DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

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ABSTRACT

Docosahexaenoic acid (DHA; 22:6-*n*3, 4Z,7Z,10Z,13Z,16Z,19Z) is a long-chain polyunsaturated omega-3 fatty acid with several health benefits. It promotes cardiovascular health and is considered essential for the normal neural and visual development of infants. It also has beneficial effects on anti-inflammatory and immune functions. Despite the well-documented health benefits, the intake of DHA is lower than recommended in most Western countries. The primary sources of DHA in the diet are fatty fish and food supplements. Due to its structure with six double bonds, DHA is highly susceptible to oxidation. Autoxidation is initiated by a radical-induced interaction between atmospheric oxygen and fatty acid, resulting in a complex and multiphase series of chemical reactions. The reactions result in fatty acid decomposition and generation of a diverse array of lipid oxidation products. Oxidation introduces off-flavors and lowers the nutritional quality and safety of food. It is a challenge to the food industry's ability to fortify foods with DHA, which would otherwise be a convenient way to increase the insufficient dietary intake. Oxidation also results in losses within the omega-3 industry supply chains. In the supplements, the fatty acids are found as esterified to triacylglycerol, ethyl ester, or phospholipid structure. These lipid structures, as well as the fatty acid position in the glycerol backbone (*sn*-1, *sn*-2, or *sn*-3), can influence the oxidative stability and antioxidant response of the oil.

This research aimed to investigate novel means to improve the oxidative stability of DHA by the choice of lipid structure and examination of a promising sphingoid base antioxidant, dihydrosphingosine. Additionally, the study aimed to elucidate the oxidation pattern of DHA because only a few previous studies are available on the topic. A new omics-type analytical approach was applied with liquid- and gas chromatographic separation coupled to mass spectrometric detection for the non-volatile and volatile oxidation products, nuclear magnetic resonance spectroscopy, and substrate and antioxidant concentration monitoring during the oxidation trials.

The results showed that the oxidative stability of DHA is influenced by the lipid structure into which it is integrated, and lipid structures were observed to interact differently with tocopherols. Tridocosahexaenoin was more stable than DHA ethyl ester without added α-tocopherol, while in the presence of 0.2% α-tocopherol, the opposite was observed. In the triacylglycerols with two palmitic acids and one DHA either at *sn*-1, *sn*-2, or *sn*-3 position, DHA at the *sn*-2 position was the most stable structure, both with and without 0.14% *RRR*-α-tocopherol. Without antioxidant, there was no significant difference between the *sn*-1 and *sn*-3 positions, while with *RRR*-α-tocopherol, the *sn*-1 position was slightly more stable than the *sn*-3 position. Increased stability might indicate favorable

diastereomeric interactions between the enantiopure *RRR*-α-tocopherol and DHA at the *sn*-1 position. This was the first study conducted on the oxidative stability of enantiopure triacylglycerols. Dihydrosphingosine (d18:0) showed an improved antioxidative effect after the initial stages of oxidation, indicating antioxidative carbonyl-amine reaction product formation from the d18:0 amine group and oxidation product carbonyls. Some of the formed imine structures could be tentatively identified for the first time. Application of d18:0 for increasing the stability of DHA-rich oils showed promise, but further research is needed for a more comprehensive understanding of the effect.

This thesis elucidated the role of lipid structure and different antioxidant strategies on the stabilization of DHA. Also, an analytical approach for comprehensive lipid oxidation product analysis from a small lipid amount was introduced. The findings can be utilized in future research on lipid oxidation and antioxidant strategies, as well as for formulating DHA-rich oils with improved stability.

SUOMENKIELINEN ABSTRAKTI

Dokosaheksaeenihappo (DHA; 22:6-*n*3, 4Z,7Z,10Z,13Z,16Z,19Z) on pitkäketjuinen omega-3 sarjan rasvahappo, johon liitetään useita terveyshyötyjä. Se edistää sydän- ja verisuoniterveyttä ja on välttämätön sikiö- ja imeväisvaiheessa olevien lasten normaalille näön ja hermoston kehitykselle. Lisäksi se vaikuttaa myönteisesti immuunipuolustukseen ja kehon tulehdustiloihin. Vaikka DHA:n terveyshyödyt tunnetaan, on sen saanti useimmissa länsimaissa suosituksia vähäisempää. DHA:n pääasiallisia lähteitä ravinnossa ovat rasvainen kala ja lisäravinteet. Useita kaksoissidoksia sisältävän rakenteensa takia DHA on erittäin herkkä hapettumiselle. Hapettumisella (auto-oksidaatio) tarkoitetaan vapaan radikaalin käynnistämää vuorovaikutusta molekulaarisen hapen ja rasvahapon välillä. Tämä vuorovaikutus laukaisee monimutkaisen ja monivaiheisen kemiallisten reaktioiden ketjun, joka johtaa rasvahapon hajoamiseen ja satojen hapettumistuotteiden muodostumiseen. Hapettuminen aiheuttaa ruokaan sivumakuja ja heikentää sen ravitsemuksellista laatua ja turvallisuutta. DHA:n hapettumisherkkyys rajoittaa sen käyttöä funktionaalisissa elintarvikkeissa, vaikka niiden avulla voitaisiin parantaa väestön DHA:n saantia. Lisäravinteissa rasvahapot voivat olla liittyneenä triasyyliglyseroli-, etyyliesteri-, tai fosfolipidirakenteeseen. Nämä lipidirakenteet, samoin kuin rasvahapon paikka glyserolirungossa (*sn*-1, *sn*-2, tai *sn*-3), vaikuttavat öljyn hapettumisherkkyyteen ja vuorovaikutukseen antioksidanttien kanssa.

Tämän väitöskirjatutkimuksen tavoitteena oli löytää uusia keinoja parantaa DHA:n säilyvyyttä tutkimalla lipidirakenteen ja mahdollisesti antioksidanttina toimivan dihydrosfingosiinin vaikutusta hapettumiseen. Lisäksi tavoitteena oli tutkia DHA:n hapettumisprofiilia, josta on aiemmin ollut saatavilla vain vähän tutkimustietoa. Tutkimuksessa sovellettiin omiikka-analytiikkaa käyttämällä neste- ja kaasukromatografista erottelua ja massaspektrometristä tunnistusta haihtuville ja ei-haihtuville hapettumistuotteille, ydinmagneettista resonanssispektroskopiaa, sekä substraatin ja antioksidantin konsentraatioiden monitorointia hapettumisen edetessä.

Tulokset osoittivat, että lipidirakenteella on merkittävä vaikutus DHA:n säilyvyyteen, ja eri lipidirakenteet vuorovaikuttavat tokoferolien kanssa eri tavoin. DHA hapettui herkemmin etyyliesterissä kuin tridokosaheksaenoyyliglyserolissa ilman lisättyä α-tokoferolia, mutta 0,2 prosentin α-tokoferololisäys johti päinvastaiseen säilyvyysjärjestykseen. Triasyyliglyseroleissa, jotka koostuivat kahdesta palmitiinihaposta ja DHA:sta joko *sn*-1, *sn*-2 tai *sn*-3 paikalla, säilyvyys oli paras *sn*-2 paikalla riippumatta 0,14 prosentin *RRR*-α-tokoferololisäyksestä. Ilman lisättyä antioksidanttia ei havaittu eroa *sn*-1 ja *sn*-3 sijaintien välillä, mutta *RRR*-α-tokoferololisäys paransi

sn-1 paikan säilyyyttä hieman enemmän verrattuna *sn*-3 paikkaan. Parantunut säilyvyys voi johtua diastereomeerisistä vuorovaikutuksista enantiopuhtaan *RRR*-α-tokoferolin ja *sn*-1 sijainnin välillä. Tässä väistöskirjassa enantiopuhtaiden triasyyliglyserolien hapettumisalttiutta tutkittiin ensimmäistä kertaa. Dihydrosphingosiini (d18:0) lisäsi tridokosaheksaenoyyliglyserolin stabiiliutta hapettumisen alkuvaiheessa, mikä viittaa d18:0:n amiiniryhmän ja hapettumistuotekarbonyylien välisissä reaktioissa muodostuneiden yhdisteiden antioksidatiiviseen vaikutukseen. Tutkimuksessa näissä reaktioissa muodostuneita imiinirakenteita pystyttiin alustavasti tunnistamaan ensimmäistä kertaa. D18:0:n käyttö antioksidanttina runsaasti DHA:ta sisältävissä öljyissä vaikuttaa lupaavalta, mutta vaikutusmekanismin syvemmäksi ymmärtämiseksi tarvitaan vielä lisätutkimusta.

Tässä väitöskirjassa esitetään uusia tutkimuslöydöksiä lipidirakenteen ja eri antioksidanttistrategioiden vaikutuksesta DHA:n hapettumiseen. Lisäksi työssä esitetään analyyttinen menettelytapa, jolla hapettumista ja muodostuneita hapettumistuotteita voidaan analysoida kokonaisvaltaisesti myös pienistä öljymääristä. Tutkimustuloksia voidaan hyödyntää lipidien hapettumisen ja antioksidanttistrategioiden tutkimuksessa, sekä nykyistä paremmin säilyvien DHA-öljyjen kehittämisessä.

LIST OF ABBREVIATIONS

α-TOC	α-Tocopherol
ALA	α-Linolenic acid
BHT	Butylated hydroxytoluene
d18:0	Dihydrosphingosine
DHA	Docosahexaenoic acid
DOPC	Dioleoyl-phosphatidylcholine
DOPE	Dioleoyl-phosphatidylethanolamine
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
EE	Ethyl ester
EPA	Eicosapentaenoic acid
FA	Fatty acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HS-SPME	Headspace solid phase microextraction
LC-QTOF	Liquid chromatography quadrupole time-of-flight mass spectrometry
LO•	Alkoxy radical
LOH	Lipid alcohol
LOO•	Peroxy radical
LOOH	Lipid hydroperoxide
MDA	Malondialdehyde
MS	Mass spectrometry
NBD-PE	N-(7-nitro-2-1,3-benzoxadiazol-4-yl-dioleoyl)-PE
NMR	Nuclear magnetic resonance spectroscopy
OH•	Hydroxyradical
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
PS	Phosphatidylserine
SPE	Solid phase extraction
TAG	Triacylglycerol
UHPLC	Ultra high-performance liquid chromatography

LIST OF ORIGINAL PUBLICATIONS

I. Ahonen, E.; Damerau, A.; Suomela, J.-P.; Kortesniemi, M.; Linderborg, K. M. Oxidative stability, oxidation pattern and a-tocopherol response of docosahexaenoic acid (DHA, 22:6*n*-3)-containing triacylglycerols and ethyl esters. *Food Chemistry*. **2022**, 387, 132882–132882.

II. Damerau, A.; Ahonen, E.; Kortesniemi, M.; Gudmundsson, H. G.; Yang, B.; Haraldsson, G. G.; Linderborg, K. M. Docosahexaenoic acid in regio- and enantiopure triacylglycerols: Oxidative stability and influence of chiral antioxidant. *Food Chemistry*. **2023**, 402, 134271–134271.

III. Ahonen, E.; Damerau, A.; Linderborg, K. M. Antioxidative effect of dihydrosphingosine (d18:0) and α-tocopherol on tridocosahexaenoin (DHA-TAG). *Journal of Agricultural and Food Chemistry*. **2023**, 71 (40), 14769-14781.

1 INTRODUCTION

Lipid autoxidation denotes a complex and multiphase series of chemical reactions initiated by a radical-induced interaction between atmospheric oxygen and a fatty acid. These reactions lead to fatty acid (FA) decomposition and formation of hundreds of different volatile and non-volatile lipid oxidation products. Lipid oxidation causes chemical deterioration of processed foods and introduces off-odors and flavors that consumers consider unpleasant. Furthermore, it lowers the nutritional quality of food and can alter physical characteristics like color and texture. In addition to food, the same lipid oxidation reactions occur in cells and tissues, having adverse effects on human health. Also, the quality of cosmetics products, pharmaceutical emulsions, and paints can be altered by lipid oxidation. The radical-induced autoxidation is the primary oxidation mechanism when considering refined oils, although lipid oxidation can also emerge via photooxidation, enzymatic oxidation, or thermal oxidation (Shahidi & Zhong, 2010).

Polyunsaturated fatty acids (PUFA) have several double bonds in their structure, which makes them highly susceptible to autoxidation. Since the health benefits of long-chain PUFA are currently well recognized and their inclusion in the diet is considered essential, the food industry is in constant search for improved ways to control lipid oxidation. One of the FAs with the lowest oxidative stability and most well-recognized health benefits is the long-chain omega-3 PUFA docosahexaenoic acid (DHA). The link between cardiovascular health and the marine mammal/fish oil-derived polyunsaturated omega-3 FAs, DHA and eicosapentaenoic acid (EPA), was initially proposed by Bang et al. in 1971. Since the pioneering studies in the 1970s, focused on the Greenland Inuit population and Danish controls, the number of studies on the health effects of omega-3 FAs has exploded. In addition to cardiovascular health, DHA is considered essential for the normal neural and visual development of infants and it also has beneficial effects on anti-inflammatory and immune functions (Calder, 2016; Djuricic & Calder, 2021; Ghasemi Fard et al., 2019). Despite the admitted health benefits, the intake of DHA is lower than recommended in most Western countries (Micha et al., 2014; Schuchardt et al., 2022; Stark et al., 2016).

The main sources of DHA in the diet are fatty fish and food supplements. In the supplements, the FAs are typically found as esterified either to triacylglycerol, ethyl ester, or phospholipid structure. These lipid structures, as well as the FA position in the glycerol backbone, can influence the oxidative stability and antioxidant response of the oil. In bulk oil conditions, the phospholipid structure appears to offer superior stability (Lyberg et al., 2005; Mozuraityte et al., 2017; Song et al., 1997), while the results concerning possible stability differences between triacylglycerol and ethyl ester structures remain controversial (Lee et

al., 2003; Martín et al., 2012; Song et al., 1997; Sullivan Ritter et al., 2015; Yoshii et al., 2002). The central position of the glycerol backbone (*sn*-2) is suggested to exhibit superior protection against oxidation compared to the side positions (*sn*-1 and *sn*-3) (Wijesundera, 2008). The possible stability differences between *sn*-1 and *sn*-3 positions have not been studied to date due to the lack of suitable model compounds. It is essential to recognize that the stability and conformation of fatty acids can vary significantly in bulk oil conditions as opposed to aqueous micelles or biological membranes. Increasing the number of double bonds in the FA acyl chain decreases oxidative stability in bulk oil and organic solvent, while the opposite is observed in aqueous micelles (Miyashita, 2014).

Currently, tocopherols are the most commonly used antioxidants in omega-3 oils, and consumers have a strong preference for natural, non-synthetic antioxidants. In addition to antioxidant strategies, the oxidation of DHA-rich oils can be controlled by employing other techniques such as micro- or nanoencapsulation and emulsification. Lipid oxidation poses a challenge to the food industry's ability to fortify foods with DHA, which would otherwise be a convenient way to increase the insufficient dietary intake. It also results in losses within the omega-3 industry supply chains, thereby resulting in financial losses, environmental load, and increased demand for fishery resources, which are already at risk of overfishing. The fraction of fishery stock at a biologically sustainable level was only 65% in 2019, and the world consumption of aquatic foods will continue to rise (FAO, 2022). Furthermore, the production of DHA by phytoplankton at the bottom of the aquatic food web is predicted to decrease due to global warming (Hixson & Arts, 2016). Oxidation can also lead to compromised quality of the omega-3 supplement products on the market (Damerau et al., 2020; Hands et al., 2023; Heller et al., 2019; Mason & Sherratt, 2017; Rundblad et al., 2017), and some of the ingested oxidation products may even be harmful to health (Sottero et al., 2019; Vieira et al., 2017).

This research aimed to investigate novel means to improve the oxidative stability of DHA by the choice of lipid structure and examination of a promising sphingoid base antioxidant, dihydrosphingosine (d18:0). Sphingoid bases are structural backbones of sphingolipids. Among foods, they are most abundantly present in eggs, meat, dairy, and aquatic products (W. Li et al., 2021). Additionally, the study aimed to elucidate the oxidation pattern of DHA because only a few previous studies are available on the topic. Thus, the study hypothesized that lipid structure and lipid structure interactions with antioxidants affect the oxidative stability and oxidation pattern of DHA. Additionally, it was hypothesized that the stability of DHA can be improved by the addition of d18:0 due to the formation of antioxidative carbonyl-amine reaction products.

To be able to study the oxidation pattern profoundly, an omics-type analytical approach was applied with liquid- and gas chromatographic separation coupled to mass spectrometric detection for the non-volatile and volatile oxidation products, NMR, as well as substrate and antioxidant concentration monitoring during the oxidation trials. Comprehensive data on lipid oxidation patterns is highly relevant for food chemists when considering the most feasible ways to analyze lipid oxidation by fast and relevant methods, in terms of sensory quality, and when choosing the most suitable antioxidants. The traditional, simple lipid oxidation analysis methods like peroxide- and para-anisidine value are not suitable for all oil types, and depending on the oxidative status, can even give misleading results (Barriuso et al., 2013; Damerau et al., 2020; Mozuraityte et al., 2017; Thomsen et al., 2013).

This literature review discusses the basic principles of autoxidation and mechanisms of antioxidant action, with a particular focus on the DHA oxidation pattern, tocopherols, and antioxidant potential of amine compounds. Also, previous DHA stability studies within various lipid structures in hydrophobic environments are reviewed.

2 REVIEW OF THE LITERATURE

2.1 Docosahexaenoic acid (22:6*n*-3)

Docosahexaenoic acid (DHA; 22:6*n*-3) is a long chain PUFA with 22 carbons and six double bonds (**Figure 1**). All the double bonds are in *cis* configuration, and present at positions 4, 7, 10, 13, 16, and 19 when counted from the carboxyl carbon. Omega-3 or *n*-3 indicates the position of the first double bond when counted from the methyl end. The cis double bonds in the acyl chain cause it to bend, hindering close packing and reducing the melting point (-44 °C). In a liquid state and solvent, free FAs tend hydrogen bond through carboxyl groups, forming dimeric structures. A recent study suggested that in addition to the classical carboxyl group hydrogen bonds, DHA carboxyl group can also hydrogen bond with the intramolecular terminal *n*-3 double bond, forming dimers with enhanced stability (Venianakis et al., 2023).

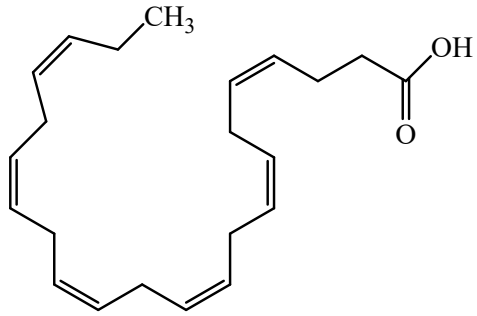


Figure 1. 4,7,10,13,16,19-Docosahexaenoic acid, (4Z,7Z,10Z,13Z,16Z,19Z)-.

The human body can synthesize DHA from α-linolenic acid (ALA; 18:3*n*-3) or its longer chain metabolites through an enzymatic pathway, or it can be obtained from dietary sources. ALA is an essential FA that the body cannot synthesize, so it must be acquired directly from the diet. The enzymatic pathway for ALA conversion to EPA and further to DHA is limited in humans. ALA conversion to EPA is estimated to be only 8-12% and to DHA less than 1% (Baker et al., 2016; Brenna et al., 2009). The conversion of linoleic acid (18:2*n*-6) to arachidonic acid (20:4*n*-6) and the conversion of ALA to EPA share the same enzymatic pathway. In a typical Western diet, the intake of linoleic acid is 5-15 times higher than that of ALA (Baker et al., 2016; Simopoulos, 2006). The prevalence of linoleic acid in the diet saturates the conversion pathway and consequently reduces the conversion of ALA to EPA and DHA (Djuricic & Calder, 2021). Dietary sources with the highest DHA contents include fatty fish (mackerel,

salmon, trout, herring, tuna, and sardines), and supplements and pharmaceutical-grade omega-3 preparations produced from livers of lean fish (cod liver), krill oil, and algae oil (Calder, 2016). Unlike fish, humans, or higher plants, microalgae have enzymatic pathways capable of converting ALA and linoleic acid effectively into EPA and DHA, and they are the primary producers of these FAs in the food chain (Remize et al., 2021).

2.1.1 DHA in different lipid structures

In fish and commercial algae oils, DHA is primarily present in triacylglycerol form (TAGs), with three FAs esterified to glycerol backbone (**Figure 2A**). Omega-3 contents in natural fish- and fish liver oils varies between species and their habitat area (Bimbo, 2013). Fish oils generally contain approximately 30% EPA+DHA combined (~12% DHA). Through transesterification, EPA and DHA concentration can be increased as high as 90% for supplement and pharmaceutical product use. When preparing a concentrate from fish oil, the FAs are typically first hydrolyzed from the TAG backbone and transesterified to ethyl esters (EE) (**Figure 2B**). Subsequently, the EEs are molecularly distilled to remove short-chain- and saturated FAs and increase the EPA and DHA contents to around 60%. Finally, the EPA- and DHA-EEs are enzymatically reconverted back to concentrated TAGs (Dyerberg et al., 2010). In addition to molecular distillation, low-temperature crystallization, urea complexation, and lipase concentration can be applied for the concentration step (Xie et al., 2022).

In krill oils, 60-70% of the omega-3 FAs are bound to phospholipids (PL) (Ramprasath et al., 2015). In the PL structure, a phosphate head group is typically found at the *sn*-3 position. PLs are named after the phosphate group, and structures include phosphatidylcholine (PC), phosphatidylethanolamine (PE) (**Figure 2C**), phosphatidylserine (PS), and phosphatidylinositol. In supplement products, DHA is available as TAG, EE, free FA, and PL. Examples of DHA in different lipid structures are illustrated in **Figure 2**.

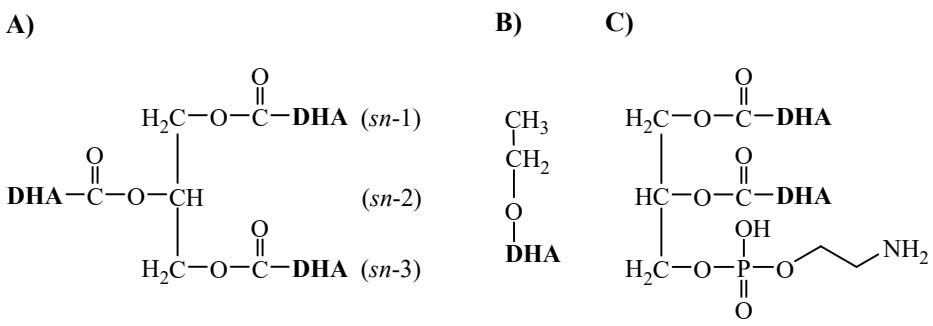


Figure 2. DHA esterified to different lipid structures. A: tridocosahexaenoin (DHA-TAG), B: DHA ethyl ester (DHA-EE), and C: 1,2-docosahexanoyl-*sn*-glycero-3-phosphoethanolamine, (DHA-PE). *Sn*-numbering (2A) designates the stereospecific numbering of glycerol carbons.

In natural TAGs, the number of possible FA combinations in the three glycerol positions is substantial. However, in the species-specific biosynthesis of TAGs, FAs are esterified to TAG *sn*-positions non-randomly. In PLs, saturated FAs are preferentially located at the *sn*-1 position, and unsaturated FAs are more often found at the *sn*-2 position (Cui & Decker, 2016). If the carbon in the glycerol *sn*-2 position has four different substituents, the molecule becomes chiral, having two spatial atomic arrangements that are superimposable mirror images of each other. For TAGs, this means that an AAB-type TAG positional isomer represents two different TAG enantiomers, one with the B FA in *sn*-3 position (*sn*-AAB, 1,2-di-A-3-B-*sn*-glycerol) and one with the B FA in *sn*-1 position (*sn*-BAA (1-B-2,3-di-A-*sn*-glycerol). Chirality is a fundamental aspect of the natural world. For instance, amino acids in proteins and sugars in DNA structures prefer one specific enantiomer, and homochirality is believed to be a prerequisite for the development of life (Blackmond, 2019; M. Liu et al., 2015). The chiral distinction is also crucial in drug development, as the desired therapeutic effects are attributed exclusively to either of the enantiomers (McConnell et al., 2007). Nagai et al. (2013) analyzed the positional distribution of DHA in marine organism TAGs with one or two palmitic acids (**Table 1**).

Table 1. Ratios (%) of TAG positional isomers and enantiomers of palmitic acid- and DHA-containing TAGs in marine mammals and fishes (Adapted from Nagai et al., 2013).

	Marine mammals		**Fishes**	
	Steller Sea Lion	Harp Seal	Skipjack Tuna	Sardine
sn-DPP	13.6	13.6	2.8	8.6
PDP	0	0	43.8	64.9
sn-PPD	86.4	86.4	53.4	26.5
sn-DDP	0	5.3	4.6	13.5
DPD	89.2	70.5	5.9	6.6
sn-PDD	10.8	24.2	89.5	79.9

Abbreviations: D (DHA), P (Palmitic acid)

In the case of one DHA and two palmitic acids in a TAG, DHA was found mainly in the *sn*-3 position in marine mammals, while in sardines it was mainly located at the *sn*-2 position. When the TAG consisted of two DHAs and one palmitic acid, the DHAs occupied mainly *sn*-1 and *sn*-3 positions in marine mammals and *sn*-2 and *sn*-3 positions in fish (Nagai et al., 2013). For salmon, anchovy, and sardine oils, also other studies reported a higher prevalence of DHA at the *sn*-2 position than at the *sn*-1/3-positions (Nwosu & Boyd, 1997; Suárez et al., 2010). The production of triacylglycerols with distinct DHA positions in the triacylglycerol backbone (*sn*-1, *sn*-2, or *sn*-3) is possible through enzymatic modification (Castejón & Señoráns, 2020).

2.1.2 Health effects

Traditionally, lipids have been linked to two fundamental roles within the body: they serve as integral components of cell membranes and provide a source of metabolic energy. In addition to these functions, DHA has a role in several other biological processes. It alters the lipid membrane structure and function, ion channel function, gene expression (nuclear receptors and transcription factors), and lipid mediator production (Mozaffarian & Wu, 2012). Due to the curved structure and 14 rotatable bonds, DHA takes more space in the membrane bilayers than the straight-chain saturated FAs or planar cholesterol molecules, having a favorable effect on membrane fluidity, flexibility, thickness, and lipid packing defects (DHA methyl end exposed to the hydrophilic surface) (Hashimoto et al., 2017; Hishikawa et al., 2017). DHA participates in the amino acids transport through the cell membrane, regulates the function of sodium channels, and initiates the response of rhodopsin to visual stimuli. DHA is the most abundant FA in the central nervous system and retina, where it promotes

neurotransmission, neuroplasticity, and signal transduction. It also increases serotonin and acetylcholine levels in nervous tissue (Djuricic & Calder, 2021). Low DHA levels in the brain and eye during the late fetal stages and infancy are associated with poorer cognitive development and visual function (Calder, 2016).

In most studies examining the effects of long-chain polyunsaturated FAs on cardiovascular health, fish oil, which contains both EPA and DHA, has been used. Nevertheless, the effects of DHA may have variations or complement those of EPA. According to a review article summarizing research conducted using fish oils with high DHA concentration, DHA can reduce blood triglycerides, improve LDL and total cholesterol, and decrease heart rate and heart rate variability (Ghasemi Fard et al., 2019). Earlier review on the cardioprotective role of DHA alone additionally reported beneficial effects on endothelial- and platelet function and blood pressure (Cottin et al., 2011). Innes & Calder (2018) summarized that DHA may decrease blood triacylglycerols more than EPA and increase the cardioprotective HDL cholesterol sub-fraction (HDL_2). On the other hand, it can increase LDL cholesterol, simultaneously increasing the particle size. The higher particle size may result in less atherogenic effect. (Jacobson et al., 2012).

The beneficial effects of DHA on inflammation, immune function, smooth muscle contraction, and platelet reactivity can be related to the regulation of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) or caused by the production of other bioactive lipid mediators, including resolvins, protectins, and maresins (Calder, 2016; Dyall et al., 2022). Resolvins and protectins can reduce peritonitis and neuronal inflammation and counter-regulate cytokines and chemokines to promote resolution of inflammation. Protectins can also have neuroprotective actions in the brain, retina, and central nervous system, and maresins are pro-regenerative, pro-repair, and neuroprotective in a wide range of tissues and organs (Dyall et al., 2022).

Based on cardiovascular benefits, the European Food Safety Authority (EFSA) recommends a 250 mg daily intake of DHA+EPA for the adult population, corresponding to 1-2 fatty fish meals per week. During pregnancy and lactation, an additional 100-200 mg/day DHA is recommended, and for infants from 6 up to 24 months, the recommendation is 100 mg/day, based on visual function (European Food Safety Authority (EFSA), 2017). In the USA, the American Heart Association recommends 4 g daily doses (EPA+DHA or only EPA) for patients with increased blood triacylglycerols (Skulas-Ray et al., 2019). Despite the recommendations and admitted health benefits, the consumption of omega-3 FAs from seafood, as well as the omega-3 status in red blood cells, is generally low in most global populations (Micha et al., 2014; Schuchardt et al., 2022; Stark et al., 2016). Nevertheless, there are some exceptions to this trend in countries

with a longstanding tradition of high fish consumption, such as Japan, Norway, Greenland, and South Korea.

2.1.3 Bioavailability

The bioavailability of DHA can be influenced by the lipid structure in which it is consumed. Generally, the bioavailability of DHA and EPA takes the following order: free FA ≈ natural oil TAG > EE (Xie et al., 2022). There are two reasons for the low bioavailability of EEs. Firstly, pancreatic lipase hydrolyzes FA-ethanol bonds 10-50 more slowly than the FA-glycerol bonds (Yang et al., 1990), and EE digestion also requires an additional carboxyl ester lipase step (bile-salt dependent lipase) (Davidson et al., 2012). Secondly, after absorption, the FAs are re-esterified to TAGs for further transportation to blood. Re-esterification requires glycerol and 2-monoacylglycerol molecules. In the case of TAG absorption, these are readily available from the substrate, while for EEs, their delivery seems to be inefficient (Schuchardt & Hahn, 2013). The low bioavailability of EE can be significantly increased by intake with a high-fat meal (Davidson et al., 2012; Lawson & Hughes, 1988). In terms of PL bioavailability, the reported results are inconclusive. In most of the human studies, no clear difference has been found between PL and TAG, while in the animal studies, PL has shown improved bioavailability compared to TAG (Ghasemifard et al., 2014; Lapointe et al., 2019; J. Li et al., 2021; Schuchardt & Hahn, 2013). However, the uptake of DHA to the brain is higher from PL than from TAG or free FAs (Chouinard-Watkins et al., 2019; Sugasini et al., 2017).

Dyerberg et al. (2010) also reported higher bioavailability of re-esterified concentrated TAG oil than natural TAG cod liver oil. Bioavailability was measured from three serum lipid classes (cholesterol esters, TAGs, and PLs) of 72 healthy subjects after ingestion of 3.1–3.6 g EPA+DHA for two weeks. The re-esterified TAG oil consisted of approximately 55–60% TAGs, 38–42% diacylglycerols, and 1–3% monoacylglycerols. According to the authors, this is a typical lipid class distribution for re-esterified TAG oils. The presence of non-TAG species was thought to promote the formation of micelles that facilitate monoacylglycerol absorption (Dyerberg et al., 2010). Dyerberg et al. (2010) also reported that the randomization of EPA and DHA in TAG during the concentration step had no negative effect on bioavailability.

In addition to lipid class, the *sn*-position of DHA in the glycerol backbone can influence absorption efficiency. During digestion, pancreatic lipase catalyzes the formation of 2-monoacylglycerol and two free FAs from *sn*-1/3-positions. The cleaved free FAs can form calcium soaps, which can lower their bioavailability (Hunter, 2001). However, the existing reviews indicate that there is only a minimal or short-term improvement in the bioavailability of FAs from the *sn*-2

position, with the exception of 16:0 and 18:0 (Mensink et al., 2016; Mu & Porsgaard, 2005; Sala-Vila et al., 2008). Only a few studies are available on the bioavailability differences between DHA at *sn*-1 and *sn*-3 positions. Linderborg et al. (2019) studied the bioavailability of DHA at either *sn*-1, *sn*-2, or *sn*-3 position of TAGs with the two remaining positions occupied by stearic acid (18:0) in a short-term feeding trial with mildly omega-3 deficient rats. Excretion to feces was lowest in the *sn*-2 DHA group, but no differences between groups were reported in terms of body weight or DHA contents in plasma or organs (Linderborg et al., 2019). In a long-term rat feeding trial with TAGs consisting of DHA and palmitic acids, plasma TAG DHA levels in the *sn*-1 DHA group were higher than in the *sn*-2 and sn-3 groups, indicating improved bioavailability from the *sn*-1 position. The *sn*-3 group, on the other hand, had the highest DHA accumulation into visceral fat (Zhang et al., 2023).

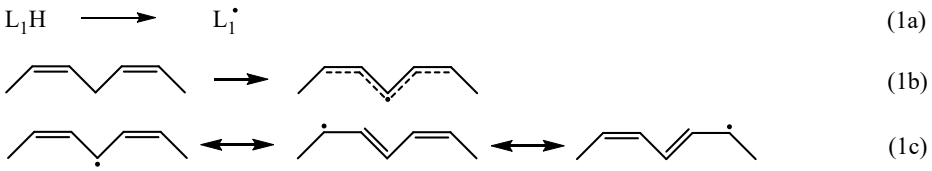
2.2 Lipid autoxidation

The first reported studies on lipid oxidation date back to the early 1800s, when Nicolas Théodore de Saussure (1767–1845) reported that linseed oil consumed 12 times its volume of oxygen during four months of storage. Nevertheless, a hundred years later, in 1910, it was still debated whether the changes related to rancidity were caused by lipid hydrolysis or oxidation (Hammond & White, 2011). Considerable progress in revealing the autoxidation mechanisms was achieved in the 1940s by the work of Ernest Harold Farmer and the Rubber Producers Research Association group (Swern et al., 1948), and since then, the research on the topic has been active and continues to evolve.

Lipid autoxidation proceeds as a free radical chain reaction through initiation, propagation, and termination steps. Several comprehensive reviews and book chapters are available on the topic (Choe & Min, 2006; Frankel, 1980, 1991, 2005; Gardner, 1989; N. A. Porter, 1986; N. A. Porter et al., 1995; Schaich, 2005, 2020; Shahidi & Zhong, 2010), and in the following discussion the course of the reaction and formed oxidation products are briefly discussed. Presented reaction schemes are adapted from publications by several authors (Bors et al., 1987; Frankel, 1980; Gardner, 1989; Schaich, 2020).

Molecular oxygen (triplet oxygen, $^3O_2$) cannot react with lipid double bonds directly due to different spin states. Thus, autoxidation initiation requires the formation of an alkyl radical (L•) (Reaction 1a). Alkyl radical reacts with triplet oxygen at a diffusion-limited rate (~ 10^9 $M^{-1}s^{-1}$) under normal oxygen pressure (Johnson & Decker, 2015). Alkyl radicals form when a radical species abstracts hydrogen from the lipid acyl chain, and the carbon is left with an unpaired electron. Abstractions are more facile from the carbons between two double bonds (bisallylic carbons) than from the carbons adjacent to double bonds or

carbons in the aliphatic chain (bond dissociation energies 75-80, 88, and 101 kcal/mol, respectively) (Gardner, 1989; Koppenol, 1990).



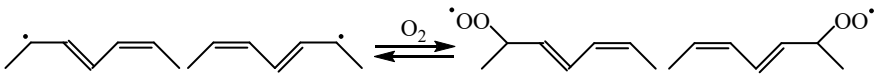
(1a)

(1b)

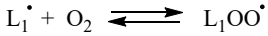
(1c)

Trace amounts of catalysts needed for the formation of initiating radicals are practically always present in foods, biological systems, and laboratory experiments. Most important catalysts include redox-active metals, ultraviolet- or visible light, heat, lipoxygenase, heme proteins, porphyrins, and ozone. Concerning edible oils, trace metals are one of the most important catalysts (Schaich (2005). Visible light can excite photosensitizers (e.g., pigments or flavins), which transfer the received energy to bonds so that free radicals form directly or to oxygen to form singlet oxygen ($^1O_2$), which can react with double bonds. Photosensitized oxidation is 1000-1500 times faster for polyenes than autoxidation (Shahidi & Zhong, 2010). Lipoxygenase catalyzes hydroperoxide formation from *cis*-non-conjugated pentadiene structures without lipid radical release. Photosensitized oxidation and enzymatic oxidation are distinguished from autoxidation due to differences in initiation and consequently, oxidation product ratios. Recently, a direct hydroxyradical (OH•) reaction with double bonds was suggested as an initiation mechanism (Zeng et al., 2020). Reaction rates of different radicals with lipids vary considerably. OH• are the most reactive (10^9-10^{10} L mol^{-1} sec^{-1}), while alkoxy radicals (LO•) and peroxy radicals (LOO•) have slower reaction rates (10^6-10^8 and 10^1-10^3 L mol^{-1} sec^{-1}, respectively) (Schaich, 2005).

After hydrogen abstraction, a pentadienyl radical with delocalized electrons forms (Reactions 1b, 1c) and oxygen adds to the less electron-dense outer carbons, forming LOO• isomers with conjugated double bonds (Reaction 2a). *Trans*- configuration for the shifted double bond is thermodynamically more stable (Choe & Min, 2009). Oxygen addition to L• establishes the free radical chain reaction and shifts autoxidation to propagation step (Reaction 2b). However, O_2 addition is reversible, and β-scission of oxygen is favored when alternate reactions of LOO• do not occur fast enough to convert it to more stable products. LOO• β-scission contributes to the formation of positional and geometric LOO• isomers and hydroperoxide isomers (N. A. Porter et al., 1981; Schaich, 2020).



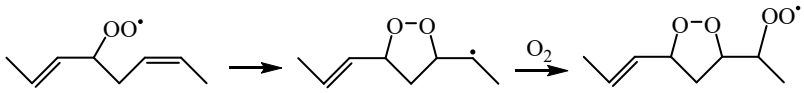
(2a)



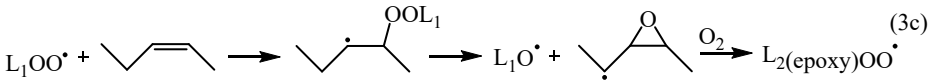
(2b)

LOO• can abstract hydrogens from other acyl chains to form lipid hydroperoxides (Reaction 3a), thus propagating the chain reaction from one acyl chain to another. Hydrogen abstraction by LOO• is the rate-limiting reaction step. LOO• can also add to adjacent *cis* double bonds, forming a cyclic radical (Reaction 3b), which, after O_2 addition and hydrogen abstraction, forms hydroperoxy epidioxide. The addition is possible also to intermolecular double bonds. This leads to the formation of two chain-propagating radical species, namely L_1O• and L_2(epoxy)OO• (Reaction 3c). In the later stages of oxidation, the addition to intermolecular double bonds leads to the formation of dimers and polymers (Schaich, 2020). Also, self-recombination and further cleavage to two alkoxy radicals is possible (Reaction 3d).

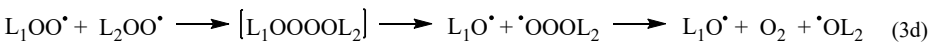
(3a)



(3b)

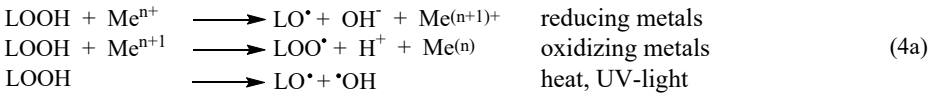


(3c)

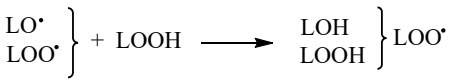


(3d)

The formed lipid hydroperoxides (LOOH) (Reaction 3a) decompose in the presence of redox-active metals, heat, and UV light (Reaction 4a) to form mainly alkoxy radicals. Reducing metals (e.g., Fe^{2+}) react faster with LOOH than oxidizing metals (e.g., Fe^{3+}). The formed LO• and LOO• can also abstract hydrogens from LOOH (Reaction 4b).



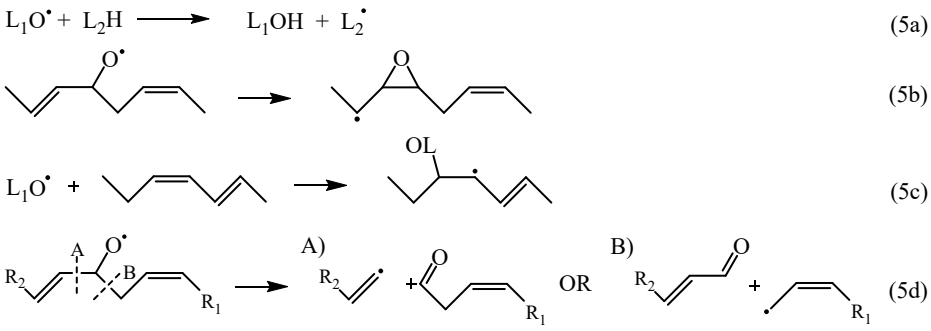
(4a)



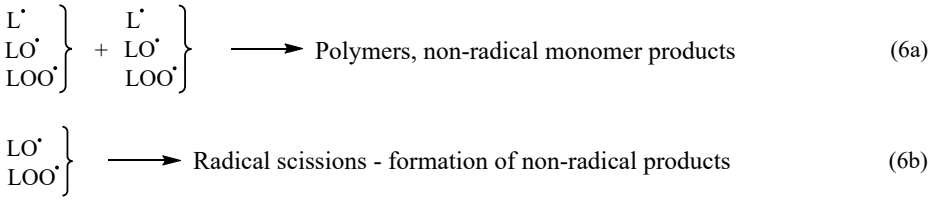
(4b)

Alkoxy radicals can react by abstracting hydrogens from other lipid acyl chains

(Reaction 5a), by forming epoxides and epoxyallylic radicals by 1,2-addition to the adjacent double bond (Reaction 5b), as well as by additions to intermolecular double bonds (Reaction 5c). Scission on either side of the alkoxy carbon is probably the most recognized LO• reaction. It leads to aldehyde formation on the LO• side and terminal radical formation on the other side (Reaction 5d). Formed radicals can react further, e.g., by hydrogen abstraction, OH• addition, or O_2 addition, and a variety of secondary lipid oxidation products, including oxo-esters or -TAGs with cleaved acyl chain and low-molecular-weight aldehydes, ketones, alcohols, and short-chain hydrocarbons are formed. These volatile secondary oxidation products are the cause for the unpleasant odor of rancid oil. In addition to alkoxy radicals, the cleavage products of cyclic peroxides, polyhydroperoxides, polymers, and preformed cleavage products can contribute to the variety of formed low-molecular weight oxidation products (Frankel, 1983).



Two radicals react to form non-radical species in the termination step (Reaction 6a). Depending on the reacting radicals, this can lead to the formation of polymers or non-radical monomer products. Formation of non-radical products is also possible through radical scission (Reaction 6b).



When the classical free radical chain reaction is discussed in a narrow sense, often only reactions 1a, 2b, 3a, 4a, 5a, 5d, and 6a are presented. Nonetheless, these reactions alone fall short in elucidating the kinetics and product mixes observed in autoxidation studies. To provide a more comprehensive

understanding of autoxidation mechanisms, it is imperative to consider also the alternate reaction pathways (Schaich, 2012). Autoxidation pathways and formed products shift according to the reaction conditions, e.g., solvent proticity, lipid concentration and conformation, temperature, oxygen pressure, surfaces, antioxidants, hemes, porphyrins, and metals (Schaich, 2012, 2020).

Considering oil or food preservation, the initiation phase preceding the exponential LOOH increase (propagation) is the most important. This slow reaction phase is known as the induction period, and it can be used as an estimate of oxidative stability (Kamal-Eldin & Pokorný, 2005). A typical kinetic curve for polyunsaturated FA autoxidation (e.g., linoleate) is presented in **Figure 3**. Lipid- and antioxidant decomposition, oxygen consumption and formation of hydroperoxides and volatile- and non-volatile oxidation products are shown. Sectors 1 and 2 represent the induction period and active (exponential) phase of autoxidation, respectively. In phase 3, hydroperoxides decompose faster than they are forming, and the levels of volatile oxidation products start to increase exponentially (Kamal-Eldin & Pokorný, 2005).

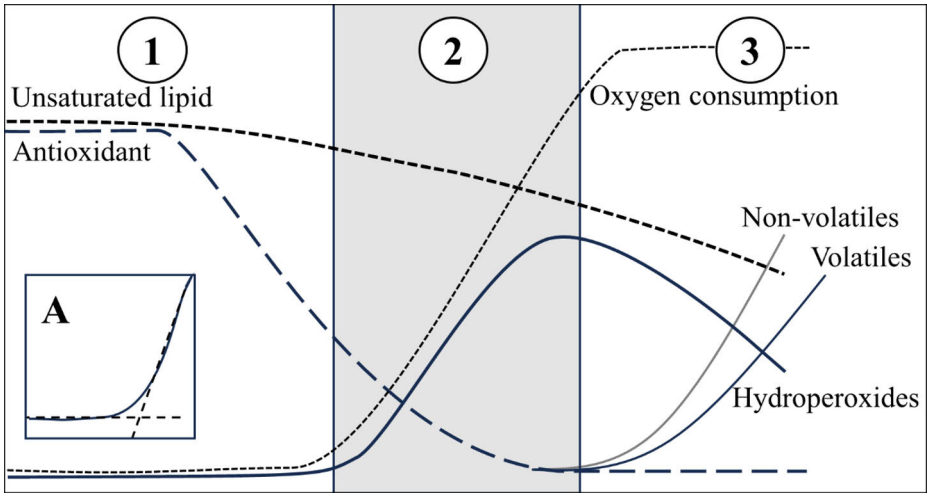


Figure 3. Typical kinetic curve for polyunsaturated FA autoxidation. Sectors 1 and 2 represent the induction period and active (exponential) phase of autoxidation, respectively. In phase 3, hydroperoxides are decomposing faster than they are forming, and the levels of volatile oxidation products start to increase exponentially. The length of the induction period can be determined by a tangent method as presented in insert A (adapted from Kamal-Eldin & Pokorný, 2005).

The induction period ends when a critical LOOH concentration or LOOH/antioxidant ratio is reached (Kamal-Eldin & Pokorný, 2005; Witting, 1969). In addition to the chemical reaction pathways, the physical location of the

molecules in bulk oil needs to be considered when evaluating the course of lipid oxidation. Even refined bulk oils still contain minor amounts of water, monoacylglycerols, diacylglycerols, free FAs, PLs, sterols, and polar oxidation products (e.g., hydroperoxides, aldehydes, ketones, and epoxides). These amphiphilic molecules aggregate to form association colloids, like lamellar structures or reverse micelles, which can alter the physical location of prooxidants, antioxidants, and oxidation substrates, hence affecting the oxidation kinetics (Chaiyasit et al., 2007; Homma et al., 2015; Xenakis et al., 2010). Brimberg (1993) suggested that the propagation phase starts when the critical micellar concentration of LOOH is reached (Brimberg, 1993; Brimberg & Kamal-Eldin, 2003). The previous impression of bulk oil oxidation mainly occurring at the oil/air interface (Frankel et al., 1994) has been revised along with new experimental evidence, postulating that oxidation mechanisms take place inside- or at the immediate vicinity of the association colloids (Budilarto & Kamal-Eldin, 2015; Villeneuve et al., 2023).

2.3 Tocopherols and amines as antioxidants in edible oil

Antioxidant is defined as a "substance that, when present at low concentrations compared with those of an oxidizable substrate, delays or prevents the oxidation of that substrate" (Halliwell, 1990). Primary antioxidants, also called "radical-scavenging" or "chain-breaking" antioxidants, react with lipid radicals by hydrogen donation, thus converting the radical to a non-radical species and inhibiting chain propagation. The formed antioxidant radical (A•) is stabilized due to the delocalization of the unpaired electron and the formation of a resonance hybrid. Antioxidants whose one-electron reduction potential (E°) is lower than that of a specific radical are capable of hydrogen donation. Primary antioxidants mainly contain mono- or polyhydroxy phenolic groups (e.g., flavonoids, tocopherols, butylated hydroxyanisole, butylated hydroxytoluene (BHT), and tert-butylhydroquinone).

Antioxidants that work by other means than free radical quenching are called secondary antioxidants. These antioxidants function by chelating transition metal catalysts (e.g., phytic acid, EDTA, and citric acid), regenerating primary antioxidants, decomposing hydroperoxides to non-radical species (e.g., to lipid alcohols), deactivating singlet oxygen, absorbing UV radiation, or acting as oxygen scavengers (Budilarto & Kamal-Eldin, 2015; Elias & Decker, 2017). A combination of antioxidants can result in three types of interaction: synergism, additive effect, or antagonism. Synergism refers to a situation where the combined effect of two antioxidants is greater than the sum of their individual effects. In additive effect, the antioxidant activity is the sum of each individual

effect, and in antagonism, two antioxidants in combination have an effect that is less than the sum of their individual effects (Olszowy-Tomczyk, 2020).

In a bulk oil system, the location of an antioxidant (reverse micelle vs. oil phase) is essential for its activity. The nano-scale reverse micelles are dynamic objects capable of trapping polar or hydrophilic molecules in their structure (Villeneuve et al., 2023). W. L. Porter et al. (1989) presented the "polar paradox" theory, suggesting that in polar emulsions, non-polar or amphiphilic antioxidants are most effective, while in non-polar bulk oil, polar antioxidants are most operative. The effect was explained by antioxidant migration to the site of oxidation in each system, with the nowadays contested assumption of bulk oil oxidation occurring at the oil/air interface. Later, it was realized that the phenomenon is more complicated, and also other attributes than polarity affect the antioxidant activity, with optimum values ("cut-off" effect) for the best protection (Laguerre et al., 2009; Shahidi & Zhong, 2011).

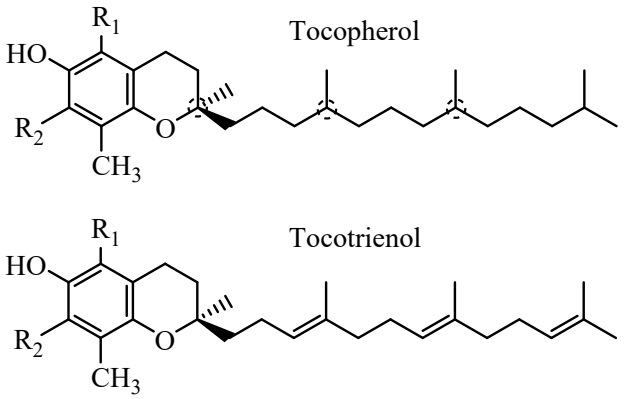
Antioxidant efficacies in different bulk oil systems cannot be predicted based on theoretical assumptions, but each case needs to be examined individually. This is due to numerous influencing factors, including the presence of association colloids, PLs, water, transition metals, metal binding constants, redox potential, antioxidant radical stability, antioxidant solubility, other antioxidant molecules (synergists/antagonists), and antioxidant concentration (Laguerre et al., 2015). There is a constant search for more effective antioxidants and antioxidant combinations to control lipid oxidation. Synthetic antioxidants like butylated hydroxyanisole and BHT were used in foods since the 1940s, but present consumers favor natural antioxidants due to health concerns related to synthetic molecules (Barouh et al., 2022).

2.3.1 Tocopherols

Tocopherols are probably the most used antioxidants on an industrial scale, including omega-3 oils. They are lipid soluble and natural, and can be used as natural extracts or synthetic molecules. In DHA-rich omega-3 fish oils, a combination of tocopherols and ascorbic acid derivatives and/or plant extracts (e.g., rosemary, oregano, tea) are known to reduce oxidation effectively (Kazuo, 2019). In Europe, tocopherols can be added to oils without an established limit on a *quantum satis* principle (Commission Regulation 1129/2011), except for addition to refined olive oils. The synthetic tocopherol forms can be esterified with different functional groups such as acetate, succinate, and nicotinate. The esterified forms are more stable but must be hydrolyzed to serve as antioxidants (Craft, 2016).

Tocopherols and their unsaturated derivatives, tocotrienols, consist of a chromane moiety and a phytyl tail (**Figure 4**). Tocopherols are naturally found

in polyunsaturated vegetable oils and germs of cereal seeds, while tocotrienols are present in aleurone and subaleurone layers of cereal seeds and palm oils (Yoshida et al., 2007). The four tocopherols (α-, β-, γ-, δ-) vary in the position and number of methyl groups on the phenolic ring. The only difference between tocotrienols (α-, β-, γ-, δ-) and the corresponding tocopherols is that they have three double bonds in their phytyl tail.



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 4. Chemical structures of tocopherols and tocotrienols. The circles with dashed line mark the three chiral centers in tocopherols (Adapted from Manolescu et al., 2008).

Tocopherols act as chain-breaking antioxidants by donating phenolic hydrogen to LOO• and forming a resonance-stabilized chroman-6-oxyl radical. Corresponding tocopherols and tocotrienols have mainly the same reactivities for radicals (Yoshida et al., 2003). LOO• reacts much faster with tocopherol (10^4-10^9 M^{-1} s^{-1}) than with lipid acyl chains (10-60 $M^{-1}s^{-1}$). In lipophilic bulk oil conditions, the formed tocopherol radicals mainly react by self-coupling to form tocopherol dimers or couple with lipid radicals (e.g., LOO• or LO•) (Barouh et al., 2022). However, for α-analogs, the rate constant for dimerization was found to be very low, probably due to steric hindrance of the two *ortho*-methyl substituents (Lucarini et al., 1994). As effective hydrogen donors, tocopherols decrease the PUFA LOO• reactions to *trans*, *trans*-hydroperoxide isomer and cyclic structure formation and increase the formation of LOOH (Peers & Coxon, 1983). In addition to radical quenching, tocopherols are able to quench reactions with singlet oxygen both physically and chemically (Clough et al., 1979; Kaiser et al., 1990).

Tocopherols have a hydrophobic tail and a more hydrophilic chromane ring. This can enable partitioning into oil/water interfaces, which increases antioxidant efficacy. Gunaseelan et al. (2006) reported that 73% of α-tocopherol (α-TOC) occupied the oil/water interface of a macroemulsion (1:1 oil/water, 2% $C_{12}E_6$ v/v), with only 26% residing at the hydrophobic phase. Pastoriza-Gallego

et al. (2009) reported interfacial/oil region partitioning constants of P_O^I = 52-75 (15-30 °C) for α-TOC in octane-$C_{12}E_6$-water emulsions (1:1 o/w). A kinetic pseudophase model was employed for the analysis. It was postulated, that the hydrophilic headgroup enters the interfacial structure while the phytyl tail remains in the octane phase (Pastoriza-Gallego et al., 2009).

At high concentrations, tocopherols may exhibit a pro-oxidative effect. Several potential explanations have been proposed for the phenomenon. In bulk oil conditions and low temperatures, tocopherols can donate hydrogens to LOOH or LOOL, decomposing them to lipid alcohols (LOH) and alkoxy radicals. This reaction is more favored at high LOOH/tocopherol ratios. Tocopherols are also known to reduce transition metals to a more pro-oxidative state (e.g., Fe^{3+} to Fe^{2+}). Oxidation can also be propagated by tocopherol radical reactions with molecular oxygen ($^3O_2$), and under certain conditions (e.g., low LOO• concentration), TOC radicals can react with lipid acyl chain hydrogens to form lipid radicals (Barouh et al., 2022; Kamal-Eldin & Appelqvist, 1996; Mukai & Okauchi, 1989; Yamamoto, 2001). Kim et al. (2007) also suggested that the oxidized, amphiphilic α-TOC molecules could reduce the oil surface tension and thus increase oxygen transfer into oil. Since the presence of tocopherols alters the oxidation pattern, the observed prooxidative effect may also depend on the methods used to determine lipid oxidation (e.g., LOOH vs. secondary oxidation products), as well as on the test system, concentration, and oxidation time (Huang et al., 1994; Martin-Rubio et al., 2018).

Tocopherols can exhibit synergistic effects when combined with various types of secondary antioxidants. They can be regenerated by ascorbic acid, glutathione (Niki et al., 1982; Yamamoto, 2001), green tea polyphenols (methyl gallate, epicatechin, epigallocatechin, epicatechin gallate) (Fujisawa et al., 2006; B. Zhou et al., 2005), myricetin (Bayram et al., 2023) and PE (Cui et al., 2015). Synergism with β-carotene can be either due to tocopherol regeneration or the sparing effect of β-carotene caused by tocopherol (Elias & Decker, 2017; Zou & Akoh, 2015). Also, the simultaneous use of metal chelators, such as PLs, amino acids, or peptides, can result in a synergistic effect (Kamal-Eldin & Appelqvist, 1996). Tocopherol synergism with amines by means of regeneration, alteration of tocopherol position in the oil, and formation of antioxidative carbonyl-amine reaction products will be discussed in more detail in section 2.3.4.

2.3.2 Possible amine reactions in oxidizing oil

Amines are compounds and functional groups characterized by a basic nitrogen atom with a lone pair of electrons. Amines originate from ammonia (NH_3) through the substitution of one or more hydrogens with organic groups. Proteins, amino acids, and other amine compounds can interact with lipid oxidation

products in a complicated manner by forming amino compound radicals, physical complexes, and new covalent bonds. These interactions can have positive or negative outcomes, altering the odor, flavor, nutritional quality, and safety of food, as well as resulting in browning and formation of antioxidative compounds. The following discussion deals with the interactions between lipid oxidation products and amines, with a focus on aminophospholipids and alkylamines.

The formation of amine radicals is facilitated by low reduction potentials or bond dissociation energies of the amine hydrogen (Gribov et al., 2004; Treyde et al., 2022). This makes them susceptible to hydrogen abstraction by lipid radicals in addition to the more reactive free radicals like OH• and reactive oxygen species. Amine radicals have been observed to form in the presence of peroxidizing lipids (Bartesaghi et al., 2010; Hidalgo & Zamora, 2019; Karel et al., 1975). These radicals can crosslink with proteins or lipid radicals at the termination stages of oxidation (Gardner, 1979; Shchepin et al., 2010).

Non-covalent oxidized lipid-amine complexes can form through hydrophobic association and/or hydrogen bonds, facilitating further chemical interaction and oxidation (Gardner, 1979; Narayan et al., 1964). LOOHs can hydrogen bond next to reactive sites (e.g., Cu^{+}, Fe^{2+}) of proteins, which leads to hydroperoxide decomposition and fast formation of alkoxy- and protein radicals (Hidalgo & Kinsella, 1989; Schaich, 2008). In addition to proteins, also other amines can hydrogen bond and go through similar concerted reactions with hydroperoxides (Le Harris & Olcott, 1966; Schaich, 2008), as shown in Scheme 7 (Schaich, 2008).

$$R_3N + R'OOH \rightarrow [R_3N \ldots HOOR'] \rightarrow R_3NO + R'O\bullet \quad (7)$$

Interactions through the formation of new covalent bonds can follow various reaction pathways. The oxidation products formed after LOOH decomposition can have several electrophilic reaction sites available for the nucleophilic amine moiety. Carbonyl-amine reactions between oxidized lipids and amines mainly produce five- or six-membered aromatic heterocyclic structures (Hidalgo & Zamora, 2017). The most studied lipid oxidation product-amine interaction is probably the reaction of secondary oxidation product carbonyls with amine compounds via Maillard-type carbonyl-amine reaction by Schiff's base (imine) formation or Michael addition. The latter requires an α,β-unsaturated aldehyde as the reacting carbonyl (**Figure 5**). The formed Schiff's base adducts can further polymerize by aldol condensation, forming brown pigments (Hidalgo & Zamora, 2000b).

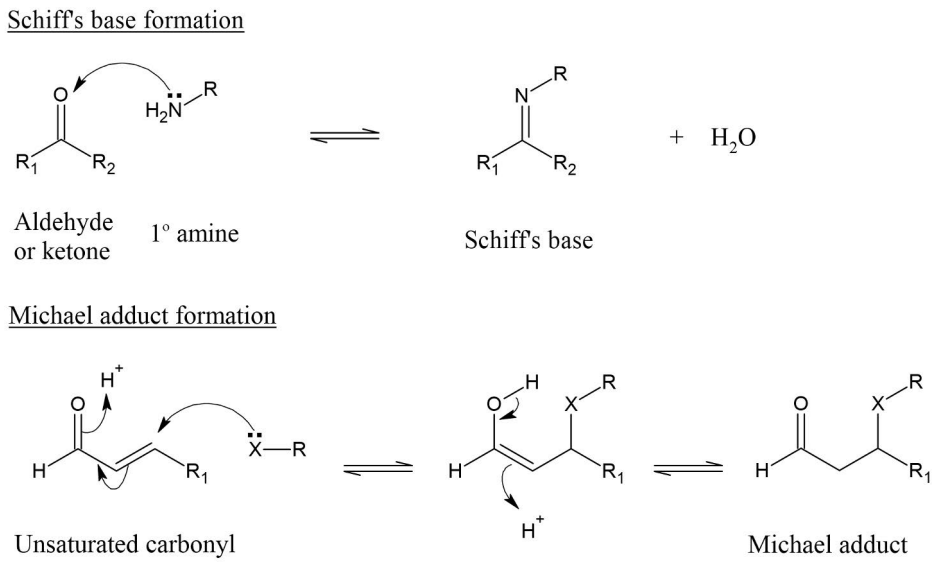


Figure 5. Schiff's base and Michael adduct formation. Michael adduct formation is shown for the nucleophilic group X (adapted from Viedma-Poyatos et al., 2021).

Schiff's base or Michael adduct formation often serves as the initial step for numerous subsequent reactions. However, other reaction routes like amide formation and S_N2 mechanism have also been proposed. The following discussion will cover the alkyl amine, amino alcohol, and PL reactions with several lipid autoxidation product types, including 4,5-epoxy-2-alkenals, α,β-unsaturated hydroxyalkenals, γ-ketoaldehydes, simple alkenals, alkanals, malondialdehyde (MDA), and γ-hydroxy-α,β-unsaturated epoxides. Studies on alkylamine, amino alcohol, and PL reactions with lipid oxidation products in solvents/solutions are summarized in **Table 2**. The table includes only studies where emulsions or liposomal structures were not intentionally prepared.

Table 2. Studies on alkylamine, amino alcohol, and PL reactions with lipid oxidation products in solvents/solutions.

Oxidation product	Amine	Reaction conditions	Reaction products	Reference
4,5-Epoxy-2-decenal 13-OOH of linoleic acid	Butylamine	Chloroform, 37°C or RT	• l-Substituted 2-(l'-hydroxyalkyl)pyrroles, l-substituted pyrroles	Zamora & Hidalgo, 1995
4,5(*E*)-Epoxy-2(*E*)-heptenal	16:0/16:0-PE Ethanolamine	Chloroform, 37°C	• Phosphatidylethanolpyrroles, phosphatidylethanol-2-(1-hydroxypropyl)pyrroles	Zamora & Hidalgo, 2003
4,5-(*E*)-Epoxy-2(*E*)-decenal 4,5-(*E*)-Epoxy-2(*E*)-heptenal	Butylamine	Acetonitrile/water (2:1), 37°C	• N-substituted 2-alkylpyrroles, alkylfurans	Zamora & Hidalgo, 2005
4,5-Epoxy-2-decenal	Octylamine Benzylamine 2-Phenylglycine methyl ester	Acetonitrile/sodium citrate or phosphate buffer (2:1), 37 or 60 °C	• Strecker degradation products: Octanal (trace amounts) from octylamine 2-Pentylpyridine & benzaldehyde (4.3%) from benzylamine 2-Oxo-2-phenylacetate (49%) from 2-phenylglycine methyl ester • Other products: N-substituted pyrroles, hexanal, 2-pentylfuran	Zamora et al., 2006
Methyl 9,10(*Z*)-epoxy-13-oxo-ll(*E*)-octadecenoate Methyl 12,13(*Z*)-epoxy-9-oxo-10(*E*)-octadecenoate	Butylamine	Chloroform, 37 °C	• Methyl 9-hydroxy-9-(5-pentyl-N-butyl-1H-pyrrole-2-)nonanoate, N-alkyl-2-pentylpyrrole • Methyl 8-[5-(1'-hydroxyhexy1)N-butyl-lH-pyrrole-2-]octanoate, Methyl 8-(N-butyl-1H-pyrrole-2-)octanoate	Hidalgo & Zamora, 1995
16:0 or 18:0/9:0(oxo)-PC	18:1/18:1-PE 16:0/16:0-PS	Chloroform/methanol (2:1), RT	• Schiff's base adducts	Ravandi et al., 1997
2-[9-oxo]nonanoyl glycerol	16:0/18:1-PE	Chloroform/methanol (2:1), RT	• Schiff's base adducts	Kurvinen et al., 1999
4-Hydroxynonenal	Benzylamine Phenylethylamine	Acetonitrile/phosphate buffer (pH 7.8) (1:1), 25 °C	• Pyrroles	Sayre et al., 1993
4-Hydroxynonenal	PE, PS	Diethylether/buffer (NaCl-HEPES pH 8.5) (1:1), vigorous stirring at 30 °C	• Michael adducts (PE, PS) and a minor Schiff's base adduct which was partly cyclized to a pyrrole derivative (PE only)	Guichardant et al., 1998
4-hydroxydodeca(2*E*,6*Z*)-dienal 4-hydroxy-2(*E*)-nonenal 4-hydroxy-2(*E*)-hexenal	18:0/20:4-PE 18:0/22:6-PE	Diethylether/buffer (NaCl-HEPES pH 8) (8:2), vigorous stirring at RT	• Michael adducts, Schiff's base adducts	Bacot et al., 2003

Oxidation product	Amine	Reaction conditions	Reaction products	Reference
4-Hydroxy-2-nonenal 4-Oxo-2-nonenal	16:0/18:1-PE	Dry dichloromethane, 40 °C	• Michael adducts (both oxidation products) and cyclization to hemiacetal derivative (only 4-hydroxy-2-nonenal) • Schiff's base adducts (both oxidation products) and cyclization to pyrrole derivative (only 4-hydroxy-2-nonenal)	Vazdar et al., 2017
γ-Ketoaldehydes (isoketals)	16:0/18:2-PE	Diethylether/buffer (NaCl-HEPES pH 8) (8:2), RT	• Schiff's base adducts, pyrroles	Bernoud-Hubac et al., 2004
Oxidized arachidonic acid	16:0/16:0-PE	Triethylammonium acetate/chloroform/ethanol (1:1:3) in a shaker bath at 37°C	• Putatively identified structures (also amide-linked): Formyl-PE, N-acetyl-PE, N-propenal-PE, N-oxoacetyl-PE, N-4-carboxybutanoyl-PE, N-hexanoyl-PE, N-7-carboxyhept-2-enoyl-PE, N-9-carboxynona-3,5-dienoyl-PE, oxHNE-PE, IsoLG-PE (hydroxylactam)	Guo et al., 2012
2-Propenal	18:1/18:1-PE	Methanol/methylene chloride/ HBSS buffer (2:1:0.8), 37 °C	• 1,2-Diradyl-*sn*-glycero-3-phosphoethanol-(3-formyl-4-hydroxy)piperidine	Zemski Berry & Murphy, 2007
2-Propenal	Sphingosine	Chloroform or phosphate buffer, stirring at RT	• Eight-membered heterocycles 2,6,9-triazabicyclo[3.3.1]nonanes and 1,5-diazacyclooctanes through imino [4+4] cycloaddition	Takamatsu et al., 2014
(*E*)-2-Octenal	*n*-Octylamine	Chloroform at 37 °C	• (*E*)-2-Octenoic acid, 1-(octylimino)-2-octene and 2-(1-carboxymethyl)-1-octyl-4-pentylpyridinium betaine	Alaiz et al., 1996
MDA • Na	Methylamine	Phosphate buffer (pH 7)	• 1,4-Dimethyl-l,4-dihydropyridine-3,5-dicarbaldehyde, methylamine-MDA-adduct	Kikugawa & Ido, 1984
MDA	16:0/16:0-PE	Chloroform/water (1:1), 37 °C	• N-1,4-dihydropyridine-3,5-dicarbaldehyde-PE, PE-N-propenal-adduct	Guo et al., 2012
Propanal Heptanal	Ethanolamine hydrochloride	Stirring at RT	• Pyridium salts	Suyama & Adachi, 1979
Heksanal	16:0/18:1-PE	Chloroform/methanol (2:1), stirring at 25, 40 and 60 °C	• 2-Pentyl-3,5-dibutyl-dihydropyridine	Goritschnig et al., 2020
(*E*)-4,5-Epoxy-(*E*)-2-hexen-1-ol	Propylamine	Tetrahydrofuran, 100 °C	• 4-(Propylamino)-*trans*-2-hexene-1,5-diol by S_N2 mechanism	Lederer, 1996

Abbreviations: RT (room temperature), PE (phosphatidylethanolamine), PC (phosphatidylcholine), PS (phosphatidylserine), HEPES (N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid), oxHNE (oxidized hydroxynonenal), IsoLG (isolevuglandin), HBSS (Hank's balanced salt solution)

Zamora and Hidalgo (Hidalgo & Zamora, 2000a; Zamora & Hidalgo, 1994, 1995, 2003, 2005) showed that 4,5-epoxy-2-alkenals react with amine compounds by forming N-substituted pyrroles and 2-(1-hydroxyalkyl)pyrroles. In the authors extensive work, 4,5-epoxy-2-decenal, 4,5(*E*)-epoxy-2(*E*)-heptenal, and 13-OOH of linoleic acid were reacted with lysine, bovine serum albumin, butylamine, PE and ethanolamine. Reactions for butylamine and PE were carried out at 37 °C in chloroform or acetonitrile/water (1:1). The formed 2-(1-hydroxyalkyl)pyrroles were suggested to be responsible for the fluorescence and color development through polymerization, while the N-substituted pyrroles were more stable. Also, the formation of a short-chain saturated aldehyde was observed. The suggested reaction mechanism starts with an imine formation, followed by further cyclization to a pyrrole ring by nitrogen addition to the epoxy carbon closer to the double bond. From this intermediate, N-substituted pyrroles and 2-(1-hydroxyalkyl)pyrroles form through electron rearrangement and proton transfer (**Figure 6**) (Zamora & Hidalgo, 2003).

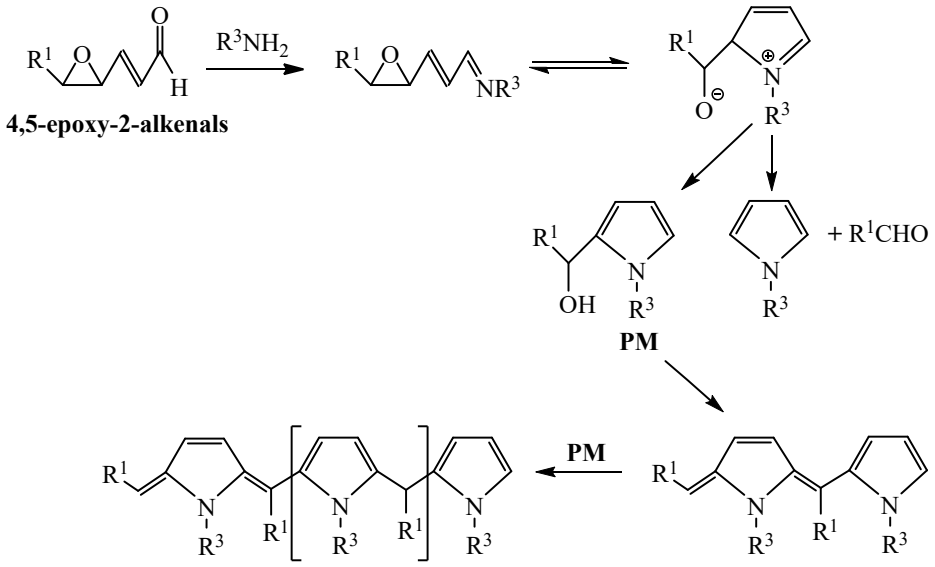


Figure 6. Reaction of 4,5-epoxy-2-alkenals with primary amino groups, forming N-substituted pyrroles, 2-(1-hydroxyalkyl)pyrroles (PM) and polymer structures (adapted from Zamora & Hidalgo, 2003).

4,5-Epoxy-2-alkenals can degrade amino acids by a Strecker-type mechanism, and similar pathway is possible for the degradation of alkylamines. The process results in the formation of pyridines and Strecker aldehydes. Zamora et al. (2006) studied the reactions of 4,5-epoxy-2-decenal with octylamine, benzylamine, and 2-phenylglycine methyl ester and found that the formation of Strecker aldehydes

was most favored for the 2-phenylglycine methyl ester (49% yield of 2-oxo-2-phenylacetate) with methoxycarbonyl group at α-position to the amino group. Octylamine produced only trace amounts of octanal, and benzylamine was converted into benzaldehyde with a 4.3% yield. The results might indicate that the generation of Strecker aldehydes is less pronounced in alkylamines compared to amino acids. Lu et al. (2013) reported Strecker aldehyde and hydrophobic pyrrole formation in PE liposomal dispersions after incubation at 60 °C.

In addition to the short-chain cleavage product, the long-chain oxidation products with intact acyl chains can react in a similar manner. Epoxyoxoene methyl esters (octadecenoates) with a similar 4,5-epoxy-l-oxo-2-pentene system as described above for 4,5-epoxy-2-alkenals, reacted with butylamine and lysine, producing N-substituted pyrrole methyl esters and short-chain pyrroles through carbon chain cleavage (Hidalgo & Zamora, 1995). The formation of Schiff's base adducts was also reported from the reactions of core aldehydes 2-[9-oxo]nonanoyl glycerol and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-PC with PE and PS (Kurvinen et al., 1999; Ravandi et al., 1997).

Besides 4,5-epoxy-2-alkenals, α,β-unsaturated hydroxyalkenals can react with aminophospholipids and alkylamines, forming Schiff's base- and Michael adducts. 4-Hydroxynonenal reacted with PE in an aqueous-organic biphasic system, forming mainly Michael adducts. Schiff's base adducts were formed in lower quantities, probably due to decreased stability. PS reacted weakly with 4-hydroxynonenal, suggesting that the close proximity of carboxyl and amine groups could prevent the reaction (Guichardant et al., 1998). In addition to PE, pyrrole formation was reported from 4-hydroxynonenal reactions with benzylamine and phenylethylamine (Sayre et al., 1993). In the detailed study on the reaction mechanisms of 4-hydroxynonenal and 4-oxo-nonenal with PE, Vazdar et al. (2017) reported that the Michael adduct of 4-hydroxynonenal can further cyclize, forming a hemiacetal derivative, while for 4-oxo-nonenal with a keto-group no cyclization was observed for either Michael- or Schiff's base adduct. The reactivity of hydroxyalkenals (4-hydroxynonenal, 4-hydroxy-2(*E*)-hexenal, 4-hydroxydodeca(2*E*,6*Z*)-dienal) towards PE was increased by the increasing hydrophobicity of the hydroxyalkenal moiety (Bacot et al., 2003).

γ-Ketoaldehydes (isoketals), which can form through further reactions of arachidonic acid or DHA bicycloendoperoxides (Aschner et al., 2021), can form Schiff's base adducts and pyrroles with ethanolamine (Bernoud-Hubac et al., 2004). Guo et al. (2012) also reported the formation of several amide-linked PE adducts. Compared to epoxy- or hydroxy alkenals, the reactions of simple alkenals with amines are more complex because stable compounds form more slowly (Alaiz et al., 1996b). 2-Propenal can react with amines forming Michael- and Schiff's base adducts (Davies & Guo, 2014; Esterbauer et al., 1991; Zemski Berry & Murphy, 2007). 2-Propenal reactions with sphingosine (d18:1) resulted

in the formation of an eight-membered heterocycle through imino [4+4] cycloaddition (Takamatsu et al., 2014). Alaiz et al. (1996) reacted (*E*)-2-octenal and *n*-octylamine in chloroform at 37 °C and reported the formation of several compounds, from which (*E*)-2-octenoic acid, 1-(octylimino)-2-octene and 2-(1-carboxymethyl)-1-octyl-4-pentylpyridinium betaine could be identified.

MDA reacts covalently with amines, including PE and PS, to form N-propenal-, N-1,4-dihydropyridine-3,5-dicarbeldehyde-, and N-propenal-crosslinks (Bhuyan et al., 1996; Guo et al., 2012; Kikugawa & Ido, 1984). Reactions of alkanals with primary amines produced pyridium salts as the main product at room temperature (Suyama & Adachi, 1979), and 2-pentyl-3,5-dibutyl-dihydropyridine was identified as the reaction product of hexanal and 16:0/18:1-PE (Goritschnig et al., 2020). Also, the non-carbonyl oxidation products can react with amines. γ-Hydroxy-α,β-unsaturated epoxides reacted with amine compounds by nucleophilic attack of the amine nitrogen to the allylic epoxy-carbon by the S_N2 mechanism (Lederer, 1996; Lederer et al., 1999). Lederer (1996) modeled the reaction with propylamine and (*E*)-4,5-epoxy-(*E*)-2-hexen-1-ol in aprotic tetrahydrofuran (100 °C, 16 h).

In all of the studies presented above, the reactions were conducted in solvents and/or buffer solutions, mainly by reacting individual amines with individual oxidation products. Actual oxidized lipids in some form were applied in two studies. Zamora & Hidalgo (1995) incubated oxygenated linoleic acid hydroperoxide (13-OOH) in chloroform with butylamine, and Guo et al. (2012) oxidized arachidonic acid in a solvent system (triethylammonium acetate/chloroform/ethanol) with PE. Currently, the possible and most prevalent reactions in oxidizing, neat TAG oils with added amines are largely unknown.

2.3.3 Antioxidative properties of amines

Proteins, peptides, and amino acids can protect lipids from oxidation by donating hydrogens to radical species. This can happen if the amino acids are oxidatively more labile than the unsaturated FAs or if the location of the amino acid is physically close to the free radical or reactive oxygen species generation site. Other means for the antioxidative effect of proteins include metal chelating, hydroperoxide reduction, enzymatical elimination of pro-oxidants, and physical separation of reactive species (Elias et al., 2008). Comprehensive discussions on the antioxidative properties of added proteins and peptides in foods are available elsewhere (Elias et al., 2008; Mardani et al., 2023; F. G. Pan et al., 2022), and in the following discussion, the primary focus will be on the antioxidative properties of aminophospholipids and other non-protein amines.

PLs can sequester prooxidative metals due to the negative charges on their phosphate head groups, thereby mitigating lipid oxidation. However, it is also

possible that the chelated metal is still active and able to promote lipid oxidation. The chelators are more effective when their concentration exceeds the metal concentration because then all the coordination sites can be bound, and the metal becomes unreactive (Cui & Decker, 2016). The iron-binding capacity of PLs was reported in several studies using liposomal systems (Dacaranhe & Terao, 2001; Vile & Winterbourn, 1987; Zago & Oteiza, 2001), while Yoon & Min, (1987) studied the antioxidative effect of different PLs, including PC, PE, phosphatidylinositol, and phosphatidic acid in a bulk oil model. Purified soybean oil with 300 ppm PL was oxidized at 60 °C in the dark with and without one ppm ferrous iron (Fe^{2+}). The antioxidative effect was observed only in the samples with added iron, while in the absence of iron, the effect was prooxidative.

Amines can delay lipid oxidation by donating hydrogens to lipid free radicals. Aromatic amines with low bond dissociation energies and resonance-stabilized structures are commonly used as antioxidants in rubbers, lubricants, and hydrocarbon fuels (Denisov & Denisova, 2015). If the amine radical does not contain a resonance-stabilized structure to lower its energy, the total oxidation is not delayed but merely partly redirected from lipids to amines. Ease of hydrogen abstraction from amines at high concentrations can lead to LOOH and LOH accumulation and temporary blockage in the formation of secondary oxidation products (Schaich, 2020).

Amines can also attack the electron-deficient oxygen of LOOH, reducing it to a more stable alcohol (Saito & Ishihara, 1997), thus delaying the oxidation propagation and formation of secondary oxidation products. The effect might be enhanced by the intramolecular hydroxy groups, which could strengthen the nucleophilicity of amines by hydrogen bonding with the amine protons (**Figure 7**).

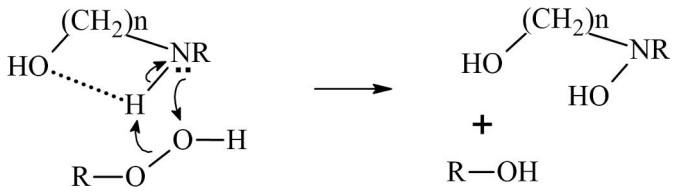


Figure 7. LOOH decomposition in the presence of amines with intramolecular hydroxyl groups (adapted from Saito & Ishihara, 1997).

LOOH decomposition and increased LOH formation in the presence of ethanolamine and choline were reported for methyl linoleate hydroperoxides in methyl linoleate, autoxidized linseed oil, and sardine oil (Miyazawa et al., 1984; Saito & Ishihara, 1997). The choline/ethanolamine levels in the studies were relatively high, varying from 1 to 3% w/w. Also, commercially available PLs (1% w/w) showed the ability to decompose LOOH into LOH in linoleic acid (X. Pan

et al., 2010). In addition to PL moieties, trioctylamine, and its analogs were able to decompose *tert*-butylhydroperoxide into corresponding LOH and hydroxylamines at 70 °C. The hydroxylamines showed antioxidant activity, which was attributed to hydrogen donation from hydroxylamine to LO• or termination reactions of the formed nitroxide radicals (Le Harris & Olcott, 1966). The latter pathway was also suggested by other authors (Tikhonov et al., 2015; Van Der Veen et al., 1970).

In addition to the antioxidative effect of amines as such, the carbonyl-amine reaction products show antioxidant activity. Oxidized PE, including heterocyclic carbonyl-amine reaction products, had higher antioxidant activity in refined soybean oil (oxidized at 60 °C in the dark) than non-oxidized PE. In the case of PC and phosphatidylinositol without primary amine groups, oxidation decreased their antioxidant activity (Hidalgo et al., 2005). Similar results for PE and PC were obtained under Rancimat conditions (110 °C) for refined olive oil (Hidalgo et al., 2006). Alaiz et al. reported the antioxidative efficacies of pyrroles and pyridium salts formed through amino acid reactions with (*E*)-2-octenal and 4,5(*E*)-epoxy-2(*E*)-heptenal (Alaiz et al., 1995a, 1995b), as well as of pyrrole, imidazole, dihydropyridine, and pyridinium salt derivative model compounds (100-200 ppm) in stripped/refined soybean oil oxidized at 60 °C (Alaiz et al., 1996a). The results suggested that compounds with a six-member ring have higher antioxidative activity than compounds with a five-member ring, and the presence of two heteroatoms gives better effectiveness than only one nitrogen atom. However, molecular weight, boiling point, and stability also influence the antioxidative properties. Later, Hidalgo et al. (2003) found that the antioxidative activity of pyrroles is increased by alkyl-substitution and lack of free α-positions and decreased by the presence of oxygenated groups. During polymerization, pyrroles undergo the loss of free α-positions and hydroxyl groups (**Figure 6**). Consequently, it was observed that pyrrole dimers had higher antioxidant activity when compared to higher dimers or monomers. Some pyrrole derivatives are associated with nonenzymatic browning through polymerization (Hidalgo et al., 2003).

An antioxidative effect was also reported for *n*-octylamine, N-methylheptylamine, and N,N-dimethylhexylamine in oxidized, stripped soybean oil and attributed to the formed carbonyl-amine reaction products. When added at 100-200 ppm, only *n*-octylamine and N-methylheptylamine could decrease thiobarbituric acid reactive substances (TBARS). N,N-dimethylhexylamine is a tertiary amine and cannot form antioxidative Schiff's base or Michael adduct products. However, at elevated concentrations (500, 1000, and 2000 ppm), all three amines had high antioxidant activity (Alaiz et al., 1996b), suggesting another antioxidant mechanism. Furthermore, the reaction products of sphingoid bases and oxidation product aldehydes showed antioxidant activity. Sphingoid

bases d18:0 and d18:1 were reacted with propanal and 2-propenal, and the reaction products delayed the oxidation of fish oil effectively with added α-TOC at 50 °C in a reaction mixture where no unreacted sphingoid base was present. The highest antioxidant activity was reported for the reaction products of d18:0 and 2-propenal (Suzuki-Iwashima et al., 2021). To conclude, PLs, alkylamines, and amino alcohols can have antioxidative properties through metal chelation, hydrogen donation, hydroperoxide reduction, and antioxidative carbonyl-amine reaction product formation. The mechanism behind the observed antioxidative effect during oxidation can be challenging to distinguish, possibly resulting from a combination of multiple simultaneous mechanisms.

2.3.4 α-Tocopherol synergism with amine antioxidants

Aminophospholipids can have a synergistic antioxidative effect when combined with tocopherols. Most commonly, the effect is explained by regeneration of oxidized forms of tocopherols or by alteration of the physical location of tocopherols to be more effective at the site of oxidation (association colloids/reverse micelles). Tocopherols can also contribute to the antioxidant activity of carbonyl-amine reaction products. The following discussion mainly focuses on α-TOC, which was applied in the current study, and is clearly the most studied tocopherol homologue concerning amine synergism.

2.3.4.1 Tocopherol regeneration

Some PLs can regenerate the oxidized form of α-TOC, α-tocopherylquinone, back to the active α-TOC form, thus increasing antioxidant efficacy. For example, PE and PS reduced α-tocopherylquinone to α-TOC at 100 °C (Doert et al., 2012; Weng & Gordon, 1993). Often, the proposed mechanism for regeneration involves direct hydrogen transfer from PLs to tocopherol radicals (Judde et al., 2003; King et al., 1992; Tang et al., 2023). Based on the chirality of the regenerated α-TOC, Doert et al. (2012) suggested that the regeneration does not occur by radical pathway but rather by carbonyl-amine reaction between PE/PS and α-tocopherylquinone (Doert et al., 2012). The formation of α-tocopherylquinone and the suggested reaction scheme for α-TOC regeneration by PE is illustrated in **Figure 8**.

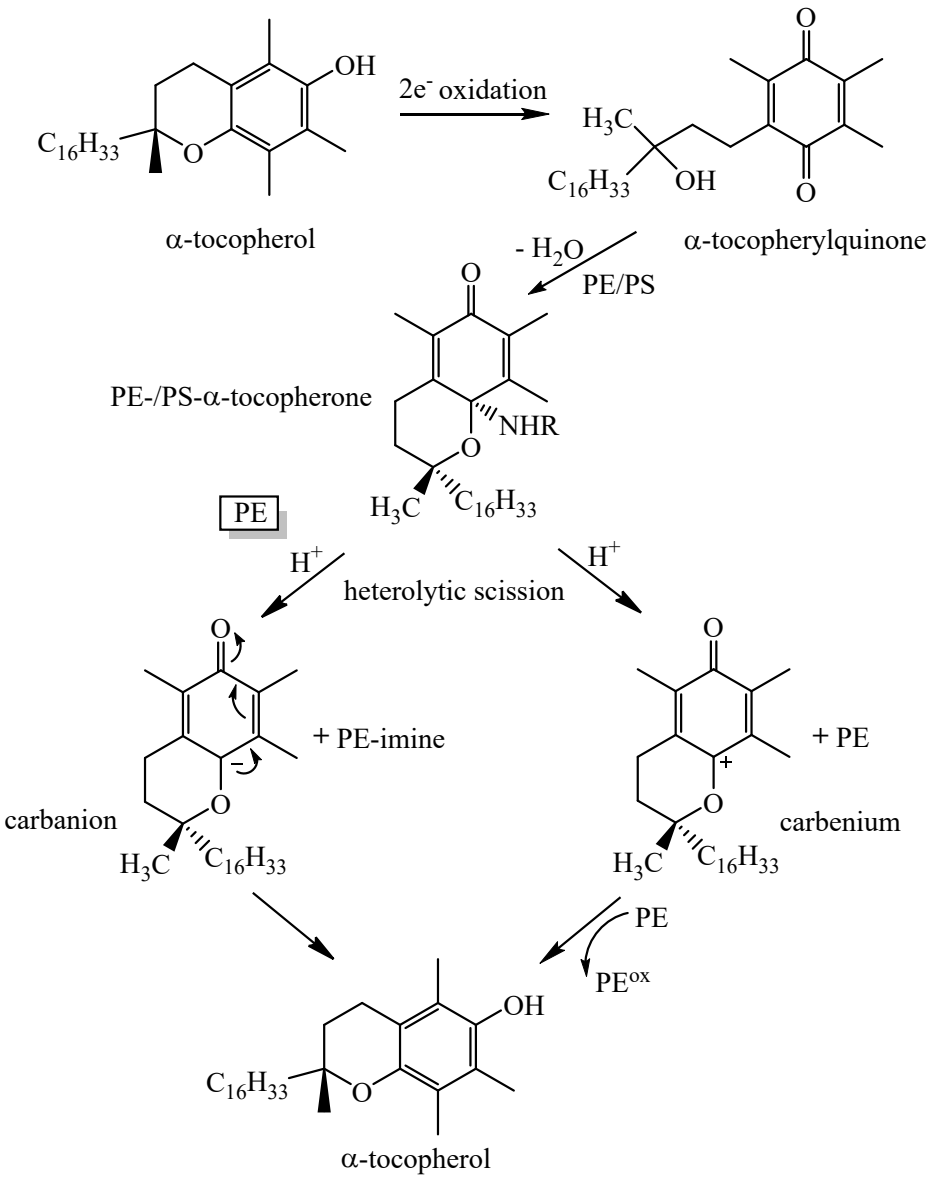


Figure 8. Formation of α-tocopherylquinone as a result of antioxidant activity and its regeneration by PE through carbonyl-amine reaction and acid heterolysis (adapted from Bayram et al., 2023; Doert et al., 2012).

In the suggested reaction pathway, α-tocopherone forms first through a carbonyl-amine reaction, and it further reacts by rearrangements and acid heterolysis (PE) or decarboxylation (PS) to form α-TOC (Doert et al., 2012). A non-radical pathway was also suggested by Cui et al. (2015) based on the reduction potential measurements. The necessity of carbonyl-amine reaction could be the cause for the incapability of PC with no primary amine group to act as α-TOC regenerator

(Cui et al., 2015) and possibly explain the negligible synergism between PC and α-TOC reported in several studies (Cui et al., 2015; Doert et al., 2012; Kashima et al., 1991; Takenaka et al., 2007; Xu et al., 2019). Schiff's base product of γ-tocopherylquinone and octylamine has been later tentatively identified in a model system (120° C, 1h) (Dragoun et al., 2022), which also supports the suggested reaction pathway through carbonyl-amine reaction. The possibility of α-TOC regeneration by d18:0 was ruled out by Shimajiri et al. (2013), since d18:0 could not preserve α-TOC in tricaprylin during incubation at 50 °C without the presence of oxidation products.

2.3.4.2 Interactions in association colloids

Above their critical micellar concentration, PLs aggregate to form reverse micelles in bulk oil, which can affect the oxidation chemistry and partitioning of oxidation products and antioxidants. Koga & Terao (1995) studied the synergistic effect of PL (100 nM) and α-TOC (10 nM) in a bulk oil model with either oil-soluble or water-soluble radical initiator. The water-soluble radical initiator was dispersed to the oil phase in water (1% v/v). The PLs could enhance the effect of α-TOC only when the oxidation was initiated in the aqueous phase. Dipalmitoyl-PC was found to be more effective than dipalmitoyl-PE. The authors postulated that PLs increase the accessibility of α-TOC to the chain-initiating radicals in the aqueous microenvironment (Koga & Terao, 1995).

Cui et al. (2015) studied whether the physical location of α-TOC in an oil mixture consisting of 25% stripped soybean oil and 75% medium chain TAGs was affected by dioleoyl-PC (DOPC) and dioleoyl-PE (DOPE) reverse micelles. For the study, they used NBD-PE (N-(7-nitro-2-1,3-benzoxadiazol-4-yl-dioleoyl)-PE), a surface-active PL with a fluorophore group, expected to be located at the interfacial area. With DOPC concentration above the critical micellar concentration (1000 μM) and increasing α-TOC concentration, the emission signal from NBD-PE decreased, while with DOPE no significant change was observed. This indicated α-TOC accumulation in the DOPC reverse micelles but not in the DOPE ones. However, despite the α-TOC accumulation, DOPC (1000 μM) reverse micelles had a pro-oxidative effect, both with and without 100 μM α-TOC (Cui et al., 2015). Also, Chen et al. (2011) reported a pro-oxidative effect for DOPC (1000 μM) as such, and with 100 μM α-TOC in stripped soybean oil, while at lower α-TOC concentration (10 μM) antioxidative effect was observed. Also, DOPE reverse micelles were reported to be pro-oxidative as such (Cui et al., 2014, 2015) in an oil mixture consisting of 25 % stripped soybean oil and 75% medium chain TAGs.

Recently, Velasco et al. (2023) studied the effect of PC reverse micelles on the efficacy of α-TOC in stripped sunflower oil. The applied α-TOC concentration (1.16 mmol kg^{-1} or 1067 μM) was higher than in the above-

mentioned studies, resembling the concentration naturally found in vegetable oil. PC reverse micelles (0 - 5000 mg kg^{-1}) had no effect on α-TOC efficacy, or its partitioning in the oil. For Trolox (polar homolog of α-TOC), a decrease in the antioxidant efficacy was observed with increasing PC concentration, but the efficacy was still greater than that of α-TOC. It was postulated that the main oxidation reactions did not occur at the PC reverse micelles but rather in the reverse micelles formed by hydroperoxides and other oxidation products. As a more polar antioxidant, Trolox would partition more to the hydroperoxide reverse micelles than α-TOC in the absence of PC, and in the presence of PC, it would also partition to the PC reverse micelles, decreasing the antioxidant activity. It was also concluded that the previous studies with model systems consisting of blends of stripped vegetable oils with medium-chain TAG oils and significantly lower α-TOC than PC concentrations overstated the pro-oxidative effect of PC (Velasco et al., 2023). Also, Rokosik et al. (2020) reported no interactions between α-TOC (10–500 μmol kg^{-1}) and DOPC reverse micelles in stripped rapeseed oil. Overall, the pro- and antioxidative interactions of α-TOC and PLs/LOOHs in association colloids are currently not fully understood, and the outcomes can't be reliably predicted without examining each condition individually. The interactions are affected by various factors, including oil substrate, PL structure, PL and α-TOC concentrations, and other amphiphilic minor components and trace metals present in the oil.

2.3.4.3 Synergism with carbonyl-amine reaction products

Carbonyl-amine reaction products can also enhance the antioxidant activity of α-TOC and/or vice versa. Ahmad et al. (1998) reported the antioxidative activities of four model compounds representing pyrroles, dihydropyridines, Michael adducts, and pyridium salts together with BHT and α-TOC in soybean oil. With the applied total antioxidant concentrations of 50-200 ppm, an additive effect of the lipid-amine reaction product with BHT/α-TOC was observed in all cases. The detected synergistic efficiency range varied mainly between ± 20% (Ahmad et al., 1998). Some studies are also suggesting that α-TOC as such is needed for the antioxidative activity of carbonyl-amine reaction products. Shimajiri et al. (2013) studied the effect of PLs, sphingolipids, and d18:0 on the oxidation of fish oil TAGs at 37 °C. The antioxidative effect for these compounds was seen only in the presence of α-TOC (0.05%) and seemed to increase with increasing concentration of amine groups in the reaction mixture. Also, Uemura et al. (2016) and Suzuki-Iwashima et al. (2021) reported strong antioxidative effect for d18:0 and mixed milk sphingoid bases with α-TOC during soybean, linseed, and fish oil oxidation at 50 °C in the dark, while with the sphingoid bases alone negligible or mild antioxidative effect was observed.

Hidalgo et al. (2007) examined the antioxidative activity of PC, PE, and lysine in tocopherol-stripped olive oil and in olive oil with added α-TOC (250 μg/g oil). None of the tested amine compounds increased the induction period of tocopherol-stripped oil, but PE and lysine had an antioxidative effect in the oil with added α-TOC. The formation of carbonyl-amine reaction products was reported in both oils after the addition of PE or lysine. Additionally, two model compounds of the reaction products were synthesized, and their antioxidant activity was tested in both oils. In the oil with added α-TOC, the derivative from lysine, 6-amino-2-(1H-pyrrol-1-yl)hexanoic acid, protected the oil with similar efficiency as BHT, while in the tocopherol-stripped oil, no effect was observed. The pyrroles formed from amino acids are likely to be hydrophilic, while pyrroles formed from PE with free amino groups are likely to be lipophilic. This can affect their antioxidative efficacy through partitioning in the oil. On the whole, α-TOC seems to have a major contribution to the antioxidative activity of carbonyl-amine reaction products, although the mechanism behind the effect is not understood.

2.4 Oxidation pattern and stability of docosahexaenoic acid in oils

The low bond dissociation energies of the five bisallylic hydrogens make DHA highly susceptible to hydrogen abstraction by LOO• and other radicals, decreasing its oxidative stability. Cosgrove et al. (1987) measured the oxygen uptake of different PUFA esters in chlorobenzene at 37 °C and reported that the oxidative stability of PUFA is linearly dependent on the number of bisallylic positions in the molecule. Thus, DHA was five times more prone to oxidation than linoleate. Similar results were reported for PUFA esters and free acids by other researchers in neat oil (Cho et al., 1987a; Holman & Elmer, 1947; Miyashita & Takagi, 1986). PUFA as free acids were more prone to oxidation than the esters, apparently due to the catalytic effect of the carboxyl group in the decomposition of LOOH and formation of free radicals (Holman & Elmer, 1947; Miyashita & Takagi, 1986). Although the increasing unsaturation decreased oxidative stability in bulk oil and organic solvent, the opposite was observed in aqueous micelles and liposomes. A possible cause for the increased stability could be the tight packing of DHA molecules and the presence of water near the double bonds, which could prevent hydrogen abstraction from the bisallylic positions (Hirano et al., 1997; Miyashita et al., 1993; Miyashita, 2014).

The number of bisallylic sites available for hydrogen abstraction in the DHA acyl chain is high, and the bent structure brings hydrogens and double bonds from opposite ends to proximity with each other. These characteristics influence the oxidation pattern of DHA in comparison to other FAs. Curved acyl chain

facilitates LOO• additions to double bonds within the same chain and the formation of cyclization products. These structures include endoperoxides, polyperoxides, bicycloperoxides, and prostaglandin-like structures formed through further reactions of endoperoxides and 6-oxo exocyclic peroxides (Funk et al., 1975; Hamberg & Samuelsson, 1966; N. A. Porter, 1986; Schaich, 2020). Some of the possible structures formed through the initial cyclization of LOO• are illustrated in **Figure 9** for arachidonic acid (FA 20:4), and reaction scheme for 6-oxo exocyclic peroxide formation is presented in **Figure 10**.

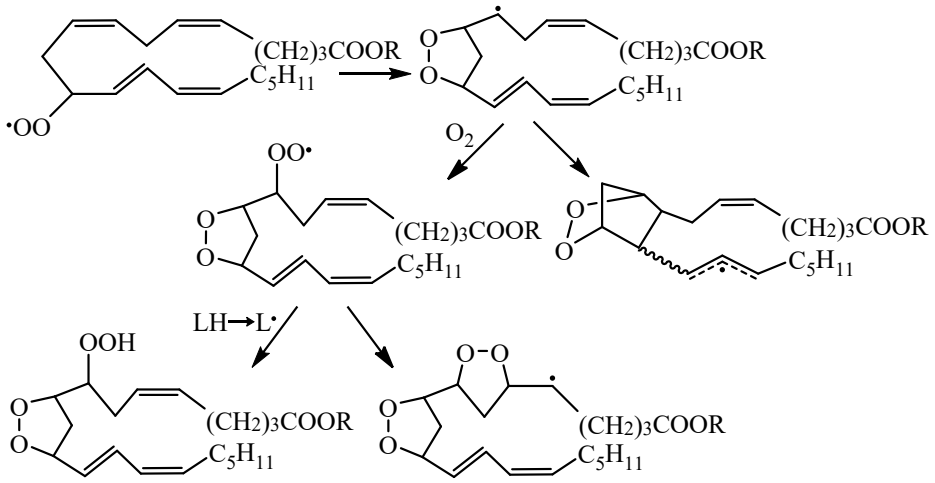


Figure 9. Arachidonic acid (FA 20:4) peroxyl radical cyclization products (adapted from Porter, 1986).

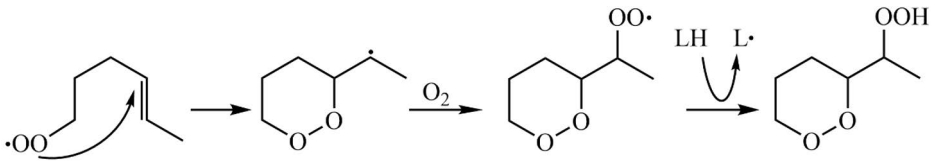


Figure 10. Formation 6-oxo exocyclic peroxide (adapted from Funk et al., 1975).

In the presence of α-TOC, hydrogen is provided to LOO• before cyclization occurs, and the reaction is directed to the formation of hydroperoxides instead of cyclic structures. Due to cyclization, autoxidation products of FAs with three or more double bonds contain less *trans*, *trans*-substituted conjugated dienes than diene FA oxidation products. This is because LOO• β-scission (loss of O_2) occurs at a slower rate than cyclization (140 s^{-1} vs. 800 s^{-1}) (N. A. Porter, 1986). In the cyclization reactions, two moles of oxygen are absorbed per FA, but only one chain-propagating radical is formed. LOO• additions to intermolecular double bonds can lead to dimerization (C-O-O-C) and polymerization especially in neat

oil where LOO• are in proximity with double bonds from neighboring acyl chains (Gardner, 1989).

The bent structure also facilitates intramolecular H abstractions by LO•, but on the other hand, the high amount of double bonds should increase the proportion of LO• scissions due to increased double bond charge polarization (Gardner, 1989; Schaich, 2020). LO• additions to intermolecular double bonds can lead to the formation of ether-linked dimers (C-O-C) also in moderate temperatures (Gardner, 1989). Overall, the oxidation of FAs with more than three double bonds is very complicated, and the formation of mono- and dicyclic peroxides and epoxy alcohols present as several stereoisomers results in a variety of hundreds of different oxidation products (N. A. Porter et al., 1995).

Studies on the autoxidation of pure DHA are scarce, and the postulated reaction mechanisms described above are mainly based on the studies conducted with other FAs, e.g., linoleic, ALA, or arachidonic acids in free form or as methyl/ethyl esters. Reports on the autoxidation products of pure DHA in different lipid structures are summarized in **Table 3**. The results indicated low stability of DHA monohydroperoxides and increased formation of polyhydroperoxides and cyclic structures already at the early stages of oxidation (Cho et al., 1987a; Lyberg et al., 2005). As expected, the instability of monohydroperoxides increased along with increasing temperature (Lyberg & Adlerceutz, 2006). For DHA-EE, also the formation of dimers and polymers was significant, comprising 70% of the total polar material after oxidation at 5 °C in the dark (Cho et al., 1987a), and polymerization was visually observed also for free DHA (Lyberg et al., 2005; Lyberg & Adlerceutz, 2006). The levels of monohydroperoxide isomers formed at the double bonds furthest and closest to the carboxyl group (C20, C4) in free DHA were higher compared to the middle positions (Lyberg et al., 2005; Lyberg & Adlerceutz, 2006). Such effect, however, was not observed for DHA-TAG, and in the case of DHA-PC and -PE, only carbons in the middle- and end positions of the chain (C20, C16+C17, C13+C14, C10) formed monohydroperoxides (Lyberg et al., 2005).

Table 3. Studies on the autoxidation products of pure DHA in different lipid structures when oxidized as neat oil.

DHA form	Oxidation	Analyses	Results	Reference
DHA-methyl ester	50 °C in the dark	High vacuum distillation & GC-MS	• 42 Volatile oxidation products identified: aldehydes, aldehyde esters, hydrocarbons, methyl esters, keto-esters, unsaturated ketones, cyclic esters, furan derivatives • Formation mechanisms suggested	Noble & Nawar, 1971, 1975
DHA-ethyl ester	5 °C in the dark	O_2 absorption, conjugated dienes, PV, TLC, HP-GPC	• Hydroperoxide oxygen/total absorbed oxygen levels lower for DHA than for α-linolenic acid and decreased rapidly → DHA hydroperoxides less stable • 70% of the formed polar material dimers and polymers	Cho et al., 1987a
DHA-FA, DHA-PC, DHA-PE, DHA-TAG	25–30 °C in the dark	CV, PV, mono- and polyhydroperoxides, and cyclic structures by HPLC-UV	• Monohydroperoxides increased most rapidly in DHA-FA (8 mol%, day 1). DHA-PC, -PE, and -TAG produced low amounts of monohydroperoxides (0.3-0.7 mol%) • DHA-TAG oxidized favorably to polyhydroperoxides/cyclic structures (14 mol%, day 3) • In DHA-TAG, LOOH isomers were formed in fairly equal amounts (~7-13%, except for C8, 18%), while in DHA-FA C20 (25%) and C4 (14%) isomers were most abundant, and in PL only carbons 20, 16+17, 13+14, and 10 were oxidized to LOOH	Lyberg et al., 2005
DHA-FA	6, 25, and 40 °C, shielded from UV light	Mono- and polyhydroxides/cyclic structures by HPLC-UV, LOOH isomers by LC-QTOF	• Double bonds closest (C4) and furthest (C20) from the carboxyl group were most prone to LOOH formation (likely because C17, 16, 14, 13, 11, 10, 8, and 7-peroxy radicals could react to form cyclic structures instead)	Lyberg & Adlerceutz, 2006
DHA-FA	25 °C with Cu (II) in a light box	GC-O, GC-MS, NMR	• Important aroma compounds (flavor dilution factor > 64) from DHA oxidation identified (16 identified oxidation products)	Hammer & Schieberle, 2013
DHA-FA	110 °C in an oil bath	HS-SPME-GC-MS	• 4-Heptenal (*Z*)- and 2,4-heptadienal (*E*,*E*)- major volatiles from thermal degradation of DHA (18 identified oxidation products)	Peinado et al., 2016

Abbreviations: GC-MS (gas chromatography-mass spectrometry), PV (peroxide value), TLC (thin layer chromatography), HP-GPC (high-performance gel-permeation chromatography), FA (fatty acid), PC (phosphatidylcholine), PE (phosphatidylethanolamine), TAG (triacylglycerol), CV (carbonyl value), HPLC-UV (high-performance liquid chromatography-UV detection), LOOH (lipid hydroperoxide), LC-QTOF (liquid chromatography quadrupole time-of-flight mass spectrometry), GC-O (gas chromatography-olfactometry), NMR (nuclear magnetic resonance spectroscopy), HS-SPME-GC-MS (headspace solid phase microextraction-gas chromatography-mass spectrometry)

Noble & Nawar (1971, 1975) identified volatile oxidation products after DHA methyl ester autoxidation at 50 °C, including aldehydes, aldehyde esters, hydrocarbons, methyl esters, keto-esters, unsaturated ketones, cyclic esters, and furan derivatives. Formation mechanisms were suggested and included both direct scissions on either side of the hydroperoxide and mechanisms related to the scissions of cyclic structures and further oxidations of cleavage products. For example, methyl ketones (e.g., 3,5-octadienone) and keto-esters were suggested to form through cleavage of hydroperoxy epidioxides, and cyclic esters through intramolecular addition to a double bond followed by cyclization to form a six-membered ring. Also, the oxidation products formed from the methyl ester end were included in the reported aldehyde-, keto-, cyclic-, and alkane/alkene methyl esters (Noble & Nawar, 1971, 1975).

Hammer & Schieberle (2013) examined the volatile oxidation products from DHA autoxidation at 25 °C and identified the major odor-active compounds by gas chromatography (GC)-olfactometry and aroma extract dilution analysis (AEDA). The highest flavor dilution factors were reported for *trans*-4,5-epoxy-(*E*,*Z*)-2,7-decadienal, (*Z*)-1,5-octadien-3-one, (*Z*)-3-hexenal and (*Z*,*Z*)-3,6-nonadienal. Peinado et al. (2016) reported 2,4-heptadienal (*E*,*E*)- and 4-heptenal (*Z*)- as the most abundant volatile oxidation products from the thermal degradation (110 °C) of DHA-FA. Also, hexanoic acid and octanal levels increased substantially during the first 15-30 min of oxidation. The volatile oxidation products of DHA (DHA-FA and DHA-methyl ester) identified in the above-mentioned studies are reported in **Table 4.**

Table 4. Identified volatile oxidation products from pure DHA-FA and DHA methyl ester.

Aldehydes	Ketones	Alkenes	Acids	Alcohols	Furans	Esters
propanal[1]	1-penten-3-one[2,3]	*n*-octadiene[1]	acetic acid[3]	1-penten-3-ol[3]	2-pentylfuran[1]	3-formyl methyl propanoate (C4:0)[1]
hexanal[1,3]	(*Z*)-1,5-octadien-3-one[2]		propanoic acid[3]	(*E*)-2-penten-1-ol[3]	2-butylfuran[1]	6-formyl methyl 4-hexenoate (C7:1)[1]
3-hexenal[1,2]	3,5-octadien-2-one[1]		butanoic acid[2]	(*E*)-3-hexen- 1-ol[3]	2-pentenyl furan[1]	5-formyl methyl 4-pentenoate (C6:1)[1]
heptanal[3]	undecatrien-2-one[1]		pentanoic acid[3]	1-octen-3-ol[3]		7-formyl methyl 4,6-heptadienoate (C8:2)[1]
(*Z*)-4-heptenal[2,3]			hexanoic acid[3]	4-hepten-1-ol[3]		9-formyl methyl nonadienoate (C10:2)[1]
(*E*,*E*) & (*E*,*Z*)-2,4-heptadienal[1,2,3]						12-formyl methyl dodecatrienoate (C13:3)[1]
octanal[3]						methyl 4- & 5-oxohexanoate[1]
2-octenal[3]						methyl 4-oxohexenoate[1]
(*Z*,*Z*)-2,5-octadienal[2]						methyl 8-oxononadienoate[1]
nonanal[3]						methyl 2-cyclohexenyl acetate[1]
(*E*,*Z*)-2,6-nonadienal[1,2]						methyl 5-cyclohexenyl pentenoate[1]
(*Z*,*Z*)-3,6-nonadienal[1,2]						methyl propanoate[1]
(*E*,*E*,*Z*)-2,4,6-nonatrienal[2]						methyl 4-hexenoate[1]
2,4-decadienal[1]						methyl nonadienoate[1]
(*E*)-4,5-epoxy-(*E*,*Z*)-2,7-decadienal[2]						methyl dodecatrienoate[1]
(*E*,*E*,*Z*)- & (*E*,*E*,*E*)-2,4,7-decatrienal[2]						methyl pentadecatetraenoate[1]
3,6,9-dodecatrienal[1]						methyl hexadecatetraenoate[1]
3,6,9,12-pentadecatetraenal[1]						butyl butyrate[3]

[1)] Noble & Nawar, 1971, 1975 [2)] Hammer & Schieberle, 2013 [3)] Peinado et al., 2016

2.5 Effect of lipid structure on the oxidation of docosahexaenoic acid

2.5.1 Fatty acid *sn*-position in glycerol backbone

The possible effect of FA *sn*-position in glycerol backbone on oxidative stability has been mainly studied by three different approaches: by comparing stabilities of randomized and non-randomized natural oils or pure TAGs, by comparing the regioisomeric distribution of primary oxidation products, or by comparing stabilities of synthesized regiopure TAGs. Especially in the case of natural oils, randomization studies have resulted in contradictory results (Kimoto et al., 1994; Lau et al., 1982; D. K. Park et al., 1983a). Discrepancies could be due to varying FA distributions (*sn*-2 vs. *sn*-1/3) and total PUFA amounts in studied oils before randomization (Wada & Koizumi, 1986), loss of antioxidants or introduction of pro-oxidants during the interesterification process (Ki Park et al., 1983; Wijesundera, 2008), or differences in the levels of non-TAG components (Ledóchowska & Wilczyńska, 1998). Overall, the research in this area has received little attention over the past two decades.

Studies on the oxidative stability of randomized pure TAGs and regiopure TAGs are reported in **Table 5**. In all reported studies the substrates were purified prior to oxidation and/or the initial PVs were reported. Randomization studies of pure saturated and unsaturated TAGs showed superior stability for randomized oil due to the decreased amount of unsaturated FAs in a single TAG (Raghuveer & Hammond, 1967; Wada & Koizumi, 1983). However, Park et al. (1983) reported equal stability for randomized and non-randomized TAG mixtures. Also, investigations with regiopure synthesized TAGs revealed that oxidative stability increases along with the decreasing molar amounts of EPA or DHA in a single TAG (Endo et al., 1997a, 1997b).

Table 5. Studies on the effect of TAG structure on the oxidative stability of synthesized, randomized pure TAGs and regiopure TAGs.

Oxidation trial & analyses	Oxidative stability	Conclusions	Reference
PV during air incubation at 37 °C	• Randomized LLL/LnLnLn/CaCaCa (1.5:0.5:97) and LLL/CaCaCa (1.5:98.5) more stable than non-randomized	• Proximity of more than one unsaturated FA on a TAG decreased stability	Raghuveer & Hammond, 1967
PV during incubation at 37 °C in the dark & dissolved O_2 during incubation with linoleic acid monohydroperoxide methyl ester	• PLnP = PPLn, PPL = PLP, PPO = POP • No difference in the stability of randomized and non-randomized LaLaLa and LLL TAGs	• Position of unsaturated FA had no effect on TAG stability	D. K. Park et al., 1983a
O_2 consumption during incubation at 50 °C, enzymatic method for *sn*-2 FA estimation	• Randomized TAGs of SSS/OOO (1:1), PPP/LLL (1:1), SSS/LLL (1:1) more stable than non-randomized. • PPL < PLP, SSL < SLS, SSO < SOS	• Randomization increased stability • Unsaturated FAs at *sn*-2 position more stable than those at *sn*-1/*sn*-3	Wada & Koizumi, 1983
Hydroperoxides after autoxidation at 40 °C by HPLC	• No difference in LLL *sn*-2 and *sn*-1/3 monohydroperoxide concentrations	• Oxidation of trilinoleoylglycerol not selective toward *sn*-1(3)- or *sn*-2 positions	Neff et al., 1990
Non-volatile oxidation products and PV after bubbling pure O_2 at 40 °C	• LLnL < LLLn • LnLnL < LnLLn	• Interactions between Ln and L higher than between two adjacent L • Interactions between two Ln higher than between adjacent Ln and L	Miyashita et al., 1990
Volatile oxidation products and PV after autoxidation at 40 °C	• LLnL = LLLn • LnLnL < LnLLn	• Interactions between two Ln higher than between Ln and L in adjacent *sn*-positions	Frankel et al., 1992

Oxidation trial & analyses	Oxidative stability	Conclusions	Reference
O_2 consumption during oxidation at 25 °C with 0.1% α-TOC.	• EEE/PPP (2:1) < EEP = EPE • EEE/PPP (1:2) < EEP/PPP 1:1 < EPP < PEP	• Stability inversely correlated with the moles of EPA in a single TAG • EPA *sn*-position 1,2 or 2,3 vs. 1,3 had no effect on stability • EPA at *sn*-2 position most stable (*sn*-1/3 vs. 2)	Endo et al., 1997a
Oxidation with oil- or water-soluble radical initiator in benzene or phosphate buffer at 37 °C. O_2 consumption monitored.	• EEE/PPP (2:1) < EEP < EPE • DDD/PPP (2:1) < DDP < DPD • EEE/PPP (1:2) < EEP/PPP (1:1) < EPP = PEP	• Stability inversely correlated with the moles of EPA in a single TAG • EPA/DHA at *sn*-1,3 more stable than at *sn*-1,2 or 2,3 • EPA *sn*-position (1/3 vs. 2) had no effect	Endo et al., 1997c
PV, DHA depletion, and volatile oxidation products during oxidation at 40 or 50 °C in the dark.	• PPD < PDP • OOD < ODO	• DHA at *sn*-2 position more stable than at *sn*-1/3	Wijesundera et al., 2008
Volatile oxidation products of TAG emulsions oxidized at 50 °C in the dark.	• SSD < SDS	• DHA at *sn*-2 position more stable than at *sn*-1/3	Shen & Wijesundera, 2009

Abbreviations: PV (peroxide value), L (linoleic acid 18:2), Ln (linolenic acid 18:3), Ca (caproic acid 6:0), FA (fatty acid), TAG (triacylglycerol), P (palmitic acid 16:0), O (oleic acid 18:1), La (lauric acid 12:0), S (stearic acid 18:0), HPLC (high-performance liquid chromatography), α-TOC (α-tocopherol), E (eicosapentaenoic acid), D (docosahexaenoic acid)

Research data on the regioisomeric oxidative selectivity of TAGs containing three unsaturated FAs is limited. Neff et al. (1990) examined the monohydroperoxide isomers of oxidized trilinoleoylglycerol and found no difference in the *sn*-1/3 and *sn*-2 monohydroperoxide concentrations. Also, Miyashita et al. (1990) reported no differences in the cyclization of internal LOO• of the Ln (linolenic acid) component in Ln and L (linoleic acid) containing TAGs at *sn*-1/3 and *sn*-2 positions. However, differences in other oxidation indicators were found. Two adjacent Ln (LnLnL) TAGs were more prone to oxidation at 40 °C than TAGs, where they were separated by L (LnLLn). Decreased stability might be due to interactions between the two adjacent Ln residues (Frankel et al., 1992; Miyashita et al., 1990). In the case of TAGs with two L, LLnL oxidized faster than LLLn, which could relate to higher oxidative interaction between Ln and L than between two L (Miyashita et al., 1990). Similarly, increased stability was reported for EPA/DHA at *sn*-1 and *sn*-3 positions compared to *sn*-2 and *sn*-1/3 in TAGs together with one palmitic acid (Endo et al., 1997c).

When considering TAGs with one unsaturated and two saturated FAs, superior stability was reported for *sn*-2 position when compared to *sn*-1 or *sn*-3 (Endo et al., 1997a; Shen & Wijesundera, 2009; Wada & Koizumi, 1983; Wijesundera et al., 2008). No differences were reported in the detected volatile and non-volatile oxidation products of DHA at *sn*-2 vs. *sn*-1/3 positions (Wijesundera et al., 2008). Most studies suggest that the chain length of saturated FA components has little or no effect on oxidative stability (Endo et al., 1997a, 1997c; Wada & Koizumi, 1983), while Park et al. (1983b) stated that shorter chain FAs improve stability. In the case of PLs, no significant difference was observed in the oxidative stability of PE and PC with DHA at *sn*-1 or *sn*-2 position together with palmitic acid (Lyberg et al., 2005).

There is no clear explanation for the described stability differences. An early theory from Raghuveer & Hammond (1967) suggested that the hexagonal packing of TAG acyl chains in a molten state (*ortho* array of the tuning-fork configuration) enables interaction between the *sn*-1 and *sn*-3 acyl chains of the same TAG, while the *sn*-2 acyl chain would be surrounded by six *sn*-1/3 acyl chains. Thus, in the case of saturated FAs at *sn*-1/3 positions, the unsaturated FA at the *sn*-2 position would be surrounded barely by saturated FAs, and a very low autoxidation rate would be expected. The theory assumed that the FA chain length and double bond number would not cause any distortion to the array. Still, to date, the ordering of TAGs in the molten state is not clearly understood. In melt TAGs aggregate to form ordered structures, for which smectic, nematic, and discotic arrangements have been proposed (Iwahashi & Kasahara, 2011; Mykhaylyk & Martin, 2009; Sadeghpour et al., 2018). The formed structures are affected by the FA chain length and unsaturation level of the TAGs involved.

Endo et al. (1997a) detected dihydroperoxides only in EPA/palmitic acid TAGs that contained more than one EPA (EEP and EEE), while monohydroperoxides and monohydroperoxy epidioxides were detected in all sample types. Authors postulated that free radical chain reaction proceeded from one EPA to the adjacent one within the same TAG, which could explain the low stabilities of TAGs with adjacent unsaturated FAs.

2.5.2 Triacylglycerols, ethyl esters and phospholipids

Based on prior research involving model compounds (Lyberg et al., 2005; Song et al., 1997) and comparisons between fish oil and krill oil or other marine PLs (Moriya et al., 2007; Mozuraityte et al., 2017; Thomsen et al., 2013), the incorporation of DHA into PLs provides better protection against oxidation compared to TAG or EE. The improved stability could be caused by the previously discussed antioxidant activities of PLs via metal-chelation, tocopherol regeneration, antioxidative carbonyl-amine reaction product formation, or antioxidant location alteration (oil/association colloid). In the case of marine PL, the presence of astaxanthin, coenzyme Q10, lutein, cholesterol, and residue amino acids can also influence the improved stability (Lu et al., 2017; Moriya et al., 2007).

Concerning the stability studies comparing TAG and EE structures contradictory findings have been reported (**Table 6**). The discrepancies could be due to varying FA and antioxidant concentrations in the compared oils, as well as differences in their initial oxidative statuses. Song et al. (1997) studied the oxidative stability of DHA containing PL (PC/PE ratio 3:1), TAG, and EE oils prepared from egg yolk lipids of laying hens fed on a fish oil-supplemented diet. Examined oils contained 10.7 mol% DHA and almost the same constituent FAs. The oils were incubated in the dark at 25 °C for ten weeks, and oxygen absorption, tocopherol and substrate depletion, and carbonyl- and peroxide values were analyzed. In the TAG oil, the oxygen uptake was clearly the most significant, followed by EE and PL oils. In the TAG oil, also the DHA content decreased most during the incubation period. After ten weeks, only 2,8% of the initial DHA was present in the TAG oil, while in the EE and PL oils, 32,7% and 89,7% of DHA, respectively, could be found. At the beginning of the trial, total tocopherol content was highest in TAG oil (35.1 mg/100 g), followed by EE (18.9 mg/100 g) and PL (6.7 mg/100 g) oils. After the oxidation period, only PL oil had some tocopherol left (Song et al., 1997).

Table 6. Studies on the stability differences between TAG and EE structures. Tocopherol contents is given as mg/g for all studies.

Compared oils & oxidation trial	Oxidation trial & analyses	Oxidative stability	Reference
• 10.7 mol% DHA-containing TAG, EE, and PL oils with almost similar FA composition • Tocopherol content (mg/g): PL 0.067, EE 0.189, TAG 0.351	Substrate and tocopherol depletion, oxygen uptake, PV, and carbonyl value after oxidation at 25 °C in the dark	• PL > EE > TAG	Song et al., 1997
• TAG (66.0% EPA+DHA) and EE (65.8% EPA+DHA) fish oils • Tocopherol content (mg/g): TAG 2.67, EE 2.04	PV and PAV value after oxidation in open vials at 5, 15, 30, 45 and 60 °C in the dark	• TAG > EE	Sullivan Ritter et al., 2015
• TAG (60% DHA/20% EPA) and EE (80% DHA/10% EPA) fish oil concentrates • Initial PV (mEq/kg): EE 11.8, TAG 4.0 • α-TOC + rosemary extract (1:1): 0.5 mg/g (both oils)	Rancimat 50-90 °C, PV, and PAV after oxidation at RT in the dark	• Rancimat: no difference • Storage conditions: TAG > EE (with and without AO)	Martín et al., 2012
• TAG (45.1% DHA, 7.2% EPA) and EE (65.7% DHA, 7.4% EPA) fish oils • AO content not analyzed/reported • Initial total volatile oxidation product level higher in EE fish oil than in TAG fish oil	Substrate depletion and SPME-GC-MS after storage at 80 °C with aeration	• TAG > EE	Lee et al., 2003
• DHA-EE (92% purity) and TAG oil (47% DHA, 5% EPA) • AO content and initial oxidative status not analyzed/reported	Oxygen uptake at 35, 50, and 70 °C	• TAG > EE	Yoshii et al., 2002

Abbreviations: DHA (docosahexaenoic acid), TAG (triacylglycerol), EE (ethyl ester), PL (phospholipid), PV (peroxide value), EPA (eicosapentaenoic acid), PAV (para-anisidine value), α-TOC (α-tocopherol), RT (room temperature), AO (antioxidant), SPME-GC-MS (solid phase microextraction-gas chromatography-mass spectrometry)

In contrast to the findings of Song et al. (1997), most of the studies indicate enhanced stability for TAG compared to EE. For example, Sullivan Ritter et al. (2015) compared the stability of molecularly distilled, steam-deodorized TAG and EE fish oils (anchovy and sardine oils from Peru) containing DHA+EPA about 65% of total FAs and a blend of mixed natural tocopherols. Concentrations of EPA and DHA in the two oils were nearly identical, for DHA 225 and 219 mg/g and for EPA 308 and 308 mg/g. Oil samples were incubated in the dark at 5 °C (21 days), 15 °C (9 days), 30 °C (4 days), 45 °C (4 days) and 60 °C (4 days). In all temperatures, EE oil oxidized more rapidly than the TAG oil. The smaller size of EE molecules, which leads to more molecules per oil volume, lower viscosity, and more collisions between molecules, was postulated as a possible reason for the higher oxidation rate of EE oil. The result may have been affected by the higher α, γ and δ-tocopherol levels in the TAG oil, 2.67 ± 57 mg/g, compared to 2.04 ± 53 mg/g in the EE oil.

Martín et al. (2012) used commercial EE fish oil concentrate (80% DHA, 10% EPA) and TAG fish oil concentrate (60% DHA, 20% EPA) obtained by enzymatic interesterification of the commercial EE concentrate for analyzing the oxidative stability at Rancimat conditions (50–90 °C) and during storage at room temperature in the dark. In the Rancimat conditions, the EE oil showed slightly longer induction times, although no statistical significance was reached. During the storage at room temperature, the TAG samples deteriorated slower than the EE samples, both without antioxidants and with 500 mg/kg added rosemary extract and α-TOC. The results may have been impacted by the higher peroxide value of the EE concentrate compared to TAG at the beginning of the trial (11,8 vs. 4,0 meq/kg). Also, the higher total concentration of DHA and EPA in the EE sample may have affected the obtained results.

Lee et al. (2003) examined the volatile oxidation products of TAG and EE fish oils during a 10-day incubation with aeriation at 80 °C. The initial DHA and EPA contents in the TAG oil were 45.1% and 7.2%, respectively. The EE oil contained 65.7% DHA and 7.35 % EPA. According to the SPME analysis data (total volatile compounds), the EE oil was more oxidized already at the beginning of the trial. During the 10-day incubation, the relative amount of DHA decreased by 35.9 % in the TAG oil and 70.5 % in the EE oil, indicating better stability for the TAG oil. In the EE oil, the intensity and number of volatile compounds were higher, 31 compounds vs. 23 compounds in the TAG oil. Authors suggested that the high level of ethanol in the EE oil, even without accelerated storage, could be a sign of the ester bond breakage, which could have led to the formation of ethanol and free FAs. Free FAs are pro-oxidative (Kittipongpittaya et al., 2014; Mistry & Min, 1987) and could be possible cause for the inferior stability of the EE oil. Also, in this study, the different DHA concentrations in the examined oils limit the possibility of drawing conclusions

based on the results. Furthermore, the antioxidant content was not analyzed/reported and the initial oxidative status of the compared oils differed. Similar weakness is related to the study by Yoshii et al. (2002). Most of the studies indicate improved stability for the TAG structure. However, drawing conclusions is challenging due to variations in the antioxidant content (either unanalyzed or differing), FA content, or initial oxidative status of the compared oils.

3 AIMS OF THE STUDY

The main aim of this study was to examine the effect of lipid structure and selected antioxidants on the oxidation of DHA in bulk oils by using formulated pure model substrates. Dihyrosphingosine was chosen for antioxidative evaluation in study **III** based on the potent protective effect it had demonstrated combined with α-tocopherol in fish oils, as well because its' antioxidant activity is still relatively unexplored (Shimajiri et al., 2013; Suzuki-Iwashima et al., 2021; Uemura et al., 2016). The focus was on elucidating the differences in oxidative stability, oxidation pattern, and antioxidant response.

I. Obtain a comprehensive view of the differences in the oxidation pattern, oxidative stability, and α-TOC response between DHA in TAGs and EEs by using an omics-type, untargeted analytical approach.

II. Compare the oxidative stability and oxidation pattern of DHA in regio- and enantiopure TAGs at positions *sn*-1, *sn*-2, and *sn*-3, and examine the possible interaction between DHA in enantiopure TAGs at *sn*-1 and *sn*-3 positions with enantiopure *RRR*-α-tocopherol.

III. Examine the effect of dihydrosphingosine and α-tocopherol, combined or one at a time, on the oxidation pattern of DHA-TAG, and identify the antioxidative carbonyl-amine reaction products and the carbonyls taking part in the reaction.

4 MATERIALS AND METHODS

4.1 Sample materials and oxidation trial setup

Pure tridocosahexaenoin > 99% and ethyl docosahexanoate > 99% for studies **I** and **III** were purchased from Larodan (Solna, Sweden). Regio- and enantiopure lipids for study **II** were synthesized from pure palmitic acid and DHA (Larodan, Solna, Sweden) at the University of Iceland Science Institute. ABA-type DHA-TAGs were synthesized via a two-step chemoenzymatic approach, utilizing highly *sn*-1,3 regioselective immobilized *Candida antarctica* lipase (CAL-B from Novozymes, Denmark) for incorporating pure palmitic acid to *sn*-1 and -3 positions. DHA was incorporated into the *sn*-2 position using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) as a coupling agent.

Enantiopure asymmetrically structured AAB-type [(*S*)-AAB and (*R*)-AAB] TAGs were synthesized with a five-step chemoenzymatic route using enantiopure *R*- and *S*-solketals (2,2-dimethyl-1,3-dioxolane-4-methanol) as chiral precursors. The *sn*-1/3 position was first protected with benzyl moiety, and palmitic acids were introduced to the benzylated glycerol adduct enzymatically with CAL-B. Lastly, the protective benzyl group was removed, and DHA incorporated into the terminal *sn*-1/3 position using EDCI. The chemical and regioisomeric purity and oxidative quality of all synthesized lipids for study **II** were verified by ^{1}H and ^{13}C NMR, infrared (IR) spectroscopy, and accurate mass spectrometry (HRMS) analyses. Enantiomeric purity was established by chiral-phase recycling high-performance liquid chromatography (HPLC). Details for both synthesis methods are reported elsewhere (Halldorsson et al., 2003; Kalpio et al., 2020; Kristinsson et al., 2014; Kristinsson & Haraldsson, 2008).

In studies **I** and **III**, α-tocopherol > 96% from Sigma-Aldrich (Steinheim, Germany) was utilized as the antioxidant, while in study **II,** a natural enantiopure *RRR*-α-tocopherol (Sigma–Aldrich, Buchs, Switzerland) was employed. Dihydrosphingosine (d18:0) > 98% for study **III** came from Larodan (Solna, Sweden). Oxidation trial setup was similar in all three studies (**I-III**). Pure oil was solvated in hexane and divided into amber 10 mL SPME vials. Each vial contained 20 mg oil, except for study **I,** where the molar amount of DHA was equalized between the TAG and EE samples (EE/TAG molar ratio 3/1; 20.00 mg /19.14 mg). In study **I**, samples were supplemented with 0.2% (w/w) α-TOC, and in study **II**, 0.14% (w/w) *RRR*-α-TOC was added. In study **III**, 0.05% α-TOC and 1% (w/w) d18:0 were used as antioxidants. In study **I**, the required amount of α-TOC was first added to a volumetric flask in ethanol, and the solvent evaporated before the addition of oil in hexane. In study **II**, *RRR*-α-TOC in hexane was added directly to SPME vials, and in study **III**, antioxidants were added in ethanol. An overview of the samples and conducted analyses in studies **I-III** are presented in **Figure 11**.

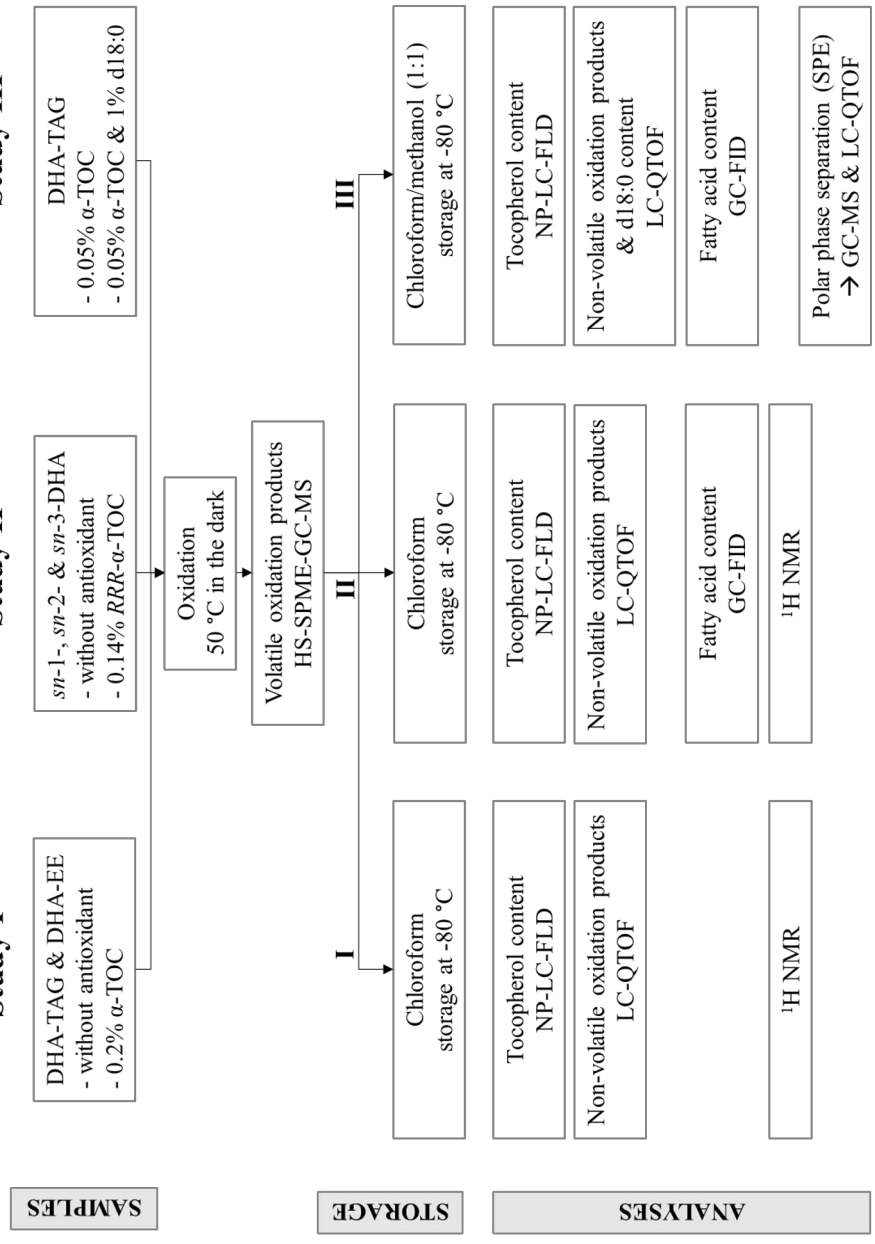


Figure 11. Overview of the analytical setup in studies **I-III**.

Samples were stored at -80 °C until oxidized. To initiate oxidation, hexane was removed from the samples under nitrogen flow, and the headspace was filled with compressed air. Samples were oxidized at 50 in °C the dark in a Hewlett Packard 6890 Series Plus G1530A GC oven (Wilmington, DE, USA), except for the non-antioxidant containing samples in study **I**, which were oxidized in GC-MS autosampler Tray Cooler MC 03–03 Rev. A (PAL System, CTC Analytics AG, Zwingen, Switzerland). Right after oxidation, the samples were analyzed with headspace solid phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) at different oxidation time points. SPME incubation and extraction times were included in the total oxidation time.

Times points for oxidation were chosen following preliminary tests using HS-SPME-GC-MS. Additionally, the oxidative quality of the pure compounds received was confirmed using the same method prior to sample preparation. Three replicates were prepared for each time point. Immediately after GC injection, the vial was cooled and 1 mL chloroform (studies **I** and **II**) or 2 mL chloroform/methanol 1:1 (study **III**) was added. In studies **I** and **II**, the vial was thereafter cooled at -20 °C, the headspace gently filled with nitrogen, and the sample stored at -80 °C for further analysis. In study **III,** 0.8 mL of the sample was filtered with a 0.2 µm PTFE syringe filter (VWR International, Radnor, PA, USA) and readily divided into autosampler vials for further analysis. From the unfiltered part (1.2 mL), the polar phase was later separated with solid phase extraction (SPE). The vials were subsequently stored at -80 °C. All sample preparation and handling were done in dim light conditions, when feasible, to protect samples from oxidation.

4.2 α-Tocopherol concentration

In all three studies (**I-III**), α-TOC concentration was analyzed by normal-phase method with Shimadzu Nexera XR LC-30 HPLC instrument and RF-20A prominence fluorescence detector (Shimadzu, Kyoto, Japan). Excitation and emission wavelengths were 292 and 325 nm, respectively. In studies **I** and **II** an HPLC method with Phenomenex OOG-4162-EO Luna 3 µm silica column (250 × 4.6 mm, pore size 100 Å; Torrance, CA, USA) was used, while for study **III** an improved UHPLC (ultra high-performance liquid chromatography) method (Aitta et al., 2023) with a Restek Pinnacle DB Silica UHPLC column (100 × 2.1 mm, 1.9 µm, Bellefonte, PA, USA) was applied. Separation was done at 30 °C by isocratic elution with heptane/1,4-dioxane 97:3 (**I**, **II**) 98:2 (**III**) mobile phases. Quantification was performed using a standard curve prepared from an α-TOC stock solution. Stock solution concentration was determined spectrophotometrically (Podda et al., 1996).

4.3 Fatty acid content

In studies **II** and **III**, FA content was analyzed by GC coupled with a flame ionization detector (FID) by using a Shimadzu GC-2030 instrument, an AOC-20i autoinjector, and a flame ionization detector (Shimadzu Corporation, Kyoto, Japan). A DB-23 column (60m × 0.25mm, 0.25 μm) from Agilent Technologies (Santa Clara, CA, USA) was used for separation. For the analysis, the FAs were converted to volatile methyl esters with an acetyl-chloride method described by Christie & Han (2010). External standards 37 Component FAME mix (Supelco, St. Louis, MO, USA) and 68D (NuCheck-Prep, Elysian, MN, USA) were used for FA identification, and quantification was done with an internal standard (triheptadecanoin from Larodan, Solna, Sweden).

4.4 Non-volatile oxidation products

Non-volatile oxidation products were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF) with an Elute UHPLC and Bruker Impact II QTOF instruments from Bruker Daltonic (Bremen, Germany). In studies **I** and **II**, an HPLC method was employed with Nucleodur C18 Isis (250 mm × 4.6 mm, 5 μm) column and solvent system consisting of acetonitrile/water and 2-propanol, both with formic acid and ammonium acetate. For study **III**, the method was developed to UHPLC with a Phenomenex (Torrance, CA, USA) Kinetex PS C18 column (100 × 2.1mm, 2.6 μm), and a solvent system consisting of water/methanol and 2-propanol/methanol, both with ammonium formate. With the UHPLC method, the running time was decreased from approximately 37 min in studies **I** and **II** to 17 min in study **III**. In all three studies (**I-III**), electrospray ionization was applied in positive mode. The capillary voltage was set to 4.5 kV, and the end plate offset to 500V. Nebulizer gas pressure, drying gas flow rate, and drying gas temperature were 1.5 bar, 4 L/min, and 350 °C, respectively. Auto MS/MS was used as a scanning mode, and internal calibration was performed with sodium formate. For d18:0 quantification in study **III**, a standard curve was prepared from the d18:0 stock solution. Otherwise, the results were semi-quantitative, based on the evolution of the integrated area data. Compound identification was done based on fragmentation patterns. In studies **I** and **II**, the data processing was mainly done with Bruker Compass DataAnalysis 5.1 (Bruker Daltonic GmbH, Bremen, Germany) and Lipid Maps database (Sud et al., 2007). In study **III**, also MS-DIAL ver.4.92 software (Tsugawa et al., 2015) was applied to detect the oxidation products with a low signal-to-noise ratio. In certain instances, when the intensities obtained from auto MS/MS scans were insufficient for

fragmentation and identification, a multiple reaction monitoring analysis (MRM) was conducted (studies **I**-**III**).

4.5 Volatile secondary oxidation products

Volatile oxidation products were analyzed with a HS-SPME injector and Thermo Scientific GC-MS instrument consisting of a Trace 1310 GC, ISQ 7000 mass spectrometer, and TriPlus RSH autosampler (Waltham, MA, USA) with a divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm fiber (Supelco, Bellefonte, PA, USA). In studies **I** and **II**, a semipolar SPB®-624 capillary column (60 m × 0.25 mm, 1.4 µm, Supelco, Bellefonte, PA, USA) was applied, while in study **III**, a polar DB WAX column (60m × 0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara, CA, USA) was used. Sample incubation (1 min) and extraction (30 min) was done at 50 °C in all three studies (**I**-**III**). Fiber desorption time in GC-port (240 °C) was 10 min in study **II** and 5 min in studies **I** and **III**. Electron ionization (EI) at 240 °C and 70 eV was applied for the MS, and mass-to-charge ratios were scanned between 40 and 300 amu. Acquired data were processed by the Chromeleon 7.2.9 Chromatography Data System (Thermo Fisher Scientific, Waltham, MA). In studies **I** and **II**, compound identification was based on external standards and the NIST MS Search library (version 2.4, National Institute of Standards and Technology, Gaithersburg, MD, USA). In Study **III**, it was also possible to utilize the retention indices available for the polar DB WAX column. Quantification was semi-quantitative in all three studies (**I**-**III**) based on the integrated area data.

4.6 Oxidation products from the polar fraction

In study **III**, the polar fraction of the oxidized samples was separated with SPE to concentrate the fraction containing carbonyl-amine reaction products. Only samples from selected time points were extracted and analyzed. SPE with International Sorbent Technology (IST) (Hengoed, Mid Glamorgan, UK) Isolute C18 (EC) 100 mg (1 mL) extraction columns with water/methanol (5:95) elution were employed. After elution samples were concentrated by evaporating at 30 °C under gentle nitrogen flow. Volatile oxidation products were analyzed by GC-MS with liquid injection, using a Thermo Scientific (Waltham, MA, USA) Trace 1310 instrument with AI 1310 autosampler and TSQ 8000 Evo mass spectrometer with a DB-WAX UI column (30 m × 0.25 mm, 0.25 µm, Agilent, Santa Clara, CA, USA). Electron ionization (EI) at 250 °C and 70 eV was applied for detection with a scanning range from 40 to 350 amu. Also, the non-volatile oxidation products were analyzed from the solid phase extracted samples. Except for the LC solvent gradient program, the method was similar to that described

for study **III** in section 4.4. For both GC-MS and LC-QTOF, the samples were injected in water/methanol (5:95).

4.7 ^{1}H NMR

^{1}H NMR was applied in an untargeted manner in studies **I** and **II** for monitoring the levels of oxidation products and degradation of the oxidized substrate during the oxidation trial. In both studies, NMR analysis was exclusively performed at specific time points. Samples were transferred to $CDCl_3$/DMSO-d6 (5:1) for the analysis. Selective gradient excitation (*selgpse*) was applied on regions δ 11.5–10.5 ppm and δ 10.0–9.0 ppm for hydroperoxides and aldehydes, respectively. A 600 MHz Bruker AVANCE-III NMR spectrometer (Bruker BioSpin, Switzerland) equipped with a Prodigy TCI CryoProbe and a SampleJet robotic sample changer operated at 600.16 MHz (^{1}H) at 298 K was used to record spectra. Sample preparation and data collection were adapted from (Merkx et al., 2018) with minor adjustments. TopSpin 4.0.6 (Bruker Biospin Corporation, Billerica, MA, USA) and Chenomx NMR Suite 8.6 (Chemomx Inc., Edmonton, AB, Canada) were used to process the NMR data.

4.8 Statistical analysis

IBM SPSS 28.0.0 (or 27.0.1.0) statistical software (IBM Corporation, Armonk, NY, USA) was used for data processing in studies **I-III**. Independent samples t-test was used to examine the differences in compound levels at each time point in studies **I** and **III**. In study **II**, one-way ANOVA and Tukey's HSD test were applied. In all studies, differences were considered statistically significant with p-values below 0.05. In studies **I** and **II**, principal component analysis (PCA) was applied for the binned and manually integrated NMR data after Pareto-scaling and mean-centering using SIMCA® 16 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). In study **III,** a similar PCA plot was produced from the integrated non-volatile and volatile oxidation product area data after UV scaling.

5 RESULTS AND DISCUSSION

5.1 Oxidation trial setup

Monitoring lipid oxidation in ambient conditions can be time-consuming, so accelerated shelf-life protocols with elevated temperatures are often applied for stability tests. Generally, the oxidation rate doubles for each 10 °C increase in temperature (Hu & Jacobsen, 2016). In studies **I-III**, oxidation of pure oils took place at 50 °C in the dark, in closed vials exposed to air at the beginning of the trial. Oxidation mechanisms can change at temperatures exceeding 40 °C (Frankel, 2005; Schaich, 2012; Sullivan et al., 2011), and hence, the slightly higher temperature may have induced some alterations in the reaction pathways. Temperatures ≥ 60 °C can impact the kinetics even more due to a rapid decrease in oxygen solubility (Chen, McClements, et al., 2011; Sullivan Ritter et al., 2015). The oxidative quality of the pure oils (**I-III**) was verified by volatile oxidation product analysis (SPME-GC-MS) before sample pretreatment. The oxidative quality of commercial standard oils is expected to be high. The regio- and enantiopure samples (**II**) were also analyzed by NMR.

At the start of the trial, the vial headspace was filled with pressurized air instead of oxygen to mimic the conditions in foods. Theoretically, there were approximately 1.5 moles of oxygen available for every DHA acyl chain in the 10 mL vials (**I**, **III**). In study **II** with regio- and enantiopure samples, the oxygen availability was even higher. Oxygen concentration becomes a limiting factor for oxidation only at very low concentrations (< 0.5% v/v) (Andersson & Lingnert, 1999). In stripped oil blends, an increase in oxygen partial pressure from 10% to 21% did not affect the onset of hydroperoxide formation at temperatures above 30 °C (Hoppenreijs et al., 2021). The reduction in oxygen concentration likely had no significant impact on the oxidation pattern, and if an effect did occur, it probably manifested in the very late stages of oxidation, when a significant amount of the oil substrate had depleted. Overall, the setup was well-suited for assessing the oxidative stability of high-priced oils with limited oil quantities.

5.2 Substrate depletion

Depletion of the substrate during the trial was used as an oxidation indicator in all three studies (**I-III**). In study **I**, the HPLC-QTOF method developed for the non-volatile oxidation product analysis was also utilized to monitor DHA-TAG and -EE depletion in a semi-quantitative manner by peak area evolution. In studies **II** and **III**, FA concentrations were analyzed by GC with a flame ionization detector (FID) after transesterification into volatile methyl esters. In study **III**, the DHA (22:6) to palmitic acid (16:0) ratio was employed as an

indicator, given that palmitic acid, being a saturated FA, is not expected to oxidize to any significant extent. Induction periods for substrate depletion in studies **I-III** are reported in **Figure 12**.

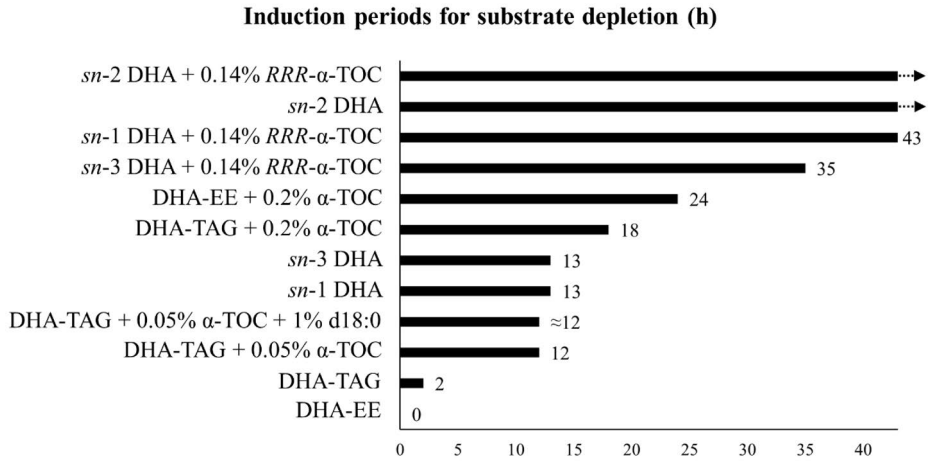


Figure 12. Substrate depletion induction periods (h) for DHA-TAG and DHA-EE depletion (DHA-TAG and -EE + 0.2% α-TOC) (**I**), depletion of 22:6/16:0 ratio (*sn*-1, *sn*-2, and *sn*-3 DHA + 0.14% α-TOC) (**II**), and DHA depletion (DHA-TAG + 0.05% α-TOC + 1% d18:0) (**III**) after oxidation at 50 °C in the dark.

α-TOC demonstrated an antioxidative effect at all applied concentrations. The addition of 0.05% α-TOC increased the induction period of DHA-TAG from 2 to 12 h, and 0.2% addition resulted in an 18 h induction period. When d18:0 was also added (0.05% α-TOC + 1% d18:0) in study **III**, the induction period determination became more complicated as the oil depletion rate decreased after 12 h. At 24 h, 44.4% and 18.8 % of the oil was left in the samples with and without d18:0, respectively. As expected, the decrease in the molar amount of DHA in the samples without antioxidant (**II)** led to increased stability compared to corresponding DHA-TAG and -EE samples (**I**). However, DHA-TAG and DHA-EE with 0.2% α-TOC (**I**) were more stable than *sn*-1 and *sn*-3 DHA without antioxidant addition. With 0.14% *RRR*-α-TOC (**II**), DHA in *sn*-1 was more stable than in *sn*-3. DHA in the *sn*-2 position accompanied by two palmitic acids was clearly the most stable structure, even without added antioxidant.

5.3 α-Tocopherol depletion

α-TOC concentrations were analyzed with normal phase HPLC (**I**, **II**) and slightly modified UHPLC (**III**) separation methods with a fluorescence detector.

Two types of α-tocopherols were used as antioxidants in the conducted oxidation trials. In studies **I** and **III**, a synthetic form of α-TOC, comprising 12.5% of all eight isomers (*RRR*, *RRS*, *RSR*, *RSS*, *SRR*, *SRS*, *SSR,* and *SSS*) (Weiser et al., 1996) was utilized. In Study **II**, which focused on the regio- and enantiopure TAGs, the natural form, *RRR*-α-TOC, consisting of a single stereoisomer, was employed. The biological activities of the non-natural isomers of synthetic α-TOC are lower compared to *RRR*-α-TOC, but they all act as antioxidants *in vitro* (Brigelius-Flohé, 2006).

Percentual α-TOC depletion from the maximum value in each study (**I-III**) is presented in **Figure 13**. The slowest consumption was observed in the regio- and enantiopure samples (**II**) with 0.14% added *RRR*-α-TOC. In Study **II**, the ratio of α-TOC to DHA was higher than in the DHA-TAG and DHA-EE samples (**I** and **III**). *Sn*-2 DHA was the only sample type where residual *RRR*-α-TOC was still left at the end of the oxidation period (54.6 h). In the *sn*-1 DHA samples, the *RRR*-α-TOC depletion was slower than in the *sn*-3 DHA samples.

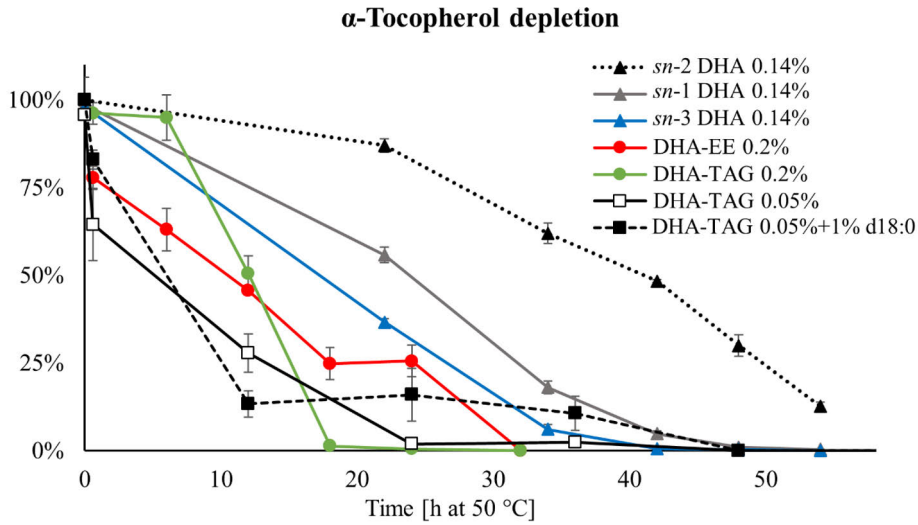


Figure 13. Percentual decrease in α-TOC concentration during the oxidation trials (**I-III**).

In the DHA-TAG samples with 0.2% α-TOC (**I**), the antioxidant depletion started only after 6 hours of oxidation, but subsequently, the level decreased rapidly to zero in 18 hours. In the DHA-EE samples, α-TOC level had decreased to approximately 78% at 0.6 h samples, which was the first analyzed time point in study **I**. However, despite the lower α-TOC level from 0.6 h to 12 h, it was longer preserved in the DHA-EE samples than in the DHA-TAG samples (32 h vs. 18 h). This indicated that DHA-TAG and DHA-EE interacted differently with α-TOC.

Level evolution in the samples with 0.05% α-TOC + 1% d18:0 differed from other sample types with a fast decrease in the beginning followed by a stabilizing level from 12 h onwards (**III**). This behavior was probably related to the relatively low α-TOC concentration for protecting pure DHA-TAG and the formation of antioxidative compounds after the oxidation had proceeded for 12 h. In the DHA-TAG samples with 0.05% α-TOC, the level dropped fast from 0 to 0.6 h. The result for the 0 h sample was based on only one replicate, and for the 0.6 h samples, the standard deviation was reasonably high (64.5 ± 10.2%). The analytical challenges with these samples might be related to the sample pretreatment step, where the original solvent was evaporated from the 100 µL glass inserts before heptane addition. For ensuring complete solvent evaporation, a better approach might be to do the evaporation in the original vial, add heptane, and subsequently transfer the sample into a glass insert. An unexpected drop in tocopherol levels after sample preparation was also reported by Doert et al. (2012) when analyzing initial 0.05% tocopherol levels during the oxidation of ethyl linoleate with HPLC coupled to fluorescence detection (Doert et al., 2012).

5.4 Non-volatile oxidation products

Oxidized TAGs are commonly analyzed by reverse phase LC methods with electrospray ionization and MS detection (Zeb, 2015). This approach allows the analysis of intact TAGs without derivatization. Electrospray ionization produces cationized, mobile phase-derived TAG adduct ions ($[M+Na]^+$, $[M+K]^+$ or $[M+NH4]^+$) without protonated molecules (Byrdwell & Neff, 2002; Holčapek et al., 2003; Sjövall et al., 2002). The relative abundance of diacylglycerol fragments necessary for TAG identification is low in electrospray ionization, so tandem MS techniques are applied (Xia & Budge, 2017). Quantification of individual TAG oxidation products, including epoxy- and hydroxy FAs and epoxidized and hydroperoxidized TAGs, is possible with methods requiring methylation and derivatization or separation of the polar phase with SPE (Grüneis et al., 2019; Xia & Budge, 2018). However, additional processing steps can result in further oxidation or other unwanted reactions in the analyte.

In studies **I**-**III**, an LC-QTOF method, developed based on the method by (Tarvainen et al., 2010), was applied to analyze the non-volatile oxidation products in an untargeted manner. The method used in studies **I** and **II** was further developed for study **III** to detect the amine compounds, shorten the running times, and decrease solvent consumption. No standards were available for non-volatile DHA-TAG or DHA-EE oxidation products, so area evolutions were monitored to detect changes in the oxidation product levels semi-quantitatively. High substrate lipid concentration compared to oxidation

products restricted injection volumes, and thus, the intensities for several detected non-volatile oxidation products were low.

Identification of oxidation products was based on their sodium or ammonium adduct fragmentation patterns, obtained through AutoMS/MS. The fragmentation patterns are described in the corresponding publications (Ahonen et al., 2022, 2023; Damerau et al., 2023). In the oxidation products with several added oxygens, the position or nature of added oxygens could not be distinguished. Thus, for example, the cyclic structures could not be separated from polyhydroperoxides. Nevertheless, given that the monohydroperoxide represents the primary oxidation product, the mass of M+2O was mainly attributed to it when discussing the findings. Pure compounds with known structures as the oxidized substrate allowed tentative identification of the oxidized TAGs with cleaved acyl chains using exact masses and the Lipid Maps structure database (Sud et al., 2007).

Identified non-volatile oxidation products for DHA-TAG (**I**, **III**), DHA-EE (**I**), and 16:0/16:0/DHA (**II**) are reported in **Table 7**. In study **III**, 25 oxidation products were detected, while in studies **I** and **II**, 19 and 16 oxidation products, respectively, were identified. The UHPLC method with Kinetex PS C18 column and solvent system comprising of water/methanol and 2-propanol/methanol with ammonium formate (**III**) allowed the separation and identification of a higher number of non-volatile oxidation products than the HPLC method with Nucleodur C18 Isis column and solvent system consisting of acetonitrile/water and 2-propanol with formic acid and ammonium acetate (**I** and **II**). Injection solvent in study **III** consisted of methanol/chloroform (1:1), while in studies **I** and **II,** chloroform was employed. Furthermore, MS-DIAL software (version 4.92) (Tsugawa et al., 2015) in study **III** greatly enhanced the screening of oxidation products with low signal-to-noise ratio, especially the acyl chain cleavage products, within the obtained data.

Table 7. Tentatively identified non-volatile oxidation products in studies (**I-III**) for DHA-TAG (**I**, **III**), DHA-EE (**I**) and 16:0/16:0/DHA (**II**). For acyl chain cleavage products, the FAs represent either 22:6 (**I**, **III**) or 16:0 (**II**).

Tentatively identified oxidation product	DHA-TAG	DHA-EE	16:0/16:0/DHA
M+O (+16 Da epoxide/hydroxide)	I & III	I	II
M+O (+14 Da ketone/epoxide)	-	-	II
M+2O	I & III	I	II
M+4O	I & III	I	II
M+6O	I & III	I	II
M+7O	-	-	II
M+8O	I & III	I	II
M+9O	III	-	II
M+10O	I	-	II
DHA-DAG	III	-	-
DHA-DAG+O	III	-	-
DHA-DAG+2O	III	-	-
DHA-DAG+4O	III	-	-
TAG FA/FA/4:0; O (oxo)	III	-	-
TAG FA/FA/5:0; O2	III	-	-
TAG FA/FA/5:1; O2 or 6:0; O	-	-	II
TAG FA/FA/6:1; O	III	-	II
TAG FA/FA/6:1; O2	III	-	II
TAG FA/FA/8:1; O2	III	-	-
TAG FA/FA/8:1; O3	III	-	-
TAG FA/FA/9:2; O (oxo)	III	-	-
TAG FA/FA/9:2; O2	III	-	-
TAG FA/FA/11:2; O3	III	-	-
TAG FA/FA/12:3; O (oxo)	III	-	
TAG FA/FA/12:3; O2	-	-	II
TAG FA/FA/13:2; O3	-	-	II
TAG FA/FA/14:2; O3	III	-	-
TAG FA/FA/15:4; O2	I	-	-
TAG FA/FA/18:5; O2	I	-	II
TAG FA/FA/19:5; O2	-	-	II
TAG FA/FA;O2/6:1; O	III	-	-
TAG FA/FA;O2/7:0; O	III	-	-
TAG FA/FA;O2/10:2	III	-	-
O – O dimers (+4O/6O/8O/10O/12O)	-	I	-
C – C dimer	-	I	-

Non-volatile oxidation products from DHA neat oil oxidation have not been reported earlier to a similar extent. Dimers were detected only in the DHA-EE samples (**I**), although it must be noted that the masses for TAG dimers would have been out of the applied MS scanning range. The formation of dimers and trimers in DHA-EE autoxidation was also reported by Cho et al. (1987a, 1987b). After oxidation under light irradiation at 5 °C (peroxide value 1150 meq/kg), approximately 80% of the DHA-EE dimers were C-O-O-C linked and 20% C-O-C linked (Cho et al., 1987b). Polymers were identified as a major secondary oxidation product in DHA-EE oxidation, comprising over 70 % of the polar material (Cho et al., 1987a). The largest variety of acyl chain cleavage products was detected in study **III** (14 pcs). Only for six of them, the formation could be postulated by reaction pathways following β-cleavage of alkoxy radicals. In study **III**, two peaks could be separated for the mass addition of one oxygen (+16 Da) to DHA-TAG. Based on fragmentation behavior and retention order (Brühl et al., 2016; Neff & Byrdwell, 1998; Suomela et al., 2011), epoxide and hydroxide could be tentatively distinguished. In studies **I** and **II** this was not possible due to weaker separation.

To generalize the non-volatile oxidation product level evolution trends reported in studies **I-III**, the monohydroperoxide levels increased faster/earlier than the levels of M+4O, and as expected, M+6O levels increased thereafter. In the presence of α-TOC, the difference between M+2O and M+4O was substantial, while in the absence of α-TOC, it was less pronounced. Also, Lyberg et al. (2005) reported later peaking of DHA free FA polyhydroperoxides compared to corresponding monohydroperoxides. Low monohydroperoxide levels for DHA-TAG indicated fast further reactions to polyhydroperoxides (Lyberg et al., 2005).

The formation of acyl chain cleavage products generally started later than the formation of M+2O and M+4O (**I**, **III**), but also some variation was observed (**II**). Later appearance is expected as the cleavage products form from LOOH decomposition. The formation of oxygen-linked EE dimers (**I**) started from the very beginning of the trial in the samples without antioxidant, while for the carbon-linked dimers, a two-hour induction period was observed. This indicated that dimerization through oxygen bridges occurs already at the early stages of DHA-EE oxidation. Also, Cho et al. (1987a) reported early dimerization of DHA-EE with an induction period similar to oxygen absorption. The epoxide/hydroxide (**I**) and epoxide (**III**) levels were behaving similarly to M+4O and M+6O in studies **I** and **III** (for **III** data not shown). However, in the enantio- and regiopure samples without antioxidant (**II**), the formation of epoxide/hydroxide occurred early, resembling the trend of M+2O.

Percentual maximum mean area distribution of the five most abundant oxidation products for each sample type are presented in **Figure 14**. In the DHA-TAG (**I**, **III**) samples, the areas of M+2O (37-41%) and M+4O (33-40%) were

the highest, followed by M+6O (12-15%), M+8O (6-7%), and epoxide/hydroxide (4-5%) (**I**)/epoxide (8%) (**III**). In the DHA-EE samples, the general pattern was similar, except for the increased proportion of M+4O (49-51%) compared to M+2O (23-25%). This could indicate higher prevalence of further reactions within the same acyl chain in DHA-EE. However, also the ionization efficiencies, influenced by various factors including the chemical and physical properties of the analyte, could vary between M+2O and M+4O. The regio- and enantiopure samples (**II**) were differentiated by higher epoxide/hydroxide levels (8-23%) and by the high level of acyl chain cleavage product 16:0/16:0/19:5;O2 (5-21%). This product could form through further oxidation after propanal cleavage from 20-LO•. Also, the levels of other acyl chain cleavage products were higher in study **II** than in study **I**. In the *sn*-2 DHA samples with 0.14% α-TOC, oxidation had not advanced as far as in the other samples, which likely explains the elevated M+2O levels compared to other products.

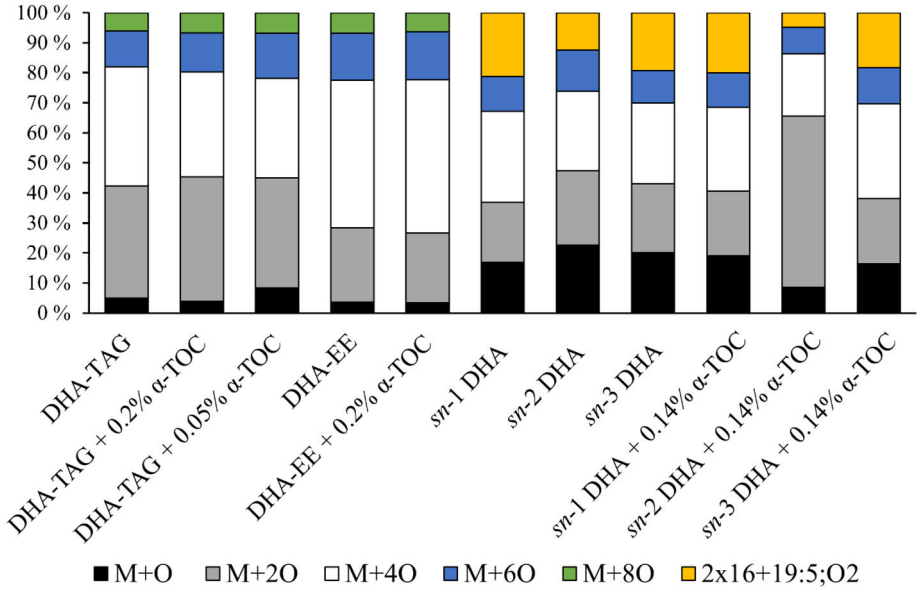


Figure 14. Percentual maximum mean area distribution of the five most abundant oxidation products for each sample type. DHA-EE dimer areas are omitted from the data.

The accelerated formation and elevated peak areas of epoxide/hydroxide in the regio- and enantiopure TAGs (**II**), containing two palmitic acids and one DHA, might be due to the lower hydrogen availability from DHA acyl chains, which favors epoxide formation over polyhydroperoxides (Schaich, 2012). The same reason might explain the high M+6O and M+8O levels in the DHA-TAG and

DHA-EE samples (**I**, **III**). Overall, the addition of antioxidants did not seem to have a notable effect on the yield of the main non-volatile oxidation products, although the timing of their appearance was strongly altered. Stability differences between different lipid structures will be discussed alongside with other oxidation indicators in section 5.8.

The LC-QTOF method would benefit from improved analyte separation, enabling more specific identification of the molecular species with added oxygens. Exact identification was challenging due to the formation of numerous oxidized TAG isomers with minor differences in their polarities. It might be possible to improve the separation by using two columns in a series (Byrdwell & Neff, 2002; Suomela et al., 2011), or normal-phase methods with polar mobile phase and sodium addition post column for ionization (Kato et al., 2021; Steenhorst-Slikkerveer et al., 2000). One option would be to separate and/or derivatize the oxidation products prior to analysis, but considering the instability of DHA, it might induce alterations in the analytes. This approach might also not be feasible when using small sample amounts.

5.5 Volatile secondary oxidation products

In studies **I** and **II,** volatile secondary oxidation products were analyzed by HS-SPME-GC-MS method with a semipolar SPB®-624 capillary column, while in study **III**, a similar method with a polar DB WAX column was employed. A stationary phase with increased polarity was chosen in study **III** to enable better separation of amine compounds. In study **III**, the volatile oxidation products were also analyzed from the SPE-extracted polar phase by GC-MS with liquid injection and a DB-WAX UI column. External standards (**I**, **II**), the NIST MS Search library (**I**-**III**), and retention indices (**III**) were used for compound identification.

Volatile secondary oxidation products detected in studies **I**-**III** are presented in **Table 8**. Only compounds with increasing levels (from close to zero level) from the start of the oxidation period were considered oxidation products. For consistency in comparing the data between the studies, only the oxidation products with NIST match numbers higher than 800 are shown. Altogether, 79 volatile secondary oxidation products were identified in studies **I**-**III**. Aldehydes (22 products), ketones (15 products), alkenes (9 products), alcohols (8 products), esters (8 products), and acids (7 products) were the most abundant compound groups.

Table 8. DHA-derived volatile oxidation products detected in studies **I-III**. Data from the d18:0-containing samples (**III**) is excluded.

Compound group	Compound	DHA TAG	DHA EE	16:0/16:0/ DHA
aldehydes (22)	acetaldehyde	I, III	I	II
	propanal [1]	I, III	I	II
	2-propenal	I, III	I	II
	butanal	I	I	II
	2-butenal, (*Z*)-	I, III	I	II
	2-butenal, (*E*)-	I, III	I	-
	2-pentenal, (*Z*)-	I, III	I	II
	2-pentenal, (*E*)-	I, III	I	II
	2-hexenal, (*E*)-	I, III	I	II
	3-hexenal [1,2]	I, III	I	II
	4-oxohex-2-enal	I, III	-	II
	4-heptenal, (Z)- [2,3]	III*	-	II
	2-octenal, (*E*)- [3]	III	-	-
	nonanal [3]	-	-	II
	2-decenal, (*E*)-	III	-	-
	2,4-hexadienal, (*E,E*)-	I, III	I	II
	2,4-heptadienal, (*E*,*Z*)- [1,2,3]	I, III	I	II
	2,4-heptadienal, (*E,E*)- [1,2,3]	I, III	I	II
	2,6-nonadienal, (*E*,*Z*)- [1,2]	I, III*	-	-
	3,6-nonadienal, (*Z*,*Z*)- [1,2]	III*	-	-
	2,4-decadienal, (*E*,*Z*)- [1]	I, III	-	-
	2,4-decadienal, (*E*,*E*)- [1]	I, III	-	-
ketones (15)	1-penten-3-one [2,3]	I, III	I	II
	3-penten-2-one	III	-	-
	2,3-pentanedione	III*	-	-
	2-hexanone	I	I	-
	3-hexanone	I	I	-
	1-hydroxy-2-propanone	III*	-	-
	1-hydroxy-2-butanone	III	-	II
	6-octen-2-one, (*Z*)-	III*	-	-
	3,5-octadien-2-one (*E,Z*)- [1]	I, III	I	II
	3,5-octadien-2-one, (*E,E*)- [1]	I, III	I	II
	2(5H)-furanone, 5-methyl-	III	-	II
	2(3H)-furanone, dihydro-5-methyl-3-methylene-	III	-	-
	2(5H)-furanone, 5-ethyl	I, III	I	II
	2(3H)-furanone, 5-acetyldihydro-	III*	-	-
	2,5-furandione, 3,4-dimethyl-	III	-	-

[1)] Noble & Nawar, 1971, 1975 [2)] Hammer & Schieberle, 2013 [3)] Peinado et al., 2016

* Detected exclusively in the SPE-GC-MS analysis with liquid injection (**III**).

Compound group	Compound	DHA TAG	DHA EE	16:0/16:0/ DHA
	2-hexene, 3,5,5-trimethyl-	III	-	-
	2,4-hexadiene, (*E*,*Z*)-	III*	-	-
alkenes	3-methyl-1,6-heptadiene	III	-	-
(9)	2,4-octadiene [1]	I, III	I	-
	3,5-octadiene, (*Z*,*Z*)-	III	-	-
	1,3-(*E*)-5(*Z*)-octatriene	III	-	-
	1,3,5-(*Z*,*Z*,*Z*)-octatriene	III	-	-
	2,4-nonadiene, (*E*,*E*)-	-	-	II
	5-dodecene, (*Z*)-	III	-	-
	ethanol	-	I	-
	1-penten-3-ol [3]	I, III	I	II
	2-penten-1-ol, (*E*)- [3]	III	-	II
	2-penten-1-ol, (*Z*)-	III	-	II
alcohols	2-methyl-2-pentanol	-	-	II
(8)	hex-5-en-3-ol	III*	-	-
	3-hexen-1-ol, (*Z*)-	III	-	-
	4-decen-1-ol, (*Z*)-	III	-	-
	octanoic acid, methyl ester	I, III	-	-
	nonanoic acid, methyl ester	I	-	-
esters	decanoic acid, methyl ester	I	-	-
(8)	undecanoic acid, methyl ester	I	I	-
	propanoic acid, ethyl ester	-	I	-
	hex-5-enoic acid, ethyl ester	-	I	-
	hex-4-enoic acid, ethyl ester	-	I	-
	decanoic acid, ethyl ester	-	I	-
	formic acid	I, III	I	II
	acetic acid [3]	I, III	I	II
acids	propanoic acid [3]	I, III	I	II
(7)	butanoic acid [2]	I, III	I	II
	3-hexenoic acid, (*E*)-	I, III	I	II
	3-heptenoic acid	III	-	-
	nonanoic acid	I	I	II
furans	2-methylfuran	I	I	II
(4)	2-ethylfuran	I, III	I	II
	2-pentylfuran [1]	III	-	-
	E-2-(2-pentenyl) furan [1]	I, III	I	II
benzene	benzaldehyde	III*	-	-
derivatives	1-phenyl-1-propanone	III*	-	-
(4)	benzene, acetyl	III	-	-
	4-ethylphenol	I	I	-
epoxides	2-methyl-3-vinyloxirane	I	I	-
(2)	2-ethyl-3-vinyloxirane	I, III	I	-

[1)] Noble & Nawar, 1971, 1975 [2)] Hammer & Schieberle, 2013 [3)] Peinado et al., 2016

* Detected exclusively in the SPE-GC-MS analysis with liquid injection (**III**).

The DB WAX column (**III**) allowed the identification of a slightly higher number of oxidation products compared to the SPB®-624 column (**I** and **II**), with the most significant difference observed in the alkene group (eight vs. two oxidation products). As an analysis method, HS-SPME is more sensitive to low-molecular-weight than high-molecular-weight compounds (D'Auria et al., 2008). It is also possible that the volatile oxidation products have different affinities for the fiber coating, potentially causing discrepancies between the analyzed results and the actual conditions in the sample (Górecki et al., 1999; Thomsen et al., 2016). The employed DVB/CAR/PDMS fiber has a mixed polarity, and it is suited for the analysis of low-molecular mass polar compounds (Kataoka et al., 2000; Mansur et al., 2018)

Generally, there were no considerable differences in the detected volatile oxidation products between 16:0/16:0/DHA (**II**) and DHA-TAG (**I**, **III**). The compound areas of analyses conducted at different times using different fibers, although of the same type, are not directly comparable. Also, the amount of DHA in study **II** was lower than in studies **I** and **III**, which could result in fewer oxidation products and less competition on the fiber. 2,4-Decadienal was detected only in the DHA-TAG samples (**I**, **III)** and 4-oxohex-2-enal in the TAG and 16:0/16:0/DHA samples (**I-III**). The suggested pathway for 4-oxohex-2-enal formation is through 16,20-dihydroperoxides (Long & Picklo, 2010), and 2,4-decadienal is theoretically expected to originate from omega-6 FAs (Frankel, 1983). Still, 2,4-decadienal was also detected by Noble & Nawar (1971) as an oxidation product of DHA methyl ester. No apparent reason for either's absence in the DHA-EE samples can be postulated.

Ethanol and ethyl esters were detected exclusively in DHA-EE samples (**I**). These compounds were also identified by Lee et al. (2003) after HS-SPME-GC analysis of EE fish oil after oxidation at 80 °C with aeration. The detected ethyl esters included saturated chains with 10, 11, and 12 carbons (Lee et al., 2003). Noble & Nawar (1971, 1975) detected several methyl ester products after DHA methyl ester oxidation at 50 °C in the dark. However, the detected methyl esters mainly contained unsaturated chains, which are expected to form in DHA oxidation. These compounds constitute the other part following LO• cleavage, corresponding to acyl chain cleavage products in the DHA-TAG samples. The formation of methyl and ethyl ester products with saturated 8-11 carbon chains in TAG and EE samples (**I**, **III**) does not adhere to any standard oxidation reaction pathway. They could theoretically form through thermal degradation (S. Park et al., 2022), which at 50 °C does not seem likely, through acid reactions (e.g., nonanoic acid **I**, **II**) with methanol/ethanol or some other route. Saturated aldehydes (C5-C9) were detected after thermal degradation of DHA (110 °C) (Peinado et al., 2016), and could be a source of corresponding acids. It is also

possible that impurities in the substrate may have reacted with oxidation products, leading to elevated levels observed during the oxidation trial.

Of the 79 detected volatile secondary oxidation products, 19 were also detected in previous studies with pure DHA oxidized as neat oil (Hammer & Schieberle, 2013; Noble & Nawar, 1971, 1975; Peinado et al., 2016). Some similar tendencies in the volatile oxidation product level evolution could be observed between the studies. For example, 2-ethylfuran levels were increasing very fast from the beginning of the trial, followed by a steep decrease (**I-III**, for **III** data not shown), and quite a similar trend was observed for acetaldehyde (**I**, **II**). 2-Ethylfuran can form 16-/17-OOH through 2-hexenal (Adams et al., 2011), and acetaldehyde can originate from alkenals, polymers, hydroperoxides, and MDA (Frankel, 1983; Vandemoortele et al., 2021). Also, propanal, 2-propenal, 1-penten-3-one, and 1-penten-3-ol had increasing levels at a relatively early point of the oxidation period (**I-III**). Propanal, 2-propenal, and 1-penten-3-ol were reported to appear also in the early stages of fish oil TAG oxidation (Shibata et al., 2018). 3,5-Octadien-2-one appeared slightly later during oxidation (**I**, **III**, for **III** data not shown). The later appearance might be due to its postulated formation through hydroperoxy epidioxides (Noble & Nawar, 1975). Also, the alkenes for which area was integrated in study **III** (data not shown) appeared at the later stages of oxidation. For the short-chain saturated acids, the levels tended to increase throughout the oxidation period (**I-III**). The behavior might be explained by the increasing number of aldehydes oxidizing further to acids as the oxidation proceeds, and the lower probability of further reactions in the case of acids.

Percentual maximum mean area distribution of 2,4-heptadienal (*E*,*Z*)- and (*E*,*E*)-, 3-penten-1-ol, 2-propenal, propanal, and acetic acid are presented in **Figure 15**. For most samples, these were among the compounds with highest maximum areas, although for the DHA-TAG samples in study **I**, 2-pentenal (*E*)//*Z*) areas were slightly higher than those of acetic acid, and also formic acid was accumulating into the samples. Formic acid areas were omitted from the data due to peak tailing.

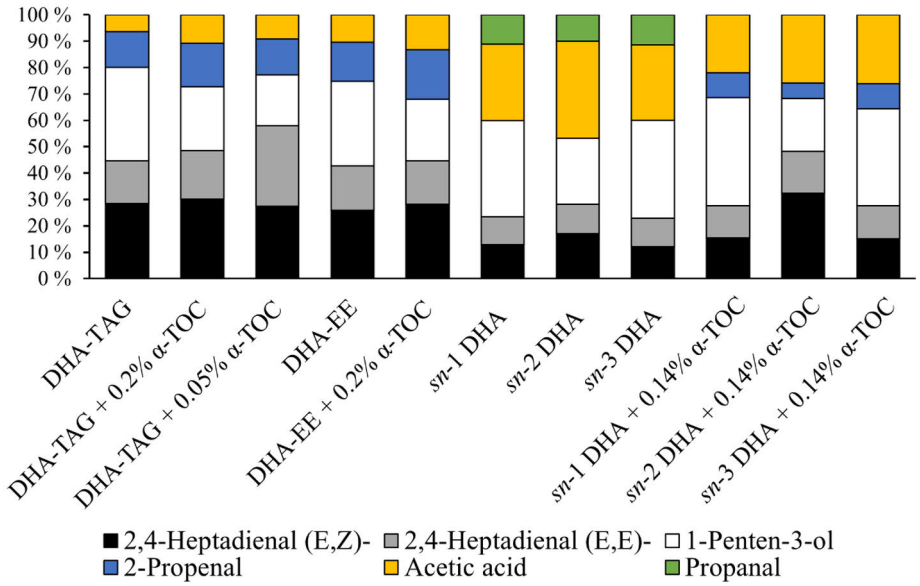


Figure 15. Percentual maximum mean area distribution of 2,4-heptadienal (*E*,*Z*)- and (*E*,*E*)-, 3-penten-1-ol, 2-propenal, propanal and acetic acid.

By maximum area, the most abundant volatile oxidation products in the DHA-TAG and -EE samples (**I**, **III**) included 2,4-heptadienal (*E*,*Z*)-, (26-30%) and (*E*,*E*)-, (16-30%) 1-penten-3-ol (19-35%), and 2-propenal (14-19%). In the regio- and enantiopure samples (**II**), the proportions of both 2,4-heptadienal isomers decreased, and acetic acid increased (22-37%). Interestingly, in the regio- and enantiopure samples without antioxidant (**II**), the maximum mean areas of propanal (10-11%) were higher than those of 2-propenal. The peak areas for both compounds reached their maximum values at either the 24-hour or 30-hour time points. The addition of 0.14% α-TOC, and thus increased hydrogen availability, resulted in an increased proportion of 2-propenal (6-9%) (**Figure 15**). The 2-propenal/propanal maximum area ratios varied in the range of 3.1-5.4 in the DHA-TAG and -EE samples (**I**, **III**), 0.6-0.8 in the regio- and enantiopure samples without α-TOC (**II**), 1.2-1.3 in the regio- and enantiopure samples with 0.14% α-TOC.

The sensitivity of the DVB/CAR/PDMS fiber to 2-propenal was reported to be lower compared to DVB/CAR fiber or static headspace GC (SHS-GC) (Shibata et al., 2018). Thus, the 2-propenal areas analyzed with DVB/CAR/PDMS fiber in the current work might be somewhat underestimated. No considerable sensitivity difference was reported for 2,4-heptadienal (*E*,*E*)- and 1-penten-3-ol (Shibata et al., 2018). Peinado et al. (2016) reported 2,4-heptadienal (*E*,*E*)- as the most abundant aldehyde after DHA heating at 110 °C, while 1-penten-3-ol had low levels, and 2-propenal was not detected. HS-SPME analysis was performed on aqueous sample extracts in saturated NaCl solution

with a PDMS/DVB fiber (Peinado et al., 2016). 2,4-Heptadienal and 1-penten-3-ol were reported as the major volatiles by peak area also in omega-3 supplement oils obtained from the Finnish market, analyzed with a similar SPME-GC-MS method as used in studies **I** and **II** (Damerau et al., 2020).

2,4-Heptadienal (*E*,*Z*)- can originate from 16-OOH (Frankel, 1983), 16,14-dihydroperoxides, and hydroperoxy bicycloendoperoxides (Frankel et al., 1984), while 1-penten-3-ol is postulated to derive from 17-OOH (Lee et al., 2003). Propanal can originate from 20-OOH, alkenals, polymers, hydroperoxy epidioxides, and hydroperoxy bisepidioxides (Frankel, 1983; Frankel et al., 1983). The increased formation of 16:0/16:0/19:5;O2 (**Figure 14**), originating from further oxidation after propanal cleavage from 20-OOH, aligns with the increased propanal formation observed in the regio- and enantiopure samples. Possible 2-propenal sources include dihydroperoxides, hydroperoxy epidioxides, 20-OOH, and 2,4-decacienal (Endo et al., 2013; Frankel et al., 1983). It has been also suggested that 2-propenal can form continuously from the DHA LOOH acyl chain after propanal cleavage (Miyashita et al., 2018). The increased generation of 2-propenal under elevated hydrogen availability would support its formation through dihydroperoxides. Acetic acid can originate from the corresponding aldehyde, acetaldehyde, or 2,4-decadienal (Frankel, 1983). Due to the complexity and multiplicity of possible formation routes for each volatile oxidation product, drawing clear conclusions on oxidation pattern differences between lipid structures based on the obtained data is challenging.

The main volatile oxidation products from DHA oxidation have different qualities in terms of sensory quality. 2,4-Heptadienal (*E*,*Z*)- contributes to the oxidized, painty, fishy, fatty, and burnt flavor notes (Hartvigsen et al., 2000; Karahadian & Lindsay, 1989), and it has odor thresholds of 4000 ppb (nasal) and 50 ppb (retronasal). Propanal has a fruity and pungent flavor, with odor thresholds of 9.4 ppb (nasal) and 68 ppb (retronasal) (Belitz et al., 2009). The flavor of 1-penten-3-ol has been described as sweet, green, and chemical (Cengiz et al., 2023; Hartvigsen et al., 2000). 2-Propenal (acrolein) is a highly reactive toxic aldehyde (Y. Zhou et al., 2023), characterized by an irritating and unpleasant odor with a low odor threshold of 3.6 ppb (Endo et al., 2013). However, apparently, these compounds do not have a significant impact on the overall odor of autoxidized DHA. Hammer & Schieberle (2013) reported that 1,5-octadien-3-one (*Z*)-, *trans*-4,5-epoxy-(*E*,*Z*)-2,7-decadienal, 3,6-nonadienal (*Z*,*Z*)-, and 3-hexenal (*Z*)- were the volatile oxidation products with highest flavor dilution (FD) factors in autoxidized DHA.

Overall, 2,4-heptadienal-, 1-penten-3-ol, and 2-propenal were the major volatile secondary oxidation products derived from DHA oxidation. Especially 2-propenal and 1-penten-3-ol formed at the relatively early stages of oxidation. The analytical approach could be further improved by analyzing part of the

volatile oxidation products quantitatively. In HS-SPME-GC-MS, quantitation using external standards diluted in simple matrices does not necessarily equal the extraction conditions from a complicated and unstable matrix like oxidizing DHA. Furthermore, there can be problems with standard curve linearity due to fiber saturation (Thomsen et al., 2016). However, the standard addition method, although laborious, could be applied for the most abundant volatile oxidation products. Also, methods with multiple internal standards have been employed (Fortini et al., 2017), although there might be differences in the fiber interactions and MS response between the standard and the analyte. When using internal standards, isotopically labeled standards would be the optimal choice (Gómez-Cortés et al., 2012, 2015).

5.6 Oxidation products from the polar fraction

In study **III**, the solid phase extracted samples were analyzed by LC-QTOF and GC-MS methods described in section 4.6. to detect the carbonyl-amine reaction products. In the LC-QTOF analysis, eight imine structures were tentatively identified based on the fragmentation patterns, which in every case included fragments from d18:0. This was the first study detecting d18:0-carbonyl reaction products in oxidized oil. Previously, Takamatsu et al. (2014) identified eight-membered heterocycles as the initial reaction products of 2-propenal and sphingosine (d18:1) in chloroform at room temperature. It was suggested that the presence of alcohol groups in the d18:1 enabled the formation of the heterocycles through imino [4+4] cycloaddition.

Several nitrogen-containing compounds were detected in the GC-MS analysis, but their intensities were too low for identification, or no library match was available. SPE was performed with water/methanol (5:95), and the concentration step included evaporation at 30 °C under nitrogen flow. It is possible that these reaction steps induced some reactions in the analyte. However, imine structures were shown to form also in the samples without SPE, as indicated by the detected d18:0-MDA-d18:0 adduct. Fragment spectra and suggested fragmentation patterns for the tentatively identified MDA-imine (A) and d18:0-MDA:d18:0 adduct (B) are presented in **Figure 16**.

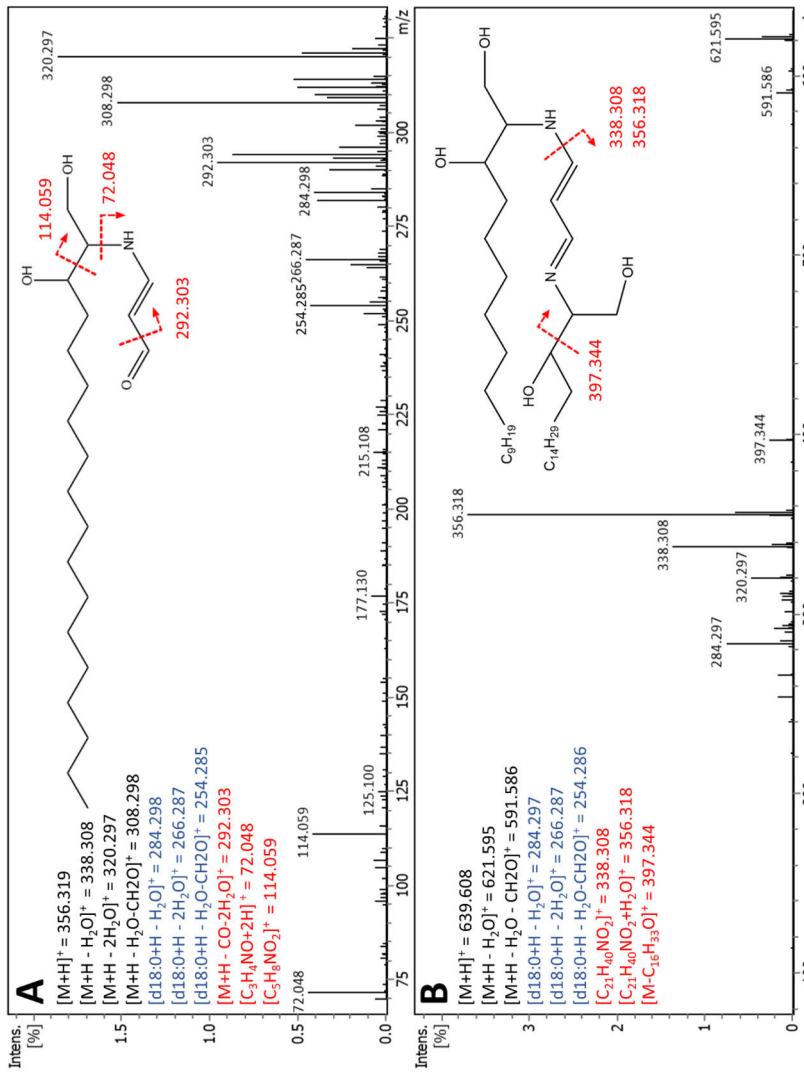


Figure 16. Fragment spectra and suggested fragmentation patterns for MDA-imine (A) and d18:0-MDA-d18:0 (B). Fragments originating from d18:0 are shown in blue, and fragments corresponding to cleavages indicated by arrows are shown in red. High-intensity molecular ions and for A also $[M+H-H_2O]^+$ are omitted from the spectra. Copyright 2023 American Chemical Society.

The formed imines were not expected to contribute to the increased antioxidant activity through radical-scavenging ability, as this would require ring structures with delocalized electrons. However, antioxidative pyrrole, dihydropyridine, and pyridinium salt derivatives could form through d18:0 reactions with lipid oxidation products, as described in section 2.3.2. It is unclear whether the formed imines as secondary amines could contribute to α-TOC regeneration by the mechanisms described for PE and PS by Doert et al. (2012) (**Figure 10**).

5.7 ^{1}H NMR

^{1}H NMR spectroscopy was applied in an untargeted manner for monitoring the levels of oxidation products and the degradation of substrate structures during the oxidation trials in studies **I** and **II**. ^{1}H NMR can be applied for simultaneous monitoring of different lipid oxidation products, including epoxides, aldehydes, hydroperoxides, hydroxides, acids, and ketones (Alberdi-Cedeño et al., 2020; Boerkamp et al., 2022; Martínez-Yusta & Guillén, 2019; Xia et al., 2016). As a method, NMR is simple and fast, it does not require substantial sample preparation prior to analysis and has high reproducibility. Unlike other methods, NMR provides data on the entire sample rather than focusing solely on certain oxidation products. However, the downside of NMR is the lower sensitivity compared to chromatographic methods (Hwang, 2017). In studies **I** and **II**, hydroperoxide and aldehyde levels were monitored by region-specific excitation (Merkx et al., 2018), by which the sensitivity could be substantially increased. PCA models were generated from the binned and manually integrated NMR data, and the results were overall in agreement with the data obtained from other analytical methods. The feasibility of NMR would benefit from even more detailed assignments for different hydroperoxide and aldehyde spectral regions, allowing accurate monitoring of oxidation progress at a compound group level.

5.8 Effect of lipid structure and d18:0 on oxidative stability

5.8.1 Samples without antioxidant

Graphical presentation of oxidation kinetics, illustrating the substrate depletion and level evolution of mono- and dihydroperoxides/cyclic structures and total volatile oxidation products on a percentual level, is presented in **Figure 17** for DHA-TAG, DHA-EE (**I**), and *sn*-1, *sn*-2, and-*sn*-3 DHA samples (**II**) without added α-TOC.

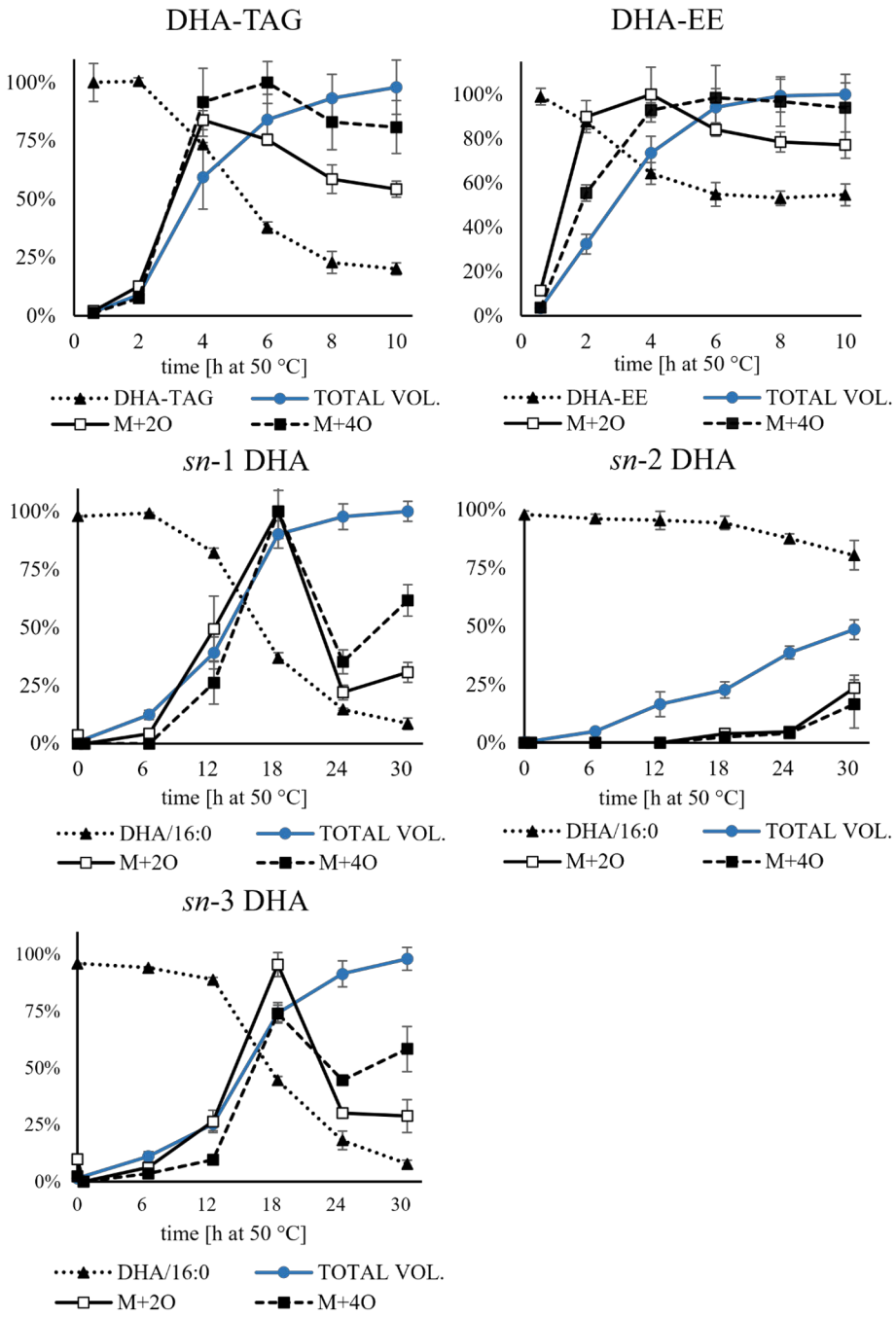


Figure 17. Oxidation kinetics of DHA-TAG, DHA-EE, and *sn*-1, *sn*-2, and *sn*-3 DHA samples without α-TOC. Data is shown as % of one maximum value for each indicator (**I**, **II**), except for DHA-TAG/-EE, M+2O, and M+4O in study **I**, for which maximum value from the α-TOC/non-α-TOC runs was used. Total volatile oxidation products (TOTAL VOL.) refers to the sum of integrated volatile oxidation product areas.

Without added antioxidant, a two-hour induction period was observed for all oxidation indicators in the case of DHA-TAG (**I**). For DHA-EE (**I**), on the other hand, no induction period was observed for any of the oxidation indicators. Therefore, it was concluded that the TAG structure was oxidatively more stable than the EE structure. This behavior could be due to lower viscosity and closer association of the molecules in EE oil, facilitating faster oxidation propagation. Also, the different structures of TAG and EE association colloids or varying tendencies for their formation might affect the observed stability difference. However, Homma et al. (2015) reported no considerable differences in the capability of stripped fish oil TAG- and EE-hydroperoxides to reduce interfacial tension (form association colloids) without surface-active components.

The stability of DHA-TAG and -EE structures has not been studied previously with pure compounds, and the TAG/EE structures overall have not been compared with equalized antioxidant and FA concentrations. In agreement with the current study, Martín et al. (2012) reported improved stability for TAG fish oil concentrate compared to EE in the absence of antioxidants. However, the FA content and initial oxidative status of the oils were not equal. Also, Lee et al. (2003) reported better stability for TAG than for EE fish oil, although it was not declared whether the compared oils contained antioxidants. The authors postulated that the high level of ethanol in the EE oil could be a sign of ester bond breakage and the formation of pro-oxidative free FAs. In study **I**, some ethanol was detected in the EE samples, but not in significant amounts.

In the TAGs with one DHA and two palmitic acids (**II**), the *sn*-2 position was the most stable. *Sn*-3 DHA appeared to be slightly more stable than *sn*-1 DHA, but overall, no clear difference was observed between *sn*-1 and *sn*-3. A similar result was reported for DHA/16:0/16:0 by Wijesundera et al. (2008) and has been observed also for other TAGs with one unsaturated and two saturated FAs (Endo et al., 1997a; Wada & Koizumi, 1983) as neat oil, and in emulsion (Shen & Wijesundera, 2009). The slower oxidation rate of DHA at the *sn*-2 position might be related to steric hindrance and less oxidative interaction between *sn*-2 DHA-TAGs than between *sn*-1 and *sn*-3 DHA-TAGs.

The indicators for substrate depletion between sample types differed since the DHA-TAG and -EE samples contained an equimolar amount of DHA, not an equimolar amount of DHA-TAG and -EE. Thus, the results from DHA-FA concentration would have been more directly comparable. Still, since the induction periods of monohydroperoxides and volatile oxidation products were in line with the ones from oil depletion (**Figures 17 and 18**), the monitoring of DHA-TAG and -EE areas instead of DHA concentration did not seem to have considerable effect on the obtained results. The moderate DHA-TAG decrease rate might indicate that oxidation proceeded mainly within the same TAG, turning monohydroperoxides into di- and trihydroperoxides and cyclic structures.

If one third of the FAs were oxidized, each in a different TAG, 100% of the DHA-TAG structures would be degraded, compared to only 1/3 for TAG-EEs.

In both sample types (**I** and **II**), the elevation in M+4O levels at the beginning of the trial mirrored the fast increase of M+2O levels **(Figure 17**), suggesting that oxidation proceeded substantially also within the same acyl chain/molecule, leading to dihydroperoxide or cyclic structure formation. Also, Lyberg et al. (2005) reported fast further reactions of monohydroperoxides into polyhydroperoxides/cyclic structures for DHA-TAG and DHA as free FA. After reaching the maximum level, M+2O and M+4O levels were more stable in the DHA-TAG and -EE samples (**I**) than in the *sn*-1 DHA and *sn*-3 DHA samples (**II**). Possibly, the formation rate of M+2O was lower in study **II** due to lower oxidative interactions in the TAGs with just one DHA, resulting in a steeper decrease once the induction period ended and fast LOOH decomposition started.

5.8.2 Samples with antioxidant

Oxidation kinetics on a percentual oxidation indicator evolution level for DHA-TAG and DHA-EE with 0.2% α-TOC (**I**) and *sn*-1, *sn*-2, and *sn*-3-DHA with 0.14% *RRR*-α-TOC (**II**) is presented in **Figure 18**. In the DHA-TAG samples (**I**), there was an 18-hour induction period for oil depletion and formation of M+4O and total volatiles. The induction period ended when α-TOC was depleted. M+2O levels increased fast from the beginning due to the increased hydrogen availability from α-TOC and started to decrease when α-TOC was consumed. In the DHA-EE samples (**I**), the corresponding induction time was 24 h, and thus it was the more stable lipid structure in the presence of 0.2% α-TOC.

The stability difference could be ascribed to the reduced viscosity of EE oil, which promoted more efficient interaction with α-TOC. However, it is also possible that the differences were due to dissimilar interactions with α-TOC in the association colloids. TAG hydroperoxides were reported to be more surface-active than EE hydroperoxides in the presence of surfactants (Homma et al., 2015). However, the nature of association colloids formed in oil with just α-TOC and DHA-TAG or -EE oxidation products is unclear. Also, Song et al. (1997) reported better stability for EE when compared to TAG in egg yolk lipids containing 10.7 mol% DHA and tocopherols. Conversely, other studies have reported better stability for TAG form in oils with added antioxidants (Martín et al., 2012; Sullivan Ritter et al., 2015).

In the presence of *RRR*-α-TOC (**II**), *sn*-2 DHA was clearly the most stable structure (**Figure 18**). Nevertheless, the rapid elevation in M+2O levels in *sn*-2 DHA occurred concurrently and reached nearly identical levels with the *sn*-1/3 samples, although M+4O and total volatile levels remained low. The stability of *sn*-1 DHA was slightly better than that of *sn*-3 DHA, which was observed in the

later peaking of M+2O and M+4O levels and slower oil and *RRR*-α-TOC depletion. Improved stability of the *sn*-1 enantiomer could indicate diastereomeric interactions between DHA in the *sn*-1 position and *RRR*-α-TOC. The oxidative stability of enantiopure TAGs and any potential variation in the antioxidant response between enantiomers have not been studied earlier. However, existing studies report different antioxidant activities for phenolic acid enantiomers *in vitro* (Liu et al., 2014) and suggest that chirality can influence oxidation kinetics in low-temperature combustion (Danilack et al., 2021; Doner et al., 2022). It could be possible that the enantiomers orientate and interact differently with *RRR*-α-TOC in the association colloids.

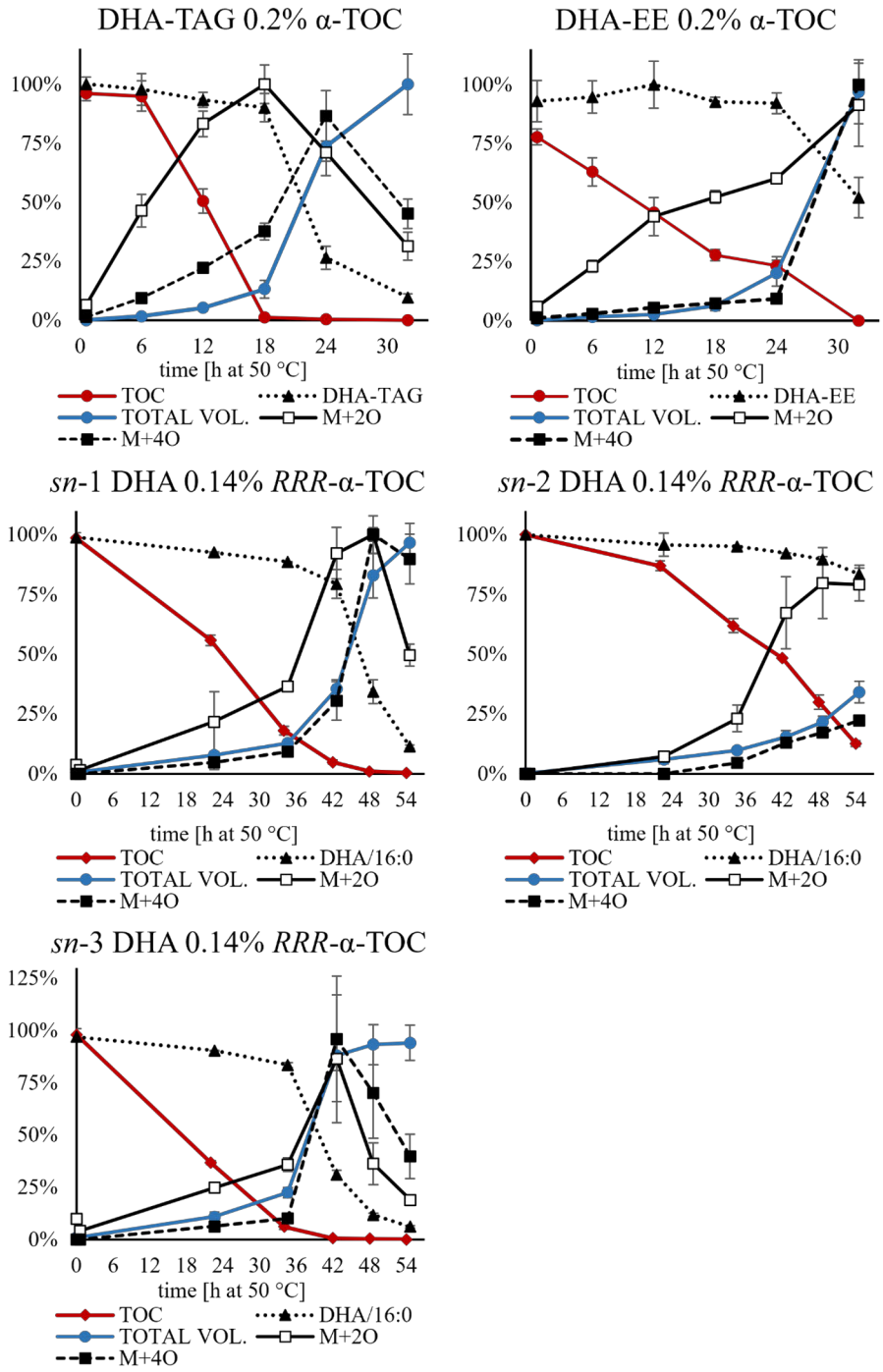


Figure 18. Oxidation kinetics of DHA-TAG/EE + 0.2% α-TOC (**I**) and *sn*-1, *sn*-2, and *sn*-3-DHA + 0.14% *RRR*-α-TOC (**II**). Data is shown as % of maximum value for each indicator (**I**, **II**), except for substrate, M+2O, and M+4O in study **I**, for which % of maximum value from α-TOC/non-α-TOC runs was used.

In study **III**, the oxidative stability of DHA-TAG was studied in the presence of 0.05% α-TOC and 0.05% α-TOC + 1% d18:0 (**Figure 19**). The 0.05% α-TOC concentration was used because previous studies (Shimajiri et al., 2013; Suzuki-Iwashima et al., 2021; Uemura et al., 2016) suggested the formation of antioxidative reaction products at this antioxidant level and 1:20 α-TOC:d18:0 ratio. The oxidation rate in the DHA-TAG samples with both antioxidants was faster than in the samples with just α-TOC during the first 12 hours of oxidation. However, after 12 h, the oxidation rate decreased, and overall, DHA-TAG with α-TOC + d18:0 was better preserved than DHA-TAG with only α-TOC.

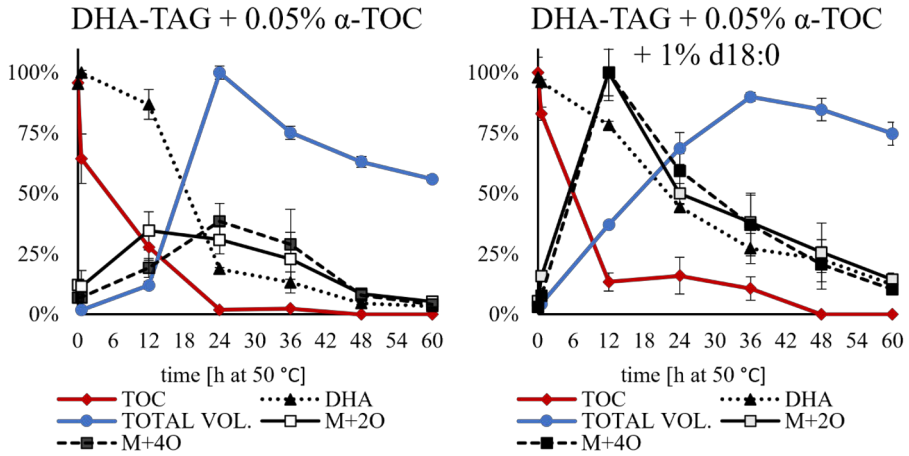


Figure 19. Oxidation kinetics of DHA-TAG with 0.05% α-TOC and 0.05% α-TOC + 1% d18:0. Data is shown as % of maximum value for each indicator.

The increased stability after 12 h indicated the formation of antioxidative compounds through carbonyl-amine reactions. Improved stability for fish oil with both α-TOC + d18:0 compared to α-TOC alone has been reported also earlier (Shimajiri et al., 2013; Suzuki-Iwashima et al., 2021; Uemura et al., 2016), and the reaction products of d18:0/d18:1 with propanal/2-propenal have shown antioxidant activity (Suzuki-Iwashima et al., 2021). Carbonyl-amine reaction products may have acted as radical-scavenging antioxidants or regenerated α-TOC, resulting in decreased α-TOC consumption after 12 h. Similar behavior in α-TOC decomposition was reported by Shimajiri et al. (2013), who also showed that d18:0 and α-TOC had no antioxidant activity in tricaprylin in the absence of oxidation products. To draw a distinct conclusion on the synergistic effect of d18:0 and α-TOC, including samples for all time points also for d18:0 alone would have been necessary.

The addition of 1% d18:0 resulted in increased formation of hydroperoxides and hydroxides, and decreased formation of volatile oxidation products. Lower levels of propanal, 2-propenal, 1-penten-3-one, 1-penten-3-ol, and 1-hydroxy-2-

butanone were observed at 24 h and thereafter in the samples with both antioxidants. A decrease in the volatile oxidation product levels was also reported earlier (Suzuki-Iwashima et al., 2021; Uemura et al., 2016). In the samples with both antioxidants, monohydroperoxide and M+4O levels increased simultaneously during the first 12 hours, which might indicate too fast radical formation for α-TOC stabilization. The low stability from zero to 12 hours may have been influenced by the amphiphilicity of d18:0, which led to slight dropleting in the bottom of the SPME vial and increased surface area exposed to the air. Applying a higher oil amount might have prevented this effect. Overall, the 0.05% α-TOC level for pure DHA-TAG stabilization resulted in quite harsh oxidation conditions, and applying a lower temperature (40 °C) might have enabled closer investigation of the kinetics at the early stages of oxidation. The α-TOC/d18:0 ratio of 1:20 was chosen after stability tests with HS-SPME-GC-MS, comparing the ratios 1:20, 1:10, and 1:5. Further investigations are needed to assess the antioxidant efficacy of d18:0 and α-TOC across varying temperatures and antioxidant concentrations.

In study **III**, the reduced quantity of α-TOC (0.05%) compared to study **I** (0.2%) with the same substrate (DHA-TAG) did not cause any significant temporal differences in the oxidation kinetics. The fast formation of total volatiles started simultaneously with the M+2O maximum and accelerated substrate depletion in both studies (**I**, **III**). Also, the maximum level of M+4O was reached later than that of M+2O. However, α-TOC was depleted later in study **III**, after the rapid oxidation phase had already started. This might be due to the high amount of preformed hydroperoxides, which started the exponential phase before α-TOC was consumed. The transition to the exponential phase is determined by the accumulation of critical hydroperoxide level ([LOOH]/[α-TOC]) (Kamal-Eldin, 2006; Witting, 1969). In the *sn*-1 and *sn*-3 DHA samples (**II)**, the M+2O and M+4O maximum levels were reached concurrently, and the difference between M+2O and M+4O level evolutions was less substantial than in the DHA-TAG and -EE samples (**I**). Observed differences in the oxidation kinetics are likely due to the higher amount of oxidizable substrate and available hydrogens in the DHA-TAG and -EE samples.

6 SUMMARY AND CONCLUSION

The applied analytical approach enabled comprehensive examination of lipid oxidation and formed non-volatile and volatile oxidation products from a small lipid amount. This kind of approach is valuable when analyzing oxidation patterns of expensive commercial or synthesized lipids. Utilizing pure compounds with equalized DHA and antioxidant concentrations helped mitigate the impact of extraneous variables on the results. One notable strength of this study was the synthesis of the regio- and enantiopure DHA standards, for which stability studies have not been conducted earlier due to their unavailability.

Studies **I** and **II** demonstrated that the oxidative stability of DHA is influenced by the specific lipid structure into which it is integrated. Moreover, lipid structures were observed to interact differently with tocopherols. Tridocosahexaenoin was found to be more stable than DHA-EE without added α-TOC, while in the presence of 0.2% α-TOC, the opposite was observed. In the TAGs with two palmitic acids and one DHA at the *sn*-1, *sn*-2, or *sn*-3 position, DHA at the *sn*-2 position was the most stable structure, both with and without 0.14% *RRR*-α-TOC. Without antioxidant, there was no significant difference between the *sn*-1 and *sn*-3 positions, while with *RRR*-α-TOC, the *sn*-1 position was slightly more stable than the *sn*-3 position. Increased stability might indicate favorable diastereomeric interactions between the enantiopure *RRR*-α-TOC and DHA at the *sn*-1 position. This study represents the pioneering investigation into the stability of enantiopure *sn*-1 and *sn*-3 TAGs. Diastereomeric interactions were revealed, but further research is needed to elucidate their role in the antioxidant response.

In study **III**, DHA-TAG stability was increased due to d18:0 addition. Stability improvement after the initial stages of oxidation supported the hypothesis of antioxidative carbonyl-amine reaction product formation from the d18:0 amine group and oxidation product carbonyls. Some of the formed imine structures could be tentatively identified for the first time. This was also the first study reporting the effects of d18:0 addition on the total oxidation pattern, including numerous volatile and non-volatile oxidation products. The low levels of several volatile oxidation products in the d18:0-containing samples, including propanal, 2-propenal, and 1-penten-3-one, indicated consumption in carbonyl-amine reactions. Application of d18:0 for increasing the stability of DHA-rich oils showed promise. However, additional research is required to determine the optimal concentrations, assess the impact of temperature on the formation of reaction products, and understand the alterations in physical properties induced by d18:0. Also, the formed antioxidants and their mechanisms of action remain undiscovered.

DHA oxidation patterns and formed oxidation products were reported comprehensively for the first time in studies **I-III**. In the absence of antioxidants, oxidation proceeded fast within the same acyl chain/ molecule, resulting in the formation of polyhydroperoxides/cyclic structures, while antioxidant addition could delay their formation (**I-II**). The higher hydrogen availability from DHA acyl chains in the DHA-TAG and -EE samples (**I**, **III**) also led to overall higher proportions of polyhydroperoxides/cyclic structures than in the regio- and enantiopure samples with just one DHA (**II**). On the other hand, the proportion of epoxides/hydroxides and acyl chain cleavage products was increased in the regio- and enantiopure samples. In the DHA-EE samples, also dimerization occurred at the relatively early stages of oxidation. These structure-related changes in the oxidation product proportions can influence the results of various analytical methods used to assess oxidative status.

2,4-Heptadienal, 1-penten-3-ol, and 2-propenal were among the main volatile oxidation products formed in DHA oxidation, and they could be potential oxidation indicators for DHA-rich oils. High hydrogen availability appeared to promote the formation of 2-propenal, whereas, under conditions with lower hydrogen availability, the formation of propanal increased. Nevertheless, given the complex nature of DHA oxidation, even the acquired data is not detailed enough to enable drawing definite conclusions on DHA oxidation reaction pathways. The multiplicity of differing reaction routes that might follow each reaction step make also kinetical analysis of lipid oxidation challenging. Even so, investigations focusing on quantified data on specific oxidation products would be valuable for further enhancing the understanding of DHA oxidation mechanisms.

In this thesis the differences in DHA stability and antioxidant interactions between lipid structures were observed, but their underlying causes could not be elucidated. Detected differences are likely attributed to diverse interactions within the association colloids. These interactions are influenced by several factors, including the structure of the lipid substrate, the nature and concentration of antioxidants, and the structure and concentration of other surface-active components, including oxidation products. Foreseeing these interactions is not feasible with the currently available analytical methods, and this area of research will likely continue to evolve. Revealing the conformational structures of different DHA-containing lipid molecules in neat oils, and the effect of TAG *sn*-position on the conformation, would be essential for understanding the observed results. Furthermore, future research should incorporate also alternative antioxidants besides α-TOC, with varying concentrations and oxidation temperatures. Also, studies with less pure substrates would contribute to validating the present findings.

This thesis elucidated the role of lipid structure and different antioxidant strategies on the stabilization of DHA. Also, an analytical approach for comprehensive lipid oxidation product analysis from a small lipid amount was introduced. The findings can be utilized in future research on lipid oxidation and antioxidant strategies, as well as for formulating DHA-rich oils with improved stability. Currently DHA-rich oils are utilized by omega-3 industry for supplement production, in fortification (e.g. of infant formulas) and as feed ingredients. Enzymatical approaches enable the production of structured lipids with DHA incorporated to desired TAG *sn*-positions.

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APPENDIX: ORIGINAL PUBLICATIONS

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DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.

36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.
41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.
44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.
45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.
55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.
56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.
57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.
58. **HEIKKI ARO (2012)** Fractionation of hen egg and oat lipids with supercritical fluids. Chemical and functional properties of fractions.
59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.
60. **MARKO TARVAINEN (2013)** Analysis of lipid oxidation during digestion by liquid chromatography-mass spectrometric and nuclear magnetic resonance spectroscopic techniques.
61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.
62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.
63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.
64. **LOTTA NYLUND (2015)** Early life intestinal microbiota in health and in atopic eczema.
65. **JAAKKO HIIDENHOVI (2015)** Isolation and characterization of ovomucin – a bioactive agent of egg white.
66. **HANNA-LEENA HIETARANTA-LUOMA (2016)** Promoting healthy lifestyles with personalized, *APOE* genotype based health information: The effects on psychological-, health behavioral and clinical factors.
67. **VELI HIETANIEMI (2016)** The *Fusarium* mycotoxins in Finnish cereal grains: How to control and manage the risk.
68. **MAARIA KORTESNIEMI (2016)** NMR metabolomics of foods – Investigating the influence of origin on sea buckthorn berries, *Brassica* oilseeds and honey.
69. **JUHANI AAKKO (2016)** New insights into human gut microbiota development in early infancy: influence of diet, environment and mother's microbiota.
70. **WEI YANG (2017)** Effects of genetic and environmental factors on proanthocyanidins in sea buckthorn (*Hippophaë rhamnoides*) and flavonol glycosides in leaves of currants (*Ribes* spp.).
71. **LEENAMAIJA MÄKILÄ (2017)** Effect of processing technologies on phenolic compounds in berry products.
72. **JUHA-MATTI PIHLAVA (2017)** Selected bioactive compounds in cereals and cereal products – their role and analysis by chromatographic methods.

73. **TOMMI KUMPULAINEN (2018)** The complexity of freshness and locality in a food consumption context
74. **XUEYING MA (2018)** Non-volatile bioactive and sensory compounds in berries and leaves of sea buckthorn (*Hippophaë rhamnoides*)
75. **ANU NUORA (2018)** Postprandial lipid metabolism resulting from heated beef, homogenized milk and interesterified palm oil.
76. **HEIKKI AISALA (2019)** Sensory properties and underlying chemistry of Finnish edible wild mushrooms.
77. **YE TIAN (2019)** Phenolic compounds from Finnish berry species to enhance food safety.
78. **MAIJA PAAKKI (2020)** The importance of natural colors in food for the visual attractiveness of everyday lunch.
79. **SHUXUN LIU (2020**) Fermentation with non-*Saccharomyces* yeasts as a novel biotechnology for berry wine production.
80. **MARIKA KALPIO (2020)** Strategies for analyzing the regio- and stereospecific structures of individual triacylglycerols in natural fats and oils.
81. **JOHANNA JOKIOJA (2020)** Postprandial effects and metabolism of acylated anthocyanins originating from purple potatoes.
82. **NIINA KELANNE (2021)** Novel bioprocessing for increasing consumption of Nordic berries.
83. **NIKO MARKKINEN (2021)** Bioprocessing of berry materials with malolactic fermentation.
84. **GABRIELE BELTRAME (2021)** Polysaccharides from Finnish fungal resources.
85. **SALLA LAITO (2022)** Bioactive compounds in oats and gut health.
86. **KANG CHEN (2022)** Multi-omics study on the effects of anthocyanin extracts from bilberries and purple potatoes on type 2 diabetes in Zucker diabetic fatty rats.
87. **WENJIA HE (2022)** Bioprocessing of alcoholic beverages from apples and pears: Effects of raw materials and processes on quality.
88. **TANJA KAKKO (2023)** Alternative approaches to improve the processing and quality of under-utilized fish.
89. **MIKAEL FABRITIUS (2023)** Mass spectrometric methodologies for analysis of triacylglycerol and phospholipid regioisomers in natural fats and oils.
90. **ELLA AITTA (2023)** Green technologies for the extraction of oil and protein from Baltic herring (*Clupea harengus membras*).
91. **AMRUTA KULKARNI (2023)** Effect of omega-3 deficiency and positional distribution of docosahexaenoic acid in triacylglycerols on tissue lipids in rats.
92. **LIZ A. GUTIÉRREZ QUEQUEZANA (2023)** Effect of cultivar, growth environment and developmental stage on phenolic compounds and ascorbic acid in potato tubers grown in Finland.
93. **MINNA ROTOLA-PUKKILA (2024)** The umami compounds in Nordic food raw materials and the effect of cooking.
94. **EIJA AHONEN (2024)** Impact of lipid structure and selected antioxidants on the oxidation of docosahexaenoic acid.

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