Journal of Chromatography B 1231 (2023) 123922

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Human blood lipid profiles after dietary supplementation of different omega 3 ethyl esters formulations

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ARTICLE INFO

Keywords:
Lipidomics
Dried blood spot
Fatty acid ethyl esters
Tuna fish industry
Blue economy
Waste valorization

ABSTRACT

The validity of omega 3 fatty acids (ω3 FAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as dietary supplements has been widely proved. It's well known in fact, that they protect against cardiovascular diseases, reduce the levels of triacylglycerides (TAGs) and cholesteryl esters (CEs) in blood, and have anti-inflammatory activity. For these reasons, in the last few years the production of dietary supplement containing ω3 has increased significantly. In this context, the possibility to obtain ω3 and other high value molecules from alternative sources as fish waste, in accordance with the principles of circular economy, becomes an enormous attractive. In addition, the opportunity of creating new products, with greater health benefits, represents an interesting challenge. The current study was focused on the extraction of ω3 fatty acids and peptides from tuna waste industry, to realize a new dietary supplement. To this purpose, a supercritical fluid extraction (SFE) method was developed to separate, isolate, and enrich the different fractions subsequently used to produce an innovative formulate. The obtained supplement was characterized in terms of fatty acids esterified ester (FAEE) composition by gas chromatography (GC) coupled to both flame ionization detection (FID) and mass spectrometry (MS), and content of heavy metals by inductively coupled plasma–mass spectrometry (ICP-MS).

The effects of ω3 supplementation on metabolism and circulating lipid profiles was tested on 12 volunteers and assessed by GC-FID analysis of whole blood collected on paper support (Dried Blood Spot, DBS) at the beginning of the study and after thirty days. The results of plasma fatty acids levels after 30 days showed a significant decrease in the ω6/ω3 ratio, as well as the saturated/polyunsaturated fatty acids (SFA/PUFA) ratio, compared to subjects who took the ω3 ethyl esters unformulated. The novel formulated supplements proved to be extremely interesting and promising products, due to a significant increase in bioavailability, that makes it highly competitive in the current panorama of the nutraceutical industry.

1. Introduction

Several studies in the past have showed how the consumption of omega 3 polyunsaturated fatty acids (ω3 PUFAs), mainly eicosapentaenoic acid (EPA, C20:5 ω3) and docosahexaenoic acid (DHA, C22:6 ω3), protects against cardiovascular diseases [1,2]. However, recent

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https://doi.org/10.1016/j.jchromb.2023.123922
Received 11 August 2023; Received in revised form 17 October 2023; Accepted 3 November 2023
Available online 4 November 2023

investigations revealed that ω3 dietary supplementation did not reduce the risk of cancer or heart strokes [3–5]. Nevertheless, the uptake of ω3 PUFAs is still highly recommended, given their beneficial effects in brain development [6], their anti-inflammatory activity [7,8], and their ability to reduce the levels of triacylglycerides (TAGs) and cholesteryl esters (CEs) in blood [9]. Moreover, in the last years, the public awareness towards a healthier lifestyle considerably increased, resulting in a greater usage of food supplements as source of vitamins, minerals, and other beneficial molecules. For these reasons, the market size of ω3 dietary supplements, containing mainly EPA and DHA, has hugely increased, with a value of 5.58 billion USD in 2020, and an expected compound annual growth rate (CAGR) of 8.6 % until 2028 [10]. Fish oil is the main source of ω3 PUFAs for supplement manufacture, accounting for more than 60 % of the global production in 2020, but it is also heavily employed as an economically viable EPA and DHA source in marine fish aquaculture industry [11]. After the COVID-19 pandemic, the scenario for fish oil production remains uncertain, since the increasing regulations on fishing activities and the high demand, mainly from the booming Chinese aquaculture industry, results in very high prices, with an increase of USD 700 per tonne from 2021 [12]. For these reasons, there is an urgent need of new sources of ω3 PUFAs for human consumption. In this context, the re-utilization of by-products derived from fishery industries as starting material to obtain fish oil and other products of economic interest is gaining more and more attention. For instance, the processing of raw tuna fish into edible goods can produce an amount of wastes as high as 70 % of the starting material [13], from which is possible to retrieve several high-value molecules, such as ω3 PUFAs, bioactive peptides, essential amino acids (AAs) and metals [14–17].

In this work, by-products from a tuna processing industry were used to extract fish oil, which was later *trans*-esterified using KOH as alkaline catalyst and food grade ethanol to produce fatty acid ethyl esters (FAEEs). The ω3 FAEEs were subsequently enriched by using supercritical fluid extraction (SFE) and formulated using peptides also derived from the same tuna wastes. Peptide-based delivery systems have been extensively pursued for biomedical applications, due to several advantages compared to synthetic systems [18]. Such benefits include better biocompatibility and bioavailability, lack of toxicity, and different biochemical and biophysical properties related to peptides [19]. The obtained supplement was characterized in terms of FAEE composition by gas chromatography (GC) coupled to both flame ionization detection (FID) and mass spectrometry (MS), and content of heavy metals, whose maximum limits in food supplements are reported in Commission Regulation (EC) 629/2008 [20], by inductively coupled plasma–mass spectrometry (ICP-MS). Then, it was administered to a cohort of 12 volunteers, and the effect on the circulating lipid profile was assessed by GC-FID analysis of whole blood collected on a dried blood spot (DBS) collection support. Blood was collected at the beginning of the study and after thirty (30) days, since several studies pointed out how the dietary intake induces important changes in the membrane composition of cells after at least 4 weeks from the intake [21,22]. The same approach was used on a control group, to which the unformulated ω3 enriched FAEEs were administered. In order to enhance sample throughput and operator safety, an automatic workstation for lipid derivatization and on-line injection into a GC-FID system was exploited [23]. The obtained lipidomic profiles were compared in terms of specific fatty acid families ratios such as ω6/ω3, saturated/monounsaturated fatty acids (SFAs/MUFAs), and saturated/polyunsaturated fatty acids (SFAs/PUFAs), and statistically significant differences were highlighted by using a two tailed *t*-test.

2. Experimental

2.1. Reagents and materials

All solvents, reagents, and standard materials, when not otherwise specified, were purchased from Merck KGaA (Darmstadt, Germany). Sodium methoxide (CH_3ONa) solution (0.5 M in methanol) and boron trifluoride (BF_3) in methanol solution (14 % *v/v*) were used as derivatizing agents. *n*-Heptane (for HPLC, ≥ 99 %) and saturated sodium chloride (NaCl) aqueous solution were used for the extraction of fatty acid methyl esters (FAMEs) from DBS samples, and FAEEs from the formulated and unformulated ω3 enriched samples. Suprapur nitric acid (HNO_3 > 69 % (*v/v*)) was used for mineralization of the ω3 supplement prior of the analysis of metals. Ultrapure water (18 MΩ cm^{-1}), generated through the Milli-Q Advantage A10 system (Merck KGaA), was employed for sample and standard dilution prior of metal analysis. Standard stock solutions containing 1000 mg/L cadmium (Cd), mercury (Hg) and lead (Pb), and internal standard solutions containing 1000 mg/L of scandium (Sc) and rhodium (Rh), were used for the building of calibration curves. A multi-element solution, composed by Be (beryllium) 1000 μg L^{-1}, Co (cobalt) 500 μg L^{-1}, Mn (manganese) 500 μg L^{-1}, Bi (bismuth) 200 μg L^{-1}, In (indium) 200 μg L^{-1}, and Ce (cerium) 200 μg L^{-1} in HNO_3 1 %, was provided by CPA Chem (Stara Zagora, Bulgaria) and used for daily instrument calibration.

A C4-C24 even carbon saturated FAMEs (1000 μg mL^{-1}) standard mixture in hexane was utilized for linear retention index (LRI) calculation. 903 Whatman Protein saver cards (Merck Life Science) were used as sampling support for DBS.

2.2. Extraction of tuna fish oil and transesterification procedure

Fish processing waste were provided from an Italian fish company, and frozen to maintain its proprieties during shipping and storage. Raw fish waste was thawed at room temperature, cut in small pieces of 5–10 cm and homogenized in a mixer. This fish paste was heated in an oven at 90–95 °C for 30 min, to remove excess of humidity, and then transferred to a glass bottle and autoclaved at 121 °C for 20 min (heating/sterilization step). The wastes were then pressed by using a dewatering screw press. After removal of solids, liquid phase was centrifuged for 60 min at 4000 rpm at room temperature (25 °C), and the upper oily phase was recovered (Figure S1).

Transesterification was performed using an alkaline catalyst (KOH) at 25 °C for 30 min. The choice to use a catalyst concentration of 1 g_{KOH}/100 mL_{oil} and molar ratio ethanol:oil of 15 arose from literature observation [24]. Lower amounts of catalysts promote the formation of free fatty acids while higher quantities, although they bring the reaction to completion in a few minutes, transform the reaction mixture into a gel [25].

2.3. Enrichment of ω3 FAEEs by SFE

ω3 FAEEs within transesterified fish oil were enriched by using supercritical carbon dioxide ($scCO_2$) extraction process. A closed-cycle SFE process was used for this purpose. Preliminary tests were made with a lab-scale plant to examine optimal conditions, before to use a semi-industrial pilot plant to produce an appropriate amount of ω3 enriched oil (Figure S2). The optimized conditions for the ω3 FAEEs enrichment were: oil flow rate 0.09 kg h^{-1}, $scCO_2$ flow rate 9 kg h^{-1}, flow back 0.4 kg h^{-1}, column temperature 60 °C, column pressure 145 bar, separator temperature 35 °C and separator pressure 42 bar [26].

2.4. Peptides production

Peptides were obtained by enzymatic hydrolysis as previously described with a slight modification [15]. Briefly, 20 g of sterilized fish processing waste were homogenized in a mixer using saline phosphate buffer (PBS, 1:10 *w/v*) acidified at pH 6.0 with HCl 1 N. Solubilized proteins were separated by centrifugation at 13000 rpm for 15 min at RT (Ohaus, Switzerland). The pH of the supernatant was adjusted to 8.0 (with 1 N NaOH) and then 12 U mg^{-1} of protease from *Bacillus licheniformis* (Merck KGaA) were added. Enzymatic digestion was carried out

for 2 h at 37 °C in water bath and then stopped by heating the solution for 10 min in a boiling water. Peptides were recovered after centrifugation at 13000 rpm for 15 min, filtered through a nylon syringe filter (0.22 μm pore diameter, VWR International, Italy), and stored at 4 °C until use.

2.5. Formulation of ω3 supplement

The Formulated ω3-ethyl esters (F) were obtained emulsifying amphiphilic peptides (1 %) derived from digested tuna proteins with purified ω3 ethyl esters (Not Formulated; NF).

F and NF ω3 ethyl esters (1 g) were enclosed in a soft gelatin shell, following Good Manufacturing Practice (GMP). All the components of the capsules included the excipients as well as the gelatin shell met the current European food regulations. Both Formulated (F) and Not Formulated (NF) capsules were identical regarding color, size, odor, and secondary packaging to ensure double-blind conditions.

2.6. Analysis of the fish oils and ω3 supplement

The extraction of FAEEs from fish oils (neat and after ω3 enrichment) and from the formulated ω3 supplement, was performed by liquid–liquid extraction. In detail, the extraction was carried out by adding 0.5 mL of *n*-heptane solvent to the same volume of sample (1:1 *v/v*). The mixture was homogenized by using a vortex mixer (10 min) and centrifuged at 5.000 rcf for 10 min at 25 °C. The upper organic phase, containing FAEEs, was collected and transferred into 2 mL glass-vial fitted with 200 μL-insert for vial. The analysis of the FAEEs profile was performed on a GC-2030 NEXIS (Shimadzu, Duisburg, Germany) gas chromatograph coupled to a single quadrupole mass spectrometer (QP2020 NX, Shimadzu). The injection was carried out in automatic manner by using an AOC-20i autosampler and a split/splitless injector kept at 280 °C. The separation of FAEEs was performed on an SLB-IL60 capillary column, 30 m x 0.25 mm ID x 0.20 μm d_f (Merck Life Science). The temperature program was as reported in previous works [27,28]: 50 °C to 280 °C at 3.0 °C min^{-1}. Helium was used as carrier gas at a constant linear velocity of 30 cm s^{-1} (initial inlet pressure of 26.6 kPa). The volume injection was 1.0 μL with a split ratio of 1:10. The MS was operated in scan mode with the following parameters: acquisition range: 40 – 650 *m/z*; interface temp: 250 °C; ion source temp: 220 °C. The GCMSsolution software (version 4.50 Shimadzu) was used for both data acquisition and processing. The identification of FAEE compounds was carried out by using a dedicated custom database, applying two different identification criteria: MS spectra similarity (≥85 %) and linear retention index (LRI) tolerance window (±5). A homologous series of C4-C24 saturated FAMEs was used for the determination of the experimental LRIs.

The quantification of FAEEs was achieved by injection of the extracted ethyl esters into a GC-2030 NEXIS gas chromatograph (Shimadzu) equipped with a FID detector and an AOC-20i auto-sampler. Capillary GC column, temperature program, carrier gas, volume injection, split ratio and injector temperature were the same as previously described for GC–MS investigations, except for the initial inlet pressure of 99.4 kPa (the average linear velocity was 30 cm s^{-1}). The temperature of FID was set at 280 °C (sampling rate: 40 ms). FID detector gas flows were as follows: 40 mL min^{-1} for hydrogen (H_2), 10 mL min^{-1} for the make-up gas (nitrogen, N_2) and 400 mL min^{-1} for air. Data were collected and processed using the LabSolution software (version 5.92, Shimadzu).

The ω3 supplement was analyzed by using an ICPMS-2030 instrument (Shimadzu), after mineralization, for the monitoring of metal content. The validated procedure was previously reported by Donnarumma et al. [17] for the analysis of tuna waste products. Briefly, an amount of 0.2 g of sample was placed in a Teflon digestion vessel with 10 mL of Suprapur concentrated HNO_3 to undergo mineralization through the MARS 6 One Touch Technology Microwave lab station (CEM Microwave Technology Ltd., NC, USA). Microwave program was as follows: 25–200 °C in 25 min at 1800 W (hold for 15 min). After cooling, digests were diluted with deionized water in a 50 mL volumetric flask and subjected to ICP-MS analysis using the following conditions: Rf power 1.20 Kw, plasma gas flow rate 8.0 L min^{-1}, carrier gas flow rate 0.70 L min^{-1}, auxiliary gas flow rate 1.10 L min^{-1}, chamber temperature 5 °C, and sampling depth 5.0 mm.

2.7. Bioavailability study

A double-blind controlled study was conducted on 24 volunteer subjects, randomly divided in a 1:1 ratio using the research randomizer (https://www.randomizer.org), into the ω3 ethyl esters F group (FG, n = 12) or the ω3 ethyl esters NF group (NFG, n = 12). All procedures for research participants were performed in accordance with the European Commission's guidance notes [29] on ethical review and food related research, including informed consent and ensuring data protection and privacy.

The inclusion criteria applied in the subject's selection included triglyceride levels = 150–200 mg dL^{-1}; total cholesterol 190–220 mg dL^{-1}; LDL = 120–160 mg dL^{-1}; HDL ≤ 45 mg dL^{-1}; body mass index (BMI) ranging between 25.5 and 29.9 (=overweight). Exclusion criteria included pregnancy, systemic disease, diabetes mellitus, hepatic steatosis, hypertension, cardiovascular disease, sleep apnea syndrome, osteoarthritis, and fish intolerance/allergy; subjects already treated with statins; with ω3 or other hypolipidemic agents.

The study protocol also included the division of the sample according to: (i) age; (ii) gender; and (iii) metabolic set-up. As reported in Table 1, demographics and baseline characteristics were similar across the groups. Each research participant took for 1 month, 1 g/day of either ω3 ethyl esters F or ω3 ethyl esters NF.

Dried blood spot (DBS) method was performed at the beginning (time 0) and at the end (time 30 days) of F and NF administration. In particular, sterile blood lancets (Heinz Herenz Hamburg, Germany) were used to take a drop of blood from the fingertip, which was spotted, in triplicate, onto Whatman Protein saver cards. The DBS cards were allowed to dry for at least 2 h and then stored at room temperature in sealed plastic bags until analysis.

2.8. Lipidomic analysis of DBS

Dual-stage derivatization of lipids from DBS was carried out, as previously reported [23], in a fully automated way by using an AOC-6000 multifunctional autosampler preparative station equipped with an automatic tool exchange arm and two park stations containing a total of 6 syringes, a vortex mixer and an oven. The procedure was initiated by adding 500 μL of CH_3ONa methanol solution (0.5 % *w/v*) and the reaction mixture was heated for 15 min at 95 °C. After, 500 μL of BF_3

Table 1
Baseline demographics and subject characteristics. All the results are expressed as mean value.

Demographics	FG (*n* = 12)	NFG (*n* = 12)	*p* value
Age in years (SD)	55.0 (1.9)	56.2 (1.7)	0.42
Sex (man %)	48	52	0.5
Height in cm (SD)	175.6 (14.2)	172.1 (13.4)	0.55
Weight in kg (SD)	76.5 (12.1)	77.9 (10.3)	0.54
BMI in kg m^{-2} (SD)	26.7 (3.5)	26.6 (3.4)	0.39
Metabolic set-up			
Triglycerides in mg dL^{-1} (min and max value)	178.2 (152.8, 203.6)	175.9 (150.6, 201.2)	0.74
Total cholesterol in mg dL^{-1} (min and max value)	196.8 (186.9, 206.7)	194.4 (184.2, 204.6)	0.95
LDL in mg dL^{-1} (min and max value)	139 (120, 158)	138.6 (118.9, 158.3)	0.58
HDL in mg dL^{-1} (min and max value)	43.5 (42, 45)	43.3 (42.3, 44.3)	0.59

methanolic solution was added to reaction mixture and again heated for 15 min at 95 °C. Finally, 350 μL of *n*-heptane and 300 μL of NaCl aqueous solution were added to the reaction mixture; the sample was vortexed at 2000 rpm for 100 sec. After gravitational separation (standby time 2 min), the upper heptanic layer containing the FAMEs was injected into the GC-FID instrumentation operated in the same condition previously described. The identification of FAME compounds was carried out by using a commercial database, namely LIPIDS GC–MS Library (version 1.0, Shimadzu), applying the same criteria as previously reported.

2.9. Statistical analysis

Considering the intrinsic variability of *in vivo* data, possible outliers were identified by using the ROUT method [30], setting the false discovery rate (Q) at 1 %. No outliers were identified in the dataset. The efficacy of the supplement was evaluated by comparing the ω6/ω3, SFA/MUFA and SFA/PUFA ratios between the two groups (F and NF) at different timepoints (0 and 30 days after administration). Statistically significant differences were evaluated by using a two tailed *t* test (*P* values < 0.05 were considered statistically significant). The statistical

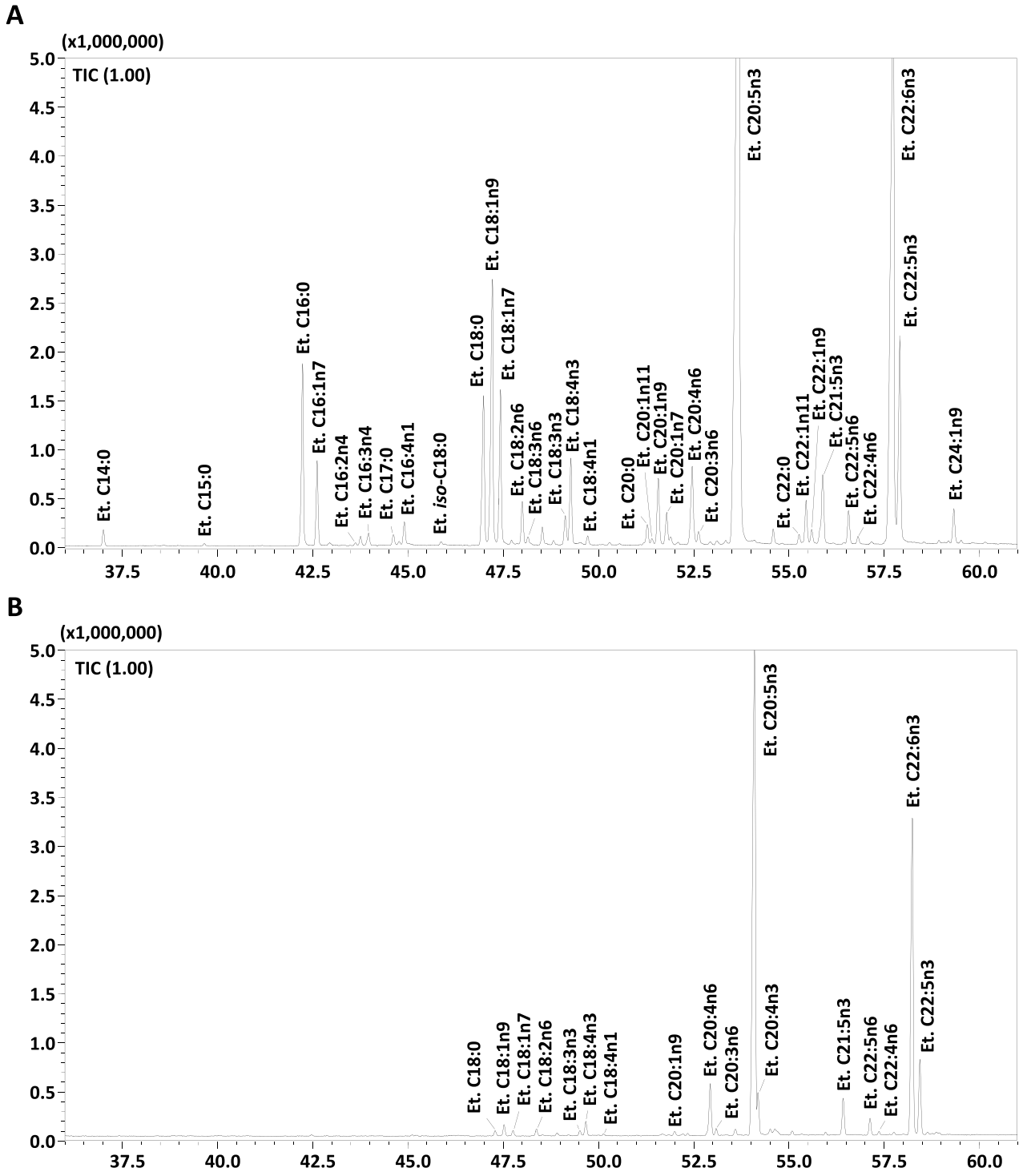

Fig. 1. FAEEs GC–MS chromatograms of transesterified oil (A) and ω3 enriched oil (B).

analysis was performed by using the Prism 9 software (GraphPad Software, Boston, MA, USA).

3. Results and discussion

3.1. Extraction and enrichment of ω3 from tuna industry by-products

Tuna processing industry generates three by-products: solid fresh fraction of innards separated during the preliminary cleaning process, solid cooked fraction mainly composed by head, fins, and skin, removed during the final cleaning process, and liquid fraction produced during the cooking step. The two solid fractions were used for tuna oil production using a wet rendering process. This process has a good efficiency with a high yield, avoiding the use of solvents [31]. This methodology includes three major steps: heating/sterilization, pressing and sedimentation. In the first step the wastes were pre-heated in order to remove the excess of water, and later autoclaved for 20 min. During this step, proteins underwent denaturation, thus liberating the oil from the tissues. Moreover, vacuum generated by the sterilization process ensures a low oxygen pressure and contributes, along with storage in the dark and at low temperatures, to minimize oxidative degradation processes. The wastes were pressed to extract the oil/water emulsion and remove any solid debris. The recovered liquid was centrifuged again to obtain two liquid phases: bottom aqueous phase containing water-soluble proteins, and upper oily phase rich in lipids. The lower phase was used to obtain peptides, while the fish oil was transesterified using food grade ethanol to generate FAEEs.

Transesterification is necessary to obtain high purity ω3 during the SFE process, since they are naturally present in fish oil in the form of TAGs, together with other fatty acids, and it is impossible to concentrate them efficiently in this form. If the extraction is preceded by a transesterification reaction, that replaces the skeleton of the triglyceride glycerol with alcohol molecules, the fatty acid chains are separated, and it is possible to obtain an enriched oil with an ω3 concentration greater than 80 % [32].

During the transesterification step, performed by using ethanol in alkaline condition at room temperature, the use of commercial ethanol at 96 % (*v/v*) caused the formation of a dense emulsion with a consequent difficulty to separate phases. Only ethanol anhydrous 100 % (*v/v*) was used for other tests, by changing temperature and reaction time variables to assess optimal conditions. The obtained transesterified oil was characterized using both GC–MS and GC-FID. FAEEs separation is showed in the chromatogram of Fig. 1A, while the relative abundances are reported in Table S1. These pretreatments allowed to obtain a fish oil with an ω3 average concentration of 68.35 %.

The SFE plant, represented in Figure S2, is characterized by an extraction packed column operating continuously with a countercurrent contact between oil from the top and $scCO_2$ from the bottom. The output from the column head is composed by the light fraction of the oil compounds, defined extract (C14-C16), dragged by the solvent. Most of the heavy fraction (C18-C22) does not solubilize in $scCO_2$ and remains within the column, cumulating in the bottom as raffinate. The extracted fraction expands in the back pressure valve and subsequently separates from gaseous solvent in the liquid–gas separator, where phase equilibrium is reached. Carbon dioxide from top is cooled and return liquid inside the hold-up tank for its recovery, in order to reduce the operating costs and render the whole process competitive with other enrichment procedures usually employed at industrial level. Part of extract fraction may be refluxed on the top of extraction column to improve ω3 enrichment. The obtained ω3 enriched oil was than characterized in terms of fatty acid content by GC–MS and GC-FID, and the results are shown in Fig. 1-B, while the relative quantification data are reported in Table S2. The total amount of ω3 FAEEs was higher than 90 %, with 56.22 % of EPA and 24.97 % of DHA. This ω3 enriched oil was used for the formulation of the food supplement.

3.2. Characterization of the ω3 supplement and bioavailability study

The ω3 enriched ethyl esters were formulated in presence of amphiphilic peptides (1 %) derived from the Tuna wastes, based on the numerous literature data reporting the ability of these peptides to act as activators of intestinal permeability [33–35]. GC-FID analysis of the final product revealed that the fatty acid composition remained unaltered compared to the ω3 enriched oil (Fig. 1B and Table S2) analyzed before formulation.

The same enriched oil without any other ingredient was used to fill colored pearls capsules and used as negative control in a double-blind study conducted on 24 volunteer subjects.

Prior to the administration to volunteers, the supplement was analyzed through ICP-MS for the monitoring of the 3 regulated heavy metals, namely Cd, Hg, and Pb. All of them were found below the legislation limits of 1.0, 0.1 and 3.0 mg Kg^{-1}, respectively. Specifically, Cd was not detected, then it was considered below the detection limit of 2.60 μg Kg^{-1}, previously assessed [17], Hg was detected at 0.08 ± 0.01 mg Kg^{-1}, and Pb was found near to the quantification limit of 0.05 mg Kg^{-1}.

Each research participant was invited to refrain from taking other types of ω3 based supplements and to compile a daily food diary indicating the foods eaten, the quantities and methods of preparation, with particular focus on the condiments used. In addition, the volunteers answered a weekly questionnaire about the occurrence of nine potential side, such as acid reflux, nausea, and abdominal pain. No side effects were reported.

The analysis of fatty acids from DBS samples was performed by using a fully automated and miniaturized approach as reported in Ferracane et al. [23]. The procedure involved the derivatization of lipids into FAME derivatives, and subsequential analytical separation by GC-FID analysis. The abundance of each FAME compounds was expressed as relative percentage (%). Also, fatty acid families (ω6, ω3, SFAs, MUFAs, and PUFAs), and specific fatty acid ratios were monitored for each analyzed DBS sample (Table S3). In Fig. 2 are shown the chromatograms obtained by the DBS analysis of the same patient administered with the ω3 ethyl esters F supplement at *t0* (Fig. 2A) and at *t30* (Fig. 2B). The sample collected from one subject of the Formulated ω3 ethyl esters (F) group (Subject 12 t30) was discarded due to the low quantity of blood present on the DBS card.

The main indicators selected to evaluate the effectiveness of the formulated ethyl esters were the ω6/ω3, SFA/MUFA and SFA/PUFA ratios, monitored at different timepoints (0 and 30 days after administration, *t0* and *t30* respectively). These indicators are commonly used to determine if the subject has the right balance between the various lipid species [36]. The ω6/ω3 ratio can indicate both ω-6 excess and/or a deficiency of ω3. The SFA/MUFA and SFA/PUFA ratios are indicative of the health state of the organism as well as of the fluidity of cell membranes. High values represent an increase and accumulation of SFAs, related to the metabolic alterations or simply to a diet rich of saturated fats, with consequent imbalance of the lipid metabolism. High amount of SFA are correlated to the increase of the cholesterol and to the potential appearance of some pathologies like diabetes, metabolic syndrome, cardiovascular disease, obesity, etc. [37,38]. No outliers were identified in the dataset by using the the ROUT method [30], while a two tailed *t* test was used to evaluate statistically significant differences (p values < 0.05). The results are shown in Fig. 3. As can be seen from the graphs, the population that received the ω3 ethyl esters formulate shows, after 30 days, a significant decrease in the ω6/ω3 ratio ($p = 0.0059$) as well as in the SFA/PUFA ratio ($p = 0.0003$), while the SFA/MUFA does not change ($p = 0.8165$), compared to the corresponding control (ω3 ethyl esters NF) at 30 days. In all cases the comparison between the two *t0* shows no difference. Furthermore, it is interesting to note that the SFA/MUFA ratio increases significantly between *t0* and *t30* both in the NF group ($p = 0.0359$) and in the F group ($p = 0.0423$), indicating that this ratio is not influenced by the assumption of the ω3 supplement object of

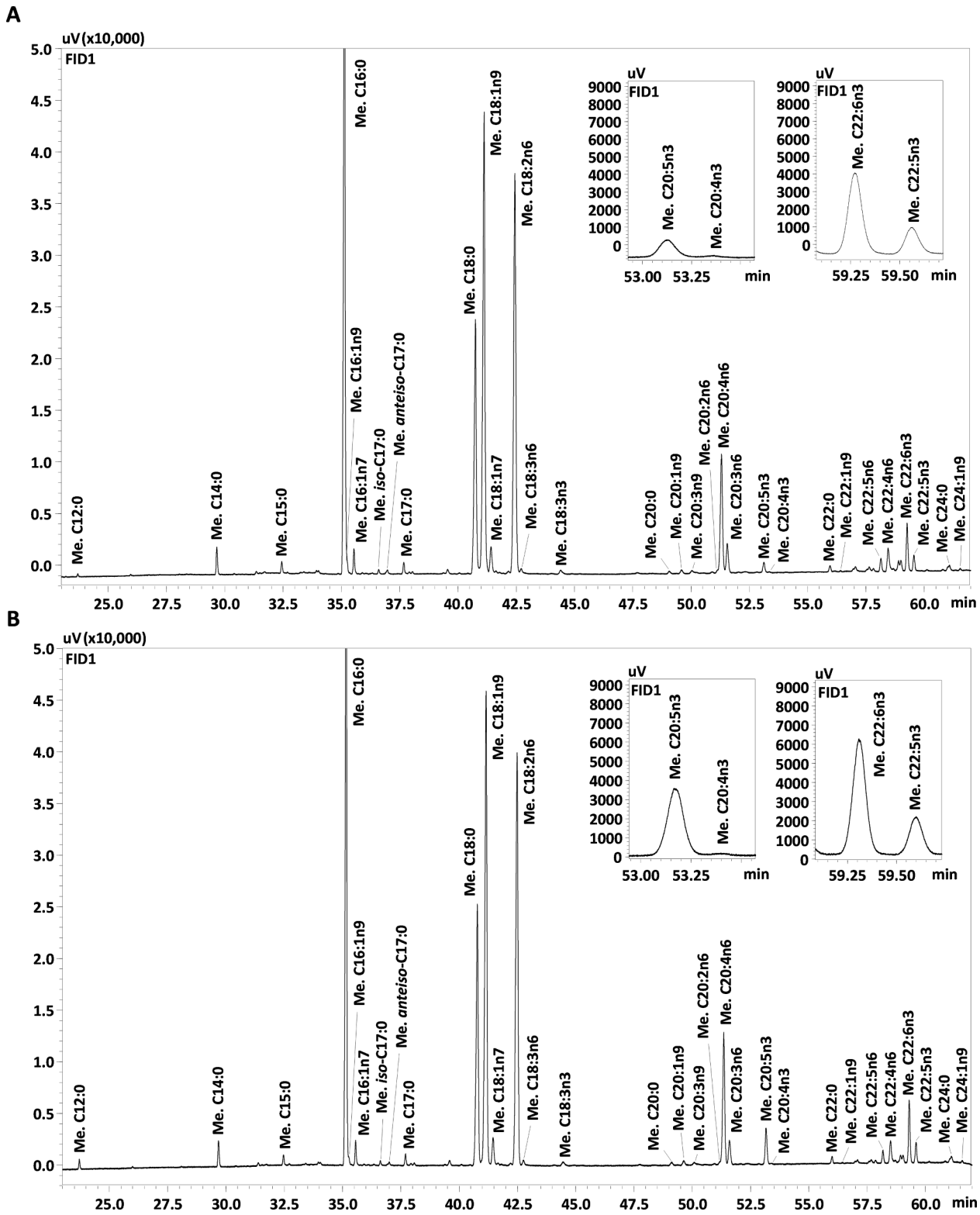

Fig. 2. Examples of FAMEs GC-FID chromatograms of DBS samples at t0 (A) and t30 (B) of ω3 ethyl esters F supplement (Subject 3 in the ω3 ethyl esters F group reported in Table S3). In the insets are reported a magnification of the EPA and DHA regions for both samples.

the study (containing almost exclusively PUFA), but only by the diet of the population under examination. In particular, the SFA/MUFA ratio increases in a very similar way between the two populations, probably due to a similar increase in SFA intake in their diet. Conversely, the SFA/PUFA ratio between *t0* and *t30* increases substantially in the NF ($p = 0.0070$), while it decreases significantly in the F group ($p = 0.0186$), indicating that the use of the supplement guaranteed a better supply of PUFA, in particular ω3, such as to counterbalance the increase in SFA due to the diet. These results reflect the high bioavailability of the ω3 ethyl esters present in the novel formulated supplement and the consequent ability to increase the nutritional intake of these fatty acids, contributing to an adequate balance in the modern western diet.

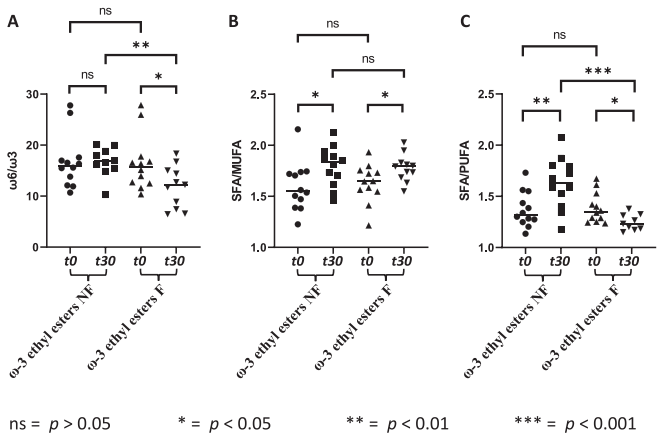

Fig. 3. Graphics reporting the differences between the Formulated ω3 ethyl esters (F) and Not Formulated ω3 ethyl esters (NF) groups in the ω6/ω3 ratio (A), the SFA/MUFA ratio (B) and the SFA/PUFA ratio (C), monitored at different timepoints (0 and 30 days after administration, *t0* and *t30* respectively). Black lines indicate the median among independent experiments. Statistical analyses were performed using a two tailed *t* test.

4. Conclusions

Tuna fish processing industry produces more than 70 % of waste and by-products (meat, scales, head, viscera, and roes) that are usually discarded or used to produce low added value products such as fish meals. However, these waste materials still contain molecules essentials for the human diet, such as, vitamins, ω3 fatty acids, and proteins of high nutritional value. The re-use of such wastes to extract high added value molecules would perfectly fit the need of new sources of ω3 PUFAs for human consumption, according to a circular economy model. In this work, the supplements formulated using the ω3 ethyl esters enriched by SFE, and peptides have proved to be extremely interesting and promising products. In fact, the study of tolerability and palatability of the product in healthy volunteers did not record any dropouts since no side effects were reported. Furthermore, analyses of plasma FAs levels on a cohort of 12 volunteers administered with the ω3 ethyl esters F for 30 days, showed a significant decrease in the ω6/ω3 ratio, as well as the SFA/PUFA ratio, compared to subjects who took the ω3 ethyl esters NF. The significant increase in bioavailability demonstrated by the formulated supplement, composed by components extracted from industry by-products and according to green chemistry processes, makes it highly competitive in the current panorama of the nutraceutical industry.

CRediT authorship contribution statement

Danilo Donnarumma: Conceptualization, Formal analysis, Writing – original draft. **Anna Di Salle:** Investigation, Writing – review & editing. **Giuseppe Micalizzi:** Formal analysis, Writing – review & editing. **Federica Vento:** Investigation, Visualization. **Roberta La Tella:** Investigation, Visualization. **Pasquale Iannotta:** Investigation, Writing – review & editing. **Emanuela Trovato:** Formal analysis, Writing – review & editing. **Mariarosa Anna Beatrice Melone:** Supervision, Visualization, Funding acquisition. **Francesca Rigano:** Conceptualization, Writing – review & editing. **Paola Donato:** Supervision, Writing – review & editing. **Luigi Mondello:** Supervision, Project administration, Funding acquisition. **Gianfranco Peluso:** Supervision, Visualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgements

Funding resources: Bando “HORIZON 2020” PON I&C 2014-2020, Project number F/050347/01-03/X32. The authors acknowledge Merck Life Science and Shimadzu Corporations for the continuous support.

Ethics and Food-Related Research

All procedures for research participants were carried out in accordance with the European Commission’s Guidance Notes (2012) on Ethical Review and Food Research, including informed consent and ensuring data protection and privacy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123922.

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