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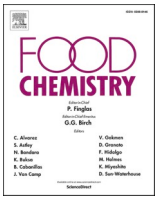

Oxidative stability, oxidation pattern and α-tocopherol response of docosahexaenoic acid (DHA, 22:6*n*–3)-containing triacylglycerols and ethyl esters

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ABSTRACT

DHA is most often used in supplements either in its triacylglycerol or ethyl ester form. Currently, there is only little published data on the differences in the oxidative stability and α-tocopherol response between the two lipid structures, as well as on the oxidation patterns of pure DHA. This study investigated the oxidative stability, α-tocopherol response and oxidation pattern of DHA incorporated in triacylglycerols and as ethyl esters with an untargeted approach after oxidation at 50 °C in the dark. Liquid and gas chromatographic methods with mass spectrometric detection and nuclear magnetic resonance spectroscopy were applied. DHA was more stable in triacylglycerols than as ethyl esters without α-tocopherol addition. With α-tocopherol added the opposite was observed. The oxidation products formed during triacylglycerol and ethyl ester oil oxidation were mostly similar, but also some structure-related differences were detected in both volatile and non-volatile oxidation products.

1. Introduction

Long-chain omega-3 polyunsaturated fatty acid (*n*–3 LC-PUFA) DHA (docosahexaenoic acid, 22:6*n*–3) is primarily obtained from marine resources into the human diet. DHA affects neuronal signaling and vision, and its sufficient intake during fetal development and infancy is necessary for normal mental and visual development. DHA also reduces inflammation, improves immune function, and optimizes cellular metabolism, hence lowering the risk of insulin resistance, metabolic syndrome, hyperlipidemia, and cardiovascular disease (Calder, 2016). Despite the well-documented health benefits of DHA, the intake is globally low (Micha et al., 2014). In addition to fatty fish and other marine resources, DHA is consumed as dietary supplements and functional foods fortified with these fatty acids. The challenge industry is facing when utilizing *n*–3 LC-PUFAs is their proneness to oxidation due to their high degree of unsaturation. Unsaturated lipids are typically oxidized by free radical chain reaction (autoxidation) through initiation, propagation, and termination steps, yielding lipid hydroperoxides and other oxygenated, volatile, and non-volatile compounds. In the initiation step, hydrogen is lost from the lipid acyl chain to a free radical and lipid radical is formed. Oxygen adds to the lipid radical in the propagation step, forming peroxyl radical, which can abstract hydrogen from another lipid acyl chain, forming lipid hydroperoxide and propagating the reaction further. In the termination step two radicals react to form non-radical products (Frankel, 1980). Oxidation causes disagreeable flavors as well as a decline in nutritional quality and safety. The addition of antioxidants, inactivation of pro-oxidant metals, and minimization of exposure to heat, light, and oxygen suppress oxidation. Tocopherols are naturally occurring antioxidants, which are commonly used to protect *n*–3 LC-PUFAs in dietary supplements and functional foods. Tocopherols scavenge free radicals and thus slow down the oxidation rate by donating hydrogen atoms from their hydroxy group to peroxyl radicals, forming resonance-stabilized tocopheroxy radicals which do not propagate the reaction (Choe & Min, 2006).

Fatty acids are naturally present in or can be chemically modified to be included in different lipid structures. In fish and algae oils, most fatty acids are bound to the triacylglycerol (TAG) structure, where three fatty acids are esterified to a glycerol backbone. Industrial processes which concentrate *n*–3 LC-PUFA require cleavage of these FAs from the TAG structures followed by their stabilization and subsequent separation in

Abbreviations: DHA, Docosahexaenoic acid; LC-QTOF, Liquid chromatography-quadrupole-time-of-flight mass spectrometry.

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the form of ethyl esters (EEs). The bioavailability of the EE form is found to be inferior when compared to the TAG form, especially from low-fat meals (Schuchardt & Hahn, 2013), which is one reason for adding an additional step to the process to transesterify the EEs to the "natural" TAG form. Previous studies about the oxidative stability differences between DHA-containing EE and TAG oils mostly suggest that the TAG structure is more stable (Lee et al., 2003; Martín et al., 2012; Sullivan Ritter et al., 2015; Yoshii et al., 2002). However, the results are somewhat disputable due to the differences in the fatty acid and α-tocopherol concentrations or oxidative state in the oils at the beginning of the oxidation trial. Also, one study with higher stability of the EE form compared to the TAG form (Song et al., 1997) exists. There are only a few studies investigating the autoxidation products of pure DHA (Kawai et al., 2007; Lyberg et al., 2005; Noble & Nawar, 1971, 1975). To our knowledge, there is currently no published data about the possible differences in the α-tocopherol response between purified DHA in EE and TAG structures.

This research hypothesized that the oxidative stability, oxidation pattern, and α-tocopherol response of DHA are influenced by the lipid structure (TAG/EE). Hence, the aim of the study was to obtain a comprehensive view of the differences in the oxidation pattern, oxidative stability, and α-tocopherol response between DHA in TAGs and EEs (hereafter DHA-TAG and DHA-EE oils) by using an omics-type, untargeted analytical approach. The oxidative stability, oxidation pattern, and α-tocopherol response of pure DHA-containing TAG and EE oils were examined during an oxidation trial at 50 °C degrees in the dark with and without added α-tocopherol. For a detailed picture of lipid oxidation pattern, both volatile and non-volatile oxidation products were analyzed using gas (GC) and liquid chromatographic (LC) methods coupled with mass spectrometry (MS), and with nuclear magnetic resonance (NMR) spectroscopy. The change in α-tocopherol concentration during oxidation was examined by normal-phase high-performance liquid chromatography with fluorescence detection (NP-HPLC-FLD).

2. Materials and methods

2.1. Samples

Tridocosahexaenoin > 99% and ethyl docosahexanoate > 99% originated from Larodan (Solna, Sweden) and α-tocopherol > 96% from Sigma–Aldrich (Buchs, Switzerland). Samples were prepared with and without added α-tocopherol. α-Tocopherol stock solution was prepared in ethanol (Altia, Rajamäki, Finland), and its concentration was determined according to Podda et al. (1996) with Evolution 300 BB UV–VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA). α-Tocopherol stock solution representing 0.2% of the oil amount was transferred into volumetric flasks and ethanol evaporated under nitrogen prior to adding the oil solution. DHA-TAG and DHA-EE oils were diluted into *n*-hexane (VWR, Gliwice, Poland) and volumes representing similar molar amounts of DHA (EE/TAG molar ratio 3/1; 20.00 mg /19.14 mg) were transferred into 10 mL amber SPME-vials. The headspace was gently covered with nitrogen and vials cooled (– 20 °C, 20 min), sealed with parafilm, and stored at – 80 °C until the beginning of the oxidation trial. Storage time was 2.5 weeks maximum. Dim lighting conditions were employed during all sample preparation to reduce sample oxidation.

2.2. Oxidation trials

Hexane was evaporated with nitrogen from the vials and replaced with compressed air to provide oxygen for the oxidation reaction. Samples without α-tocopherol were oxidized for 10 h (sampling at 0.6 h, 2 h, 4 h, 6 h, 8 h, and 10 h) in GC–MS autosampler Tray Cooler MC 03–03 Rev. A (PAL System, CTC Analytics AG, Zwingen, Switzerland), which was set to 50 °C (at the vial bottom). Samples with α-tocopherol were oxidized in Hewlett Packard 6890 Series Plus G1530A GC oven (Wilmington, DE, USA) at 50 °C for 32 h (sampling at 0.6 h, 6 h, 12 h, 18 h, 24 h and 32 h). Different oxidation times were chosen to be able to examine all the kinetical phases of lipid oxidation including induction period, exponential increase of hydroperoxides, and decomposition of hydroperoxides/exponential increase of secondary oxidation products, in both oil types (with and without α-tocopherol). Oxidation was carried out in the original SPME-vial without stirring. SPME-GC–MS analysis started immediately after the oxidation period. Oxidation temperature of 50 °C was chosen to have a reasonably short oxidation time for ensuring repeatable SPME-GC–MS conditions and to still have a fairly similar oxidation pattern when compared to ambient temperatures. After GC-injection the sample vial was cooled to room temperature, 1 mL chloroform from Sigma–Aldrich (St. Louis, MO, USA) added, and vial placed to – 20 °C for 20 min. After cooling, the headspace was gently filled with nitrogen and the vial moved to – 80 °C for further analysis. Three replicates were prepared for each time point.

2.3. Volatile oxidation products (HS-SPME-GC–MS)

Volatile secondary oxidation products (VSOPs) were analyzed to track the differences in the oxidative stability and oxidation pattern between DHA in TAG and EE oils in real-time as the oxidation proceeded. VSOPs were analyzed with a method presented previously (Damerau et al., 2020). HS-SPME injector and Thermo Scientific GC–MS instrument consisting of Trace 1310 GC, ISQ 7000 mass spectrometer, TriPlus RSH autosampler (Waltham, MA, USA), SPB®-624 capillary column (60 m × 0.25 mm × 1.4 μm, Supelco, Bellefonte, PA, USA) and DVB/CAR/PDMS 50/30 μm (Supelco, Bellefonte, PA, USA) fiber were employed. The temperature for sample incubation (1 min) and extraction (30 min) was 50 °C. Desorption (5 min) temperature in GC-injector port was 240 °C (splitless injection) and column temperature: 40 °C held for 6 min, 5 °C/min to 200 °C, held for 10 min. Helium (1.4 mL/min) was used as carrier gas. Electron ionization at 240 °C and 70 eV was employed for the MS and mass to charge ratios were scanned between 40 and 300 amu. Compound identification was based on external standards and NIST MS Search library (version 2.4, National Institute of Standards and Technology, Gaithersburg, MD, USA). External standards included propanal, 2-methylfuran, 2-ethylfuran, 1-penten-3-ol, 2,4-hexadienal, *(E,E)*-2,4-heptadienal, and *(E)*-2-nonenal from Sigma–Aldrich (Steinheim, Germany), butanal, *(E)*-2-hexenal, and *(E,Z)*-2,6-nonadienal from Acros Organics (New Jersey, USA) and (*E*)-2-pentenal from Fluka (Buchs, Switzerland). Alkane standard (C7–C30, Supelco, Bellefonte, PA, USA) was used for calculating retention indexes. Acquired data was processed by Chromeleon 7.2.9 Chromatography Data System (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Non-volatile oxidation products (HPLC-QTOF)

HPLC-QTOF was applied in an untargeted manner for analysis of hydroperoxides and other non-volatile oxidation products as well as for observing the decrease in the levels of DHA-TAG and -EE during the oxidation trial. Compounds were analyzed with Elute UHPLC and Bruker Impact II QTOF instruments from Bruker Daltonic (Bremen, Germany) and Macherey-Nagel (Düren, Germany) HPLC column Nucleodur C18 Isis (250 mm × 4.6 mm, 5 μm particle size). The applied LC method was modified from the method developed by Tarvainen et al. (2010). Solvent A consisted of acetonitrile and water from Fisher Scientific (Loughborough, UK) and formic acid from VWR (Leuven, Belgium) in 50:50:0.1 by volume, in addition to 10 mM ammonium acetate from Sigma–Aldrich (Steinheim, Germany). Solvent B consisted of 2-propanol from Honeywell/Riedel de Haën (Seelze, Germany), water, and formic acid in 100:0.1:0.1 by volume, in addition to 10 mM ammonium acetate. The proportion of solvent B was increased from the initial level (60:40 A:B) to 60% in 4 min, to 95% in 23 min, kept for 5 min, reduced back to 40% in 0.5 min, and kept for 4.5 min. Run time was 37 min and total flow 0.8 mL/min, of which 0.37 mL/min was directed to the MS. The column oven temperature was set to 30 °C and autosampler cooler temperature

to 4 °C. Samples were injected in chloroform (2 μL injection volume). Electrospray ionization (ESI) with positive ionization mode was applied for the MS. ESI capillary voltage was set to 4.5 kV, end plate offset voltage to 500 V, dry gas flow (N_2) to 4 L/min, dry gas temperature to 350 °C and nebulizer pressure to 1.5 bar. Auto MS/MS scanning mode with a range from 60 to 2000 *m/z* was employed. The instrument was calibrated with sodium formate. Bruker Compass DataAnalysis 5.1 (Bruker Daltonic GmbH, Bremen, Germany) was applied for data processing.

2.5. NMR spectroscopy

^{1}H NMR spectroscopy was applied in an untargeted manner for monitoring the levels of oxidation products and degradation of the original molecule during the oxidation trial, as well as the development of the levels of lipid hydroperoxides and aldehydes by region-specific excitation. Selected time points were analyzed for samples without α-tocopherol (0.6 h, 4 h, and 10 h) and with α-tocopherol (0.6 h, 24 h, and 32 h). For the NMR analysis, an aliquot of 500 μL was taken from the samples and chloroform evaporated under nitrogen flow. 200 μL of $CDCl_3$ (Merck, Darmstadt, Germany) / DMSO-d_6 (VWR, Leuven, Belgium) (5:1) was added according to Merkx et al. (2018) and 180 μL of this solution transferred into 3-mm NMR tube. The samples were stored in – 20 °C (in a desiccator) until analysis. $CDCl_3$/DMSO-d_6 was dried prior to use with 4 Å molecular sieves (Sigma–Aldrich, Steinheim, Germany). The spectra were recorded at 298 K with a 600 MHz Bruker AVANCE-III NMR spectrometer (Bruker BioSpin, Switzerland), operating at 600.16 MHz (^{1}H), and equipped with a Prodigy TCI CryoProbe and a SampleJet robotic sample changer. The data collection was adopted from Merkx et al. (2018) with minor adjustments. Proton spectra (*zg30*) were collected with 32 scans, sweep width of 14 ppm, receiver gain of 32, acquisition time of 4 s, and relaxation time of 5 s. Selective gradient excitation (*selgpse*) was applied to collect selective ^{1}H spectra with 128 scans, 4 dummy scans, receiver gain of 32, acquisition time of 2.7 s, and relaxation time of 5 s. The length of the 180-degree shaped pulse was 1566.15 μs. The excitations were performed on regions δ 11.5–10.5 ppm and δ 10.0–9.0 ppm for hydroperoxides and aldehydes, respectively. NMR data was processed by TopSpin 4.0.6 (Bruker Biospin Corporation, Billerica, MA, USA) and Chenomx NMR Suite 8.6 (Chemomx Inc., Edmonton, AB, Canada).

2.6. α-Tocopherol concentration

α-Tocopherol concentrations were analyzed according to Schwartz et al. (2008) by NP-HPLC-FLD 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. Phenomenex OOG-4162-EO Luna 3 μm silica column (250 × 4.6 mm, pore size 100 Å; Torrance, CA, USA) was applied for 12 min separation run at 30 °C. Tray cooler temperature of SIL-20AC autosampler (Shimadzu, Kyoto, Japan) was set to 4 °C. Mobile phase (isocratic, 2 mL/min) consisted of 3% 1,4-dioxane (Sigma–Aldrich, Steinheim, Germany) and 97% heptane (Honeywell/Riedel de Haën, Seelze, Germany). Samples were transferred to heptane for analysis. For quantification, α-tocopherol standard curve was prepared using the α-tocopherol stock solution. Chromatographic data was analyzed by LabSolutions 5.93 (Shimadzu Corporation, Kyoto, Japan).

2.7. Statistical analysis

Data of volatile oxidation product levels (areas) and α-tocopherol concentrations were analyzed statistically using the IBM SPSS 28.0.0 statistical software (IBM Corporation, Armonk, NY, USA). Independent samples T-test was used to examine the differences in compound levels at each time point. P-values of $p \leq 0.05$ were considered statistically significant. For the NMR data principal component analysis (PCA) was performed to binned, log-transformed,mean-centered and Pareto-scaled data by SIMCA® 16 from Sartorius Stedim Data Analytics AB (Umeå, Sweden).

3. Results and discussion

3.1. Volatile oxidation products (HS-SPME-GC–MS)

The main route for the formation of VSOPs is the scissions of alkoxy radicals formed from different hydroperoxides, cyclic peroxides, and bicyclic endoperoxides. Also, breakdown products of polymers and further oxidized scissions products contribute to the formation of volatile compounds (Frankel, 1984). As an example, C20 alkoxy radical, formed from 20-OOH through O—O cleavage can cleave on either side of the alkoxy carbon, forming propanal and other volatile compounds (**Supplementary** Fig. 1). However, this is only one possible route for propanal formation, other sources for propanal are cleavage reactions of alkenals, polymers, hydroperoxy epidioxides, and hydroperoxy bisepidioxides (Table 1). In total 42 VSOPs were identified of which aldehydes, followed by esters, acids, and ketones were the most abundant compound groups (Table 1). Also furans, alcohols, epoxides, dienes, and phenols were detected. Suggested sources of identified VSOPs and related literature are reported in Table 1.

Production of VSOPs was detected to be structure-dependent. 4-Oxo-hex-2-enal, 2,4-decadienal, and methyl ester of decanoic acid were detected only in DHA-TAG oils (with and without α-tocopherol), whereas ethanol and ethyl esters of propanoic, hex-4-enoic and hex-5-enoic acid were only found in DHA-EE oils (with and without α-tocopherol). Also Lee et al. (2003) detected ethanol and ethyl ester acids only in EE oil when they compared the VSOPs of DHA-enriched EE and TAG fish oils during accelerated storage at 80 °C with aeration. The addition of α-tocopherol affected little the type of volatiles formed in both DHA-TAG and -EE oil. However, 2,6-nonadienal and methyl esters of octanoic and nonanoic acid were exclusively observed in the DHA-TAG oil without α-tocopherol, and ethyl ester of decanoic acid was only detected in the DHA-EE oil without α-tocopherol (Table 1). As the oxidative state was less advanced in the trial with α-tocopherol addition than in the one without when the tracking was terminated (Fig. 1), certain volatiles may not have been yet formed in detectable quantities in the samples with added α-tocopherol. All other identified VSOPs were present in all oxidized samples. By the peak area the most abundant VSOPs were (*E,Z/E,E*)-2,4-heptadienal, 1-penten-3-ol, and formic acid in both DHA-TAG and -EE oils. Percentual area development of total and selected VSOPs (acetaldehyde, 2-propenal, acetic acid, 2-ethylfuran, 1-penten-3-ol, (*E,Z/E,E*)-2,4-heptadienal, and (*E,Z/E,E*)-3,5-octadien-2-one) is presented in Fig. 1.

In the case of both DHA-TAG and -EE oil without α-tocopherol addition, the amounts of acetaldehyde and 2-ethylfuran (Fig. 1**A and 1D**) first increased rapidly (2–4 h) and started to decrease quickly after that. This indicates rapid further reactions after initial formation for both compounds, which in case of acetaldehyde could mean oxidation to acetic acid (Fig. 1**C**). No induction period was detected for 2-ethylfuran in either DHA-TAG or -EE oil. 2-Propenal, 1-penten-3-ol, and (*E,Z/E,E*)-2,4-heptadienal showed similar formation behavior (Fig. 1**B, 1E, 1F**). For all compounds, the formation was most rapid until 6 h and at 8 to 10 h the amounts stabilized. The similar behavior can be related to similar formation route directly from monohydroperoxides (Table 1). For (*E,Z/E,E*)-3,5-octadien-2-one (Fig. 1**G**) and acetic acid (Fig. 1**C**) no leveling was perceived by 10 h. The total volatiles (Fig. 1**H**), acetaldehyde, 2-propenal, acetic acid, 1-penten-3-ol, (*E,Z/E,E*)-2,4-heptadienal and (*E,Z/E,E*)-3,5-octadien-2-one all displayed an induction period for up to 2 h for DHA-TAG oil, while for the DHA-EE oil no induction period could be observed. At 2 h, the levels of all VSOPs in Fig. 1, except for 2-ethylfuran, showed a significant difference between DHA-TAG and -EE. First VSOPs to show significantly higher levels for DHA-EE were 2-propenal ($p < 0.05$), 2-ethylfuran ($p < 0.01$) and acetic acid ($p < 0.01$), already at 0.6

Table 1
Volatile compounds identified by SPME-GC–MS in oxidized DHA triacylglycerol (TAG) and ethyl ester (EE) oils (T = DHA-TAG, E = DHA-EE, TA = DHA-TAG + α-tocopherol, EA = DHA-EE + α-tocopherol; (+) = detected in the current study, (-) = not detected in the current study).

Compound group	Compound	T	E	TA	EA	Match [a]	RT [b]	RI [c]	Possible source [d]	Source reference [e]
Acids (6)	Formic acid	+	+	+	+	957	16.1	–	Formaldehyde Malondialdehyde	Frankel, 1982; Vandemoortele et al., 2021
	Acetic acid	+	+	+	+	955	17.3	–	Acetaldehyde	Frankel, 1982
	Propanoic acid	+	+	+	+	951	21.6	785	Propanal	Frankel, 1982
	Butanoic acid	+	+	+	+	904	25.3	871	Butanal	Frankel, 1982
	3-Hexenoic acid	+	+	+	+	876	33.5	1084	Hexenal	Frankel, 1982
	Nonanoic acid	+	+	+	+	862	42.4	1346	Nonanal	Frankel, 1982
Alcohols (2)	Ethanol	–	+	–	+	939	8.9		EE ethyl group	Lee et al., 2003
	1-Penten-3-ol	+	+	+	+	895 *	19.3	737	17-OOH	Lee et al., 2003
Aldehydes (14)	Acetaldehyde	+	+	+	+	967	6.4	–	Alkenals, polymers Malondialdehyde	Frankel, 1982; Vandemoortele et al., 2021
	2-Propenal	+	+	+	+	902	9.7	–	17,20-Dihydroperoxides, hydroperoxy epidioxides, hydroperoxy bisepidioxides	Endo et al., 2013; Frankel et al., 1983
	Propanal	+	+	+	+	867 *	9.8	–	20-OOH, alkenals, polymers, hydroperoxy epidioxides, hydroperoxy bisepidioxides	Frankel, 1982; Frankel et al., 1983
	Butanal	+	+	+	+	877 *	14.4	–	Alkenals, hydroperoxy epidioxides, hydroperoxy bisepidioxides	Frankel, 1982; Frankel et al., 1983
	2-Butenal	+	+	+	+	876	18.0	708	Hydroperoxy bisepidioxides, alkenals	Frankel et al., 1983, 1984
	2-Pentenal (*Z*)	+	+	+	+	922	22.3	799	17-OOH, alkenals, polymers	Frankel et al., 1981; Frankel, 1982
	2-Pentenal (*E*)	+	+	+	+	797 *	22.9	814	17-OOH, alkenals, polymers	Frankel et al., 1981; Frankel, 1982
	3-Hexenal	+	+	+	+	925	24.3	848	16-/17-OOH	Frankel et al., 1981
	2-Hexenal (*E*)	+	+	+	+	891 *	27.2	915	16-/17-OOH	Frankel et al., 1981
	2,4-Hexadienal (*E*, *E*)	+	+	+	+	893 *	29.9	984	Dihydroperoxides	Frankel et al., 1984
	4-Oxohex-2-enal	+	–	+	–	923	32.4	1054	16,20-Dihydroperoxides	Long & Picklo, 2010
	2,4-Heptadienal	+	+	+	+	920	33.1 33.7	1073 1089	16-OOH, dihydroperoxides, hydroperoxy epidioxides, bicycloendoperoxides	Frankel et al., 1981; Frankel et al., 1983; Frankel et al., 1984
	2,6-Nonadienal	+	–	–	–	796 *	38.4	1232	13-/14-OOH	Frankel et al., 1981
	2,4-Decadienal	+	–	+	–	906	44.8	–		
Ketones (5)	1-Penten-3-one	+	+	+	+	918	19.1	733	17-OOH	Hammer & Schieberle, 2013
	3-Hexanone	+	+	+	+	945	23.7	833		
	2-Hexanone	+	+	+	+	937	24.1	843		
	3,5-Octadien-2-one	+	+	+	+	895	35.5 36.4	1144 1171	14-OOH-13,16-epidioxide	Noble & Nawar, 1975
	5-Ethyl-2(5*H*)-furanone	+	+	+	+	825	36.3	1168	3-Hexenal	Buttery & Takeoka, 2004
Furans (3)	2-Methylfuran	+	+	+	+	822	14.0	–	2-Pentenal	Adams et al., 2011
	2-Ethylfuran	+	+	+	+	921 *	18.7	724	2-Hexenal Hydroperoxy epidioxides	Adams et al., 2011 Frankel et al., 1983
	(*E*)-2-(2-Pentenyl) furan	+	+	+	+	901	31.6	1031	13-OOH	Pan et al. 2005
Esters (8)	Propanoic acid, ethyl ester	–	+	–	+	920	19.5	742	4-OOH	Noble & Nawar 1971
	Hex-5-enoic acid, ethyl ester	–	+	–	+	880	31.3	1022		
	Hex-4-enoic acid, ethyl ester	–	+	–	+	866	32.1	1045	7-OOH	Noble & Nawar 1971
	Octanoic acid, methyl ester	+	–	–	–	869	36.0	1159		
	Nonanoic acid, methyl ester	+	–	–	–	826	39.2	1257		
	Decanoic acid, methyl ester	+	–	+	–	936	42.8	1356		
	Decanoic acid, ethyl ester	–	+	–	–	944	45.8	–		
	Undecanoic acid, methyl ester	+	+	+	+	944	47.3	–		
Dienes (1)	2,4-Octadiene	+	+	+	+	888	23.3	824	14-OOH	Noble & Nawar, 1971
Epoxides (2)	2-Methyl-3-vinyl-oxirane	+	+	+	+	920	16.7	–		
	2-Ethyl-3-vinyl-oxirane	+	+	+	+	875	21.3	779		
Phenols (1)	4-Ethylphenol	+	+	+	+	857	28.6	952		

[a] NIST match number (* = external standard).
[b] RT = retention time (min).
[c] RI = Kováts retention index.
[d] Possible source for the compound formation.
[e] Reference for the suggested formation pathway.

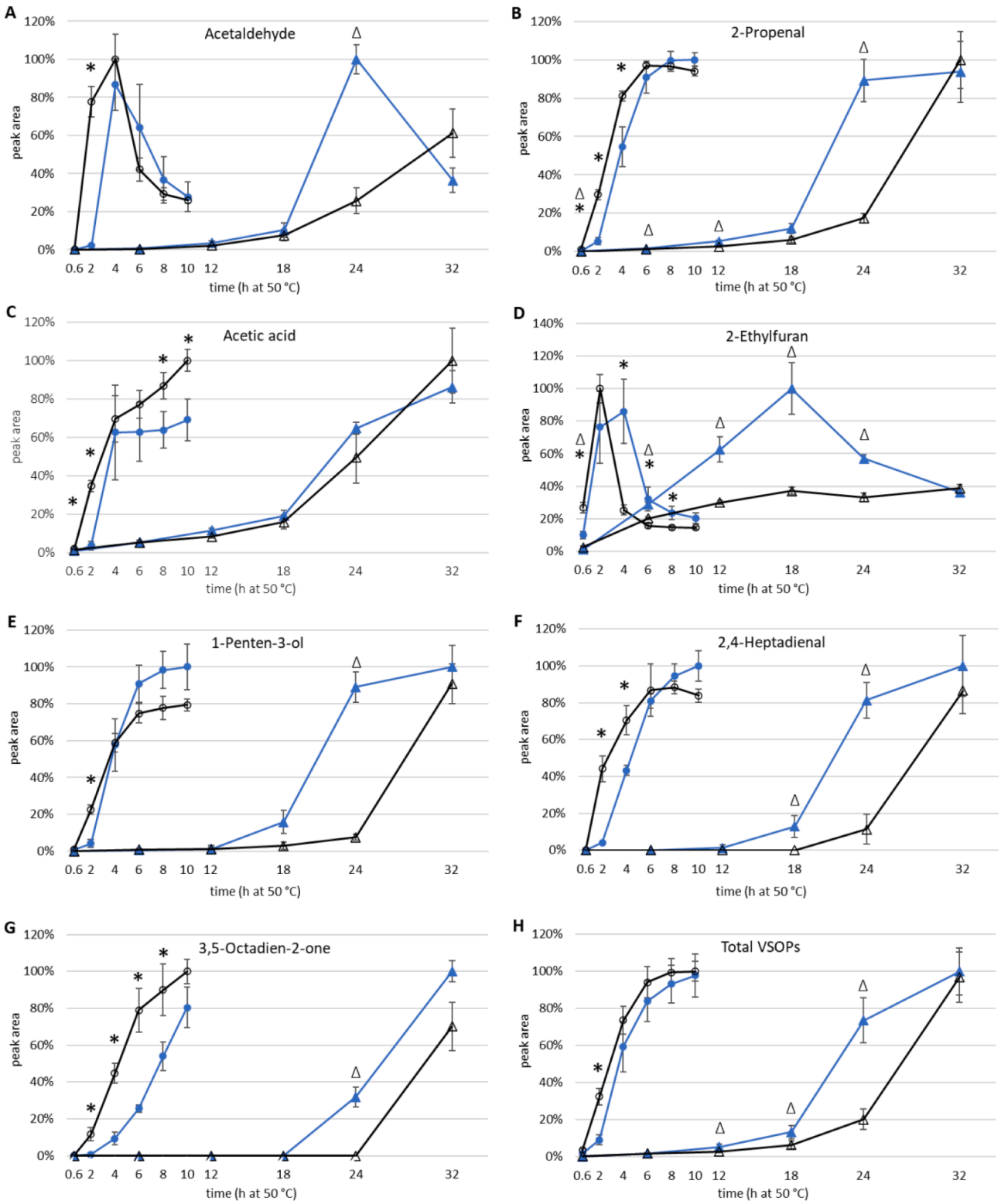

Fig. 1. Percentual area development of acetaldehyde (**A**), 2-propenal (**B**), acetic acid (**C**), 2-ethylfuran (**D**), 1-penten-3-ol (**E**), (*E,Z/E,E*)-2,4-heptadienal (**F**), (*E,Z/E,E*)-3,5-octadien-2-one (**G**) and total volatile secondary oxidation products (VSOPs) (**H**) of DHA-TAG (blue line, filled marker) and DHA-EE (black line, open marker) during oxidation at 50 °C in the dark without (circle) and with (triangle) 0.2% α-tocopherol. Values are mean ± standard deviation of three replicates. There is one 100% value (largest mean area) for the 0–10 h samples (without tocopherol) and one for 0–32 h samples (with tocopherol) for each volatile compound. Statistically significant difference ($p < 0.05$) between oxidation product levels in DHA-TAG and DHA-EE samples is marked with star (samples without antioxidant) and triangle (antioxidant-containing samples).

h. It can be concluded that without α-tocopherol the DHA-TAG oil had higher oxidative stability than DHA-EE oil.

The formation of VSOPs is delayed with the addition of α-tocopherol. For total VSOPs, acetaldehyde, 2-propenal, 1-penten-3-ol and (*E,Z/E,E*)-2,4-heptadienal nearly no formation was observed until 12 h, for both DHA-TAG and -EE oil with α-tocopherol addition. The (*E,Z/E,E*)-3,5-octadien-2-one levels started to increase later when compared to other analyzed VSOPs. No induction period in the formation of 2-ethylfuran was found for either DHA-TAG or -EE oil, and in the DHA-TAG oil a decrease was observed after 18 h. A similar decrease was seen for acetaldehyde after 24 h in the DHA-TAG oil. For all other volatiles presented in Fig. 1 no decline in the amount was noticed during the oxidation period of 32 h. For 2-propenal, 1-penten-3-ol and (*E,Z/E,E*)-2,4-heptadienal the rapid increase started at 18 h for the DHA-TAG oil and at 24 h for the DHA-EE oil. Also, the total VSOP formation was in line with these observations. An induction period of 18 and 24 h for DHA-TAG and DHA-EE oil, respectively, could be perceived. At 24 h 2-propenal ($p < 0.001$), 2-ethylfuran ($p < 0.001$), acetaldehyde ($p < 0.001$) 1-penten-3-ol ($p < 0.001$), 2,4-heptadienal ($p = 0.001$), 3,5-octadien-2-one ($p = 0.001$), and total volatile ($p < 0.001$) levels were higher in DHA-TAG. Acetic acid did not show any statistically significant difference between the two oil types at any point of the trial. Similarly, as in the case of non-antioxidant-containing samples, 2-propenal was among the first VSOP to show a significant difference between the oil types ($p < 0.05$) already at 0.6 h, 6 h, and 12 h, for higher levels in DHA-TAG. It can be concluded that the oxidative stability of DHA-EE oil compared with DHA-TAG oil was higher in the presence of α-tocopherol.

3.2. Non-volatile oxidation products (HPLC-QTOF)

The analytes detected with HPLC-QTOF typically formed sodium $[M + 23]^+$, ammonium $[M + 18]^+$, and potassium $[M + 39]^+$ adducts, of which the ammonium adducts were the most abundant for the non-oxidized compounds and the sodium adducts for the oxidation products. Due to the low signal-to-noise ratio and low concentration of oxidation products when compared to the non-oxidized compounds, extracted-ion chromatograms (EIC) were applied for collecting the peak area data (**Supplementary** Fig. 2). Altogether eight oxidation products were identified based on the fragmentation patterns for DHA-TAG oil and 11 for DHA-EE oil. Identified oxidation products are presented in Table 2.

Ammoniated non-oxidized DHA-TAG formed diacylglycerol (*m/z* 695.5) and DHA acyl chain ([RC = O + 74] *m/z* 385.3) fragments by auto MS/MS, while the corresponding DHA-EE fragments included DHA acyl chain ([RC = O] *m/z* 311.2), acyl chain with loss of water (18 Da) *m/z* 293.2 and acyl chain carbon–carbon bond cleavage fragments. For both DHA-TAG and -EE oils, compounds with additions of two oxygens (32 Da) were detected until + 10O for the DHA-TAG and + 8O for the DHA-EE. In addition to hydroperoxides these compounds can include additions of individual oxygens (e.g., epoxides and hydroxides) as well as cyclic hydroperoxy epidioxide, bicycloendoperoxide and polyperoxide structures. In the DHA-TAG M + 2O, the fragments consisted of loss of DHA ([M + Na-DHA] *m/z* 749.5) or loss of DHA + 2O ([M + Na-DHA-2O] *m/z* 717.5) from the sodiated molecular ion, as well as [DHA + Na + 2O] *m/z* 383.2. Also fragments from acyl chain cleavage beside the hydroperoxy-carbon with losses of e.g., 126 and 86 Da were detected, corresponding to 14-OOH and 17-OOH hydroperoxide isomers, as described by Ito et al. (2015). The DHA-TAG M + 4O fragmentation pattern was similar to M + 2O. For structures with additions of six or more oxygens, no fragmentation was observed in the auto MS/MS mode. The sodiated DHA-EE hydroperoxides formed distinctive fragments with losses of 46 Da [M + Na-CH_3CH_2OH] and 18 Da [M + Na-H_2O], as well as acyl chain cleavage fragments from the hydroperoxide site as described above. For both DHA-TAG and -EE also compounds with the addition of one oxygen (16 Da) [M + Na + O] were detected. In the DHA-TAG, the fragments consisted of loss of DHA ([M + Na-DHA] *m/z* 733.5) or loss of DHA + O ([M + Na-DHA-O] *m/z* 717.5), and [DHA + Na + O] *m/z* 367.2. In the DHA-EE a loss of water ([M + Na-H_2O] *m/z* 377.3) was detected, otherwise the sodium adduct fragments could not be connected to certain cleavage products. By the mass addition of one oxygen, the compound could be either an epoxide which is formed by the addition of peroxy radical to a double bond or through the cyclization of alkoxy radical (Xia & Budge, 2017), or a hydroxide. Since no significant amounts of hydroxides were detected in the NMR analysis described later, but instead epoxides were detected, it seems more likely that the compound was an epoxide.

Among the DHA-TAG oxidation products acyl chain cleavage products or "2 ½ glycerides" were detected, namely TG 22:6/22:6/(15:4 + 2O) and TG 22:6/22:6/(18:5 + 2O). The ammoniated molecular ion fragments consisted of diacylglycerols with either of the possible acyl chain combinations (*m/z* 695.5 with *m/z* 633.4 or *m/z* 673.4), and for the oxygenated acyl chain also with a loss of 18 Da (H_2O). In addition, individual acyl chains fragments were detected (*m/z* 249.1 for 15:4 + 2O and *m/z* 289.2 for 18:5 + 2O), similarly for the oxygenated acyl chain also with the loss of water. TAGs with one shortened fatty acyl chain can be formed from mono- and polyhydroperoxides or cyclic peroxides by the cleavage reactions described by Frankel (1984). Acyl chain cleavage products were not detected in the DHA-EE samples. For

Table 2
Compound mass, ammonium $[M + 18]^+$ and sodium $[M + 23]^+$ adduct masses (*m/z*), retention times (min) and main fragments (*m/z*, of the underlined adduct) for DHA-TAG, DHA-EE and identified oxidation products analyzed by HPLC-QTOF.

Compound	*m/z*	$[M + 18]^+$	$[M + 23]^+$	RT	Main fragments
DHA-TAG	1022.7	1040.7	1045.7	23.9	695.5 / 385.3
M + 2O	1054.7	1072.7	1077.7	18.8	383.2 / 749.5 / 717.5
M + 4O	1086.7	1104.7	1109.7	16.2	415.2 / 781.5 / 717.5
M + 6O	1118.7	1136.7	1141.7	14.4	–
M + 8O	1150.7	1168.7	1173.7	9.4	–
M + 10O	1182.7	1200.7	1205.7	8.6	–
Epoxide	1038.7	1056.7	1061.7	21.0	367.2 / 733.5 / 717.5
TG 22:6/22:6/(15:4 + 2O)	960.6	978.6	983.6	14.0	695.5 / 633.4 / 615.4 / 311.2 / 249.1 / 231.1
TG 22:6/22:6/(18:5 + 2O)	1000.6	1018.6	1023.6	15.0	695.5 / 673.4 / 655.4 / 311.2 / 289.2 / 271.1
DHA-EE	356.3	374.3	379.3	11.0	67.1 / 81.1 / 93.1 / 107.1 / 311.2 / 293.2
M + 2O	388.3	406.3	411.3	6.5	365.2 / 393.2 / 245.1 / 285.1
M + 4O	420.3	438.3	443.3	4.0	397.2 / 426.1 / 357.2 / 245.1 / 317.1
M + 6O	452.2	470.2	475.2	3.5	429.2 / 357.2 / 277.1
M + 8O	484.2	502.2	507.2	3.2	461.2 / 317.1 / 277.1
Epoxide	372.3	390.3	395.3	7.8	377.3
O—O dimer (+4O)	776.5	794.5	799.5	8.4	–
O—O dimer (+6O)	808.5	826.5	831.5	7.5	–
O—O dimer (+8O)	840.5	858.5	863.5	6.2	397.2 / 443.3 / 459.3 / 425.2
O—O dimer (+10O)	872.5	890.5	895.5	5.8	397.2 / 429.4 / 443.3 / 459.3 / 475.2
O—O dimer (+12O)	904.5	922.5	927.5	3.6	397.2 / 429.2 / 443.3 / 459.3 / 475.2
C—C dimer	710.5	728.5	733.5	16.0	355.3 / 711.5

DHA-EE masses corresponding to dimers with additions of two oxygens from + 4O to + 12O were detected. The main sodium adduct fragment for the dimers was EE + 4O with a loss of 46 Da ([M + Na-CH_3CH_2OH] *m/z* 397.2). EE + 4O was also found among the fragments as such (*m/z* 443.3) and with loss of water. Also, EE + 6O (*m/z* 475.2), DHA-EE + 6O with loss of 46 Da ([M + Na-CH_3CH_2OH] *m/z* 429.2) and EE + 5O (*m/z* 459.3) were detected, as well as acyl chain cleavage fragments. The formation of oxygen-linked dimers can occur by the addition of peroxy radical to a double bond or by the recombination of two alkoxy radicals (Schaich, 2005). Corresponding dimeric oxygen- and carbon-linked structures from methyl linolenate autoxidation at 40 °C are described by Neff et al. (1988). DHA-EE carbon-linked dimer (2EE-2H) ammonium adduct formed fragments of DHA-EE with loss of one hydrogen (*m/z* 355.3) in addition to the protonated molecular ion [M + H] *m/z* 711.5. Carbon-linked dimers form through the recombination of two alkyl radicals (Schaich, 2005). Sodium, ammonium, and potassium adducts were also detected for masses corresponding to possible trimers, e.g. 3EE + 10O (*m/z* 1228.7) and 3EE + 12O (*m/z* 1260.7) which were detectable in trace amounts. Masses of dimeric TAG structures were beyond the detection limits of the applied method. Dimerization has been previously shown to be significant only for methyl esters of PUFA at ambient conditions, where intermolecular condensations of peroxyl radicals are favored. For TAG further oxidation to di- and trihydroperoxides is the preferred reaction (Neff et al., 1990). For both DHA-TAG acyl chain cleavage products and DHA-EE dimers, the auto MS/MS did not fragment any ions originally due to the low signal-to-noise ratio, but they were analyzed by multiple reaction monitoring (MRM). Percentual area development of the non-oxidized compound and the non-volatile oxidation products during the oxidation trial is presented in Fig. 2.

In the samples without α-tocopherol the level of non-oxidized DHA-TAG started to decrease after 2 h induction period, while the DHA-EE was rapidly decreasing from 0.6 h onwards (Fig. 2**A**). A similar trend with 2 h induction period in the compound formation for DHA-TAG oil was noticeable in the M + 2O, M + 4O, and M + 6O as well as in the epoxides (Fig. 2**B, 2C, 2D, 2E**), while for the DHA-EE oil the levels increased directly from the start of the trial. The formation of DHA-TAG fatty acyl chain cleavage products (Fig. 2**F**) requires the prior formation of hydroperoxides, which could explain the practically zero increase during the 2 h induction period. The DHA-EE oxygen-linked dimers started to increase right from 0.6 h (Fig. 2**G**), while for the carbon-linked dimer there is a 2 h induction period (Fig. 2**H**). In a study by Neff et al. (1988), 88% of the dimers produced in methyl linolenate autoxidation at 40 °C were peroxy-linked, and 12% ether- and/or carbon-linked, suggesting a lower amount for the carbon-linked dimers. Overall, the results of the non-volatile oxidation product analysis support the findings from the analysis of VSOPs, where the DHA-TAG oil has a 2 h induction period and the DHA-EE is less stable with rapidly increasing oxidation product levels right from the beginning of the trial.

Results from the non-volatile oxidation product analysis confirmed that the samples with 0.2% α-tocopherol were more stable than the ones without. The DHA-TAG level was slightly decreasing from 0.6 h to 18 h, after which the oil started to decompose rapidly. The DHA-EE level stayed constant until the start of the decomposition at 24 h. The DHA-TAG M + 2O increased constantly until 18 h, after which the level started to decline, while the DHA-EE M + 2O level was increasing more slowly and quite constantly until 32 h. One route for the loss of M + 2O is also their further oxidation to polyhydroperoxides and cyclic peroxides. This could for its part explain the DHA-TAG M + 4O and M + 6O reaching the highest level later when compared to M + 2O. Similar development with a decrease from 24 h onwards for DHA-TAG and rapid increase from 24 h onwards for DHA-EE was perceived for M + 4O, M + 6O, and epoxides. The DHA-TAG FA chain cleavage product levels increase later than those for hydroperoxides, similarly as in the samples without α-tocopherol. In the oxygen-linked dimer levels, the rapid increase started at 24 h, and similar behavior with a slower increase rate is observed for the carbon-linked dimer. The results indicate better stability for the DHA-EE oil when compared to the DHA-TAG in the presence of 0.2% α-tocopherol.

3.3. NMR spectroscopy

The PCA models of ^{1}H NMR data in Fig. 3 show the comparison of binned data from total spectra (plots A and B) and manually integrated data of selected regions (plots C and D) for the four sample types. Due to the lack of previous studies on the oxidation products of pure DHA analyzed by ^{1}H NMR, the compound identifications presented here are tentative and based on previous studies of trilinolenin (Frankel et al., 1990; Xia et al., 2016) and other edible oils (Martínez-Yusta et al., 2014; Merkx et al., 2018).

The sample grouping along the first principal component (PC1) in plot A is explained by the increasing oxidation level from negative to positive half, while the PC2 is dividing the DHA-EE samples to the positive half and DHA-TAG samples to the negative half. The grouping of the more oxidized samples to the positive half of PC1 was mostly explained by the signals from methylene group (CH_2) between two epoxy groups at bin 1.7 ppm (Xia et al., 2016), signal from CH group from epidioxide ring at bin 4.42 (Frankel et al., 1990) and signal from double bonds associated with hydroperoxides (CHOOH-CH=C<u>H</u>–) at bin 5.7 ppm (Martínez-Yusta et al., 2014). The negative half of PC1 was characterized by the signals from double bond hydrogens (–C<u>H</u>=C<u>H</u>–) and bisallylic hydrogens, both of which were lost as the oxidation proceeded. The grouping of EE samples to the positive half of PC2 was mostly explained by $CH_3C\underline{H}_2$-O- structure from the ethyl group. The grouping of TAGs to the negative half was explained by the oxidation products related to epoxides and hydroperoxides (e.g. bins at 1.7 and 5.7 ppm), but also the hydrogens from glycerol moiety (gly CH_2, at bin 4.3 ppm) and hydrogens from the beginning of the acyl chain (-C═O) $C\underline{H}_2CH_2CH$=, at bin 2.38) grouped on the negative half. Plot A shows that the DHA-EE samples with α-tocopherol were only slightly oxidized still at 24 h (EA24), which was also noticed by the other applied analytical methods. Also, at 0.6 h the α-tocopherol-containing EA0 samples were not as oxidized as the corresponding E0 samples without antioxidant. This could indicate that the E0 samples were somewhat oxidized before the first sampling point of SPME-GC–MS analysis at 0.6 h. Slightly higher oxidation levels of the DHA-EE samples without α-tocopherol at 0.6 h, when compared to the α-tocopherol-containing DHA-EE samples, were also noticeable in some of the HPLC-QTOF and SPME-GC–MS results. The lower oxidation level of the DHA-TAG samples without antioxidant at 4 h (T4) when compared to the DHA-EE samples (E4) as seen in plot A was also detected in the VSOP analysis results. The data from the manually integrated spectral areas is presented in PCA plot C, where the oxidation mostly proceeds from the right-down corner (+ PC1, – PC2) of the plot to the left-up corner (– PC1, + PC2). According to the loadings plot D the proceeding of oxidation was related to the loss of bisallylic and double bond hydrogens and formation of aldehydes and lipid hydroperoxides. The PCA model for the data from region-specific excitation of hydroperoxides (11.5–10.5 ppm) is presented in plots E and F (Fig. 3) and corresponding spectra in **Supplementary** Fig. 4. The 0.6 h oxidized DHA-TAG samples with and without α-tocopherol (T0, TA0) and 0.6 h and 24 h oxidized DHA-EE samples with α-tocopherol (EA0, EA24) are grouping to three different regions mostly in the positive side of PC1 while the more oxidized samples are grouping together on the negative side of PC1, closer to the origin. Also, the E0 samples are grouping with the more oxidized samples, indicating higher oxidation level as discussed above. The different 0.6 h oxidized sample types (T0, TA0, EA0) are correlating positively with different cyclic hydroperoxide (exact structures are unknown) and isomeric monohydroperoxide structures in spectral areas presented by Merkx et al. (2018). This could indicate that both the lipid structure and the presence of α-tocopherol influence the variety of formed hydroperoxide structures in the early stages of oxidation. The spectra for region-specific excitation of hydroperoxides (**Supplementary** Fig. 4**)** shows

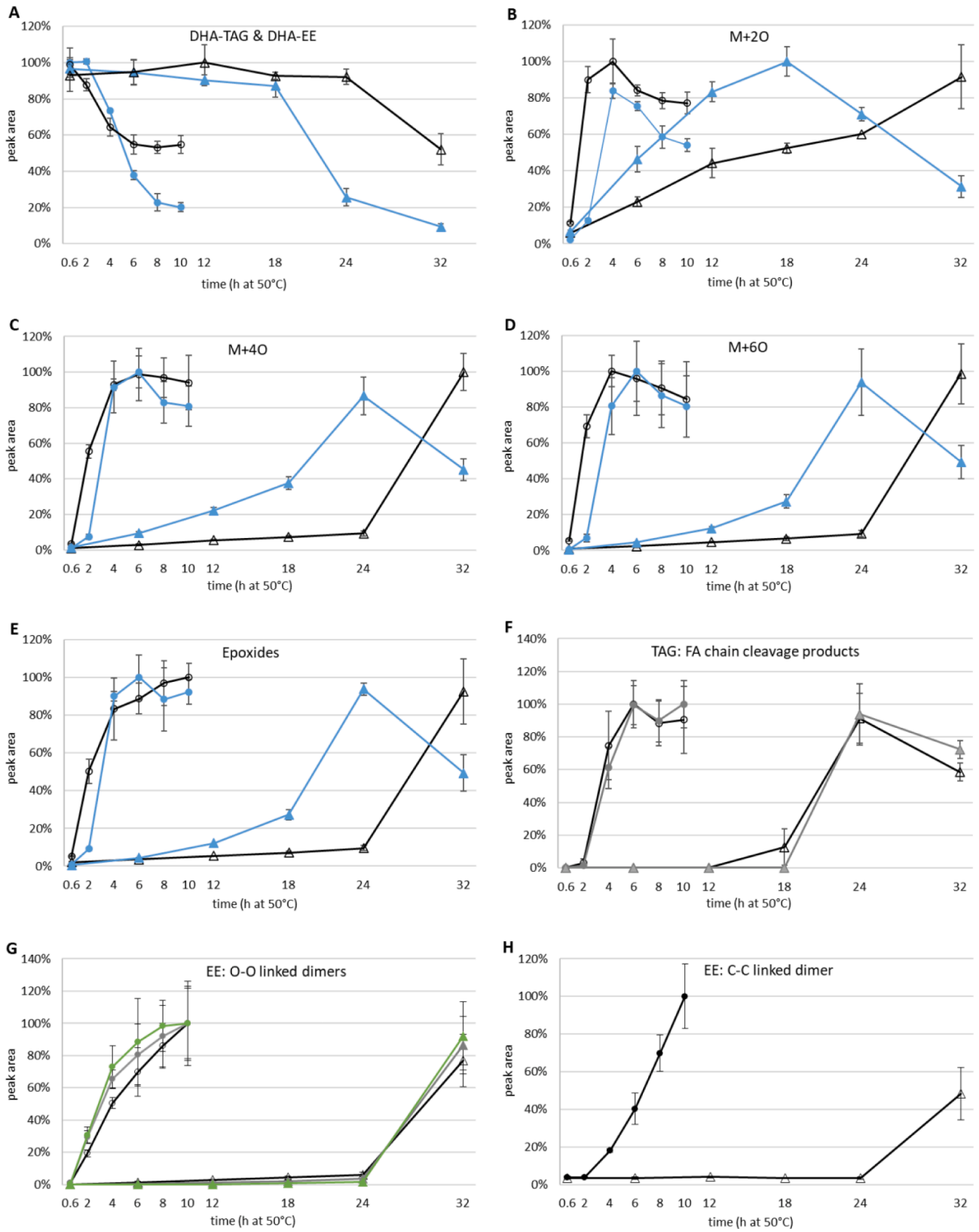

Fig. 2. Percentual area development of the non-oxidized compounds DHA-TAG and DHA-EE (**A**) M + 2O (**B**), M + 4O (**C**), M + 6O (**D**) and epoxides (**E**) for the DHA-TAG (blue line, closed marker) and DHA-EE (black line, open marker), DHA-TAG fatty acid chain cleavage products TG 22:6/22:6/(18:5 + 2O) (black line) and TG 22:6/22:6/(15:4 + 2O) (gray line) (**F**), DHA-EE O—O linked dimers 2 M + 4O (black line), 2 M + 6O (gray line) and 2 M + 8O (green line) (**G**) and DHA-EE C—C linked dimer (black line) (**H**) during oxidation at 50 °C in the dark without (circle) and with (triangle) 0.2% α-tocopherol. Values are mean ± standard deviation of three replicates. There is one 100% value (largest mean area) for the DHA-TAG samples and one for the DHA-EE samples for each compound (as DHA-TAG and DHA-EE areas were not comparable).

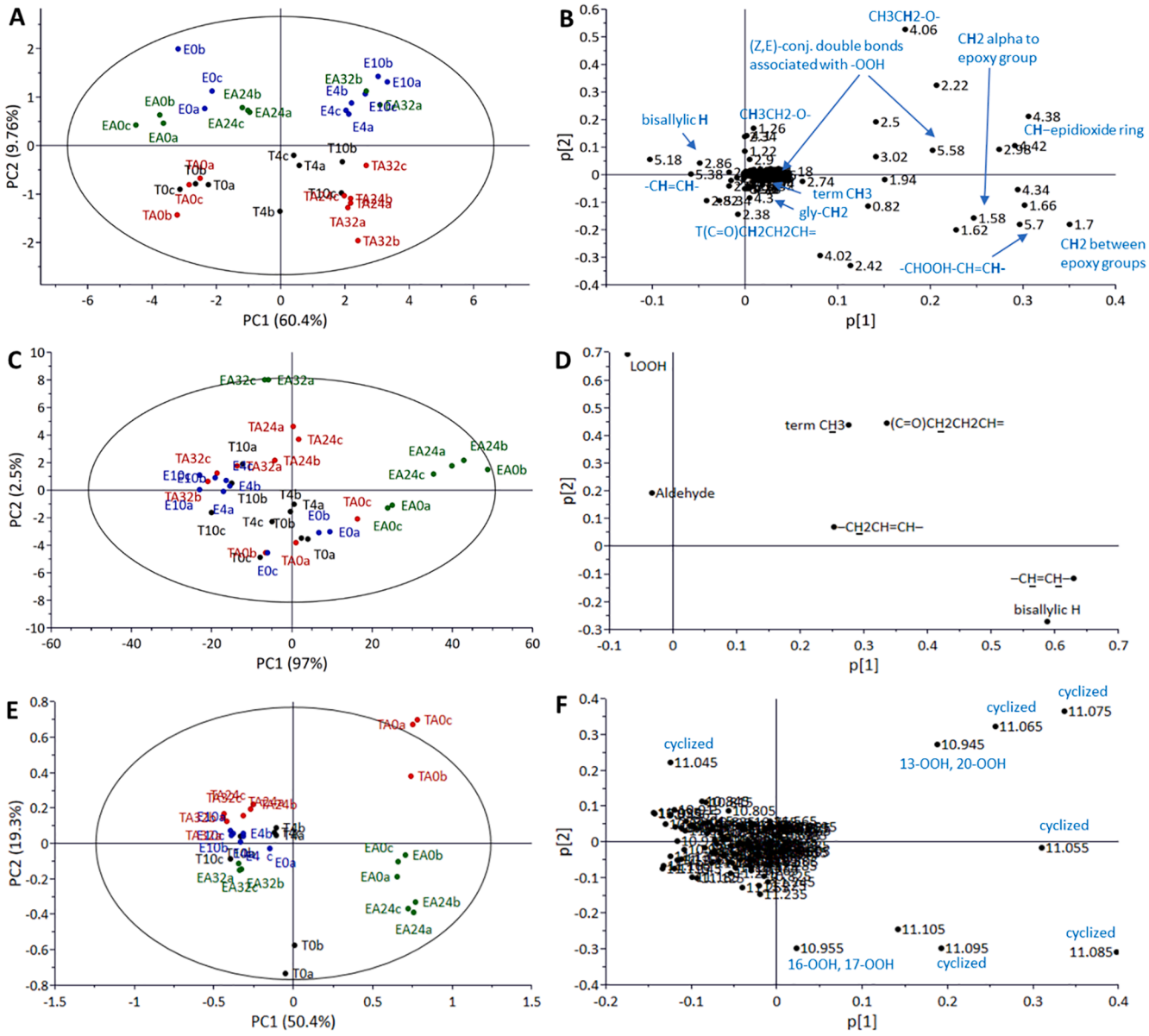

Fig. 3. PCA model of the binned, Pareto-scaled and mean-centered NMR data of total spectral area (PC1 vs. PC2; $R^2X[1] = 0.604$, $Q^2[1] = 0.564$; $R^2X[2] = 0.097$, $Q^2[2] = 0.068$) with scores plot (**A**) and loadings plot (**B**), PCA model for the manually integrated, Pareto scaled and mean-centered NMR data of the aldehyde (10–9 ppm), hydroperoxide (11.5–10.5 ppm) and selected lipid structural regions (PC1 vs. PC2; $R^2X[1] = 0.97$, $Q^2[1] = 0.948$; $R^2X[2] = 0.025$, $Q^2[2] = 0.72$) with scores plot (**C**) and loadings plot (**D**) and PCA model for the binned, Pareto-scaled and mean-centered NMR data of selectively excited spectral region 11.5–10.5 ppm for hydroperoxides (PC1 vs. PC2; $R^2X[1] = 0.504$, $Q^2[1] = 0.401$; $R^2X[2] = 0.193$, $Q^2[2] = 0.192$) with scores plot (**E**) and loadings plot (**F**). Sample time points 0 h (=0.6 h), 4 h and 10 h for samples without α-tocopherol (DHA-TAG = T, black and DHA-EE = E, blue) and time points 0 h (=0.6 h), 24 h and 32 h for the samples with α-tocopherol (DHA-TAG = TA, red and DHA-EE = EA, green).

that the DHA-EE sample without α-tocopherol contains small amounts of hydroperoxides already at 0.6 h and at 4 h the level is as high as at 10 h, while in the corresponding TAG sample there is practically no hydroperoxides at 0.6 h and the level is rising slower. In the samples with α-tocopherol the DHA-EE has only small amounts of hydroperoxides still at 24 h, while in the DHA-TAG sample the level at 24 h is close to the 32-h level.

The PCA model for the data from the selective excitation of aldehyde regions (10.0–9.0 ppm) is presented in plots A and B in **Supplementary Fig. 3** and corresponding spectra in **Supplementary Fig. 5**. In scores plot A the sample oxidation roughly proceeds from right up (+ PC1, + PC2) part of the PCA through right-down part (+ PC1, – PC2) to left-up part (– PC1, + PC2). Small *n*-alkanals (bin 9.785 ppm, Merkx et al., 2018) formed in the early stages of oxidation (e.g. propanal, acetaldehyde) are correlating with the slightly oxidized EA24, T4, and also E0 samples and most oxidized 10 h and 32 h samples correlate with the more oxidized aldehydes, namely (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-*E*-2-alkenals at bins 9.505, 9.515 and 9.525 ppm and 4-hydroxy (pero)xy-(*E*)-2-alkenals at bins 9.575, 9.565 and 9.555 ppm (Merkx et al., 2018). The spectra for region-specific excitation of aldehydes (**Supplementary Fig. 5**) shows the later development of aldehydes in the DHA-TAG sample without α-tocopherol when compared to the corresponding EE sample. In the samples with α-tocopherol, there is practically no aldehydes detected at 24 h in the DHA-EE sample, while in the DHA-TAG sample the 24 h and 32 h levels are almost equal. Similarly, as in the case of other applied analytical methods, also the NMR results indicate better stability for the DHA-EE structure in the presence of α-tocopherol, while without α-tocopherol the DHA-TAG structure was

found to be more stable.

3.4. α-Tocopherol concentration

α-Tocopherol concentration (mg/mol DHA) development during the 32-h oxidation trial is shown in Fig. 4. In the DHA-TAG oil, the concentration remained at the initial level for the first 6 h of oxidation and then dropped rapidly to zero by 18 h, while in the DHA-EE oil the α-tocopherol concentration was lower than in DHA-TAG oil at 0.6 h, it was consumed at a quite constant rate from 0.6 h onwards and dropped to zero after 24 h. The lower α-tocopherol concentration of the DHA-EE sample at 0.6 h when compared to DHA-TAG could be caused by its consumption before the 0.6 h time point. Some of the NMR, HPLC-QTOF and SPME-GC–MS results are showing higher oxidation level of the DHA-EE sample without α-tocopherol already at 0.6 h, which is consistent with the overall conclusion that the DHA-EE without α-tocopherol was most susceptible to oxidation of the four sample types analyzed. Despite the 6 h induction period for α-tocopherol decomposition in the DHA-TAG oil it was depleted faster than in the DHA-EE oil. The results are indicating a clear difference in the α-tocopherol response between the DHA-EE and -TAG structures. Faster depletion of tocopherol in TAG oil, when compared to EE, has been similarly reported by Song et al. (1997) for δ-tocopherol during autoxidation of DHA-containing oils at 25° C in the dark. Statistically significant difference between α-tocopherol levels was observed at time points 0.6 h ($p < 0.05$), 6 h ($p < 0.01$), 18 h ($p < 0.01$), and 24 h ($p = 0.001$).

3.5. Overall oxidation behavior

Results from the comprehensive set of applied analytical methods indicate better stability of the DHA-TAG oil when no α-tocopherol is added, while with 0.2% α-tocopherol addition the DHA-EE oil was found to be more stable than DHA-TAG oil. In the free radical chain reaction mechanism, the chain propagation occurs through hydrogen abstraction by peroxyl radicals from the bisallylic site of another acyl chain. This is also considered as the rate-limiting step of the reaction (Porter et al., 1995). For a chemical reaction to happen the reacting atoms need to collide from a favorable angle and with sufficient energy (collision theory). This is more probable in less viscous material where the molecules move faster and more easily past each other. When considering the DHA-TAG and -EE oils without α-tocopherol, it can be postulated that the hydrogen abstractions are facilitated by the lower viscosity and closer association of the acyl chains in the DHA-EE oil when compared to DHA-TAG with larger and more complex molecules, thus resulting in a direct oxidation propagation for the DHA-EE oil and 2 h induction period for the DHA-TAG oil. The viscosity difference between TAG and EE oils is considerable, e.g., for eicosapentaenoic acid 12.5 cp vs. 2.88 cp at 37 °C, respectively (Hamazaki et al., 1985). When α-tocopherol is present in the oil it serves as the main source of hydrogens for the peroxy radicals instead of acyl chains and the chain propagation is delayed. Presumably, the lower viscosity of DHA-EE oil also promotes its interaction with the hydrogens from α-tocopherol molecules, thus leading to immediate and constant consumption of α-tocopherol as seen in Fig. 4, and better preservation of the oil.

The results reported here are in line with the work from Song et al. (1997), who compared the oxidative stability of oils containing 10.6 mol % DHA in PL, EE, and TAG form with initial total tocopherol content of 6.7, 18.9, and 35.1 mg/100 g, respectively. After 5-week oxidation at 25 °C in the dark, the EE oil had a total tocopherol content of 1.4 mg/100 g and TAG oil 0.1 mg/100 g, thus indicating a faster consumption of tocopherols in the TAG oil and better oxidative stability of the EE oil. Most previous studies report better oxidative stability for the TAG oil with added tocopherols. Sullivan Ritter et al. (2015) reported higher oxidation rates for EE oil after incubation of oils containing DHA and EPA approximately 65% of the total fatty acids in the dark in temperatures between 5 and 60 °C. The initial total tocopherol content of the TAG and EE oils were 2.67 and 2.04 mg/g, respectively. Also, Martín et al. (2012) reported better stability for the TAG oil during storage at room temperature in the dark with 500 mg/kg added α-tocopherol and rosemary extract. Also without antioxidant addition, the TAG oil was found to be more stable. However, in their study, the peroxide value of the EE oil was higher already at the beginning of the trial (11.8 vs. 4.0 meq/kg) and also the higher total concentration of LC-PUFA in the EE oil might have affected the results. Better stability of TAG has also been reported by Lee et al. (2003) and Yoshii et al. (2002). However, in both of these studies, the LC-PUFA content differed significantly in the compared oils and no antioxidant content was reported.

Mostly similar oxidation products were formed during DHA-TAG and -EE oil oxidation, but also some structure-related differences were detected for both volatile and non-volatile oxidation products. 2-Propenal was one of the first volatile oxidation products with significantly differing levels in both sample types already in the very early stages of oxidation, showing possible applicability as an oxidation marker compound for LC-PUFA oils. NMR, which measures all the formed oxidation products and the decomposition of the original molecule simultaneously, has been seen as a more reliable and accurate method for lipid oxidation analysis when compared to the traditional methods, which concentrate only on one oxidation product. Results from the ^{1}H NMR measurements were in line with other applied analytical methods and especially the selective excitation of aldehyde and hydroperoxide spectral regions showed promise as a fairly sensitive analytical method for lipid oxidation analysis. However, most of the spectral areas in

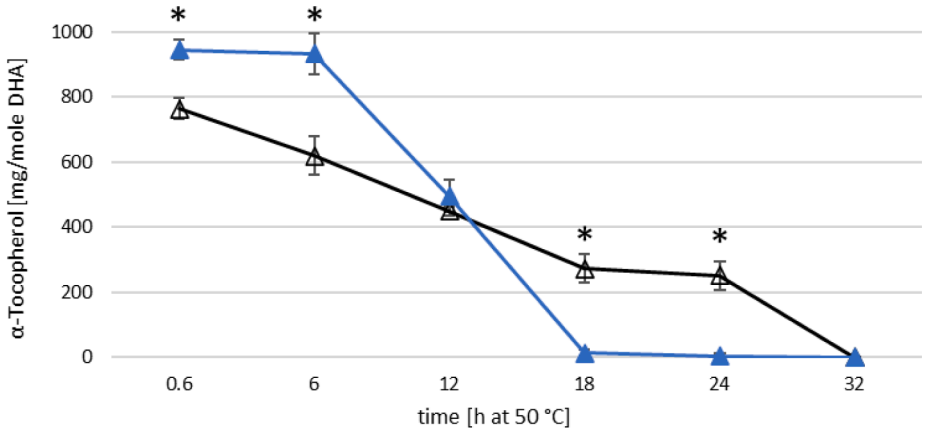

Fig. 4. α-Tocopherol concentration (mg/mole DHA) development during 32 h oxidation trial for DHA-TAG (blue line, filled marker) and DHA-EE (black line, open marker). Statistically significant differences ($p < 0.05$) are marked with star.

hydroperoxide and aldehyde regions could not be assigned to specific molecular structures. A lot of compound-specific analysis and spectral data is still needed in order to be able to identify the specific oxidation product structures from mixtures by ^{1}H NMR. The formation of the majority of the identified products can be explained by the mechanisms of free radical chain reaction, although not all of the VSOPs fit into this scheme. To the authors knowledge current study is the first one applying an omics-type approach to DHA oxidation analysis, with the target of reporting the formed oxidation products and their level development during the three kinetical phases of oxidation as widely as possible. This kind of data is highly relevant for food chemists when considering the best ways to analyze lipid oxidation, sensory quality (formed volatiles), and choosing the most suitable antioxidants. However, still only a part of all possible oxidation products were detected and identified by the applied methods. As there are differences in the oxidation reaction pathways in neat oils when compared to solvent systems and the reaction can be easily shifted by temperature, fatty acid types, metals, concentration, and solvent type among other things, the results are not directly applicable to other conditions. Thus, further research on how the observed oxidation behavior translates to other oxidation conditions and food models/systems is needed. Although kinetical analysis is known to be difficult in terms of PUFA oxidation due to the numerous differing reaction routes that can follow each step, also data from such studies with the focus on a limited amount of quantified oxidation products and several oxidation temperatures would be highly useful in further increasing the understanding of DHA oxidation.

4. Conclusion

The lipid structure (TAG/EE) affected the oxidative stability, oxidation pattern, and α-tocopherol response of DHA. Under the applied oxidation conditions pure DHA-TAG oil was more oxidatively stable than pure DHA-EE oil without α-tocopherol addition. In the presence of α-tocopherol the opposite was observed, i.e. DHA-EE was more stable than DHA-TAG. This behavior was likely influenced by molecular size, structure and viscosity differences. The detected volatile and non-volatile oxidation products were largely similar. The majority were reaction products of autoxidation of DHA. Nevertheless, also structure-related differences were found in oxidation products mainly related to products formed from the ester end of the molecules. This study highly contributes to the knowledge of oxidation products and pattern of DHA, previously only investigated in few studies. Further, oxidation trial set-up and analytical methods applied showed great potential to be used in studies of oxidation behavior of other lipid structures in the future.

CRediT authorship contribution statement

Eija Ahonen: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Annelie Damerau:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Jukka-Pekka Suomela:** Methodology, Writing – review & editing, Supervision. **Maaria Kortesniemi:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Kaisa M. Linderborg:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Adams, A., Bouckaert, C., Van Lancker, F., De Meulenaer, B., & De Kimpe, N. (2011). Amino acid catalysis of 2-alkylfuran formation from lipid oxidation-derived α, β-unsaturated aldehydes. *Journal of Agricultural and Food Chemistry, 59*(20), 11058–11062. https://doi.org/10.1021/jf202448v

Buttery, R. G., & Takeoka, G. R. (2004). Some unusual minor volatile components of tomato. *Journal of Agricultural and Food Chemistry, 52*(20), 6264–6266. https://doi.org/10.1021/jf040176a

Calder, P. C. (2016). Docosahexaenoic Acid. *Annals of nutrition and metabolism* 69.1, 8–21. Web.

Choe, E., & Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety, 5*(4), 169–186. https://doi.org/10.1111/j.1541-4337.2006.00009.x

Damerau, A., Ahonen, E., Kortesniemi, M., Puganen, A., Tarvainen, M., & Linderborg, K. M. (2020). Evaluation of the composition and oxidative status of omega-3 fatty acid supplements on the Finnish market using NMR and SPME-GC–MS in comparison with conventional methods. *Food Chemistry, 330*, Article 127194. https://doi.org/10.1016/j.foodchem.2020.127194

Endo, Y., Hayashi, C., Yamanaka, T., Takayose, K., Yamaoka, M., Tsuno, T., & Nakajima, S. (2013). Linolenic acid as the main source of acrolein formed during heating of vegetable oils. *Journal of the American Oil Chemists' Society, 90*(7), 959–964. https://doi.org/10.1007/s11746-013-2242-z

Frankel, E. N. (1980). Lipid oxidation. *Progress in Lipid Research, 19*(1–2), 1–22. https://doi.org/10.1016/0163-7827(80)90006-5

Frankel, E. N. (1982). Volatile lipid oxidation products. *Progress in Lipid Research, 22*(1), 1–33. https://doi.org/10.1016/0163-7827(83)90002-4

Frankel, E. N. (1984). Lipid oxidation: Mechanisms, products and biological significance. *Journal of the American Oil Chemists' Society, 61*(12), 1908–1917. https://doi.org/10.1007/bf02540830

Frankel, E. N., Neff, W. E., & Selke, E. (1981). Analysis of autoxidized fats by gas chromatography-mass spectrometry: VII. Volatile thermal decomposition products of pure hydroperoxides from autoxidized and photosensitized oxidized methyl oleate, linoleate and linolenate. *Lipids, 16*(5), 279–285. https://doi.org/10.1007/bf02534950

Frankel, E. N., Neff, W. E., & Selke, E. (1983). Analysis of autoxidized fats by gas chromatography-mass spectrometry: VIII. Volatile thermal decomposition products of hydroperoxy cyclic peroxides. *Lipids, 18*(5), 353–357. https://doi.org/10.1007/bf02537231

Frankel, E. N., Neff, W. E., & Selke, E. (1984). Analysis of autoxidized fats by gas chromatography-mass spectrometry. IX. Homolytic vs. Heterolytic cleavage of primary and secondary oxidation products. *Lipids, 19*(10), 790–800. https://doi.org/10.1007/bf02534473

Frankel, E. N., Neff, W. E., & Miyashita, K. (1990). Autoxidation of polyunsaturated triacylglycerols. *II. Trilinolenoylglycerol. Lipids, 25*(1), 40–47. https://doi.org/10.1007/bf02562426

Hamazaki, T., Kobayashi, S., Urakaze, M., Yano, S., & Fujita, T. (1985). Viscosities of some triglycerides and ethylester of fatty acids frequently found in cell membranes. *Biorheology, 22*(3), 221–226. https://doi.org/10.3233/bir-1985-22306

Hammer, M., & Schieberle, P. (2013). Model studies on the key aroma compounds formed by an oxidative degradation of ω-3 fatty acids initiated by either copper (II) ions or lipoxygenase. *Journal of Agricultural and Food Chemistry, 61*(46), 10891–10900. https://doi.org/10.1021/jf403827p

Ito, J., Mizuochi, S., Nakagawa, K., Kato, S., & Miyazawa, T. (2015). Tandem mass spectrometry analysis of linoleic and arachidonic acid hydroperoxides via promotion of alkali metal adduct formation. *Analytical Chemistry, 87*(9), 4980–4987. https://doi.org/10.1021/acs.analchem.5b00851

Kawai, Y., Takeda, S., & Terao, J. (2007). Lipidomic analysis for lipid peroxidation-derived aldehydes using gas chromatography-mass spectrometry. *Chemical Research in Toxicology, 20*(1), 99–107. https://doi.org/10.1021/tx060199e

Lee, H., Kizito, S. A., Weese, S. J., Craig-Schmidt, M. C., Lee, Y., Wei, C. I., & An, H. (2003). Analysis of headspace volatile and oxidized volatile compounds in DHA-enriched fish oil on accelerated oxidative storage. *Journal of Food Science, 68*(7), 2169–2177. https://doi.org/10.1111/j.1365-2621.2003.tb05742.x

Long, E. K., & Picklo, M. J. (2010). Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid peroxidation: Make some room HNE. *Free Radical Biology and Medicine, 49*(1), 1–8. https://doi.org/10.1016/j.freeradbiomed.2010.03.015

Lyberg, A., Fasoli, E., & Adlercreutz, P. (2005). Monitoring the oxidation of docosahexaenoic acid in lipids. *Lipids, 40*(9), 969–979. https://doi.org/10.1007/s11745-005-1458-1

Martín, D., Terrón, A., Fornari, T., Reglero, G., & Torres, C. F. (2012). Oxidative stabilization of ultra-high omega-3 concentrates as ethyl esters or triacylglycerols. *Food Research International, 45*(1), 336–341. https://doi.org/10.1016/j.foodres.2011.09.022

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A Review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: Influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 838–859. https://doi.org/10.1111/1541-4337.12090

Merkx, D. W. H., Hong, G. T. S., Ermacora, A., & van Duynhoven, J. P. M. (2018). Rapid quantitative profiling of lipid oxidation products in a food emulsion by ^{1}H NMR. *Analytical Chemistry, 90*(7), 4863–4870. https://doi.org/10.1021/acs.analchem.8b00380

Micha, R., Khatibzadeh, S., Shi, P., Fahimi, S., Lim, S., Andrews, K.G., Engell, R.E., Powles, J., Ezzati, M., & Mozaffarian, D. (2014). Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys. (2015b). *BMJ*, *350*(mar26 22), h1702. https://doi.org/10.1136/bmj.h1702.

Neff, W. E., Frankel, E. N., & Miyashita, K. (1990). Autoxidation of polyunsaturated triacylglycerols. I. Trilinoleoylglycerol. *Lipids, 25*(1), 33–39. https://doi.org/10.1007/bf02562425

Neff, W. E., Frankel, E. N., & Fujimoto, K. (1988). Autoxidative dimerization of methyl linolenate and its monohydroperoxides, hydroperoxy epidioxides and dihydroperoxides. *Journal of the American Oil Chemists' Society, 65*(4), 616–623. https://doi.org/10.1007/bf02540690

Noble, A. C., & Nawar, W. W. (1971). The autoxidation of highly unsaturated fatty acids: Methyl 4, 7, 10, 13, 16, 19-docosahexaenoate. *Journal of the American Oil Chemists Society, 48*(12), 800–803. https://doi.org/10.1007/bf02609286

Noble, A. C., & Nawar, W. W. (1975). Identification of decomposition products from autoxidation of methyl 4, 7, 10, 13, 16, 19-docosahexaenoate. *Journal of the American Oil Chemists' Society, 52*(3), 92–95. https://doi.org/10.1007/bf02633045

Pan, X., Kaneko, H., Ushio, H., & Ohshima, T. (2005). Oxidation of all-cis-7, 10, 13, 16, 19-docosapentaenoic acid ethyl ester. Hydroperoxide distribution and volatile characterization. *European Journal of Lipid Science and Technology, 107*(4), 228–238. https://doi.org/10.1002/ejlt.200501135.

Podda, M., Weber, C., Traber, M. G., & Packer, L. (1996). Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. *Journal of Lipid Research, 37*(4), 893–901. https://doi.org/10.1016/s0022-2275(20)37587-8

Porter, N. A., Caldwell, S. E., & Mills, K. A. (1995). Mechanisms of free radical oxidation of unsaturated lipids. *Lipids, 30*(4), 277–290. https://doi.org/10.1007/bf02536034

Schaich, K. M. (2005). Lipid oxidation: Theoretical aspects. In F. Shahidi (Ed.), *Bailey's industrial oil and fat products* (6th edition, pp. 269–355). New York: John Wiley & Sons Inc.

Schuchardt, J. P., & Hahn, A. (2013). Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 89*(1), 1–8. https://doi.org/10.1016/j.plefa.2013.03.010

Schwartz, H., Ollilainen, V., Piironen, V., & Lampi, A.-M. (2008). Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. *Journal of Food Composition and Analysis, 21*(2), 152–161. https://doi.org/10.1016/j.jfca.2007.07.012

Song, J. H., Inoue, Y., & Miyazawa, T. (1997). Oxidative Stability of docosahexaenoic acid-containing oils in the form of phospholipids, triacylglycerols, and ethyl esters. *Bioscience, Biotechnology, and Biochemistry, 61*(12), 2085–2088. https://doi.org/10.1271/bbb.61.2085

Sullivan Ritter, J. C., Budge, S. M., Jovica, F., & Reid, A. J. M. (2015). Oxidation rates of triacylglycerol and ethyl ester fish oils. *Journal of the American Oil Chemists' Society, 92*(4), 561–569. https://doi.org/10.1007/s11746-015-2612-9

Tarvainen, M., Suomela, J. P., Kuksis, A., & Kallio, H. (2010). Liquid chromatography–light scattering detector–mass spectrometric analysis of digested oxidized rapeseed oil. *Lipids, 45*(11), 1061–1079. https://doi.org/10.1007/s11745-010-3474-4

Vandemoortele, A., Heynderickx, P. M., Leloup, L., & De Meulenaer, B. (2021). Kinetic modeling of malondialdehyde reactivity in oil to simulate actual malondialdehyde formation upon lipid oxidation. *Food Research International, 140*, Article 110063. https://doi.org/10.1016/j.foodres.2020.110063

Xia, W., Budge, S. M., & Lumsden, M. D. (2016). ^{1}H-NMR Characterization of epoxides derived from polyunsaturated fatty acids. *Journal of the American Oil Chemists' Society, 93*(4), 467–478. https://doi.org/10.1007/s11746-016-2800-2

Xia, W., & Budge, S. M. (2017). Techniques for the analysis of minor lipid oxidation products derived from triacylglycerols: Epoxides, alcohols, and ketones. *Comprehensive Reviews in Food Science and Food Safety, 16*(4), 735–758. https://doi.org/10.1111/1541-4337.12276

Yoshii, H., Furuta, T., Siga, H., Moriyama, S., Baba, T., Maruyama, K., Misawa, Y., Hata, N., & Linko, P. (2002). Autoxidation kinetic analysis of docosahexaenoic acid ethyl ester and docosahexaenoic triglyceride with oxygen sensor. *Bioscience, Biotechnology and Biochemistry, 66*(4), 749–753. https://doi.org/10.1271/bbb.66.7