jung and Min, 1990; Huang *et al.*, 1994), including fish oils (Bragadóttir *et al.*, 1995; Burkow *et al.*, 1995; Hamilton *et al.*, 1998). No prooxidant effect was observed for any of the tocopherols in the present study. These results support the findings in purified rapeseed oil TAG (Lampi *et al.*, 1999) and

purified sunflower oil TAG (Fuster *et al.*, 1998), 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 the oxidation of triacylglycerols (Lampi *et al.*, 1997). Purification by column chromatography leaves the control oil used in this study devoid of any tocopherol, and the autoxidation is very rapid and with no apparent induction period.

The influence of method used for the evaluation of antioxidant activity on the results that may be obtained is illustrated in Figure 4.6, where the induction periods, as well as the initial rates of formation of conjugated dienes and C3-aldehydes, are plotted versus initial αTOH concentration. Above about 250 ppm αTOH there appeared to be a linear relationship between the initial rate of hydroperoxide formation and the initial αTOH concentration, in accordance with the linear relationship observed between the rate of αTOH consumption and the amount of αTOH added (Fig. 4.5). The induction period, on the other hand, increased in a curvilinear fashion with αTOH concentration, and the extension at high antioxidant concentrations (αTOH > 500 ppm) was small. This can be explained by the high rate of αTOH consumption in this concentration range (Fig. 4.5). The stabilization of hydroperoxides by αTOH and the retardation of C3-aldehyde formation is also apparent from Figure 4.6. The C3-aldehyde curve appears to be a mirror image of the induction period curve, and at relatively high rates of hydroperoxide formation (αTOH > 500 ppm), the inhibition of hydroperoxide decomposition by αTOH was somewhat reduced. This trend was also evident later in the storage period when the C3-aldehyde levels were higher. From these observations it appears that, when αTOH is the only antioxidant added, an αTOH level of 500 ppm is a good compromise between the formation of hydroperoxides and the formation of volatile secondary oxidation products.

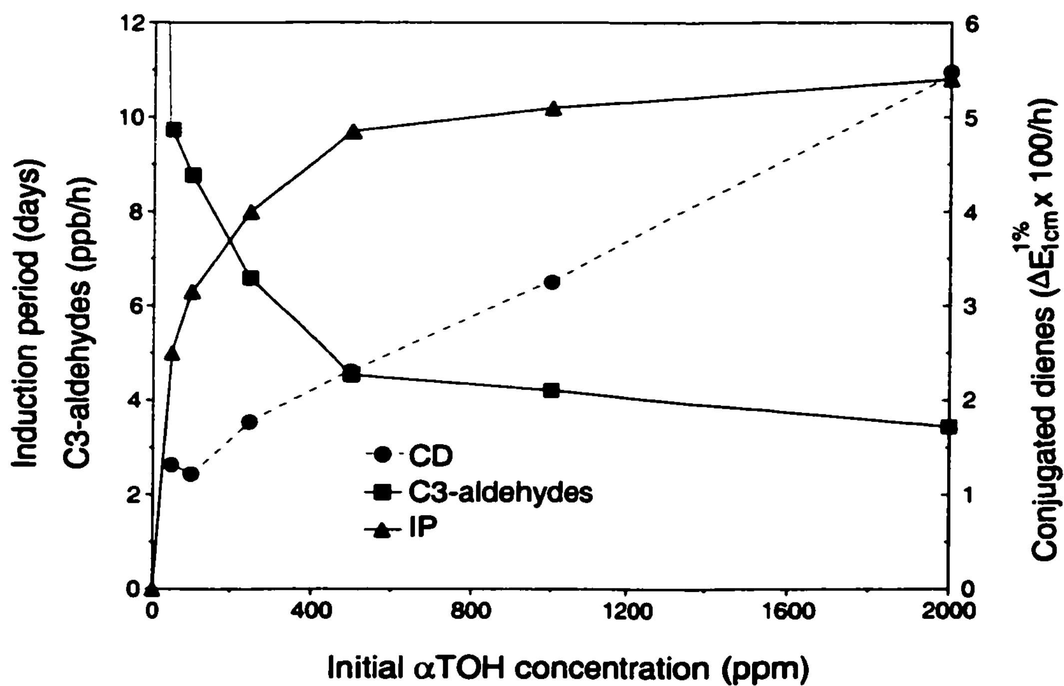


Figure 4.6. Comparison of the induction period (IP) and initial rates of formation of conjugated dienes (CD) and C3-aldehydes in purified fish oil TAG with different concentrations of αTOH. Initial rates of formation of oxidation products were estimated from the measurements obtained after two days of storage.

4.3.2 Tocopherol Mixtures

Formation of hydroperoxides and C3-aldehydes

The rate of formation of hydroperoxides decreased with increasing ratio of (γTOH + δTOH) to that of αTOH (Fig. 4.7). The presence of ascorbyl palmitate (500 ppm) markedly reduced the formation of hydroperoxides in fish oil with 500 ppm αTOH, γTOH and δTOH, and this antioxidant combination was the most effective of those tested at retarding the formation of hydroperoxides (results not shown). A sample with 500 ppm αTOH only was also included in the study, and the rate of formation of hydroperoxides in this system was similar to that in fish oil with 200 ppm αTOH + 650 ppm γTOH + 650 ppm δTOH (results not shown). The addition of more tocopherol, in the form of 500 ppm γTOH and 500 ppm δTOH, to fish oil already containing 500 ppm αTOH, thus had a negative impact on the oil stability on the basis of hydroperoxide formation

The relative effect of the tocopherol mixtures on the rate of formation of volatile secondary oxidation products, as C3-aldehydes, was not in accordance with their relative abilities in retarding hydroperoxide formation. All the tocopherol mixtures tested had a strong inhibitory effect on the rate of formation of C3-aldehydes when compared to purified fish oil TAG without any antioxidant (Fig. 4.8). Due to technical problems, data from day 2 of storage were not obtained. Initially, the formation of C3-aldehydes was slowest in the purified fish oil TAG with 1500 ppm αTOH. In contrast, the tocopherol mixture with the lowest αTOH concentration was the most effective in retarding C3-aldehyde formation later in the storage period. The data for the remaining tocopherol combinations were intermediate and are not included in the graph for legibility reasons. The formation of volatile secondary oxidation products is dependent on the ability of the antioxidant to retard the formation of hydroperoxides, as well as its ability to protect the hydroperoxides from decomposition. The findings in this study suggest that αTOH is the most effective tocopherol homologue in stabilizing the fatty acid hydroperoxides from further reactions. The αTOH in the 1500 ppm-oil was rapidly oxidized during storage,

and as the concentration of hydroperoxides became very high, the degree of inhibition of hydroperoxide decomposition decreased.

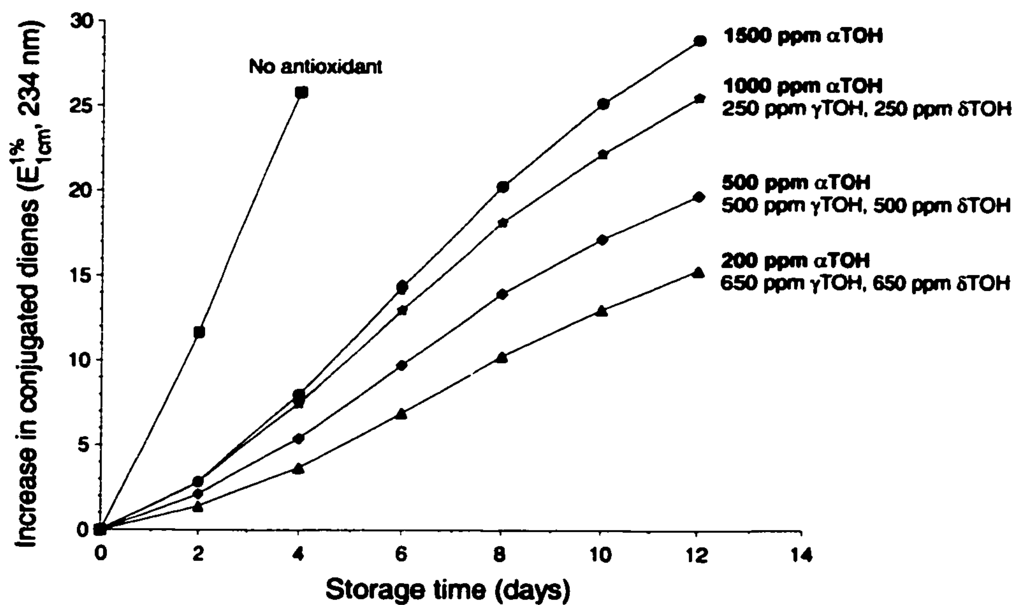


Figure 4.7. Effect of antioxidant composition on the formation of hydroperoxides in purified fish oil TAG with 1500 ppm mixed tocopherols at 30°C. Data points are means ± SD (n = 3). Error bars not visible are smaller than the symbol size.

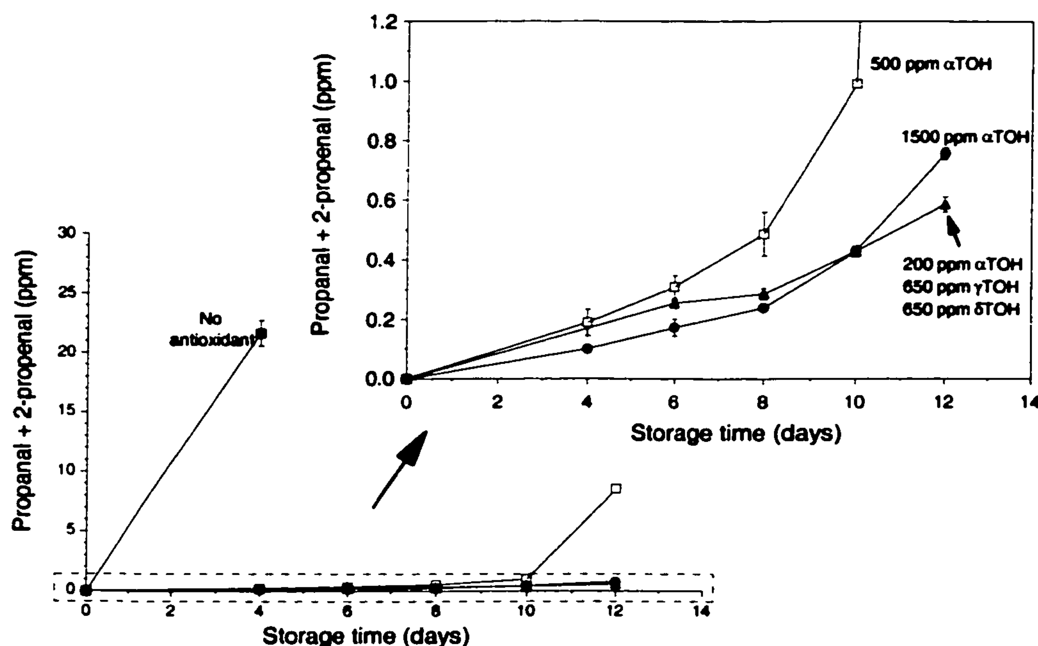


Figure 4.8. Formation of C3-aldehydes in purified fish oil TAG without and with 500 ppm αTOH and 1500 ppm mixed tocopherols during storage at 30°C. Data points are means ± SD (n = 3).

The tocopherol concentration used in this study is somewhat high considering the results obtained when studying the effects of the tocopherols individually, particularly the accumulation of hydroperoxides at high αTOH-levels. On the other hand, tocopherols are often added at concentrations of 1000-2000 ppm in the commercial production of fish oils and n-3 concentrates. Moreover, this level of addition is relevant considering the physiological requirements for vitamin E with high intakes of polyunsaturated fatty acids (Muggli, 1994).

At low initial tocopherol concentrations (≤100 ppm), αTOH effectively retarded the formation of primary oxidation products in purified fish oil TAG (Section 3.3.2 and 4.3.1), and substituting some of this αTOH with the less active hydrogen-donors γTOH and δTOH is expected to reduce the oxidative stability of a fish oil. On the other hand,

the rapid formation of hydroperoxides at high levels of addition of αTOH, makes the substitution of some αTOH with γTOH and/or δTOH increasingly effective at reducing the formation of primary oxidation products.

The determination of residual tocopherols, plotted against storage time for the mixture with 500 ppm of each of the homologues in Figure 4.9, showed that αTOH was consumed more rapidly than γTOH, which again was consumed more rapidly than δTOH. This is in accordance with their relative rates of reaction with lipid peroxyl radicals (Burton and Ingold, 1981). The data presented in Figure 4.9 also shows that complete consumption of αTOH was not a prerequisite for the oxidation of γTOH, and likewise, there was simultaneous loss of γTOH and δTOH from the oil. The somewhat more rapid consumption of αTOH observed when added (at 500 ppm) in a mixture with γTOH and δTOH than when added at 500 ppm as the only antioxidant, is most likely related to this co-oxidation of the antioxidants. Using an ESR technique, Niki *et al.* (1986) showed that αTOH reacted rapidly with γTO• and δTO•, thereby regenerating them from their tocopheroxyl radicals. This suggests that αTOH in the purified fish oil TAG substrate was consumed predominantly in the reactions with lipid peroxyl radicals, but also to some extent by the regeneration of γTOH from γTO•. Buettner (1993) elegantly used the thermodynamic properties of the free radicals to predict an activity (or "pecking") order for the hydrogen atom transfer reactions involved in the protection of PUFA by αTOH and in the regeneration of αTOH by ascorbic acid. In a co-oxidizing situation, their one-electron reduction potentials support a mechanism where γTOH (and δTOH) are regenerated from their tocopheroxyl radicals by remaining αTOH, and not the other way around. The somewhat more rapid consumption of αTOH when added together with γTOH and δTOH found in this study, is in accordance with the findings by Lampi *et al.* (1999) when comparing the antioxidant activities of 500 ppm αTOH, 500 ppm γTOH and 500 ppm αTOH + 500 ppm γTOH in purified rapeseed oil.

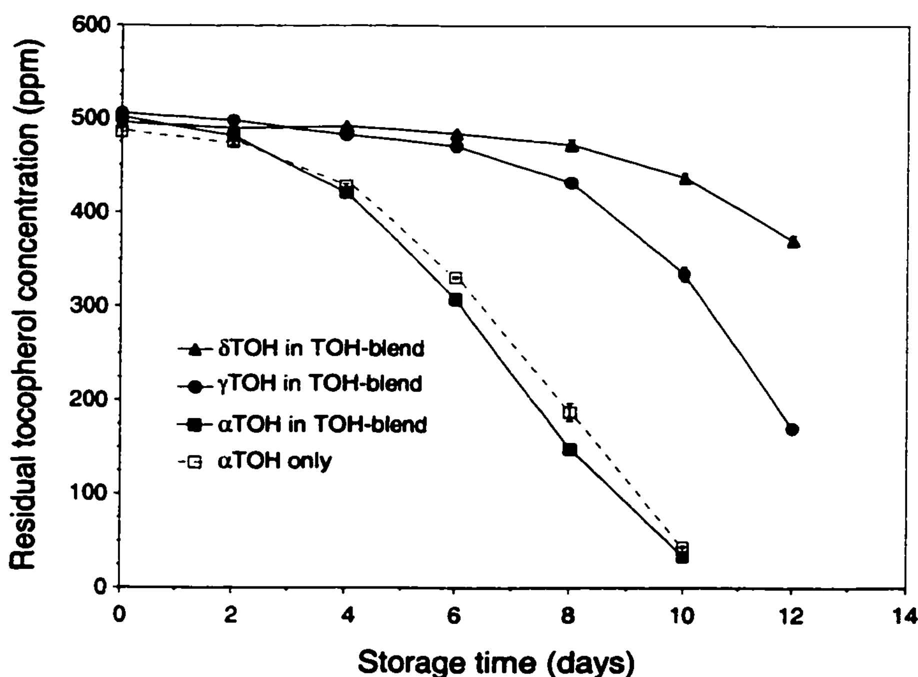


Figure 4.9. Consumption of tocopherols in purified fish oil TAG with 500 ppm αTOH and with 1500 ppm mixed tocopherols (500 ppm αTOH, γTOH and δTOH) during storage at 30°C. Data points are means ± SD (n = 3).

The average rate of consumption (ppm/day) of the different tocopherols in the tocopherol mixtures was calculated from the initial concentrations and the tocopherol remaining at day 10 of storage (Fig. 4.10). At this oxidation stage there was some αTOH left in all the fish oil samples. In accordance with the results obtained when adding αTOH alone to purified fish oil TAG (Fig. 4.5), the rate of αTOH consumption increased markedly with increasing level of addition. As expected from the rapid oxidation of αTOH when added to high concentrations to purified fish oil TAG (Fig. 4.5), and the lack of protection of αTOH by the other tocopherol homologues (Fig. 4.9), the rate of total tocopherol consumption also increased with increasing αTOH concentration in the tocopherol mixtures.

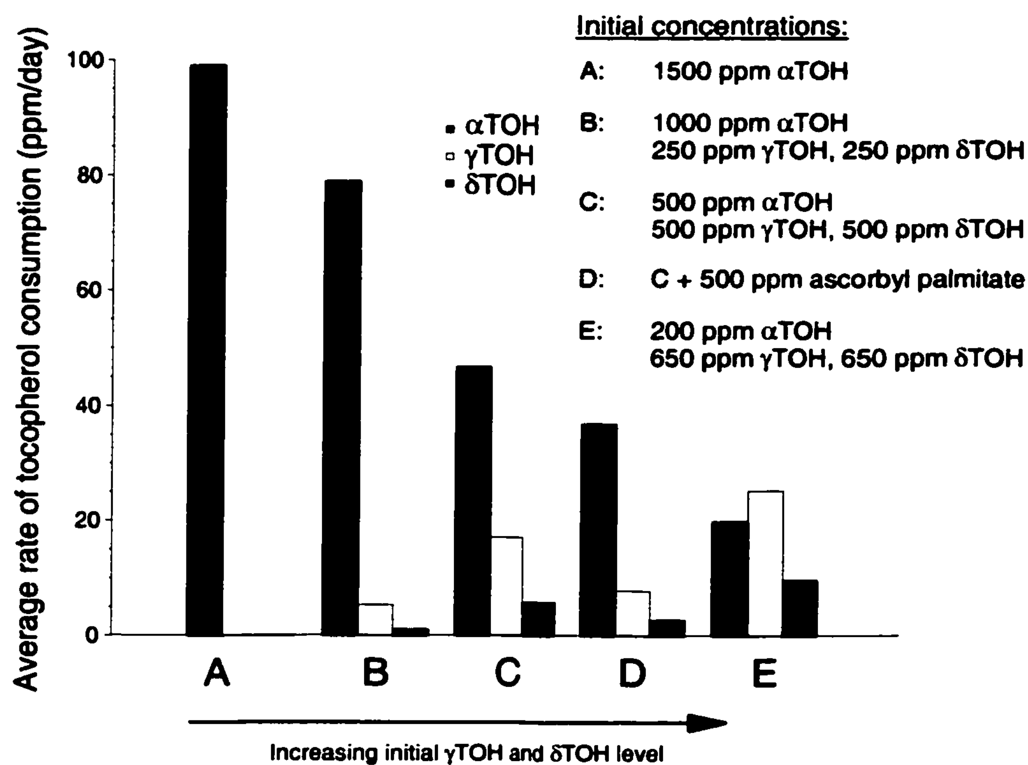


Figure 4.10. Average rate of tocopherol consumption in purified fish oil TAG with 1500 ppm initial tocopherol concentration during storage at 30°C.

The addition of ascorbyl palmitate (treatment D, Fig. 4.10) to the fish oil with 500 ppm of each of the tocopherols (treatment C) appeared to reduce the rate of oxidation of all three tocopherols. The consumption of the three tocopherols during storage of the purified fish oil TAG with and without ascorbyl palmitate is shown in Figure 4.11. In the presence of ascorbyl palmitate, the concentration of γTOH and δTOH was not reduced until late in the storage period. Chemical evidence for the regeneration of αTOH from αTO• by ascorbyl palmitate was presented by Lambelet *et al.* (1985). In contrast, information on the combined antioxidant properties of ascorbyl palmitate and γTOH/δTOH is scarce. The sparing effect of ascorbyl palmitate on γTOH and δTOH observed in this study may be due to a reduced requirement for these when the concentration of the best hydrogen donor, αTOH, remained relatively high, or direct interaction of γTO•/δTO• with ascorbyl palmitate, or a combination of these processes. An increased degree of regeneration of γTOH and δTOH by αTOH may also have contributed to their slower consumption.

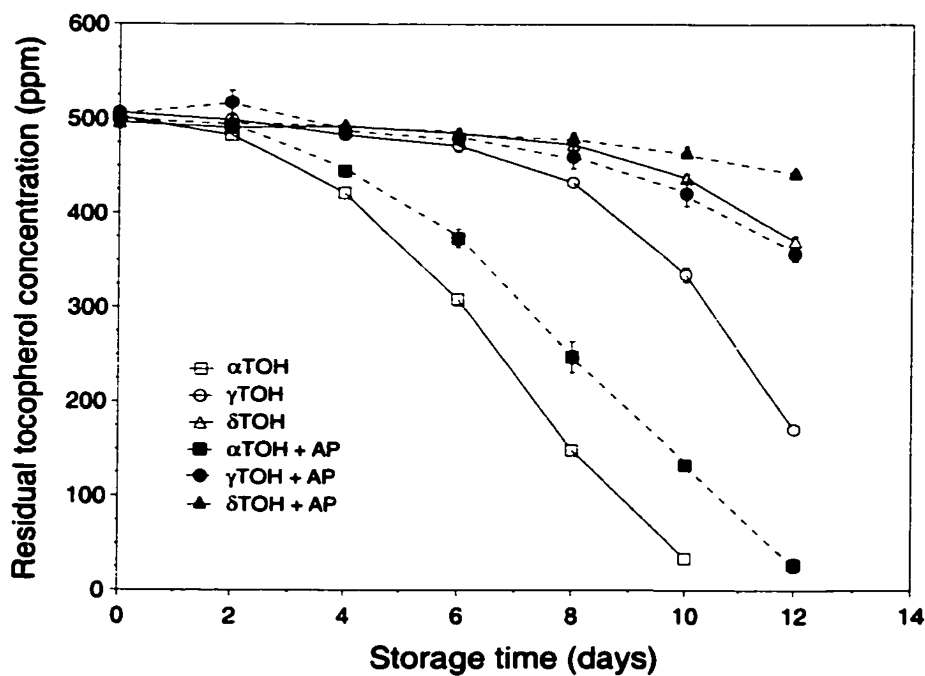


Figure 4.11. Effect of ascorbyl palmitate (AP; 500 ppm) on the consumption of tocopherols in a mixture of αTOH (500 ppm), γTOH (500 ppm) and δTOH (500 ppm) in purified fish oil TAG. Data points are means ± SD (n = 3).

5 Effect of Tocopherols on the Relationship Between Two Methods for Determination of Primary Oxidation Products

5.1 Introduction

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 method Cd 8-53 and Cd 8b-90) for POV determination is a measure of the fatty acid peroxides formed during oxidation. They are based on the measurement of the iodine produced from potassium iodide added as a reducing agent to the sample dissolved in a chloroform-acetic acid (or isooctane-acetic acid) mixture. The iodine is titrated with standard sodium thiosulphate to a starch endpoint, and the result is expressed as milliequivalents of iodine per kg oil (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 as either % conjugated dienoic acid (AOCS method Ti 1a-64) or as the sample extinction coefficient ($E^{1\%}_{1cm}$; IUPAC, 1987). 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 types of fats and oils (White, 1995). The UV absorbance at 268 nm is a measure of conjugated trienes and secondary oxidation products such as conjugated ketodienes and dienals (Noor and Augustin, 1984; IUPAC, 1987).

The CD values measured during oxidation of fats and oils have been found to correlate fairly well with POV (Shahidi *et al.*, 1994; Hahm and Min, 1995; Khan and Shahidi, 2000). 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 αTOH, affect the distribution of oxidation products formed during storage of unsaturated fatty acids (Peers *et al.*, 1981; Karahadian and Lindsay, 1989b; Mäkinen and Hopia, 2000). Hopia *et al.* (1996) 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 objective of this work, when studying the antioxidant effectiveness of α-, γ- and δTOH in fish oil, was 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, it was also aimed at obtaining a relative measure of the contribution from oxidation products other than hydroperoxides in these tests.

5.2 Materials and Methods

Materials

Freshly processed South American Pacific fish oil (refined, deodorized and winterized; EPAX3000TG; POV < 1 meq/kg), with a composition similar to that of anchovy oil, 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 GC according to the procedure described in Section 3.2.2. The initial concentration of αTOH in the oil was 0.12 mmol/kg oil (50 ppm), as determined by HPLC according to the method described in Section 3.2.2. Menhaden oil, refined and bleached, was provided by Omega Protein (Reedville, VA). Immediately upon arrival, the oils were stored at -30°C. 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, whereas the γTOH and δTOH

contained 1.5% and 3.3% of other tocopherols, respectively, as determined by HPLC. All solvents were analytical grade.

The anchovy oil was used without further treatment, whereas the menhaden oil was purified by column chromatography to remove the αTOH originally present, as well as other minor constituents, as described in Section 3.2.1. The purified menhaden oil TAG fraction was practically colourless and odourless, and the results from the characterization (POV, *p*AV, colour, %TAG, iron, copper) of a typical purified batch is given in Table 3.1 (p. 40). No αTOH could be detected in the oil (detection limit 1 ppm). The major fatty acids in a typical purified menhaden oil TAG fraction 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 n-3 (DHA; 13.1%).

Oxidation tests

Two oxidation tests were performed, one comparing the storage stability of fish oils with 2.32 mmol α-, γ- or δTOH per kg oil (carried out at the Norsk Hydro Research Centre), and one testing the storage stability of fish oils with different concentrations of αTOH (carried out at Dalhousie University). Purified menhaden oil TAG samples with 2.32 mmol αTOH/kg oil (1000 ppm), γTOH (967 ppm) or δTOH (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 as described in Section 3.2.3.

Fish oil samples (42 g each for the purified menhaden oil TAG, 75 g each for the anchovy oil) were stored in triplicate in 100 mL uncapped brown glass bottles (9 x 4 cm i.d.) in the dark in an oven at 30°C. The storage time was 32 days for the purified menhaden oil TAG and 23 days for the anchovy oil. The bottles were sampled at regular intervals for CD and POV analysis. The POV was determined according to AOCS method Cd 8-53, based on half of the weight of oil described in that procedure. The 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 at Dalhousie University and a Shimadzu UV-1200 spectrophotometer at the Norsk Hydro Research Centre. The $E^{1\%}_{1cm}$ was calculated from the absorbance reading and the sample oil concentration, and the result is 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 considered different when $P < 0.05$.

5.3 Results and Discussion

The order of antioxidant activity of the tocopherols (2.32 mmol/kg oil; approx. 1000 ppm) in the purified menhaden oil TAG was δTOH > γTOH > αTOH on the basis of both POV and CD measurements (Fig. 5.1). This is in agreement with the expected order of activity based on the study of the properties of the three tocopherols in purified anchovy oil TAG (Section 4.3.1). The addition of more 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 (Fig. 5.2). These findings are in accordance with the αTOH-mediated accumulation of hydroperoxides, at levels of addition above 100 ppm, observed in purified menhaden oil TAG (Section 3.3.2) and in purified anchovy oil TAG (Section 4.3.1).

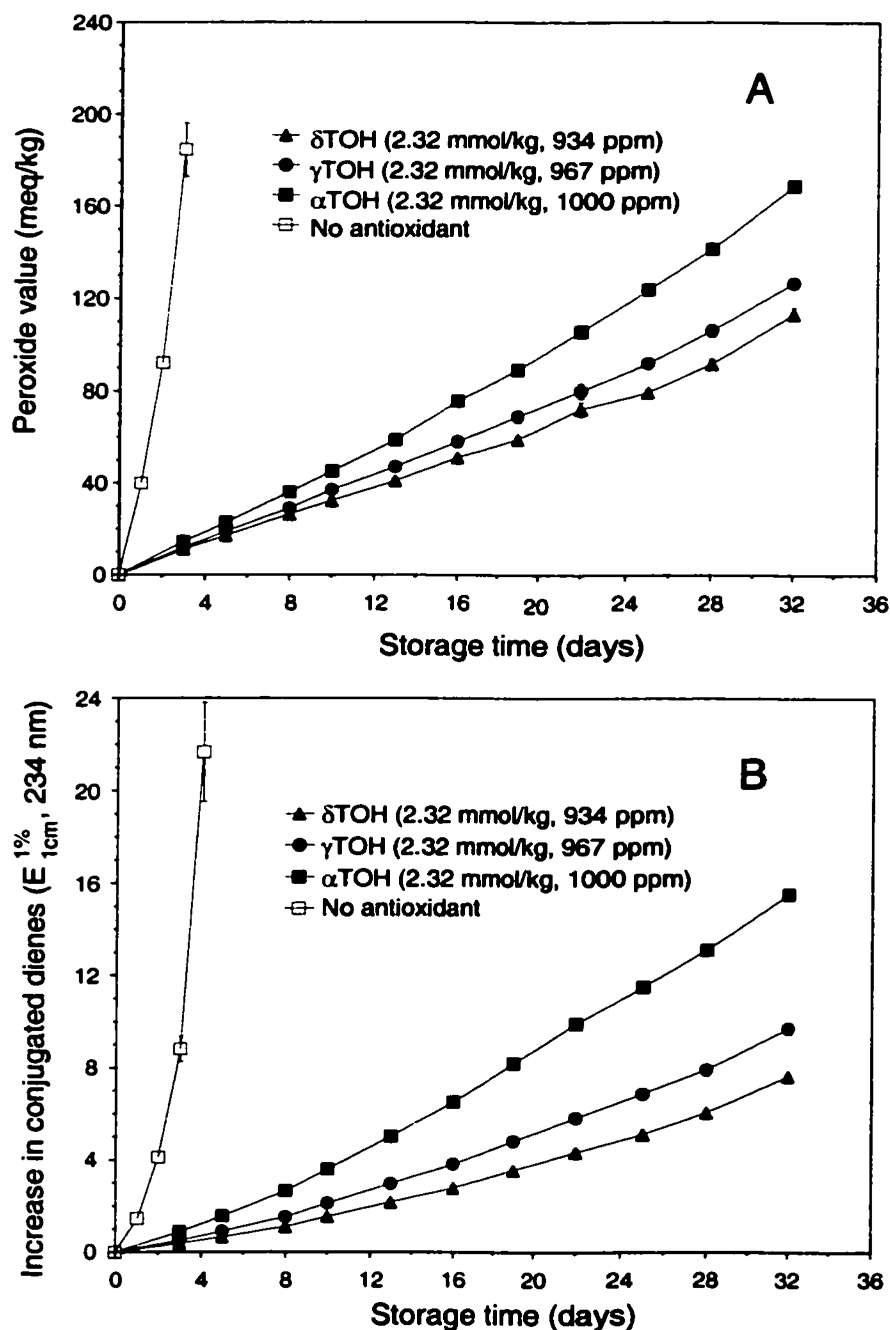


Figure 5.1. Formation of primary oxidation products in purified menhaden oil TAG with and without 2.32 mmol α-, γ- or δ-tocopherol per kg oil: (A) Peroxide value, (B) Conjugated dienes. Data points are means ± SD (n = 3).

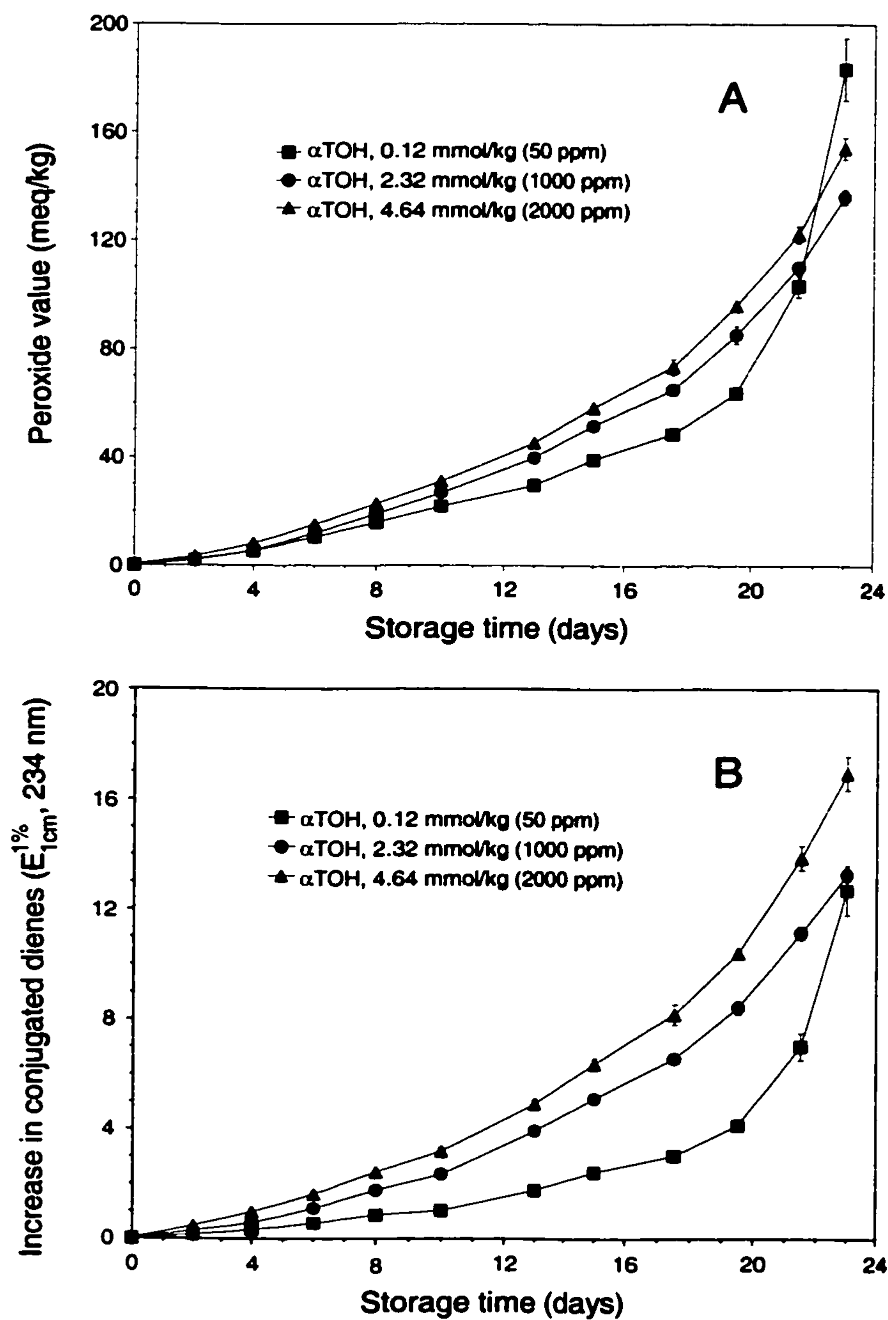


Figure 5.2. Formation of primary oxidation products in anchovy oil with different concentrations of αTOH: (A) Peroxide value, (B) Conjugated dienes. Data points are means ± SD (n = 3).

The POV and CD values measured during storage of the fish oils were strongly correlated (Fig. 5.3 and 5.4, Table 5.1), and linear relationships between the two data sets were found for all tocopherols, as well as the levels of αTOH, tested. The value of the slope of the regression lines, 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 5.1), despite the difference in substrate composition and the fact that the two storage experiments were conducted at different times. Anchovy oil with 0.12 mmol αTOH/kg reached the end of the induction period after 18-20 days of storage (Fig. 5.2). Only POV and CD data points obtained before the end of the induction period were included in the linear regression analysis (Fig. 5.4).

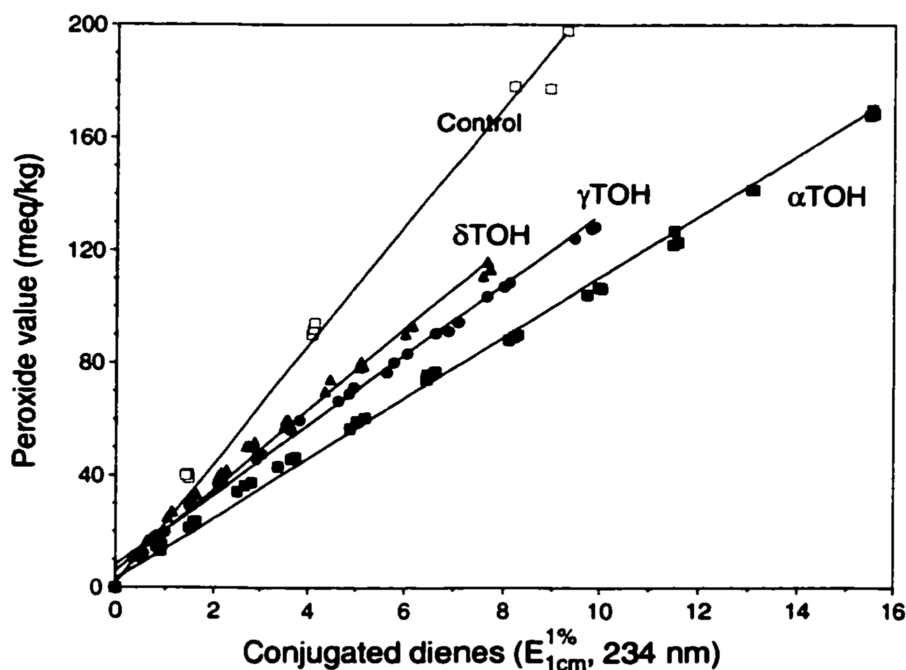


Figure 5.3. Relationships between peroxide value and conjugated diene measurements in purified menhaden oil TAG with and without 2.32 mmol α-, γ- or δ-tocopherol per kg oil.

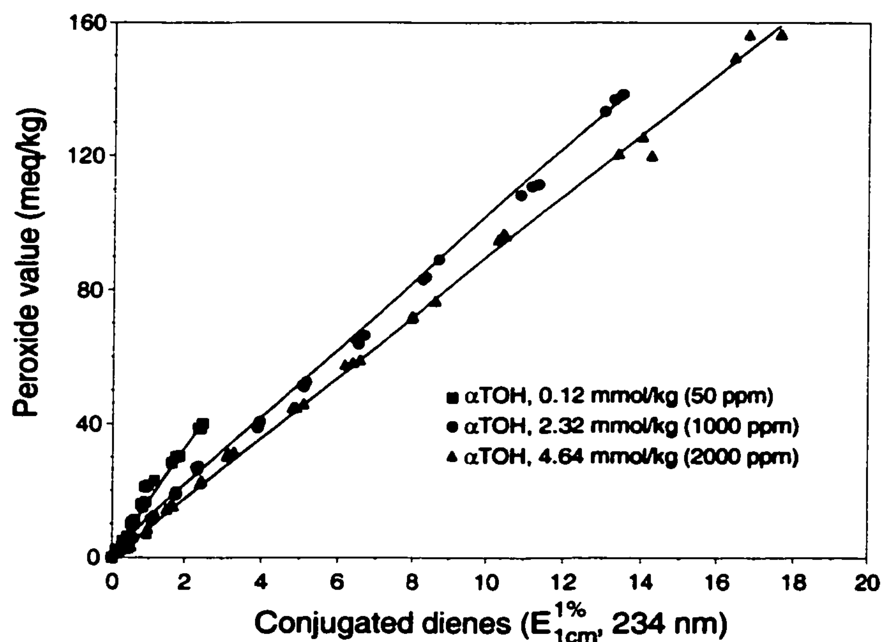


Figure 5.4. Relationships between peroxide value and conjugated diene measurements in anchovy oil with different concentrations of αTOH. Only POV and CD data points obtained before the end of the induction period are included.

Table 5.1 Regression coefficients (± standard error) and coefficients of determination (r^2) from 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 ($P < 0.05$)

The tocopherols are chain-breaking antioxidants and act mainly by donating their phenolic hydrogen to lipid free radicals. 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. In the present study, the value of the slope of the regression lines presented in Figure 5.3 decreased in the order δTOH > γTOH > αTOH. From these results it appears that an increased 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 (Fig 5.4).

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 on tocopherol homologue and concentration, namely fatty acid hydroxy compounds and hydroperoxy epidioxides. 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 hydroperoxides (Grosch, 1987). In addition to the reaction with fatty acid peroxyl radicals, hydrogen-donating antioxidants react with alkoxyl radical intermediates in an analogous way to form fatty acid hydroxy compounds (Pokorný, 1987; Fig. 5.5). In accordance with this view, Hopia *et al.* (1996) found that the decomposition of methyl linoleate hydroperoxides was effectively inhibited by αTOH and Trolox™ with the simultaneous formation of methyl linoleate hydroxy compounds and no hexanal formation. 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 analysed 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 (Frankel, 1998). In a study of the oxidative stability of lipids

in frozen mackerel, Saeed and Howell (1999) recently reported that the fatty acid hydroperoxides formed during frozen storage 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 and similar molar absorptivities as their corresponding hydroperoxides (Chan and Levett, 1977), but do not contribute to the POV. Increased tocopherol hydrogen-donating power may direct the decomposition of hydroperoxides towards the formation of fatty acid hydroxy compounds.

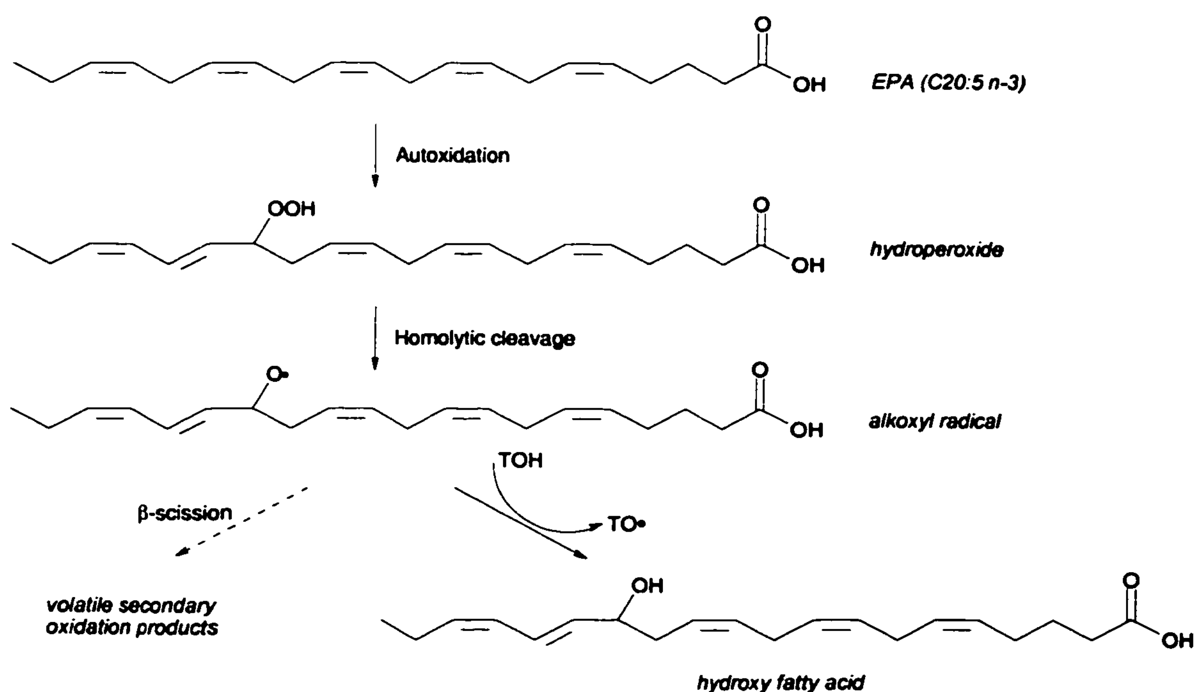


Figure 5.5. Reaction pathway for the formation of a typical hydroxy compound from a hydroperoxide during autoxidation of unsaturated fatty acids.

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, DHA), by rapid intramolecular peroxyl radical

addition to a homoallylic double bond and subsequent reaction with a second oxygen molecule (Porter *et al.*, 1981; Frankel, 1991). This leads to the formation of oxidation products with two peroxide groups per conjugated diene unit. Both peroxide groups are expected to contribute to the POV, the cyclic peroxide unit to a lesser extent than the hydroperoxide group due to a lower reactivity of dialkyl peroxides with iodide ion compared to that of hydroperoxides (Sanchez and Myers, 1996). The hydrogen-donating power of αTOH (concentration 5%) was found to completely inhibit 1,3-cyclization of methyl linolenate peroxyl radicals by trapping the peroxyl radicals as monohydroperoxides (Peers *et al.*, 1981). Based on 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 as shown in Figure 5.6, 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 (Frankel, 1998). Epoxy hydroperoxides are structurally expected to contribute to the POV only, while epoxy hydroxy compounds are structurally 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 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 (Frankel, 1998). It therefore seems likely that the hydroperoxy epidioxides are the most influential of these groups of oxidation products in fish oils as well.

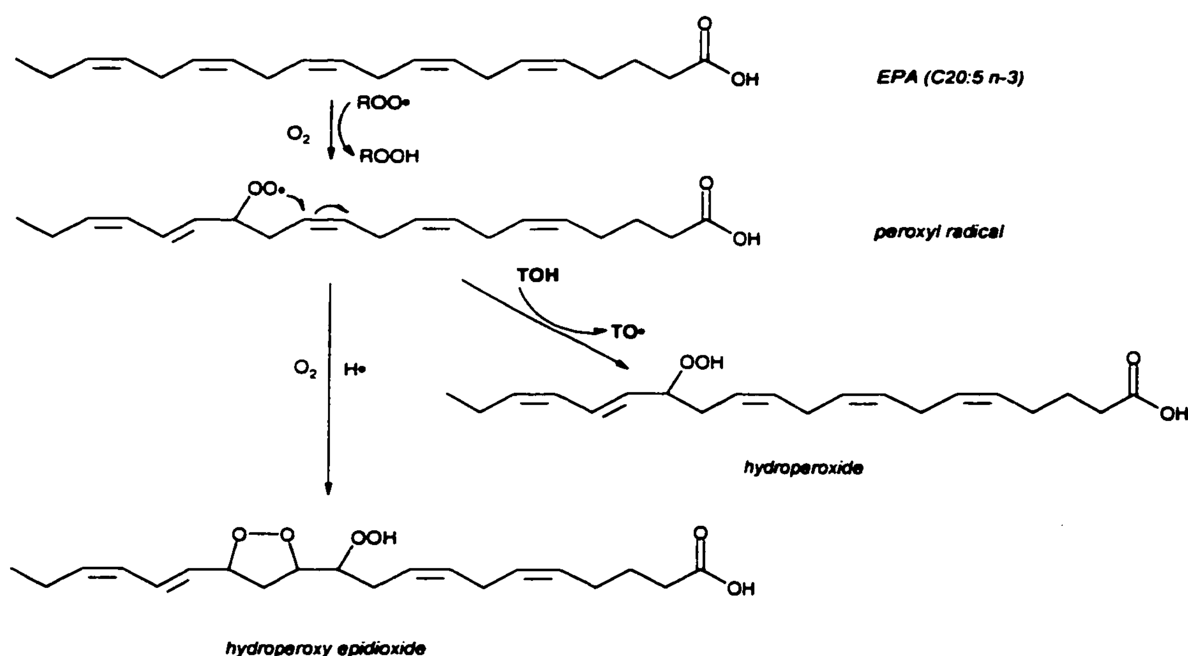


Figure 5.6. Inhibition of formation of hydroperoxy epidioxides by tocopherol hydrogen-donation to peroxyl radicals.

During autoxidation the fish oil fatty acids may cross-link through any of 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 (Frankel, 1998), and it is difficult to predict whether the antioxidant hydrogen-donating power will affect the rate of formation of these relative to that of hydroperoxides. In order to investigate the relative ability of the tocopherol homologues to affect the formation of the different groups of oxidation products discussed, and thereby verify the importance of hydroperoxy epidioxides and hydroxy compounds, a simpler model system such as methyl linolenate could be studied.

According to the interpretation of the findings in the present study, the POV overestimates the concentration of primary oxidation products (hydroperoxides) by being influenced by the level of hydroperoxy epidioxides, whereas the CD method

overestimates the concentration of primary oxidation products by including the fatty acid hydroxy compounds. The relative contribution from the two groups of oxidation products is not known. However, considering the competing reactions available to alkoxyl radicals in addition to hydroxy formation (Gardner, 1987), and the high concentrations of hydroperoxy epidioxides (25% of total peroxides) found in autoxidized methyl linolenate (Neff *et al.*, 1981), the concentration of the hydroperoxy epidioxides is expected to be higher than that of the fatty acid hydroxy compounds.

The magnitude of the differences between the data points for the antioxidant treatments in this study were smaller when POV were used as a measure of primary oxidation products than when CD points were plotted. Autoxidation of polyunsaturated fatty acids results in the formation of complex and dynamic mixtures of unreacted fatty acids and different classes of oxidation products. Most established analytical methods detect only a limited range of these oxidation products. It has previously been demonstrated that the effectiveness of antioxidants may depend on whether primary oxidation products or secondary oxidation products are analysed as a measure of lipid oxidation (Section 4.3.1; Huang *et al.*, 1994 and 1995), and it is therefore essential to use more than one specific method to determine the extent of oxidation. The results from the present study suggest that, in addition to analysing for both primary and secondary oxidation products, tests for antioxidant activity may also benefit from the determination of primary oxidation products by more than one procedure, particularly when studying oils with a high concentration of fatty acids with more than three methylene-interrupted double bonds.

6 Effect of α-, γ- and δ-Tocopherol on the Distribution of Volatile Secondary Oxidation Products

6.1 Introduction

6.1.1 Volatile Oxidation Products Formed in Fish Oils

The many different fatty acid hydroperoxide positional and geometrical isomers that are formed during oxidation of the n-3 PUFA in fish oils give rise to a complex mixture of secondary oxidation products according to the reaction scheme for homolytic cleavage of monohydroperoxides presented in Figure 2.4 (p. 13). Secondary oxidation products from other unsaturated fatty acids present in fish oil, such as C18:1 n-9 and C18:2 n-6, are also expected to be formed, but to a lesser extent. The formation of volatile secondary oxidation products in fish oils has been studied by dynamic headspace analysis by several investigators (Hsieh *et al.*, 1989; Karahadian and Lindsay, 1989a; Rørbæk, 1994), and a variety of different aldehydes, alcohols, hydrocarbons, ketones and short-chain acids have been identified. The hydroperoxide precursors of some of the volatile aldehydes formed from n-3 PUFA are shown in Figure 6.1.

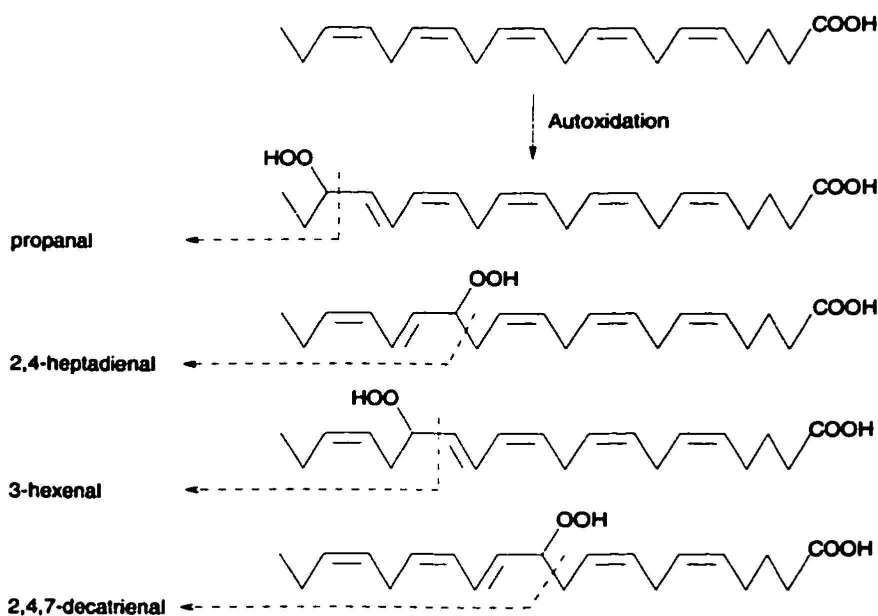


Figure 6.1. Autoxidation sites associated with major aldehydes expected to be formed from EPA.

The formation of many of the volatile secondary oxidation products identified in fish oils, as well as in other fats and oils, cannot be explained by the classical hydroperoxide cleavage mechanism (Fig. 2.4, p. 13). Unsaturated aldehydes and ketones are further oxidized, by autoxidation or α-hydroperoxide formation, and accumulation of saturated and low molecular weight carbonyls is expected during the more advanced stages of oxidation (Frankel, 1998). Nonvolatile secondary oxidation products, such as dimers and oligomers, dihydroperoxides, epoxy hydroperoxides, and hydroperoxy epidioxides, also undergo decomposition, and provide additional sources of volatile compounds.

6.1.2 Flavour Significance of Volatiles in Fish Oil

Oxidized fish oil may be described as painty, rancid, burnt or fishy. The disagreeable flavour is most likely caused by a complex mixture of oxidation products, but certain volatile carbonyls have emerged as the principal contributors.

The low flavour threshold values of unsaturated aldehydes with an n-3 double bond, formed upon autoxidation of n-3 fatty acids, makes these likely contributors to the rapid flavour deterioration of fish oils during storage. Meijboom and Stroink (1972) characterized *t,c,c*-2,4,7-decatrienal as having a whale oil-like flavour and proposed that it was an important contributor to the objectionable fishy flavour of oxidized fish oils and other oils containing long-chain n-3 fatty acids. Ke *et al.* (1975) also identified 2,4,7-decatrienal isomers in severely oxidized samples of mackerel oil. Hsieh *et al.* (1989), using GC-olfactometry, described the odours of two decatrienal isomers as oxidized fish oil-like. Karahadian and Lindsay (1989a) evaluated the combined flavour of the *t,c,c*- and *t,t,c*-isomers of 2,4,7-decatrienal by addition to bland canola oil and found that the flavour progressed from a mild plant-like to a very distinct burnt flavour with increasing concentration. This flavour note was recognized from oxidizing fish oils, and the authors concluded that the 2,4,7-decatrienal isomers are important contributors to adverse flavour formation.

The sensory profile of sand launce oil showed a development from green and fresh fish-like characters, via a trainy (fish oil) odour and flavour into a painty character during storage (Rørbæk, 1994). In an aroma extract dilution analysis based on GC-olfactometry, 1,5-octadien-3-one, characterized by a geranium-like, metallic odour, was suggested to be the most important aroma compound in oxidized fish oil, followed by *c*-4-heptenal, 1-penten-3-one, hexanal, 2,3-butanedione and nonanal. The author did identify 2,4,7-decatrienal in the fish oil samples, but its concentration was too low to be evaluated by GC-olfactometry. 1,5-Octadiene-3-one and *c*-4-heptenal are both extremely potent with flavour threshold values in oil of 0.02 ppb and 0.5-1.6 ppb, respectively (Frankel, 1982).

Karahadian and Lindsay (1989a) monitored the concentration of compounds thought to be associated with fishy flavour development during storage of cod liver oil, and the concentration of the different oxidation products was correlated with the flavour changes in the oil. They concluded that the initially developed green notes were caused principally by *t,c*-2,6-nonadienal, but some green-type notes were also contributed by *t*-2-hexenal and 1,5-octadien-3-one. The formation of *c*-4-heptenal, *t,c,c*- and *t,t,c*-2,4,7-decatrienal resulted in the characteristic burnt/fishy flavour of oxidized fish oil. Hexanal, the 2,4-heptadienal isomers and the 2,4-decadienal isomers were thought to contribute general oxidized, painty flavours to fish oils. Nakamura *et al.* (1980), on the other hand, described 1-penten-3-one as the most noticeable oxidation product in rancid sardine oil.

In a recent study, Hu and Pan (2000) sought to reduce the undesirable rancid odours in fish oil by treating it with lipoxygenase to produce specific volatile compounds *via* position-specific cleavage of hydroperoxides. Undesirable odour components included 2,4-heptadienal isomers, 2,4-decadienal isomers, as well as acetic acid and several unidentified compounds with fishy odour notes. The lipoxygenase treatment preferentially increased the concentration of volatile secondary oxidation products with

odour notes classified as desirable (e.g. fresh fish, melon and oyster) and included *t,c*-2,6-nonadienal, 2,4-octadienal isomers, 3,5-octadien-2-one isomers and 1-penten-3-ol.

In summary, important contributors to the disagreeable flavour development during storage of fish oils appear to be the 2,4,7-decatrienal isomers, *c*-4-heptenal, the 2,4-heptadienal isomers, the 2,4-decadienal isomers, and perhaps also 1-penten-3-one, while 1,5-octadiene-3-one and *t,c*-2,6-nonadienal appear to impart less objectionable green-type notes. Trimethylamine, although contributing fishiness to seafood flavours, is not important in the flavour development in refined fish oils of high initial quality (Karahadian and Lindsay, 1989a). The identification of potent flavour compounds in food lipids depends on the ability of the procedure used to detect components present at very low concentrations. Synergistic or antagonistic effects for mixtures of compounds may also be important (Grosch, 1987). Other secondary oxidation products in addition to those mentioned, or combinations of compounds, may thus also have an impact on flavour formation in fish oils.

6.1.3 Influence of Antioxidants on the Mechanism(s) for the Formation of Volatile Oxidation Products

The antioxidant activity of phenolic antioxidants is highly dependent on their ability to scavenge lipid free radicals. This influences the overall formation of primary and secondary oxidation products, but is also likely to affect the proportion of geometrical hydroperoxide isomers formed, as well the relative formation of nonvolatile secondary oxidation products such as hydroperoxy epidioxides, which again will influence the composition of volatile secondary oxidation products, and possibly flavour formation during storage.

Mechanistic studies have shown that the oxygen addition to pentadienyl radicals to form peroxyl radicals is reversible, and hydrogen-donors affect the relative rates of the forward and reverse reactions (Chan *et al.*, 1982). In the absence of a hydrogen donor, the initially

formed *cis, trans*-peroxyl radical isomerizes to the more stable *trans, trans*-configuration. In the autoxidation of methyl linoleate and methyl linolenate the presence of αTOH reduced the proportion of *trans, trans*-monohydroperoxides, and at a very high αTOH concentration (5%) only *cis, trans*-isomers were formed (Peers *et al.*, 1981). Moreover, Mäkinen and Hopia (2000) recently demonstrated that high levels of αTOH also inhibit the isomerization of already formed *cis, trans* methyl linoleate hydroperoxides.

In the autoxidation of fatty acids with three or more methylene-interrupted double bonds, the peroxyl radicals undergo rapid 1,3-cyclization and further oxygenation to form hydroperoxy epidioxides (Section 5.3). At a very high concentration (5%), αTOH was found to completely inhibit the formation of hydroperoxy epidioxides of methyl linolenate by rapid hydrogen-donation to the peroxyl radicals (Peers *et al.*, 1981). In polyunsaturated fatty acids able to form hydroperoxy epidioxides upon autoxidation, the *cis, trans*-hydroperoxides are not as readily isomerized to the *trans, trans*-configuration as are those of e.g. methyl linoleate, apparently because 1,3-cyclization is favoured over geometric isomerization (Frankel, 1991).

6.1.4 Objectives

The general objective of this study was to assess the relative impact of the different tocopherols on the composition of volatile secondary oxidation products formed in fish oil upon storage. The presence of αTOH has been demonstrated to affect the formation of geometrical isomers of monohydroperoxides as well as the relative formation of hydroperoxy epidioxides when compared to an oil without any antioxidant. The relative rate of tocopherol hydrogen-donation to peroxyl radicals is 1 : 0.68 : 0.28 (αTOH:γTOH:δTOH) (Burton and Ingold, 1981), and γTOH and δTOH may therefore affect the formation of these oxidation products to a different extent compared to αTOH.

According to Karahadian and Lindsay (1989b), the *t,c,c*-2,4,7-decatrienal contributes a pronounced fishy/burnt flavour note in oxidizing fish oils, while the *t,t,c*-isomer exhibits a

more green-fishy character. High concentrations of αTOH directed the formation of the *t,c,c*-isomer. The less active hydrogen donors γTOH and δTOH may influence this ratio in favour of the less flavour potent *t,t,c*-isomer. The formation of several different hydroperoxy epidioxides is possible during the autoxidation of EPA and DHA, and the decomposition of at least some of these may theoretically give rise to a higher proportion of aldehydes with a low molecular weight (C2, C3) compared to the decomposition of the corresponding monohydroperoxides. Unsaturated carbonyls, formed upon cleavage of the hydroperoxides of unsaturated fatty acids, are further oxidized during storage, and short and saturated aldehydes accumulate (Frankel, 1998). It is likely that antioxidants to different extents also retard this process, and, based on their relative hydrogen-donating abilities, αTOH is expected to be a better inhibitor of oxidation of unsaturated carbonyls than γTOH and δTOH.

6.1.5 Principal Component Analysis

A large amount of data is acquired when analysing fish oil samples by dynamic headspace analysis, and such data sets are difficult to evaluate by traditional statistical methods. Principal component analysis (PCA) is a procedure for observing sources of variation in complex data sets (Massart *et al.*, 1997). This is done by reducing the number of variables by making linear combinations of them. The new variables are called latent variables or principal components (PC).

PC1 represents the direction in the data containing the largest variation, i.e. accounts for most information. In a data matrix consisting of n objects (i.e. fish oil samples) for which m variables (i.e. volatile oxidation products) have been determined, the projections of the data points from the original m-dimensional space on PC1 are called the scores (of the objects) on PC1. The PC2 is orthogonal to PC1 and represents the direction of the largest residual variation around PC1. One can continue extracting principal components, containing less and less variation and therefore less and less information, theoretically until m principal components have been obtained. The projections of the data on the

plane of PC1 and PC2 can be computed (or sometimes also on the PC1-PC3 or the PC2-PC3 plane) and shown in a score plot.

The scores on the principal components are weighted sums of the values of the original variables, and these weights are called loadings. The directions of the principal components are thus highly influenced by variables with high loadings. While the relationships among objects can be visualized in score plots, the relationships among the variables can be visualized in two-dimensional loading plots. The interpretation of a loading plot is based on the direction in which the variables lie as seen from the origin of the plot, and two variables are strongly correlated when there is a small angle between the lines connecting them with the origin.

The relative order of tocopherol antioxidant activity in fish oil was previously found to be concentration-dependent. At a low level of addition (100 ppm) the relative order of antioxidant activity was αTOH > γTOH > δTOH on the basis of both formation of hydroperoxides and C3-aldehydes (Section 4.3.1). When increasing the initial αTOH-concentration to above 100 ppm, an increase in the rate of hydroperoxide formation was observed , and at a relatively high level of addition (1000 ppm) the order of inhibition of hydroperoxide formation was δTOH > γTOH > αTOH (Section 5.3). The influence of the different tocopherols on the distribution of volatile secondary oxidation products was therefore studied at two antioxidant concentrations: 100 ppm (low-level) and 1000 ppm (high-level). In addition, the oils were analysed at several stages of oxidation.

6.2 Materials and Methods

This work was carried out at the Norsk Hydro Research Centre, Norway.

Materials

Freshly processed South American Pacific fish oil (refined, deodorized and winterized; EPAX3000TG), with a composition similar to that of anchovy oil, was provided by

Pronova Biocare (Sandefjord, Norway). The oil was purified by column chromatography to remove minor components as described in Section 3.2.1. The purified fish oil TAG was practically colourless and odourless, and the results from the characterization (POV, *p*AV, colour, %TAG, iron, copper) of a typical batch of purified EPAX3000TG is shown in Table 3.1 (purified anchovy oil). No αTOH could be detected in the oil (detection limit 1 ppm). The fatty acid composition of a typical purified oil fraction is given in Appendix A (purified anchovy TAG-2). The *d*-α-tocopherol (purity ≥ 99%), the *d*-γ-tocopherol (purity ≥ 95%) and the *d*-δ-tocopherol (purity ≥ 93%) were all supplied by Acros (Ghent, Belgium). The αTOH product contained no other tocopherols, while the γTOH and the δTOH contained 1.5% and 3.1% of other tocopherols, respectively, as determined by HPLC. Tenax GR (60-80 mesh) and Tenax TA (60-80 mesh) was from Alltech (Deerfield, IL), and Carbosieve SIII (60-80 mesh) was from Supelco (Bellefonte, PA). Dynamic headspace analysis standards of propanal and 2-propenal (acrolein) was from Sigma (St. Louis, MO), 1-penten-3-ol from Aldrich (Stockholm, Sweden) and *t,t*-2,4-heptadienal from Tokyo Kasei (Tokyo, Japan). Soybean oil was obtained from a local supermarket. All solvents were analytical grade.

Oxidation test

Antioxidant was added to the oil samples as previously described (Section 3.2.3). When adding antioxidants, particularly in non-crystalline form, to oil samples, it is difficult to add an exact predetermined amount. In this study, it was important to make sure that any observed difference between oil treatments was due to the properties of the tocopherol homologue itself and not a result of slight differences in the tocopherol concentration at each level of addition. Instead of testing exact triplicate oil samples, the concentrations aimed at were 97, 100 and 103 ppm (nominal concentration 100 ppm) for the low-level samples and 985, 1000 and 1015 ppm (nominal concentration 1000 ppm) for the high-level samples. The actual initial tocopherol oil concentrations, when determined by HPLC, were found to deviate somewhat from the intended amounts, particularly for the high-level samples. They were, however, within the same concentration range, on a

molar basis, at both levels of additions for all tocopherols. The three samples within each concentration range are denoted a "treatment group" instead of replicates. Triplicate samples of a control oil without any antioxidant were also tested.

The fish oil samples (6.0 g) were stored in uncovered Petri dishes (50 mm i.d.) in the dark in an oven at 30°C. Triplicate portions of a control oil without any antioxidant were removed from the oven after two days of storage. Three low-level samples of each tocopherol were removed from the oven on days 2 and 5 of storage, and three high-level samples of each tocopherol were removed from the oven on days 2, 5 and 8 of storage. The oil intended for dynamic headspace analysis (5.1 g) was transferred to 10 mL glass vials which were flushed with N_2 for 6 s, crimp-sealed and kept at -80°C until the end of the storage experiment. The remaining oil from each Petri dish was frozen separately at -80°C until the end of the test, when its tocopherol concentration was determined. Two additional fish oil samples (6.0 g) from each treatment group were included, and these were sampled periodically for the determination, in duplicate, of conjugated dienes.

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 Shimadzu UV-1200 spectrophotometer. The $E^{1\%}_{1cm}$ was calculated from the absorbance reading and the sample oil concentration, and the result is reported as the increase from the measurement obtained at time zero. Tocopherol concentrations were determined by normal phase HPLC with fluorescence detection as described in Section 3.2.2. The HPLC system consisted of a Partisil 5 µm column (11 cm x 4.7 mm; Whatman, Clifton, NJ), equipped with a guard column, and connected to a Waters 715 Ultra Wisp sample processor, a Waters 600E system controller, and a Waters 470 scanning fluorescence detector. Linear regression curves for each of the tocopherols were plotted from the peak response from the analysis of three standard solutions of different concentrations, and the sample tocopherol concentrations were calculated using an Atlas chromatography data collection and processing system (LabSystems, Cheshire, UK).

Dynamic headspace analysis

The volatile secondary oxidation products in the fish oil samples were analysed by dynamic headspace GC-mass spectrometry (MS) at Matforsk (Norwegian Institute of Food Research) at Ås, Norway, with the assistance of Elisabeth Olsen.

Fish oil (4.90 g) with an internal standard of ethyl heptanoate (0.10 g of a 52.6 μg/g soybean oil solution) was weighed into a glass tube, and volatiles were stripped from the oil with N_2 (purity > 99.996%; flow rate 100 mL/min) at 70°C for 20 min after a conditioning period of 10 min at the same temperature. The samples were heated in a water bath holding four samples. The outlet of the glass tube was connected, via a washing bottle head, to a stainless steel adsorbent tube (8.9 cm x 0.6 cm i.d.) which had been sequentially filled with two adsorbents, Tenax GR and Carbosieve SIII (1:1 v:v). The Tenax GR had been found to preferably adsorb volatiles with carbon chain-lengths greater than C7, while the Carbosieve SIII preferably adsorbed the short-chain volatiles (C2-C5) (E. Olsen, personal communication). The adsorbent tubes had been conditioned at 300°C before use. After volatile adsorption, the adsorbent tubes were disconnected, and placed in a Perkin Elmer Automatic Thermal Desorption System (ATD 400) for desorption at 225°C and continuous cryofocusing onto Tenax TA at -30°C for 5 min. The Tenax TA was then rapidly heated, and the volatiles were transferred by the GC carrier gas, helium, to a Hewlett Packard 5890 GC equipped with a DB-WAXetr column (J&W Scientific, Folsom, CA; polyethylene glycol stationary phase, 30 m, 0.25 mm i.d., 0.5 μm film). The temperature program was an initial hold of 10 min at 30°C, a rise to 40°C at a rate of 1°C/min, a subsequent rise to 70°C at a rate of 3°C/min, a rise to 220°C/min at a rate of 6.5°C/min, and a final hold of 6 min. This gave a total GC run time of 59 min. The detector was a Hewlett Packard 5970 mass spectrometer. The volatiles were tentatively identified by comparison of their MS data with those of an on-line computer library (Wiley), except for propanal, 2-propenal, 1-penten-3-ol, and *t,t*-2,4-heptadienal which were identified by their MS data and by comparison of the retention time with that of a standard dissolved in soybean oil and analysed as described for the fish oil samples.

The concentrations of the different volatile oxidation products in the fish oil samples were calculated by comparison of their peak response with that of a known concentration of the internal standard of ethyl heptanoate. The response factors for each component were not determined, and this procedure therefore underestimates the concentration of components with a lower vapour pressure than the internal standard and overestimates the concentration of components with a higher vapour pressure (e.g. propanal). The concentrations are therefore listed as relative ppm.

Statistical analysis

PCA was performed using The Unscrambler™ 7.5 (Camo ASA, Oslo, Norway). The variables were pre-treated using column-standardization (1/standard deviation) to eliminate scale effects.

6.3 Results and Discussion

6.3.1 Volatile Secondary Oxidation Products

The major volatiles found in the oxidizing fish oil were propanal, 2-propenal, 1-penten-3-ol, 2,4-heptadienal isomers and an unidentified compound with retention time 36.7 min (Table 6.1). Proper identification of a volatile oxidation product requires a GC retention time identical to that of a standard, in addition a match with the MS fragmentation pattern of the standard or with that of a library spectrum. The identification by MS was confirmed by the comparison of the retention time with that of a standard for a few compounds only. The tentative MS identification is, however, considered strengthened by the fact that the different volatile oxidation products, with the exception of 1-heptene, are all predicted based on the reaction mechanisms for fatty acid hydroperoxide decomposition (Fig. 2.4, p. 13), further oxidation of unsaturated carbonyls (Frankel, 1982), and/or previous identification in other studies of oxidation of oils high in PUFA by dynamic headspace analysis (Hsieh *et al.*, 1989; Karahadian and Lindsay, 1989a; Rørbæk, 1994). A typical chromatogram from the dynamic headspace GC-MS analysis of the oil is shown in Appendix H. The concentrations of the different volatiles

determined during storage of purified fish oil TAG with an initial γTOH concentration of 1000 ppm is given in Appendix I, as an example of the amounts encountered.

Two peaks were identified as 2,4-heptadienal by their MS data. One of them was identified as the *t,t*-2,4-heptadienal by comparison of its retention time with that of a standard. The other compound identified as 2,4-heptadienal by MS was therefore regarded as *t,c*-2,4-heptadienal. A preferable formation of the *t,c*-isomer was also reported by Karahadian and Linsay (1989a), and this is also in accordance with the preferable formation of the *t,c*-hydroperoxide geometrical isomers of methyl linolenate (Frankel, 1991). The elution of the *t,c*-2,4-alkadienal isomer ahead of the *t,t*-2,4-alkadienal isomer has also been reported in several other studies with similar GC column liquid phases (Hsieh *et al.*, 1989; Karahadian and Lindsay, 1989a).

Previous results from the analysis of volatile secondary oxidation products in fish oil vary quite substantially with regard to the compounds identified, their relative concentrations, as well as the overall level of oxidation. The oxidation products formed are a complex mixture of compounds with different molecular weights and polarities. In addition to the actual concentration of these in the fish oil samples, their recovery by dynamic headspace analysis is influenced by the stripping time and temperature, the properties of the adsorbent, and the properties of the GC column. The identification of short-chain acids is for example likely to require both a relatively high overall level of sample oxidation and the use of a polar GC column. A sampling temperature of 70°C was chosen in this study as a compromise between degree of recovery of the oxidation products and hydroperoxide decomposition. Some thermal hydroperoxide decomposition is expected at this temperature, however, Rørbæk (1997) claimed that fish oil sample degradation in dynamic headspace analysis was of minor importance below 75°C for the sampling time used in the present study.

Table 6.1. Volatile secondary oxidation products found in oxidizing fish oil TAG.

Retention time (min)	Compound	Coding of variable for PCA[a]	Suggestion (MS)
2.28	pentane	C5AN	
2.37	1-pentene	C5EAN	
2.43	2-methyl-1-butene	METBU	
2.96	1,3-pentadiene	PENTDI	
3.24	acetaldehyde	ACAL	
3.61	1-heptene	C7EAN	
4.52	propanal	C3AL	
6.11	2-propenal	C3EAL	
7.16	butanal	C4AL	
9.09	octadiene	OCTDI	2,4-octadiene
9.76	3-methyl-1,4-heptadiene	MEHE	
10.61	ethanol	C2OL	
11.82	2-ethylfuran	ETFU	
13.68	pentanal	C5AL	
17.53	1-penten-3-one	C5EON	
19.52	2-butenal	C4EAL	
23.54	hexanal	C6AL	
24.96	octatriene	OCTRI	1,4,6-octatriene
25.92	2-pentenal	C5EAL1	
27.62	2-pentenal	C5EAL2	
30.60	1-penten-3-ol	C5EOL	
31.38	heptanal	C7AL	
33.31	hexenal	C6EAL	2-hexenal/3-hexenal
34.94	alcohol	ALC1	1-hexen-3-ol/2-buten-1-ol
35.07	unknown1	UNKN1	
36.66	unknown2	UNKN2	
37.66	ethyl heptanoate (i.s.)		
37.93	unknown3	UNKN3	
39.58	nonanal	C9AL	
41.02	alcohol	ALC2	1-nonen-3-ol/1-octen-3-ol
41.17	acetic acid	C2AC	
41.55	unknown4	UNKN4	
41.73	*t,c*-2,4-heptadienal	*c,t*-HEPDI	
42.49	*t,t*-2,4-heptadienal	*t,t*-HEPDI	
42.60	formic acid	C1AC	
42.88	unknown5	UNKN5	
43.23	propanoic acid	C3AC	
44.00	nonadienal	NONDI	
45.18	butanoic acid	C4AC	
45.74	2-decenal	C10EAL	
47.91	2-undecenal	C11EAL	
49.38	hexanoic acid	C6AC	

[a]Coding of the variables in the initial principal component analysis of the data (Fig. 6.7)

The flavour of oxidized fish oil is likely caused by a complex mixture of carbonyl compounds, to which the 2,4,7-decatrienals are considered important contributors. Neither of the two expected 2,4,7-decatrienal isomers were detected in this study, and the concentrations of other diunsaturated aldehydes were also lower than expected from the overall oxidative state of the samples, particularly late in the storage period. Unsaturated aldehydes have been shown to undergo faster autoxidation than the unsaturated fatty acids from which they are formed (Grosch, 1987; Frankel, 1998). The presence of these longer chain aldehydes at concentrations above the limit of detection appears to be dependent on the availability of oxygen, and only by reducing the concentration of oxygen in the system, by purging with nitrogen, was 2,4,7-decatrienal detected as a decomposition product from the 9-hydroperoxide of methyl linolenate (Grosch, 1987). The high fish oil surface-to-volume ratio and the moderate storage temperature used in the present study assured the availability of oxygen, and rapid further oxidation of the 2,4,7-decatrienals is considered the main reason these were not detected. In addition, the temperature (70°C) used for the stripping of the volatiles from the oil in the dynamic headspace analysis may have been too low for enrichment of these to occur on the adsorbent.

Autoxidation at ambient conditions of 2,4-decadienal, one of the major unsaturated aldehydes expected from the autoxidation of n-6 PUFA, was studied by Matthews *et al.* (1971), and the complex mixture of volatiles included hexanal, 2-propenal, butenal, 2-octenal, glyoxal, and 2-buten-1,4-dial. The formation of 2-octenal and glyoxal supported olefinic oxygen attack on the Δ2 double bond, while the formation of hexanal and 2-buten-1,4-dial supported hydroperoxide formation at the Δ4 double bond. Accordingly, the oxidation 2,4-heptadienal affords propanal, 2-propenal, 2-butenal, 2-pentenal, glyoxal and 2-buten-1,4-dial, and according to the reaction scheme supported by the findings of Matthews *et al.* (1971), also 1-pentene/2-pentene, 1-penten-3-ol/2-penten-1-ol and acetaldehyde (Fig. 6.2).

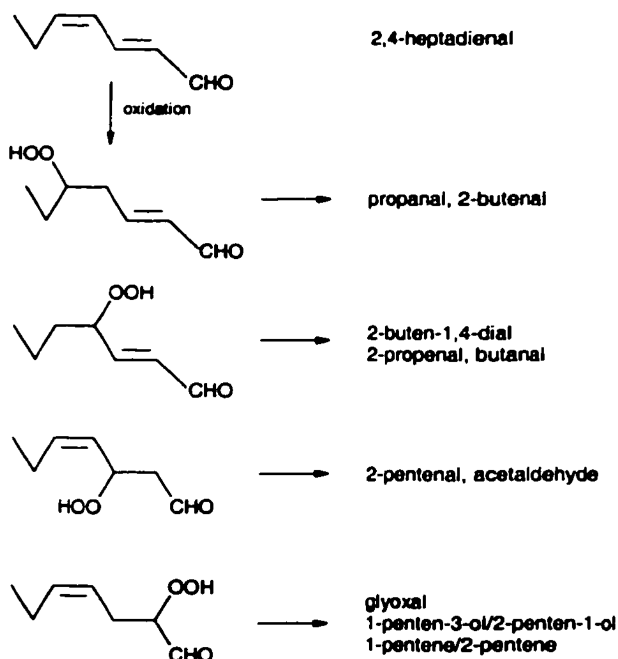


Figure 6.2. Volatile oxidation products expected from 2,4-heptadienal.

In this study, the compound found in the highest concentration relative to the internal standard was 1-penten-3-ol. 1-Penten-3-ol was not identified in a study of the volatile components in menhaden oil (Hsieh *et al.*, 1989), but it was present at a relatively low concentration in cod liver oil (Karahadian and Lindsay, 1989a). It was one of the major volatile oxidation products in moderately oxidized sand launce oil (Rørbæk, 1994) and also present at relatively high levels in stored sardine mince (Yoshiwa *et al.*, 1996). 2-Penten-1-ol is an expected cleavage product from the decomposition of the 15-hydroperoxide of EPA or the 17-hydroperoxide of DHA according to the accepted reaction pathway for hydroperoxide decomposition (Fig. 2.4, p. 13). It is proposed that the 1-penten-3-ol isomer is formed by double bond migration according to Figure 6.3. This is in accordance with the observed formation of 1-octen-3-ol from the 10-hydroperoxide of methyl linoleate (Frankel, 1982). 1-Penten-3-ol is also a likely oxidation product of the unsaturated aldehydes, as shown for 2,4-heptadienal in Figure 6.2, which may account for the high concentrations found. The flavour of 1-penten-3-ol

was described as sharp and irritating by Badings (1970) and the odour (from GC olfactometry) as gluish and sweet by Rørbæk (1994). Its threshold value in oil is relatively high, 4.2 ppm (Badings, 1970).

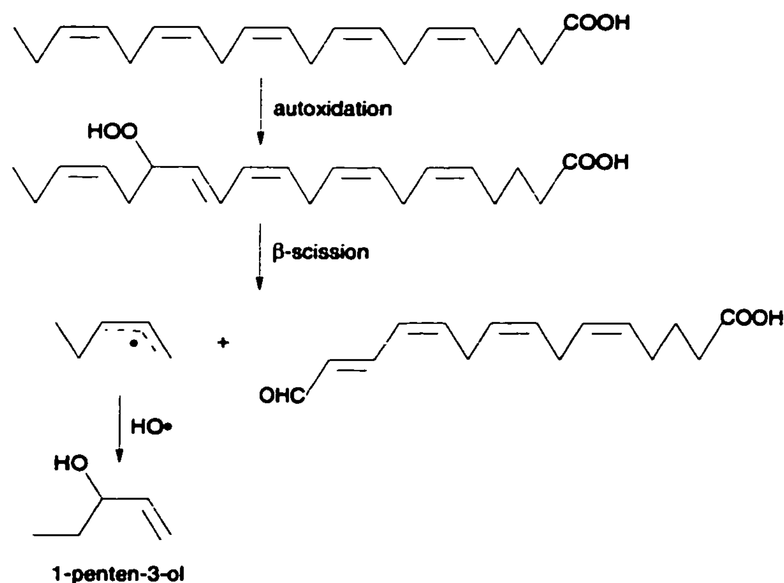


Figure 6.3. Proposed mechanism for the formation of 1-penten-3-ol.

Two of the major peaks in the GC chromatogram (Appendix H) were not identified. It is likely that the compound with a retention time of approximately 51.7 min is an artefact from the equipment used (e.g. plastic liners). Its concentration was not related to the total volatile concentration, and a compound with this retention time had often appeared in the chromatogram when employing this dynamic headspace procedure (E. Olsen, personal communication). The MS pattern recognition algorithm suggested 2-butanone for the compound with t_r 36.7 min, but at a relatively low degree of fragment match with the library spectrum, and 2-butanone was ruled out based on the GC retention time. The molecular peak was missing in the mass spectrum, and this complicated the identification. The possibility exists that the compound is 2- or 3-heptanone or an octadienone. 3-Heptanone was the volatile compound present in the highest concentration in crude

menhaden oil (Hsieh *et al.*, 1989), while 2-heptanone was formed from hydroperoxy epidioxides of photosensitized oxidized methyl linoleate (Frankel, 1982). Unsaturated ketones, such as 3,5-octadien-2-one and 1,5-octadien-3-one, are commonly found in oxidized fish oils, but their concentrations have generally been relatively low, particularly that of the 1,5-octadien-3-one (Hsieh *et al.*, 1989; Karahadian and Lindsay, 1989a; Rørbæk, 1994).

6.3.2 Influence of Tocopherol Homologue on the Distribution of Volatile Secondary Oxidation Products

Formation of hydroperoxides

Purified fish oil TAG with αTOH, γTOH or δTOH was oxidized at 30°C. Figure 6.4 shows the formation of primary oxidation products, as conjugated dienes, during storage of two low-level (nominal concentration 100 ppm) and two high-level (nominal concentration 1000 ppm) oils from each tocopherol treatment. The third oil in each treatment group was not evaluated for the formation of primary oxidation products due to lack of substrate. The control oil without any antioxidant oxidized very rapidly, and all the treatments retarded hydroperoxide formation. At nominal concentration 100 ppm, the relative order of tocopherol activity was αTOH > γTOH > δTOH, in accordance with the results presented in Sections 4.3.1 and 7.3. At nominal concentration 1000 ppm, the order of activity of the tocopherols was reversed: δTOH > γTOH > αTOH, also in accordance with results previously obtained (Section 5.3). It is noteworthy that even when the tocopherol concentration was increased by a few ppm only in the low-level samples, the rate of hydroperoxide formation was markedly reduced, particularly for γTOH and δTOH (Fig 6.4A). When the αTOH and γTOH concentration was high, i.e. above that for inversion of activity determined in Section 4.3.1 (100 ppm for αTOH and 500 ppm for γTOH, of the concentrations tested), a slight increase in the tocopherol concentration caused a somewhat higher rate of hydroperoxide formation (Fig. 6.4B). The addition of a few ppm δTOH to oil with a relatively high δTOH concentration, on the other hand, appeared to have a weak stabilizing effect (Fig. 6.4B). These results also emphasize the

sensitivity of the conjugated diene method for the determination of primary oxidation products.

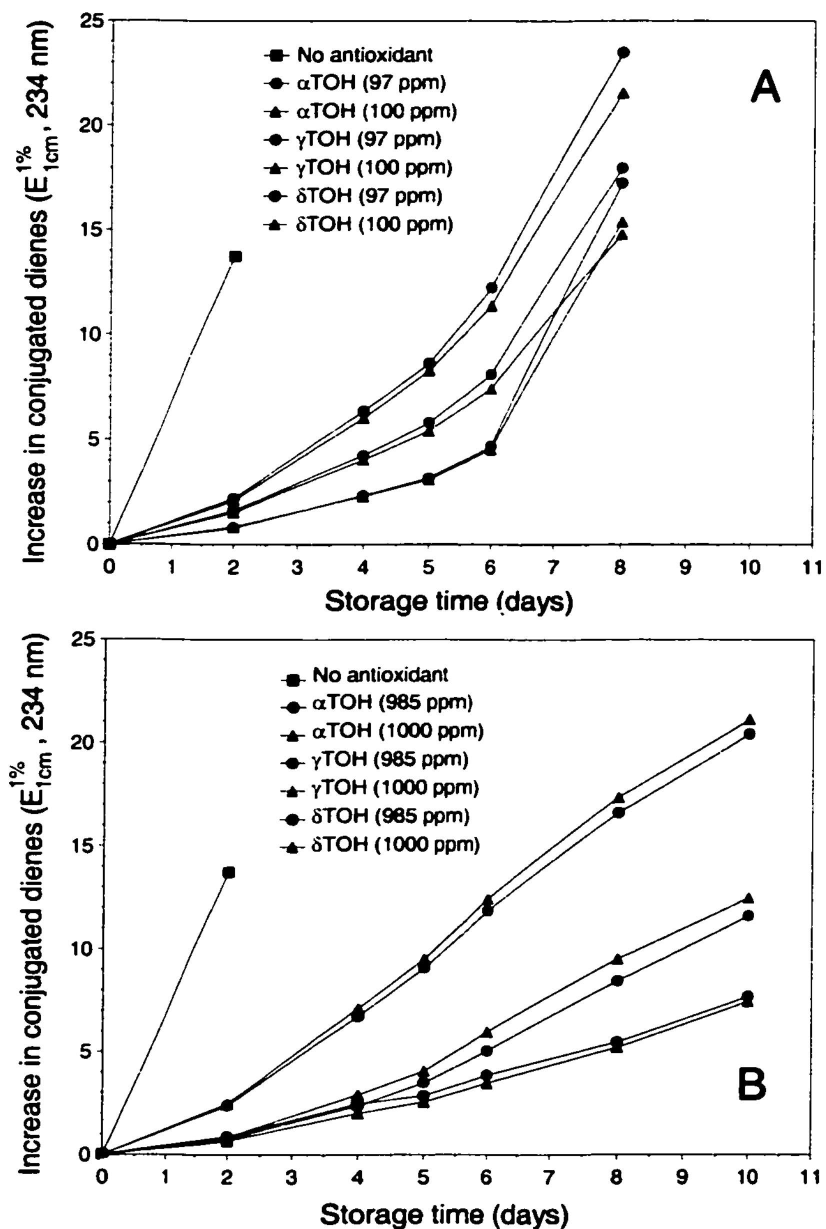


Figure 6.4. Formation of conjugated dienes during storage of purified fish oil TAG with αTOH, γTOH or δTOH at 30°C: (A) Nominal initial tocopherol level 100 ppm, (B) Nominal initial tocopherol level 1000 ppm. Each data point is the mean of two determinations.

Total volatile formation

The sum of the concentrations of volatile oxidation products in the different oil samples, as analysed by dynamic headspace GC, are shown in Figure 6.5. The samples were analysed in random order, and they are listed in the order of increasing initial tocopherol concentration within each treatment group. The result from the analysis of one sample (αTOH, nominal concentration 1000 ppm, day 5) was discarded due to technical problems. The only exact replicates are the three samples without any antioxidant, and the total volatile concentrations in these shows that the precision of the method is somewhat low (%CV = 5.2). The precision of the method appears to be too low to distinguish between samples from the same treatment group. All the treatments retarded the formation of both primary and volatile secondary oxidation products. The relative order of tocopherol activity at the 100 ppm nominal tocopherol level was in accordance with that found for the formation of primary oxidation products: αTOH > γTOH > δTOH. At the 1000 ppm nominal tocopherol level the total volatile concentration was similar for all three tocopherols, except for late in the storage period when it appeared to be somewhat higher for αTOH than for γTOH and δTOH. In order to better illustrate these results, the mean total volatile concentrations for each fish oil treatment group early in the storage period (day 2) were plotted versus the nominal tocopherol concentration (Fig. 6.6). The difference in the ability of the three tocopherols to retard the overall formation of volatile secondary oxidation products appears to decrease with increasing tocopherol level. The rate of formation of volatile secondary oxidation products is influenced by both the rate of formation of the primary oxidation products, and by the ability of the antioxidant to protect the hydroperoxides from decomposition. Due to the superior free radical scavenging properties of αTOH, this antioxidant is likely to be a better inhibitor of hydroperoxide decomposition than the other tocopherols, but as the difference in the concentration of hydroperoxides increases, the ability of αTOH to retard the formation of secondary oxidation products decreases. Late in the storage period of the 1000 ppm nominal tocopherol level samples, the concentration of primary oxidation products was very high in the αTOH samples compared to the samples with γTOH and δTOH (Fig.

6.4B). The high hydroperoxide concentration and low concentration of residual αTOH available for hydroperoxide protection, resulted in a somewhat higher final average concentration of volatile secondary oxidation products than in the samples with γTOH and δTOH. At this stage of oxidation the oils are not suitable for human consumption. None of the fish oil samples analysed by dynamic headspace GC, except for the control samples, had reached the end of the induction period. This was confirmed by the presence of tocopherol, as determined by HPLC.

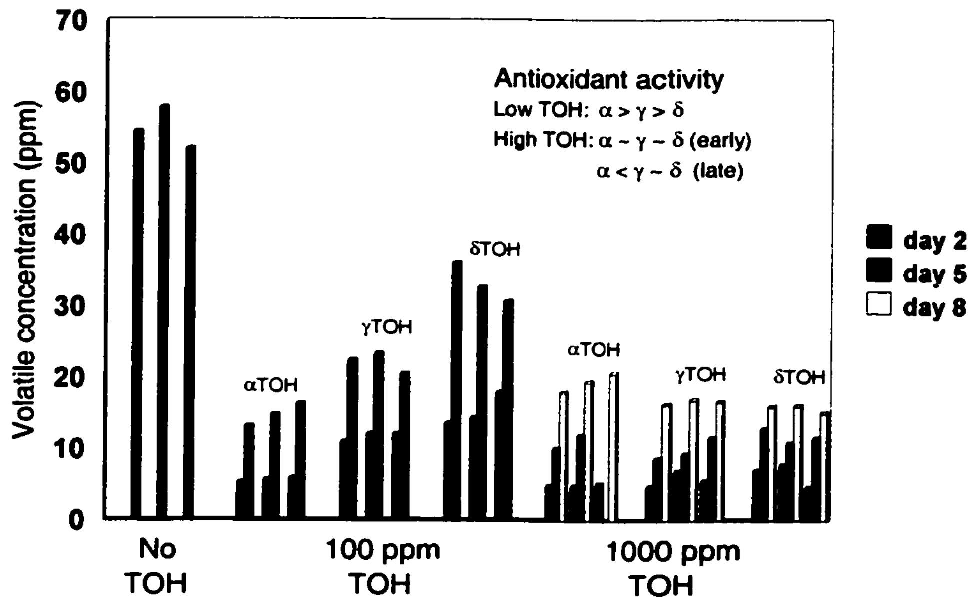


Figure 6.5. Total volatile concentrations in oxidized fish oil with αTOH, γTOH or δTOH as determined by dynamic headspace GC.

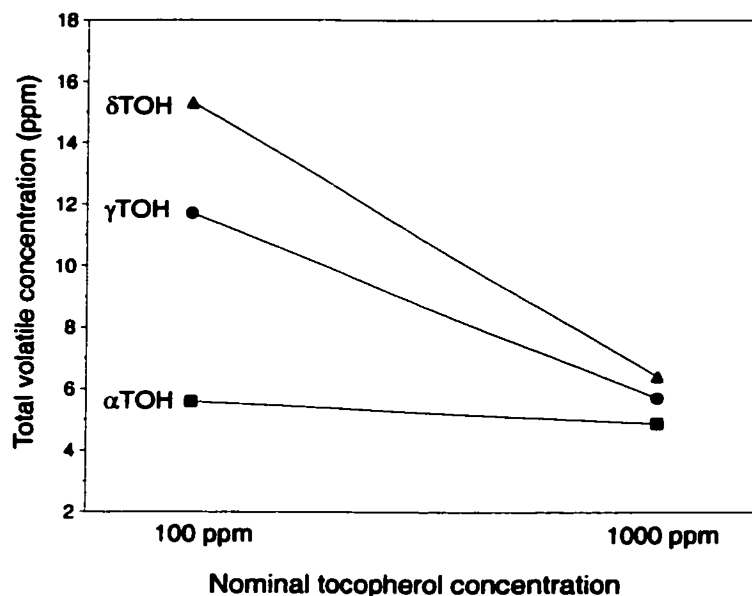


Figure 6.6. Effect of tocopherol concentration on the formation of volatile secondary oxidation products (day 2 of storage) in purified fish oil TAG with αTOH, γTOH or δTOH. The data points are the mean values for each treatment group.

Distribution of volatile secondary oxidation products

The composition of volatile secondary oxidation products in an oil is likely to be influenced by the sample oxidative state, and as antioxidants reduce the rate of oxidation to different extents, samples should ideally be compared after having reached the same overall level of oxidation. This is difficult to achieve, particularly as there is no simple definition of oxidative state. By using PCA to evaluate the results, the impact of the tocopherols on the overall level of oxidation could be separated from the effect of the tocopherols on the composition of volatile oxidation products. Principal component analysis has not previously been employed in a study of the effect of antioxidants on the composition of volatile secondary oxidation products in fats and oils.

The data obtained from the dynamic headspace analysis of the fish oil samples were evaluated by PCA (47 samples and 40 variables). The variable abbreviations are listed in

Table 6.1 (p. 107). The peak corresponding to *t,t*-2,4-heptadienal was not separated from that of formic acid for the most oxidized samples (control + 100 ppm δTOH, day 5), and *t,t*-2,4-heptadienal was therefore not included in this initial data analysis. The samples were coded as follows: tocopherol homologue (aT, gT, dT) or control without antioxidant (C), concentration (10 is 1000 ppm, the remaining are 100 ppm samples) - sample number (1, 2 or 3), days of storage (2, 5 or 8). Sample 1 had the lowest initial tocopherol concentration and sample 3 the highest within each treatment group, except for the control oil. A fish oil sample with an initial nominal αTOH concentration of 100 ppm, having the highest concentration within that treatment group, after 5 days of storage is according to this denoted "aT-3,5". The score plot and the loading plot from the PCA is shown in Fig. 6.7 (p. 125). The PC1 explained the majority (72%) of the variability in the data set and is a latent variable describing the overall volatile formation, i.e. the values of most of the original variables increased along PC1. The concentrations of nonanal and ethanol did not appear to increase with storage time to the same extent as most of the other oxidation products. The PC2 was dominated by some aldehydes (nonanal, 2-decenal and 2-undecenal) present at very low concentrations and only in some of the samples in certain treatment groups, particularly gT10-2,2 and aT10-3,2, and this information dominated over any information in the data set related to the effect of the different tocopherols. To eliminate such within-treatment group-variations, the variables were grouped as shown in Table 6.2 (p. 118), and a new PCA using the grouped variables was performed.

Again, PC1 was an indicator of general size and described the overall oxidative state based on volatile secondary oxidation product formation (Fig. 6.8A and B, p. 126 and 127). Samples with a high score on PC1 had high concentrations of most of the original variables. The sample scores on PC1 are strongly correlated with the total volatile concentrations given in Figure 6.5 (p. 114). As expected, the majority of the variability in the data (88%) was explained by PC1. While PC1 was an indicator of general size, PC2 expressed contrast and described differences in sample volatile composition. The PC2

apparently mainly differentiated between samples with relatively high hydrocarbon levels and samples with relatively high alcohol (mainly 1-penten-3-ol) concentrations (Fig. 6.8B, p. 127), and the PCA indicated that the relative formation of these are influenced by the hydrogen-donating ability of the tocopherol added as antioxidant. High tocopherol hydrogen-donating power (αTOH, high-level) directed the formation of hydrocarbons, while low tocopherol hydrogen-donating power (δTOH, low-level) directed the formation of alcohols. The low-level tocopherol samples, with the exception of αTOH, day 2 of storage, were distributed in the lower right corner of the score plot (Fig. 6.8A, p. 126), and associated with high total volatile formation (PC1) and the formation of alcohol, and to a lesser extent with 1-penten-3-one and with short aldehydes (PC2). When comparing the hydrocarbon and alcohol concentrations in the αTOH-samples (high-level, day 5; high score on PC2) with those in the δTOH-samples (low-level, day 2; low score on PC2), it was found that the hydrocarbon concentrations from the αTOH-treatment were more than twice those in the δTOH-samples. For the alcohol-variable, the reverse was found.

The most oxidized samples, control and 100 ppm δTOH day 5, were not included in this PCA. As mentioned earlier, the concentration of *t,t*-2,4-heptadienal was not recorded in these oils. Due to their high total volatile concentrations, these samples also tended to dominate the PCA score plots and thus mask the effect of the different tocopherol homologues on volatile formation. One of the samples from several of the treatment groups had quite high concentrations of ethanol and 2-ethylfuran, and these samples dominated PC2 in an original PCA of grouped variables. These samples had been analysed consecutively within a few hours on the same day. The glass tubes used for stripping the volatiles from the oil were rinsed with ethanol and subsequently dried in an oven before use. Unusually high ethanol concentrations may be the result of inadequate drying. When ethanol and 2-ethylfuran were excluded as variables, the samples in question behaved similar to the remaining samples in the different treatment groups in the PCA.

Table 6.2. Grouping of volatile oxidation products in PCA.

Group	Compounds included
Short-chain aldehydes	Acetaldehyde, propanal, 2-propenal, butanal, 2-butenal, pentanal, 2-pentenal isomers
Long-chain aldehydes	Hexanal, hexenal, heptanal, 2,4-heptadienal isomers, nonanal, nonadienal, 2-decenal, 2-undecenal
Diunsaturated aldehydes	2,4-Heptadienal isomers, nonadienals
Saturated aldehydes	Acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, nonanal
Ketones	1-Penten-3-one
Alcohols	Alcohol t_r 34.9 min, alcohol t_r 41.0 min, 1-penten-3-ol
Hydrocarbons	Pentane, 1-pentene, 2-methyl-1-butene, 1,3-pentadiene, 1-heptene, 3-methyl-1,4-heptadiene, octadiene, octatriene
Acids	Formic acid, acidic acid, propanoic acid, butanoic acid, hexanoic acid
Unknown	t_r 36.7 min
Total	All volatile compounds

A PCA of the grouped-variable data set including the ratios of diunsaturated/saturated aldehydes, long-chain/short-chain aldehydes and *t,c-/t,t*-2,4-heptadienal, as well as the conjugated diene results and the amount of tocopherol consumed was performed (Fig. 6.9A and B, p. 128 and 129). Only two samples from each treatment group were included due to the limited CD data obtained. The samples were less clustered in the score plot of Figure 6.9A (p. 128) compared to Figure 6.8A (p. 126). The CD value and the tocopherol consumption were strongly correlated and related to the formation of the majority of the volatiles, as described by PC1 (Fig. 6.9B, p. 129). The samples with long storage times had high loadings for these two variables on PC1, particularly those with αTOH, in accordance with results previously obtained (Section 4.3.1). The ratios of diunsaturated to saturated aldehydes and long-chain to short-chain aldehydes decreased upon storage, as unsaturated aldehydes were oxidized to shorter aldehydes with a lesser degree of

unsaturation. The high-level αTOH samples were characterized by high scores for these ratios on PC2. The 2,4-heptadienals are important contributors to both the diunsaturated and the long-chain aldehyde group. The high-level αTOH samples were also characterized by relatively high *t,c-/t,t*-2,4-heptadienal ratios, in accordance with the hypothesized more rapid scavenging of the *t,c*-peroxyl radicals by αTOH compared to γTOH and δTOH. Most of the low-level samples were, as in Figure 6.8A (p. 126), located in the lower right corner of the score plot, having negative scores on PC2, and thus relatively low values for the aldehyde-ratios. The extraction of more than two principle components from the data set (or any of the sub-sets analysed) did not provide any additional useful information, as they described within-treatment group-variations.

Effect of tocopherol homologue

To better understand the impact of the type of tocopherol added on sample volatile distribution, a separate PCA was performed of the samples with 1000 ppm nominal initial tocopherol level (Fig. 6.10A and B, p. 130 and 131). As seen in Figure 6.5 (p. 114), the total volatile concentration increased to about the same extent during storage for the three treatments, with αTOH-samples having a somewhat higher total concentration on day 8 of storage. In the score plot with colour code according to tocopherol homologue (Fig. 6.10A, p. 130), the samples were distributed as three bands along PC2 in the same order as their hydrogen-donating powers, with the exception of sample gT10-1,8, which appeared to be an outlier with an unusually high propanal concentration. In accordance with the results presented in Figure 6.8 (p. 126 and 127), the αTOH samples were characterized by relatively high hydrocarbon concentrations and also relatively high diunsaturated and long-chain aldehydes levels (Fig. 6.10B, p. 131). The δTOH samples, on the other hand, were characterized by preferable formation of the short aldehydes and the saturated aldehydes, particularly propanal. The difference between the treatments increased with storage time. The formation of acids did not appear to be influenced by the type of tocopherol used.

As for the complete grouped-variable data set, a PCA of the high-level tocopherol samples was also performed using ratios of diunsaturated/saturated aldehydes, long-chain/short-chain aldehydes and *t,c-/t,t*-2,4-heptadienal as variables, as well as including the conjugated diene results and the amount of tocopherol consumed (Fig. 6.11A and B, p. 132 and 133). As expected, the CD value and the tocopherol consumption were strongly correlated with each other and with αTOH on PC2, and related to the formation of most of the volatiles as described by PC1. 2-Ethylfuran was also included as a variable in this PCA, and in contrast to the other volatiles, it had a positive loading on PC1, i.e. its concentration tended to decrease upon storage (Fig. 6.11B, p. 133). The PCA also suggested a preferable formation of 2-ethylfuran in the δTOH samples. The αTOH samples were characterized by high scores for the aldehyde-ratios on PC2, in accordance with the PCA results presented in Figure 6.9 (p. 128 and 129).

The results from this PCA were verified by comparing the values for the most important variables from the αTOH-treatment (samples aT10-1,5 and -2,5) and the δTOH-treatment (samples dT10-2,5 and -3,5) after 5 days of storage (Table 6.3). These samples have similar scores on PC1 (Fig. 6.10A, p. 130), and the difference between them detected in the PCA is therefore mainly caused by a difference in the volatile composition.

Table 6.3. Values for variables found to be important in the PCA of high-level tocopherol samples[a].

PCA variable	**αTOH (1000 ppm, day 5)**	**δTOH (1000 ppm, day 5)**
Total volatiles	11.0 ± 0.9 ppm	11.2 ± 0.4 ppm
Hydrocarbons	1.46 ± 0.08 ppm	0.71 ± < 0.01 ppm
Propanal	0.37 ± 0.07 ppm	0.70 ± 0.02 ppm
t,c/*t,t*-2,4-Heptadienal	7.3 ± 1.5[b]	5.0 ± 0.5[c]
Diunsaturated / saturated aldehydes	0.61 ± 0.07	0.29 ± 0.04

[a]The values are mean of two samples ± the difference between the mean and each sample value. Concentrations are relative to the ethyl heptanoate internal standard.
[b]Day 8: 7.5 ± 0.5 (mean ± SD, n = 3)
[c]Day 8: 5.1 ± 0.3 (mean ± SD, n = 3)

Effect of tocopherol concentration

The effect of tocopherol concentration on the distribution of volatile oxidation products was studied in more detail by performing a separate PCA of the samples containing αTOH (Fig. 6.12A and B, p. 134 and 135). The loading plot (p. 135) was similar to that from the PCA of the 1000 ppm tocopherol samples (Fig. 6.11B, p. 133), i.e. decreasing the system hydrogen-donating power by decreasing the αTOH-concentration appeared to affect the volatile distribution in the same direction as decreasing the tocopherol hydrogen-donating power by using a different tocopherol homologue. A high initial αTOH-level was characterized by a higher *t,c-/t,t*-2,4-heptadienal ratio, hydrocarbon concentration and higher levels of long and diunsaturated aldehydes compared to shorter and saturated aldehydes, respectively. The impact of the tocopherol concentration on the distribution of volatile secondary oxidation products in the fish oil appeared to decrease in the order: αTOH > γTOH > δTOH (Fig. 6.9A, p. 128), and separate data analyses of the high- and low-level samples of γTOH and δTOH were not carried out.

The purpose of including the major unidentified peak (t_r = 36.7 min; Appendix H) in the PCAs, was, in addition to relating the formation of this compound to the type of tocopherol added, to obtain information about its identity by comparison of its loadings with those of the other volatile classes. Its PCA-behaviour was similar to that of acids/ketone/total volatiles and thus only to a limited extent influenced by the type of tocopherol.

The ultimate goal in a study like this is to use the findings of the impact of antioxidants on the autoxidation process to make assumptions about (or explain) the overall flavour formation. Many of the volatile secondary oxidation products assumed to be important for adverse flavour formation in fish oil were not identified, and are probably present in very low concentrations. However, based on the results obtained in this study, some general conclusions about the different properties of the tocopherols can be made.

It may be speculated that the preferential formation of hydrocarbons at conditions of high antioxidant hydrogen-donating ability is due to rapid hydrogen-donation to alkyl and vinyl radicals formed upon β-scission of the intermediate alkoxyl radicals during hydroperoxide decomposition (Fig. 2.4, p. 13). Low antioxidant hydrogen-donating power may instead direct the formation of alcohols, as this involves the reaction with a hydroxyl radical instead of hydrogen-abstraction. On the other hand, the formation of secondary oxidation products from PUFA is a very complex process, and other mechanisms/precursors may be of equal or greater importance. The relatively high final hydrocarbon concentrations in the high-level αTOH samples may also be the result of the accumulation of these compounds in the oil, as they are not expected to be further oxidized to the same extent as e.g. the unsaturated carbonyls.

Alcohols and hydrocarbons are quite neutral with regard to their flavour impact More relevant for fish oil flavour is the influence of tocopherol type and concentration on the formation of long-chain and unsaturated aldehydes relative to that of short-chain and

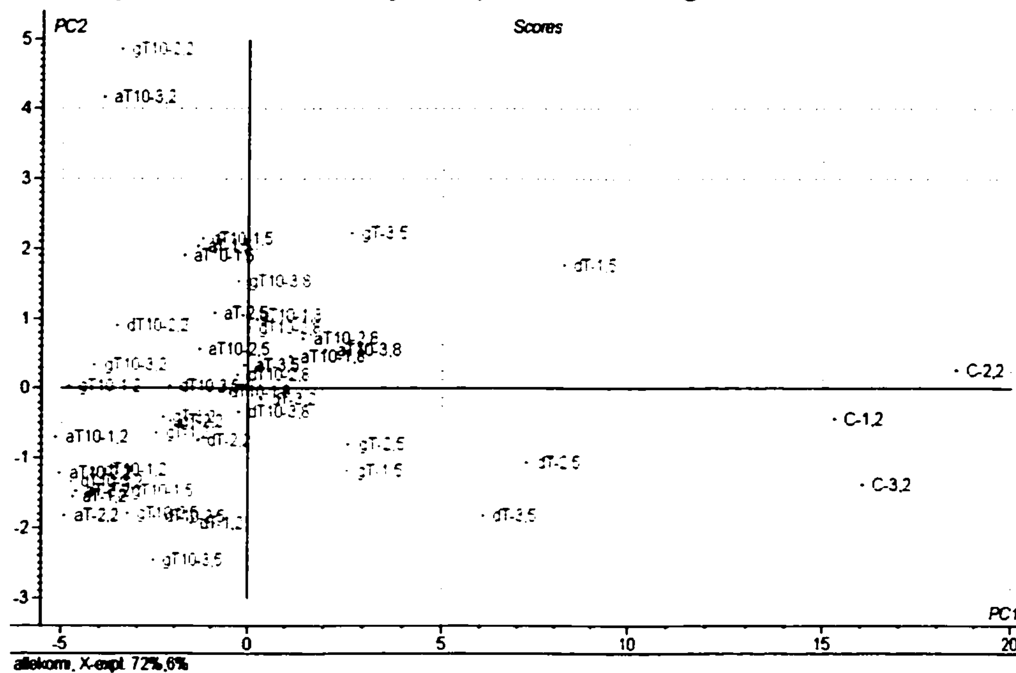
saturated aldehydes, as well as the relative formation of geometrical isomers of unsaturated aldehydes. In this study, the PCA suggested that the formation of *t,c*-2,4-heptadienal increased relative to the *t,t*-isomer with increasing hydrogen-donating power of the tocopherol, as well as with αTOH concentration. This is in accordance with what was expected based on the observed effect of αTOH on the formation of methyl linoleate and methyl linolenate hydroperoxides geometrical isomers (Peers *et al.*, 1981). It is considered likely that the tocopherols influence the relative formation of the geometrical isomers of other unsaturated aldehydes expected to be formed in fish oils, particularly the 2,4,7-decatrienals, in the same direction. Karahadian and Lindsay (1989b) found that *t,c,c*-2,4,7-decatrienal contributed a more disagreeable flavour note to fish oil than its *t,t,c*-isomer. *c*-3-Hexenal and *c*-4-heptenal are also much more flavour intense than the corresponding *t*-3-hexenal and *t*-4-heptenal isomers (Frankel, 1982).

The many routes suggested for the formation of lower saturated aldehydes have been reviewed by Frankel (1982). In addition to being formed by the classical hydroperoxide cleavage mechanism, aldehydes are produced from the decomposition of shorter primary hydroperoxides, formed upon oxygen addition to an alkyl or vinyl radical (Fig. 2.4, p. 13). Unsaturated aldehydes with methylene-interrupted double bonds are expected to oxidize further *via* the formation of pentadienyl radicals. In addition, α-hydroperoxidation of 2-alkenals/2,4-alkadienals/2,4,7-alkatrienals yields shorter aldehydes with fewer double bonds, together with dialdehydes. Oxidative degradation of aldehydes may also proceed through a peracid intermediate with simultaneous formation of formic acid. In this study, the relative formation of lower saturated aldehydes was dependent on both the type of tocopherol added as antioxidant and the αTOH concentration, and increased with decreasing antioxidant hydrogen-donating power. This suggests that tocopherol hydrogen-donating power influences the oxidative degradation of unsaturated aldehydes (and/or decomposition of primary hydroperoxides). The oxidation of unsaturated aldehydes may also produce 1-penten-3-ol (Fig. 6.2, p. 109), and this pathway may explain the high levels of this alcohol encountered in all the samples, and at conditions of

low hydrogen-donating power in particular. An expected preferable formation of hydroperoxy epidioxides, as well as bicycloendoperoxides, in the presence of γTOH and δTOH may also have contributed to the directed formation of the short and saturated aldehydes.

The results from this study suggest that αTOH, especially at high levels of addition, directs the formation of the more flavour-potent aldehyde geometrical isomers and of the more flavour-potent unsaturated aldehydes compared to γTOH and δTOH. These findings should be tested by sensory analysis. Of particular importance is the relation between the chemical findings and sensory data of fish oils and the storage time of their suitability for human consumption. In the PCAs performed in this work, the variation in the data related to volatile composition (explained by PC2) was generally much smaller than the variation in the data related to overall volatile concentration (explained by PC1). At low tocopherol levels, both the rate of formation of hydroperoxides and the overall rate of formation of volatile secondary oxidation products were highly dependent on the type of tocopherol added as antioxidant, and the antioxidant activity decreased in the order αTOH > γTOH > δTOH. Any influence of tocopherol type on the distribution of the volatile secondary oxidation products is considered to be of minor importance compared to the effect of the tocopherol added on the overall oxidative state of the oil. At a high initial tocopherol level the tocopherols also inhibited the formation of hydroperoxides to different extents, but the order of activity was reversed. The overall rates of formation of volatile secondary oxidation products, on the other hand, were similar for the three tocopherols. The influence of the particular tocopherol used on the distribution of the volatiles formed may therefore be of greater importance for flavour formation at high tocopherol concentrations.

Score plot: Colour code by tocopherol homologue



Loading plot

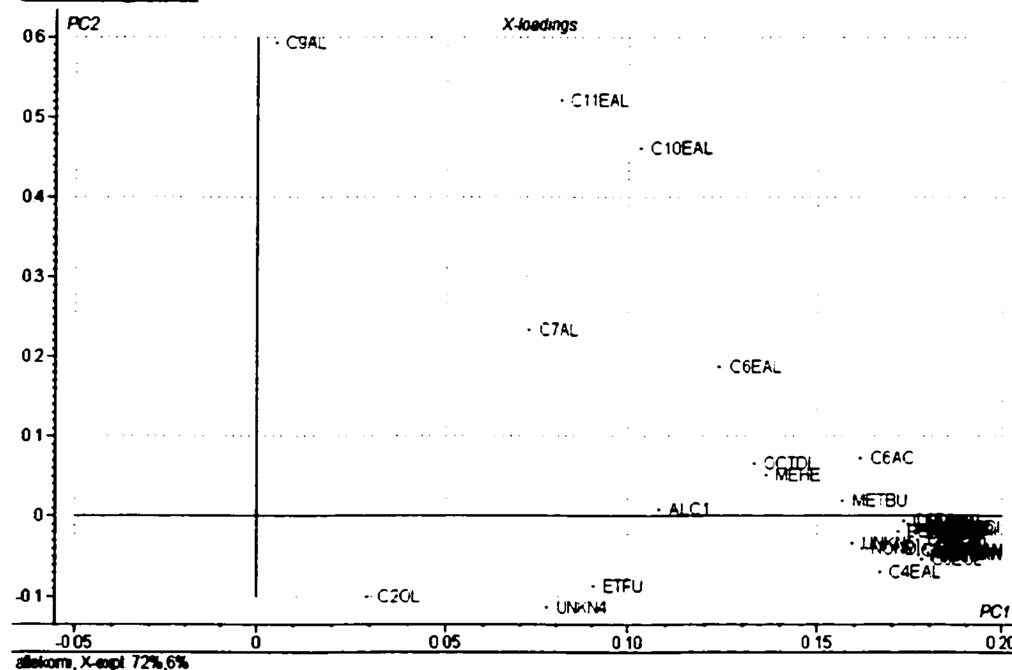


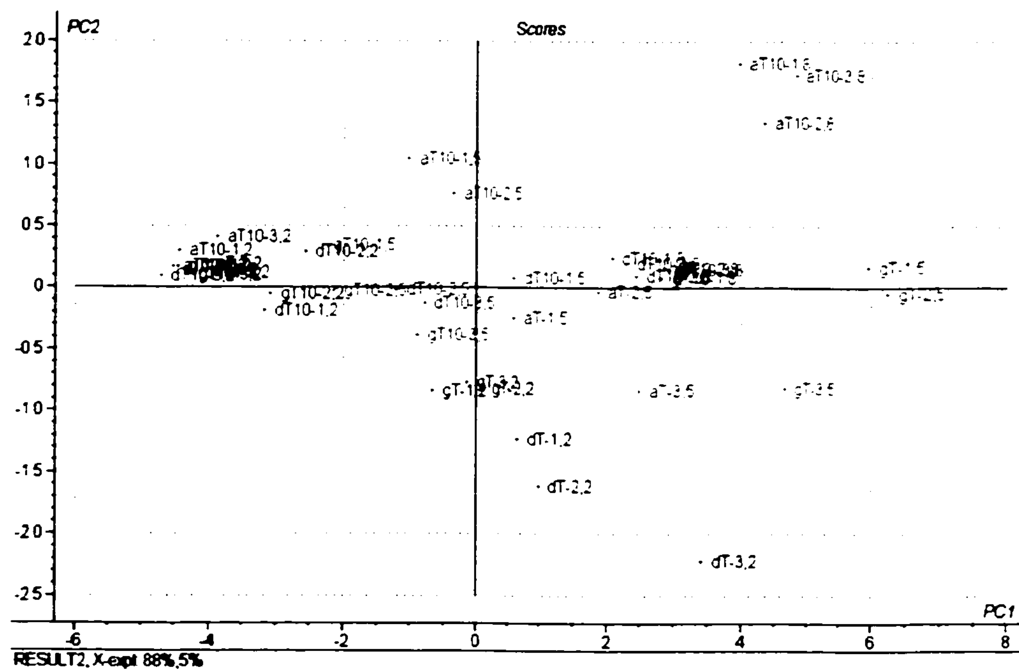
Figure 6.7. PCA of volatile secondary oxidation products formed during storage of purified fish oil TAG: Score plot and loading plot.

Score plot: Colour code by tocopherol homologue

Score plot: Colour code by tocopherol level

Figure 6.8A. PCA of grouped variables: Score plots. Control and 100 ppm δTOH, day 5 samples excluded.

Score plot: Colour code by storage time



Loading plot

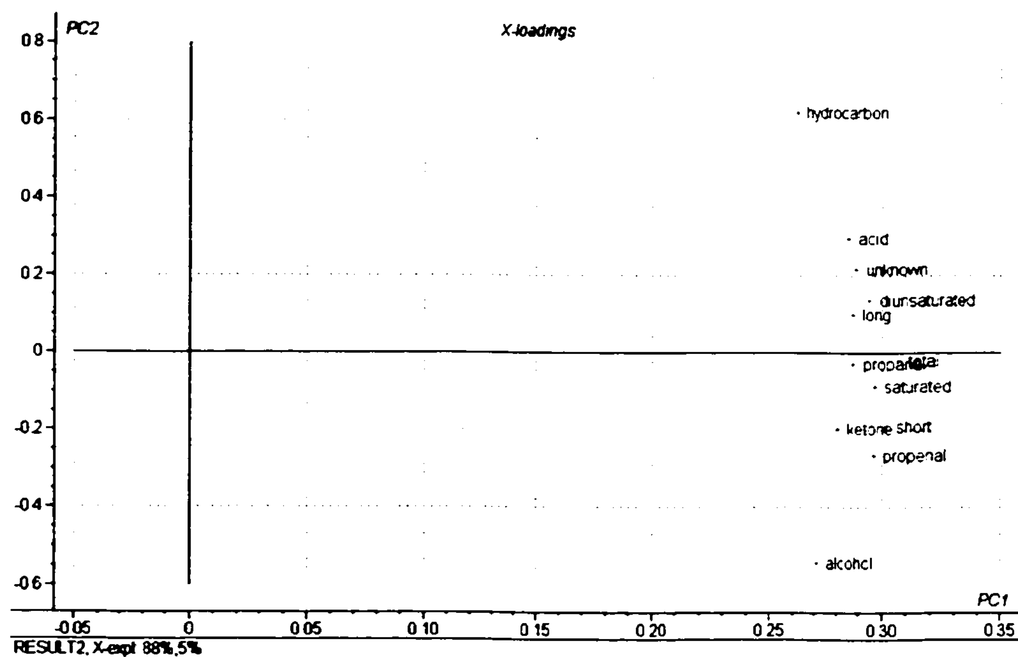
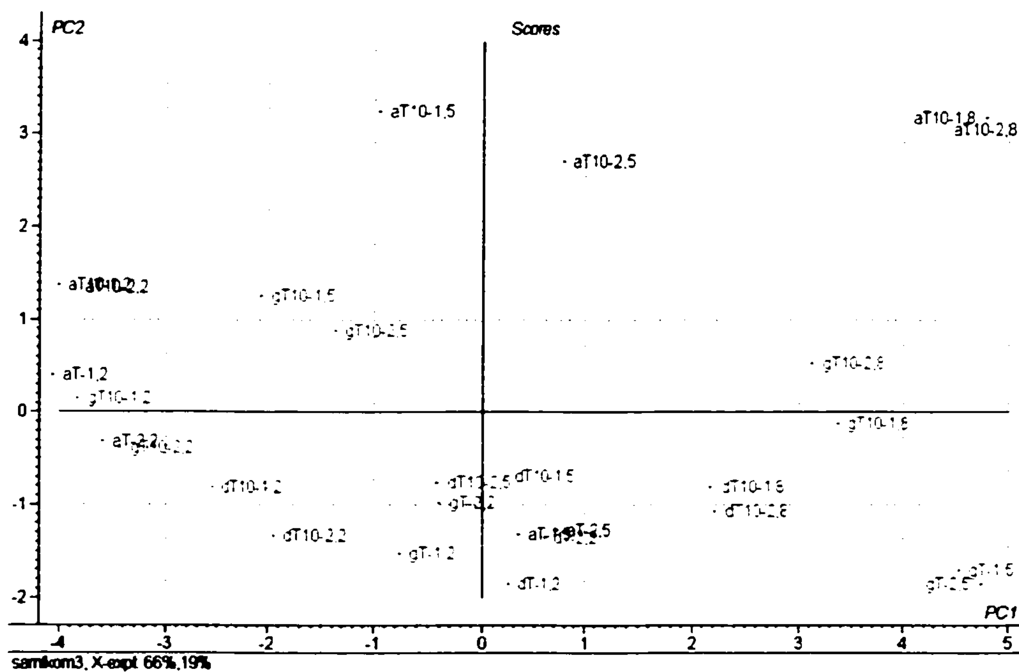


Figure 6.8B. PCA of grouped variables: Score plot and loading plot. Control and 100 ppm δTOH, day 5 samples excluded.

Score plot: Colour code by tocopherol homologue



Score plot: Colour code by tocopherol level

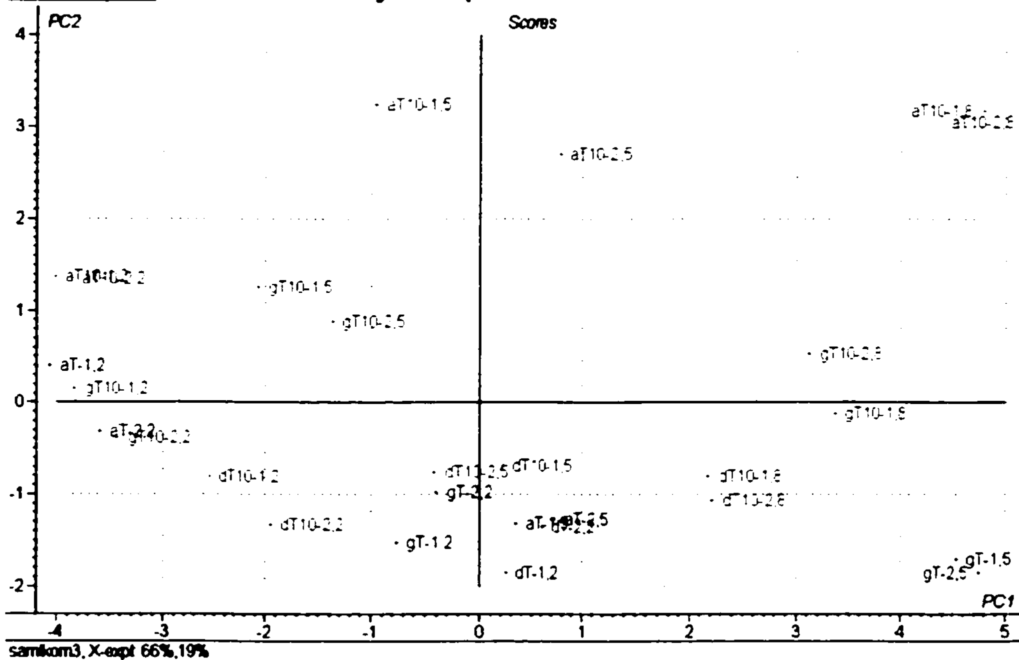


Figure 6.9A. Score plots from the PCA of grouped variables, including ratios between groups, conjugated dienes (CD) and tocopherol consumption (tocloss). Control and 100 ppm δTOH, day 5 samples excluded.

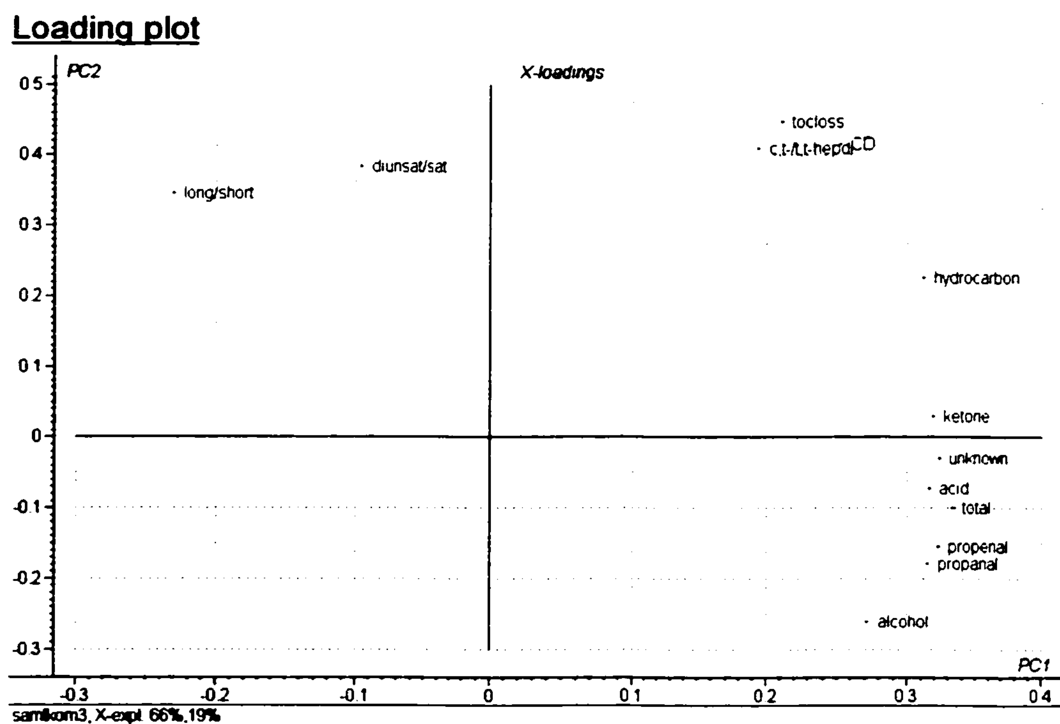
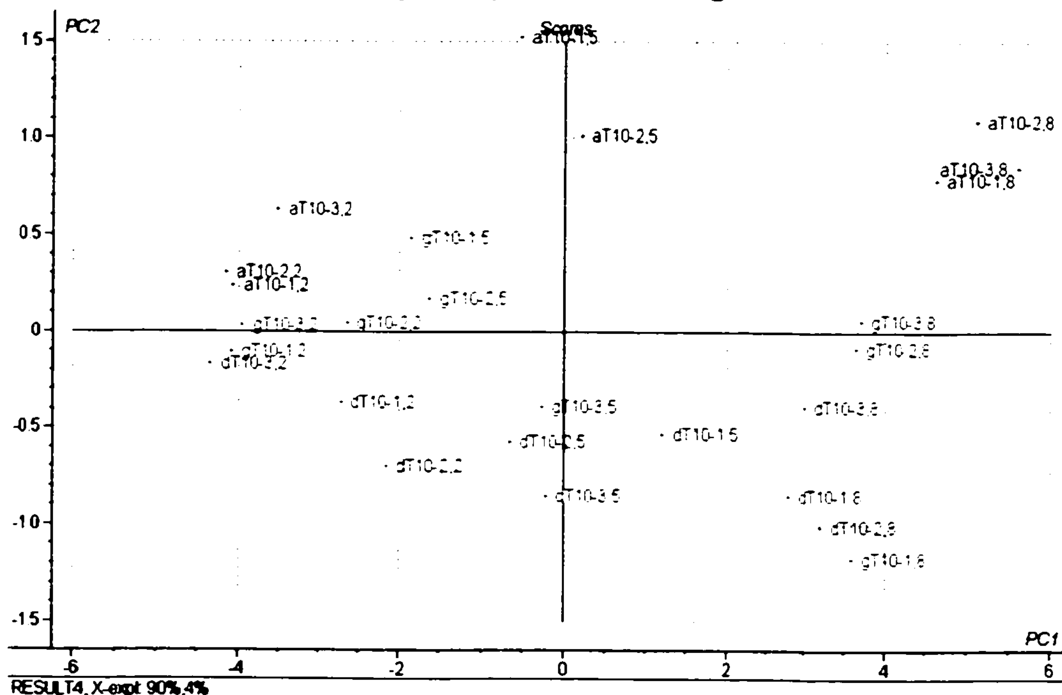


Figure 6.9B. Loading plot from the PCA of grouped variables, including ratios between groups, conjugated dienes (CD) and tocopherol consumption (tocloss). Control and 100 ppm δTOH, day 5 samples excluded.

Score plot: Colour code by tocopherol homologue



Score plot: Colour code by storage time

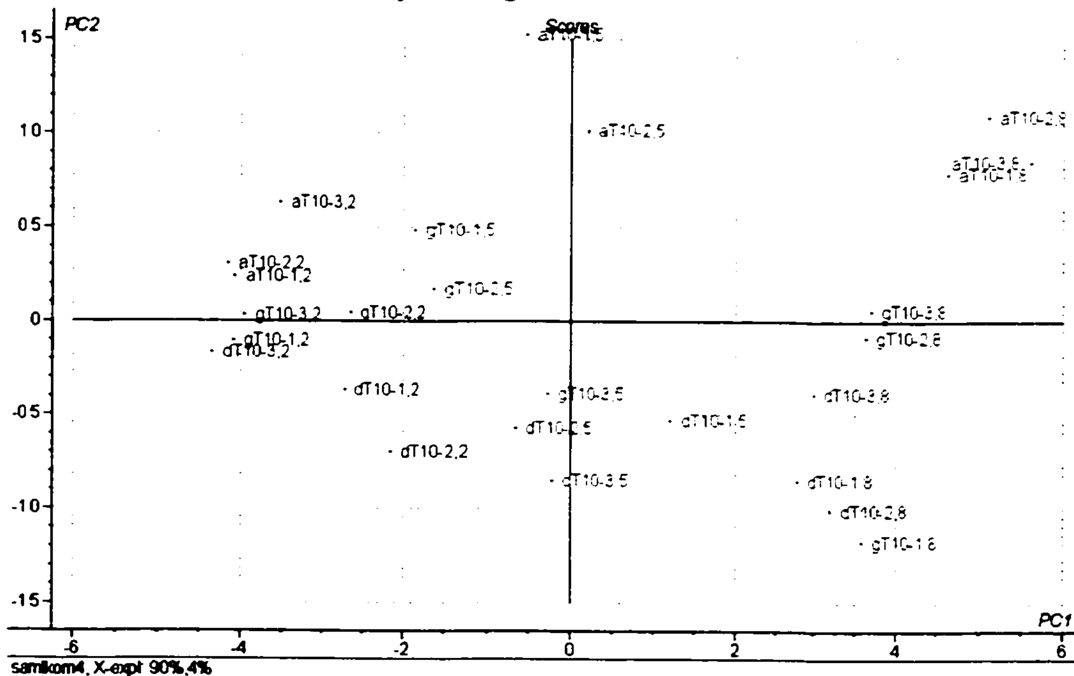


Figure 6.10A. Score plots from the PCA of grouped variables; 1000 ppm tocopherol.

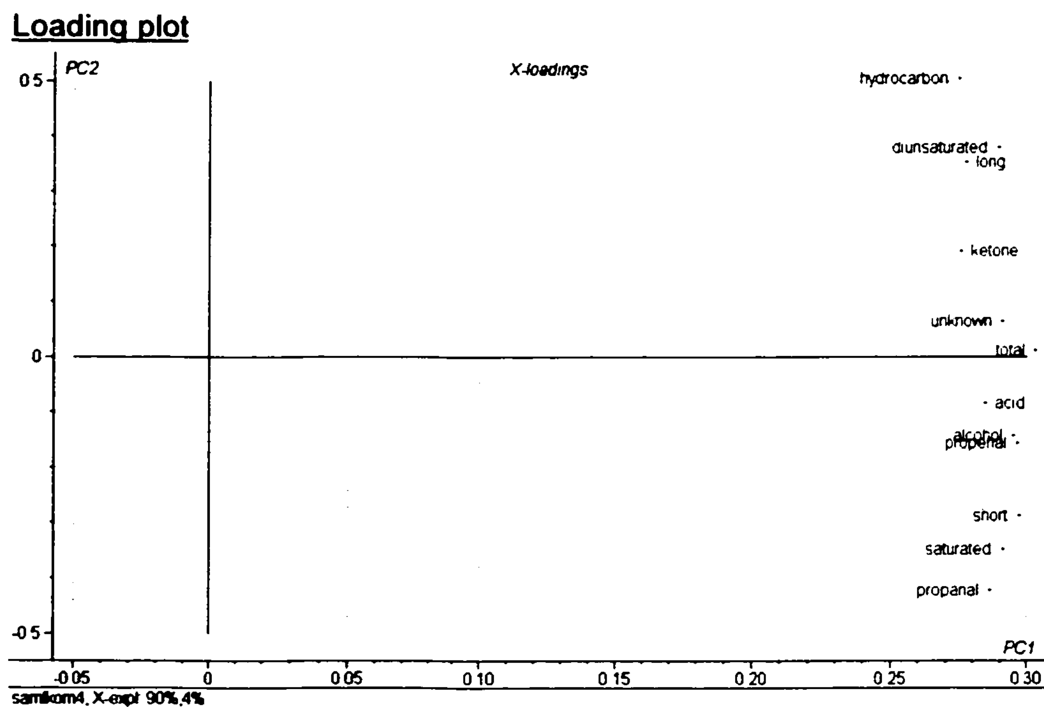
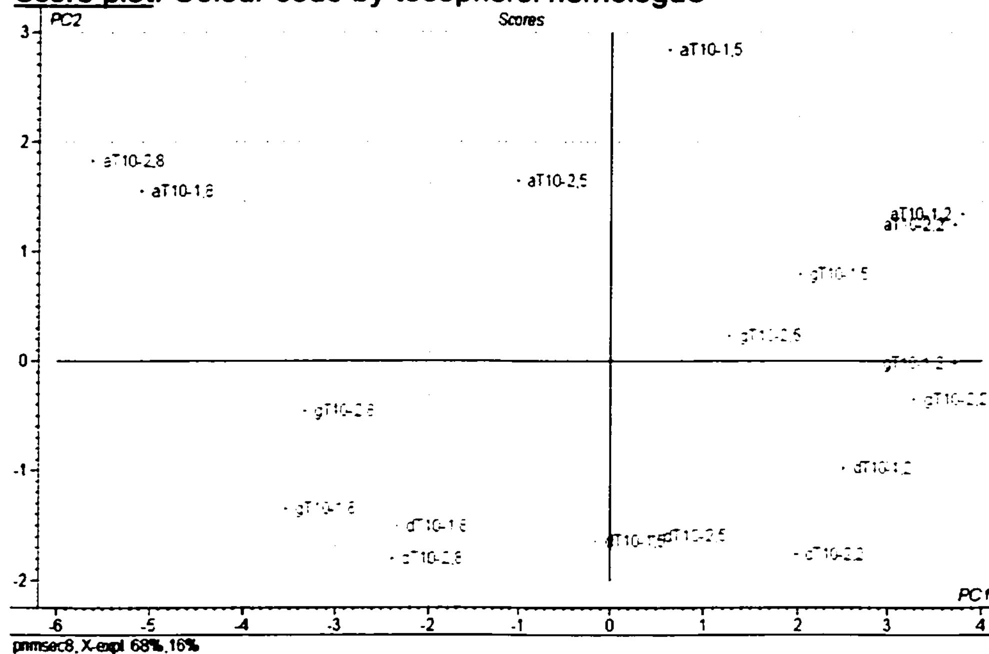


Figure 6.10B. Loading plot from the PCA of grouped variables; 1000 ppm tocopherol.



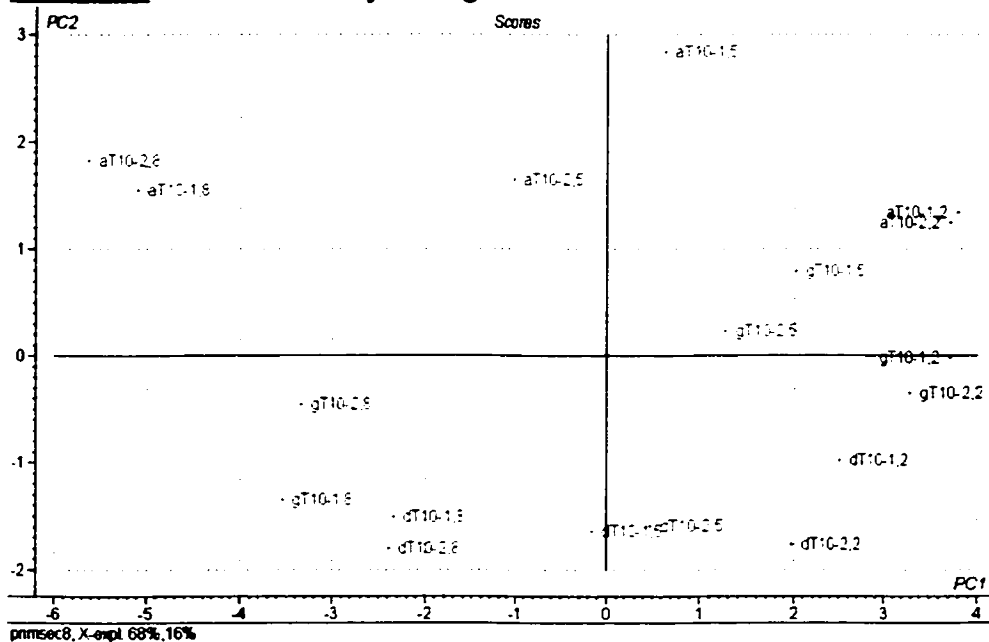


Figure 6.11A. Score plots from the PCA of grouped variables, including ratios between groups, conjugated dienes (CD) and tocopherol consumption (tocloss) for the 1000 ppm αTOH, γTOH and δTOH samples.

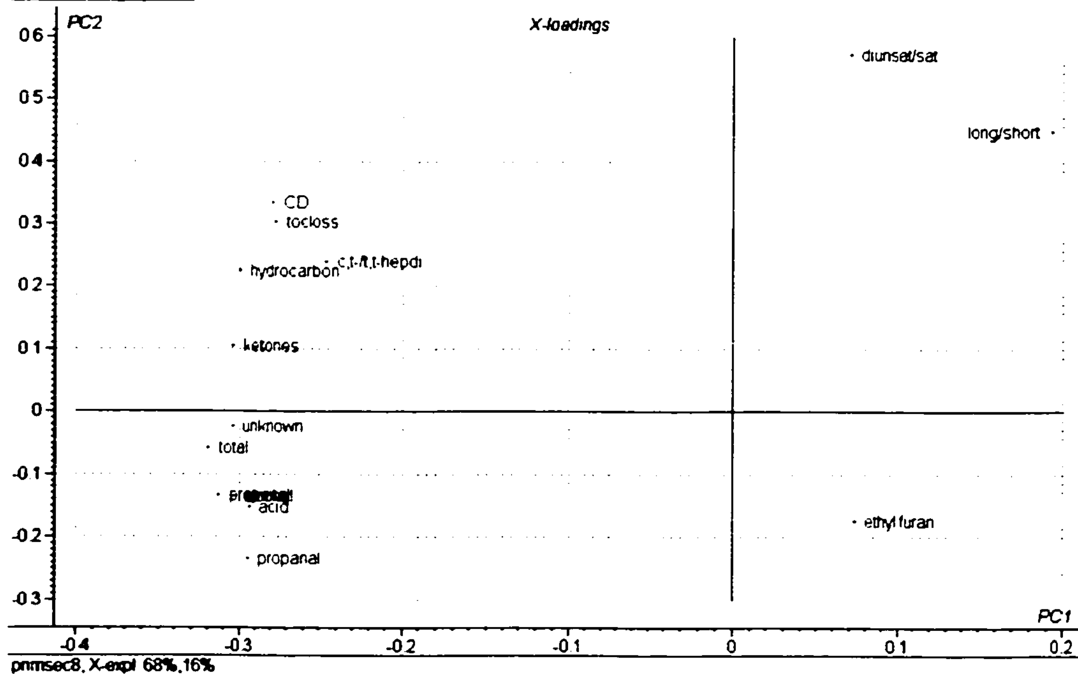
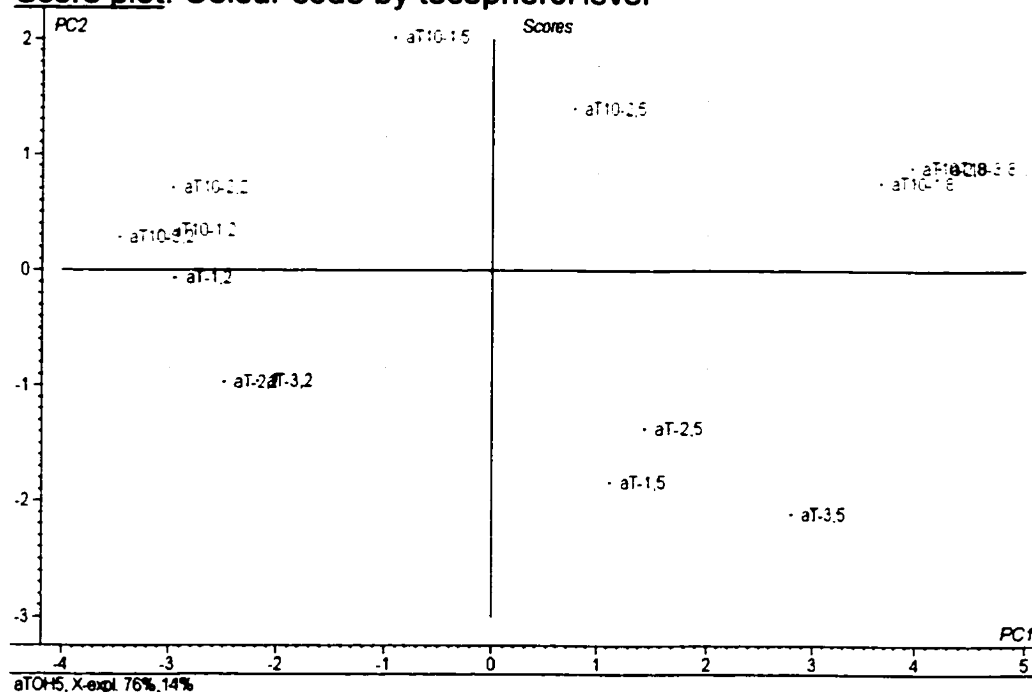


Figure 6.11B. Loading plot from the PCA of grouped variables, including ratios between groups, conjugated dienes (CD) and tocopherol consumption (tocloss) for the 1000 ppm αTOH, γTOH and δTOH samples.



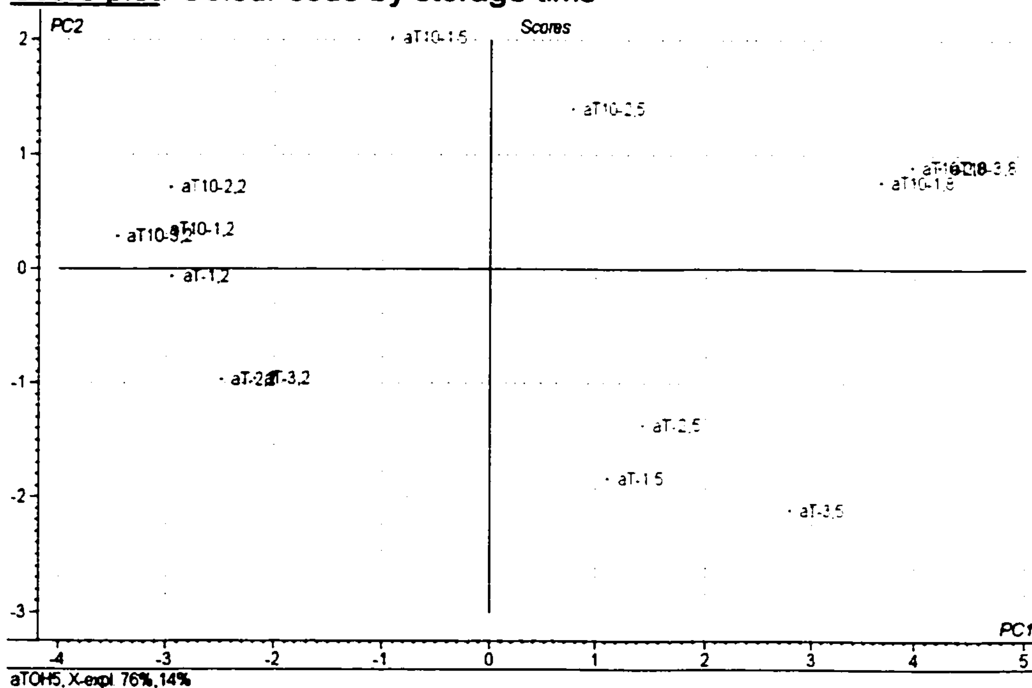


Figure 6.12A. Score plots from the PCA of grouped variables, including ratios between groups, for samples with αTOH.

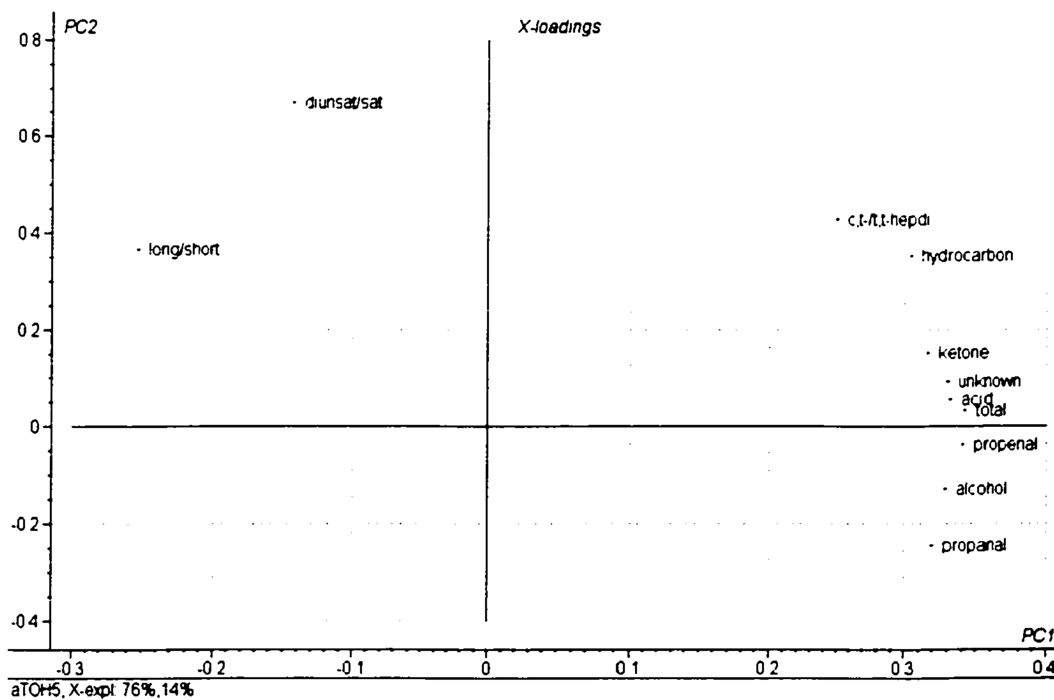


Figure 6.12B. Loading plot from the PCA of grouped variables, including ratios between groups, for samples with αTOH.

6.3.3 Propanal as a Marker of Volatile Secondary Oxidation Products

Propanal is one of the major volatile secondary oxidation products formed from n-3 fatty acids (Fig. 6.1), and has been analysed by static headspace GC as a marker of this group of oxidation products in fish oils (Frankel and Tappel, 1991; Boyd *et al.*, 1992; Frankel, 1993a; Frankel *et al.*, 1996). When studying the properties of the different tocopherols in purified fish oil TAG (Section 4), a static headspace GC peak with the same retention time as an authentic reference compound of propanal was easily monitored (Fig. 6.13A). When analysing fish oil volatiles by dynamic headspace GC in the present study, a polar column (DB-WAXetr) with a polyethylene glycol stationary phase was used instead of the medium polar SPB-1701 with a poly (14% cyanopropylphenyl 86% dimethylsiloxane) stationary phase used in the static headspace GC procedure. Two C3-aldehydes, propanal and 2-propenal (acrolein), were then identified (Fig. 6.13B), both as major volatile oxidation products. Standards of propanal and 2-propenal were subsequently found to coelute on the SPB-1701 column. Based on these observations, it is likely that the peak monitored as propanal in other studies of fish oil oxidation by static headspace GC analysis with a column of medium polarity (Frankel and Tappel, 1991; Frankel, 1993a; Frankel *et al.*, 1996) have actually been propanal + 2-propenal. In accordance with this view, 2-propenal was not identified by dynamic headspace analysis of oxidized sand launce oil by Rørbæk (1994) when using a medium polarity GC column (DB-1701), nor when employing static headspace GC analysis with DB-1701 to determine oxidation of fish muscle lipids during thermal processing (Medina *et al.*, 1999). By using a nonpolar column (DB-5), on the other hand, propanal may be separated from 2-propenal, with 2-propenal eluting first (Snyder *et al.*, 1985; Boyd *et al.*, 1992). When using a medium polarity column such as the DB-1701, the marker of volatile secondary oxidation products should therefore be denoted e.g. propanal/2-propenal or C3-aldehydes instead of propanal.

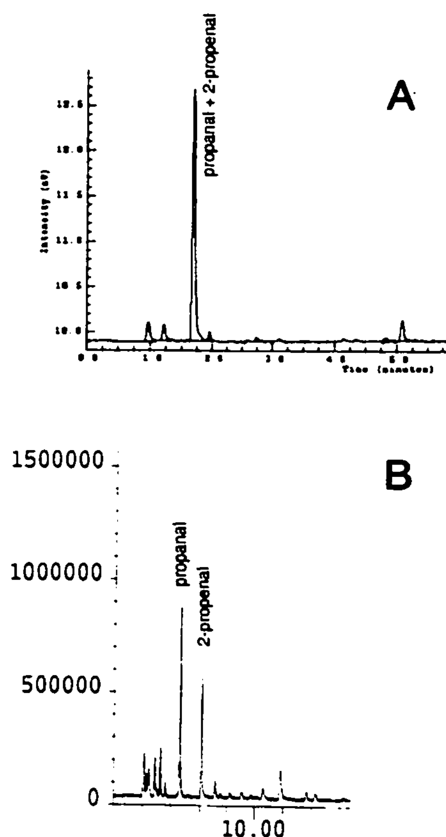


Figure 6.13. Analysis of propanal/2-propenal: (A) Static headspace analysis of fish oil with separation on a medium polarity GC column (SPB-1701), (B) Dynamic headspace analysis of fish oil with separation on a polar GC column (DB-WAXetr).

The formation of propanal, from the n-3 hydroperoxide of e.g. DHA, EPA or linolenic acid, is easily explained from the accepted reaction pathway for hydroperoxide decomposition (Grosch, 1987; Fig. 2.4, p. 13). The 16-hydroperoxide of methyl linolenate has been suggested as the source of "propanal/2-propenal" by several investigators (Frankel, 1982; Przybylski and Eskin, 1995), but no specific reaction pathway has been proposed for the 2-propenal. As discussed in Section 6.3.2, unsaturated aldehydes from the decomposition of hydroperoxides have been demonstrated to rapidly oxidize to lower molecular weight compounds under oxygen-sufficient conditions

(Frankel, 1982). A possible source of 2-propenal is 2,4,7-decatrienal, by hydroperoxide formation at carbon number 4 and subsequent hydroperoxide decomposition according to the classical pathway for decomposition of primary oxidation products (Fig. 6.14). This pathway for the formation of 2-propenal also leads to the formation of 2,4-heptadienal, one of the major volatile oxidation products found in the oxidized fish oil samples in the present study. Alternatively, the 8-hydroperoxide of 2,4,7-decatrienal may form, and subsequent decomposition yields propanal. 2-Propenal is also an expected oxidation product of 2,4-heptadienal (Fig. 6.2, p. 109).

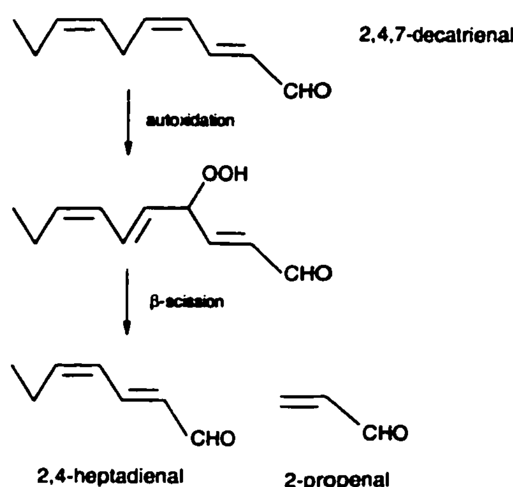


Figure 6.14. Suggested pathway for the formation of 2-propenal from 2,4,7-decatrienal.

In this study, the relative formation of propanal compared to 2-propenal and compared to the overall volatile formation, was dependent on the type and concentration of tocopherol used as antioxidant and the storage time of the fish oil. The preferential formation of propanal compared to that of 2-propenal, as well as compared to the formation of diunsaturated aldehydes and total volatiles, when using δTOH (high-level) and when using a low initial αTOH concentration is illustrated in Figure 6.10 (p. 130 and 131) and 6.12 (p. 134 and 135), respectively. At conditions of low tocopherol hydrogen-donating

power the static headspace determination of propanal only as a marker of secondary oxidation, would have resulted in an overestimation of the overall volatile formation, as well as of the longer-chain unsaturated aldehydes.

Table 6.4 gives the concentrations (mean values for each treatment group) of propanal, 2-propenal and total volatiles in the fish oil samples with 100 ppm and 1000 ppm initial nominal αTOH concentration. These values were used in the PCA of the αTOH-data presented in Figure 6.12. The effect of increasing the initial concentration of αTOH was quite substantial on the basis of the formation of propanal (concentration lowered by about 67%), but much smaller on the basis of total volatiles (concentration lowered by 12-25%). The formation of 2-propenal was initially much more rapid than the formation of propanal, and increasing the αTOH concentration appeared to have little effect on the initial rate of formation of this oxidation product. Upon further storage, and as the αTOH was consumed, the degree of inhibition of propanal formation by αTOH decreased, and the difference in the concentration of the two C3-aldehydes became smaller. Based on a better agreement with the total volatile concentration, as well as with the formation of 2,4-heptadienal, the C3-aldehydes (i.e. propanal + 2-propanal), or 2-propenal only, both appear to be more suitable markers of volatile secondary oxidation products than propanal alone. This is also supported by a higher coefficient of determination obtained for the linear relationship between C3-aldehydes and total volatiles than between propanal on total volatiles (Fig. 6.15).

Table 6.4. Mean concentrations of propanal, 2-propenal and total volatiles (TV) in fish oil with 100 ppm and 1000 ppm αTOH during storage at 30°C.[a]

Storage time (days)	100 ppm αTOH			1000 ppm αTOH		
	Propanal (ppm)	*2-Propenal (ppm)*	*TV (ppm)*	*Propanal (ppm)*	*2-Propenal (ppm)*	*TV (ppm)*
2	0.24	0.40	5.63	0.08	0.42	4.93
5	1.14	1.16	14.7	0.37	0.82	11.0
8				1.11	1.35	19.4

[a]Concentrations are relative to the ethyl heptanoate internal standard

The suitability of propanal as a marker of volatile secondary oxidation products has now been discussed based on its rate of formation relative to the overall formation of volatile secondary oxidation products. As previously mentioned, the unpleasant flavour of oxidized fish oil appears to be dominated by relatively high molecular weight unsaturated carbonyls with low flavour thresholds. The evaluation of the suitability of propanal/2-propenal as markers of adverse flavour formation requires sensory analysis and remains to be investigated.

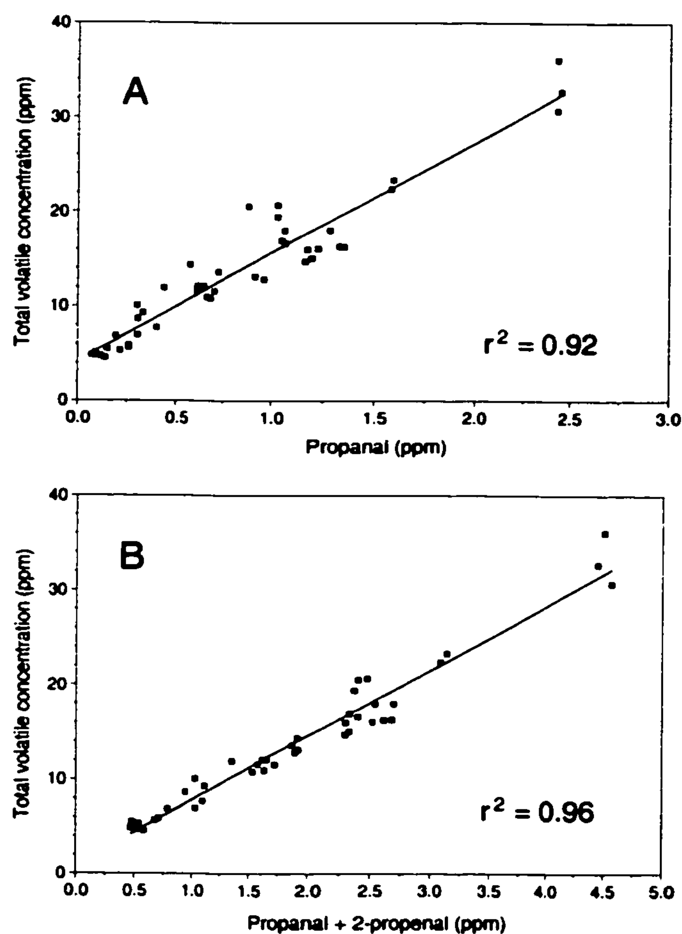


Figure 6.15. Relationships between propanal and total volatile concentration (A) and between C3-aldehydes and total volatile concentration (B) in oxidizing fish oil TAG.

7 Properties and Protection of Astaxanthin in Autoxidizing Fish Oil Triacylglycerols

7.1 Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) (Fig. 7.1), a carotenoid pigment, is the principal pigment of salmonids, imparting the attractive colour to the flesh, skin and eggs. The biological functions of astaxanthin in fish are not fully understood but may include provitamin A activity, antioxidant activity, protection from light, fertilization horomone, as well as a source of pigment for the chromatophores (Torrissen *et al.*, 1989).

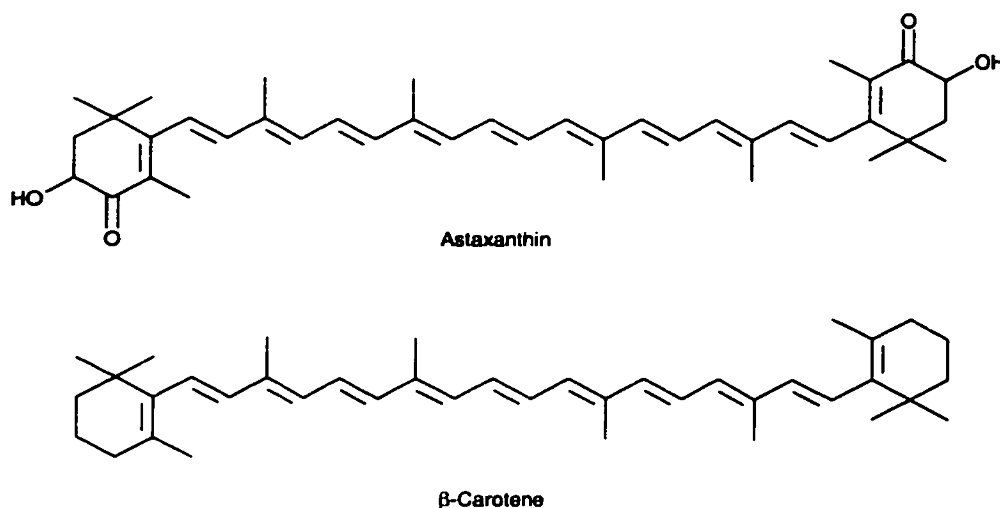


Figure 7.1. Structure of astaxanthin and β-carotene.

7.1.1 Astaxanthin in Salmonid Aquaculture

Since salmonids cannot synthesize astaxanthin *de novo*, the pigment must be supplemented in the feed of the farmed fish. To achieve adequate pigmentation of the fish flesh, a dietary astaxanthin concentration of 35-75 ppm is used by most fish farmers. The predominant pigment source is synthetic astaxanthin supplied in a gelatin-matrix (e.g. Carophyll Pink™ from Hoffman-La Roche, Basle, Switzerland). Astaxanthin is easily

degraded when subjected to oxygen, heat or light, and a substantial amount of it is lost during production and storage of the fish feed. Fish feed manufacturers therefore have to exceed the label claim of astaxanthin when preparing the feed to ensure that their final product specifications are met. As astaxanthin constitutes up to 20% of the feed costs in salmonid aquaculture, decreasing the pigment waste is of great economic importance.

Aquaculture feeds are usually fortified with synthetic tocopherol in the form of α-tocopherol acetate. However, natural tocopherol may become a wiser alternative to synthetically prepared tocopherol in the light of findings in mammalian tissues regarding the greater retention of the natural αTOH (RRR-configuration) compared to synthetically produced αTOH (Ingold *et al.*, 1987). In addition, and possibly of greater importance when the stability of the feed itself is considered, α-tocopherol acetate is, due to its esterified hydroxyl group, not an antioxidant *in vitro*. It will therefore not protect the fatty acids and the astaxanthin in the feed from oxidative degradation during feed processing and storage.

Oil from fatty fish like sardine, capelin and herring constitutes an important part of the diet of farmed salmonids. The irregular availability of fish oil world-wide, however, may become a limiting factor in the expected continued increase in the salmonid aquaculture production. Moreover, certain consumer groups have recently demanded a higher ratio of vegetable ingredients in the fish feed to reduce the stress on the wild marine resources. Replacing some of the fish oil with vegetable oils will introduce other tocopherol homologues besides αTOH, as non-esterified components, to the feed of the farmed salmonids. Recent studies suggest that dietary γTOH can be readily deposited in salmon muscle (Sigurgisladottir *et al.*, 1994a; Parazo *et al.*, 1998). The δ- and βTOH are deposited to a lesser extent (Sigurgisladottir *et al.*, 1994a). Both αTOH and γTOH have been shown to be effective in preserving the quality of frozen-stored salmon fillets (Parazo *et al.*, 1998).

Salmonid aquaculture is a rapidly growing industry, and satisfactory treatment of the waste from the fish processing plants, preferably into value-added products, has become important. An attractive red salmon oil is currently being manufactured in Norway from the remains of the fish after filleting. Rapid handling of the fish after slaughter, as well as pressing under mild conditions, produces an oil of high initial quality that is used in n-3 health supplements and as a food ingredient. The composition of the salmon oil is partially dependent on the composition of the fish feed, and an increased use of vegetable ingredients is likely to increase the level of tocopherols other than αTOH, particularly γTOH, in this fish oil.

7.1.2. Antioxidant Properties of Carotenoids

Carotenoids may function as antioxidants in lipid systems by singlet oxygen quenching and by reacting with lipid peroxyl radicals. Their antioxidant properties have been documented in a number of *in vitro* studies, including lipids in homogenous solutions, liposomes, isolated membranes and intact cells, and direct evidence of carotenoids functioning as antioxidants *in vivo* has been reported in animal models (reviewed by Palozza and Krinsky, 1992).

Carotenoids prevent damage in photobiological systems by quenching of singlet oxygen ($^1O_2$) as well as excited sensitizer molecules, and the antioxidant activity of the carotenoids in photoxidation of fatty acids has been found to depend on the number of conjugated double bonds in the molecule (Krinsky, 1989). The anticarcinogenic properties of carotenoids indicated in many reports has, on the other hand, been related to their ability to interact with various radical species that can be generated within cells (Burton and Ingold, 1984; Krinsky, 1989). Data from intervention trials have, however, indicated that high dose levels of β-carotene increase the risk of lung cancer in smokers, these findings being possibly related to a low vitamin C status of this high-risk population (Siemensma, 1997). The mechanism of the radical scavenging action of the carotenoids is still not fully understood. The carotenoids do not have the structural features commonly

associated with radical chain-breaking antioxidants, and most investigators support a mechanism where a resonance-stabilized carbon-centered radical (ROOCar•) is formed by the addition of a peroxyl radical (ROO•) to the conjugated system of the carotenoid (Car) (Burton and Ingold, 1984; Kennedy and Liebler, 1992; Palozza and Krinsky, 1992; Tsuchihashi *et al.*, 1995) [7.1]. A carotenoid hydrogen atom transfer mechanism [7.2] has also been suggested (Liebler and McClure, 1996; Mortensen and Skibsted, 1998) to be of importance.

$$ROO\bullet + Car \rightarrow ROOCar\bullet \qquad [7.1]$$

$$ROO\bullet + Car(H) \rightarrow ROOH + Car\bullet \qquad [7.2]$$

The available literature on the effect of carotenoids on the rate of oxidation in fats and oils is conflicting. Carotenoids, of which the functions of β-carotene (Fig. 7.1) are the most extensively studied, have been shown to improve the oxidative stability of oils (Warner and Frankel, 1987; Suzuki *et al.*, 1989; Lee and Min, 1990). However, prooxidative effects have frequently been observed in oils stored in the dark (Suzuki *et al.*, 1989; Haila *et al.*, 1996; Heinonen *et al.*, 1997; Haila *et al.*, 2000) as well as under exposure to light (Haila and Heinonen, 1994; Haila *et al.*, 1996). The properties of carotenoids in lipid oxidation appear to be highly dependent on the experimental conditions, particularly the presence of other antioxidants and the availability of oxygen.

The singlet oxygen-quenching effect of carotenoids is complicated by their susceptibility to oxidation by lipid radicals. In soybean oil, β-carotene was an effective inhibitor of photooxidation only when it was protected from interacting with peroxyl radicals by the tocopherols naturally present (Warner and Frankel, 1987). Similarly, β-carotene promoted hydroperoxide formation under light in purified rapeseed oil TAG both in the absence and presence of a photosensitizer, while a combination of β-carotene and γTOH inhibited hydroperoxide formation more effectively than γTOH alone (Haila and

Heinonen, 1994). Prooxidant effects of β-carotene was also observed in an oil-in-water emulsion of purified rapeseed oil TAG in the dark (Heinonen *et al.*, 1997). In this system, both αTOH and γTOH protected the β-carotene and the oil from oxidative deterioration.

The effectiveness of β-carotene as a free radical scavenging antioxidant in methyl linoleate solution was markedly enhanced at lower pO_2 (Burton and Ingold, 1984), and these findings were suggested to be relevant to physiological conditions. The improved antioxidant activity of β-carotene with decreasing pO_2 has later been confirmed in other studies (Kennedy and Liebler, 1992; Jørgensen and Skibsted, 1993). The antioxidant efficiency of β-carotene (and other carotenoids) is thought to depend on the balance between free radical trapping by and autoxidation of the carotenoid, and the partitioning of β-carotene between one radical trapping and two autoxidation pathways has been suggested to be a function of pO_2 (Kennedy and Liebler, 1992; Fig. 7.2). Pathway A, the formation of an epoxide from the peroxyl-carotenoid adduct, is an autoxidative pathway for β-carotene, since no net consumption of radicals occurs. On the other hand, the addition of a second peroxyl radical (pathway B) is considered an antioxidant pathway. In pathway C, oxygen adds reversibly to the adduct to form another peroxyl radical, and the net radical consumption is zero. According to this theory, the extent to which pathway C contributes to the carotenoid consumption is determined by pO_2. At low pO_2, the trapping of a second peroxyl radical (pathway B) is favoured, and the result is an antioxidant effect.

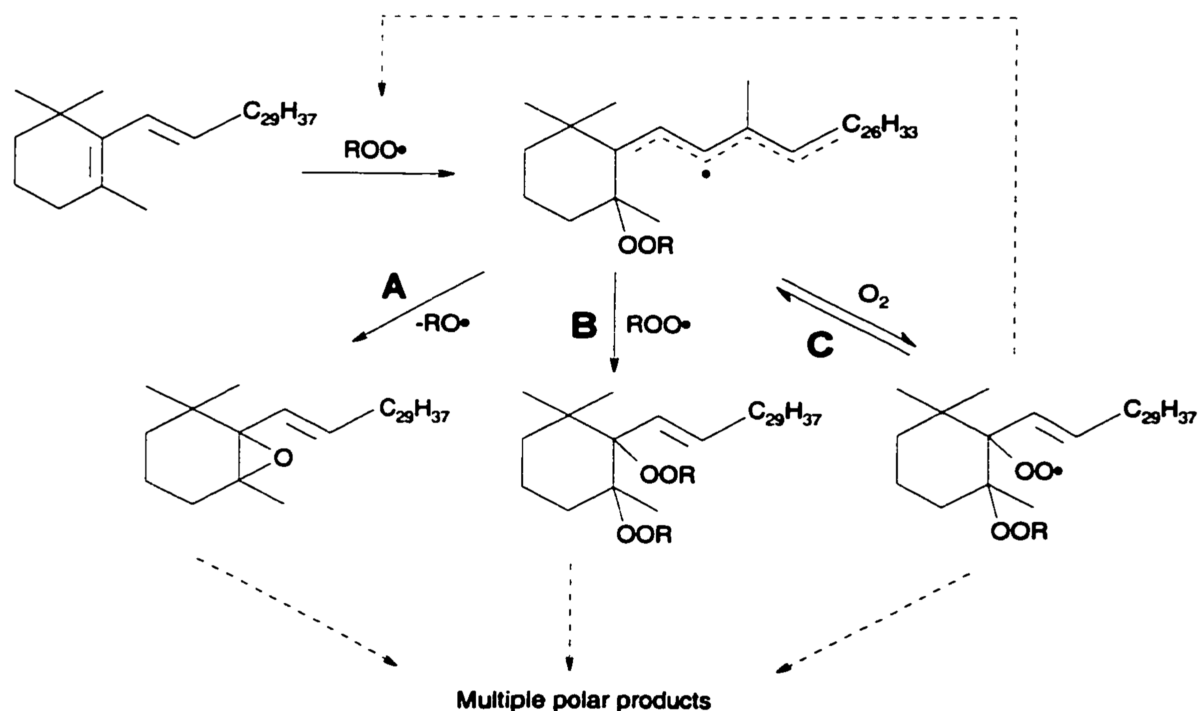


Figure 7.2. Proposed reaction pathways for β-carotene during lipid autoxidation (Adapted from Kennedy and Liebler, 1992).

Most studies of the effects of carotenoids on lipid oxidation have focused on the properties of β-carotene, and limited data are available for the behaviour of astaxanthin. The presence of a conjugated carbonyl group with an electron-withdrawing character in astaxanthin is expected to enhance the stability of its radical compared to that of β-carotene by reducing the unpaired electron density on the carbon skeleton. Superior radical-scavenging properties of astaxanthin was confirmed for the oxidation of methyl linoleate in organic solvents initiated by radical-type initiators (Terao, 1989; Jørgensen and Skibsted, 1993). The antioxidant effectiveness of carotenoids on the chlorophyll-sensitized photooxidation of soybean oil was found to increase with the number of conjugated bonds in the carotenoid, astaxanthin being the most active of the carotenoids tested (Lee and Min, 1990). No antioxidant effect of astaxanthin was observed in salmon muscle upon storage in air at 35°C (Sigurgisladottir *et al.*, 1994b).

The effect of astaxanthin on the peroxyl radical-induced autoxidation in fats and oils has not been studied.

As previously mentioned, increasing the stability of astaxanthin during processing and storage of fish feed is of importance in the effort to reduce costs in salmonid aquaculture. An objective in this work was, by using a fish oil model system, to investigate the ability of α-, γ- and δTOH to retard the oxidative degradation of astaxanthin. A related issue is the preservation of the attractive red colour of salmon oil by the addition of tocopherols. The effect of the presence of astaxanthin on the formation of primary and secondary oxidation in the fish oil was also studied. As fish feed and salmon oil are generally stored in the dark, only the radical scavenging properties of astaxanthin are considered important.

7.2 Materials and Methods

This work was carried out at the Norsk Hydro Research Centre, Porsgrunn, Norway.

Materials

Freshly processed South American Pacific fish oil (refined, winterized and deodorized; EPAX3000TG), with a composition similar to that of anchovy oil, was provided by Pronova Biocare (Sandefjord, Norway). The fish oil was purified by column chromatography as described in Section 3.2.1. The purified TAG fractions were practically colourless and odourless, and the results from the characterization (POV, *p*AV, colour, %TAG, iron, copper) of a typical batch of purified EPAX3000TG is given in Table 3.1 (p. 40). No αTOH could be detected in the purified oil (detection limit 1 ppm). The fatty acid composition of a typical purified fish oil TAG fraction is given in Appendix A (purified anchovy TAG-2), determined as described in Section 3.2.2. Synthetic astaxanthin was from Hoffman-La Roche (Basle, Switzerland). Sunflower oil was obtained from a local supermarket. The *d*-α-tocopherol (purity ≥ 99%), the *d*-γ-tocopherol (purity ≥ 95%) and the *d*-δ-tocopherol (purity ≥ 93%) were supplied by

Acros (Ghent, Belgium). The αTOH product contained no other tocopherols, while the γTOH and δTOH contained 1.5% and 3.1% of other tocopherols, respectively, as determined by HPLC. All solvents were analytical grade.

Oxidation tests

The tocopherols were added to the oil samples immediately after purification as previously described (Section 3.2.3). Astaxanthin was added to a small part of the oil as a solution of known concentration in acetone, and acetone was removed by N_2-stripping. The same amount of acetone was also added to and stripped from the oil samples that were not to contain any astaxanthin. Portions (1.5 g) of the different oil treatments were stored in 17 mL (40 mm x 23 mm i.d.) uncapped glass vials in the dark in an oven at 30°C. Three vials of each oil treatment were removed from the oven at regular intervals for analysis of conjugated dienes, C3-aldehydes, and remaining tocopherol and astaxanthin. One analytical determination was performed for each oil portion.

The following studies were conducted:

1. The determination of the effect of astaxanthin (100 ppm) on the formation of primary (as conjugated dienes) and secondary (as C3-aldehydes) oxidation products when added to purified fish TAG alone and in combination with different levels of αTOH. The disappearance of astaxanthin and αTOH was also studied.

2. The effect of astaxanthin at different concentrations (5, 20 and 100 ppm) on autoxidation in fish oil without tocopherol and with α-, γ- or δTOH (100 ppm) was compared by the determination of conjugated dienes. The loss of astaxanthin was also determined.

3. The ability of the different tocopherols (α-, γ- and δTOH), as well as a mixture of these, at a total tocopherol concentration of 500 ppm, to protect astaxanthin from being

oxidized was studied. The tocopherol blend had 100 ppm αTOH, 200 ppm γTOH and 200 ppm δTOH. Primary oxidation in the oils was determined by conjugated diene analysis.

Analytical methods

Conjugated dienes were determined spectrophotometrically, using a Shimadzu UV-1200 spectrophotometer, as described in Section 3.2.2. The addition of 100 ppm astaxanthin to the freshly purified fish oil TAG did not increase the $E^{1\%}_{1cm}$ at 234 nm. Tsuchihashi *et al.* (1995) recorded the change in the UV-Visible absorption spectrum during free radical initiated oxidation of β-carotene. They observed a small increase in the absorption at 290-330 nm, but no difference was noted in the spectrum at lower wavelengths upon carotenoid degradation. It it therefore considered unlikely that astaxanthin oxidation products contribute to the conjugated diene value. The tocopherol concentration was determined by HPLC, as described in Section 3.2.2, except that the HPLC system consisted of a Waters 715 Ultra Wisp sample processor controlled by a Waters 600E system controller and connected to a Waters 470 scanning fluorescence detector. The C3-aldehydes were determined by static headspace GC using a Dani 3950 autosampler (Monza, Italy) connected to a Varian 3700 GC (Palo Alto, CA) according to the method used at the Norsk Hydro Research Centre (Section 4.2, p. 60). The astaxanthin concentration in the oil was determined spectrophotometrically using a Shimadzu UV-1200 spectrophotometer. The fish oil TAG samples containing astaxanthin were diluted with sunflower oil (approx. 0.1 g fish oil to 3.0 g sunflower oil), and the sample absorbance was read at 485 nm against the corresponding fish oil TAG samples without astaxanthin after dilution of these with the same amount of sunflower oil. The astaxanthin concentration was determined from a standard curve ($r^2 = 0.999$) prepared from the analysis of known concentrations of astaxanthin in sunflower oil.

Statistical analysis

The concentrations of oxidation products, as well as of the remaining astaxanthin, for the different treatments were compared using a *t*-test (StatMost™, DataMost Corporation, Salt Lake City, UT). The data from study 2 were analysed by one-way analysis of variance, followed by Tukey's test whenever applicable, using StatMost™. Differences in treatment means were considered significant when $P < 0.05$.

7.3 Results

Study 1

The effect of astaxanthin, alone and in combination with αTOH, on the autoxidation of purified fish oil TAG, was investigated, together with the ability of αTOH to protect astaxanthin from oxidative degradation. Astaxanthin (100 ppm) was an antioxidant in oil without αTOH but had a weak prooxidant effect when added to oil with 100 ppm and with 500 ppm αTOH (significant ≥ day 4 of storage), as well as in oil with αTOH and ascorbyl palmitate, when the formation of hydroperoxides was measured (Fig. 7.3A). In accordance with the results presented in Section 3.3.2, 500 ppm αTOH promoted the formation of hydroperoxides compared to 100 ppm αTOH, and ascorbyl palmitate eliminated this αTOH-mediated accumulation of hydroperoxides. As expected from the previous work on αTOH-properties in fish oil (Section 4.3.1), αTOH reduced the rate of formation of C3-aldehydes in a concentration-dependent manner (Fig. 7.3B). As for primary oxidation products, astaxanthin was an antioxidant when added alone to purified fish oil TAG on the basis of the formation of volatile secondary oxidation products, but appeared to have a slight prooxidant effect when added together with αTOH (Fig. 7.3B). A somewhat higher concentration of C3-aldehydes in the oil with astaxanthin + αTOH compared to the oil with αTOH, was only apparent shortly after the end of the induction period, indicating a shorter induction period in the presence of astaxanthin. The large standard deviation for the concentration of C3-aldehydes in the oil with 500 ppm αTOH + ascorbyl palmitate at day 15 of storage is thought to reflect slightly different lengths of the

induction period for the different replicates. The effect of astaxanthin on the rate of formation of primary oxidation products, as conjugated dienes and POV, was verified in a repeat experiment testing fish oil TAG with and without 100 ppm αTOH.

Astaxanthin was rapidly destroyed in oil without αTOH, and αTOH protected astaxanthin in a concentration-dependent manner (Fig. 7.4). Figure 7.5 shows that when astaxanthin and αTOH were added together, the tocopherol was preferentially consumed. When all the αTOH had been consumed, about 50% of the pigment had been destroyed, and a subsequent increase in the rate of astaxanthin breakdown was observed. Complete consumption of αTOH corresponded with the end of the induction period as determined by the formation of oxidation products (Fig. 7.3). Figure 7.6 shows that αTOH was consumed slightly faster in the presence of astaxanthin ($P < 0.05$ for data points > day 2 for samples without ascorbyl palmitate), and this may be related to the weak prooxidant effect of astaxanthin.

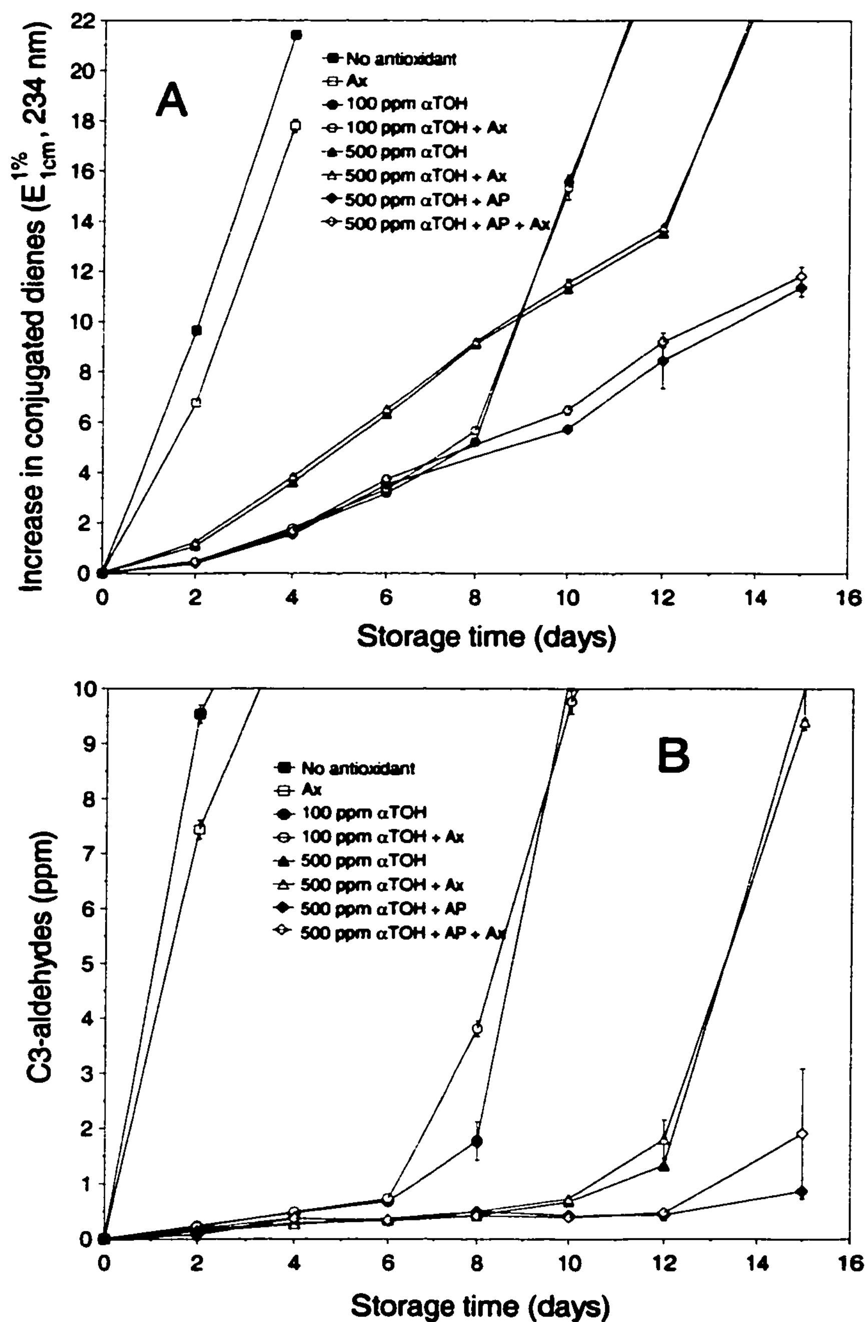


Figure 7.3. Effect of astaxanthin (Ax) on the autoxidation of purified fish oil TAG with and without αTOH and ascorbyl palmitate (AP) at 30°C in the dark: (A) Formation of conjugated dienes, (B) Formation of C3-aldehydes. Data points are means ± SD (n = 3 for days 2, 4, 6, 8 and n = 2 for days 10, 12, 15).

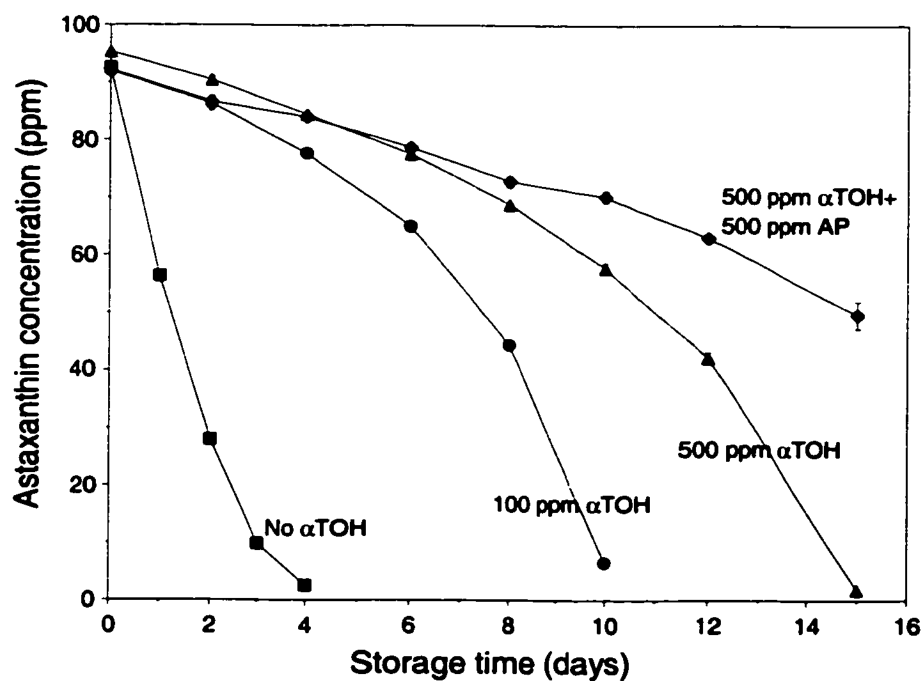


Figure 7.4. Loss of astaxanthin during autoxidation of purified fish oil TAG with and without αTOH and ascorbyl palmitate (AP) at 30°C in the dark. Data points are means ± SD (n = 3 for days 2, 4, 6, 8 and n = 2 for days 10, 12, 15).

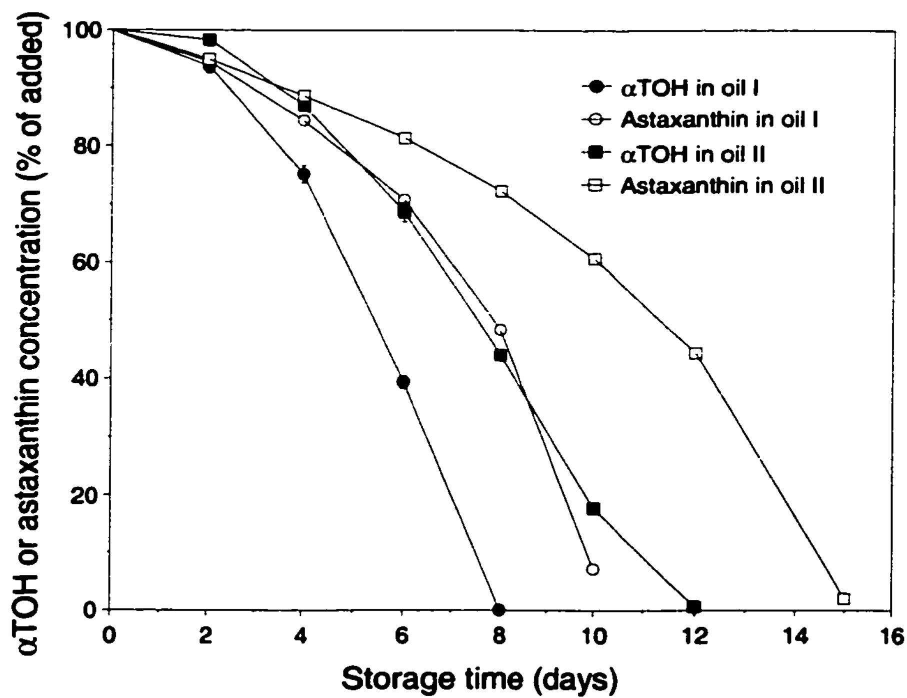


Figure 7.5. Comparison of the rates of loss of astaxanthin and αTOH in purified fish oil TAG stored at 30°C in the dark.
Oil I: 100 ppm αTOH, 100 ppm astaxanthin
Oil II: 500 ppm αTOH, 100 ppm astaxanthin

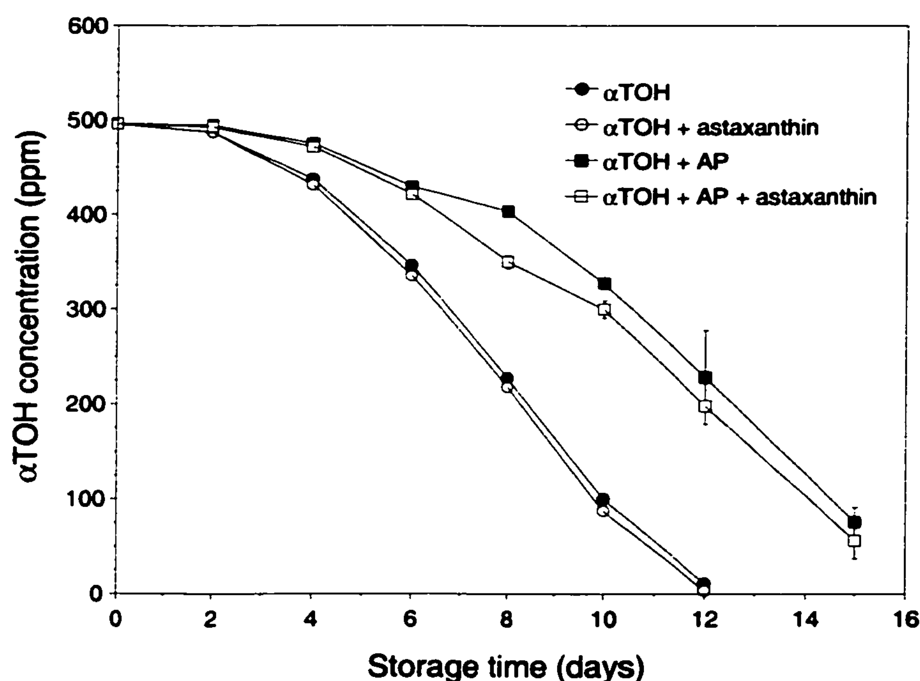


Figure 7.6. Effect of astaxanthin on the concentration of αTOH during storage of purified fish oil TAG at 30°C in the dark. Data points are means ± SD (n = 3 for days 2, 4, 6, 8 and n = 2 for days 10, 12, 15).

Study 2

The impact of astaxanthin concentration on the oxidative stability of fish oil TAG without tocopherol and with α-, γ- or δTOH (100 ppm), as well as the relative ability of the tocopherols to protect astaxanthin from oxidative degradation, was studied. Oil without tocopherol oxidized very rapidly and was sampled after 2 days of storage, while the oils with tocopherol were analysed after 4.5 days of storage. All three tocopherols protected the oil and astaxanthin from oxidation, and their order of activity was αTOH > γTOH > δTOH (Table 7.1 and 7.2). Under the experimental conditions studied, astaxanthin did not display any antioxidant activity at any of the pigment concentrations tested in the presence of tocopherol (Table 7.1). The small prooxidant effect observed at 100 ppm

astaxanthin in oil with αTOH, as well as the antioxidant effect of 100 ppm astaxanthin in oil without tocopherol, is in accordance with the findings in study 1. Lower astaxanthin concentrations (5 and 20 ppm) had no effect on the rate of formation of hydroperoxides in these systems. A small, but increasing, prooxidant effect of astaxanthin with level of addition was observed when added together with γTOH. In contrast, astaxanthin had no effect on the rate of formation of primary oxidation products in fish oil TAG with 100 ppm δTOH. In accordance with their relative ability to retard the formation of fatty acid hydroperoxides, αTOH was more effective at protecting astaxanthin than γTOH and δTOH (Table 7.2). The rate of astaxanthin breakdown was dependent on its initial concentration.

Table 7.1. Effect of astaxanthin concentration on formation of conjugated dienes ($\Delta E^{1\%}_{1cm}$) in fish oil TAG without tocopherol and with 100 ppm α-, γ- or δ-tocopherol after 4.5 days of storage at 30°C.[a]

Astaxanthin (ppm)	αTOH (100 ppm)	γTOH (100 ppm)	δTOH (100 ppm)	No tocopherol
0	2.81 ± 0.03[a]	4.16 ± 0.11[a]	6.5 ± 0.1[a]	14.9 ± 0.2[a]
5	2.85 ± 0.05[a]	4.86 ± 0.10[b]	6.4 ± 0.2[a]	14.6 ± < 0.1[a]
20	2.89 ± 0.06[a]	5.05 ± 0.04[c]	6.4 ± < 0.1[a]	14.7 ± 0.4[a]
100	3.12 ± 0.08[b]	5.23 ± 0.07[d]	6.8 ± 0.2[a]	12.5 ± 0.4[b]

[a]Means ± standard deviation, n = 3. Values in each column followed by a different letter are significantly different ($P < 0.05$).

Table 7.2. Loss of astaxanthin in fish oil TAG without tocopherol and with 100 ppm α-, γ- or δ-tocopherol after 4.5 days of storage at 30°C.[a]

Astaxanthin added (ppm)	Loss of astaxanthin (ppm)			
	αTOH	*γTOH*	*δTOH*	*No tocopherol*
5	1.19 ± 0.03	2.9 ± 0.1	3.6 ± 0.1	4.8 ± 0.1
20	5.1 ± 0.2	11.3 ± 0.1	14.2 ± 0.2	19.6 ± 0.1
100	25 ± 1	58.1 ± 0.8	70 ± 1	93.1 ± 0.5

[a]Means ± standard deviation, n = 3.

Study 3

The order of antioxidant activity of the tocopherol homologues in purified fish oil TAG was found to depend on their levels of addition (Section 4.3.1 and 6.3.2). In the previous study, when testing a tocopherol concentration of 100 ppm, αTOH was a more effective inhibitor of both fatty acid and astaxanthin oxidation than γTOH and δTOH. In the present study, the relative ability of the different tocopherols, as well as a tocopherol blend, to protect astaxanthin at a higher initial tocopherol concentration, 500 ppm, was tested.

In contrast to the findings in study 2, the antioxidant activity of the tocopherols decreased in the following order: δTOH > γTOH > mixed tocopherols > αTOH on the basis of formation of hydroperoxides (Fig. 7.7A). At this level of addition of tocopherol (500 ppm), their relative ability to retard autoxidation of the fatty acids in the oil was not in accordance with their relative ability to protect astaxanthin from oxidative degradation. Astaxanthin was rapidly destroyed in the oil without tocopherol, and αTOH and γTOH were the most efficient tocopherols in retarding astaxanthin breakdown at storage times less than 10 days (Fig. 7.7B). There was no difference between the astaxanthin concentrations in the oils with αTOH and with γTOH determined for storage times of < 10 days, likewise, there was no difference between the astaxanthin concentrations in the oils with mixed TOH and with δTOH at storage times of < 10 days. The rapid decrease in

astaxanthin concentration in the oil with αTOH after 8-10 days of storage reflects depletion of αTOH and the end of the induction period. The other tocopherol-samples apparently remained in the induction period throughout the experiment, but the loss of astaxanthin in these oils was very high (70-80% after 12 days of storage).

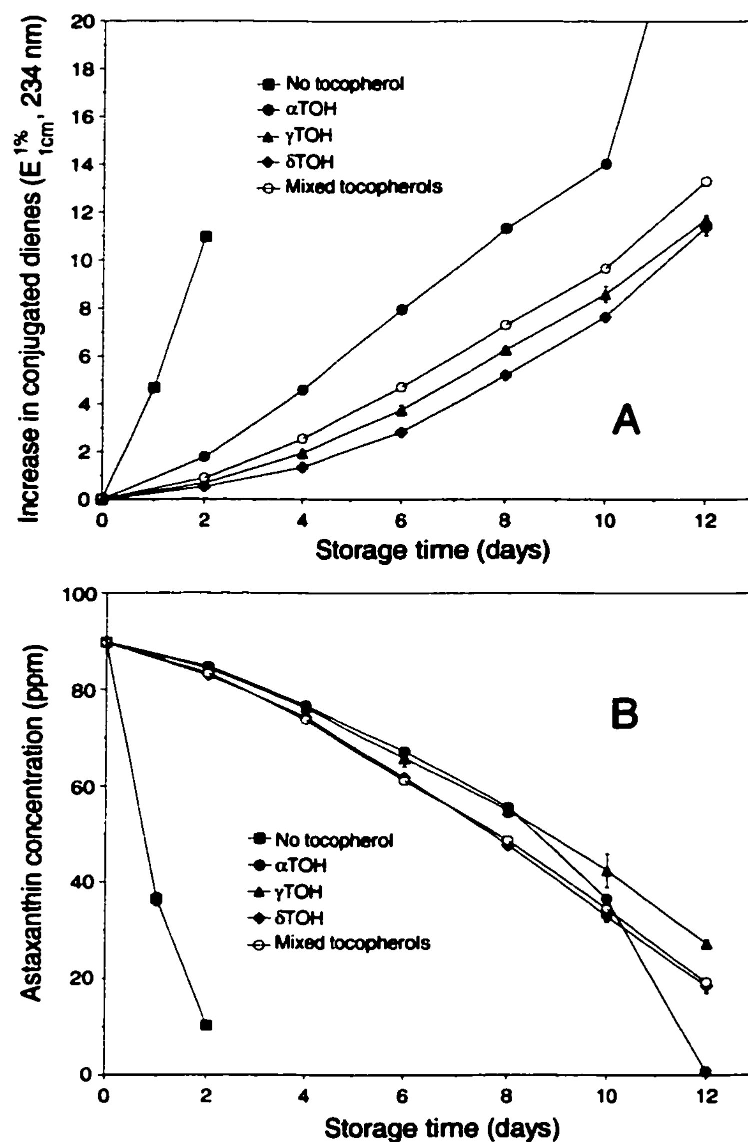


Figure 7.7. Storage of purified fish oil TAG with and without 500 ppm α-, γ-, δ- or mixed tocopherol and with 100 ppm astaxanthin at 30°C in the dark: (A) Formation of conjugated dienes, (B) Loss of astaxanthin. Data points are means ± SD (n = 3).

7.4 Discussion

Astaxanthin was either a weak prooxidant, an antioxidant, or had no effect on the rate of oxidation in purified fish oil TAG depending on its level of addition, whether it was added in combination with tocopherol, as well as the type of tocopherol used. Generally, the protection of the fatty acids in an oil by a chain-breaking antioxidant involves slow consumption of the antioxidant itself as it is being preferentially oxidized. According to the mechanism proposed by Kennedy and Liebler (1992; Fig. 7.2), carotenoids are consumed in both autoxidative and radical trapping pathways, and the impact of astaxanthin on oil stability is therefore not necessarily related to the rate of its consumption.

Astaxanthin was an antioxidant at a relatively high level of addition (100 ppm) in purified fish oil TAG without tocopherol. In contrast, most of the available literature reports a prooxidant effect of carotenoids during autoxidation in oils in the absence of other antioxidants (Suzuki *et al.*, 1989; Haila *et al.*, 1996; Heinonen *et al.*, 1997; Haila *et al.*, 2000). Purified fish oil TAG without any tocopherol oxidized very rapidly as shown in Figure 7.3. However, based on the susceptibility of the PUFA towards oxidation and the autocatalytic nature of the oxidation process, an even more rapid rate of hydroperoxide formation may have been expected. The fact that the oxidation curve is not steeper is suggested to be due to a combination of decomposition of the primary oxidation products and restricted oxygen access. Although autoxidation of the oil samples was conducted under air and with a high oil surface-to-volume ratio, the rapid fatty acid oxidation may induce oxygen diffusion control. The proposed reaction pathways for carotenoids during lipid autoxidation was presented in Section 7.1 (Fig. 7.2). Oxygen-deficient conditions in the oil may reduce the rate of formation of a second carotenoid peroxyl radical (pathway C), and subsequent hydrogen-abstraction from an intact fatty acid, and rather direct the reaction towards radical trapping (pathway B).

In the present work, astaxanthin had either no effect or showed a weak prooxidant effect in the autoxidation of purified fish oil TAG with added tocopherol, when the rate of autoxidation was substantially reduced compared to that in fish oil TAG without tocopherol. Similarly, β-carotene (1-20 ppm) was a weak prooxidant in soybean oil with natural tocopherols when stored in the dark (Warner and Frankel, 1987), and β-carotene (10 ppm) was a weak prooxidant in methyl linoleate with a tocopherol mixture of αTOH and δTOH (150 ppm) (1:1 by weight) (Suzuki *et al.*, 1989). In contrast, β-carotene and αTOH acted synergistically in retarding autoxidation in a 10% oil-in-water emulsion of purified rapeseed oil (Heinonen *et al.*, 1997). An antioxidant effect of carotenoids can be explained from the distribution of the carotenoid to the different oxidation pathways presented in Figure 7.2. Prooxidant behaviour, on the other hand, has been suggested to involve the oxidative breakdown products of the carotenoids (Suzuki *et al.*, 1989; Haila *et al.*, 2000).

The oxidation tests in this work were performed using a high oil surface-to-volume ratio and uncapped vials. In addition to the studies presented in the previous section, a separate storage test of purified fish oil TAG with αTOH (100 ppm) with and without astaxanthin (100 ppm) was carried out. Hydroperoxide formation in these oils (1.0 g samples) stored either in uncapped or in crimp-sealed vials (10 mL) with a 5% O_2 atmosphere was compared. The rate of formation of hydroperoxides was reduced by approximately 30% with reduced pO_2 for both oils, and no antioxidant effect of astaxanthin was evident. The precision of the data obtained for the oil samples stored under 5% O_2 was too low for a proper statistical evaluation, but the results indicate that even under conditions of restricted oxygen access, an antioxidant/prooxidant effect of astaxanthin is of little importance for oil stability compared to the effect of the tocopherols.

It is well known that tocopherols suppress β-carotene breakdown (Warner and Frankel, 1987; Suzuki *et al.*, 1989; Haila and Heinonen, 1994; Heinonen *et al.*, 1997). In accordance with these findings, the present data showed that the rate of oxidation of

astaxanthin in fish oil is substantially reduced in the presence of α-, γ- or δTOH or their combination. An important finding in this work was that in spite of the increase in the rate of formation of hydroperoxides observed upon increasing the initial αTOH concentration from 100 ppm to 500 ppm (Fig. 7.3A), the higher αTOH concentration more effectively retarded astaxanthin oxidation (Fig. 7.4). The participation of αTO• in reactions other than with peroxyl radicals, promoting the formation of hydroperoxides, was discussed in Section 3.4. The addition of ascorbyl palmitate to oil with αTOH had an additional stabilizing effect on astaxanthin, most likely primarily by regenerating αTOH from αTO•, and thereby reducing the rate of αTOH consumption.

A related finding was the discrepancy observed between the relative ability of the tocopherol homologues (at 500 ppm) to protect astaxanthin from oxidation and to protect the fatty acids from oxidation. At a low level of addition (100 ppm), the tocopherols reduced the rate of fatty acid autoxidation and astaxanthin breakdown in accordance with their established order of reactivity with peroxyl radicals (αTOH > γTOH > δTOH; Burton and Ingold, 1981). At a higher initial tocopherol concentration (500 ppm), the tocopherols inhibited fatty acid hydroperoxide formation in the order of δTOH > γTOH > mixed tocopherols > αTOH. In contrast, their relative ability to retard astaxanthin oxidation was αTOH ≈ γTOH > mixed tocopherols ≈ δTOH. The relative ability of the tocopherols, as well as αTOH at different concentrations, to reduce the rate of astaxanthin oxidation thus appears to be more closely related to their hydrogen-donating power than to their ability to retard the formation of fatty acid primary oxidation products.

It is likely that αTOH protects astaxanthin by being preferentially oxidized (Fig. 7.5). This is in agreement with the findings of Tsuchihashi *et al.* (1997), who reported that αTOH was 32 times more reactive toward peroxyl radicals than β-carotene in a free radical initiated oxidation of methyl linoleate in benzene. In their system of study, the oxidation of β-carotene was almost completely inhibited until αTOH had been consumed.

As mentioned earlier, astaxanthin is, based on the stabilizing effect of the carbonyl group on the carotenoid radical formed, expected to be a somewhat better fatty acid peroxyl radical scavenger than β-carotene, and accordingly a better competitor to the αTOH for peroxyl radicals. This may account for the relatively high rate of astaxanthin breakdown observed even when it was added together with αTOH (Fig. 7.5). In accordance with this view, a recent study by Naguib (2000) concluded that the peroxyl radical scavenging ability of astaxanthin was intermediate to those of αTOH and β-carotene, when using an azo peroxyl radical generator in an octane/butyronitrile medium.

The tocopherols may interfere with astaxanthin oxidation at several stages of the oxidation process by scavenging fatty acid peroxyl radicals (ROO•), peroxyl-carotenoid adduct radicals (ROOCar•) and carotenoid peroxyl radicals (ROOCarOO•) (Fig. 7.8). The high reactivity of the tocopherols toward peroxyl radicals suggests that the tocopherols, and αTOH in particular, protect astaxanthin mainly by rapid hydrogen donation to ROO•, thereby reducing the amount of ROO• available for reaction with astaxanthin. Since γTOH and δTOH are less active than αTOH as hydrogen-donors, the differences in the rates of peroxyl radical scavenging between these tocopherols and astaxanthin are likely smaller than that between αTOH and astaxanthin, and astaxanthin is therefore more rapidly destroyed.

At antioxidant concentrations above 500 ppm, the order of tocopherol activity on the basis of hydroperoxide formation is expected to remain δTOH > γTOH > αTOH, and the difference between the rates of hydroperoxide formation affected by the tocopherols is expected to increase. The rate of formation of hydroperoxides in fish oil, as well as the rate of αTOH consumption, was found to increase in a linear manner with initial αTOH concentration (Fig. 4.6 and 4.5, respectively). Accordingly, the relative ability of the tocopherols to protect astaxanthin may change with tocopherol concentration, and at sufficiently high tocopherol levels, γTOH, and eventually δTOH, may become better

stabilizers of astaxanthin than αTOH due to their more effective inhibition of the formation of fatty acid hydroperoxides and their lower rates of consumption.

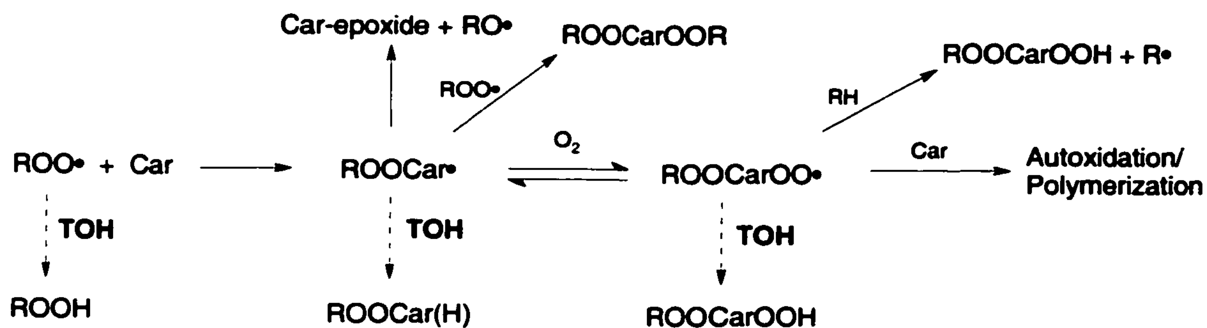


Figure 7.8. Possible reactions of tocopherols during oxidative degradation of astaxanthin.

8 Overall Discussion and Conclusions

Natural antioxidants are designed by evolution for optimum activity *in vivo*. Factors, in addition to the rate of reaction with peroxyl radicals and the availability of oxygen, that are important for their physiological activity include their ease of transport and incorporation into cells, placement in cell membranes, mobility and lipophilicity, as well as interplay with other *in vivo* defence systems against lipid peroxidation. These conditions change dramatically when the fatty acids and antioxidants are extracted, processed and stored *in vitro*, and it is not surprising that the properties of natural antioxidants change as well. α-Tocopherol is the main lipid soluble antioxidant in humans and animals, and it is generally the only tocopherol found in wild fish and in the fish oils produced from such fish. The tocopherols (as αTOH or blends of α-, γ- and δTOH), and to a lesser extent ascorbyl palmitate, are commonly used as antioxidants for fish oils, but without detailed knowledge about their modes of action. The overall purpose of this research project was to obtain more information about the properties of the tocopherols in fish oils. The antioxidant activities of individual tocopherols in fish oils, over a wide range of concentrations and with regard to the formation of both primary and secondary oxidation products, have not previously been evaluated.

Most of the oxidation studies presented in this thesis were conducted using fish oil purified by column chromatography to remove minor oil components, including antioxidant and prooxidant molecules. This allowed better control over factors known to influence the autoxidation process, and the purified TAG system provide a basis for comparison of the effectiveness of any antioxidant or antioxidant mixture in highly refined fish oils. Of particular importance was the removal of αTOH to below the limit of detection by HPLC. It is likely that the effect of tocopherols originally present in fats and oils is an important contributing factor to previous conflicting results in the literature with regard to the relative activities of added antioxidants. The concentration of metal catalysts (Fe and Cu) in the purified fish oil was very low, and citric acid, an efficient metal chelator in vegetable oils, had no effect on the rate of fatty acid oxidation.

The work presented in this thesis has demonstrated that the tocopherols affect many stages of the autoxidation process in purified fish oils, including the formation of hydroperoxides, the decomposition of hydroperoxides, the formation of nonvolatile secondary oxidation products, as well as the composition of the volatile secondary oxidation products formed. In all the oxidation tests addressing these different aspects of lipid autoxidation, as well as in a study of the effect of the tocopherols on the oxidative degradation of astaxanthin, the results obtained could be related to the relative hydrogen-donating ability of the tocopherol homologues, which decreases in the order of αTOH > γTOH > δTOH.

The number of methyl groups on the chroman ring of the tocopherols had a remarkable effect on their antioxidant properties in fish oil. The relative ability of the tocopherols to retard the formation of hydroperoxides in purified fish oil TAG 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 500 ppm and above. This dependence of relative antioxidant activity on tocopherol concentration was caused by the existence of concentrations for maximum antioxidant activity of αTOH and of γTOH. An inversion of activity, on the basis of hydroperoxide formation, was observed for αTOH at 100 ppm and for γTOH at 500 ppm, while the antioxidant activity of δTOH increased with level of addition up to 1500-2000 ppm. Maximum antioxidant activity of αTOH at 100 ppm has also been observed by several investigators in vegetable oils (Jung and Min, 1990; Huang *et al.*, 1994; Blekas *et al.*, 1995; Lampi *et al.*, 1999), despite the big differences in oil composition and susceptibility to oxidation among the different vegetable oils and between vegetable oils and fish oil. Moreover, removal of minor components from menhaden oil in the present work reduced the rate of fatty acid autoxidation but did not appear to influence the concentration of αTOH for maximum antioxidant activity. These results suggest that the oil composition has little impact on the αTOH-concentration for inversion of activity. The order of the rate of consumption of the different tocopherols was concentration dependent, and the reverse of their relative ability

to retard the formation of hydroperoxides, i.e. the tocopherol consumed most rapidly was the least effective one in retarding primary oxidation. Contrary to many previous reports on the effects of the tocopherols on the formation of lipid hydroperoxides, none of the tocopherols displayed any prooxidant activity.

Antioxidants are commonly evaluated by their ability to retard the formation of primary oxidation products, however, the volatile secondary oxidation products may better reflect their ability to reduce flavour deterioration. Studies of the relative impact of tocopherol homologue and concentration on the formation of secondary oxidation products are complicated by the many reaction routes available for hydroperoxide decomposition. In this work, all three tocopherols retarded the formation of C3-aldehydes (propanal + 2-propenal), determined as a marker of volatile secondary oxidation products, in a concentration-dependent manner. When studying tocopherol blends at a total tocopherol concentration of 1500 ppm, a high αTOH-content was the most effective at retarding the initial formation of C3-aldehydes. In contrast, tocopherol mixtures with a high γTOH+δTOH-concentration were the most active in the later stages of storage. The formation of volatile secondary oxidation products depends on both the rate of formation of primary oxidation products and the degree of antioxidant inhibition of their decomposition. Results obtained in this work suggest that αTOH is better than γTOH and δTOH in retarding the decomposition of hydroperoxides. This view is based on the observation that, although αTOH was at certain levels of addition the least active of the tocopherols in retarding the formation of hydroperoxides, the concentrations of volatile secondary oxidation products were similar or lower than in oils with γTOH or δTOH.

It has previously been demonstrated that αTOH inhibits the decomposition of hydroperoxides of methyl linoleate in a concentration-dependent manner (Hopia *et al.*, 1996; Mäkinen and Hopia, 2000). Based on this finding, as well as those of the present work, it is considered likely that protection of the already formed hydroperoxides has

contributed to the induced accumulation of hydroperoxides observed at tocopherol concentrations above 100 ppm for αTOH and above 500 ppm for γTOH.

Data from the dynamic headspace analysis of the volatile secondary oxidation products formed during storage of purified fish oil TAG were evaluated by principal component analysis, and both the overall formation and composition of volatiles were markedly affected by the type and concentration of tocopherol added as antioxidant. Multivariate analysis has not previously been used in an effort to relate the formation of specific volatile secondary oxidation products to the antioxidants added. The PCA suggested that high tocopherol hydrogen-donating power (i.e. a high tocopherol concentration or the use of αTOH as opposed to γTOH and δTOH) directs the formation of hydrocarbons and of unsaturated carbonyl compounds of higher molecular weight, as well as the formation of *trans, cis* isomers of diunsaturated aldehydes. Although an active inhibitor of overall formation of volatile secondary oxidation products, a high αTOH concentration may thus direct the formation towards the more flavour-potent carbonyls.

Propanal is one of the major volatile oxidation products formed from n-3 fatty acids and has been analysed by static headspace GC as a marker of this group of oxidation products in fish oils (Boyd *et al.*, 1992, Frankel *et al.*, 1996). Principal component analysis of volatile secondary oxidation products from fish oil in the present work, suggested that the C3-aldehydes together (propanal + 2-propenal) is a more suitable marker of secondary oxidation of n-3 fatty acids than propanal alone. These two oxidation products coelute on medium polarity GC columns (e.g. SPB-1701).

The determination of conjugated dienes as a measure of primary oxidation was found to correlate very well with the more widely used peroxide value. The CD analysis is fast and simple, requires very little sample, and is considered particularly useful for stability studies with oils. Fatty acid hydroxy compounds and hydroperoxy epidioxides are considered secondary lipid oxidation products. From a comparison of CD and POV

measurements, it is suggested that the formation of these oxidation products relative to that of hydroperoxides is affected by the hydrogen-donating ability of the tocopherol added as antioxidant. The relative formation of hydroperoxy epidioxides, expected to be the most abundant of these two groups of secondary oxidation products in fish oils, appears to decrease with increasing tocopherol hydrogen-donating ability and with αTOH-concentration. These results are of particular importance when evaluating the activity of antioxidants, by the established methods for the determination of primary oxidation, in oils rich in PUFA with three or more methylene-interrupted double bonds.

In this work, addition of ascorbyl palmitate to fish oil also containing αTOH, eliminated the αTOH-mediated accumulation of hydroperoxides. By maintaining a high αTOH concentration for a longer time, the initial rate of hydroperoxide formation was reduced in the oils with an αTOH concentration above 100 ppm, which emphasizes the importance of the αTO•, as opposed to αTOH itself, in reactions inducing hydroperoxide formation. The question remains as to why the initial rate of formation of αTO• apparently increases with αTOH concentration (Lambelet and Löliger, 1984), when a low αTOH level (e.g. 100 ppm) appears to be adequate for the scavenging of the peroxyl radicals generated by chain initiation and propagation. The tocopherols react with lipid peroxyl radicals mainly via reactions [8.1] and [8.2], and, accordingly, a stoichiometric factor of approximately two has been experimentally confirmed in solutions using artificial radical initiators (Burton and Ingold, 1981; Liebler and Burr, 1995).

$$ROO\bullet + TOH \rightarrow ROOH + TO\bullet \qquad [8.1]$$

$$ROO\bullet + TO\bullet \rightarrow \text{nonradical products} \qquad [8.2]$$

Due to the higher rate of reaction of αTOH with fatty acid peroxyl radicals compared to those of γTOH and δTOH, it is speculated that at high initial αTOH concentrations, reaction [8.1] dominates. This implies that most of the ROO• generated are being rapidly

consumed in a reaction with αTOH, as opposed to with αTO•, and the accumulation of αTO• is induced. A preferential scavenging of peroxyl radicals by γTOH, as opposed to γTO•, becomes important at a higher initial tocopherol concentration compared to that for αTOH, and accordingly a higher γTOH concentration for inversion of activity is observed.

In a study of the oxidation products of αTOH in methyl linoleate, Yamauchi *et al.* (1995) observed that the formation of αTOH dimers occurred at a high αTOH concentration only, and was accompanied by an increase in the rate of formation of methyl linoleate hydroperoxides. The rate constant for dimer formation is 5 orders of magnitude lower than that for the formation of 8a-peroxy-tocopherones (Fig. 8.1), and for dimer formation to occur to any significant extent, the ratio [αTO•] : [ROO•] must be high. The findings of Yamauchi *et al.* (1995) thus appear to support the view just presented, namely that at high initial αTOH concentrations, peroxyl radicals are scavenged preferentially by αTOH hydrogen donation, and to a lesser extent by radical coupling with αTO•, despite the higher rate constant for this last reaction (Fig. 8.1). In this scenario, some αTOH is likely consumed after having scavenged only one peroxyl radical. This implies a theoretical reduction in the stoichiometric factor, which may lower the antioxidant power.

For γTOH and δTOH, which have lower rate constants than αTOH for the hydrogen donation to lipid peroxyl radicals, the resulting tocopheroxyl radicals may be better competitors for the lipid peroxyl radicals [8.2], and the concentration of the TO• remains relatively low. Higher rate constants for the bimolecular coupling reactions to form tocopherol dimers for γTO• (4.5×10^4 $M^{-1}s^{-1}$) and for δTO• (1.5×10^5 $M^{-1}s^{-1}$) compared to that for αTO• (3×10^3 $M^{-1}s^{-1}$) (Burton *et al.*, 1985) may also contribute to maintaining a lower tocopheroxyl radical concentration.

Rapid generation of TO• may lead to hydroperoxide formation via the abstraction of hydrogen from an intact fatty acid by the TO•, and this chain-transfer reaction was

suggested to induce an observed prooxidant effect of αTOH by Terao and Matsushita (1986) and Pokorný (1987). However, the rate constant for this reaction is very low, in the order of 10^{-2} $M^{-1}s^{-1}$ (Mukai *et al.*, 1993), compared to the αTO• coupling reaction (Fig. 8.1), and it seems likely that other reactions involving the αTO• also contribute to αTOH-mediated peroxidation in fats and oils *in vitro*.

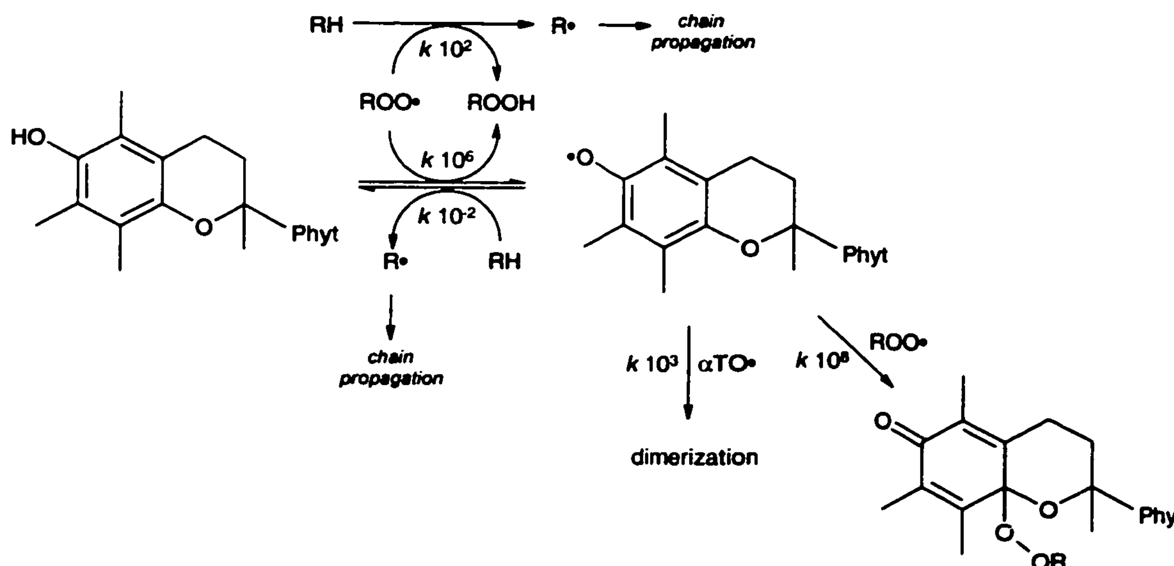


Figure 8.1. Rate constants (k, $M^{-1}s^{-1}$) for the reactions of αTOH/αTO• with free radicals. Summarized from Burton and Ingold (1981), Mukai *et al.* (1993) and Bowry and Ingold (1995).

Astaxanthin is generally regarded as an efficient peroxyl radical scavenger(Terao, 1989; Jørgensen and Skibsted, 1993; Naguib, 2000). However, as is also the case with αTOH, the excellent antioxidant activities observed in solutions using artificial azo radical generators, is not necessarily relevant for bulk oils. In a purified fish oil model system with αTOH, astaxanthin was a weak prooxidant on the basis of the formation of both primary and volatile secondary oxidation products. Considering its negative impact on hydroperoxide formation at concentrations above 100 ppm, αTOH was surprisingly

effective in protecting astaxanthin from oxidative degradation compared to the other tocopherol homologues.

The focus of this research has been to study the properties of the tocopherols, and not to develop an optimum antioxidant system for fish oils. However, based on the findings in this work, some general recommendations can be made. Antioxidants may be evaluated by their ability to retard both the formation of hydroperoxides and the formation of secondary oxidation products, as well as the extent to which they increase the induction period of an oil. An antioxidant for oils high in PUFA should also be active *in vivo*. Ideally, an antioxidant should perform well according to all these criteria. α-Tocopherol is very active in retarding the formation of secondary oxidation products, but induces hydroperoxide formation at concentrations relevant for addition, and should not be used alone as an antioxidant additive to fish oils, particularly not at high concentrations. In addition, it appears to direct the formation of the more flavour-potent volatile secondary oxidation products. In contrast, δTOH at high levels of addition is an efficient antioxidant on the basis of both primary and secondary oxidation, as well as the duration of the induction period, but its biological activity is very low. Due to its high activity *in vivo*, αTOH should continue to be used as an antioxidant additive to fish oils. Methods to protect αTOH, as well as γTOH, from rapid consumption and corresponding induction of hydroperoxide formation, and, moreover, to make the most of its excellent peroxyl radical scavenging activity, therefore become important. Ascorbyl palmitate substantially increased the antioxidant strength of αTOH in fish oil. The addition of ascorbyl palmitate also improved the antioxidant activity of a tocopherol blend. Its behaviour in binary systems with γTOH or δTOH was not studied.

Antioxidant addition is an important factor in any attempt to increase the stability of fish oils. However, for antioxidants to substantially reduce flavour deterioration, the oil to which they are added must be of high initial quality with regard to oxidative deterioration. This implies the use of freshly produced oil that has been subjected to a gentle refining

process and kept in the absence of light and oxygen. The use of high temperature and/or oxygen access will induce the formation of artefacts such as dimers and oligomers, as well as conjugation and isomerization of double bonds. Although not detected by the most common methods for determining fatty acid oxidation, these products are likely to contribute to a more rapid formation of fishy off-flavours.

Bibliography

Ackman, R.G. 1994. Animal and marine lipids. In *Technological Advances in Improved and Alternative Sources of Lipids*, B.S. Kamel and Y. Kakuda (Eds.), p. 292. Blackie Academic & Professional, Chapman & Hall, Glascow.

Ackman, R.G. and Cormier, M.G. 1967. α-Tocopherol in some Atlantic fish and shellfish with particular reference to live-holding without food. *J. Fish. Res. Bd. Canada* 24: 357-373.

Aminullah Bhuiyan, A.K.M., Ratnayake, W.M.N. and Ackman, R.G. 1993. Nutritional composition of raw and smoked Atlantic mackerel (*Scomber scombrus*): Oil- and water-soluble vitamins. *J. Food Comp. Anal.* 6: 172-184.

Azzi, A. and Stocker, A. 2000. Vitamin E: Non-antioxidant roles. *Prog. Lipid Res.* 39: 231-255.

Badings, H.T. 1970. Cold-storage defects in butter and their relation to the autoxidation of unsaturated fatty acids. *Nethlds. Milk Dairy J.* 23-24: 146-256, 1969/70.

Barlow, S.M. 1997. Fish oils and their future role in international fats and oils scene. Paper presented at LIPIDEX, Antwerp, March 1997. IFOMA, Hertfordshire, UK.

Bimbo, A.P. 1990. Processing of fish oils. In *Fish Oils in Nutrition*, M.E. Stansby (Ed.), p. 181. Van Nostrand Reinhold, New York.

Blekas, G., Tsimidou, M. and Boskou, D. 1995. Contribution of α-tocopherol to olive oil stability. *Food Chem.* 52: 289-294.

Boyd, L.C., King, M.F. and Sheldon, B. 1992. A rapid method for determining the oxidation of n-3 fatty acids. *J. Am. Oil Chem. Soc.* 69: 325-330.

Bowry, V.W. and Ingold, K.U. 1995. Extraordinary kinetic behaviour of the α-tocopheroxyl (vitamin E) radical. *J. Org. Chem.* 60: 5456-5467.

Bragadóttir, M., Þórisson, S. and Hjaltason, B. 1995. Effects of different antioxidants in fish oils, compared by Warburg manometric technique, chemical measurement and sensory analysis. In *Proceedings of the 18th Nordic Lipid Symposium, Reykjavik, Iceland*, pp. 101-104. Lipidforum, Bergen.

Buettner, G.R. 1993. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* 300: 535-543.

Burkow, I.C., Vikersveen, L. and Saarem, K. 1995. Evaluation of antioxidants for cod liver oil by chemiluminescence and the Rancimat method. *J. Am. Oil Chem. Soc.* 72: 553-557.

Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L. and Ingold, K.U. 1985. Autoxidation of biological molecules. 4. Maximizing the antioxidant activity of phenols. *J. Am. Chem. Soc.* 107: 7053-7065.

Burton, G.W., Hughes, L. and Ingold, K.U. 1983. Antioxidant activity of phenols related to vitamin E. Are there chain-breaking antioxidants better than α-tocopherol? *J. Am. Chem. Soc.* 105: 5950-5951.

Burton, G.W. and Ingold, K.U. 1981. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J. Am. Chem. Soc.* 103: 6472-6477.

Burton, G.W. and Ingold, K.U. 1984. β-Carotene: An unusual type of lipid antioxidant. *Science* 224: 569-573.

Burton, G.W. and Ingold, K.U. 1989. Vitamin E as *in vitro* and *in vivo* antioxidant. *Ann. N.Y. Acad. Sci.* 570: 7-22.

Chahine, M.H. 1978. Effect of cupric chloride and ferric chloride on decreasing the oxidative stability of herring oil treated with phenolic antioxidants. *Grasas y Aceites* 29: 423-430.

Chan, H.W.-S. 1987. The mechanism of autoxidation. In *Autoxidation of Unsaturated Lipids*, H.W.-S. Chan (Ed.), pp. 1-16. Academic Press, London, England.

Chan, H.W.-S. and Coxon, D.T. 1987. Lipid hydroperoxides. In *Autoxidation of Unsaturated Lipids*, H.W.-S. Chan (Ed.), pp. 17-50. Academic Press, London, England.

Chan, H.W.-S., Coxon, D.T., Peers, K.E. and Price, K.R. 1982. Oxidative reactions of unsaturated lipids. *Food Chem.* 9: 21-34.

Chan, H. W.-S. and Levett, G. 1977. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids* 12: 99-104.

Chow, C.K. and Draper, H.H. 1974. Oxidative stability and antioxidant activity of the tocopherols in corn and soybean oils. *Internat. J. Vit. Nutr. Res.* 44: 396-403.

Cillard, J. and Cillard, P. 1980. Behavior of alpha, gamma, and delta tocopherols with linoleic acid in aqueous media. *J. Am. Oil Chem. Soc.* 57: 39-42.

Cillard, J., and Cillard, P. 1986. Inhibitors of the prooxidant activity of α-tocopherol. *J. Am. Oil Chem. Soc.* 63: 1165-1169.

Cort, W.M. 1974. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. *J. Am. Oil Chem. Soc.* 51: 321-325.

Cort, W.M., Mergens, W. and Greene, A. 1978. Stability of α- and γ-tocopherol: Fe^{3+} and Cu^{2+} interactions. *J. Food Sci.* 43: 797-798.

Elmadfa, I. and Wagner, K.-H. 1997. Vitamin E and stability of vegetable oils. *Fett/Lipid* 99: 234-238.

El-Magoli, S.B., Karel, M. and Yong, S. 1979. Acceleration of lipid oxidation by volatile products of hydroperoxide decomposition. *J. Food Biochem.* 3: 111-123.

Evans, H.M. and Bishop, K.S. 1922. On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science* 56: 650-651.

Fitch Haumann, B. 1990. Antioxidants: firms seeking products they can label as 'natural'. *INFORM* 1: 1002-1013.

Frankel, E.N., 1982. Volatile lipid oxidation products. *Prog. Lipid Res.* 22: 1-33.

Frankel, E.N. 1991. Recent advances in lipid oxidation. *J. Sci. Food Agric.* 54: 495-511.

Frankel, E.N. 1993a. Formation of headspace volatiles by thermal decomposition of oxidized fish oils *vs.* oxidized vegetable oils. *J. Am. Oil Chem. Soc.* 70: 767-772.

Frankel, E.N. 1993b. In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol.* 4: 220-225.

Frankel, E.N. 1996. Antioxidants in lipid foods and their impact on food quality. *Food Chem.* 57: 51-55.

Frankel, E.N. 1998. *Lipid Oxidation.* The Oily Press, Dundee, Scotland.

Frankel, E.N. and Gardner, H.W. 1989. Effect of α-tocopherol on the volatile thermal decomposition products of methyl linoleate hydroperoxides. *Lipids* 24: 603-608.

Frankel, E.N., Huang, S.-W., Prior, E. and Aeschbach, R. 1996. Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. *J. Sci. Food Agric.* 72: 201-208.

Frankel, E.N. and Tappel, A.L. 1991. Headspace gas chromatography of volatile lipid peroxidation products from human red blood cell membranes. *Lipids* 26: 479-484.

Fuster, M.D., Lampi, A.-M., Hopia, A. and Kamal-Eldin, A. 1998. Effects of α- and γ-tocopherols on the autoxidation of purified sunflower triacylglycerols. *Lipids* 33: 715-722.

Gardner, H.W. 1987. Reactions of hydroperoxides - products of high molecular weight. In *Autoxidation of Unsaturated Lipids*, H.W.-S. Chan (Ed.), pp. 51-94. Academic Press, London, England.

Gordon, M.H. 1990. The mechanism of antioxidant action *in vitro*. In *Food Antioxidants*, B.J.F. Hudson (Ed.), pp. 1-18. Elsevier Applied Science, Essex, England.

Gottstein, T. and Grosch, W. 1990. Model study of different antioxidant properties of α- and γ-tocopherol in fats. *Fat Sci. Technol.* 92: 139-144.

Grosch, W. 1987. Reactions of hydroperoxides - products of low molecular weight. In *Autoxidation of Unsaturated Lipids*, H.W.-S. Chan (Ed.), pp. 96-140. Academic Press, London, England.

Hahm, T.S. and Min, D.B. 1995. Analysis of peroxide values and headspace oxygen. In *Methods to Assess Quality and Stability of Oils and Fat-Containing Foods*, K. Warner and N.A.M. Eskin (Eds.), pp. 146-158. AOCS Press, Champaign, IL.

Haila, K.M. and Heinonen, M. 1994. Action of β-carotene on purified rapeseed oil during light storage. *Lebensm.-Wiss. u.-Technol.* 27: 573-577.

Haila, K.M., Hopia, A. and Heinonen, M. 2000. Effects of β-carotene and retinal on the formation of methyl linoleate hydroperoxides. *Eur. J. Lipid Sci. Technol.* 31-36.

Haila, K.M., Lievonen, S.M., Heinonen, M.I. 1996. Effects of lutein, lycopene, annatto, and γ-tocopherol on autoxidation of triglycerides. *J. Agric. Food Chem.* 44: 2096-2100.

Hamilton, R.J., Kalu, C., McNeill, G.P., Padley, F.B. and Pierce, J.H. 1998. Effects of tocopherols, ascorbyl palmitate, and lecithin on autoxidation of fish oil. *J. Am. Oil Chem. Soc.* 75: 813-822.

Han, D.H., Yi, O.-S. and Shin, H.-K. 1991. Solubilization of vitamin C in fish oil and synergistic effect with vitamin E in retarding oxidation. *J. Am. Oil Chem. Soc.* 68: 740-743.

Heinonen, M., Haila, K.M., Lampi, A.-M. and Piironen, V. 1997. Inhibition of oxidation in 10% oil-in-water emulsions by β-carotene with α- and γ-tocopherols. *J. Am. Oil Chem. Soc.* 74: 1047-1052.

Hemre, G.-I., Juell, J.E., Hamre, K., Lie, Ø., Strand, B., Arnesen, P. and Holm, J.C. Cage feeding of Atlantic mackerel (*Scomber scombus*): Fatty acid composition, oxidation status and vitamin E concentration. *Aquat. Living Resour.* 10: 365-370.

Hopia, A., Huang, S.-W. and Frankel, E.N. 1996. Effect of α-tocopherol and Trolox on the decomposition of methyl linoleate hydroperoxides. *Lipids* 31: 357-365.

Hsieh, T.C.Y., Williams, S.S., Vejaphan, W. and Meyers, S.P. 1989. Characterization of volatile components of menhaden fish (*Brevoortia tyrannus*) oil. *J. Am. Oil Chem. Soc.* 66: 114-117.

Hu, S.-P. and Pan, B.S. 2000. Modification of fish oil aroma using macroalgal lipoxygenase. *J. Am. Oil Chem. Soc.* 77: 343-348.

Huang, S.-W., Frankel, E.N. and German, J.B. 1994. Antioxidant activity of α- and γ-tocopherols in bulk oils and in oil-in-water emulsions. *J. Agric. Food Chem.* 42: 2108-2114.

Huang, S.-W., Frankel, E.N. and German, J.B. 1995. Effects of individual tocopherols and tocopherol mixtures on the oxidative stability of corn oil triglycerides. *J. Agric. Food Chem.* 43: 2345-2350.

Hudson, B.J.F. and Ghavami, M. 1984. Stabilising factors in soyabean oil - natural components with antioxidant activity. *Lebensm.-Wiss. u. -Technol.* 17: 82-85.

Ingold, K.U., Burton, G.W., Foster, D.O., Hughes, L. Lindsay, D.A. and Webb, A. 1987. Biokinetics and discrimination between dietary RRR- and SRR-α-tocopherol in the male rat. *Lipids* 22: 163-172.

IUPAC method 2.505. 1987. Evidence of purity and deterioration from ultraviolet spectrometry. In *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th ed., C. Paquot and A. Hautfenne (Eds.). Blackwell Sci.. Publ., Oxford.

Jung, M.Y. and Min, D.B. 1990. Effects of α-, γ- and δ-tocopherols on oxidative stability of soybean oil. *J. Food Sci.* 55: 1464-1465.

Jørgensen, K. and Skibsted, L.H. 1993. Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidant activity. *Z. Lebensm. Unters. Forsch.* 196: 423-429.

Kaitaranta, J.K. 1992. Control of lipid oxidation in fish oil with various antioxidative compounds. *J. Am. Oil Chem. Soc.* 69: 810-813.

Kamal-Eldin, A. and Appelqvist, L.-Å. 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31: 671-701.

Karahadian, C. and Lindsay, R.C. 1989a. Evaluation of compounds contributing characterizing fishy flavors in fish oils. *J. Am. Oil Chem. Soc.* 66: 953-960.

Karahadian, C. and Lindsay, R.C. 1989b. Action of tocopherol-type compounds in directing reactions forming flavor compounds in autoxidizing fish oils. *J. Am. Oil Chem. Soc.* 66: 1302-1308.

Ke, P.J., Ackman, R.G. and Linke, B.A. 1975. Autoxidation of polyunsaturated fatty compounds in mackerel oil: Formation of 2,4,7-decatrienals. *J. Am. Oil Chem. Soc.* 52: 349-353.

Ke, P.J., Nash, D.M. and Ackman, R.G. 1977. Mackerel skin lipids as an unsaturated fat model system for the determination of antioxidant potency of TBHQ and other antioxidant compounds. *J. Am. Oil Chem. Soc.* 54: 417-420.

Kennedy, T.A. and Liebler, D.C. 1992. Peroxyl radical scavenging by β-carotene in lipid bilayers. Effect of oxygen partial pressure. *J. Biol. Chem.* 267: 4658-4663.

Khan, M.A. and Shahidi, F. 2000. Oxidative stability of stripped and nonstripped borage and evening primrose oils and their emulsions in water. *J. Am. Oil Chem. Soc.* 77: 963-968.

Kinsella, J.E. 1987. *Seafoods and Fish Oils in Human Health and Disease*. Marcel Dekker, New York.

Koschinski, I. and Mcfarlane, N. 1993. Stability of marine oils by treatment with silica and addition of lecithin, ascorbyl palmitate and tocopherol. International Patent WO 93/10207.

Koskas, J.P., Cillard, J. and Cillard, P. 1984. Autoxidation of linoleic acid and behavior of its hydroperoxides with and without tocopherols. *J. Am. Oil Chem. Soc.* 61: 1466-1469.

Krinsky, N.I. 1989. Antioxidant functions of carotenoids. *Free Radic. Biol. Med.* 7: 617-635.

Lambelet, P. and Löliger, J. 1984. The fate of antioxidant radicals during lipid autoxidation. I. The tocopheroxyl radicals. *Chem. Phys. Lipids* 35: 185-198.

Lambelet, P., Saucy, F. and Löliger, J. 1985. Chemical evidence for interactions between vitamins E and C. *Experientia* 41: 1384-1388.

Lampi, A.-M., Hopia, A., Ekholm, P. and Piironen, V. 1992. Method for the preparation of triacylglycerol fractions from rapeseed and other oils for autoxidation studies. *Lebensm.-Wiss. u.- Technol.* 25: 386-388.

Lampi, A.-M., Hopia, A. and Piironen, V. 1997. Antioxidant activity of minor amounts of γ-tocopherol in natural triacylglycerols. *J. Am. Oil Chem. Soc.* 74: 549-555.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A. and Piironen, V. 1999. Antioxidant activities of α- and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols. *J. Am. Oil Chem. Soc.* 76: 749-755.

Lampi, A.-M. and Piironen, V. 1998. α- and γ-Tocopherols as efficient antioxidants in butter oil triacylglycerols. *Fett/Lipid* 100: 292-295.

Lea, C.H. 1960. On the antioxidant activities of the tocopherols. II. Influence of substrate, temperature and level of oxidation. *J. Sci. Food Agric.* 11: 212-218.

Lea, C.H. and Ward, R.J. 1959. Relative antioxidant activity of the seven tocopherols. *J. Sci. Food Agric.* 10: 537-548.

Lee, S.H. and Min, D.B. 1990. Effects, quenching mechanisms, and kinetics of carotenoids in chlorophyll-sensitized photooxidation of soybean oil. *J. Agric. Food Chem.* 38: 1630-1634.

Liebler, D.C., Baker, P.F. and Kaysen, K.L. 1990. Oxidation of vitamin E: Evidence for competing autoxidation and peroxyl radical trapping reactions of the tocopheroxyl radical. *J. Am. Chem. Soc.* 112: 6995-7000.

Liebler, D.C. and Burr, J.A. 1995. Antioxidant stoichiometry and the oxidative fate of vitamin E in peroxyl radical scavenging reactions. *Lipids* 30: 789-793.

Liebler, D.C. and McClure, T.D. 1996. Antioxidant reactions of β-carotene: Identification of carotenoid-radical adducts. *Chem. Res. Toxicol.* 9: 8-11.

Mäkinen, E.M. and Hopia, A.I. 2000. Effects of α-tocopherol and ascorbyl palmitate on the isomerization and decomposition of methyl linoleate hydroperoxides. *Lipids* 35: 1215-1223.

Marinova, E.M. and Yanishlieva, N.V. 1992a. Effect of temperature on the antioxidative action of inhibitors in lipid autoxidation. *J. Sci. Food Agric.* 60: 313-318.

Marinova, E.M. and Yanishlieva, N.V. 1992b. Inhibited oxidation of lipids III: On the activity of ascorbyl palmitate during the oxidation of two types of lipid systems in the presence of α-tocopherol. *Fat Sci. Technol.* 94: 448-452.

Massart, D.L., Vandeginste, B.G.M., Buydens, L.M.C., de Jong, S., Lewi, P.J. and Smeyers-Verbeke, J. 1997. *Handbook of Chemometrics and Qualimetrics: Part A*, pp. 519-538. Elsevier, Amsterdam, Netherlands.

Matthews, R.F., Scanlan, R.A. and Libbey, L.M. 1971. Autoxidation products of 2,4-decadienal. *J. Am. Oil Chem. Soc.* 48: 745-747.

Medina, I., Satué, M.T. and Frankel, E.N. 1999. Static headspace gas chromatographic analysis to determine oxidation of fish muscle lipids during thermal processing. *J. Am. Oil Chem. Soc.* 76: 231-236.

Meijboom, P.W. and Stroink, J.B.A. 1972. 2-*trans*,4-*cis*,7-*cis*-Decatrienal, the fishy off-flavor occurring in strongly autoxidized oil containing linolenic acid or ω3,6,9, etc., fatty acids. *J. Am. Oil Chem. Soc.* 49: 555-558.

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E. and Kinneary, J.F. 1996. *The Merck Index. An encyclopedia of chemicals, drugs, and biologicals*. Merck Research Laboratories, Whitehouse Station, NJ.

Morrison, W.R. and Smith, L.M. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipid with boron fluoride-methanol. *J. Lipid Res.* 5: 600-608.

Mortensen, A. and Skibsted, L.H. 1998. Reactivity of β-carotene towards peroxyl radicals studied by laser flash and steady-state photolysis. *FEBS Lett.* 426: 392-396.

Muggli, R. 1994. Polyunsaturated fatty acids and requirements of vitamin E. *Fat Sci. Technol.* 96: 17-19.

Mukai, K., Sawada, K., Kohno, Y. and Terao, J. 1993. Kinetic study of the peroxidizing effect of tocopherol. Hydrogen abstraction from lipid hydroperoxides by tocopheroxyls in solution. *Lipids* 28: 747-752.

Murkovic, M., Wiltschko, D. and Pfannhauser, W. 1997. Formation of α-tocopherolquinone and α-tocopherolquinone epoxides in plant oil. *Fett/Lipid* 99: 165-169.

Naguib, Y.M.A. 2000. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Sci.* 48: 1150-1154.

Nakamura, K. Iida, H. and Tokunaga, T. 1980. Separation and identification of odor in oxidized sardine oil. *Bull. Japan Soc. Sci. Fish.* 46: 355-360.

Neff , W.E., Frankel, E.N. and Weisleder, D. 1981. High-pressure liquid chromatography of autoxidized lipids: II. Hydroperoxy-cyclic peroxides and other secondary products from methyl linolenate. *Lipids* 16: 439-448.

Nieto, S., Garrido, A., Sanhueza, J. Loyola, L.A., Morales, G., Leighton, F. and Valenzuela, A. 1993. Flavonoids as stabilizers of fish oil: an alternative to synthetic antioxidants. *J. Am. Oil Chem. Soc.* 70: 773-777.

Niki, E., Tsuchiya, J., Yoshikawa, Y., Yamamoto, Y. and Kamiya, Y. 1986. Oxidation of lipids. XIII. Antioxidant activities of α-, β-, γ-, and δ-tocopherols. *Bull. Chem. Soc. Jpn.* 59: 497-501.

Noor, N. and Augustin, M.A. 1984. Effectiveness of antioxidants on the stability of banana chips. *J. Sci. Food Agric.* 35: 805-812.

Odumosu, O.T. and Hudson, B.J.F. 1978. Chemical factors determining the catalytic activity of iron in the autoxidation of edible oils. *J. Sci. Food Agric.* 29: 1095.

Olcott, H.S. and van der Veen, J. 1968. Comparison of antioxidant activities of tocol and its methyl derivatives. *Lipids* 3: 331-334.

Palozza, P. and Krinsky, N.I. 1992. Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview. *Methods in Enzymol.* 213: 403-421.

Parazo, M.P.M., Lall, S.P., Castell, J.D. and Ackman, R.G. 1998. Distribution of α- and γ-tocopherols in Atlantic salmon (*Salmo salar*) tissues. *Lipids* 33: 697-704.

Peers, K.E., Coxon, D.T. and Chan, H.W.-S. 1981. Autoxidation of methyl linolenate: The effect of α-tocopherol. *J. Sci. Food Agric.* 32: 898-904.

Perricone, N., Nagy, K., Horvath, F., Uray, I. and ZsNagy, I. 1999. The hydroxyl free radical scavenging reactions of ascorbyl palmitate as measured in various *in vitro* models. *Biochem. Biophys. Res. Comm.* 262: 661-665.

Pokorný, J. 1987. Major factors affecting the autoxidation of lipids. In *Autoxidation of Unsaturated Lipids*, H.W.-S. Chan (Ed.), pp. 141-206. Academic Press, London, England.

Pongracz, G. 1984. γ-Tocopherol as natural antioxidant. *Fette Seifen Anstrichmittel* 86: 455-460.

Pongracz, G., Weiser, H. and Matzinger, D. 1995. Tocopherols - antioxidants in nature. *Fat Sci. Technol.* 97: 90-104.

Porter, N.A., Caldwell, S.E. and Mills, K.E. 1995. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* 30: 277-289.

Porter, N.A., Lehman, L.S., Weber, B.A. and Smith, K.J. 1981. Unified mechanism for polyunsaturated fatty acid autoxidation. Competition of peroxy radical hydrogen atom abstraction, β-scisssion, and cyclization. *J. Am. Chem. Soc.* 103: 6447-6455.

Porter, N.A., Weber, B.A., Weenen, H. and Khan, J.A. 1980. Autoxidation of polyunsaturated lipids. Factors controlling the stereochemistry of the product hydroperoxides. *J. Am. Chem. Soc.* 102: 5597-5601.

Rossel, B. 1991. How to measure oxidative rancidity in fats and fatty foods. *Lipid Technol.* October-December: 122-126.

Przybylski, R. and Eskin, N.A.M. 1995. Methods to measure volatile compounds and the flavour significance of volatile compounds. In *Methods to Assess Quality and Stability of Oils and Fat-Containing Foods*, K. Warner and N.A.M. Eskin (Eds.), pp. 107-133. AOCS Press, Champaign, IL.

Rørbæk, K. 1994. *Oxidation and Flavours in Fish Oil*, Ph.D. Thesis, Technical University of Denmark, Lyngby, Denmark.

Rørbæk, K. and Jensen, B. 1997. Optimizing headspace sampling temperature and time for analysis of volatile oxidation products in fish oil. *J. Am. Oil Chem. Soc.* 74: 1607-1609.

Saeed, S. and Howell, N.K. 1999. High-performance liquid chromatography and spectroscopic studies on fish oil oxidation products extracted from frozen Atlantic mackerel. *J. Am. Oil Chem. Soc.* 76: 391-397.

Sagredos, A.N. 1991. Fatty acid composition of fish oil capsules. *Fat Sci. Technol.* 93: 184-191.

Sagredos, A.N. 1992. On the quality and contamination of fish oil capsules. *Fat Sci. Technol.* 94: 101-111.

Saito, H. and Nakamura, K. 1990. Antioxidant effect of sesamol on fish oil oxidation. *Bull. Japan. Soc. Sci. Fish.* 56: 1893.

Sanchez, J. and Myers, T.N. 1996. Peroxides and compounds (organic). In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed., vol. 18, J.I. Kroschwitz and M. Howe-Grant (Eds.), pp. 230-310. John Wiley & Sons, New York.

Satue, M.T., Huang, S.-W. and Frankel, E.N. 1995. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and deodorized olive oil. *J. Am. Oil Chem. Soc.* 72: 1131-1137.

Schuler, P. 1990. Natural antioxidants exploited commercially. In *Food Antioxidants*, B.J.F. Hudson (Ed.), pp. 99-170. Elsevier Applied Science, Essex, England.

Scott, G. 1997. *Antioxidants in Science, Technology, Medicine and Nutrition*. Albion Publishing, Chichester, England.

Shahidi, F. and Wanasundara, P.K.J.P.D. 1992. Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr.* 32: 67-103.

Shahidi, F. and Wanasundara, U.N. 1997. Measuring oxidation and evaluating antioxidant activity. In *Natural Antioxidants. Chemistry, Health Effects and Applications*, F. Shahidi (Ed.), pp. 379-396. AOCS Press, Champaign, IL.

Shahidi, F., Wanasundara, U.N. and Brunet, N. 1994. Oxidative stability of blubber of harp seal (*Phoca groenlandica*) as assessed by NMR and standard procedures. *Food Res. Int.* 27: 555-562.

Shukla, V.K.S. and Perkins, E.G. 1998. Rancidity in encapsulated health-food oils. *INFORM* 9: 955-961.

Siemensma, A.D. 1997. Functional carotenoids. Part 2. *Int. Food Ingred.* 2: 15-20.

Sigurgisladottir, S., Parrish, C.C., Ackman, R.G. and Lall, S.P. 1994a. Tocopherol deposition in muscle of Atlantic salmon (*Salmo salar*). *J. Food Sci.* 59: 256-259.

Sigurgisladottir, S., Parrish, C.C., Lall, S.P. and Ackman, R.G. 1994b. Effects of feeding natural tocopherols and astaxanthin on Atlantic salmon (*Salmo salar*) fillet quality. *Food Res. Int.* 27: 23-32.

Snyder, J.M., Frankel, E.N. and Selke, E. 1985. Capillary gas chromatography analyses of headspace volatiles from vegetable oils. *J. Am. Oil Chem. Soc.* 62: 1675-1679.

Suzuki, T., Usuki, R. and Kaneda, T. 1989. The role of carotenoids in the oxidative deterioration of edible oils. *J. Jpn. Oil Chem. Soc.* 6: 486-491.

Terao, J. 1989. Antioxidant activity of β-carotene-related carotenoids in solution. *Lipids* 24: 659-661.

Terao, J. and Matsushita, S. 1986. The peroxidizing effect of α-tocopherol on autoxidation of methyl linoleate in bulk phase. *Lipids* 21: 255-260.

Torrissen, O.J., Hardy, R.W. and Shearer, K.D. 1989. Pigmentation of salmonids - carotenoid deposition and metabolism. *Rev. Aquatic Sci.* 1: 209-225.

Totani, Y. 1997. Antioxidative effect of nitrogen-containing phospholipids on autoxidation of polyunsaturated oil. *J. Jpn. Oil Chem. Soc.* 46: 3-15.

Tsuchihashi, H., Kigoshi, M., Iwatsuki, M. and Niki, E. 1995. Action of β-carotene as an antioxidant against lipid peroxidation. *Arch. Biochem. Biophys.* 323: 137-147.

Wada, S. and Fang, X. 1992. The synergistic antioxidant effect of rosemary and α-tocopherol in sardine oil model system and frozen-crushed fish meat. *J. Food Process. Pres.* 16: 263-274.

Wanasundara, U.N. and Shahidi, F. 1998. Stabilization of marine oils with flavonoids. *J. Food Lipids* 5: 183-196.

Warner, K. and Frankel, E.N. 1987. Effects of β-carotene on light stability of soybean oil. *J. Am. Oil Chem. Soc.* 64: 213-218.

White, P.J. 1995. Conjugates diene, anisidine value, and carbonyl value analysis. In *Methods to Assess Quality and Stability of Oils and Fat-Containing Foods*, K. Warner and N.A.M. Eskin (Eds.), pp. 159-178. AOCS Press, Champaign, IL.

Yamauchi, R. 1999. Oxidation products of vitamin E. *Yukagaku* 48: 95-102.

Yamauchi, R., Kato, K. and Ueno, Y. 1995. Free-radical scavenging reactions of α-tocopherol during the autoxidation of methyl linoleate in bulk phase. *J. Agric. Food Chem.* 43: 1455-1461.

Yamauchi, R., Miyake, N., Kato, K. and Ueno, Y. 1993. Reaction of α-tocopherol with alkyl and alkylperoxyl radicals of methyl linoleate. *Lipids* 28: 201-206.

Yi, O.-S., Han, D. and Shin, H.-K. 1991. Synergistic antioxidant effects of tocopherol and ascorbic acid in fish oil/lecithin/water system. *J. Am. Oil Chem. Soc.* 68: 881-883.

Yoshiwa. T., Morimoto, K., Sakamoto, K., Ishikawa, Y., Tokita, M. and Morita, M. 1996. Volatile compounds of fishy odor in sardine by simultaneous distillation and extraction under reduced pressure. *Nippon Suisan Gakkaishi* 63: 222-230.

Appendix A
Fatty acid composition of fish oils and purified fish oil TAG fractions

Table A1. Fatty acid composition of fish oils and purified fish oil TAG fractions.

Fatty acid	Original menhaden oil (% w/w)	Purified menhaden TAG (% w/w)	Purified anchovy TAG-1 (% w/w)	Purified anchovy TAG-2 (% w/w)
iso 13:0	0.04 ± 0.01	0.02 ± < 0.01	0.02 ± < 0.01	0.06 ± 0.03
12:0	0.13 ± < 0.01	0.12 ± 0.02	0.11 ± 0.01	0.16 ± 0.01
13:0	0.07 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.07 ± 0.01
iso 14:0	0.06 ± < 0.01	0.06 ± < 0.01	0.05 ± 0.01	0.08 ± < 0.01
14:0	**7.52 ± 0.03**	**7.40 ± < 0.01**	**6.9 ± 0.3**	**8.8 ± 0.3**
14:1	0.11 ± 0.01	0.10 ± < 0.01	0.08 ± 0.03	0.16 ± 0.01
iso 15:0	0.35 ± 0.01	0.35 ± 0.010	0.22 ± 0.01	0.26 ± 0.01
anteiso 15:0	0.18 ± 0.01	0.18 ± < 0.01	0.12 ± 0.01	0.09 ± 0.01
15:0	0.60 ± 0.01	0.59 ± < 0.01	0.55 ± 0.02	0.55 ± 0.01
15:1	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.03	0.05 ± 0.02
iso 16:0	0.14 ± 0.01	0.14 ± 0.01	0.10 ± < 0.01	0.11 ± 0.01
anteiso 16:0	0.03 ± 0.01	0.02 ± < 0.01	0.01 ± 0.01	-
Pristanic	0.04 ± < 0.01	0.04 ± 0.01	0.02 ± 0.02	0.05 ± 0.01
7-methyl-16:0	0.31 ± < 0.01	0.31 ± < 0.01	0.20 ± 0.01	0.20 ± 0.01
16:0	**18.96 ± 0.05**	**19.09 ± 0.05**	**16.1 ± 1.1**	**17.1 ± 0.2**
16:1 n-9	-	-	-	-
16:1 n-7	**9.39 ± 0.03**	**9.42 ± 0.01**	**9.2 ± 0.4**	**10.0 ± 0.2**
16:1 n-5	trace	-	0.18 ± 0.02	0.51 ± 0.01
16:2 n-4	1.21 ± 0.01	1.18 ± < 0.01	1.00 ± 0.04	1.66 ± 0.04
16:3 n-4	1.71 ± 0.01	1.66 ± 0.01	1.59 ± 0.06	1.89 ± 0.06
16:3 n-3	0.07 ± < 0.01	0.06 ± < 0.01	trace	0.18 ± 0.03
16:4 n-3	0.23 ± 0.01	0.20 ± < 0.01	0.13 ± 0.01	0.13 ± 0.02
16:4 n-1	0.86 ± < 0.01	0.79 ± 0.02	1.78 ± 0.09	2.18 ± 0.06
iso 17:0	0.77 ± 0.02	0.74 ± 0.01	0.52 ± 0.02	0.41 ± 0.01
anteiso 17:0	0.18 ± < 0.01	0.18 ± < 0.01	0.10 ± 0.01	0.03 ± 0.03
Phytanic	0.33 ± 0.01	0.34 ± < 0.01	0.37 ± 0.03	0.45 ± 0.01
17:0	trace	trace	trace	trace
17:1	0.25 ± 0.01	0.23 ± 0.01	0.37 ± 0.04	0.10 ± 0.03

Table A1 (cont.). Fatty acid composition of fish oils and purified fish oil TAG fractions.

Fatty acid	Original menhaden oil (% w/w)	Purified menhaden TAG (% w/w)	Purified anchovy TAG-1 (% w/w)	Purified anchovy TAG-2 (% w/w)
iso 18:0	trace	trace	trace	trace
18:0	**3.40 ± 0.02**	**3.45 ± 0.02**	**3.0 ± 0.1**	**3.01 ± 0.03**
18:1 n-9	**8.58 ± 0.05**	**8.86 ± 0.02**	**8.9 ± 0.3**	**9.34 ± < 0.01**
18:1 n-7	**2.86 ± 0.02**	**2.93 ± 0.01**	**3.2 ± 0.1**	**2.93 ± 0.01**
18:1 n-5	0.16 ± 0.01	0.16 ± 0.01	0.10 ± 0.01	0.12 ± 0.02
18:2 Δ5,11	0.12 ± 0.01	0.11 ± 0.01	0.17 ± 0.01	0.28 ± 0.04
18:2 n-7	0.08 ± < 0.01	0.07 ± 0.01	trace	-
18:2 n-6	1.23 ± < 0.01	1.25 ± 0.01	1.30 ± 0.05	1.05 ± 0.01
18:2 n-4	0.35 ± 0.01	0.34 ± < 0.01	0.36 ± 0.01	0.45 ± 0.03
18:3 n-6	0.32 ± 0.01	0.31 ± < 0.01	0.30 ± 0.02	0.32 ± 0.02
18:3 n-4	0.42 ± < 0.01	0.42 ± 0.01	0.1 ± 0.1	0.26 ± 0.01
18:3 n-3	1.61 ± < 0.01	1.59 ± 0.03	0.98 ± 0.03	0.57 ± 0.01
18:3 n-1	trace	trace	trace	0.10 ± 0.01
18:4 n-3	**3.26 ± 0.02**	**3.18 ± 0.04**	**2.91 ± 0.09**	**2.19 ± 0.05**
18:4 n-1	0.29 ± 0.05	0.24 ± 0.02	0.26 ± 0.02	0.26 ± 0.02
19:0	trace	trace	0.06 ± 0.06	trace
20:0	0.22 ± 0.01	0.21 ± < 0.01	0.31 ± 0.01	0.34 ± 0.03
20:1 n-11	-	-	-	trace
20:1 n-9	1.35 ± 0.01	1.41 ± 0.01	1.71 ± 0.05	0.87 ± 0.01
20:1 n-7	0.28 ± 0.01	0.29 ± 0.01	0.25 ± 0.01	0.35 ± 0.01
20:2 NMID[a]	0.22 ± 0.02	0.23 ± 0.01	0.24 ± 0.01	0.19 ± 0.01
20:2 n-6	0.26 ± 0.01	0.30 ± 0.04	0.24 ± 0.01	0.18 ± < 0.01
20:3 n-6	0.20 ± 0.02	0.21 ± 0.02	0.21 ± < 0.01	-
20:3 n-3	0.26 ± 0.01	0.25 ± 0.03	0.13 ± 0.05	-
20:4 n-6	0.54 ± < 0.01	0.56 ± 0.02	1.17 ± 0.04	0.96 ± 0.04
20:4 n-3	1.64 ± 0.02	1.64 ± 0.01	1.1 ± 0.1	0.82 ± 0.04
20:5 n-3	**10.4 ± 0.1**	**10.28 ± 0.02**	**15.6 ± 0.4**	**16.6 ± 0.1**
21:5 n-3	0.8 ± 0.2	0.65 ± 0.01	1.0 ± 0.4	0.9 ± 0.1
22:0	0.23 ± 0.02	0.20 ± 0.02	1.4 ± 0.2	0.24 ± 0.01
22:1 n-11+13	-	-	-	0.25 ± 0.02

[a]20:2 nonmethylene-interrupted diene

Table A1 (cont.). Fatty acid composition of fish oils and purified fish oil TAG fractions.

Fatty acid	**Original menhaden oil (% w/w)**	**Purified menhaden TAG (% w/w)**	**Purified anchovy TAG-1 (% w/w)**	**Purified anchovy TAG-2 (% w/w)**
22:1 n-9	0.22 ± 0.02	0.21 ± 0.01	0.36 ± 0.06	0.44 ± 0.03
22:1 n-7	0.3 ± 0.1	0.17 ± < 0.01	0.20 ± 0.08	-
22:4 n-6	0.13 ± 0.01	0.16 ± 0.01	0.3 ± 0.2	0.42 ± 0.05
22:4 n-3	0.26 ± 0.07	0.23 ± 0.02	0.5 ± 0.4	0.17 ± 0.05
22:5 n-6	0.55 ± 0.01	0.62 ± 0.05	0.6 ± 0.2	0.41 ± 0.05
22:5 n-3	**2.21 ± 0.03**	**2.21 ± 0.03**	**2.2 ± 0.3**	**1.82 ± 0.08**
22:6 n-3	**13.1 ± 0.1**	**13.3 ± 0.1**	**9.8 ± 0.3**	**8.8 ± 0.4**
24:0	0.12 ± 0.02	0.13 ± 0.01	trace	0.10 ± 0.05
24:1 n-9	0.48 ± 0.02	0.49 ± 0.03	1.2 ± 0.6	trace

Appendix B
Effect of purification on anchovy lipid composition

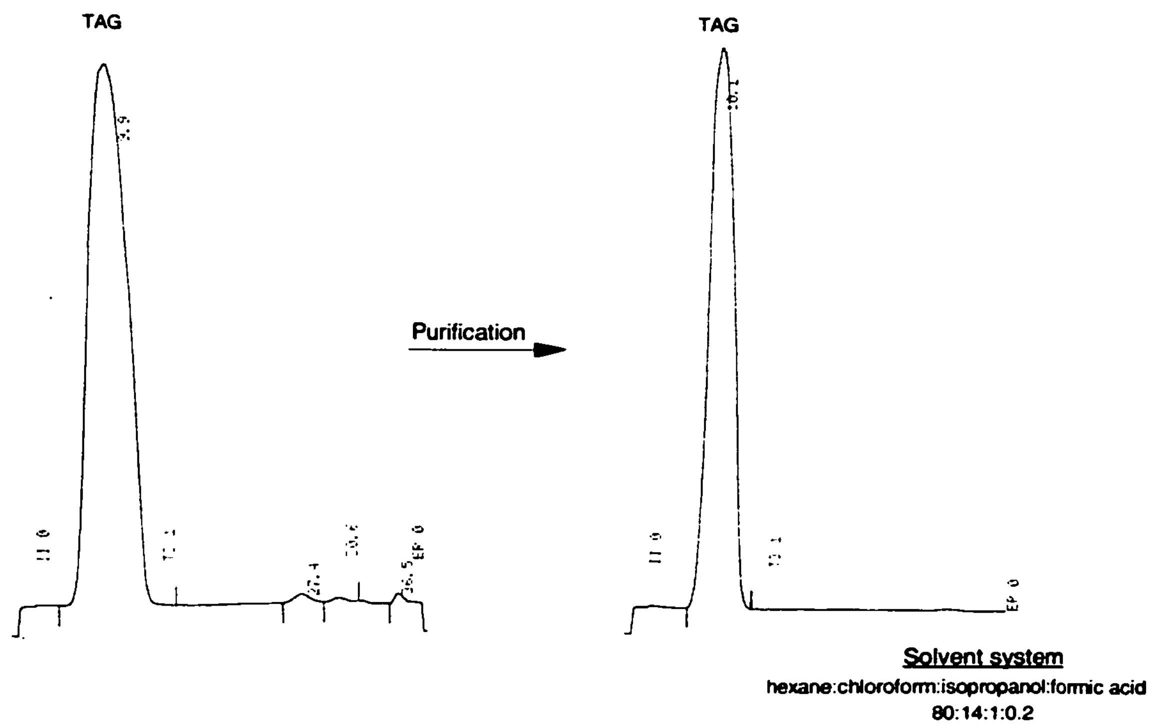


Figure B1. Iatroscan TLC-FID analysis of anchovy oil and purified anchovy oil TAG.

Appendix C
UV-spectra of freshly purified and of oxidized menhaden oil TAG

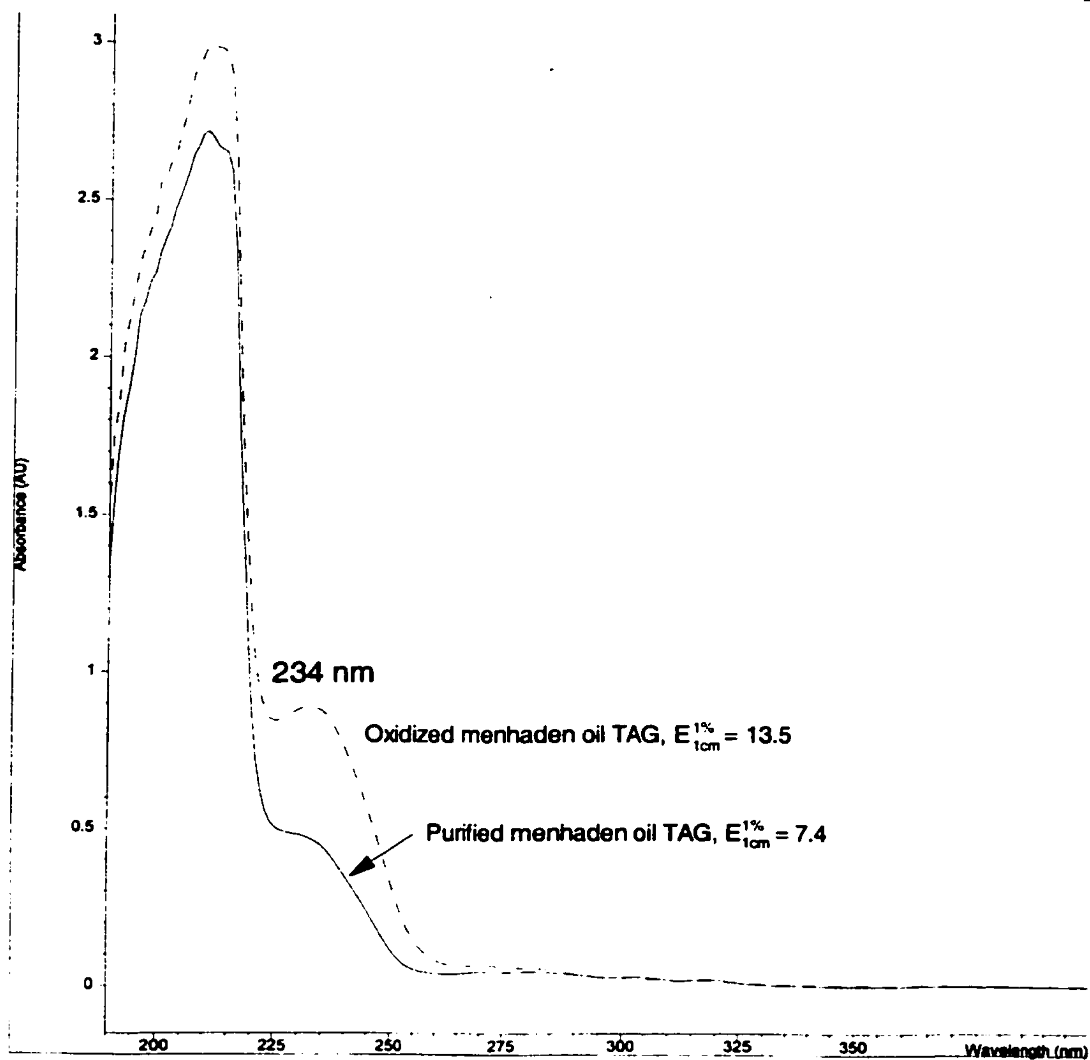


Figure C1. UV-spectra of freshly purified and of oxidized menhaden oil TAG.

Appendix D
Effect of citric acid on the formation of hydroperoxides in a commercial fish oil

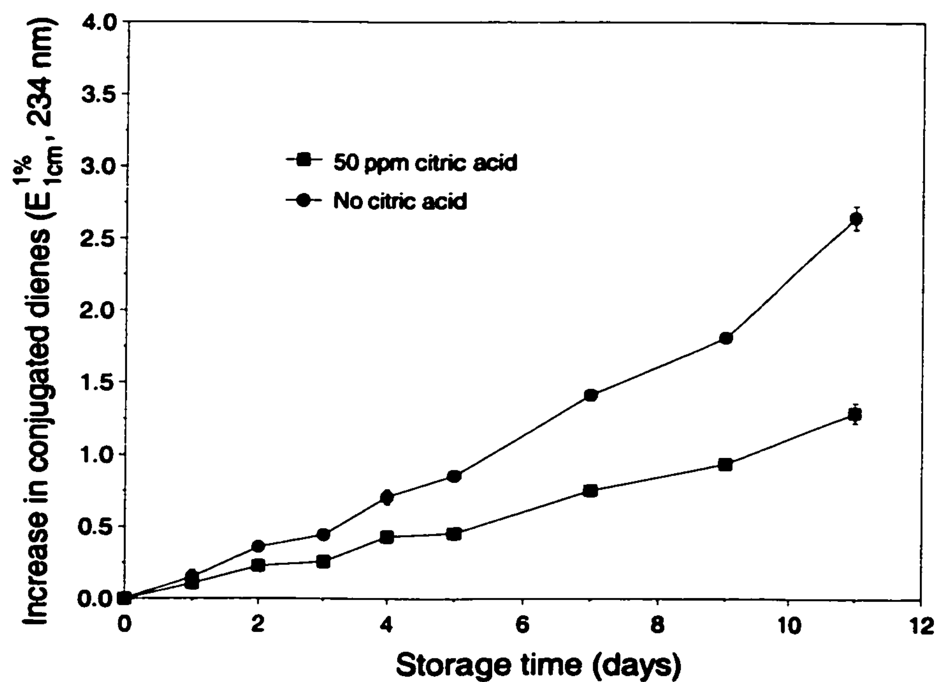


Figure D1. Effect of citric acid on the formation of hydroperoxides in a commercial fish oil. Data points are means ± SD (n = 3)

Appendix E
Equilibration curve for propanal in sunflower oil

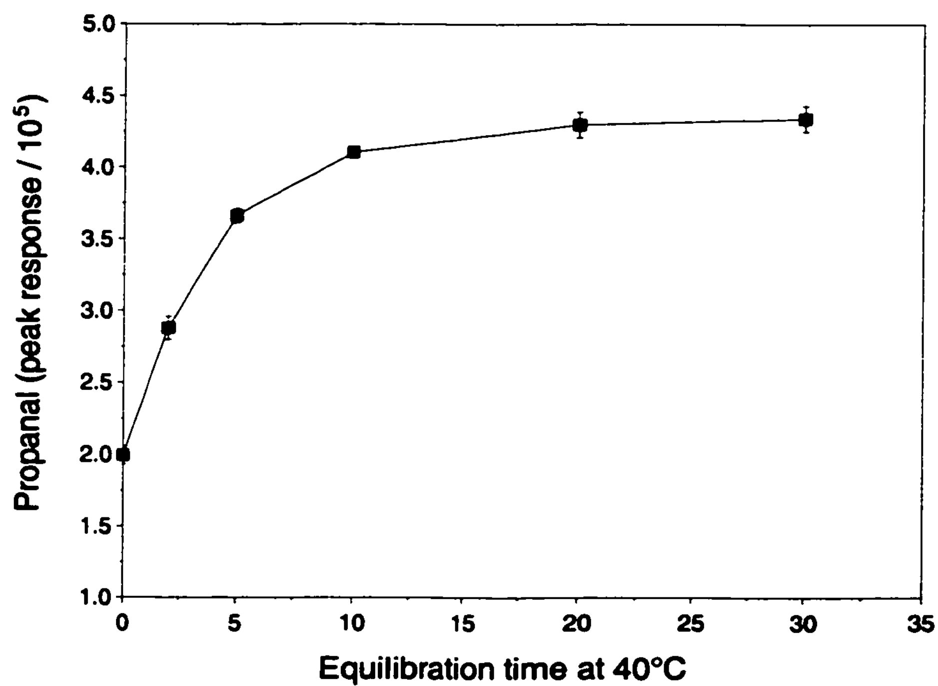


Figure E1. Equilibration curve for a propanal standard (0.79 mg/g) in sunflower oil. Data points are means ± SD (n = 3 or 4)

Appendix F
Typical static headspace GC chromatograms from the analysis of purified fish oil TAG

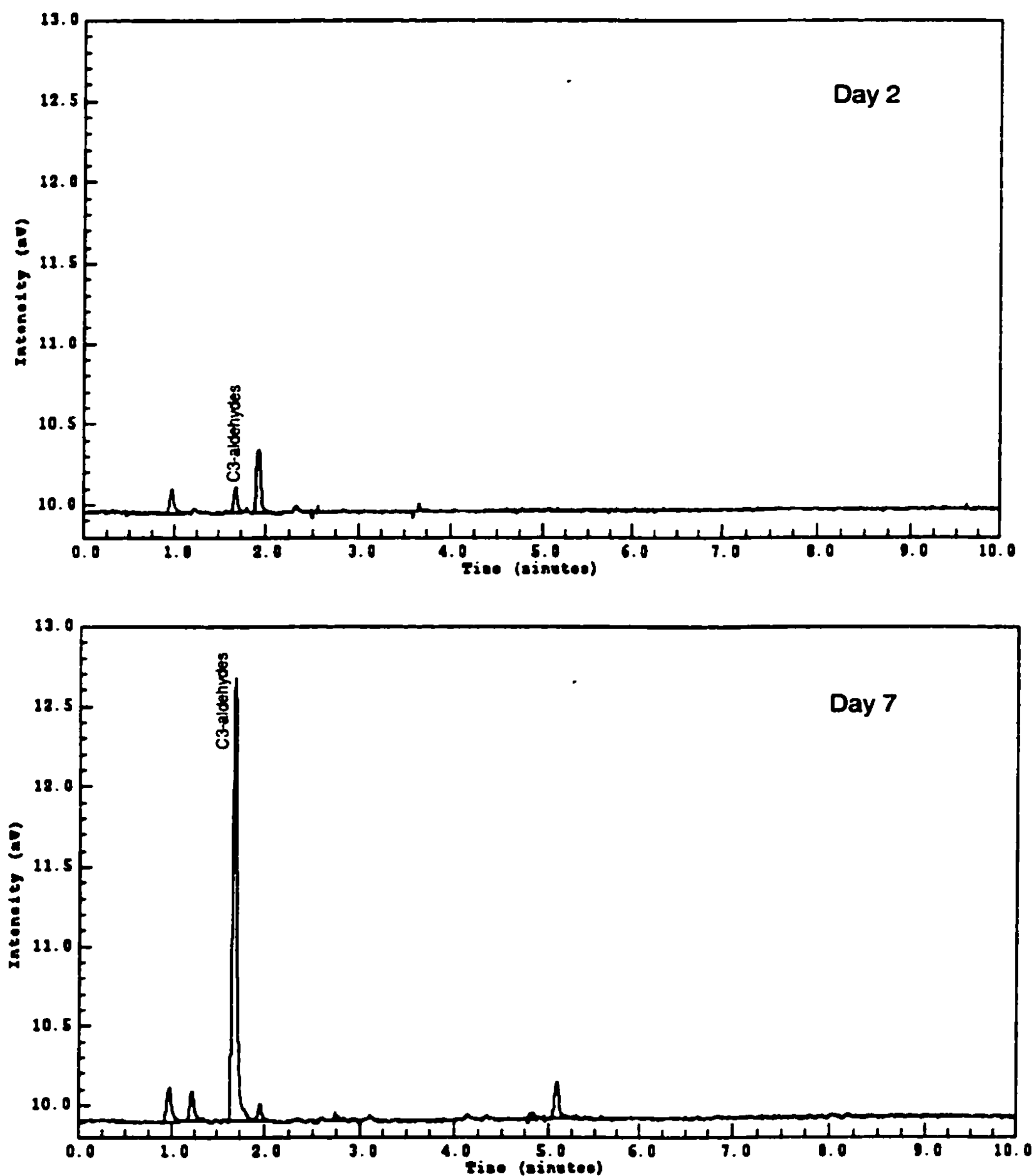


Figure F1. Formation of C3-aldehydes upon storage of anchovy oil with 50 ppm αTOH at 30°C at the Norsk Hydro Research Centre.

Appendix G

Formation of C3-aldehydes in purified fish oil TAG at 30°C with and without tocopherols (αTOH, γTOH or δTOH)

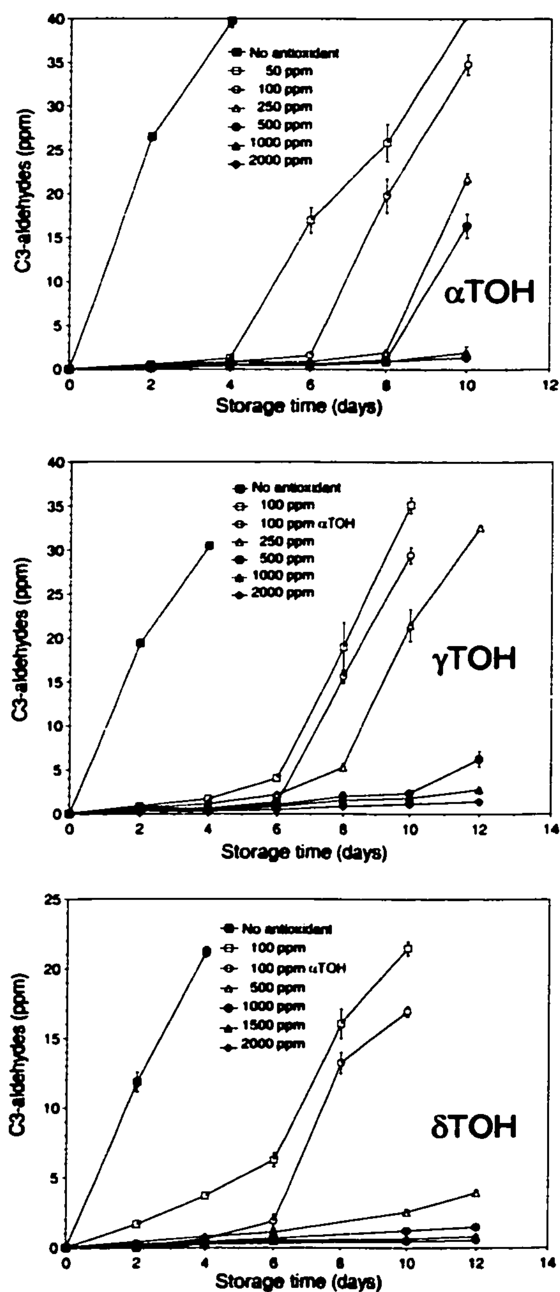


Figure G1. Formation of C3-aldehydes in purified fish oil TAG at 30°C with and without tocopherols (αTOH, γTOH or δTOH). Data points are means ± SD (n = 3).

Appendix H
Typical chromatogram obtained from the dynamic headspace analysis of fish oil

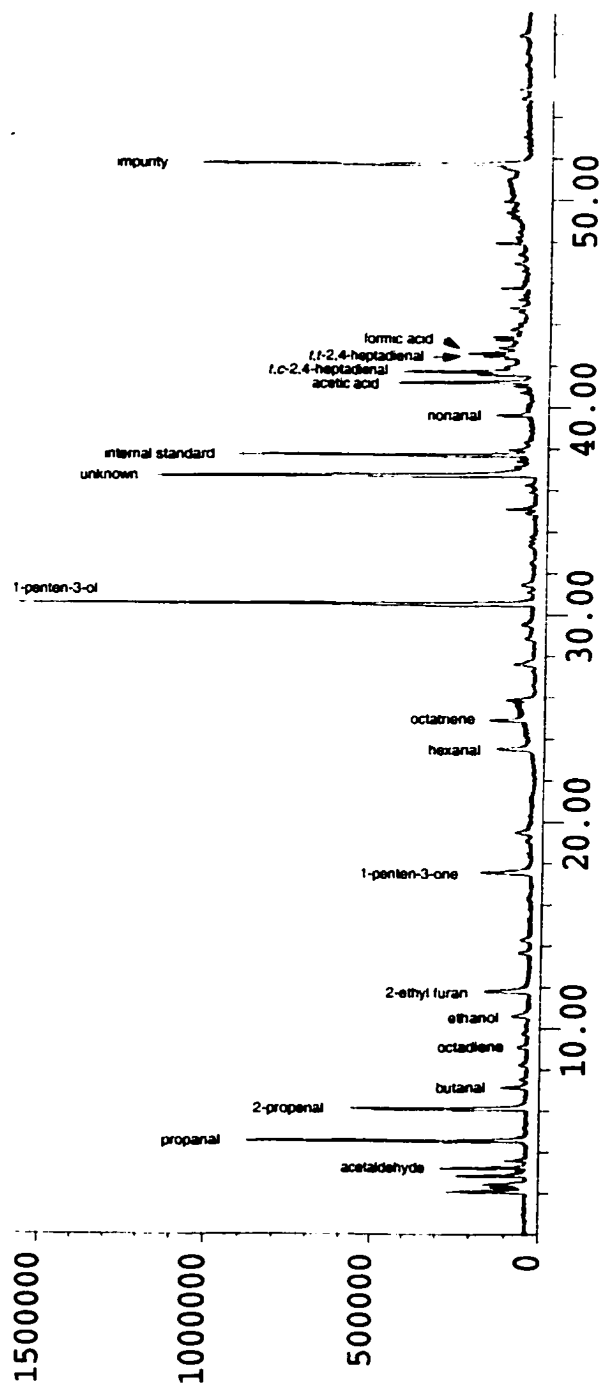


Figure H1. Dynamic headspace GC-MS chromatogram from the analysis of purified fish oil TAG with αTOH (100 ppm, 5 days of storage)

Appendix I

Formation of volatile secondary oxidation products during storage of purified fish oil TAG*

Table I1. Volatiles formed during storage of purified fish oil TAG with 1000 ppm γTOH at 30°C.

Retention time (min)	Compound	Day 2 (ppm)	Day 5 (ppm)	Day 8 (ppm)
2.28	pentane	ND	0.06	0.15
2.37	1-pentene	ND	0.03	0.14
2.43	2-methyl-1-butene	ND	0.19	0.42
2.96	1,3-pentadiene	ND	ND	ND
3.24	acetaldehyde	0.13	0.19	0.37
3.61	1-heptene	ND	0.05	0.11
4.52	propanal	0.15	0.41	1.16
6.11	2-propenal	0.45	0.80	1.29
7.16	butanal	0.07	0.08	0.15
9.09	octadiene	ND	0.08	0.16
9.76	3-methyl-1,4-heptadiene	ND	ND	0.07
10.61	ethanol	0.11	0.62	0.18
11.82	2-ethylfuran	0.48	0.49	0.36
13.68	pentanal	0.07	0.06	0.13
17.53	1-penten-3-one	0.26	0.41	0.61
19.52	2-butenal	0.07	0.09	0.14
23.54	hexanal	0.14	0.15	0.26
24.96	octatriene	0.07	0.17	0.34
25.92	2-pentenal	0.08	0.14	0.29
27.62	2-pentenal	0.13	0.12	0.16
30.60	1-penten-3-ol	1.02	1.64	2.59
31.38	heptanal	0.11	0.08	0.11
33.31	hexenal	0.07	0.03	0.05
34.94	alcohol	ND	0.05	0.09
35.07	unknown1	0.07	0.10	0.16
36.66	unknown2	0.87	1.94	3.79
37.93	unknown3	0.07	0.09	0.13
39.58	nonanal	0.18	0.10	0.20
41.02	alcohol	0.04	0.06	0.08
41.17	acetic acid	0.32	0.47	0.80
41.55	unknown4	0.06	0.17	0.18
41.73	*t,c*-2,4-heptadienal	0.19	0.45	0.69
42.49	*t,t*-2,4-heptadienal	0.07	0.09	0.12
42.60	formic acid	0.07	0.14	0.49
42.88	unknown5	0.06	0.07	0.11
43.23	propanoic acid	0.07	0.06	0.10
44.00	nonadienal	ND	0.03	0.04
45.18	butanoic acid	ND	0.03	0.06
45.74	2-decenal	0.07	ND	0.06
47.91	2-undecenal	0.08	ND	0.06
49.38	hexanoic acid	ND	ND	0.05

*The concentrations are relative to the internal standard (ethyl heptanoate).