

Research Article

Applicability of traditional and advanced methods for oxidative quality and stability on marine phospholipids

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The objective of this study was to evaluate oxidative status and stability measurements of cod roe phospholipid (PL) extracts obtained by different extraction methods, and of two refined fish oils. The oxidative status varied depending on the method and on the extraction methods for the PL extracts. Peroxide value (PV) and anisidine value (AV) were not measurable in PL extracted by ethanol due to dissolution issues and precipitation occurring in the reaction mixture, the latter were attributed to co-extracted compounds. The thiobarbituric acid reactive substances (TBARS) values were significantly lower for determinations in the presence of an antioxidant in the reaction mixture for both fish oils and PL indicating that marine lipids may become oxidized in the course of the analysis. The accelerated oxidation revealed major differences in the pattern of oxidation in bulk fish oil and bulk PL. While for fish oils, the level of omega-3 fatty acids decreased and the level of both peroxides and aldehydes increased, PL showed minor loss of omega-3 fatty acids, minimal presence of the oxidation markers accompanied by disappearance of phosphatidylethanolamine group. This study shows that the applicability of classical oxidation status methods on marine phospholipids is limited. Variations in the methods, such as the choice of lipid solvent/reaction medium, may also lead to different results. ¹H high resolution magnetic resonance spectroscopy NMR proved to be a valuable tool to study the different oxidation patterns of fish oils and PLs.

Practical applications: The oxidative status and stability of marine omega-3 lipids are important quality parameters. The marine lipid industry usually relies upon two standard analyses to describe the quality of their products: PV by the AOCS Official method Cd 8b-90 and AV by the AOCS Official Method Cd 18-90. This work studies the applicability of these traditional and other methods to describe oxidative status and stability of marine phospholipids compared to classical fish oils. The results show that depending on the processing and composition, some of the methods are not applicable for phospholipids and, therefore, can give a misleading picture of the quality of the product. Moreover, the paper also shows that oxidative pathway for phospholipids are different compared to triacylglycerides and that an analytical tool like NMR can be used to describe the quality of marine phospholipids.

Keywords: Anisidine value / Fish oil / Marine phospholipids / NMR / Peroxide value / Stability

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Abbreviations: AV, anisidine value; CD, conjugated dienes; CLO, cod liver oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MDA, malondialdehyde; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanol amine; PL, phospholipid; PUFA, polyunsaturated fatty acids; PV, peroxide value; TAG, triacylglycerols; TBARS, thiobarbituric acid reactive substances; TLC, thin-layer chromatography

1 Introduction

Over the past decades, marine lipids have gained considerable interest in the health supplement market owing to their high content of healthy omega-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Marine phospholipids have the potential to become a “new generation” omega-3 supplement because of possibly higher bioavailability of EPA and

DHA in phospholipids (PL) compared to triacylglycerols (TAG) [1, 2]. However, due to the multiple double bonds, PUFAs are prone to development of rancidity (oxidation) at relatively mild conditions (room temperature, ambient light, and exposure to air). It has not yet been firmly established whether PUFA in PL are more stable against oxidation than PUFA in TAG. In addition, the state of the lipid system, i.e., bulk lipids versus aqueous systems (emulsions, liposomes), and the overall composition of the system are important factors determining the oxidative stability.

Rancidity of PUFA has been linked to increased health risks. Even though food safety authorities, like the European Food Safety Authority [3] and the Norwegian Scientific Committee for Food Safety [4], have reported in their health risk assessments on consuming oxidized fish oil that solid conclusion about the harmful effects of oxidized fish oil could not be drawn, essentially due to lack of relevant studies.

To deliver high quality marine phospholipids on the market, reliable means of monitoring the quality and stability of their products are required. Nowadays, conventional measurements of peroxide value (PV), anisidine value (AV), and additional methods such as conjugated dienes (CD), and TBA/TBARS assays, are used to determine the oxidative quality, independent of whether the PUFAs are in PL or TAG. Recently, it has been reported that classical PV and AV methods were not applicable for phospholipid rich krill oil [5, 6].

To evaluate oxidative stability of lipids, as well as the effect of added antioxidants, various stability tests, where oxidation is accelerated by increased temperature (typically 40–90°C) and access to oxygen, are often employed. The most common methods are Schaal oven test, Rancimat, Oil Stability Instrument (OSI), and Oxipres. Rancimat and OSI measure conductivity in deionized water that increases due to volatile acid accumulation as a result of lipid oxidation. Oxipres accelerates oxidation by incubating the sample at elevated temperature in a closed vessel and monitors the pressure drop in oxygen as a result of oxygen consumption (lipid oxidation). Schaal oven test monitors the weight increase of a sample as a result of binding oxygen during the initial stage of oxidation, i.e., peroxide formation [7]. In the Rancimat and OSI tests, the samples must be in a liquid form. However, phospholipids are usually semi solids and the use of these tests is, therefore, very problematic.

Marine phospholipids are more complex in composition compared to marine oils as they are not refined and deodorized. PUFAs are the primary targets of oxidation in both marine TAG and marine PL forming a great variety of oxidation compounds: Primary oxidation products – lipid peroxides, secondary oxidation products – both non-volatile and volatile (low molecular weight) aldehydes, ketones, organic acids and alcohols, and tertiary oxidation products – polymers. Several studies [5, 6, 8, 9] investigated oxidative changes in marine phospholipids and suggested that higher temperatures during extraction of PL lead to formation of unsaturated epoxy keto fatty esters, epoxyalkenals, and

hydroxyalkenals that later may react with the primary amino groups present in phosphatidylethanolamine (PE) or residual amino acids and cause non-enzymatic browning of PL. It was also proposed that reactive α -dicarbonyl products formed during lipid oxidation may react with (i) the primary amino group in PE or amino acids to form pyrroles or (ii) amino acid residues (if present) to form Strecker degradation products [10]. The same study also proposed that the presence of two oxygenated groups, namely one carbonyl and one epoxy or hydroxyl group, is required for the Strecker degradation or pyrrolization to occur. Non-enzymatic browning reaction can also occur between α,β -unsaturated aldehydes ((E)-2-hexenal and (E,E)-2,4-decadienal) and amino acids (e.g., glycine, lysine) [11]. The amino group of PE undergoes pyrrolization more readily than the amino acids due to close proximity the generation place of lipid oxidation products to the amino group of PE [10].

Moreover, the products from the Strecker degradation and pyrrolization may have antioxidative properties [8, 12]. Therefore, high oxidative stability of marine phospholipids is usually attributed to three different factors. The first is the presence of PUFA in sn2 position of PL, which could provide tightly packed molecular conformation [13] making it difficult for free radicals and oxygen to attack PUFAs. The second is the presence of antioxidative carbonyl-amine compounds formed by reaction between amino phospholipids/amino acids and fatty acid oxidation products [8, 12] and the third is the presence of other antioxidative constituents like amino acids, astaxanthin, and tocopherol [14]. Slightly oxidized PE produces pyrroles in a dimer form, which have better antioxidative properties than pyrroles in the polymer form, which is formed during gradual polymerization of dimers [15]. Because of these more complex changes that may occur in marine phospholipids, PV and AV may not be sufficient to measure the oxidation status in marine phospholipid products.

This study investigates whether the classical methods mentioned above are suitable for marine phospholipids isolated from cod roe by different extraction techniques and whether these marine phospholipids in bulk are more stable than bulk fish oil. Nuclear magnetic resonance (^1H NMR) measurements are used as a tool to characterize changes in the lipids during an accelerated stability test.

2 Materials and methods

2.1 Material

Mature roe from North Atlantic cod (*Gadus morhua*) obtained from Nergård AS (Tromsø, Norway) was used for extraction of marine phospholipids (PL). The intact roe glands were stored at -27°C before the extraction. Cod liver oil (CLO) was purchased from a local retailer (antioxidant content declared on the product label was 2 mg/mL of

tocopherols) and stored at 4°C. Fish oil without added antioxidants was donated by a fish oil processing plant (Norway) and stored at −20°C. Soy phospholipids (Type II-S from soy beans) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and stored at 4°C.

2.2 Chemicals

All chemicals and solvents were of analytical grade (p.a.), unless specified otherwise, and were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), Merck KGaA (Darmstadt, Germany), or Fluka Chemie (Buchs, Germany). Nitrogen (99.99% N₂), hydrogen (99.99% H₂), and helium gas (99.99% He) were provided by AGA AS, Oslo. Analytical standards of fatty acid methyl esters used in GC-FID analysis and lipid classes standard mixes used in TLC-FID analysis were purchased at Nu-Check Prep Inc. (Elysian, MN). Phospholipid standards used in HPLC-CAD were purchased at Avanti Polar Lipids Inc. (Alabama, USA). Solvents used in TLC-FID, GC-FID, and HPLC-CAD were of chromatography grade. Distilled water was used for preparing aqueous solutions, and deionized (MilliQ) water was used in GC-FID and HPLC-CAD.

2.3 Phospholipid extracts

Three phospholipid extracts (PL-BA, PL-EA, and PL-E) were obtained from cod roe (Fig. 1).

PL-BA were isolated as follows: Total lipids were extracted from thawed roe according to the method of Bligh & Dyer [16] which uses chloroform, methanol, and water as extraction media. Phospholipids were further isolated from

the total lipids by cold acetone precipitation as described by Kates [17] and modified by Mozuraityte et al [18]. Briefly, an aliquot of total lipids (4 g lipids dissolved in 8–10 mL chloroform) was mixed with 200 mL of acetone and placed into a freezer at −20°C overnight. The acetone was decanted and the precipitated PL were dissolved in chloroform and collected. The precipitation was repeated once more with the collected PL to increase the purity of the PL. The final PL precipitate (PL-BA) was kept in chloroform at −20°C in a dark bottle to eliminate lipid oxidation.

PL-EA and PL-E were isolated according to the patent [19] with some modifications using 96% ethanol as the extraction medium. The roe was freeze-dried (Christ Alpha 1–4 LO Plus freeze-drier) at −60°C and 0.02 mbar, and the dried roe was mixed with ethanol in 1:5 w/v ratio. The mixture was homogenized for 30 s with an Ultra Turrax homogenizer (T25 digital, IKA, Staufen, Germany) followed by centrifugation (4080×g, 15 min). The ethanol phase was filtered via glass wool and collected. The extraction was repeated two more times with the remaining sediments, using sediments-to-ethanol ratios 1:3 and 1:2 in the second and third extraction round, respectively. The ethanol was removed from the extracts by a laboratory rotavapor (Laborta 4000, Heidolph Instruments, Germany) coupled to a vacuum pump (CVC 2-PC511, Vacuubrand, Germany) at 60°C under near vacuum. The dried phospholipids (PL-E) were dissolved in chloroform, pooled and stored at −20°C in a dark bottle.

PL-EA were obtained by further purification of PL-E by single cold acetone precipitation as described above for PL-BA. The collected precipitate was kept in chloroform at −20°C in a dark bottle.

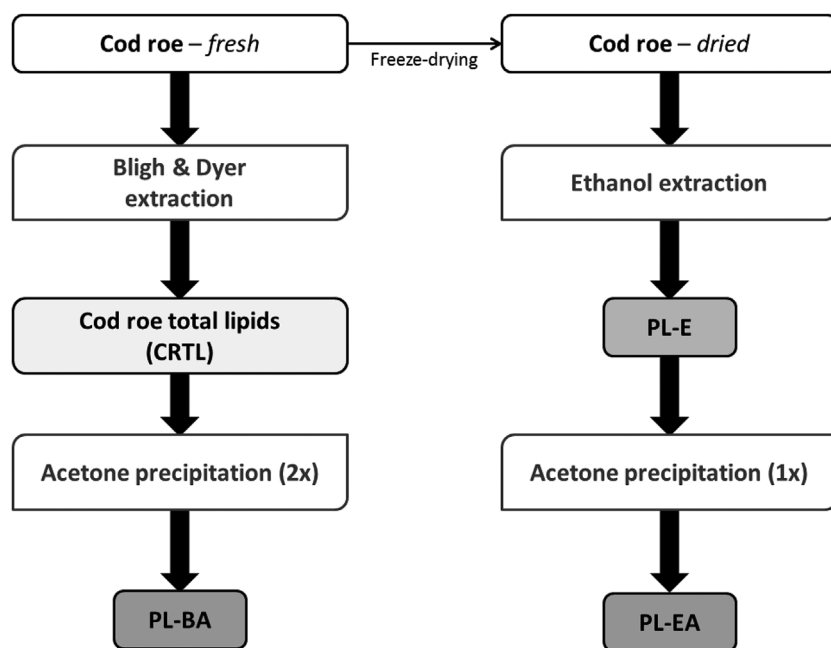


Figure 1. Isolation of phospholipids from cod roe. PL-BA – phospholipids isolated by double acetone precipitation from cod roe total lipids (CRTL) extracted by Bligh & Dyer method, PL-E – phospholipids extracted by ethanol, PL-EA – phospholipids purified by single acetone precipitation from phospholipids extracted by ethanol.

2.4 Purity of phospholipids/lipid classes

Lipid classes were determined by thin-layer chromatography (TLC) with a flame ionization detection (FID) system (Iatroscan TLC-FID analyser TH-10 MK-IV, Iatron Laboratories, Tokyo, Japan) as described by Fraser and colleagues [20]. The results were expressed in area% as the mean value \pm standard deviation of three replicates.

2.5 Fatty acid composition by GC-FID

The fatty acid (FA) profile of the lipids was determined by Agilent Technologies 7890A gas chromatograph (GC) with a FID. The methylation step and GC-FID analysis were performed as described in detail by Kristinova and colleagues [21]. Fatty acids were identified by comparison with fatty acid standards, and quantified by an internal standard. The results were expressed as relative amount (%) of each FA to a total FA amount. Two replicates were run for each sample. The results are presented as pooled values for saturated, mono- and poly-unsaturated fatty acids, omega-3 fatty acids, and the content of eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) acid.

2.6 Phospholipid classes

The composition of the isolated PL was analyzed by the Agilent 1260 Infinity HPLC system (Agilent Technologies, Germany) coupled to the Corona Ultra RS charged aerosol detector (CAD) (Thermo Scientific/Dionex, USA) according to [22]. The PL were dissolved in methanol/chloroform (1:1, v/v) to the concentration of 1–2 mg/mL, and separated on Agilent Prep-SIL Scalar 10 μ m column, 4.6 \times 150 mm (Agilent Technologies, Santa Clara, CA) kept at a constant temperature (30.0°C). For the isocratic elution, a ternary gradient having a constant flow rate of 1.25 mL/min and consisting of degassed solvents A = 13% formic acid in deionized water, B = 2-propanol, and C = hexane was used with the following timetable: At 0.00 min 3:40:57 (%A:%B:%C); at 4 min 10:40:50; at 9.00 min 10:40:50; at 9.1 min 3:40:57; and at 19 min 3:40:57. The sample temperature was 4°C and the injected volume was 10 μ L. The PL classes were identified by comparison to the retention times of commercial standards and quantified from standard curves measured at the same conditions. Each PL sample was analyzed in duplicate and the results are reported as% w/w \pm standard deviation.

2.7 Oxidative status and oxidative stability methods

Peroxide value (PV) and conjugated dienes (CD) were determined to characterize the level of primary oxidation products. Anisidine value (AV) and thiobarbituric acid reactive substances (TBARS) were determined to characterize the level of secondary oxidation products. Nuclear

magnetic resonance (NMR) was used to further characterize composition – omega-3 content and changes during oxidation. Schaal oven test was performed to assess the oxidative stability of the lipids. Before each analysis, the storage solvent (chloroform) was removed from PL-BA, PL-EA, PL-E by flushing with nitrogen gas, followed by evaporation in vacuum (2 h). Unless stated otherwise, the results are presented as average value \pm standard deviation of *n* replicates (specified under each analysis).

2.7.1 Peroxide value

Lipid hydroperoxides were determined by three different methods which are based on the following methods:

- (i) Iodometric titration method described in the AOCS Official method **Cd 8b–90** [23] which uses glacial acetic acid/iso-octane (3:2, v/v) as lipid solvent. Potentiometric determination of the titration end point was employed. The titration was performed using a TitraLab980 automatic titrator coupled with a single platinum electrode (M21Pt) and a reference electrode (REF 921) (all equipment from Radiometer Analytical ASA, Copenhagen, Denmark) according to a titration protocol [24]. The ratio between the lipid solvent and water in the titration mixture was 5:3 v/v, 0.01 mL standardized Na₂S₂O₃ was used as titrant. The method gives results in meq/kg oil. The analysis was performed with 3–14 parallels.
- (ii) Iodometric titration method described in the AOCS Official method **Cd 8–53** [25] which uses acetic acid/chloroform (3:2, v/v) as lipid solvent. The same automatic titrator and electrodes and the titration protocol was used for the titration as for (i). The ratio between the lipid solvent and water in the titration mixture was 1:3 v/v, 0.01 mL standardized Na₂S₂O₃ was used as titrant. The method gives results in meq/kg oil. The analysis was performed with 3–10 parallels. For purposes of comparison, the results from the iodometric titration methods (both i and ii) were converted to mmol/kg. According to Frankel [26], the conversion between meq/kg and mmol/kg is as follows: Meq/kg = 2 \times mmol/kg.
- (iii) Spectrophotometric ferro-method was performed according to [27] and [28] with some adjustments. Iron (II) chloride solution was made fresh by vortexing 3 mL of 0.2 mM BaCl₂ dissolved in 0.4 mL HCl with 3 mL of 36 μ M FeSO₄ dissolved in water for 1 min. The mixture was centrifuged for 3 min at 9100 $\times g$. The upper clear phase was pipetted and used as an iron (II) chloride solution. A total of 100 mg of lipids was dissolved in 10 mL of ice-cold mixture of chloroform and methanol (1:1, v/v). An aliquot of the sample (3.33 mL) was mixed with 33.4 μ L of ammonium thiocyanate solution (30%, w/v) and 33.4 μ L of the iron (II) chloride solution. The

final mixture was vortexed for 2 s and incubated at room temperature for 20 min before measuring the absorbance at 500 nm against distilled water. The blank sample contained only the mixture of chloroform and methanol (1:1, v/v). The PV was quantified using a linear standard curve prepared with cumene hydroperoxide standard (0–20 μM). The analysis was performed in triplicate.

2.7.2 Conjugated dienes

For the measurement of conjugated dienes [29], the lipids were dissolved in isooctane or chloroform (1–8 mg/mL), further diluted with the solvent (1:2–1:8, v/v) if the absorbance exceeded 0.8 AU, and absorption at 233 nm was measured against the solvent. The amount of conjugated dienes was calculated using extinction coefficient of linoleic acid hydroperoxide ($2.525 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [29]. The results are expressed as CD values in mmol/kg. Three replicates were measured for each sample, each replicate was measured twice, and the results were pooled ($n = 6$).

2.7.3 Thiobarbituric acid reactive substances

The concentration of TBARS in the samples was determined by a spectrophotometric method according to Ke and Woyewoda [30]. All amounts were reduced to one half relative to the given procedure. For a series with addition of antioxidant into the reaction mixture, 2% w/v ethanolic solution of butylated hydroxytoluen (BHT) was added into the 2-thiobarbituric acid (TBA) reagent in a ratio 3:100 v/v. Four replicates were measured for each sample and the results are expressed in $\mu\text{mol TBARS/g}$.

2.7.4 Anisidine value

The AV was determined according to AOCS Official Method Cd 18–90 [31]. The analysis was also performed using chloroform instead of iso-octane as a solvent. Each sample was analyzed in triplicate.

2.7.5 Schaal oven test

Schaal oven test was used to evaluate the oxidative stability of the lipids. The lipids ($5.00 \pm 0.01 \text{ g}$) were weighed into an open glass Petri-dish (inner diameter 8 cm, height 1.2 cm) and placed into a drying oven (TS 8136, Termaks AS, Bergen, Norway) at $70 \pm 1^\circ\text{C}$ in the dark with no air circulation. The dishes were regularly weighed (four decimals) for a period of 238 h (~ 10 days). For the weighing, the dishes were cooled to an ambient temperature in a desiccator (5 min). Two replicates were measured for each sample and the weight gain curves were constructed from the average values.

During the incubation period, all the lipids were sampled ($\sim 500 \text{ mg}$) from an extra replicate at 0, 17, 43, 70, and 91 h for analyses by GC-FID and NMR. The samples were kept in plastic 1.5 mL eppendorf tubes at -80°C .

2.7.6 ^1H High resolution nuclear magnetic resonance (HR NMR)

PL-BA, PL-E, and CLO sampled at 0, 17, and 70 h during the Schaal oven test were screened for composition by ^1H NMR. Approximately, 100 mg of the sample were dissolved in 350 μL of a mixture of deuterated chloroform and dimethyl-sulfoxide (DMSO) (5:1, v/v) (as described in [32] for optimal resolution of hydroperoxide signals), and transferred to 3 mm NMR-tubes. NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) at ambient temperature (25°C) with cryo-probe operating at a ^1H frequency of 600.23 MHz.

For ^1H NMR the following acquisition parameters were used: Pulse program zg30, time domain 64k, spectral width 20.5 ppm, acquisition time 3.0 s, relaxation delay 2.0 s, number of scans 128, dummy scans 4. Zero filling and exponential line broadening (0.30 Hz) was applied before Fourier transformation. The chemical shift scale is referred indirectly to tetramethylsilane (TMS) by the triplet of the methyl group protons of non-omega-3 fatty acids at 0.87 ppm.

The content of omega-3 fatty acids (mol% of total FAs) was evaluated by integration of ^1H NMR peaks arising from the terminal methyl group in fatty acids (resonance at 0.96 ppm for omega-3 fatty acids and 0.87 ppm for other fatty acids) [33, 34]. Peak assignments were done according to literature [35–37] and by comparison of NMR spectra from pure compounds (SDBS Web [38]) and in house databases (PE and PC). The spectra are normalized to the methyl group of non-omega-3 fatty acids at 0.87 ppm.

2.8 Calculations and statistics

Minitab 17.1.0 statistical program and Microsoft Office Excel 2010 were used for statistical analysis and data processing. The data were subjected to analysis of variance (one-way ANOVA) followed by a Tukey test. The means were accepted as significantly different at 95% confidence level ($p < 0.05$).

3 Results and discussion

3.1 Phospholipid extraction and characterization of the extracts

In this study, different isolation techniques were used for extraction of PL from cod roe – extraction with chloroform/

methanol/water, i.e., Bligh & Dyer extraction, followed by precipitation in cold acetone (PL-BA), extraction with ethanol (PL-E), and extraction with ethanol followed by precipitation in cold acetone (PL-EA) (Fig. 1). The yields of total lipids extracted from fresh cod roe (CRTL) were fairly similar between the B&D method (CRTL = $14.4 \pm 0.4\%$ ww) and ethanol extraction (PL-E = $13.6 \pm 1.0\%$ ww). The yields of PL obtained after acetone precipitation were also similar between the B&D (PL-BA) method and ethanol extraction (PL-EA), giving 5–6 g PL/100 g roe (dw).

However, the compositional analyses of the extracts revealed differences (Table 1). PL extracts obtained after acetone precipitation, i.e., PL-BA and PL-EA, contained predominantly phospholipids (98–99% PL) and traces of cholesterol (1–2%). Ethanol extracted PL (PL-E) contained the largest proportion of non-phospholipid matter (among others cholesterol, triacylglycerols), ca. 15%, and the extract was similar in the composition of both lipid classes and fatty acids to the cod roe total lipids (CRTL) extracted by the Bligh & Dyer method. The data show that both the Bligh & Dyer method (chloroform-based extraction) and the

extraction by ethanol are not specific for phospholipids and additional purification is needed to obtain purer PL fractions. Ethanol is a protic solvent capable of dissolution of not only polar lipids, but a series of other components, such as ionic compounds, pigments, and to some degree also proteins/peptides and saccharides. It is, therefore, expected that other compounds of non-lipid nature are present in the different ethanol extracts.

The analysis of PL classes revealed differences in both the amounts and ratios between the individual PL classes in the phospholipid extracts. The main PL classes in the extracts were phosphatidylcholine (PC) and phosphatidylethanolamine (PE), followed by lysoPC. All three phospholipid extracts contained higher amount of both lysoPC and lysoPE compared to the total lipids extracted from cod roe (CRTL sample). This indicates that some hydrolysis reactions occur during the production of the phospholipid extracts. The PC content was much higher in CRTL and PL-BA than in PL-EA and PL-E, indicating better extractability of PC by chloroform than by ethanol or that some other compounds other than lipids (discussed above) are co-extracted by

Table 1. Composition of lipids in terms of dominant lipid classes (area%), phospholipid classes and fatty acids

Sample	Cod roe total lipids (CRTL)	Phospholipids extracted from cod roe				Soy PL	Fish oil	Cod liver oil (CLO)
		PL-BA	PL-E	PL-EA				
Isolation method	Bligh & Dyer extraction	Bligh & Dyer extraction followed by double acetone precipitation	Ethanol extraction	Ethanol extraction followed by single acetone precipitation	Commercial product	Commercial product	Commercial product	
TAG	5.6 ± 0.6	<LOD	5.2 ± 0.1	<LOD	<LOD	97.7 ± 1.2	99.0 ± 0.1	
PL	78.3 ± 2.2	98.7 ± 0.4	79.6 ± 2.2	97.7 ± 1.4	98.0 ± 2.5	<LOD	<LOD	
Chol	10.5 ± 0.8	1.1 ± 0.2	9.7 ± 0.1	1.5 ± 0.6	<LOD	<LOD	<LOD	
PE + PI	8.7 ± 0.2	14.0 ± 0.4	5.9 ± 0.0	8.8 ± 0.4	13.3 ± 0.9	n.a.	n.a.	
PC	44.5 ± 1.4	55.3 ± 2.9	33.1 ± 0.8	32.3 ± 1.8	15.7 ± 0.4	n.a.	n.a.	
PS	0.02 ± 0.01	0.04 ± 0.00	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.2	n.a.	n.a.	
LysoPE	0.4 ± 0.0	2.4 ± 0.0	1.3 ± 1.1	2.4 ± 0.1	0.9 ± 0.1	n.a.	n.a.	
LysoPC	3.6 ± 0.8	5.6 ± 0.6	6.2 ± 0.8	6.7 ± 0.9	3.1 ± 0.8	n.a.	n.a.	
Saturated	23.9 ± 0.1	30.7 ± 0.7	23.6 ± 0.1	29.1 ± 0.2	23.6 ± 0.0	31.5 ± 0.2	15.9 ± 0.3	
Mono-unsaturated	28.0 ± 0.1	23.0 ± 0.2	28.4 ± 0.1	25.7 ± 0.1	7.6 ± 0.0	24.8 ± 0.2	54.8 ± 0.2	
Poly-unsat. (C=C ≥ 3)	46.4 ± 0.1	45.1 ± 0.5	46.6 ± 0.2	43.9 ± 0.4	9.1 ± 0.0^a	41.5 ± 0.4	26.5 ± 0.1	
EPA	16.3 ± 0.1	14.1 ± 0.3	15.7 ± 0.1	13.9 ± 0.1	0.12 ± 0.01	19.2 ± 0.2	8.6 ± 0.0	
DHA	25.6 ± 0.1	26.5 ± 0.6	25.6 ± 0.2	25.4 ± 0.3	0.34 ± 0.02	13.8 ± 0.2	11.6 ± 0.1	

n.a., not analyzed; LOD, limit of detection; TAG, triacylglycerols; PL, phospholipids; Chol, cholesterol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; FA, fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

^aThe predominant poly-unsaturated fatty acid in soy PL was C18:3 n3 (α -linolenic acid; $7.7 \pm 0.1\%$).

ethanol. These results show that the extraction techniques will influence the PL profile in the extracts. The following ratios between PC and PE, 5.1 and 5.6 was obtained in CRTL and PL-E sample, respectively. This shows that the extractability of the main phospholipids (PC and PE) was similar both in chloroform and in ethanol. The absolute concentration was lower in the PL-E extract only because of more impurities extracted by ethanol. However, when precipitating the extracts by cold acetone, the ratio PC/PE was reduced to 3.9 and 3.8 for PL-BA and PL-EA samples, respectively. This indicates that PC is more soluble in cold acetone compared to the PE as the ratio decreases after acetone precipitation. Zhao et al. [39] also obtained a PC soluble fraction when extracting krill oil with acetone.

3.2 Primary oxidation products

3.2.1 Peroxide value

Peroxide value characterizing the level of lipid hydroperoxides was measured by three different methods based on: AOCS Cd 8b–90, AOCS Cd 8–53, and so called Ferro method. The latter method is a spectrophotometric method, while the AOCS methods are iodometric titration methods which differ in the lipid solvent, employing iso-octane/acetic acid (2:3, v/v) and chloroform/acetic acid (2:3, v/v), respectively. Even though the AOCS Cd 8–53 (chloroform version) is a surplus method no longer recommended for use, we included it for the sake of evaluation of the effect of the solvent on the determination of PV in phospholipids.

The AOCS titration methods gave significantly different results for PL-BA, CLO, and fish oil, while no difference was found for PL-EA and soy PL. The measurements of PL-E had very low titrant consumptions (similar to blank) in both AOCS methods, which resulted in negative PV values (Fig. 2A).

The spectrophotometric Ferro method quantifies peroxides in mmol/kg lipids while the titration methods quantify peroxides in meq/kg. To be able to compare the results, the values from the titration methods were converted to mmol/kg. Significant differences were found between the Ferro method and either one or both AOCS methods (Figure 2A).

The overall results show some inconsistency in the determination of PV in the same lipid sample by the different methods. One of the reasons for this inconsistency can be attributed to the different sensitivity of the technique and the associated detection limits. The PL-E gave negative values in both the AOCS methods which would suggest extremely low oxidation level, basically “non-measurable” by these methods. The Ferro method gave a positive value (1.6 ± 0.3 mmol/kg), and a positive, yet relatively low, degree of oxidation was also determined by the measurement of conjugated dienes (Fig. 2B) and TBARS (Fig. 3A). The inconsistencies may have several reasons. In the Ferro method, i.e., spectrophotometric measurements, different lipid hydroperoxides may have different molar extinction coefficients [40], therefore, giving variable response to the development of color. In the titration methods, more stable peroxides may not react with iodide [41, 42].

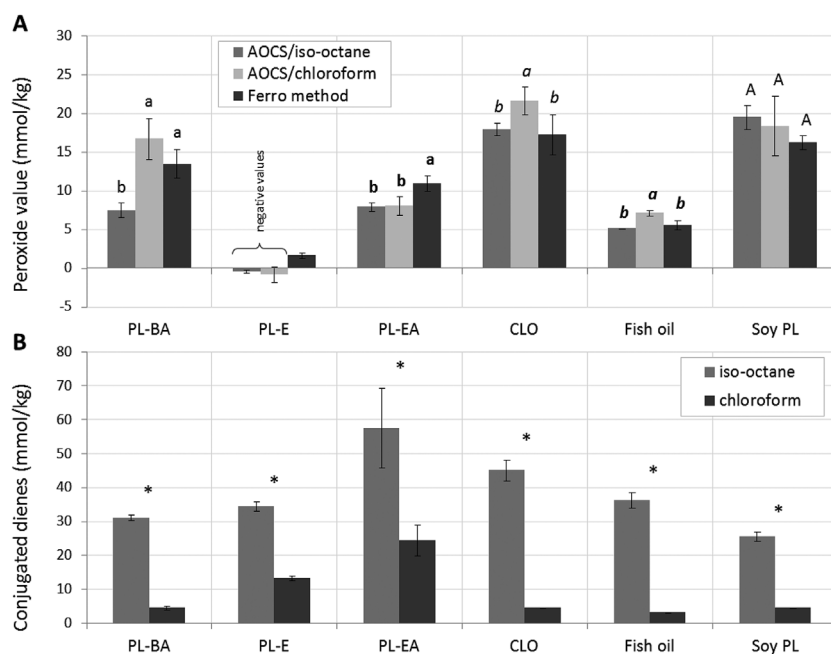


Figure 2. Measurements of primary oxidation products. Plot A: Peroxide value determined by the AOCS titration methods and the Ferro spectrophotometric method. Values that do not share a letter of the same type are significantly different ($p < 0.05$). Plot B: Conjugated dienes values for measurements in iso-octane or chloroform as the sample solvent. The asterisk (*) indicate significant difference ($p < 0.05$) between the two results.

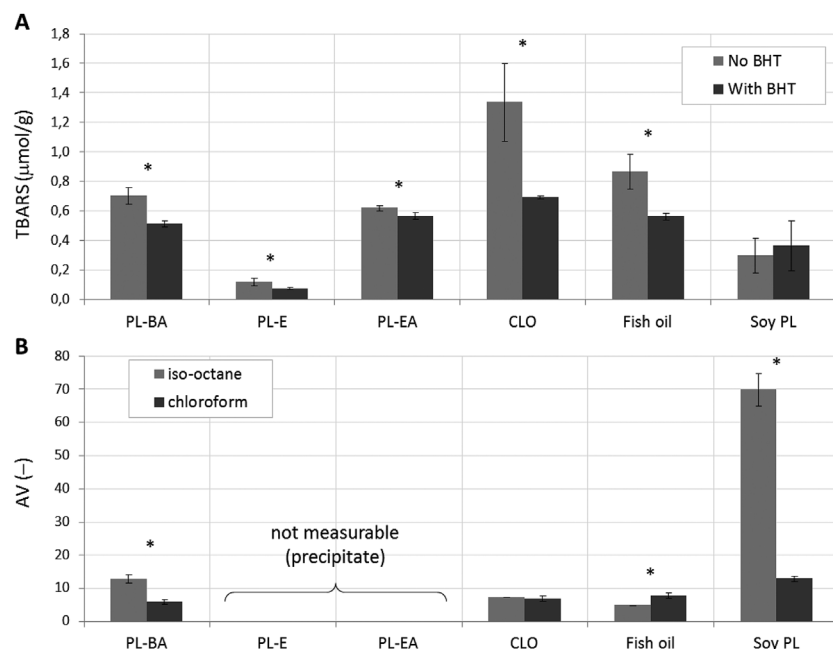


Figure 3. Measurements of secondary oxidation products. Plot A: TBARS values for measurements performed with or without antioxidant (BHT) added into the reaction mixture. Plot B: Anisidine values for measurements where the lipids were dissolved either in isooctane or chloroform. The asterisk (*) indicates a significant difference ($p < 0.05$) between the two results.

Nevertheless, all three methods showed either low or even negative PV for phospholipids extracted with ethanol (PL-E). The following theories could be raised for the very low/negative PV in PL-E: (i) The level of lipid hydroperoxides was below the detection limit of the AOCS methods, or (ii) impurities in the extract may disturb the titration end-point determination giving underestimated/negative results.

Acetone precipitated phospholipids, PL-BA and PL-EA, had significantly higher PV values than PL-E. This could be explained by some oxidation development during the acetone precipitation step. Moreover, some antioxidants could be lost during the precipitation. A loss of tocopherol was observed during purification of phospholipids from krill oil by acetone precipitation [14]. Less processing steps involved in the PL-E sample led to overall compositional difference compared to PL-BA and PL-EA (Table 1) which could lead to better oxidative stability of the PL-E extract.

3.2.2 Conjugated dienes

Diene conjugation and hydroperoxide formation result from a fatty acid undergoing hydrogen (H^\bullet) abstraction and stabilisation of the formed alkyl free radical (L^\bullet) by molecular rearrangement to form a conjugated diene structure. In the presence of oxygen, the alkyl free radical reacts with O_2 to form a peroxy radical (LOO^\bullet) which is then capable of abstraction of hydrogen from an adjacent fatty acid forming lipid hydroperoxide ($LOOH$) with diene conjugation formed in the first step. Thus, in early stages of oxidation, the level of conjugated dienes may be correlated to the peroxide content, and be a complementary analysis to peroxide value determination.

To measure CD, isooctane is commonly used to dissolve the lipids. In order to evaluate the importance of the solvent for the measurement of CD, the lipid samples were dissolved in isooctane or chloroform and the absorption was measured. Significantly higher values were obtained in isooctane for all the samples (Fig. 2B). The ratio between $CD_{\text{isooctane}}$ and $CD_{\text{chloroform}}$ varied in the range of 2.4–6.9 for the phospholipid samples, and 10.1–11.1 for the oils, showing greater difference for the oils, but no distinct trend between the CD values was found. The CD values were calculated using a molar absorption coefficient of linoleic acid hydroperoxide, which was determined by iodometric PV determinations of an ethanolic solution of linoleic acid hydroperoxide [29]. Extinction coefficients are often solvent dependent. Extinction coefficients suitable for iso-octane or chloroform are currently not known. This could result in the different values determined in iso-octane and chloroform.

However, the CD values did not follow the same trend as the PV determinations. This could be due to several reasons. Many compounds, such as sugars, hem proteins, chlorophylls, purines, and pyrimidines may interfere with the CD measurement because they absorb strongly in the UV region of CD (230–235 nm). In the early stage of oxidation, the development of conjugated dienes and peroxides follow each other. However, different correlation factors between CD and PV for different oils were observed in a study of Marmesat *et al.* [43]. At a later oxidation stage, decomposition of peroxides may occur but the conjugated dienes can stay intact in the molecule, also in addition some peroxide development without formation of conjugated diene may also occur.

3.3 Secondary oxidation products

3.3.1 TBARS

The TBARS assay measures the level of malondialdehyde (MDA) which is formed as a split product of an endoperoxide of unsaturated fatty acids. It is postulated that the formation of MDA from fatty acids with less than three double bonds occurs via secondary oxidation of carbonyl compounds (e.g., non-2-enal). The MDA is reacted with thiobarbituric acid to form a pink pigment which is measured spectrophotometrically. However, the reaction is not specific and the reaction conditions (unsaturation of aldehydes, presence of other substituents and oxidation compounds) have a significant effect on color development [44]. One step in the protocol involves boiling the reaction mixture. It was hypothesized that polyunsaturated marine lipids might become further oxidized during the boiling phase, which would lead to overestimated results.

Therefore, the TBARS measurements were performed in two series: (i) following the original protocol where no external antioxidant was added to protect the lipids against oxidation during the measurement, and (ii) adding BHT (butylated hydroxytoluene) into the TBA reagent solution. The results are shown in Fig. 3A. Significantly higher values were determined for the marine phospholipids and oils in the series without the presence of the antioxidant. These results suggest that some TBARS can be formed in the marine lipids during the measurement, presumably during the boiling step. This effect was more pronounced in the fish oils than in the marine PL. This could be due to additional protection of endogenous antioxidants in the PL. The TBARS were measurable in all the samples, a very low level ($0.07\text{--}0.09\text{ }\mu\text{mol/g}$) was determined in PL-E, suggesting low degree of oxidation. No difference between the result with and without antioxidant was found for soy PL. Since the soy PL are poor in PUFA (Table 1), addition of BHT might not have a large effect on further oxidation during the measurement.

3.3.2 Anisidine value

Anisidine value (AV) represents the level of non-volatile aldehydes present in the lipids. In the standard AOCS method, lipids are dissolved in isooctane, mixed with *p*-anisidine dissolved in glacial acetic acid, and the color development is measured after 10 min of reaction time. During the measurement of PL-EA and PL-E, precipitation in the reaction mixture occurred after addition of the *p*-anisidine/acetic acid solution, causing cloudy solution leading to unstable absorbance measurements (Fig. 3B). Therefore, the reaction mixture was centrifuged to remove the precipitate. Afterwards, the absorbance was more stable, but the AV for PL-EA and PL-E were 111 ± 10 and 51.0 ± 0.5 , respectively. This indicates that the samples

contained some aldehydes, but the values are likely to be incorrect as the reaction time was significantly prolonged due to the additional centrifugation step and the difficulties to ensure that cloudiness was entirely removed.

The precipitate in PL-EA and PL-E occurred also when chloroform was used to dissolve the lipids. The nature of the precipitates was not investigated. However, these phenomena indicate that ethanol extracted (PL-E) and ethanol extracted + acetone precipitated (PL-EA) phospholipids contained some impurities (possibly protein, salts, pigments, etc.) which were either not soluble in the reaction mixture or underwent precipitation reactions in the reaction mixture, leading to highly disturbed spectrophotometric measurements. It was possible to determine AV in PL-BA and soy PL (Fig. 3B) which indicates that these samples were devoid of the non-soluble fraction. When using chloroform as a solvent, significantly lower AV were measured for PL-BA, fish oil, and soy PL compared to using isooctane.

The findings that standard PV and AV methods are difficult to apply on the marine phospholipid extracts are in agreement with the findings of [5] who also concluded that standard oxidation methods are not applicable for phospholipid rich krill oil.

3.4 Stability of phospholipids: Schaal oven test

Schaal oven test was used to evaluate the oxidative stability of the different phospholipids and fish oils. The principle of the method is monitoring weight increase during storage in air-atmosphere at an elevated temperature, which is caused by peroxidation, i.e., binding of oxygen to fatty acids [7].

Both cod liver oil and fish oil started to gain weight rapidly after ca. 4 and 20 h of incubation, respectively. Phospholipids, on the other hand, were gaining weight considerably slower (PL-BA); and for some samples the weight was decreasing (PL-EA and PL-E) or remained constant (Soy PL) during the whole incubation period (Fig. 4).

The decrease in the weight in PL-EA and PL-E could be due to several reasons, such as evaporation of solvent traces in the samples, or the decomposition of oxidized lipids into low weight oxidation compounds that were evaporating from the samples gradually. Since traditional methods for describing oxidative quality of marine phospholipid extracts have some limitations, discussed above, the changes in fatty acid composition and ^1H NMR analysis were performed in order to explain the different oxidation patterns for marine phospholipids and oil in the Schaal oven test.

3.5 Changes in omega-3 FA content during the Schaal oven test

The omega-3 polyunsaturated fatty acids are a primary target for oxidative reactions [45] and their content, therefore, decreases during lipid oxidation as they are gradually

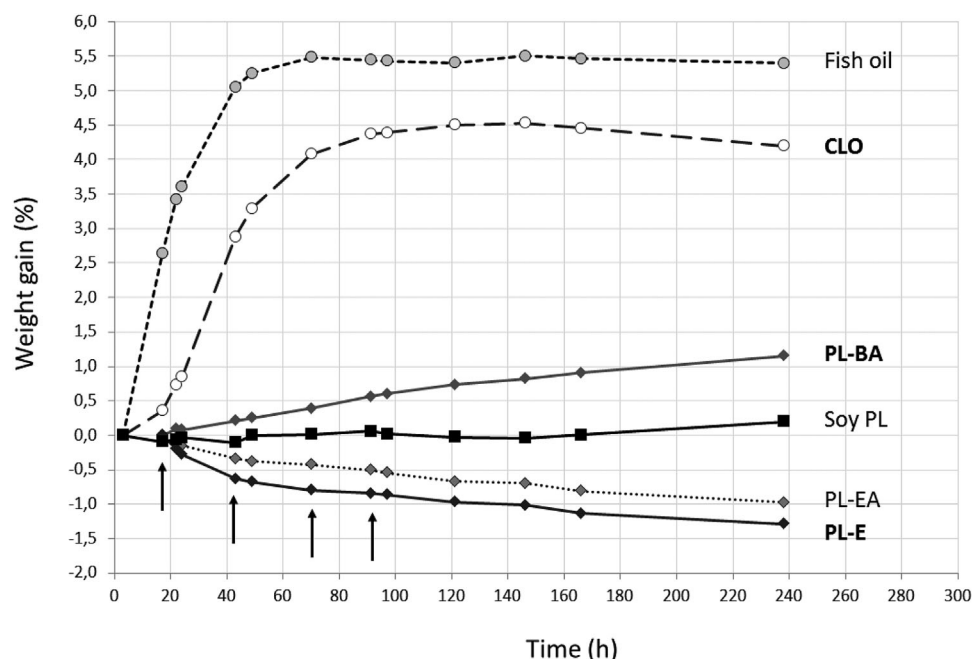


Figure 4. Schaal oven test – weight gain curves for the lipids incubated at 70°C for 238 h (~10 days). The arrows indicate sampling points of PL-BA, PL-E, and CLO for the analysis of fatty acid classes.

decomposed into secondary oxidation products. This process is dramatically enhanced by stressing conditions (high temperature, exposure to oxygen), such as in the Schaal oven test. Therefore, changes in omega-3 fatty acid content were monitored during the incubation in the Schaal oven test in CLO, PL-BA, and PL-E.

While the amount of omega-3 PUFA decreased significantly in CLO despite the presence of tocopherol in the oil, it decreased less in PL-BA and it was stable in PL-E during the whole incubation period. This trend was measured both by GC-FID and NMR (Fig. 5). The lack of weight gain in the Schaal oven test for PL-BA and PL-E (Fig. 4) could, therefore, be explained by a low degree of oxidative decomposition of omega-3 fatty acids in these samples.

The remarkably good oxidative stability of omega-3 fatty acids in bulk phospholipids could be attributed to several reasons outlined in the introduction: (i) Tightly packed molecular confirmation protecting PUFAs from free radicals and oxygen attack; (ii) the presence of antioxidative carbonylamine compounds resulting from non-enzymatic browning reactions; or (iii) presence of other antioxidative constituents like amino acids, astaxanthin, or tocopherol. Very high viscosity of the bulk phospholipids, which may hinder penetration and diffusion of oxygen into the PL mass, may also increase the resistance to oxidation. Color changes were indeed observed in the bulk phospholipids during the incubation, from yellow/orange to deep brown, showing that browning reactions take place.

3.6 Changes in oxidation products during the Schaal oven test (^1H NMR)

^1H NMR is a valuable technique for following changes in lipids due to lipid oxidation as shown in studies with both vegetable oils [32, 46] and marine lipids [47, 48].

Therefore, three lipid samples in this study (CLO, PL-BA, PL-E) were also analyzed by ^1H NMR to gain a deeper insight into the changes that take place during the accelerated oxidation in the Schaal oven test and to elucidate whether the bulk marine PL are oxidatively more stable than triacylglycerols as the preceding results imply. The ^1H NMR spectral region where peroxides and aldehydes give characteristic signals are shown in Fig. 6.

For CLO, some peroxides were present at 0 h (several peaks between 10.9 and 11.1 ppm) which is in accordance with the PV measurements. During the incubation at 70°C, the number and intensity of peroxide peaks (10.5–11.2 ppm) increased, and several new peaks assigned to aldehydes (9.4–10.0 ppm) appeared [32]. In addition, a singlet at 8.22 ppm, assigned to formic acid, appeared and increased during the oxidation. Formate has been identified as a degradation product from lipid oxidation and its presence is explained by the oxidation of the corresponding aldehyde (formaldehyde) [49].

In the PL-BA samples, two small broad peaks can be seen at 10.1 and 11.2 ppm at the beginning of the incubation period (0 h), these peaks are assigned to peroxides and aldehydes, respectively, in accordance with the PV and AV

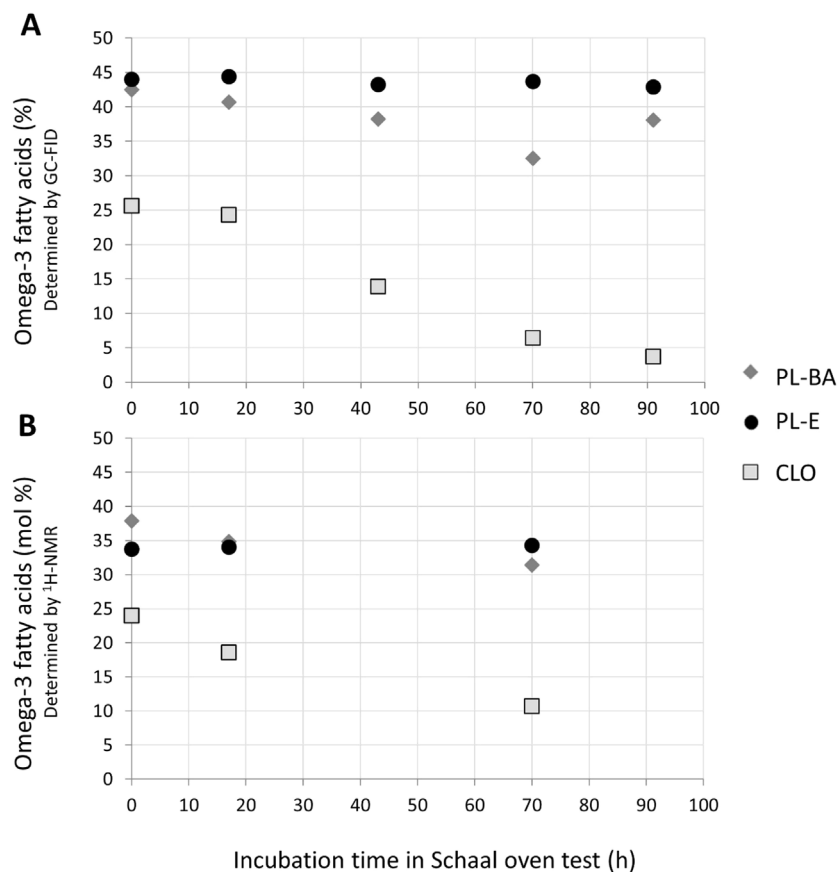


Figure 5. Amount of omega-3 fatty acids (%) in the lipids during the Schaal oven test as a function of incubation time (0, 17, 70, and 91 h) for PL-BA, PL-E, and CLO. Plot A: Measured by GC-FID (% w/w). Plot B: Measured by ¹H NMR (mol%).

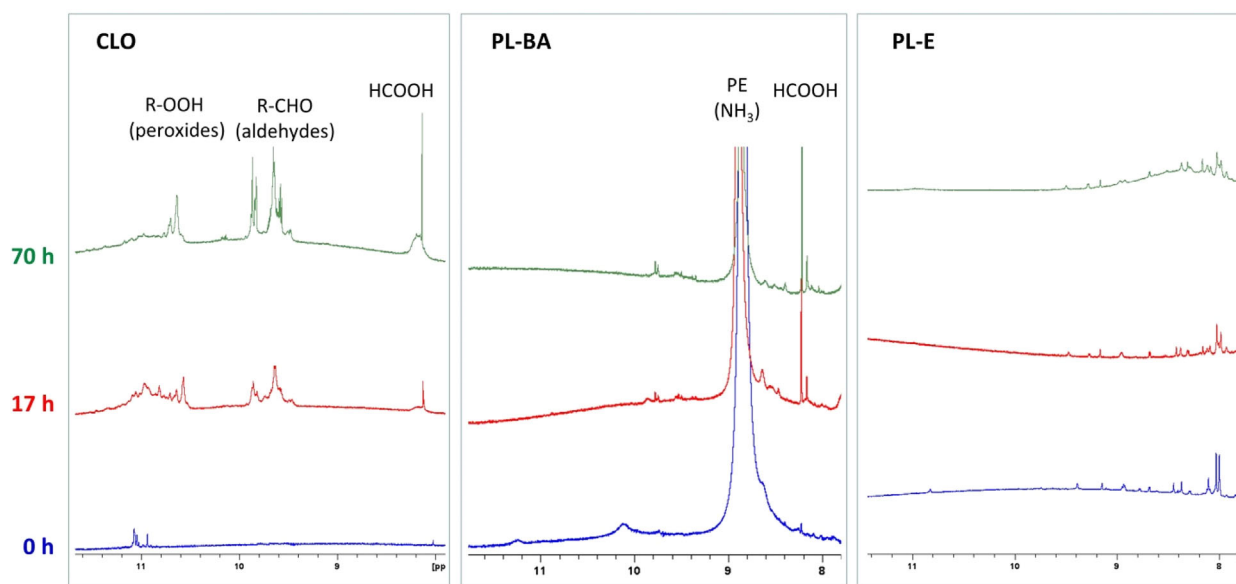


Figure 6. Comparison of ¹H NMR (600 MHz) spectral regions where peroxides (R-OOH), aldehydes (R-COH) and formic acid (HCOOH) give signals (8.0–12.0 ppm) for cod liver oil (CLO), and phospholipids (PL-BA and PL-E) sampled during the Schaal oven test at 0, 17, and 70 h.

measurements of this sample as discussed previously. The reason for these resonances appearing as broad peaks in this sample is likely due to chemical exchange (proton transfer processes). Previous results have shown that, e.g., peaks from peroxides may broaden or even disappear due to chemical exchange (proton transfer processes) with protic groups such as alcohols, water, or free fatty acids [32]. The solvent used was CDCl_3 with DMSO- d_6 added to slow down this proton exchange by formation of hydrogen bonds between the hydroperoxide group and the $S=O$ group of DMSO, but the optimal concentration might have varied due to the compositional differences of the samples during the Schaal oven test (e.g., traces of extraction solvent or – water present at 0 h). After 17 and 70 h at 70°C, the resonances from peroxides disappeared, while the signals from aldehydes (9.4–10.0 ppm) became better defined. In addition, a peak from formic acid (8.22 ppm) appeared, but the levels were lower than for the CLO sample.

Compared to CLO and PL-BA, different changes were observed in this region in the PL-E samples during the storage at 70°C. No peaks from aldehydes nor peroxides was observed and this observation is in accordance with PV and AV values that were very low. A broad resonance appeared at 7.8–9.0 ppm after 70 h of oxidation. This resonance may be due to polymerization since large molecules generally give broad peaks in ^1H NMR due to their low molecular mobility, but it may also be due to other effects such as, e.g., chemical exchange (described above). The observed broad signal in this region is in the same chemical shift range as

reported by a previous study on aromatic polymers [50] (in DMSO- d_6).

3.7 Changes in lipid classes and other observations (^1H NMR)

NMR analysis of phospholipid extracts did not indicate such a clear development of peroxides and aldehydes during accelerated storage, as observed in the fish oil. However, also other changes were observed in the samples during the test. After 70 h broad resonances could be observed at 5.2–6.5 ppm (CLO and PL-BA) (spectra not shown) and between 7.8–9.0 ppm (PL-E, Fig. 6). New resonances may stem from polymerization, or pyrrolization, or oxidation products such as epoxides and conjugated dienes. The broad resonance observed at 5.2–6.5 ppm may, e.g., stem from conjugated dienes-hydroxydienes (reported to give signals in the region 5.4–6.7 ppm in CDCl_3 [51, 52], but also vinylic hydrogens from aldehydes gives signals in this region (reported to give signals between 6.2 and 6.8 ppm [53]).

Recently, it has been proposed/shown that pyrrolization takes place in phospholipids [54]. However, pyrroles, characterized by several peaks in the region between 6 and 7 ppm in CDCl_3 (SDBS Web) [38], could not be identified in the PL-E sample. Epoxides are reported to display peaks in the region between 4.35 and 4.5 ppm in CDCl_3 [53], but the content of epoxides was difficult to evaluate due to the crowding of peaks in the samples investigated for this region [32].

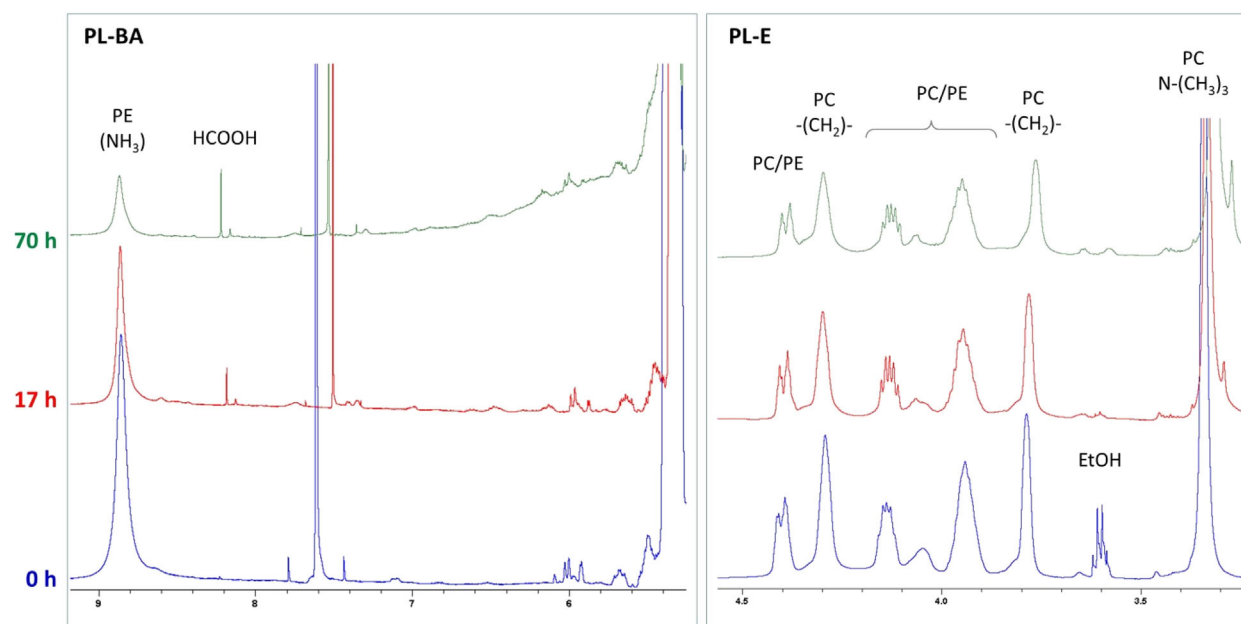


Figure 7. Comparison of ^1H NMR (600 MHz) spectral regions where phospholipids give signals (3.0–9.5 ppm) for PL-BA and PL-E sampled during the Schaal oven test at 0, 17, and 70 h.

During oxidation of marine phospholipids, the tertiary lipid oxidation products such as unsaturated epoxy keto fatty esters, epoxyalkenals and hydroxyalkenals may react with primary amino group present in PE or amino acids in marine PL and cause non-enzymatic browning [8, 54]. This scenario involves the amine group, and this change could be observed by NMR measurement. When it comes to the relative levels of the dominant phospholipid classes (PC and PE), it can be seen from several regions in the ^1H NMR spectra that PC and PE amounts in PL-BA decrease during the incubation period. PE decreases more dramatically (relative content PC: PE was 1:0.085 at the beginning of the reaction. 1: 0.015 at the end of the reaction). While in PL-E the levels of PC and PE stay more or less constant. The decreasing amount of PE is, e.g., evident by observing the intensity decrement of the broad singlet from NH_3 -group of PE at 8.85 ppm and the methylene group of ethanol amine at 3.35 ppm (Fig. 7). The decreasing amount of PE in the PL-BA sample could be due to non-enzymatic browning reactions. In the study by Thomsen et al. [5], when krill oil was stored at 40°C in the dark, the content of PE and PC declined in krill oil with a higher relative decline for PE. However, during storage of krill oil (at 20 and 40°C for up to 42 days) no change in lipid classes was obtained [6].

For the PL-E sample, traces of ethanol was observed (identified from [55]) at the beginning of the experiment, but disappeared in later stages, due to evaporation). This could be one of the reasons for the slight weight decrease in PL-E samples in the Schaal oven test. The PL-E sample contained several other compounds (not identified per date), e.g., resonances at 8.0–9.6 ppm (Fig. 6), it was difficult to observe any clear change in the intensities of these resonances due to the broad peak observed in this region.

4 Conclusions

In this study, the standard oxidative status methods were applicable for pure marine phospholipids – isolated with the Bligh & Dyer method and further purified by double acetone precipitation. However, ethanol isolated phospholipids (industrial technology) even with additional purification by cold acetone contained some impurities that disturbed the measurements and made the methods not feasible or less reliable.

The stability Schaal oven test revealed different weight gain patterns for PL and TAG. No significant increase in weight was obtained for the marine phospholipid extracts. Fatty acid analyses showed that omega-3 fatty acids were more stable in PL than in TAG at the test conditions (air atmosphere, $70 \pm 1^\circ\text{C}$ in the dark for 10 days).

The NMR spectra showed that the traditional increase in peroxide and aldehydes obtained during oxidation of fish oils, could not be observed for marine phospholipids. This indicates that different oxidation patterns are involved

during oxidation of marine phospholipids compared to marine oils (triacylglycerols) and more research should be performed to clarify the oxidation schemes in phospholipids. For more pure extract of marine phospholipids (PL-BA sample), the decrease in the phosphatidylethanolamine was observed by NMR analysis which indicates the possible non-enzymatic browning reactions do take place. Some non-identified peaks were obtained during oxidation of the phospholipid extracts and, therefore, should be investigated in future studies in order to better understand the oxidative changes in phospholipids.

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The authors have declared no conflicts of interest.

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