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ORIGINAL PAPER

One-Step Extraction and Concentration of Polyunsaturated Fatty Acids from Fish Liver

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Abstract The fatty acids (FA) eicosapentaenoic acid (20:5ω-3; EPA) and docosahexaenoic acid (22:6ω-3; DHA), which have several health benefits, have been concentrated from mako shark liver (*Isurus oxyrinchus*). The process was carried out in one single step, in which fish liver oil was simultaneously extracted, saponified and concentrated. Additionally, the polyunsaturated fatty acids (PUFA) concentrate was winterized to crystallize the remaining saturated FA, resulting in a further increase in the concentration of DHA and EPA. Two variables, temperature and water concentration in the saponification mixture, were optimized to increase the concentration of ω-3 PUFA. Best results were obtained at 12 °C and 0% water content in the mixture, obtaining 17.8% purity and 77.6% yield of EPA; DHA purity and yield were 33.3 and 82.2%, respectively.

Keywords Mako shark · *Isurus oxyrinchus* · Eicosapentaenoic acid · Docosahexaenoic acid · ω-3 PUFA concentration · Oil fractionation · Fish liver oil

Introduction

Health benefits of fish oils consumption are related to their high polyunsaturated fatty acid (PUFA) levels, especially ω-3 family. Eicosapentaenoic acid (20:5ω-3; EPA) and docosahexaenoic acid (22:6ω-3; DHA) are two ω-3 PUFA that have been investigated for their role in human health; they are abundant in fish (mackerel, salmon, mako shark, sardines...) and microalgae oils. Their absence in a normal diet may contribute to the development of a wide variety of diseases, such as coronary heart disease [1], stroke [2], hypertension [3], cardiac arrhythmias [4], diabetes [5], an inadequate development of nervous and reproductive systems [6], and several inflammatory disorders, such as Crohn's disease [7].

The methods used to concentrate PUFA from fish oil are commonly based on the differences in the polarity and/or spatial configuration of the FA. This way, PUFA can be separated according to their degree of unsaturation [8]. Some common procedures employed to obtain PUFA concentrates are enzymatic purification [9] and urea complexation [10]. Nevertheless, the urea complexation method should be avoided for human consumption because of the reported production of two potent carcinogens, ethyl- or methyl-carbamates [11]. Argentated silica gel chromatography is another common procedure to obtain higher purities of EPA and DHA [12, 13].

The sodium salts of the FA can be separated according to their solubility differences in ethanol [14]. The procedure is based on the solubility differences of FA sodium soaps in ethanol at ambient temperature, so that the higher the number of double bonds, and the shorter the chain length for the same degree of unsaturation, the higher the solubility of a given FA. By this method, PUFA concentrates containing approximately twice the amount of EPA and DHA than crude oil could be obtained from fish oil [15], and seed oil PUFA concentration has also been carried out in a

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single step (seed oil extraction/saponification/FA concentration) [16]. This article reports on the simultaneous extraction–concentration of EPA and DHA from mako shark liver biomass by solubility differences.

Experimental Procedures

Mako shark was acquired from local markets in Almería (Spain) in June 2004. Livers were lyophilized and stored at –70 °C until processed.

Simultaneous liver oil extraction and PUFA concentration was carried out in a 50 mL controlled temperature reactor (Afora, Barcelona). Four different proportions of H_2O in ethanol were essayed as extraction solvents (0, 0.5, 1 and 3%, v/v). In a typical experiment, 1 g of ground and lyophilized mako shark liver was treated with 7 mL of freshly prepared solvent containing 0.3 g of NaOH for simultaneous lipid extraction and saponification. This process was carried out at 82 °C for 2 h, with constant agitation under an argon atmosphere. Thereafter, the saponified solution was cooled at the different tested temperatures (0–20 °C) and then, a liquid and solid phase (LF and SF, respectively) were obtained.

The LF obtained was separated, while the remaining SF was filtered through sintered glass filters (POBE, 100 μm pore size) and thoroughly washed with absolute ethanol. The resulting filtrate was then added to the LF and this liquor was concentrated in a vacuum rotary evaporator at 35 °C. The upper layer, containing the unsaponifiable material, was separated after adding 2.5 mL of water and 1 mL hexane. The hydroalcoholic phase, containing the soaps, was acidified to pH 2 with HCl:H_2O (1:1, v/v), and the FA were extracted with hexane (8×20 mL). Then, the extract was washed with water until neutral pH was obtained. The hexane layer containing the PUFA concentrate was finally evaporated in a vacuum rotary evaporator at 35 °C.

The final winterization step was carried out by storing the PUFA concentrates at –70 °C for 20 h, as previously reported [17].

Index to Qualify the Purification Process

For a process oriented to recover a given FA from a biomass A (the mako shark liver) to a solution B (the liver extract), the process A–B can be defined by the following expression:

$$R_t = \frac{B}{A} \times 100,$$

where R_t is the recovery of the total FA in the process A–B; B is the total amount of FA in the extract, and A is the total amount of FA in the fish liver biomass.

Another expression that indicates the recovery of the process for an individual FA is:

$$R_i = \frac{X_B}{X_A} \times R_t,$$

where R_i is the recovery of a given FA in the process A–B; X_B is the percentage of the FA in the extract after processing, while X_A is the percentage of the FA in the initial the fish liver biomass.

FA analyses Methyl esters were prepared by treatment of the lipidic fraction with acetyl chloride and methanol [18]. The FA methyl esters (FAME) of the mixture were analyzed by gas chromatography and were identified by comparing their retention times with those for standards (''Rapeseed oil mix'' and ''PUFA-1'', from Sigma®), in a Hewlett-Packard HP5890 series II chromatograph provided with a flame ionization detector and an HP3394 integrator. A capillary column of high polarity fused silica was used (Supelco SP2330; length: 30 m; internal diameter: 0.25 mm; thickness of the film: 0.2 μm). The flow of carrier gas (N_2) was 0.75 L/min, and the split ratio of the injector was 100:1. The injector temperature was 240 °C and the detector temperature was 260 °C. The starting temperature of the oven was 205 °C and was increased at a rate of 6 °C/min until 240 °C was reached (5.83 min). The injection volume was 5 μL and a blank was run after every two analyses. Pentadecanoic acid (15:0) methyl ester was used as the internal standard for quantitative analyses.

Statistical analyses The software package Statgraphics for Windows ver. 4.1 (Manugistics, Rockville, MD, USA) was used to calculate the mean, the standard deviation (SD), and the variance. The significance level was $p < 0.05$. All assays were performed at least in triplicate, and the mean values for a representative sampling are shown in the tables and figures.

Results and Discussion

A new process to obtain PUFA concentrates has been optimized in this work. The basis of the concentration process employed here is solubility differences among FA dependent on the degree of unsaturation. When a solution of saponified FA is cooled, saturated FA (SAT) and monounsaturated FA (MON) salts crystallize before PUFA salts. Our modification of the

prior technique [15] consisted of starting the saponification directly from the fish liver biomass; this way extraction, saponification and PUFA concentration could be accomplished in one single step. Temperature and water content in the saponification mixture has been optimized, as the efficiency of this method depends on solubility differences of the FA salts in water [15, 16].

Previously, an adequate amount of the liver biomass had been directly methylated to determine its FA profile [18]. Mako shark liver contained 6.5% EPA and 13.2% DHA of total FA. This profile was used as a standard to determine differences in PUFA percentages among the various concentrates obtained by the procedure employed. For a global view of the results, FA were grouped as SAT (14:0, 16:0, 18:0), MON (16:1ω-7, 18:1ω-9, 18:1ω-7, 20:1ω-9), EPA and DHA.

Table 1 shows the FA purities and yields of the saponified solutions cooled at different temperatures (0–20 °C), with no added water. For all assayed temperatures, SAT decreased significantly ($p < 0.05$) with respect to crude oil; MON purities did not change significantly, while EPA and DHA purities increased significantly at all temperatures tested ($p < 0.05$). Higher PUFA concentrations were obtained at +12 °C, with EPA and DHA purities rising up to 17.8 and 33.3% (yielding 77.6 and 82.2% of EPA and DHA, respectively). Above this temperature, concentration effectiveness decreased. Nevertheless, an analysis of the variance (ANOVA) showed that the selected temperature had a lack of statistical significance in the final PUFA purities ($p > 0.05$). On the other hand, the cooling temperatures did have a significant effect on FA yields (F-ratio = 3.02; $p < 0.0124$), ranging from 63.5 to 82.7% of total EPA, and from 58.6 to 83.6% of total DHA, considering all temperatures assayed.

The influence of water content in the saponification mixture was also evaluated, four different water proportions (0, 0.5, 1 and 3%, v/v) were assayed in the saponification mixture, at +12 °C cooling temperature (Fig. 1). After the complete process, a significant increase ($p < 0.05$) in EPA and DHA purities in the PUFA concentrate was achieved with respect to crude oil for all water contents evaluated with the highest PUFA level reached with no added water (0%). The unextractable water in the liver after lyophilization might be enough to complete the saponification, so the addition of water seems to be unnecessary.

Figure 2 shows the final winterization step applied to further increase EPA and DHA purities by storing the PUFA concentrates at –70 °C. The use of winterization to concentrate PUFA from seed oils has been recently optimized in our laboratory [18]. According to our results, hexane seemed to be the most efficient organic solvent to increase PUFA purities by winterization. Taking into account that after the process the PUFA were finally extracted from the LF in this solvent, no additional dilution operation was needed prior to winterization. However an adjustment of the hexane:PUFA relation is recommended [18].

After the winterization process, an increase in the EPA and DHA purities was obtained, with EPA purity rising to 22.1% for a 94% yield, and DHA to 35% purity for a 100% yield.

Table 1 Temperature influence on fish liver FA extraction–concentration process, with 0% water added to the saponification mixture

FA % in liver oil	Purity of fatty acids[1,2]					Yield of fatty acids[2,3] (R_i)				Total yield (R_t)
	SAT	MON	EPA	DHA		SAT	MON	EPA	DHA	
	24.5 ± 2.3	25.6 ± 2.4	6.5 ± 0.2	13.2 ± 1.1						
Processing T[a4]										
0	16.1 ± 0.9	25.8 ± 1.2	14.5 ± 1.1	27.2 ± 1.7	0	18.7 ± 1.4	28.6 ± 2.3	63.5 ± 5.3	58.6 ± 4.3	28.4 ± 1.9
2	11.6 ± 0.8	26.2 ± 1.9	14.6 ± 1.2	26.6 ± 1.8	2	14.2 ± 1.0	30.7 ± 2.5	67.4 ± 6.0	69.3 ± 5.7	30.0 ± 2.4
4	12.9 ± 1.1	27.6 ± 1.8	14.8 ± 9.8	25.4 ± 2.0	4 [a, b]	15.1 ± 1.1	30.9 ± 2.5	65.3 ± 5.1	63.1 ± 5.4	28.6 ± 1.9
6	11.3 ± 0.9	22.9 ± 1.7	16.1 ± 1.3	30.3 ± 2.7	6 [a, c]	14.5 ± 1.2	28.2 ± 2.3	77.8 ± 6.4	83.1 ± 7.2	31.5 ± 2.8
8	10.5 ± 0.8	21.5 ± 2.0	16.2 ± 1.3	32.6 ± 3.1	8 [d, e]	11.9 ± 1.0	23.3 ± 2.0	69.1 ± 5.3	78.4 ± 7.8	27.7 ± 2.0
10	11.2 ± 1.0	20.8 ± 1.8	16.8 ± 1.3	32.9 ± 2.8	10 [a, f]	13.4 ± 1.3	23.8 ± 1.8	75.5 ± 6.0	83.8 ± 7.2	29.3 ± 1.9
12	9.1 ± 0.6	20.1 ± 1.8	17.8 ± 1.5	33.3 ± 2.5	12 [c, f, g, h]	10.5 ± 0.8	22.3 ± 1.7	77.6 ± 6.7	82.2 ± 6.7	28.4 ± 2.8
15	10.5 ± 0.7	20.3 ± 2.0	17.1 ± 1.4	30.7 ± 2.6	15 [c, g]	13.8 ± 0.9	25.6 ± 2.1	85.0 ± 7.2	86.1 ± 6.0	32.3 ± 2.1
18	9.5 ± 0.7	21.9 ± 1.8	16.5 ± 1.7	31.1 ± 2.8	18	11.8 ± 1.1	26.1 ± 2.2	77.3 ± 6.3	82.5 ± 5.9	30.5 ± 2.6
20	9.8 ± 0.6	23.1 ± 1.8	15.9 ± 1.3	28.5 ± 2.1	20 [b, e, h]	13.5 ± 1.1	30.5 ± 2.8	82.7 ± 6.9	83.6 ± 8.1	33.8 ± 2.7

[1] Percentage of each FA on total FA area, detected by GLC

[2] SAT = 14:0, 16:0 and 18:0; MON = 16:1ω-7, 18:1ω-9, 18:1ω-7, 20:1ω-9 and 20:1ω-11; Other FA accounting for 100%: 16:2ω-4, 18:2ω-6, 18:3ω-3, 18:3ω-6, 18:4ω-3, 20:2ω-6, 20:3ω-6, 20:4ω-3, 22:3ω-3, 22:4ω-6

[3] Yields of the FA after processing with respect to the original amount of FA in the fish liver biomass (25 g/100 total FA on dry matter)

[4] Temperatures of processing followed by the same letter are different ($p < 0.05$) according to the Duncan's Multiple Range Test

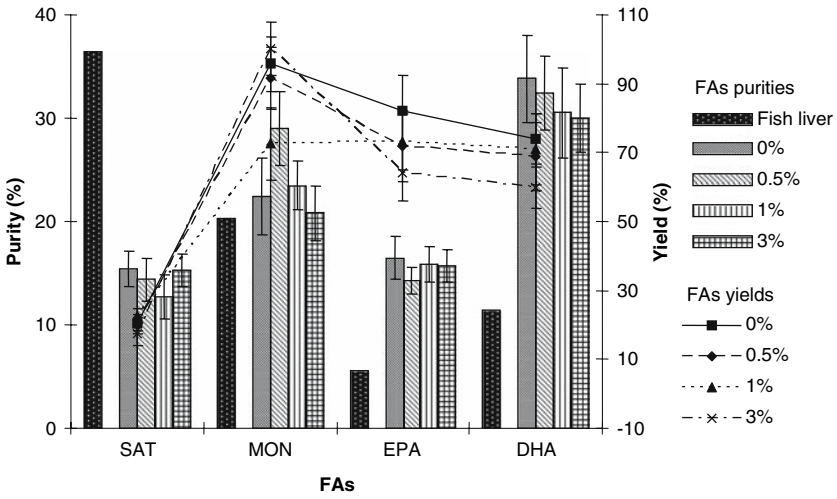

Fig. 1 Influence of different proportions of ethanol:water in the saponification mixture on the fish liver PUFA extraction–concentration process. Cooling temperature after saponification: +12 °C

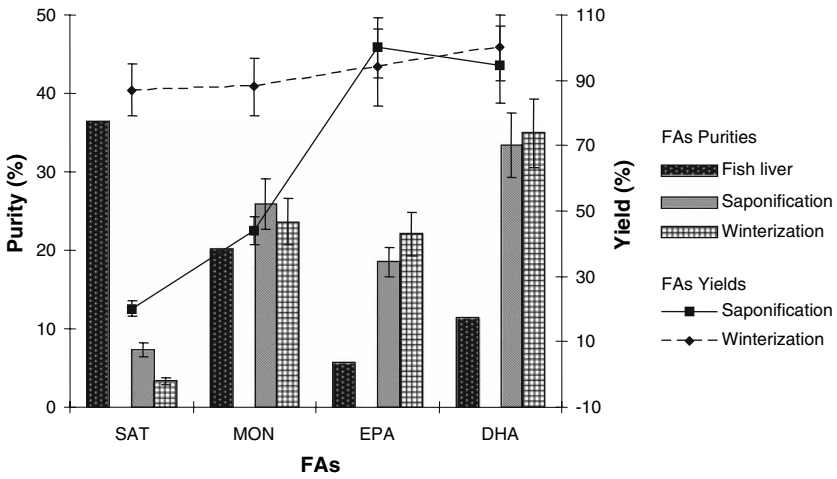

Fig. 2 FA purities and yields obtained in the simultaneous oil extraction/saponification/PUFA concentration process followed by winterization at –70 °C from fish liver oil. Cooling temperature after saponification: 12 °C. Water in the saponification mixture: 0%. Winterization temperature: –70 °C

Another factor affecting the process was the type of hydroxide used in the saponification. Besides sodium, the use of other hydroxides such as lithium, magnesium and potassium was assayed (data not shown), but only sodium hydroxide gave reasonable results.

In conclusion, the process described here is a rapid and versatile technique, easily adaptable through slight adjustments for the concentration of PUFA from different fish oils with diverse FA profiles. In addition, the method has been successfully scaled up to semi-preparative level by a factor of 300 increasing the amounts of oil and reagents, obtaining very similar EPA and DHA concentration efficiencies to those obtained at analytical level (data not shown). These results suggest that it is possible to accomplish high-scale EPA and DHA production by this procedure.

All PUFA purities obtained in this work exceed PUFA purities obtained from fish oil in previous works by the solubility differences procedure [15], and are very close to results obtained by the urea inclusion method [19]. The method tested can be considered an alternative to the urea inclusion method for obtaining PUFA concentrates for the food industry, taking into account not only the comparable efficiencies in PUFA concentration by both methods, but the additional advantage of using a safer and more bio-compatible method. Results from this study suggest that concentrates can be commercialized to produce dietary supplements that can be used to help balance the ω-3/ω-6 PUFA in the diet, or as raw material for the food or pharmaceutical industry to further concentrate EPA and/or DHA at higher purities.

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