

Fatty Acid Content, Oxidation Markers and Mercury in Fish Oil Supplements Commercialized in Brasília, Brazil

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Abstract:

Fish oil supplements are good sources of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which are important in the prevention and treatment of hypertriglyceridemia. The purpose of this study was to examine the content of EPA and DHA, oxidation markers and mercury of fish oil supplements marketed in Brasília, Brazil. Fatty acid contents were determined by gas chromatography using internal (C23:0) and external methyl ester standards. For this analysis, samples were prepared by alkali-catalyzed methylation with boron trifluoride (14% in methanol). Mercury was determined by direct vapor detection method. Oxidation markers were evaluated by measuring peroxide value (PV), anisidine value (AV) and by calculating TOTOX. The adequacy of EPA and DHA ranged from 75.9 to 105.1% and from 88.9 to 137.4%, respectively, compared to the information in the label. Mercury concentration was above limit of quantification levels, between 11 and 15 µg/kg in 14.4% of the products. Twenty percent of the products exceeded recommended levels of PV and TOTOX. Despite the high percentage of adequacy of the parameters analyzed, about 2/3 of the products showed some inadequacies according to the law. These data deserve concern due to the potential side effects of oxidized and contaminated fish oils to their proclaimed health benefits. This survey shows the relevance of constant monitoring of fish oil quality, considering current legislation and scientific advance.

Keywords: omega-3; peroxide value; anisidine value; heavy metals

1. Introduction

The omega-3 (ω -3) fatty acids (FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to play preventive and treatment roles in hypertriglyceridemia and other cardiovascular diseases [1]. Hypertriglyceridemia is defined as serum triglyceride levels above 150 mg/dL and is related to increased risk of cardiovascular diseases [1]. The main dietary sources of EPA and DHA are cold water fishes, which are not consumed routinely by Westerners in general [2]. Fish oil supplements are an efficient alternative for the intake of these ω -3 FAs.

Fish oil products are classified as dietary

supplements and do not require medical prescription [3-5]. As a product, guidelines from the US Council for Responsible Nutrition, Global Organization for EPA and DHA - GOED and Health Canada, among others, state the maximum amount of heavy metals, degree of oxidation and environmental contaminants in ω -3 FA products [6-8]. Organic contaminants, such as methylmercury (MeHg), are a globally dispersed pollutant with high potential for bioaccumulation in fish tissues. MeHg is a neurotoxin capable of causing serious deleterious effects on the developing nervous system, kidneys, and liver; and has also been associated with increased risk of acute myocardial infarction [9]. Oxidized fatty acids may cause many deleterious health effects, such as oxidative stress, inflammation, vascular

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dysfunction, high cholesterolemia, and atherosclerosis [10].

Worldwide, fish oil composition has been analysed in many countries, mostly in developed countries. In Latin America, and particularly in Brazil, few studies analysed fatty acid composition and oxidation markers in fish oil products. These studies were conducted over a decade ago and found inadequacies in products sold in Brazilian market [11, 12]. The main global suppliers of fish oil are Peru, Scandinavia, Chile, United States, and Japan [13]. The fish oil supplements sold in Brazil have either been made abroad or made in Brazil with imported raw material. When these supplements are stored or transported in adverse conditions, the FAs present in the fish oil may oxidize, producing harmful substances, reducing the amount of ω -3s, and decreasing the health benefit of the product [14].

In addition, the evaluation of fish oil has generally considered EPA and DHA contents as one single ω -3 entity [15]. However, recent studies have reported different metabolic effects and functions of these two FA, with the emergence of new products such as those more concentrated in DHA than EPA or in both FA [1, 16]. Thus, updated assessment of fish oil supplement quantity and quality is relevant [14, 15].

In this context, the main goal of the present study was to assess the composition of FA, degree of oxidation and mercury content in fish oil supplements marketed in Brazil, based on recommendations made by organizations such as US Council for Responsible Nutrition, GOED, Health Canada and ANVISA (The Brazilian Health Regulatory Agency).

2. Results and Discussion

Twenty-eight products met the study inclusion criteria. Of those, 22 (78.6%) were Brazilian brands made of imported bulk oil or capsules, and six were brands imported from USA. One sample was excluded from FA analysis because it had expired (Figure 1).

The amount of fish oil in the capsules varied from 0.5 g to 1.4 g, but the most prevalent amount was 1.0 g, present in 18 (64.3%) products. None of the 28 products mentioned sources other than

fish oil, such as algae, in the list of ingredients.

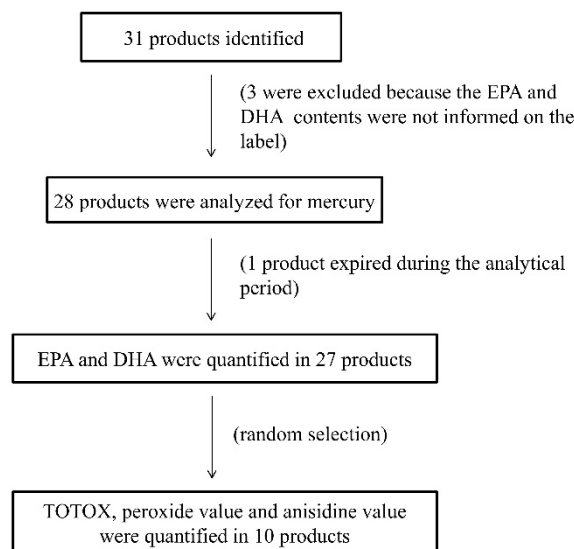


Figure 1. Flowchart of the study supplements and chemical analyses of the fish oil in the soft-gel capsules.

The coefficient of variation of the six injections was below 10%, and the calibration curves of the external standards were $r^2 = 0.9922$ and $r^2 = 0.9995$ for EPA and DHA, respectively. The amount of EPA + DHA in the products varied from 26% to 78%, being 14.8 to 41.2% for EPA and from 10.6% to 56.7% for DHA, relative to total %FA. The EPA content per capsule ranged from 83 to 578 mg, the median was 94.6 mg for the capsules with <1.0 g of oil and 234.3 mg for the capsules with ≥ 1.0 g of oil. The DHA content per capsule ranged from 60 to 340 mg, the median was 71.0 mg for the capsules with <1.0 g of oil and 168.8 mg for the capsules with ≥ 1.0 g of oil.

To further investigate the variability in the ω 3 contents of the samples, fish oil supplements were subdivided into four groups according to EPA and DHA contents (Table 1): a group with EPA and DHA contents compatible with triacylglycerol formulation (TAG, G1, $n=18$); a group with high EPA content (G2, $n=2$); a group with high DHA content (G3, $n=2$); and a group with high EPA and DHA contents (G4, $n=5$). The median EPA and DHA contents in G1 (44.70%) were significantly lower ($p<0.05$) than those of G2 (86.10%), G3 (86.81%), and G4 (74.69) (Table 1). Most of the products evaluated in this study had EPA and DHA concentrations compatible with TAGs [16,17]. The G2, G3 and G4 formulations

were consistent with fish oil concentrates, similar to those found by Nichols *et al.* [8]. Tatarczyk *et al.* [19] analysed 6 products compatible to standard fish oil and 3 products high in EPA and DHA, with EPA + DHA varying from 55.6% to 95.8%, EPA varying from 32.9% to 53.8%, and DHA varying from 22.7% to 42.0% on those three products [30]. Other recent reports on fish oil concentrates have identified EPA + DHA ranging from 53% to 59% [20-22].

TAG-based fish oils can be interesterified in the laboratory to obtain a product with higher contents of the FAs of interest [23]. Concentrated formulations include ethyl ester (EE) and re-esterified triacylglycerol (rTAG). As a product, the

advantage of rTAG is that it has higher EPA and DHA content and higher bioavailability than EE [24]. Formulations with free fatty acids (FFA) are more bioavailable than those with EE, rTAG, or TAG, but more susceptible to oxidation and are most likely to cause gastrointestinal symptoms [25, 26]. For this reason, FFAs are removed from fish oil composition during the deacidification process [27]. Consumers should bear in mind these differences between fish oil supplements and that products based on fish oil concentrates have the advantage of containing less saturated fatty acids, which can be as much as ten times lower than the amount found in TAG based products (Table 1).

Table 1. Fatty acid profile of commercial fish oil supplements.

| Fatty acid (%) ^a | G1 ^d | G2 ^e | G3 ^f | G4 ^g |
|-----------------------------|------------------------------------|--------------------|--------------------|------------------------------------|
| 4:0 | 0.00 (0.00 - 1.16) | 0.00 | 0.92 | 0.00 (0.00 - 0.46) |
| 14:0 | 5.87 (4.92 - 7.12) ^b | 0.00 ^c | 0.03 ^c | 0.08 (0.00 - 0.25) ^c |
| 16:0 | 14.79 (13.62 - 16.83) ^b | 0.00 ^c | 0.18 ^c | 1.89 (0.96 - 2.25) ^c |
| 16:1 C | 7.23 (6.44 - 8.19) ^b | 0.00 ^c | 0.05 ^c | 0.46 (0.00 - 0.82) ^c |
| 17:1 | 0.97 (0.81 ± 1.13) | 0.00 | 0.00 | 0.00 (0.00 - 0.00) |
| 18:0 | 3.41 (3.21 - 3.60) ^b | 0.19 ^c | 1.17 ^c | 2.93 (2.58 - 4.10) ^b |
| 18:1 9C | 8.60 (8.02 - 9.49) ^b | 0.29 ^c | 1.27 ^c | 6.17 (4.37 - 6.67) ^c |
| 18:1 9T | 1.12 (0.00 - 1.36) | 0.00 | 0.00 | 0.00 (0.00 - 0.00) |
| 18:1 11T | 2.93 (2.71 - 3.16) | 0.00 | 0.38 | 2.14 (1.57 - 2.26) |
| 18:2 C | 1.17 (0.00 - 1.39) | 0.00 | 0.25 | 0.89 (0.70 - 1.06) |
| 20:0 | 0.00 (0.00 - 0.00) | 0.13 | 0.00 | 0.40 (0.00 - 0.75) |
| 20:1 + 18:3 n3 | 1.72 (1.57 - 1.89) ^b | 0.57 ^c | 1.61 ^b | 2.05 (1.01 - 3.43) ^b |
| 20:2 | 2.90 (2.47 - 3.11) ^b | 0.13 ^c | 3.09 ^b | 1.66 (0.37 - 2.74) ^c |
| 20:3 n6 | 0.73 (0.00 - 1.28) | 7.14 | 0.33 | 1.25 (0.37 - 1.79) |
| 22:1 | 0.00 (0.00 - 0.00) ^b | 1.14 ^b | 1.96 ^c | 0.00 (0.00 - 1.24) ^b |
| 20:4 n6 | 1.17 (1.08 - 1.37) ^b | 1.30 ^b | 0.94 ^b | 2.11 (1.92 - 2.30) ^c |
| 22:2 n6 | 0.46 (0.00 - 1.96) ^b | 0.61 ^b | 1.00 ^b | 1.57 (0.74 - 1.90) ^c |
| 20:5 n3 (EPA) | 23.95 (22.40 - 24.91) ^b | 14.89 ^b | 53.66 ^c | 38.93 (35.72 - 39.38) ^c |
| 22:6 (DHA) | 20.91 (19.49 - 21.92) ^b | 71.21 ^c | 33.15 ^c | 34.93 (30.41 - 35.89) ^c |
| % EPA + DHA | 44.70 (42.78 - 46.53) ^b | 86.1 ^c | 86.81 ^c | 74.69 (66.88 - 76.27) ^c |
| % saturated FA | 24.79 (22.77 - 28.70) ^b | 2.46 ^c | 2.3 ^c | 5.55 (5.22 - 5.87) ^c |

^aValues expressed as medians (25th to 75th percentiles) when n > 2 and median when n = 2.

^{b,c} different letters indicate significant differences between the groups (p < 0.05).

^dFish oil (n = 18)

^e High DHA content (n = 2)

^f High EPA content (n = 2)

^g High EPA & DHA contents (n = 5)

The percent adequacy of the FA contents compared to the indication on the label also varied among brands, ranging from 75.9 to 105.1% for EPA, from 88.9 to 137.4% for DHA, and from 85.0 to 109.3% for EPA + DHA (Figure 2). Wider variation in the percentage adequacy were reported by Tatarczyk *et al.* [19] in Austria, Ritter

et al. [20] in the United States, and Albert *et al.* [21] in New Zealand: Tatarczyk *et al.* [19] found percent adequacy of 100 to 144% (EPA + DHA); Ritter *et al.* [20] of 27 to 132% (EPA) and 64 to 495% (DHA); and Albert *et al.* [21], of 32 to 144% (EPA + DHA).

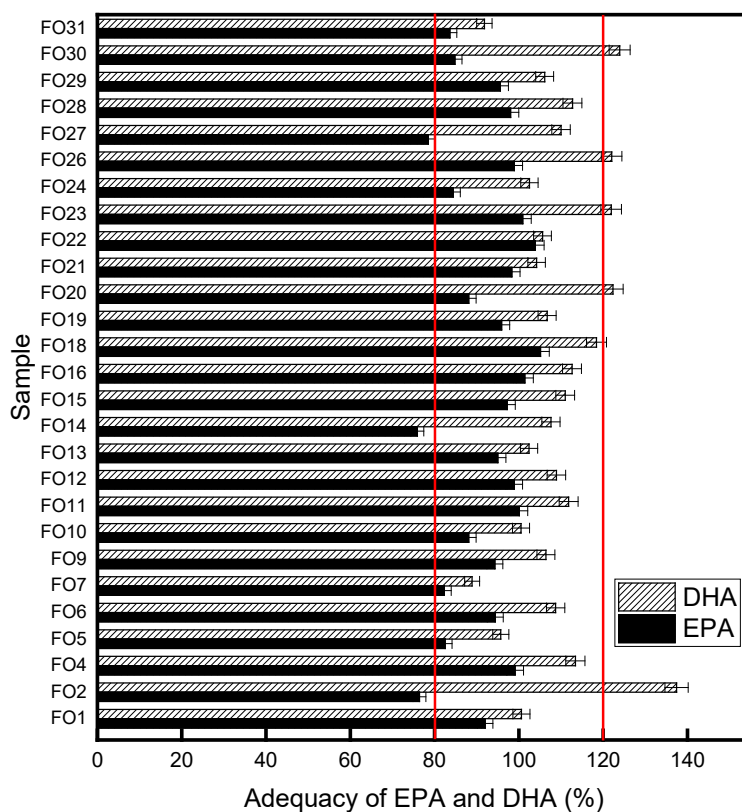


Figure 2. Percent adequacy of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in fish oil supplements (FO) and the amounts indicated on the label.

The lower variability of FA contents found in the present study agrees with the recent legislation in Brazil which states that contents of nutritional supplements may differ by no more than $\pm 20\%$ from that indicated on the label [28]. The parameter used by Kleiner et al. [22], for example, was that established by the Food and Drug Administration (FDA); in which product should contain at least 80% of the nutrient contents stated on the label, with no specification on the upper limit [29]. The analytical method used in the present study had acceptable internal validity based on the low coefficient of variation between the six replicates of each sample [30]. Thus, the results indicated that most of the fish products evaluated in the present study were in conformity with FA content informed on the label.

The reporting of EPA and DHA contents, in separate, is valuable since recent studies are demonstrating differing biological roles for these two $\omega 3$ FA [16,31]. DHA has an important role in neural and vision development and function and in cancer cells' death, while DHA and EPA play important, but different roles in cardiovascular diseases prevention and treatment [16,31]

The LOD and LOQ of mercury determined by calibration curve ($r^2 = 0.9948$) were 3.46 and 11.54 $\mu\text{g}/\text{kg}$, respectively. Seventeen (60.7%) samples presented mercury content below the LOD, and seven samples (25%), below the LOQ. The mercury contents of only four samples were above the LOQ but below the maximum limit established by international guidelines, increasing from 11.62 to 14.48 $\mu\text{g}/\text{kg}$ in samples FO23, FO15, FO4, and FO9. Interestingly, three of these four samples were products high in EPA and/or DHA contents. Thus, less than 15% ($n = 4/28$) of the study products had quantifiable mercury levels, and all products complied with the current regulations, which allow mercury levels up to 100 $\mu\text{g}/\text{kg}$ [6-8]. The LOD and LOQ of the study products were at least nine fold lower than the current limit established by law. High EPA and/or DHA content in samples with mercury above LOQ suggest that the concentration of this heavy metal may have increased during the manufacturing process.

Foran et al. [32] quantified mercury by cold vapour atomic absorption spectroscopy and found that 40% ($n = 2/5$) of the samples had mercury

levels between 10 and 12 $\mu\text{g}/\text{kg}$. Similarly, the present study found mercury levels between 11.62 to 14.48 $\mu\text{g}/\text{kg}$, but in a smaller percentage of samples (15%). The LOD found by Foran et al. [32] was 6 $\mu\text{g}/\text{kg}$, which is higher than the study LOD, and they did not report LOQ. Using a similar method used herein, with LOQ of 1.5 $\mu\text{g}/\text{kg}$, Levine et al. [33] investigated the mercury levels in many types of supplements, among them three samples of fish oil, including salmon oil. They found mercury levels of 9.89, 38.8, and 123 $\mu\text{g}/\text{kg}$, the highest being from the salmon oil. The method used herein has been validated to quantify mercury in fish tissues in relation to the cold vapour atomic absorption spectroscopy method. The method of direct detection of mercury vapour has the advantage of being faster and easier to perform than cold vapour atomic absorption spectroscopy, and of producing fewer environmentally-unfriendly substances [34].

Seafood is the main dietary source of mercury in humans [35]. Methylmercury (MeHg) is how mercury is stored in fish tissues after biotransformation, and mercury levels may range from 0.4 to 5 mg/kg, increasing with the trophic level of the fish species in the food chain [9]. Hence, older and larger fish in the food chain, such as swordfish, sharks, and some marine mammals, have the highest levels of mercury [9]. Fish oil is vapour extracted from fish tissues, and its refining process is similar to that of vegetable oils. Then, fish oil undergoes an essential purification stage: molecular distillation or supercritical fluid extraction, which removes organic contaminants, such as MeHg [36, 37]. The mercury levels detected in the products of the present study were lower than 15 $\mu\text{g}/\text{kg}$, indicating appropriate purification.

The provisional tolerable weekly intake of MeHg established in 2003 is 1.6 $\mu\text{g}/\text{kg}$ of body weight/week, also valid for children and fetuses [9]. The estimated amount of mercury ingested by people who take 2 g of the study fish oils per day range from 40.6 to 99.9 ng, or 280 to 700 ng of Hg per week. It is suggested, that one to two 50 g servings of certain fish species per week may exceed these limits and advocate the use of other more restrictive limits, such as that of the US Environmental Protection Agency of 0.1 $\mu\text{g}/\text{kg}$ of body weight/day [38]. This subject is of great public health interest because fish with high EPA

and DHA content usually have the highest levels of MeHg in the food chain [39].

AV levels were within acceptable limits in 100% of the samples (Figure 3) whereas only 80% of the samples had PV (Figure 4) and TOTOX (Figure 5) in accordance with the recommended levels by US Council for Responsible Nutrition, GOED and Health Canada. The imported brands had higher PV and TOTOX values than Brazilian brands ($p = 0.044$). TOTOX values were positively correlated with PV ($\rho = 0.903$; $p < 0.000$) and AV ($\rho = 0.952$; $p < 0.000$). Moreover, PV and AV were positively correlated ($\rho = 0.818$; $p = 0.004$). Vitamin E, generally used as antioxidant, was listed on the label of 50.0% of the products evaluated, and it was not associated with any oxidation-related variables (Table 2).

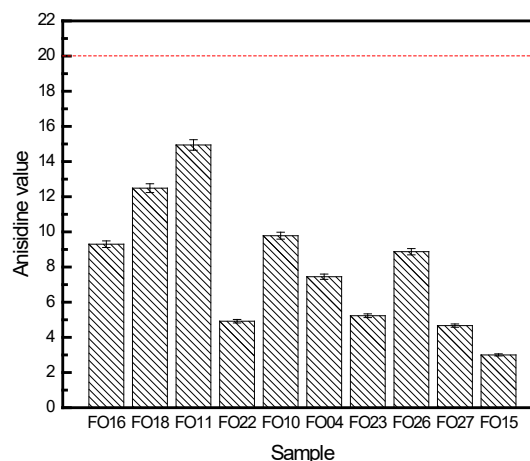


Figure 3. Anisidine value in fish oil supplements (FO).

These results are comparable to recent reports by Nichols et al. [18] on fish oil supplements from Australia and New Zealand markets, that all the products met the recommended PV and 80%, met the recommended AV levels. On the other hand, Albert et al. [21] found that only 8% of the products from New Zealand market complied with the internationally recommended limits of PV, AV, and TOTOX. Other previous studies by Fantoni et al. [11] in Brazil, Ritter et al. [20] in USA, and Opperman et al. [40] in South Africa, respectively, found that 62%, 68%, and 16% of the products analysed complied with the internationally recommended limit of PV. These results suggest that the oxidation state of these supplements in different countries is highly variable and needs to be closely monitored.

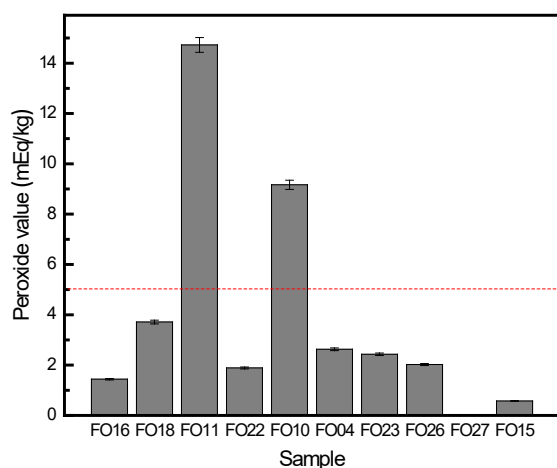


Figure 4. Peroxide value in fish oil supplements (FO).

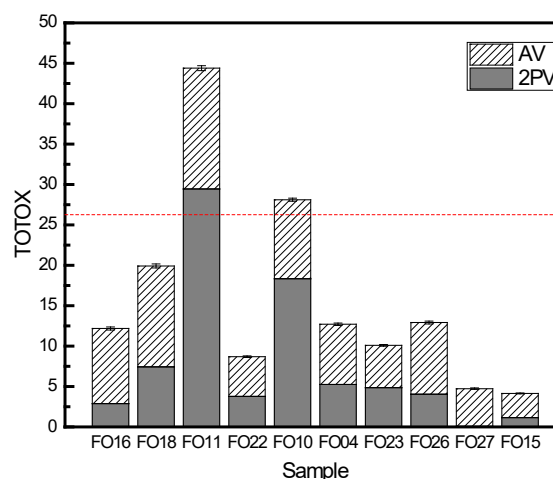


Figure 5. TOTOX in fish oil supplements (FO). TOTOX corresponds to 2 PV (Peroxide value) + 1 AV (Anisidine value).

Table 2. Degree of oxidation in the samples with and without vitamin E listed on the label.

| | samples with vitamin E (n = 5) | samples without vitamin E (n = 5) | p ^a |
|-----------------------------|--------------------------------|-----------------------------------|----------------|
| peroxide value ^b | 1.89 (0.74 – 5.60) | 2.63 (1.50 – 9.22) | 0.310 |
| anisidine value | 8.87 (4.79 – 9.54) | 7.45 (4.12 – 13.72) | 0.841 |
| TOTOX | 12.18 (6.71 – 20.52) | 12.71 (7.12 – 32.16) | 0.841 |

^aSignificant difference ($p < 0.05$)

^bValues expressed as medians (25th – 75th percentiles)

According to Ritter et al. [20], products containing fish oil ethyl esters had higher PV than TAG-based products, suggesting that formulation may affect susceptibility to oxidation. FA oxidation is multifactorial. Fish oils must be produced in environments protected from oxygen, light, and heat. The oil may oxidize during the encapsulating process or during the addition of antioxidants [41]. Extensive oxidation, especially to peroxides, may also be caused by inappropriate transportation and storage of the end product [20].

In the present study, PV and TOTOX levels above the acceptable limits were found in 20% of the products examined, suggestive of recent oxidation, compatible to poor storage conditions in the market. During the analytical period, fish oil samples were stored at $-18\text{ }^{\circ}\text{C}$, protected from light and oxygen, and when necessary, transported in a cooler with ice, in order to largely avoid sample oxidation.

TOTOX provides a complete picture of the degree of oxidation of oils because it takes into account the initial stages of oxidation, indicated by the presence of peroxides (PV values), and their possible decomposition into secondary oxidation compounds (AV values), such as aldehydes, ketones, hydroxyl acids, and hydrocarbons [42]. However, high peroxide level (high PV) is not always related to high production rate of secondary oxidation products (high AV), and vice-versa [43]. For example, determination of AV is useful for assessing the oxidation of cooking oils, which generally have low PV due to the degradation of primary oxidation products into further peroxide products, by the repeated exposure of the oil to high temperatures [44]. However, in the present study, the results of PV, AV, and TOTOX were all positively correlated, suggesting that the products were not exposed to hydroperoxide degradation into secondary compounds.

The present study has some limitations. The analysis of oxidative markers included only a subsample. The samples were not representative of all the products in the market, but the analyses were conducted with rigor to guarantee high data quality. Additionally, since most supplements sold globally contain fish oil from fish caught in defined and restricted regions [13], the results are pertinent locally and globally.

3. Material and Methods

3.1. Chemicals

The following pro analyse (P.A.) grade reagents were used: Sodium chloride (Cromoline® Química Fina – Brazil); Suprapur® nitric acid 60% (by mass), chloroform, hexane, methanol (Merck Millipore® - Germany); isooctane, glacial acetic acid, sulfuric acid, soluble starch, potassium iodate, sodium carbonate, potassium hydroxide, p-anisidine, sodium thiosulfate, potassium iodide, methyl tricosanoate (C23:0) 99,0% (by mass), boron trifluoride-methanol solution (BF₃) 14% (m/V) in methanol, fatty acids methyl ester Supelco® 37 Component FAME Mix standard (10 mg/mL in methylene chloride), docosahexaenoic acid methyl ester (10 mg/mL in heptane), eicosapentaenoic acid methyl ester (10 mg/mL in heptane) and mercury standard (1000 µg/mL Hg in 10% HNO₃) (Sigma-Aldrich® - USA).

3.2. Sample Acquisition and Transportation

The fish oil samples were acquired at the largest health commercial center in Brasília, Brazil, which contains twenty-one drugstores. All these drugstores were visited in order to purchase a variety of fish oil brands. The inclusion criteria were: products presented in soft-gel capsules form, being registered at ANVISA and not having expired. The exclusion criteria were: label not indicating the lipid content and/or presence of oils other than fish oil. The products were placed in a refrigerated container, free of light and moisture, transported to the Laboratory of Biochemistry and Nutrition at the University of Brasilia, Brazil and stored at -18°C in their original receptacles. The products were identified by sequential numbers in the order of purchase. The brand names were omitted.

3.3. Preparation of the Composite Sample

Ten capsules were individually weighed in an analytical balance and the oil was transferred to an amber glass bottle using a sterile and disposable syringe and named "composite sample". The amount of oil in each capsule was determined by subtracting the weight of the empty soft-gel capsule from the intact soft-gel capsule and the final weight was given by averaging the amounts found in ten capsules. The composite oil was stored in the amber glass bottle filled with an

inert gas (nitrogen) and sealed with a rubber bung, lid, parafilm® and wrapped in tinfoil to protect it from light. The sample was homogenized at 200 rpm for five minutes by an orbital shaker (Gehaka®) and stored at -18 °C until further analyses.

3.4. Fatty Acid Derivatization and Analysis

Firstly, 1 mL of C23:0 internal standard (IS) at a concentration of 1 mg/mL in isooctane was added to an aliquot of fish oil of roughly 20 mg. The IS was used for compensating possible analyte losses during sample preparation. The FAs were methylated with 14% (m/V) boron trifluoride (BF₃) in methanol [45]. The fatty acid methyl esters (FAME) samples were re-suspended in 1 mL of isooctane.

Each sample was prepared in duplicate and a triplicate of 1 µL from each methylated sample was injected in the gas chromatography, totalling six runs per sample. The analyses were carried out in a gas chromatography (GC model 17A – Shimadzu®) with flame ionization detector (FID) equipped with fused-silica capillary column (Supelco SP® 2560, 100 m x 0.25 mm x 0.2 mm), and hydrogen as the carrier gas. The detector and injector (split ratio of 1:50) were kept at 250 °C. The oven temperature program was: initial temperature of 125 °C for three minutes; temperature increase to 170 °C at 10 °C/min; 170 °C maintained for five minutes; temperature increase to 175 °C at 5 °C/min; 175 °C maintained for one minute; temperature increase to 185 °C at 2 °C/min; 185 °C maintained for one minute; temperature increase to 195 °C at 1 °C/min; 195 °C maintained for one minute; temperature increase to 240 °C at 5 °C/min; and once stable, the temperature was maintained at 240 °C for another eight minutes, totalling 48 minutes for each sample.

3.5. Fatty Acid Profile and Quantification of EPA and DHA

Retention times of FAME standards (Supelco 37 Component FAME Mix, Sigma-Aldrich® - USA) and relative retention times (20:5ω3 FAME as reference) were used to identify the individual FAs in the chromatograms. The results were expressed as the area percentage of each FA in

relation to the total area of the FAs in the chromatograms.

EPA and DHA were quantified by calibration curves of EPA (Sigma-Aldrich® - USA) and DHA (Sigma-Aldrich® - USA) as external standards (ES), using five known concentrations: 0.625 to 10 mg/mL, with addition of IS at 1 mg/mL concentration. EPA and DHA content adequacy were assessed according to ANVISA regulation, that is, EPA and DHA contents should be within $\pm 20\%$ of the amounts indicated on the label [46].

3.6. Determination of Mercury Content

Mercury in the samples was quantified by a portable mercury analyzer (Zeeman RA-915+, Lumex®) with PYRO-915 pyrolysis attachment. This method of direct detection of mercury vapor is based on vaporization of the sample with no pretreatment, by two-stage pyrolysis, that is, the sample is heated to 350 °C and then to 450 °C. An aliquot of oil of known mass (approximately 0.01 g) was taken from each sample. Mercury was quantified using a calibration curve with five known concentrations (5 – 20 mg/L) of the mercury standard dissolved in nitric acid. The limit of detection (LOD) and the limit of quantification (LOQ) of the device were calculated from the calibration curve data.

To calculate LOD and LOQ, a 0.25 µg/kg solution of mercury in nitric acid was used (the lowest point in the curve was diluted twenty times). The guidelines of the US COUNCIL FOR RESPONSIBLE NUTRITION, GOED, and HEALTH CANADA were used as reference for adequacy. Those guidelines suggest a maximum tolerable limit of <0.1 mg/L of mercury for fish oils [6-8].

3.7. Analysis of Sample Oxidation

Ten products were randomly selected (Random Number Generator® software, version 2.1.4) for this analysis. The peroxide value (PV) and anisidine value (AV) were determined and total oxidation (TOTOX) was calculated.

The AOCS Cd 8-53 method adapted [47] was used to measure the primary oxidation product, expressed as the peroxide value (PV) [48]. PV was determined by iodometric titration with a

sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) solution (0.001 mol/L). $\text{Na}_2\text{S}_2\text{O}_3$ was standardized with potassium iodate (KIO_3) by the AOAC #942.27 method [49]. The upper peroxide limit for fish oil used herein was 5 mEq of O_2 /kg of oil. For the anisidine value (AV) the AOCS Cd 18-90 method was used [50]. The upper anisidine limit for fish oil used herein was 20.

TOTOX corresponds to the general degree of oxidation of the oil, and it should be below 26 in fish oil. TOTOX is given by the formula:

$$\text{TOTOX} = 2 \cdot \text{PV} + \text{AV}, \quad (1)$$

where PV is the peroxide value and AV is the anisidine value.

The guidelines of the US COUNCIL FOR RESPONSIBLE NUTRITION, GOED, and HEALTH CANADA were used as reference for adequacy of PV, AV and TOTOX [6-8].

3.8. Statistical Analyses

Initially, the Shapiro-Wilk test was used to evaluate whether the variables had normal distribution. Accordingly, non-parametric Wilcoxon-Mann-Whitney test and the Spearman's rank correlation test were used. The software R® (version 3.3.0) was used for the statistical analyses and the significance level was set at 5% ($p < 0.05$).

4. Conclusions

In conclusion, EPA and DHA contents of more than 20% of fish oils in soft-gel capsules are not in compliance with either the national or international guidelines. The degree of oxidation in 20% of the samples exceeded the tolerable limits, suggestive of recent oxidation. Mercury was detected in almost 15% of the samples but at acceptable levels for human consumption. The FA contents found in the present study agrees with the recent legislation in Brazil which states that contents of nutritional supplements may differ by no more than $\pm 20\%$ from that indicated on the label.

Although the percentages of many study variables were compliant with the regulations, about 40% of the products had at least one noncompliant variable, suggesting the relevance

of constant monitoring of fish oil quality and characteristics, in light of current legislation and scientific advance.

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