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

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Keywords

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Highlights

- Study of composition and oxidation of 49 omega-3 supplements on the Finnish market
- In 24% of studied products oxidation was detected using conventional methods
- *P*-anisidine value was only suitable analysis method for 73% of the studied products

- Volatile oxidation products indicated severe lipid oxidation in two products
- 2,4-Heptadienal and 1-penten-3-ol were the most suitable indicator compounds

Abstract

Previous studies disagree on the oxidative status of omega-3 supplements. The great deviation raises concerns about quality and the methods used to monitor it. This study investigated 49 omega-3 products for their fatty acid content, lipid class and oxidative status using official methods, gas and liquid chromatography with mass spectrometry and nuclear magnetic resonance spectroscopy. With minor deviations, omega-3 fatty acid content and lipid class of all products were as declared. 24% of studied products exceeded thresholds set by The Global Organization for EPA and DHA Omega-3s for peroxide and/or *p*-anisidine value suggesting a compromised oxidative status. However, peroxide and/or *p*-anisidine value were only suitable for detection of lipid oxidation in 90% or 73%, respectively, of the products. Analysis of volatile oxidation compounds can be an alternative method for *p*-anisidine value. Nuclear magnetic resonance spectroscopy was shown to be a rapid method for determination of oil type and lipid class.

1. Introduction

Long chain omega-3 polyunsaturated fatty acids docosahexaenoic acid (22:6*n*-3, DHA) and eicosapentaenoic acid (20:5*n*-3, EPA) are essential for human health and vital for growth and development. They play an important role in the cardiovascular system and take part in the inflammatory balance (Saini & Keum, 2018). DHA is crucial for the brain development of infants, and for the brain functions associated with cognitive and emotional health as well as for the eye sight (Hashimoto, Hossain, Al Mamun, Matsuzaki, & Arai, 2017). Despite these

known health benefits, the intake of omega-3 fatty acids is inadequate; e.g. in Europe the recommended intakes for DHA and EPA are only met by 26% of the population (Sioen et al., 2017). Further, in Western countries the intake ratio between omega-3 and omega-6 can be up to 1:15, which is considerably higher than the ratio in the areas with less chronical diseases, which can be as low as 1:1 (Saini & Keum, 2018).

Fish is the primary source of DHA and EPA in the human diet. Additionally, commercial supplements are widely used to balance intake deficits. However, there are questions about the quality, especially in regard to the oxidative status, of omega-3 supplements. Oxidized supplement do not have promised health benefits (Rundblad, Holven, Ottestad, & Myhrstad, 2017). On the contrary, chronical exposure to oxidized lipids can cause negative health effects (Serini, Fasano, Piccioni, Cittadini, & Calviello, 2011; Vieira, Zhang, & Decker, 2017). Several studies worldwide have investigated the oxidative status of omega-3 supplements. In many of the studies (Albert et al., 2015; Halvorsen & Blomhoff, 2011; Heller, Gemming, Tung, & Grant, 2019; Jackowski et al., 2015; Mason & Sherrat, 2017; Opperman & Benade, 2013) the oxidative status has been poor. However, other studies found the oxidative status of omega-3 supplements to be adequate (Bannenberg et al., 2017; De Boer, Ismail, Marshall, Bannenberg, Yana, & Rowe, 2018; Ismail, Bannenberg, Rice, Schutt, & MacKay, 2016; Kolanowski, 2010; Sprague, Cooper, Tocher, & Betancor, 2018). The highly variable results raise concern about the nutritional and chemical quality of the supplements and the methods used to monitor the quality of the products.

Omega-3 supplements can be produced from oil of different marine sources, which affects the lipid class in which DHA and EPA are present. Krill oil contains both phospholipids (PLs) and triacylglycerols (TAGs) while fish and algae oil consist of TAGs. Fish or algae oil concentrates

in supplements can be in the form of TAGs but often also ethylesters (EEs) are used to avoid re-esterification after a concentration step. There are indications that unsaturated fatty acids in PLs are more oxidatively stable than those in TAGs or EEs (Mozuraityte Kristinova, Standal, Evensen, & Rustad, 2017). However, especially in case of oxidation studies of TAGs and EEs different proportions of fatty acids as well as varying concentration of other compounds, such as antioxidants, often make a direct comparison of the oxidative stability not possible.

The oxidative status of omega-3 supplements is commonly determined by analyzing the peroxide (PV) and the *para*-anisidine value (PAV). The Global Organization for EPA and DHA Omega-3s (GOED) representing the worldwide EPA and DHA omega-3 industry has set thresholds for PV, PAV as well as the total oxidation value (TOTOX = 2 × PV + PAV) to be followed by their members. It is suggested by the GOED Voluntary Monograph (GOED, 2019) that the PV must be under 5 meq/kg, the PAV under 20 and the TOTOX below 26 in the final product to be of acceptable quality. While PV and PAV are widely used to determine the quality of omega-3 supplements, they have limitations. PV is determined by indirect measurement based on the ability of the hydroperoxide group of hydroperoxides to oxidize other compounds and be reduced itself to a hydroxy group (Barriuso, Astiasaran, & Ansorena, 2013). The most common methods in industry are based on iodometry e.g. IUPAC official method 2.501 (IUPAC, 1987). It has its drawbacks, mainly because iodide can also be oxidized in the presence of light and oxygen. Further, hydroperoxides are semi-stable. They are formed and/or decomposed easily during sample pretreatment and analysis. This can cause over- or underestimation (Barriuso et al., 2013). PAV is based on the reaction of aldehydes (mainly 2-alkenals and 2,4-alkadienals) formed during lipid oxidation with *p*-anisidine to a Schiff base, with an absorption maximum at 350 nm. The PAV is defined as 100 times the absorbance of a solution containing 1 g of fat or oil in 100 mL of solvent. The PAV has several limitations.

First, the absorbance intensity is dependent on the unsaturation level of the aldehyde, which especially can cause overestimation of PAV measured from EPA and DHA rich oils. Secondly, the *p*-anisidine reacts with all kinds of aldehydes present and is therefore not selective towards aldehydes originating from lipid oxidation (Barriuso et al., 2013). Thus, method development for analysis of oxidative stability is urgently needed. Alternative approaches include modern gas (GC) or liquid chromatographic (LC) methods with mass spectrometric (MS) detection which allow direct analysis of volatile and non-volatile lipid oxidation compounds (Damerau, Kamlang-ek, Moisio, Lampi, & Piironen, 2014, Tarvainen, Suomela, & Kallio, 2011). Additionally, nuclear magnetic resonance (NMR) spectroscopy can be used to elucidate structures of lipids (Williamson & Hatzakis, 2017).

The hypotheses of this research were that omega-3 supplements can represent a risk to Finnish consumers based on their poor oxidative status and, PV and PAV, currently used as markers of oxidation by the industry, are not sufficient for determining oxidative quality of omega-3 products. Therefore, the aim of this study was to clarify the composition and oxidative status of omega-3 supplements on the Finnish market. Another objective of the investigation was to compare conventional analysis methods for lipid oxidation to more advanced novel approaches, with a focus on rapid methods, so far mainly used in scientific studies. In this study 48 omega-3 supplements, including fish, krill and micro algae oil products in different forms, and one DHA fortified margarine have been examined. Their content of omega-3 fatty acids, their lipid class and their oxidative status were determined.

2. Materials and methods

2.1 Reagents

For all analysis commercial, analytical grade solvents and reagents were used. For the di-sodium citrate buffer citric acid monohydrate and sodium hydroxide from J.T.Baker (Deventer, Netherlands) and VWR Chemicals (Leuven, Belgium), respectively, were used. Potassium chloride used for lipid extraction was obtained from Merck (Darmstadt, Germany). For the determination of fatty acid content acetyl chloride, potassium carbonate, butylated hydroxytoluene, boron trichloride and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Trimethylpentane and anhydrous sodium sulfate were obtained from Merck (Darmstadt, Germany) and sodium chloride from VWR Chemicals (Leuven, Belgium). Triheptadecanoin and methyl tricosanoate from Larodan (Solna, Sweden) were used as internal standards. External standards included 68D and GLC-490 from Nu-Check-Prep (Elysian, MN, USA) as well as 37 Component FAME Mix from Supelco (St. Louis, MO, USA). MS grade ammonium acetate (Sigma–Aldrich, Steinheim, Germany) methanol (Merck, Darmstadt, Germany) and 2-propanol (Honeywell, Seelze, Germany) were required for the LC-MS analysis of lipid classes. Standards for the lipid class analysis included oleic acid (FFA18:1), oleoyl monoacylglycerol (MAG18:1), dioleoyl diacylglycerol (DAG18:1), triolein (TAG18:1), dioleoyl phosphatidylcholine (PC18:1), cholesteryl oleate (CE18:1) and ethyl DHA from Larodan (Solna, Sweden). For the NMR analysis chloroform-*d* (99.96% D) containing 0.03% tetramethylsilane (TMS) as internal standard (Eurisotop, Saint-Aubin Cedex, France) was employed. For the PV analysis and sodium thiosulphate standardization potassium iodide (VWR, Leuven, Belgium), sodium thiosulphate (J.T.Baker, Deventer, Netherlands) starch (Riedel-de Haën, Seelze, Germany), potassium dichromate (Merck, Darmstadt, Germany) and hydrochloric acid (J.T.Baker, Deventer, Netherlands) were employed. The PAV reagents included *p*-anisidine (Sigma–Aldrich, Steinheim, Germany) and isooctane (Merck, Darmstadt, Germany). HPLC-grade hexane (VWR, Gliwice, Poland), chloroform (VWR Fontenay Sous

Bois, France), methanol (Sigma-Aldrich, Steinheim, Germany) and glacial acetic acid (VWR, Fontenay Sous Bois, France) were used in several analysis.

2.2 Sample materials

Most of the DHA containing omega-3 supplements and the DHA fortified margarine were purchased from supermarkets and pharmacies in Turku, Finland or ordered from Finnish web stores on September 2018. Samples included 40 fish- and fish liver oil products of which 26 were non-flavored (NF1-26) and 14 flavored (FF1-14), 5 krill oil products of which 3 were non-flavored (NK1-3) and 2 flavored (FK1-2), 3 micro algae oil products of which 2 were non-flavored (NA1-2) and 1 flavored (FA1) and 1 DHA containing margarine with micro algae oil (M1) (**Supplementary table 1**). The amount of samples was altogether 49, representing 27 different suppliers. Products included 35 capsule products, 10 liquid products, 3 gummies and 1 margarine. The margarine was chosen to be included in study because it is the only DHA fortified food item on the Finnish market, which is not intended for a specific consumer group. From the margarine (M1), all gummy products (FF3, FF4 and FF12) and three capsule products (NF4, NF12 and NF24) the oil was extracted by modified Folch method (Folch, Lees, & Stanley, 1957). Di-sodium citrate buffer (0.1 mol/L, pH 5.0) was added to products NF4, NF12 and NF24 prior to extraction to soften the capsules and to assist extraction. For improvement of the oil yield products FF3, FF4 and FF12 were milled to a powder with help of liquid nitrogen before extraction. Afterwards, lipids were extracted with chloroform:methanol (2:1 v/v) according to Folch et al. (1957). From all other capsules the needed sample oil for all analysis was collected with needle and syringe at dim light conditions to glass vials which were protected from light with foil and airspace was flushed with nitrogen. Liquid and extracted oil samples were stored similarly, i.e. under nitrogen at −80 °C. All the products had shelf life left at the time of sampling and analysis.

175

176 *2.3 Fatty acid content*

177 For the fatty acid content confirmations the amount of oil per capsule was checked by weighing
178 the capsule, removing the oil with needle and syringe and weighing the empty capsule shell.
179 For the capsules with high viscosity oil the capsule was cut in half and the oil was removed
180 with a cotton bud. For the liquid products the fish oil density 926 mg/mL was used for
181 calculations, unless the supplier had announced the density of the oil. The announced densities
182 varied from 915 to 1000 mg/mL. Because the extractions were not necessarily quantitatively
183 reliable the supplier declared oils amounts per capsule were used for the fatty acid content
184 calculations for the extracted products. For products NF4, NF25 and FF3 for which no oil
185 amount was declared, no fatty acid content per capsule were calculated.

186

187 For the GC analysis fatty acids were converted into volatile methyl esters using
188 borontrichloride in methanol according to European Pharmacopeia method Ph.Eur. 2.4.29
189 (European Pharmacopeia, 2005). The method was compared to methanolic hydrogen chloride
190 method using acetyl chloride in methanol (1:10) and incubating the samples in the oven at 50
191 °C overnight (Christie & Han, 2010). The fatty acid methyl esters were separated by Shimadzu
192 GC-2030 equipped with AOC-20i auto injector, flame ionization detector (Shimadzu
193 Corporation, Kyoto, Japan) and capillary column DB-23 (60 m × 0.25 mm × 0.25 μm, Agilent
194 technologies, J.W. Scientific, Santa Clara, CA, USA). Helium was used as a carrier gas.
195 Splitless injection with 0.5 μL sampling volume and 1 min sampling time were employed. The
196 temperatures were: inlet 270 °C; oven 130 °C held 1 min, 6.5 °C/min to 170 °C, 1.85 °C/min
197 to 205 °C, held for 12 min, 30 °C/min to 230 °C and held for 2 min; detector 280 °C. For the
198 acetyl chloride method the instrument setting was similar as described above, with the
199 exception of the temperature ramp from 170 °C to 205 °C, which was 2.75 °C/min with an 18

min hold. The analysis was performed in duplicate. The peaks were identified by using external standards Supelco 37 Component FAME mix, 68D, and GLC-490 in addition to previous literature (Christie & Han, 2010). Correction factors and internal standards were used for quantification.

2.4 Lipid classes

Lipid classes were analyzed by ultra-high performance liquid chromatography combined with electrospray ionization and mass spectrometer (UHPLC-ESI-MS) equipment including Waters Acquity UPLC (Waters co., Milford, MA) with Waters Cortecs UPLC C18 1.6 µm, 2.1 × 100 mm column, Waters Quattro Premier triple quadrupole MS. 10 mM ammonium acetate in MeOH:water (1:1 v/v) was used as mobile phase A and 10 mM ammonium acetate in 2-propanol:water (1000:1 v/v) as mobile phase B. Flow rate was 0.250 mL/min and gradients for A and B were as follows: until 33 min A:99% B:1%, 33–36 min A:1% B:99% and 36–38 min A:99% B:1%. Partial loop injections of 1 µL were done. The column oven temperature was set to 60 °C.

The MS was tuned with mass scans from 225 to 975 *m/z*. The capillary voltage was set to 4.90 kV, cone voltage to 40 V, extractor voltage to 6 V and RF lens voltage to 0 V. Source temperature was set to 120 °C and desolvation temperature to 400 °C. Desolvation gas flow was 749 L/h and cone gas flow 196 L/h. Mass scans from 100 to 1500 *m/z* were applied in both positive and negative ionization mode. Scan time was 0.7 s and interscan time 0.01 s. Compounds were identified with help of standards and known *m/z* values. The proportions of lipid classes were quantified by comparison to standards of known concentrations.

2.5 NMR

High-resolution ^{1}H NMR was used in untargeted manner. Oil samples (à 100 mg) were weighed and combined with 300 µL of chloroform-*d* (99.96% D) containing 0.03% tetramethylsilane (TMS) as internal standard for chemical shift calibration. An aliquot of 180 µL was transferred to a 3-mm NMR tube and analyzed with 600 MHz Bruker Avance-III NMR spectrometer equipped with a TCI-cryoprobe and pre-cooled SampleJet automatic sample changer unit (Bruker BioSpin AG, Fällanden, Switzerland). Standard proton experiments (*zg30*) were performed at 298 K with time domain 64k, sweep width 20 ppm, 128 scans, 4 dummy scans, acquisition time 2.726 s, and relaxation delay 2.0 s. The Fourier-transformed NMR data was phase-, baseline-, and shim-corrected (to 0.9 Hz) with Chenomx NMR Suite Professional v8.3 (Chenomx Inc., Edmonton, AB). The spectral data from 0 to 12 ppm was binned (0.04 ppm, with exclusion of solvent peak at 7.24–7.32 ppm) and then normalized to total binned area.

2.6 Peroxide and para-anisidine values

Due to the incomplete dissolution of some of the krill oil samples into isooctane the chloroform method IUPAC 2.501 (IUPAC, 1987) was employed for the PV determination. PAV was determined spectrophotometrically according to AOCS official method Cd 18-90 (AOCS, 2011). Analysis were performed in triplicate.

2.7 Volatile oxidation products

Volatile secondary oxidation products (VSOPs) were analyzed with headspace solid-phase micro extraction (HS-SPME) injector and GC-MS instruments Thermo Scientific Trace 1300 GC, TSQ 8000 Evo triple quadrupole MS and TriPlus RSH autosampler (Waltham, MA, USA) with SPB®-624 capillary column (60 m × 0.25 mm × 1.4 µm, Supelco, Bellafonte, PA, USA). For the analysis 20 mg of sample oil was incubated in 10 mL SPME vial for 20 min after which

30 min extraction to DVB/CAR/PDMS 50/30 µm (Supelco, Bellafonte, PA, USA) fiber took place. An incubation and extraction temperature of 40 °C were used based on our previous study (Damerau et al., 2014). Temperature for 5 min desorption in GC-injector port was 240 °C (splitless injection) and column oven temperature program as follows: 40 °C held for 6 min, 5 °C/min to 220 °C, held for 10 min. Helium (1.4 mL/min) was used as carrier gas. Electron ionization at 220 °C and 70 eV was employed for the MS and mass to charge ratios were scanned between 40–300 amu. Analysis were performed in triplicate. Compounds were tentatively identified by the database NIST MS Search library (version 2.3, National Institute of Standards and Technology, Gaithersburg, Maryland, U.S.A.). Data was processed with Xcalibur software (Thermo Fischer Scientific, Waltham, MA, USA).

2.8 Statistical analysis

For comparison of the two used methylation methods and for correlation tests between data sets RStudio 3.6.2 software (R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS 26.0 software (IBM Corporation, New York, USA) were used. Differences were considered statistically significant if *p*-value was below 0.05. For multivariate data analysis of NMR data, principal component analysis (PCA) was applied on the Pareto-scaled and mean-centered NMR data using the SIMCA v15 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). The compounds contributing the PCA loadings were identified with the help of the Chenomx NMR Suite's library and literature.

3. Results and Discussion

3.1 Content of Omega-3 fatty acids

The content of omega-3 fatty acids in the supplements was analyzed and compared with package information when possible (**Table 1**). The content for EPA was given for 40 of the 49 analyzed products. The analyzed content of EPA was 83% to 168% of claimed content with a median of 95%. The total concentration of EPA ranged from 0 in M1 to 452 mg per g of oil in NF21. Three products announced a combined EPA and DHA content only. For these products, the analyzed combined content of EPA and DHA was 93%, 86% and 114% of claimed content (NF2, NF3 and NF18, respectively). For DHA the analyzed content was 85% to 159% of the claimed content with a median of 109% for the 43 products with known content. NF26 had the highest DHA concentration with 551 mg/g. Similar as for EPA M1 had the lowest DHA concentration with 3 mg/g. It was not surprising that M1 had the lowest concentration of EPA and DHA as M1 was a margarine fortified with DHA and most likely for stability and cost reasons the DHA content was kept low but adequate to fulfill the health claim. In case of fish, micro algae and krill oil supplements the EPA and DHA concentrations varied greatly. However, it is obvious that supplements aimed at different consumer groups can have different compositions and indicated doses.

Table 1

The total omega-3 fatty acid content was stated for 35 of 49 analyzed products. The analyzed amounts of total omega-3 fatty acids in the products were 45% to 140% of the claimed amounts with a median of 97% and range from 58 to 792 mg/g. The legal limit for the omega-3 fatty acid concentration is sited to be 80% of claimed concentration in the United States of America and 90% in Australia (Ismail et al., 2016). Everything underneath these limits would be considered fraud. While Europe has no set limit on how much the actual concentration can deviate from claimed concentration, the European Commission's Guidance document relating

to food supplements advises a tolerance of +40% and –measurement uncertainty of label specified amount for polyunsaturated fatty acids (Regulation (EU) No 1169/2011). Considering a limit of 80%, all products were above the limit for EPA and/or DHA concentration. However, three products, NF16, NF22 and NA1 were below 80% in the total omega-3 content although the claimed EPA and DHA content were above the limit. Therefore, the content of other omega-3 fatty acids than EPA and DHA was too low in 6% of the 49 studied supplements. In case of NF16 this is may be due to miss-labeling as the supplement was claimed to have 1 g of omega-3 fatty acids per one gram of oil, i.e. to have omega-3 fatty acids only. The results for obliging to labeled content of EPA and DHA are similar as Kolanowski (2010) and Sprague et al. (2018) reported for supplements on the market in Poland and the United Kingdom, respectively. However, up to 69% of tested supplements marketed in New Zealand and South Africa has had EPA and DHA contents under 80% of those labeled (Albert et al., 2015; Opperman & Benade 2013).

The data obtained by the official Ph.Eur. 2.4.29 method (European Pharmacopeia, 2005) (**Table 1**) was compared to data acquired by methanolic hydrogen chloride method according to Christie and Han (2010). Methanolic hydrogen chloride method is commonly used and is less time consuming than most of the official methods. Therefore, there is an interest how the method performs compared to Ph.Eur. 2.4.29 method. In our study using the methanolic hydrogen chloride method for fish oil products 84% to 112% of EPA, 75% to 102% of DHA and 75% to 107% of total omega-3 fatty acids, and for micro algae oil products 97% to 102% of EPA, 88% to 92% of DHA and 95% to 101% of total omega-3 fatty acids, respectively, were obtained compared to Ph.Eur. 2.4.29 method. This meant that EPA, DHA and omega-3 fatty acids content was lower for 70%, 95% and 82% of all fish and micro algae oil products, respectively, using the methanolic hydrogen chloride method compared to the Ph.Eur. 2.4.29

method. However, the differences between methods was not statistically significant. In case of krill oil products the differences between methods were statistically significant, as only 66% to 88% of EPA (p = 0.0138), 61% to 80% of DHA (p = 0.0126) and 67% to 83% of total omega-3 fatty acids (p = 0.0233) were found using the methanolic hydrogen chloride methylation method compared to Ph.Eur. 2.4.29 method. The low results for the methanolic hydrogen chloride method for the krill oil products is most likely explained by solubility in the used solvent (hexane vs. trimethylpentane). Also, the extra saponification step before methylation in case of the Ph.Eur. 2.4.29 method compared to the methanolic hydrogen chloride method and the difference in internal standard (methyl tricosanoate vs. triheptadecanoin) may contributed to the difference between methods. Further, the multiple extraction steps of the methylesters in case of Ph.Eur. 2.4.29 method compared to the single extraction step in methanolic hydrogen chloride method could have improved the yield. Previous study by Carvalho and Malcata (2005) also found significant differences between methylation methods. They contributed the differences mainly to dissimilar polarities of the reaction and extraction medium. To avoid false underestimation, the use of the Ph.Eur. 2.4.29 method is important especially in the case of krill oils. In case of TAG- or EE-type marine oils the difference between methylation methods was not significant. However, Ph.Eur. 2.4.29 method gave for most products higher results than the methanolic hydrogen chloride method. This may also contribute to the negative results for EPA and DHA content in the studies by Albert et al. (2015) and Opperman and Benade (2013) as they used methanolic hydrogen chloride methods similar to the one tested in this study.

3.2 Lipid class analysis

Lipid class analysis was conducted to compare lipid class information to package information of omega-3 supplements and to determine possible impact of different lipid classes on oxidative

stability of the product. For 24 of the 49 analyzed products the lipid class type of the omega-3 source was declared. Of these 10 products were declared as TAGs, 9 products as EEs and 5 products as PLs (**Supplementary table 1**). Lipid classes were analyzed by UHPLC-ESI-MS and the data was confirmed by NMR. The analyzed proportion of lipid classes in **Table 2** showed that 32 products consisted largely of TAGs and 12 products were in majority EEs (≥ 80%). For the remaining 5 products a mixture of mainly two lipid classes was found. In 11 products 5% to 15% of diglycerides (DAGs) were detected. In all these products TAGs were identified as the main lipid class, highly indicating that these products consist of re-esterified TAGs generated by transesterification used after fractionation and concentration of omega-3 fatty acids.

Free fatty acids (FFAs), monoglycerides (MAGs) and DAGs were present in traces (≤ 3%) in 70% of all products. The detected FFAs, MAGs and DAGs are most likely remnants of refining and re- or inter-esterification processes. Traces of FFAs are a concern for lipid oxidation, as FFAs are known to be oxidatively less stable than esterified forms (Miyashita & Takagi, 1986). Profiles of fish oil products were comparable to data previously reported by Galuch et al. (2018), Kutzner et al. (2017) and Sprague et al. (2018). All micro algae oil products were largely TAGs as found also by Kutzner et al. (2017). They concluded that the algae oil products contained purified and refined algae oil containing natural TAGs based on the lack of EEs and the lower omega-3 fatty acid content than in fish oil products. Compared to the study by Kutzner et al. (2017) the omega-3 fatty acid content in micro algae oil was not significantly lower than for the fish oil products in the presented study. However, no significant remnants of re- or inter-esterification processes could be found, which points towards the content of natural TAGs in the micro algae oil products. In both studies the sample size for the algae oil

products was small compared to fish oil products, as vegan alternatives based on algae oil are only a minor proportion of omega-3 supplement market.

Table 2

For fish and algae oil products the analyzed lipid class type was in majority (≥ 95%) the declared lipid class type, expect for NF15. NF15 was declared as TAG type but contained also ≤ 25% of EEs (**Table 2**, **Supplementary Figure 1**), which are most likely residues from an incomplete transesterification process. This may effect bioavailability if one-fourth of oil are EEs instead of TAGs. Interestingly, all flavored fish and all micro algae oil products were in majority of TAG form, which could be considered to be positive as these products are often marketed for children and the bioavailability of EPA and DHA is higher from TAGs than EEs in general (Salem & Eggersdofer, 2015).

Krill oils are less refined than fish oils in order to sustain the content of PLs as source of EPA and DHA. Therefore, krill oil containing omega-3 supplements mainly display natural distribution of lipid classes in krill (Kutzner et al., 2017). All krill oil products except NK2 had TAGs and PLs as their main lipid classes type with ≤ 5% FFAs and traces of DAGs and MAGs (**Table 2**). The ratio between TAGs and PLs was similar as reported previously for krill oil supplements (Kutzner et al., 2017). The FFAs proportion was significantly higher than in fish and micro algae oil products. Kutzner et al. (2017) found 3 to 7% FFAs in krill oils. De Boer et al. (2018) reported the highest acid value for krill oil which was ten times higher than for fish oil products. Therefore, our results of FFAs in krill oil products are in line with the previous reported data. In NK2 mainly EEs were detected. Only small amounts (≤ 2%) of TAGs and PLs were present. The package information of NK2 stated that NK2 is a mixture of krill and fish oil. The lipid class analysis highly indicated that NK2 contains ≥ 95% fish oil in EE form

and ≤ 5% krill oil. As the krill oil should not contain any EEs, declaring NK2 as krill oil product as the package suggested, is misleading with such low content of krill oil. Galuch et al. (2018) previously also reported misdeclaration based on lipid class analysis.

3.3 ^{1}H NMR analysis

High-resolution ^{1}H NMR metabolomics was applied to study the lipid profiles of the omega-3 supplements in untargeted manner. The PCA model in **Figure 1** shows the similarities and differences in lipid profiles as analyzed with NMR. Grouping along the first principal component is mostly explained by the relative proportions of omega-3 vs. non-omega-3 lipids. The EE-type and krill oil products contributed to the positive half of the PC2 (explaining 10.3% of the total variance), while the TAG-type/fish oil-based, micro algae, mixed-type (containing both fish and vegetable oil) and flavored products contribute to the negative half. The presence of flavoring agents or α-tocopherol [triplet at 2.59 ppm (J = 6.8 Hz)] had little or no effect on the model loadings. ^{1}H NMR is applicable in analyzing both primary and secondary oxidation products (e.g. from fish oils or dispersed food systems following accelerated oxidation) (Mozuraityte et al., 2017; Merkx, Hong, Ermacora and van Duynhoven, 2018) but none were detected here. This may be due to the methodological approach and the type and oxidative status of the analysed samples. Band-selective pulse excitation could be used to improve the detection and quantification of hydroperoxides and aldehydes (Merkx et al., 2018).

Figure 1

The samples with the highest proportion of non-omega-3 fatty acids were M1 and a liquid supplement containing FF8 mostly plant oils (**Supplementary table 1**). The liquid products

and most of the supplements that declared to contain rosemary extract as an antioxidant lay in the bottom right quadrant of the PCA. The variation in the lipid composition of the capsule-type supplements is considerable especially among the TAG-type oils. The EE-type products were characterized having the highest levels of EPA, while the krill oils were characterized by the presence of phospholipids and generally higher levels of cholesterol. The spatial grouping of NF18 with the krill oil-type samples was explained by its relatively high $(CH_2)_n$-signal at 1.26 ppm (contributing to bin 1.28 in **Figure 1A**). The likeness of NK2 with fish oil samples was again explained by the high presence of EEs as previously discussed.

The qualitative examination of NMR spectra revealed the presence of monomethyl and dimethyl furan fatty acids in relatively low but varying levels at 1.88 and 1.82 ppm, respectively (Gottstein, Müller, Günther, Kuballa, & Vetter, 2019). These minor constituents (characteristic to fish oil) may exhibit antioxidative and radical scavenging properties. Although the impact of furan fatty acids on the PCA model loadings was not substantial, the samples on the left half of the PC1 contained the highest relative amounts of them.

NMR can be used as a rapid method to determine the oil type and the lipid class (EE/TAG/PL) of the omega-3 supplements. Successful relative and absolute quantitative analysis of lipids based on ^{1}H NMR have been reported, with good correlation to either label information or conventional methods (e.g. GC) (Williamson & Hatzakis, 2017). However, the number of samples used in these studies has been very limited or not mentioned (Dais, Misiak & Hatzakis, 2015; Williamson & Hatzakis, 2017). The spectral data from the 49 products analysed here indicate that there may be more limitations of the integration-based quantitative analysis of lipids in omega-3 supplements than what e.g. Williamson and Hatzakis (2017) indicated. Integration for many of the signals used in the calculations, for example the Hα/Hβ methylene

protons of DHA at δ 2.38 ppm and the terminal methyl groups at δ 0.88 and 0.97 ppm, is often compromised by overlapping peaks. ^{13}C NMR can provide more accurate quantitation for omega-3 supplements, as well as information on the *sn*-positions of the fatty acids (Williamson & Hatzakis, 2017).

3.4 Lipid oxidation analysis using PV and PAV

For determination of oxidative status of omega-3 supplements PV and PAV were analyzed using official methods from IUPAC and AOCS, respectively. Ten fish oil and one micro algae oil product exceeded the limit of 5 meq/kg for PV recommended by GOED (**Table 3**). FF10 and NA1 showed the highest PVs with 16.29 and 14.99 meq/kg, respectively, which is more than double of GOED limit. In general, 11 products exhibited a PV over 5 meq/kg, but only 2 products had a PV over 10 meq/kg, which is the limit set by European and British Pharmacopeias for type I fish oil (the limit for type II is same as GOED recommend limit) (Ismail et al., 2016). The determination of PV for the krill oil products was challenging as no color change prior to titration was observed, which could be due to not enough peroxides to induce the color reaction or the method not being suitable for krill oils because of disturbances. The method was tested with an increased sample amount and an oxidized krill oil. The oxidized sample resulted in a slight color change allowing the determination of the PV. However, even tripling the sample amount the result for most krill products was 0 meq/kg. De Boer et al. (2018) also had significant lower PVs for krill oils than fish oils with an average of 0.56 for krill oils and 2.66 for fish oils. Mozuraityte et al. (2017) had issue concerning the repeatability of PV analysis of krill oils even using potentiometric end point detection, which did not require a visible color change.

Table 3

The GOED-threshold of 20 for PAV was only exceed by NF24. The European and British Pharmacopeias threshold of 30 for type I fish oil was not surpassed by any sample. For type II fish oil the limit is lower with 15, which was exceed by NF18, NF24 and FF13. However, the official method used was only suitable for 73% of the studied products. Majority of the flavored and krill oil products were excluded because of disturbances causing over- or underestimation of PAV. Many of the added flavors are known to contain compounds with aldehyde structures, e.g. citral (3,7-dimethyl-2,6-octadienal) in lemon aroma, which show a similar reaction as aldehydes formed from lipid oxidation and therefore increases PAV compared to an oil without flavor (Ismail et al., 2016). Some krill oil containing supplements did not fully dissolve in the isooctane, as they showed a clear phase separation. Further, the color of krill oil caused by natural containing carotenoids disturbed the photometric determination resulting in data with a high variance and negative values. Therefore, the method was deemed to be unsuitable for the krill oil products. Earlier studies have encountered similar issues (Ismail et al., 2016; Jackowski et al., 2015; Mozuraityte et al., 2017; Thomsen et al., 2013). The TOTOX threshold of the GOED was exceeded by NF24, FF7, FF8 and NA1. All of them also exceed either the threshold for PV or PAV. In general, 12 of 49 studied products (24%) were, based on one or more parameters, of not acceptable quality according to GOED. This is a comparable percentage of studied products not complying to GOED-thresholds, in studies by Bannenberg et al. (2017), Kolanowski (2010), and Sprague et al. (2018) analyzing products on market in New Zealand, Poland and the United Kingdom, respectively. Significant higher non-compliances for GOED limits for PV and/or PAV have been reported by Albert et al. (2015), Heller et al. (2019), Jackowski et al. (2015), Mason and Sherrat (2017) as well as Opperman and Benade (2013), for products on the market in New Zealand, Australia, North America, United States of America and South Africa. However, Albert et al. (2015) and Jackowski et al. (2015) did not

exclude flavored products, although, Jackowski et al. (2015) discussed limitation in their PAV analysis. In the study from Opperman and Benade (2013) only primary oxidation was studied. Mason and Sherrat (2017) had a small sample size compared to all other mentioned studies. Of all previously cited publications De Boer et al. (2018) reported the biggest sample size with data of 1900+ globally-sourced fish oil samples. For fish oil products 13.8% exceed PV of 5 meq/kg, 6.1% exceeded PAV of 20 (only considering unflavored oil) and 8.8% exceeded TOTOX limit of 26. The study showed that the fish oil products predominantly had a low oxidative status. However, it needs to be taken into account that most samples were submitted directly from the manufacturers, which may have influenced the outcome of the study by pre-selection and remaining shelf-life. Another factor not considered in this study or any of the above cited studies is how the overall diet and the conditions in the digestive track affect the oxidative status of omega-3 supplements. Tirosh, Shpaizer and Kanner (2015) studied a supplement with a starting PV of 1 meq/kg under stomach conditions, in the presence of red meat. In the acidic stomach conditions the hydroperoxide content increased to an equivalent of PV of 9 meq/kg, which is higher than the PV threshold of the GOED. This raises concerns for even slightly oxidized omega-3 supplement especially when ingested by consumers on a diet low in fish and high in meat. Further studies in diet-supplement interactions in in-vivo conditions are needed. Since, clearly, oxidized supplements have been shown to not provide the promised health benefits and may even be harmful (Rundblad et al., 2017).

3.5 Analysis of VSOPs

As an alternative method for lipid oxidation determination, analysis of VSOPs using HS-SPME-GC-MS was chosen based on being a rapid method with minimal sample preparation. Therefore, it has the potential to be used in industry in the future. The lack of sample handling prevents promotion of oxidation. A maximum of 70 volatiles were identified in the analyzed

products. For determination of lipid oxidation 20 known VSOPs were selected based on their abundance and being mainly formed from the oxidation of EPA and DHA (**Figure 2 and 3**). Similar VSOPs were found in previous oxidation studies of fish, algae and krill oils under accelerated oxidation conditions (Gómez-Cortés, Sacks, & Brenna, 2015; Lee et al., 2003; Thomsen et al., 2013; Yang, Cheng, Chen, Tseng, Lin, & Chiang, 2017). The obtained results were compared to a test fish oil, which was oxidized for 48 h at 50 °C (data not shown). Only traces of VSOPs were found in 20 analyzed products (**Figure 2**). In case of 27 products a total peak intensity between 0.5 and 1.5 $\times 10^9$ and a maximum of 7 VSOPs was detected. This level of lipid oxidation was still considered moderate. Some level of VSOPs is expected even from a fish oil, which is considered to be non-oxidized, as 0 time data of previous oxidation studies showed (Lee et al., 2003; Thomsen et al., 2013; Yang et al., 2017).

Figure 2

The total peak area of the selected volatiles and the number of VSOPs (**Figure 2**) showed that NF24 was the most oxidized product with the highest peak area and 13 VSOPs detected. NF24 was also the only product with a PAV over 20. As aldehydes are one of the major classes of VSOPs some correlation to PAV may be expected as PAV is an indirect method for aldehyde analysis. However, considering the whole data set no correlation between VSOPs and PAV could be found, also not if only volatile aldehydes were considered. Besides the NF24 also FF6 was considered significantly oxidized based on VSOPs content. Although, only two VSOPs, octanal and ethylfuran **(Supplementary table 2),** of the selected VSOPs were detected in FF6 the peak area was over 1.5×10^9. The main contributing VSOP was octanal. Octanal is a main VSOP produced by oxidation of oleic acid (C18:1). While FF6 contained 65 mg/g of oleic acid it also contained tutti frutti aroma, which can be comprised partly of citrus oil, which naturally

548 contains octanal. However, none of other flavored products, even the citrus or lemon flavored
549 ones, had a significant octanal peak (over the limit for quantification). Therefore, it is difficult
550 to determine the source of octanal in FF6. It can also be a combination of both lipid oxidation
551 and added aroma. For FF6 no PAV could be analyzed because of interfering flavor compounds
552 present. However, the PV for FF6 was above 7 meq/kg. In general, no correlation between PV
553 and VSOPs was found, which was also not expected as PV detects primary lipid oxidation and
554 while VSOPs are formed in the later stages of lipid oxidation. In summary, 98% of all analyzed
555 omega-3 supplements were considered acceptable based on VSOPs content.
556
557 Flavor compounds were detected in flavored products. Flavor compounds can disturb the HS-
558 SPME analysis by competing with VSOPs for space on the fiber, which can result in reduced
559 amounts of VSOPs extracted and therefore false interpretation of oxidative status. However, in
560 this study after volatile participation tests a low sample amount of 20 mg was selected to reduce
561 the overall volatile amount and allow enough space on the fiber. No significant difference
562 between VSOP amount and number was observed for NF1 and FF1, which were the same type
563 of fish oil either non-flavored or flavored based on the package information. In general, no
564 trend for reduction of VSOPs compared to non-flavor products was observed.
565
566 In majority of the krill oil products not only VSOPs and added flavor compound were detected
567 but also volatiles formed from non-enzymatic browning reactions. Strecker aldehydes 2-
568 methylbutanal and 3-methylbutanal were found in NK3 and FK2. In NK1 and FK1 dimethyl
569 disulfide, dimethyl trisulfide, pyridine and trimethylpyrazine were detected. 2-methylbutanal,
570 3-methylbutanal, dimethyl disulfide and pyridine were also detected in krill oil but not in fish
571 oil by Thomsen et al. (2013) in an accelerated oxidation test. No non-enzymatic browning
572 reaction products were identified in NK2, which was not surprising as none of the fish oil

products contained any non-enzymatic browning reaction products and NK2 was in majority fish oil as discussed previously. The non-enzymatic browning reaction products may increase oxidative stability of krill oil products as discussed by Mozuraityte et al. (2017) and Thomsen et al. (2013). This may compensate the higher susceptibility to lipid oxidation based on higher FFA content compared to fish and micro algae oils. However, as almost all of the tested products showed low signs of oxidation and no storage / long-term oxidation test was conducted, no differences in oxidative stability between krill, fish and micro algae oil was observed, unlike by Thomsen et al. (2013) under accelerated oxidation conditions. Further, no significant differences in oxidative stability between products in TAG- or EE-form were found as previously described by Lee et al. (2003). However, the great differences in omega-3 fatty acid content, especially EPA and DHA, in 49 studied products made comparison difficult. For different delivery forms of the omega-3 fatty acids also no significant differentiation in oxidative status were noticed.

Figure 3

The abundance of each selected VSOP in the 49 products showed that 2,4-heptadienal (*E,Z/E,E*), 1-penten-3-ol, 2-hexenal (*E*), 2-ethylfuran, hexanal and propanoic acid were detected in more than 25 products (**Figure 3**). The sum peak area of 49 products was highest for 2,4-heptadienal (*E,Z/E,E*) followed by 1-penten-3-ol, 2-hexenal (*E*) and 2-ethylfuran (**Figure 3**). 2,4-Heptadienal (*E,Z/E,E*) is one of the main VSOP of long chain omega-3 polyunsaturated fatty acids and is formed from 14-hydroperoxide of EPA and in case of DHA from 16-hydroperoxide (Lee et al., 2003). 2-Ethylfuran is also formed from 14-hydroperoxide of EPA and 16-hydroperoxide of DHA by cyclization of the vinyl hydroperoxide after loss of a hydroxyl radical (Gómez-Cortés et al., 2015). 1-Penten-3-ol and 2-hexenal (*E*) are the main

VSOPs produced by degradation of 15-hydroperoxide of EPA and 17-hydroperoxide of DHA (Lee et al., 2003). The abundance and concentrations of 2,4-heptadienal (*E*,*Z*/*E*,*E*), 1-penten-3-ol, 2-hexenal (*E*) and 2-ethylfuran in the different products make them possible marker compounds for omega-3 oil quality. 2,4-Heptadienal was also recommended as marker compound by Yang et al. (2017). Using measurement of different VSOPs as marker/indicator compounds for the determination of the oxidative state is possible alternative to PAV in regard to oil quality analysis, as this study showed other VSOPs than aldehydes e.g. 1-penten-3-ol or 2-ethylfuran can be present in significant concentrations and not be detected by PAV methods. Further, direct analysis of VSOPs is more reliable than indirect assays like PAV as likelihood of false positives through disturbances is extremely low compared to PAV. However, VSOPs analysis must be standardized with clear thresholds for selected indicator compounds to be reliable alternative method to PAV. So far, comparisons between numerous studies is difficult due to differences in analysis method and equipment used.

4. Conclusions

All of the tested 49 products complied with information given by producer in regard to omega-3 fatty acid content and lipid class, expect for three products in case of total omega-3 fatty acid content and one product regarding the lipid class distribution. ^{1}H NMR was useful as a rapid method for oil type and lipid class determination of the omega-3 supplements. However, this study showed that the capacity to use ^{1}H NMR for relative and absolute quantitative analysis of lipids may be more limited as reported earlier for smaller sample sizes. No indication of oxidation was found based on the NMR profiles. GOED-thresholds for PV and PAV were exceeded by 24% of the studied products indicating increased lipid oxidation. However, PV and PAV analysis showed great limitation especially in regard to krill oil and flavor products,

showing to be not sufficient for determining oxidative quality of omega-3 supplements as hypothesized. Based on VSOP two of 49 products showed severe and 27 moderate sign of lipid oxidation. Analysis of VSOP seems to be a good alternative for PAV analysis for omega-3 supplements, but needs standardization. 2,4-Heptadienal, 1-penten-3-ol and 2-hexenal showed the highest potential to be used as indicator compounds for lipid oxidation in products with high EPA and DHA content. Further research into rapid analysis methods to replace PV and PAV as methods used by industry for oil quality is highly recommended.

Overall, the analysis showed that the quality of omega-3 supplements on the Finnish market is improvable as nearly one fourth of all studied products were oxidized based on one or more measured parameters compared with the GOED recommendations. Improvement in quality for these products is highly suggested. However, a vast majority (>75%) was acceptable according to all measured parameters, and thus alteration of supplements could be recommended to consumers using them frequently.

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Conflict of interest statement

Authors declare no conflicts of interest.

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Figures

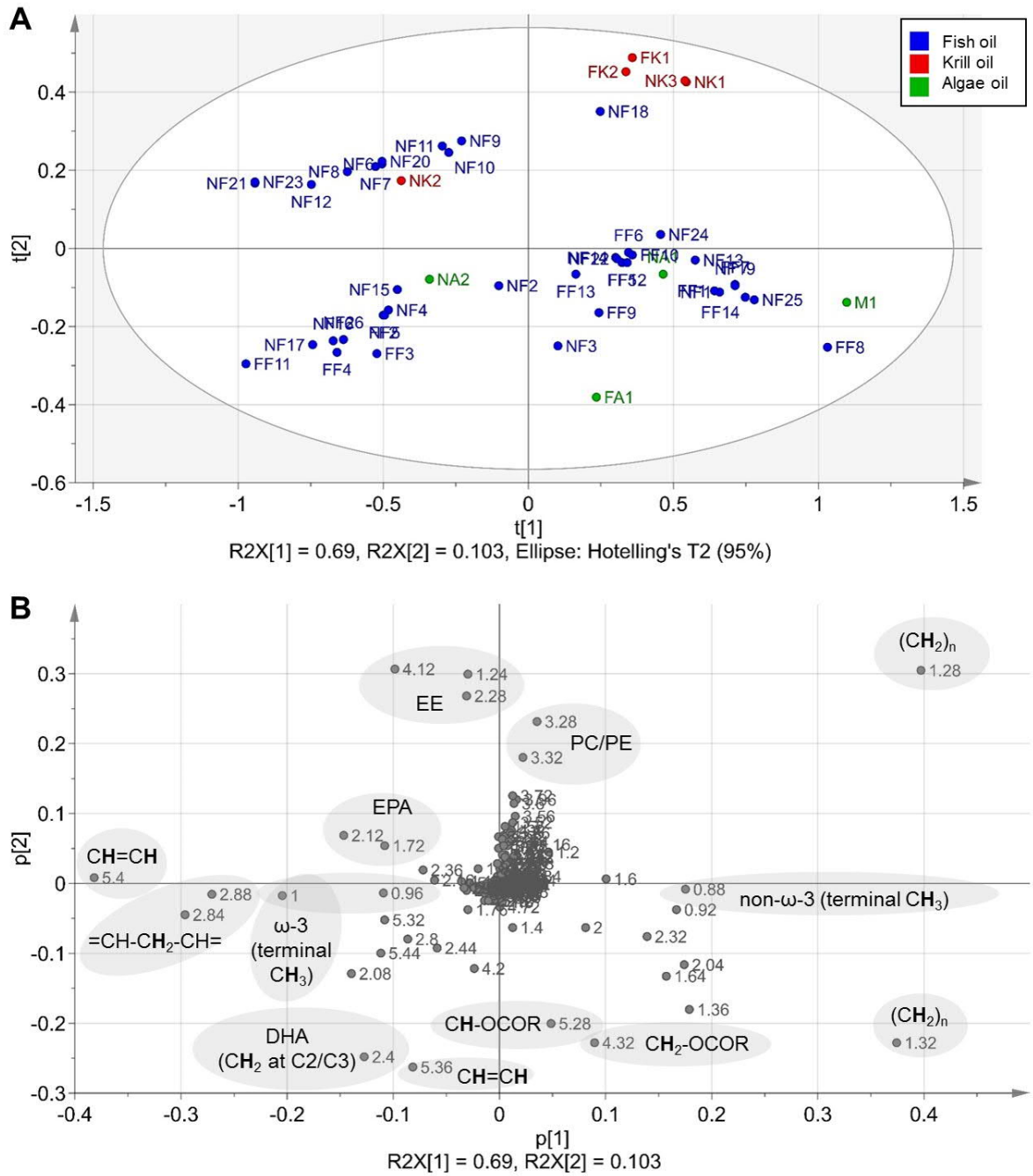


Figure 1. PCA model (t[1] vs. t[2]; R^2X[1]=0.690, Q^2[1]= 0.651; R^2X[2]=0.103, Q^2[2]= 0.134) representing the binned, Pareto-scaled NMR data. A) Scores plot. Observations (n = 49) are coloured and labelled according to the primary source of EPA/DHA. B) Loadings plot.

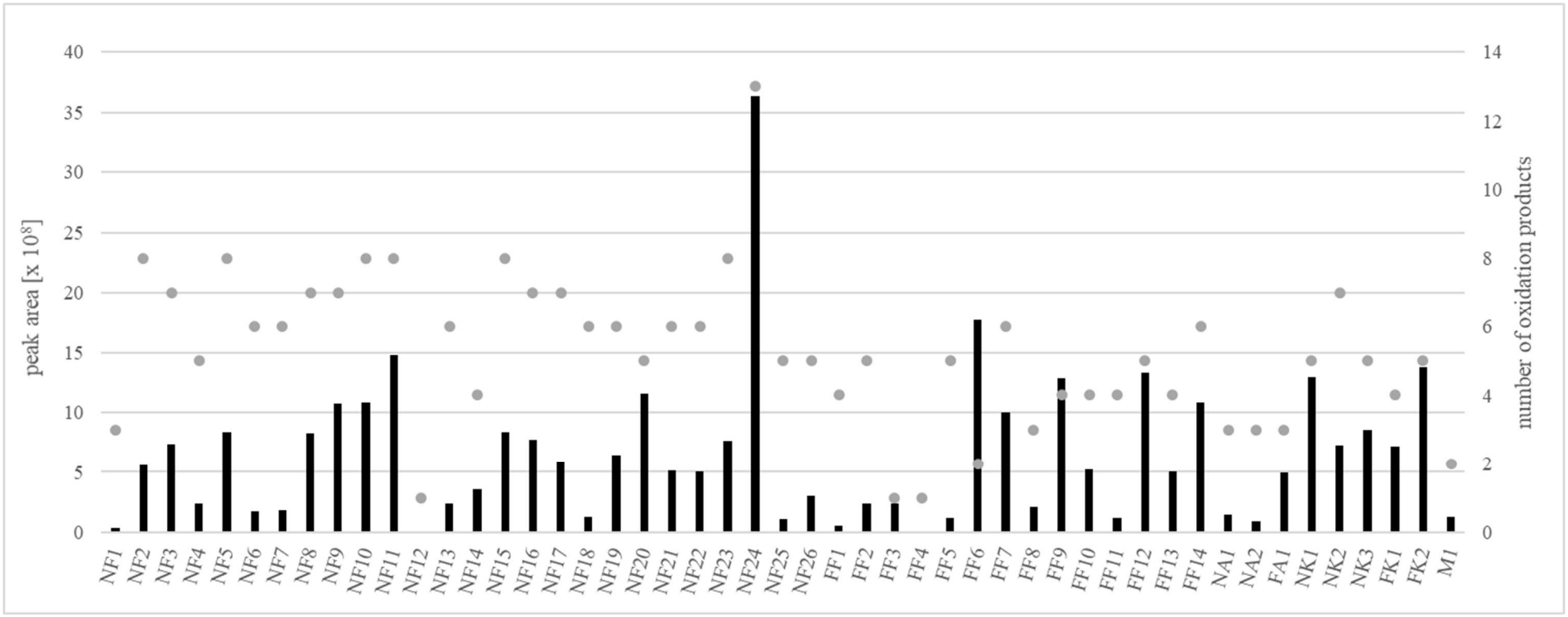


Figure 2. Sum of peak area (bars) and number of volatile oxidation products (dots) (max. 20; propanal, 2-propenal, propanoic acid, butanoic acid, 1-penten-1-ol, 1-penten-3-one, pentanal, 2-pentenal (*Z*), hexanal, 2-hexenal (*E*), benzaldehyde, 2,4-hexadienal (*E,Z*), 4-heptenal (*Z*), 2,4-heptadienal (*E,Z/E,E*), octanal, 3,5-octadien-2-one (*E,Z*), 2-nonanone, 2,6-nonadienal (*E,Z*), 2-ethylfuran, 2-pentyfuran) of 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 micro

algae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1) (n = 3) analyzed by headspace solid-phase micro extraction with gas chromatography-mass spectrometry (HS-SPME-GC-MS).

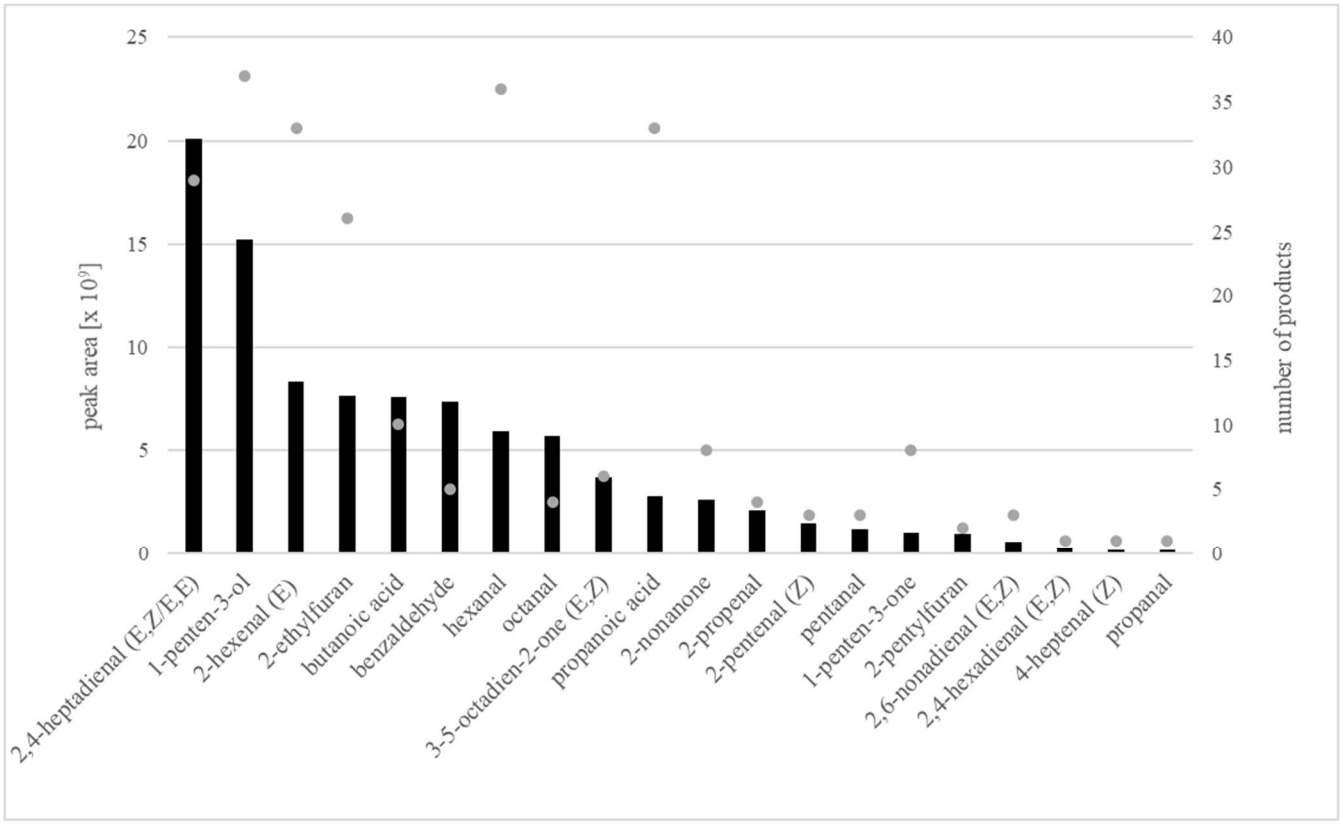


Figure 3. Sum of peak areas of individual volatile compounds of all 49 analyzed omega-3 supplements (bars) and number of products in which the individual volatile compounds were detected over the quantification limit (dots).

Tables

Table 1. Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total omega-3 fatty acid content of 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 micro algae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1) given in mg per g of oil (mean ± standard deviation; n = 2). The analyzed content was compared to the package information (**Supplementary table 1.**) and given in percent [%] of stated content (100%). The presented data was obtained according to European Pharmacopeia method Ph.Eur. 2.4.29.

Sample	**EPA**		**DHA**		**EPA + DHA**	**total omega-3**	
	mg/g	**%**	**mg/g**	**%**	**%**	**mg/g**	**%**
NF1	76 ± 2	88	118 ± 4	91		245 ± 8	95
NF2	206 ± 6		207 ± 11		93	485 ± 19	
NF3	111 ± 3		127 ± 4		86	474 ± 16	90
NF4*	294 ± 4		229 ± 8			595 ± 13	
NF5	296 ± 3	91	247 ± 4	101		622 ± 7	93
NF6	317 ± 1	98	255 ± 6	110		651 ± 7	
NF7	308 ± 1	96	267 ± 1	116		665 ± 2	100
NF8	159 ± 2	91	431 ± 4	99		653 ± 7	99
NF9	265 ± 4	85	189 ± 4	91		534 ± 9	85
NF10	261 ± 1	84	194 ± 1	95		534 ± 1	86
NF11	260 ± 2	83	206 ± 2	99		548 ± 4	87
NF12	105 ± 2		546 ± 16	118		732 ± 23	
NF13	94 ± 1	116	114 ± 2	157		281 ± 4	140
NF14	161 ± 0	99	122 ± 0	121		352 ± 1	
NF15	271 ± 8	95	240 ± 6	125		585 ± 16	
NF16	226 ± 2	94	342 ± 3	93		684 ± 5	66
NF17	241 ± 2	99	350 ± 4	98		706 ± 8	107
NF18	164 ± 2		121 ± 1		114	369 ± 30	
NF19	167 ± 12	91	124 ± 9	101		362 ± 26	119

NF20	301 ± 1	93	243 ± 7	112		626 ± 7	103
NF21	452 ± 3	91	263 ± 2	106		792 ± 5	94
NF22	173 ± 2	106	114 ± 0	112		358 ± 3	59
NF23	441 ± 2	88	248 ± 1	99		772 ± 5	90
NF24*	127 ± 0		90 ± 0			271 ± 1	
NF25	31 ± 0	113	88 ± 2	117		168 ± 1	97
NF26	93 ± 0	168	551 ± 6	120		685 ± 7	124
FF1	72 ± 1	84	110 ± 1	85		231 ± 2	89
FF2	305 ± 7	94	245 ± 4	102		629 ± 15	94
FF3*	116 ± 2		472 ± 2			664 ± 2	
FF4	122 ± 3	121	417 ± 4	100		664 ± 9	105
FF5	156 ± 1	98	120 ± 0	121		337 ± 1	101
FF6	156 ± 1	96	117 ± 1	110		349 ± 28	104
FF7	103 ± 1	90	55 ± 0	105		193 ± 10	95
FF8	102 ± 0	89	54 ± 0	104		197 ± 0	97
FF9	39 ± 0	96	11 ± 0	92		55 ± 0	
FF10	197 ± 2	107	64 ± 1	113		315 ± 3	
FF11	151 ± 0	94	115 ± 0	115		325 ± 0	93
FF12	248 ± 2	104	480 ± 3	101		791 ± 5	101
FF13	106 ± 2	138	225 ± 2	115		398 ± 4	127
FF14	32 ± 1	116	90 ± 1	127		184 ± 3	120
NA1	11 ± 0	102	294 ± 4	109		311 ± 4	45
NA2	160 ± 3	84	409 ± 8	128		629 ± 12	118
FA1	4 ± 0		135 ± 1	112		490 ± 2	
NK1	98 ± 1	106	59 ± 1	115		187 ± 4	130
NK2	192 ± 1	89	339 ± 3	93		607 ± 4	95
NK3	126 ± 1	86	64 ± 1	92		220 ± 8	
FK1	129 ± 1	103	91 ± 1	159		285 ± 0	124
FK2	139 ± 10	94	82 ± 12	120		274 ± 24	104
M1	0		3 ± 0	89		58 ± 0	

* no package information on EPA, DHA and omega-3 fatty acid content is known in mg per g oil due to no information on the amount of oil in one capsule or gummy.

Table 2. Proportions [%] of lipid classes (triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs), ethyl esters (EEs), phospholipids (PLs) and free fatty acids (FFAs) of 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 micro algae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1) based on ultra-high performance liquid chromatography combined with electrospray ionization and mass spectrometric (UHPLC-ESI-MS) analysis (example chromatogram see Supplementary Figure 1) and nuclear magnetic resonance (NMR) spectroscopy.

Sample	**TAGs**	**DAGs**	**MAGs**	**EEs**	**PLs**	**FFAs**
NF1	≥ 98%	-	-	-	-	≤ 1%
NF2	≥ 95%	≤ 3%	≤ 1%	-	-	-
NF3	≥ 98%	-	-	-	-	≤ 1%
NF4	≥ 85%	≤ 15%	≤ 1%	-	-	-
NF5	≥ 85%	≤ 15%	≤ 1%	-	-	-
NF6	-	-	-	≥ 99%	-	-
NF7	-	-	-	≥ 99%	-	-
NF8	-	-	-	≥ 98%	-	≤ 1%
NF9	-	-	-	≥ 99%	-	-
NF10	-	-	-	≥ 99%	-	-
NF11	-	-	-	≥ 98%	-	≤ 1%
NF12	≤ 1%	-	-	≥ 98%	-	-
NF13	≥ 99%	-	-	-	-	-
NF14	≥ 99%	-	-	-	-	-
NF15	≥ 60%	≤ 15%	≤ 1%	≤ 25%	-	-
NF16	≥ 80%	≤ 15%	≤ 1%	≤ 3%	-	≤ 1%
NF17	≥ 85%	≤ 15%	≤ 1%	-	-	-
NF18	≥ 99%	-	-	-	-	-
NF19	-	-	-	≥ 99%	-	-
NF20	-	-	-	≥ 99%	-	-
NF21	-	-	-	≥ 99%	-	-
NF22	≥ 99%	-	-	-	-	≤ 1%

NF23	-	-	-	≥ 99%	-	-
NF24	≥ 98%	≤ 1%	≤ 1%	-	-	≤ 1%
NF25	≥ 98%	≤ 1%	-	-	-	≤ 1%
NF26	≥ 80%	≤ 15%	≤ 1%	≤ 3%	-	-
FF1	≥ 99%	-	-	-	-	-
FF2	≥ 85%	≤ 15%	≤ 1%	-	-	-
FF3	≥ 93%	≤ 5%	≤ 1%	-	-	≤ 1%
FF4	≥ 93%	≤ 5%	≤ 1%	-	-	-
FF5	≥ 99%	-	-	-	-	-
FF6	≥ 99%	-	-	-	-	-
FF7	≥ 99%	-	-	-	-	-
FF8	≥ 99%	-	-	-	-	-
FF9	≥ 99%	-	-	-	-	-
FF10	≥ 97%	≤ 3%	-	-	-	-
FF11	≥ 98%	≤ 1%	-	-	-	-
FF12	≥ 85%	≤ 15%	≤ 1%	-	-	-
FF13	≥ 90%	≤ 10%	≤ 1%	-	-	-
FF14	≥ 98%	≤ 1%	≤ 1%	-	-	-
NA1	≥ 98%	≤ 1%	-	-	-	-
NA2	≥ 98%	≤ 1%	-	-	-	-
FA1	≥ 99%	-	-	-	-	≤ 1%
NK1	≥ 50%	≤ 1%	≤ 1%	-	≥ 40%	≤ 5%
NK2	≤ 2%	-	-	≥ 95%	≤ 2%	≤ 1%
NK3	≥ 40%	≤ 1%	≤ 1%	-	≥ 50%	≤ 5%
FK1	≥ 40%	≤ 3%	≤ 1%	-	≥ 50%	≤ 5%
FK2	≥ 40%	≤ 3%	≤ 1%	-	≥ 50%	≤ 5%
M1	≥ 99%	-	-	-	-	-

Table 3. Peroxide (PV) and para-anisidine values (PAV) of 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 micro algae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1) (mean ± standard deviation; n = 3), and the calculated total oxidation value (TOTOX = PAV + 2 * PV).

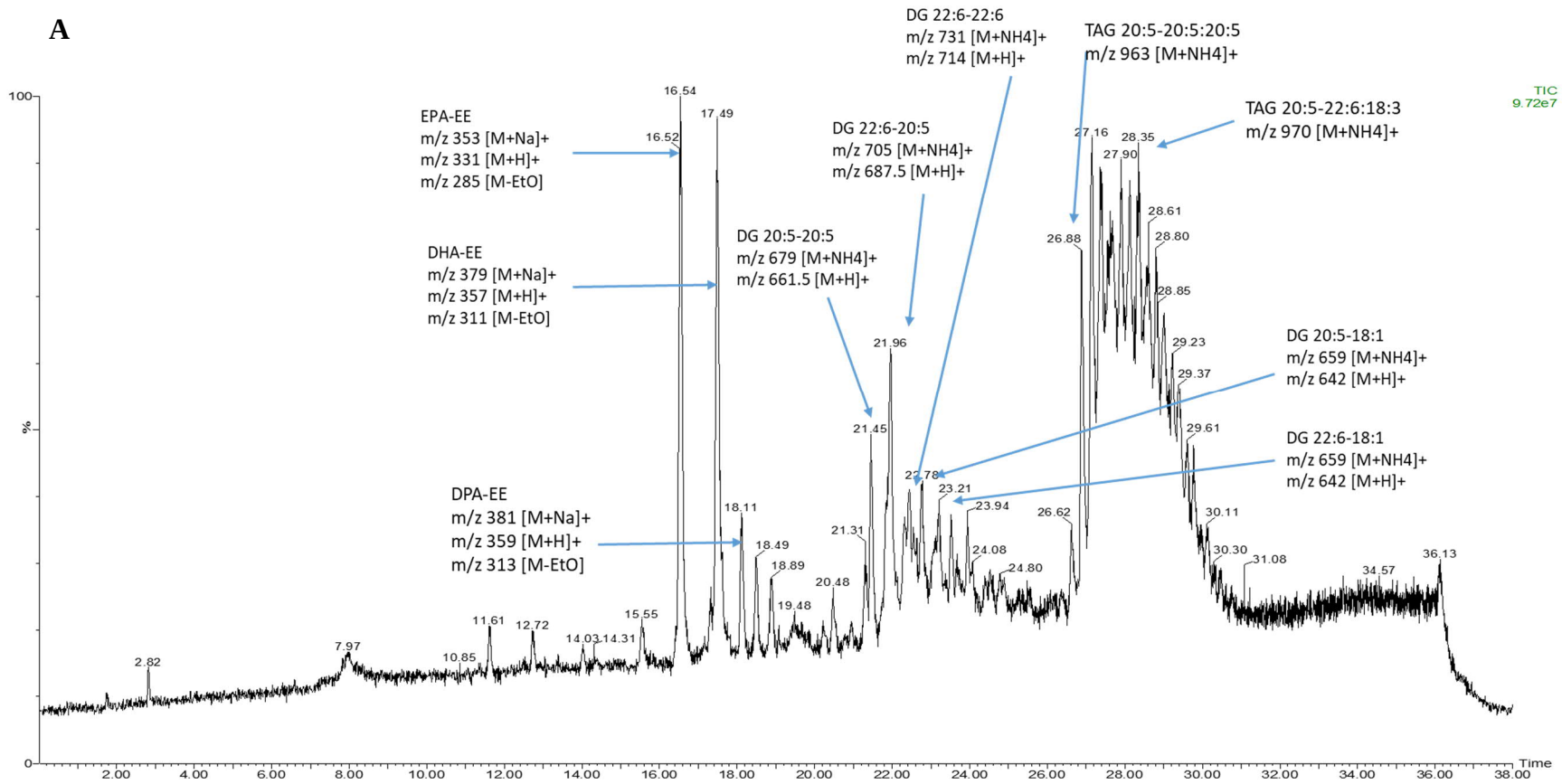
Sample	PV [meq/kg]	PAV	TOTOX
NF1	1.01 ± 0.05	4.14 ± 0.31	6.2
NF2	4.08 ± 0.18	11.52 ± 0,13	19.7
NF3	2.33 ± 0.04	3.16 ± 0.27	7.8
NF4	0.33 ± 0.06	9.23 ± 0.11	9.9
NF5	2.34 ± 0.08	11.75 ± 0.47	16.4
NF6	4.57 ± 0.16	7.93 ± 0.77	17.1
NF7	3.56 ± 0.13	4.76 ± 0.17	11.9
NF8	3.95 ± 0.16	6.65 ± 0.35	14.6
NF9	4.64 ± 0.20	8.26 ± 0.15	17.5
NF10	5.15 ± 0.09	9.56 ± 0.17	19.9
NF11	6.15 ± 0.13	12.23 ± 0.08	24.5
NF12	0.55 ± 0.11	3.48 ± 0.10	4.6
NF13	4.65 ± 0.09	12.72 ± 0.14	22.0
NF14	6.71 ± 0.13	10.12 ± 0.28	23.5
NF15	2.81 ± 0.13	12.86 ± 0.40	18.5
NF16	4.75 ± 0.13	10.85 ± 0.12	20.3
NF17	4.02 ± 0.20	6.15 ± 0.06	14.2
NF18	1.98 ± 0.10	16.48 ± 1.00	20.4
NF19	3.02 ± 0.05	10.89 ± 0.18	16.9
NF20	6.22 ± 0.04	11.11 ± 0.26	23.6
NF21	3.41 ± 0.14	9.20 ± 0.12	16.0
NF22	2.04 ± 0.04	8.40 ± 0.36	12.5
NF23	4.11 ± 0.21	12.91 ± 0.22	21.1
NF24	3.54 ± 0.07	22.48 ± 0.12	29.6
NF25	2.05 ± 0.06	4.66 ± 0.14	8.8
NF26	4.24 ±0.08	6.89 ± 0.58	15.4
FF1	1.36 ± 0.06	n.a.*	
FF2	2.11 ± 0.12	4.21 ± 0.05	8.4
FF3	6.83 ± 0.04	n.a.*	
FF4	1.80 ± 0.08	n.a.*	
FF5	3.55 ± 0.10	n.a.*	
FF6	7.36 ± 0.22	n-a*	
FF7	6.65 ± 0.16	13.13 ± 0.46	26.4
FF8	6.70 ± 0.07	14.58 ± 0.10	28.0
FF9	5.14 ± 0.09	n.a.*	
FF10	16.29 ± 0.11	n.a.*	

FF11	0.96 ± 0.03	13.57 ± 0.23	15.5
FF12	3.76 ± 0.05	n.a.*	
FF13	1.17 ± 0.05	18.09 ± 0.30	20.4
FF14	2.02 ± 0.07	n.a.*	
NA1	14.99 ± 0.46	6.18 ± 0.17	36.1
NA2	2.11 ± 0.25	11.95 ± 0.33	16.2
FA1	1.93 ± 0.20	0.76 ± 0.29	4.6
NK1	0.00 ± 0.00	n.a.*	
NK2	0.76 ± 0.05	5.03 ± 0.37	6.5
NK3	0.00 ± 0.00	n.a.*	
FK1	0.00 ± 0.00	n.a.*	
FK2	0.00 ± 0.00	n.a.*	
M1	1.41 ± 0.04	1.65 ± 0.10	4.5

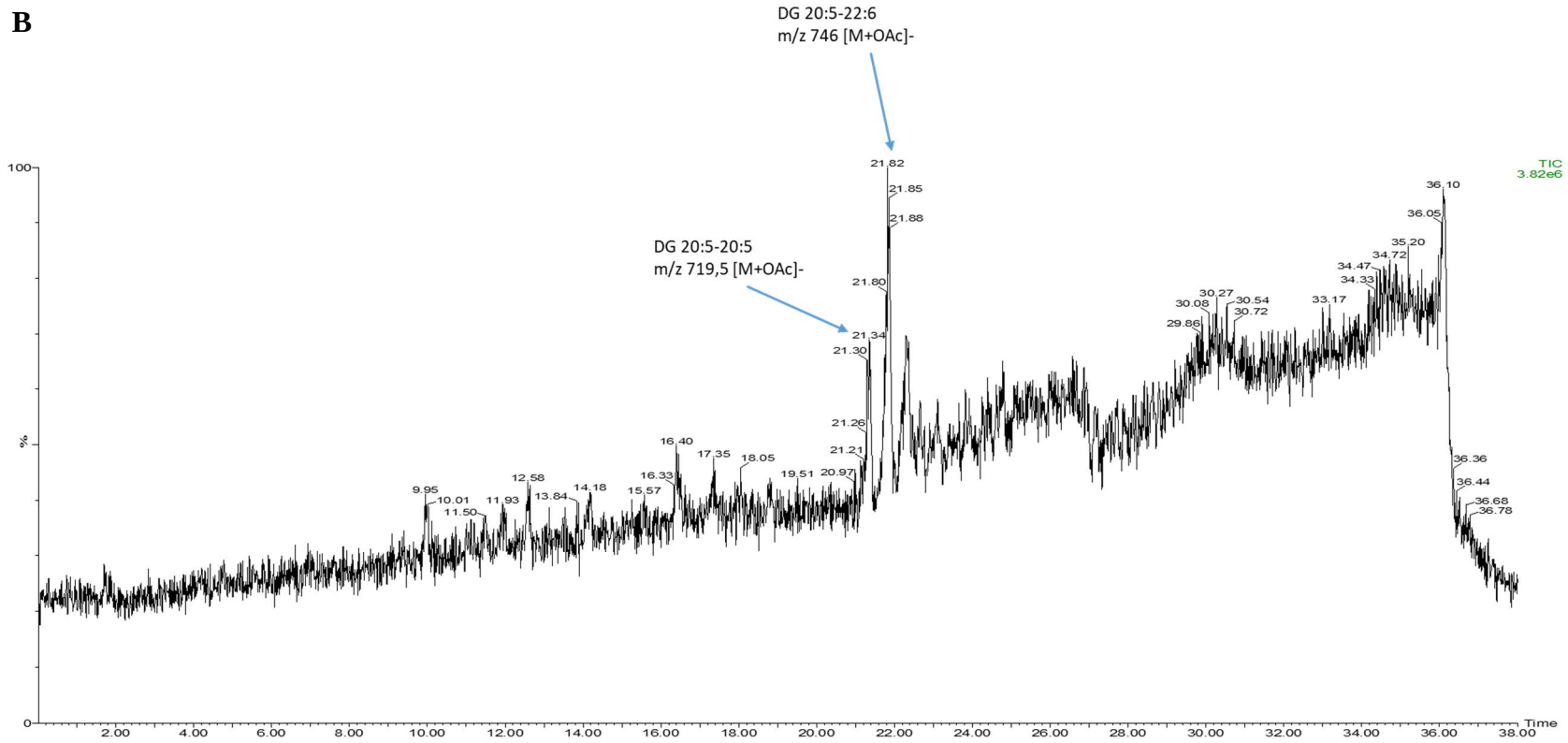
* not analyzed (n.a.) because of disturbances to the PAV method by added aroma compounds or in case of the krill oil products dissolving issues.

Supplementary material

Supplementary Figure 1. Chromatograms of lipid class analysis of fish oil supplement NF15 by ultra-high performance liquid chromatography combined with electrospray ionization and mass spectrometry (UHPLC-ESI-MS) in positive ionization mode (A) and negative ionization mode (B) with partial peak identification based on mass spectras and standards (TAG = triglyceride, DG = diglyceride, EE = ethyl ester, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, DPA = docosapentaenoic acid).


A
EPA-EE
m/z 353 [M+Na]+
m/z 331 [M+H]+
m/z 285 [M-EtO]
DHA-EE
m/z 379 [M+Na]+
m/z 357 [M+H]+
m/z 311 [M-EtO]
DPA-EE
m/z 381 [M+Na]+
m/z 359 [M+H]+
m/z 313 [M-EtO]
DG 20:5-20:5
m/z 679 [M+NH4]+
m/z 661.5 [M+H]+
DG 22:6-20:5
m/z 705 [M+NH4]+
m/z 687.5 [M+H]+
DG 22:6-22:6
m/z 731 [M+NH4]+
m/z 714 [M+H]+
TAG 20:5-20:5:20:5
m/z 963 [M+NH4]+
TAG 20:5-22:6:18:3
m/z 970 [M+NH4]+
DG 20:5-18:1
m/z 659 [M+NH4]+
m/z 642 [M+H]+
DG 22:6-18:1
m/z 659 [M+NH4]+
m/z 642 [M+H]+
TIC
9.72e7
%
Time

B


DG 20:5-22:6
m/z 746 [M+OAc]-
DG 20:5-20:5
m/z 719,5 [M+OAc]-
TIC
3.82e6
100
%
0
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00
9.95
10.01
11.50
11.93
12.58
13.84
14.18
15.57
16.33
16.40
17.35
18.05
19.51
20.97
21.21
21.26
21.30
21.34
21.80
21.82
21.85
21.88
29.86
30.08
30.27
30.54
30.72
33.17
34.33
34.47
34.72
35.20
36.05
36.10
36.36
36.44
36.68
36.78

Supplementary Table 1. Product information provided on the package for the studied omega-3 supplements; 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 microalgae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1). Source oil for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), delivery form, EPA, DHA and omega-3 fatty acid content [mg per g oil], lipid class (triacylglycerol = TAG or ethyl ester EE) and other oil present are listed as far as the information was provided.

code	EPA and DHA source	flavored**	delivery form	EPA [mg/g]	DHA [mg/g]	omega-3 [mg/g]	lipid class	other oils present in the product
NF1	fish oil *	-	liquid	86	130	259		
NF2	fish oil*	-	capsule	453			TAG	
NF3	fish oil*	-	capsule	281		526	TAG	linseed oil
NF4	fish oil*	-	capsule	***	***	***	TAG	
NF5	fish oil*	-	capsule	325	244	670	TAG	
NF6	fish oil*	-	capsule	324	233		EE	
NF7	fish oil*	-	capsule	321	230	667	EE	
NF8	fish oil*	-	capsule	175	433	661	EE	
NF9	fish oil*	-	capsule	314	208	628	EE	
NF10	fish oil*	-	capsule	310	205	620	EE	
NF11	fish oil*	-	capsule	313	208	626	EE	
NF12	fish oil*	-	capsule		463		EE	
NF13	fish oil*	-	capsule	81	72	201		
NF14	fish oil*	-	capsule	162	101			
NF15	fish oil*	-	capsule	287	191		TAG	
NF16	fish oil*	-	capsule	249	369	1039	TAG	

NF17	fish oil*	-	capsule	243	359	658	TAG	
NF18	fish oil*	-	capsule		251			
NF19	fish oi*	-	capsule	183	122	305		
NF20	fish oil*	-	capsule	325	217	610		
NF21	fish oil*	-	capsule	497	248	844	EE	
NF22	fish oil*	-	capsule	163	102	611		
NF23	fish oil*	-	capsule	502	251	853	EE	
NF24	fish oil*	-	capsule	***	***	***		
NF25	fish oil*	-	capsule	28	76	173		
NF26	fish oil*	-	capsule	55	458	553		
FF1	fish oil*	+	liquid	86	130	259		
FF2	fish oil*	+	capsule	323	240	671	TAG	
FF3	fish oil*	+	gummy	***	***	***		
FF4	fish oil*	+	gummy	101	470	631	TAG	
FF5	fish oil*	+	liquid	159	99	333		
FF6	fish oil*	+	capsule	162	106	337		
FF7	fish oil*	+	liquid	115	52	203		olive oil
FF8	fish oil*	-	liquid	115	52	203		olive oil
FF9	fish oil*	+	liquid	40	13			sunflower oil; evening primrose oil
FF10	fish oil*	+	capsule	183	57		TAG	evening primrose oil
FF11	fish oil*	+	liquid	160	100	350		
FF12	fish oil*	+	gummy	238	476	781		
FF13	fish oil*	+	liquid	77	196	314		
FF14	fish oil*	+	liquid	27	71	153		
NA1	microalgae oil	-	liquid	11	270	684		medium-chain triglycerides oil
NA2	microalgae oil	-	capsule	192	320	534		sunflower oil

FA1	microalgae oil	+	capsule		120			linseed oil
NK1	krill oil	-	capsule	93	51	144	PL	
NK2	krill oil / fish oil*	-	capsule	216	366	639	PL	
NK3	krill oil	-	capsule	150	70		PL	
FK1	krill oil	+	capsule	125	57	230	PL	
FK2	krill oil	+	capsule	147	68	264	PL	
M1	microalgae oil	+	margarine		4			rapeseed oil, vegetable fat (palm oil and coconut oil)

* fish- and/or fish liver oil.

** flavored product contains some type of natural or artificial aroma.

*** EPA, DHA and omega-3 fatty acid content could not be given in mg per g oil due to no information on the amount of oil in one capsule or gummy.

Supplementary Table 2. Occurrence of volatile secondary oxidation compounds in the studied omega-3 supplements; 40 fish- and fish liver oil products (26 non-flavored = NF1-26 and 14 flavored = FF1-14), 5 krill oil products (3 non-flavored = NK1-3 and 2 flavored = FK1-2), 3 microalgae oil products (2 non-flavored = NA1-2 and 1 flavored = FA1) and 1 DHA containing margarine (M1). Volatile compounds detected over the quantification limit are marked with an x.

Sample	1-penten-3-ol	hexanal	propanoic acid	2-hexanal (*E*)	2,4-heptadial (*E,Z/E,E*)	2-ethylfuran	butanoic acid	1-penten-3-one	2-pentylfuran	2-nonanone	3,5-octadien-2-one (*E,Z*)	2-propenal	benzaldehyde	pentanal	2-pentenal (*Z*)	octanal	2,6-nonadienal (*E,Z*)	propanal	2,4-hexadienal (*E,Z*)	4-heptenal (*Z*)
NF1	x	x	x																	
NF2	x	x	x	x	x	x			x							x				
NF3	x	x		x	x	x			x		x					x				
NF4			x		x		x	x				x								
NF5	x	x	x	x	x	x			x							x				
NF6	x	x	x	x	x	x														
NF7	x	x	x	x	x			x												
NF8			x	x	x	x				x	x						x			
NF9	x	x		x	x	x			x						x					
NF10	x	x	x	x	x	x	x		x											
NF11	x	x		x	x	x			x	x							x			
NF12			x																	
NF13	x	x	x	x	x	x														
NF14	x	x		x	x															
NF15	x	x	x	x	x					x				x			x			
NF16	x	x	x	x	x	x								x						
NF17	x	x	x	x	x		x							x						
NF18	x	x	x	x	x								x							
NF19	x	x	x	x	x	x														
NF20			x	x	x	x				x										
NF21	x	x	x	x	x	x														
NF22	x	x	x	x	x		x													
NF23	x	x	x	x	x	x		x												x
NF24	x	x	x	x	x	x		x	x		x	x	x		x				x	
NF25	x	x	x	x	x															
NF26	x	x		x	x			x												
FF1	x	x		x		x														
FF2	x	x	x	x		x														
FF3							x													
FF4			x																	
FF5		x	x	x		x												x		
FF6						x										x				
FF7	x	x	x	x		x									x					
FF8	x	x			x															
FF9	x	x	x		x								x							
FF10	x				x		x						x							
FF11	x	x	x	x																

FF12		x	x	x			x					x							
FF13		x	x	x		x													
FF14	x	x	x	x		x						x							
NA1	x				x			x											
NA2	x	x						x											
FA1	x	x				x													
NK1	x					x	x		x				x						
NK2			x	x	x	x	x	x		x									
NK3	x	x	x							x	x								
FK1	x		x							x	x								
FK2	x	x				x				x	x								
M1				x			x												

0

1

2