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Basic nutritional investigation

Aldehydes identified in commercially available ω-3 supplements via 1 H NMR spectroscopy

Pim Jansson M.Sc. [a], Bartholomew Kay M.Sc. [b,*]

[a] Independent nutritionist and biomedical scientist, Mapua, New Zealand
[b] Independent physiologist and nutritionist, Mapua, New Zealand

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ABSTRACT

Objectives: Cardiovascular disease (CVD) is the leading cause of mortality globally. Studies have suggested that supplementary ω-3 oils may provide cardiovascular protection, although the literature is equivocal. Recently, it has been established that many commercially available ω-3 supplements are unacceptably oxidized, leading to myriad potential health risks. One oxidation product of concern is aldehydes, which have been shown to have mutagenic, cytotoxic, and inflammatory properties that may contribute to many different disease processes, including CVD. The aim of this study was to assess the prevalence of aldehyde contamination in commercially available ω-3 supplements.

Methods: We tested 12 different ω-3 oils (6 fish, 4 krill, 2 algae), using 1 H-nuclear magnetic resonance scanning. This work is of a pilot nature, as such we randomly selected and purchased 12 different oils over the counter from various local retailers according to the sales representatives' recommendations.

Results: The four krill products contained aldehydes at concentrations between 5.652 (±0.496) and 6.779 (±1.817) mMol/L. Both algae samples contained aldehydes: 1.235 (±0.111) and 1.565 (±0.618) mMol/L. Two of the six fish oils contained aldehydes 1.568 (±0.291) and 4.319 (±2.361) mMol/L. There is currently no standard for aldehyde content nor for labeling of ω-3 supplements. Two-thirds (8 of 12) of the ω-3 supplements tested in this study contained aldehydes. Aldehydes have the potential to precipitate serious health problems even at very low absolute intake volumes. These findings may provide reason for sober reflection.

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality globally [1]. CVD is demonstrably causally related to chronic inflammation [2–4], among other factors. Studies have suggested that ω-3 fatty acids (FAs) have anti-inflammatory and therefore cardioprotective effects [5–9]. The long-chain polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main ω-3 FAs that have been attributed to cardioprotective effects [8]. Because the Western diet is usually low in ω-3 FAs, supplementation is generally recommended [10,11]. Worldwide, ω-3 FAs are one of the most commonly consumed supplements [12].

Despite the popularity of supplementation with ω-3 oils, benefits for cardiovascular health are unclear [5,10,12,13]. Supplementation with ω-3 may not actually be associated with a lower risk for mortality ascribable to cardiac death, sudden death, myocardial infarction, or stroke [10,13,14]. One possible explanation for this equivocality may be in the propensity for PUFAs, of which ω-3 is a form, to oxidize. PUFAs tend to oxidize because of their large numbers of carbon–carbon double bonds and owing to the position of these bonds [15]. Aldehydes are a potential oxidation product. Aldehydes have been shown to have mutagenic, cytotoxic, immune system aggravating, and inflammatory properties in addition to direct injurious effects on endothelial cells that may contribute to many different disease processes [15–37]. Therefore, it is important to determine whether oxidation is a common problem in commercially available ω-3 FA supplements.

Several existing studies have concluded that lipid peroxidation is indeed a common problem in ω-3 FA supplements [15,20,38,39]. The oxidative state of oils has been commonly determined by measurement of the peroxide and anisidine values. Although these methods can determine the total amount of primary and secondary oxidation products, the composition of these lipid peroxides (LOPs) in terms of groupings of types of LOPs is undetermined by these methods. Because different aldehydes have distinct modes of action and various grades of reactivity, it is important to determine

* Corresponding author: Tel.: +642 7537 2220.
E-mail address: bart.kay.nz@gmail.com (B. Kay).

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the type(s) of aldehydes present. Nuclear magnetic resonance (NMR) spectroscopy often is used to study chemical structures. 1 H NMR can be used to identify the carbon-hydrogen structures present in organic compounds, and thus the group of any aldehydes present [40]. As NMR is a very sensitive detection method, it is possible to detect very small concentrations that may not be detectable using other methods. Because even small amounts of aldehydes may be harmful [26], this study is of great importance. Therefore, we investigated the hypothesis that aldehydes are indeed present in many commercially available ω-3 FA supplements. The focus of this study was exclusively on commercially available EPA- and DHA-rich encapsulated ω-3 FA supplements because these are the most common mediums for their consumption. Because of resource constraints, it was not the purpose of this study to identify the exact molecular species present. That task falls to future studies. This work is of a pilot nature, thus we randomly selected and purchased 12 different oils over the counter from various local retailers according to the sales representatives' recommendations. Further study is required to assess the prevalence of aldehyde contamination in commercially available ω-3 supplements more widely.

Materials and methods

Materials

All reagents were purchased from Sigma-Aldrich (U.K.), unless otherwise stated. Twelve different commercially available encapsulated ω-3 FA supplements were analyzed in this study. All ω-3 supplements were within their use-by dates. The samples were fish, krill, or algae oils. The use of the NMR machine was negotiated with a local university professor.

Sample preparation

Oil capsules were incised at one end and the oil collected in a clean glass tube. We immediately transferred 200 μL of each oil to another clean glass tube and mixed with 400 μL deuterated chloroform containing 0.05% v/v tetramethylsilane (TMS) as a calibration and reference signal. The samples were rotamixed thoroughly with a Vortex Genie 2 (Scientific Industries) and then immediately transferred to clean standard 5-mm diameter NMR glass tubes. The samples were analyzed immediately.

NMR analysis

Proton 1 H spectra were obtained from a Bruker AV 400 MHz spectrometer operating at a frequency of 399.94 MHz. The 1 H spectra were acquired with 128 scans comprising 6.5 μs pulses with a spectral width of 8278.15 Hz. The NMR spectrometer output data was collected using Topspin 2.1 software (Bruker, UK) and processed using ACD/Spectrus Processor 2014 software. The spectra were Fourier transformed by the software to provide data in the proton energy domain. Peaks in the proton energy domain spectra were used to identify the types of hydrogen bonds present in the sample and thus to identify what functional group species were present and in what chemical concentration(s).

Identification of FAs and aldehydes present

The proton energy spectra take the form of an energy "spike" or series of spikes at a level expressed as an energy difference in parts per million with respect to the TMS signal. Identification of ω-3 FA species, non-ω-3 FA species, and aldehyde signals was achieved where possible via comparison of the acquired 1 H NMR signal with that collected previously by others [26,41–43], who were able to positively identify these using secondary methodologies, such as carbon-based NMR as a corroborator.

Quantification of chemical concentrations present

Three repeated measures of each sample were used for the quantification of chemical concentrations. The relative intensity of the NMR signal for each identified molecule was then determined by integration and the concentration was thereby determined by comparing the area under the curve for the selected molecule with that of the known internal standard (TMS). The following equation was used for this purpose:

$$\mathrm{RI} \times \mathrm{CTMS} \times V = [\text{Selected molecule}](\mathrm{mole/L})$$

where RI is the relative intensity of the selected molecule, CTMS is the concentration of TMS in the sample (mmol/L); 12 is the number of protons present in TMS, and V is the total volume of the sample divided by the volume of oil (mL).

Statistical analysis

All data are expressed as a mean value of three repeated measures unless specified otherwise. A two-way analysis of variance and intraclass correlation coefficients (ICCs) were calculated using SPSS Statistics version 21 (IBM, Armonk, NY, USA).

Results

A typical 1 H NMR spectrum of krill oil with magnifications of its 0.80 to 1.02 and 9.75 to 9.83 ppm regions is shown in Figure 1. ω-3

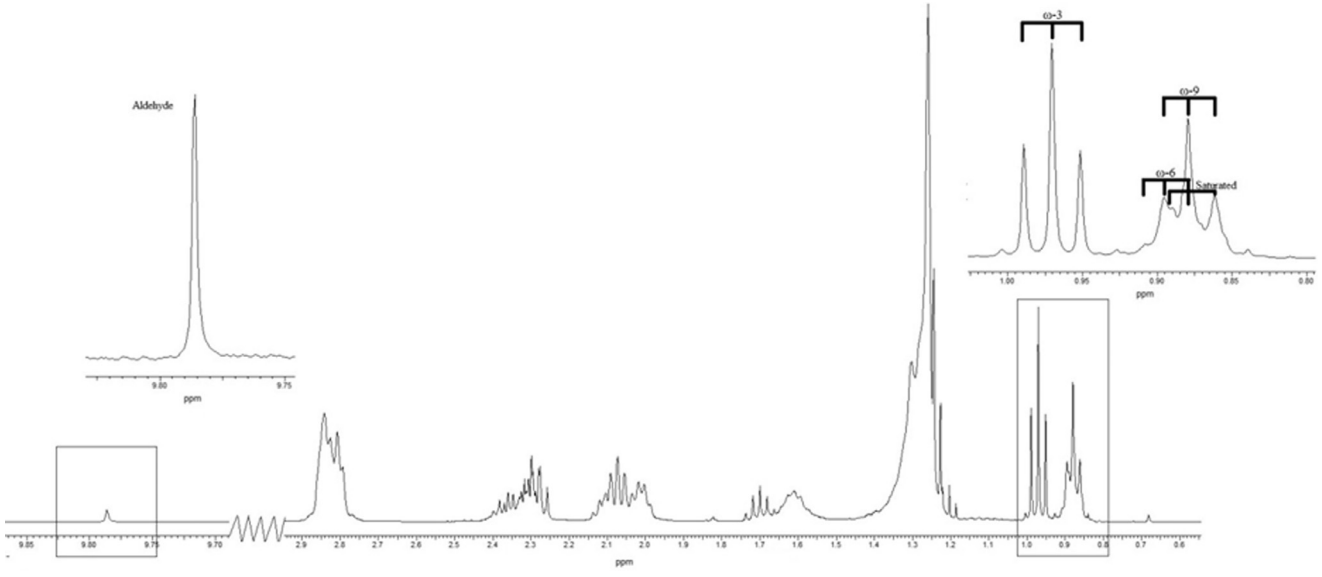

Fig 1. Typical 1 H NMR 400 mHz spectrum of a krill oil in $CDCl_3$ in this case. The spectrum shows the ω-3 fatty acid signal (0.94–1 ppm), the non-ω-3 fatty acid signal (0.83–0.92 ppm), and an aldehyde signal (9.78–9.80 ppm). On the *x* axis, ppm refers to the NMR energy shift, and not to a chemical concentration. The chemical concentration of given molecules is determined as described in the methods section. $CDCl_3$, deuterated chloroform; NMR, nuclear magnetic resonance.

Table 1
Known energy shift values of ^{1}H NMR 400 mHz signals in $CDCl_3$ of ω-3 fatty acid supplements

Functional group		Energy shift (ppm)
Saturated, monounsaturated ω-9 and ω-7 acyl groups	$-CH_3$	0.88
Unsaturated ω-6 acyl groups	$-CH_3$	0.89
Unsaturated ω-3 acyl groups	$-CH_3$	0.97
Trans-2-alkenal	-CHO	9.49
n-alkanal	-CHO	9.75
Unidentified aldehyde	-CHO	9.79

$CDCl_3$, deuterated chloroform; CHO, carbohydrate; NMR, nuclear magnetic resonance

FAs give rise to a signal at 0.97 ppm, ω-6 FAs at 0.89 ppm, ω-9 and saturated fatty acids (SFAs) both appear at 0.88 ppm (Table 1 provides further details). The difference between these signals is due to the carbon–carbon double bond at the ω-3 position in the ω-3 FAs compared with the position of double bonds in other unsaturated FAs. Quantification of ω-3 as a proportion of total FAs or indeed in mol/L can therefore be determined (Table 2).

The various aldehydes typically produce signals between 9 and 10 ppm. Three different types of aldehydes were observed in the present study. Of these, one was present in all four krill oil products tested. The signal for this aldehyde is shown in Figure 1 at a chemical shift value of 9.78 ppm. This signal has not been previously positively identified to our knowledge. Therefore, it was compared with the signal derived post hoc from the following compounds: glyoxal solution, glyoxylic acid, trans-2-octenal, trans, trans-2,4-decadienal, hexanal, and furfural. It did not correspond to any of the compounds tested and therefore remains unidentified at the time of writing. The other two aldehydes were found in both fish and algae oils: at chemical shift values of 9.49 and 9.75, respectively. These have previously been determined to belong to the trans-2-alkenals and *n*-alkenal aldehyde groups respectively [26,42].

Repeatability of measures

The ICC for single measures for aldehyde chemical concentration was 0.894 (95% confidence interval [CI], 0.750–0.965; $P = 0.000$). The ICC for average measures for aldehyde signals was 0.962 (95% CI, 0.900–0.988; $P = 0.000$). The ICC for single measures for ω-3 signals was 0.969 (95% CI, 0.921–0.990; $P = 0.000$). The ICC for average measures for ω-3 signals was 0.989 (95% CI, 0.972–0.997; $P = 0.000$). The ICC for single measures for non-ω-3 signals was 0.834 (95% CI, 0.629–0.944; $P = 0.000$). The ICC for average measures for non-ω-3 signals was 0.938 (95% CI, 0.836–0.981; $P = 0.000$).

Discussion

Disclaimers

The present study did not make any inferences with respect to purified EPA or DHA oil products because the samples were not purified (i.e., fish, krill, or algae extracts). The study did not make inferences with respect to products containing antioxidants, as none of the samples tested contained these. This study was of a pilot nature; as such, more data is needed to determine the prevalence of oxidation in commercially available ω-3 supplements more widely.

Krill oils

Three different groups of aldehydes were found in the ω-3 oil supplements tested in this study. Each sample containing aldehydes contained one type of aldehyde only. An unknown aldehyde was present in all four krill oil samples tested. One study has shown that *n*-alkanals of low-molecular weight such as ethanal and propanol have a signal at 9.79 [42]. However, these do not correspond exactly to the signal acquired in this study, meaning further analysis is needed to positively identify this aldehyde signal. This aldehyde was, however, of particularly high concentration compared with the other identified aldehydes. This also has been observed in previous studies of lipid oxidation in ω-3 supplements [15,38]. Both krill and fish oil naturally contain EPA and DHA. However, the EPA and DHA found in fish oil exist in a triacylglycerol molecular format [44]. In krill oil, EPA and DHA are mainly bound to phospholipids; in particular: phosphatidylcholine (PC) [44]. The binding of ω-3 FAs to PC is believed to contribute to the increased health benefits of krill oil compared with fish oil [44–46]. However, studies have shown that PC is highly susceptible to oxidative degradation [47,48]. Oxidation of PC also may contribute to the total oxidative load. The findings of this study may lend weight to the idea that krill oils are indeed more susceptible to oxidation than are fish or algae oils.

Fish and algae oils

Half of the fish and algae samples tested in the present study contained LOPs. Specifically, two species were identified, trans-

Table 2
Concentration of aldehyde in mMol/L and mMol/mol FAs, ratio of ω-3 to non-ω-3 FAs and class of aldehyde

Sample	Aldehyde (mMol/L)	Aldehyde (mMol/mol FAs)	Aldehyde species	ω-3:non-ω-3 FAs
Krill oil 1	6.542 (±0.525)	0.251	Unknown	0.8:1
Krill oil 2	5.920 (±0.760)	0.228	Unknown	0.9:1
Krill oil 3	5.652 (±0.496)	0.216	Unknown	0.6:1
Krill oil 4	6.779 (±1.817)	0.212	Unknown	0.7:1
Fish oil 1	1.568 (±0.291)	0.053	*n*-alkanal	0.5:1
Fish oil 2	0 (±0.000)	0		0.5:1
Fish oil 3	0 (±0.000)	0		0.7:1
Fish oil 4	0 (±0.000)	0		1.7:1
Fish oil 5	0 (±0.000)	0		2.3:1
Fish oil 6	4.319 (±2.361)	0.112	Trans-2-alkenal	1.8:1
Algae oil 1	1.235 (±0.111)	0.033	Trans-2-alkenal	1.7:1
Algae oil 2	1.565 (±0.618)	0.041	Trans-2-alkenal	1.3:1

FA, fatty acid

2-alkenals, and *n*-alkanals. Three of the samples (one fish oil and both algae oil) contained aldehydes of the trans-2-alkenal type. As with most aldehydes, trans-2-alkenals have been found to be atherogenic, mainly by adduct formation to various biomolecules [49]. Although no specific species were identified; adduct formation from ω-3 FAs primarily lead to acrolein and crotonaldehyde adducts [49]. Trans-2-pentenal exposure from ω-3 oxidation has also been shown to produce cyclic DNA adducts [49]. However, as the reactivity decreases with decreasing chain length; acrolein followed by crotonaldehyde are seemingly the most concerning trans-2-alkenals formed [49]. These belong to the α,β-unsaturated aldehydes that are known to be highly reactive aldehydes [26,37,50].

One of the fish oil samples analyzed contained one or several species of *n*-alkanals. These are saturated aldehydes that are prone to forming adducts with nitrogen groups on lysine residues [33,50–53]. All *n*-alkanals (from propanal to decanal) can react with the primary amino group of dipalmitoyl-phosphatidylethanolamine (DPPE) to form adducts [33]. These type of adducts have been detected in atherosclerotic plaques of both rats and humans [31,33]. Other types of adducts such as dimeric, linear, and cyclic trimeric adducts, also have been identified after exposure to *n*-alkanals of different lengths [33]. The biological effects of these type of adducts to date are poorly researched. Some early research shows that hexanal DPPE adduct formation can induce a concave shape in phospholipid bilayers [33]. Such changes in membrane curvature could lead to hardening of the membrane and loss of stability and permeability, all of which may contribute to CVD [33].

Ratio of ω-6 to ω-3 in oils

As shown in Figure 1, the ω-3 supplements contained different mixtures of FAs. The presence of other unsaturated FAs, especially ω-6, may have resulted in detection of different LOPs to those expected from ω-3 FA oxidation alone. It appears however, that ω-3 FAs may be more susceptible to oxidation than ω-63 FAs. This hypothesis is supported by a report of an inverse relation between days until expiration date and the peroxide value of ω-3 supplements; which is not seen in vegetable oils [15]. An explanation for this finding is that the position of the carbon–carbon double bond in ω-3 FAs is more prone to oxidation than the ω-6 double bond [15]. However, there are two highly reactive aldehydes that are typically found in oxidized ω-6 oils: 4-hydroxy-trans-2-nonenal (4-HNE) and malondialdehyde (MDA) [3,54]. These have been extensively researched because of their toxicity and ubiquity. Similarly, 4-hydroxy-trans-2-hexenal (4-HHE), which has some similar mechanisms to 4-HNE, is typically derived from ω-3 FAs [20,55]. None of these aldehydes were identified in the present study. There remains a possibility nonetheless that the consumption of these oils could lead to endogenous formation of these products. Indeed, several studies have shown that digestion of both ω-3 and ω-6 PUFAs do result in endogenous aldehyde formation [55–58]. Notwithstanding, reasons to keep the ratio of ω-6 to ω-3 in the diet as low as possible have been described previously [59]. For these reasons alone, consumers who choose ω-3 supplementation may consider favoring an ω-3 supplement with the lowest possible ω-6 content.

Potential aldehyde exposure risks from ω-3 supplementation

The potentially immediate toxic mechanisms of acute aldehyde exposure appear to mainly involve adduct formation with nucleophilic residues on macromolecules such as proteins and DNA. Common targets are lysine, cysteine, and histidine residues. Aldehyde exposure may cause immediate oxidative stress, lipid membrane peroxidation, cell damage, platelet-leukocyte aggregation, thrombosis, inflammation, increased heart rate, increased blood pressure, and increased susceptibility of vasospasm [18–25]; whereas over time, such factors may present as increased atherosclerotic lesions [21–23,60,61]. The long-term pathogenic mechanisms of these adducts are not fully understood; however, they likely differ depending on the structure of the aldehyde.

Risk-level assessment

Bioavailability of lipid oxidation products

When assessing the potential health hazard of aldehydes, it is important to consider their bioavailability. The rates at which these are absorbed naturally differ between species. Notwithstanding this, studies have shown that ingestion of oxidized lipids lead to increased urinary excretion of MDA and other lipophilic carbonyl compounds in both animals and humans [56,62]. Absorption of both hydroperoxides and aldehydes such as 4-HHE and 4-HNE have been observed [3,55,63]. Although not all aldehydes are completely absorbed, they can cause local damage in the gastrointestinal tract by adduct formation [55]. Furthermore, primary LOPs or nonoxidized lipids may become oxidised during digestion [57,58,62].

Does this study suggest a significant potential health hazard?

There has been concern about the safety of oxidized fish oils for >60 y, yet there are no requirements for their storage or labeling. There are currently no recommendations about upper limits of LOPs in oil supplements; however, upper limits of peroxide values have been suggested in previous literature [15,28,64]. Voluntary international industry standards also have recommended that peroxide aniside and total oxidation values <5 mEq/kg [20,26], respectively, should be applied for ω-3 supplements to be considered safe for consumption [38]. A typical serving of fish oil is about 1 to 1.5 mL. Presuming that the average oxidized ω-3 oil contains around 3 mmol/L aldehyde, the daily intake of aldehydes would be ~4 to 5 μmol. As discussed earlier, all aldehydes have different absorption rates, toxicity, and propensity to form adducts. Acrolein is perhaps the most studied dietary aldehyde and one that has been assessed for its toxicity. The World Health Organization has established a tolerable daily oral acrolein intake of 7.5 μg/kg, which equals 525 μg or 9.4 μmol for an average body weight of 70 kg [26,65]. If the aldehyde present in the supplement is exclusively made up of acrolein, this would be half of the tolerable daily intake. Considering most people consume fried or deep-fried food regularly and additional PUFAs in the form of vegetable oils or margarine, supplementing with oxidized ω-3 FAs could indeed pose significant potential health hazards.

Conclusion

Of the 12 ω-3 supplements tested in this study, 75% two-thirds, (8/12) contained aldehydes. Aldehydes are potentially problematic owing to the risks for multiple types of adduct formation with biological molecules, leading potentially to inflammation, dysfunction, CVD, DNA damage, cancers, and a myriad of other health concerns. Krill oils tested in this study invariably contained aldehydes, and at relatively very high concentrations. Fish and algae oils contained aldehydes in four of the eight samples. Although four samples did not contain any detectable aldehydes with 1 H NMR, these could still contain primary oxidation products, which would have to be tested using other methods. All groups of aldehydes that were identified

promoted CVD. It is also important to consider endogenous formation of both primary and secondary LOPs, which may be different from those detected. More oxidized oils also may enhance the formation of LOPs during the digestion process. There are, however, no studies confirming such a relationship. As dietary ω-3 supplements are generally recommended in conjunction with food intake, the presence of antioxidants and prooxidants also will affect the amount of endogenously produced LOPs [56]. This adds another level of complexity when determining the potential health hazard of oxidized ω-3 supplements. Not only may consumption of oxidized ω-3 FAs result in consumption of LOPs already present in the supplements, it also may increase endogenous production of LOPs. These findings provide perhaps for sober reflection on the practice of ω-3 oil supplementation.

Previous similar studies have concluded that the quality of different ω-3 supplements vary between brands [15,38,39]. The rate at which lipid oxidation can take place is influenced by several different conditions. Light, heat, oxygen concentration, antioxidants, protein. or heavy metal contamination are factors that affect the rate of oxidation. Therefore, it would be warranted to investigate how different processing of the fish, addition of antioxidants, presence of heavy metals, extraction, packaging, and storage methods affect the quality of the oil. Once oxidation has started, it will quickly propagate. Addition of antioxidants and storage in cool temperatures can slow this process but will not prevent it [39]. Therefore, the quality of the oil is highly dependent on the processing and packaging in the factory. Improving the way these oils are processed by avoiding high-temperature deodorization, exposure to oxygen, and storage at room temperature may improve the quality of the oils.

Considering that the process of oxidation will break down the ω-3 FAs, hence making them unable to exert their beneficial functions, and produce aldehydes and other LOPs that are inflammatory, there is a possibility that the state of the oil affects its performance in clinical trials. Although the aim of this study was to measure aldehyde levels, these samples also may contain primary oxidation products that contribute to inflammation. There is a real possibility that this outweighs the proposed benefits of the intact ω-3 FAs in the samples and contributes to the initiation and progression of CVD. Future clinical trials should investigate the possible health benefits of ω-3 supplements with reported primary and secondary LOP values. These studies should use a standardized method to determine the oxidation status of the oils. This would allow for an assessment on whether oxidation of these supplements affects the efficacy of the ω-3 FAs and whether it may be harmful to consume these. Future studies also should investigate how processing and storage affects the amount of lipid oxidation. Testing and labeling requirements for ω-3 supplements should be developed and enforced.

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