Purification Process for Cod Liver Oil Polyunsaturated Fatty Acids

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ABSTRACT: The polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which have several pharmaceutical properties, have been purified from cod liver oil. The process consisted of four main steps: (i) saponification of the oil, (ii) use of urea inclusion adducts method to obtain PUFA, (iii) PUFA methylation, and (iv) argentation silica gel column chromatography of the methylated PUFA. Argentation silica gel chromatography yielded highly pure DHA in the process (100% purity, 64% yield). For EPA, the recovery in the combined process was 29.6%, and the final purity was 90.6%, owing to the simultaneous elution of other polyunsaturated fatty esters. The recovery in the urea inclusion method was strongly enhanced by application of orbital agitation during the crystallization process, in which EPA yield increased from 60–70% without agitation to 90–97% at 800 rpm; stearidonic acid (18:4n-3) yield ranged from 60–75% without agitation to 87–95% at 800 rpm, and DHA yield varied from 53–73% without agitation to 85–99% at 800 rpm.

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KEY WORDS: Argentation silica gel column chromatography, cod liver oil, docosahexanoenoic acid, eicosapentaenoic acid, polyunsaturated fatty acids, urea inclusion adducts.

Cod liver oil (CLO) is an inexpensive source of several polyunsaturated fatty acid (PUFA) types, thus it is widely used for isolation and purification of PUFA. The main fatty acids it contains are palmitic acid (PA, 16:0), palmitoleic acid (POA, 16:1n-7), oleic acid (OA, 18:1n-9), stearidonic acid (SA, 18:4n-3), gadoleic acid (20:1n-9), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3). Microalgae are another source of PUFA; they have a simpler fatty acid profile, and different species show characteristic fatty acid types. Because the microalgal biomass constitutes the main food for fish, the complexity of the fatty acid profile of CLO may be attributed to this fact.

The n-3 PUFA α-linolenic acid (LA, 18:3n-3), SA, EPA, and DHA, are the most interesting from the standpoint of human nutrition. Among these, LA and SA are present in seed plants, the others being obtained from marine sources. Therefore, the traditional sources for EPA and DHA are fish and oils from microalgae. EPA and DHA are attracting increasing attention because of their importance to human health. EPA has several beneficial effects regarding coronary heart diseases (1), hypertriglyceridemia (2), blood platelet aggregation and lowered blood cholesterol, thus reducing the risk of arteriosclerosis (1,3), inflammation (4) and several carcinomas (5). The structural fatty acid DHA prevails in the grey matter of the brain and retinal tissues and is also an essential component of human milk, contributing to the normal development of the brain and of the nervous system (6).

Currently, purification of n-3 PUFA from fish and microalgal oils is usually accomplished through a three-stage process: oil saponification, PUFA concentration, and PUFA fractions separation by any appropriate method, such as by high-performance liquid chromatography (HPLC) (7).

Urea concentration seems to be the most appropriate method for n-3 PUFA enrichment (8): it allows the handling of large quantities of material in simple equipment; inexpensive solvents such as methanol can be used; milder conditions are required (e.g., room temperature); the separation is more efficient than with other methods such as fractional crystallization or selective solvent extraction; and its cost is lower. The urea method is a versatile process, because fractionation characteristics can be altered simply by changing the amounts of either solvent or urea (7,9–12). This procedure was established by Bengen in 1940 (13,14) for the separation of straight-chain compounds from branched or cyclic compounds.

The tendency of saturated and monounsaturated fatty acids to form inclusion compounds with urea increases with decreasing temperature; the optimal temperature depends on the particular PUFA. The PUFA recovery is maximal at about 4°C. This temperature is particularly suitable for concentrating SA and DHA. The higher the temperature, the lower the tendency of fatty acids to form urea adducts, and therefore the PUFA yields are lower. PUFA also form inclusion compounds at lower temperatures; however, when EPA is the desired fatty acid, crystallization at around 20–28°C is preferable because concentrations and yields in the mother liquor are higher (7,11,12). Although this method has been carefully evaluated, some variables affecting the crystallization process, such as flowing–dynamic conditions like orbital agitation, have still to be tested.

Another procedure for fatty acid methyl ester separation, which is based on number, position, and geometric configuration of double bonds, employs a silver nitrate-impregnated silica column (15). Özcimder and Hammers (16) separated fatty acid methyl esters from CLO by using 5% Ag^+ as stationary phase in HPLC; hexane containing 0.4% acetonitrile

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was the mobile phase. In the resulting chromatogram, all of the esters with the same number of carbon atoms overlapped. The authors concluded that the method was suitable for separating PUFA, but not for separating saturated, mono- or diunsaturated fatty acid esters.

Belarbi *et al.* (17) used a chromatographic technique for recovering EPA esters from microalgae. The process consisted of a one-step extraction–transesterification of fatty acids in a microalgal biomass followed by fractionation on a silver-silica gel chromatography column. The one-step extraction–transesterification was made with biomass of *Phaeodactylum tricornutum* and *Monodus subterraneus*. EPA ester of up to 96% purity was recovered in yields exceeding 70%. The process was also applied to recover EPA ester from fish oil. Depending on the source—CLO, *P. tricornutum*, or *M. subterraneus*—the purity of the EPA was 83, >96, or >91%.

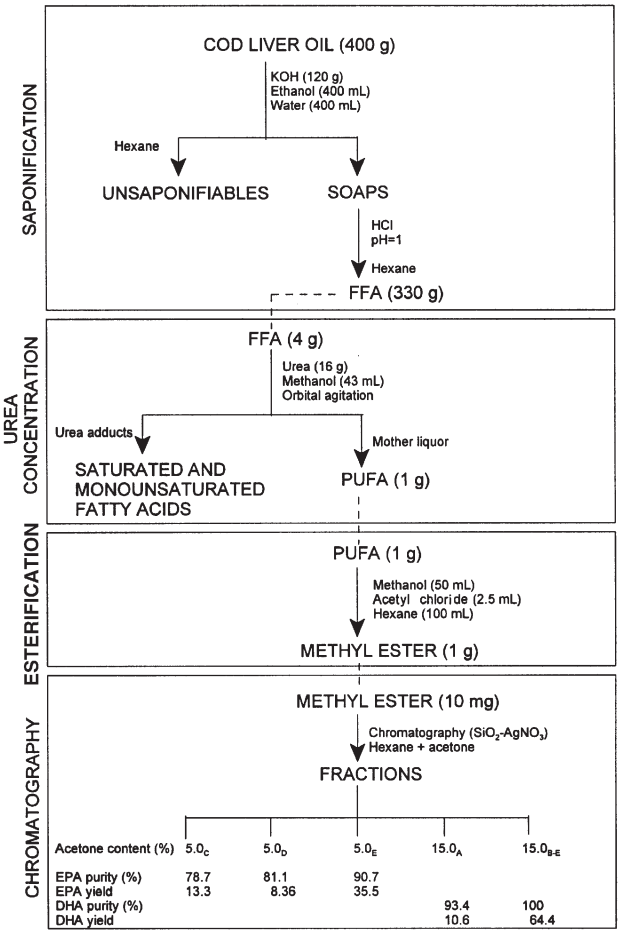
This work reports recovery of highly pure DHA and pure EPA from CLO by means of a process including CLO saponification and concentration of the fatty acids by urea inclusion with orbital agitation during the crystallization process. Afterward, PUFA were methylated, and DHA and EPA methyl esters were purified by using argentatiom silica gel column chromatography.

MATERIAL AND METHODS

CLO saponification. CLO (Acofarma, Barcelona, Spain) had the following properties: density, 925 kg·m^{-3}; refractive index, 1.47, acidity value, 0.16; iodine value (Hanus), 150.3; and unsaponifiable content, 1.46% (w/w). In a typical experiment (Scheme 1), CLO (400 g) was mixed in the reactor with 800 mL of a saponifying solution comprising KOH (120 g), H_2O (400 mL), and ethanol (400 mL; 96%, vol/vol). The saponification was carried out in a 2.5-L temperature-controlled reactor at 60ºC for 1 h, with constant agitation under an argon atmosphere. After saponification, 200 mL water was added. Unsaponifiables were separated by extraction with hexane (2 L). The aqueous alcohol phase, containing the soaps, was acidified to pH 1 with 480 mL of HCl (23%, vol/vol), and the free fatty acids (FFA) were recovered by extraction with hexane. The extract was washed with distilled water to neutral pH, and the total volume was made up to 500 mL. This solution (2 mL) was used to determine the fatty acid extraction yields by gas chromatography (GC) (18).

Fractionation with urea. The FFA (4 g) were mixed with urea in a ratio of 1:4 (w/w) (12), to obtain crystal urea adducts of saturated and monounsaturated fatty acids, and PUFA in the mother liquor (Scheme 1). The urea solvent was methanol (43 mL). Crystallization temperatures were in the range 0–30°C. Urea and urea complexes were allowed to crystallize overnight. In the presence of an orbital agitator (Type AK 15; Infors A.G., Bottmingen, Switzerland) operating at 100–800 rpm, with a 1-cm orbital diameter (maximum × 1.7 *g*). The solution was filtered under vacuum, and the filtrate was kept at 4°C for 3 h and then filtered again.

Extraction of n-3 fatty acids. The filtrate was mixed with 12 mL HCl (10% vol/vol) and 12 mL hexane, and the mixture was thoroughly stirred for 1 h. The hexane layer was separated. This layer was extracted again with hexane and hydrochloric acid, and the extracts, containing the PUFA, were combined.



SCHEME 1

Esterification of PUFA. PUFA (1 g) were mixed with absolute methanol (50 mL), acetyl chloride (2.5 mL), and hexane (100 mL) (Scheme 1). The resulting solution was transferred to a pressure vessel that was placed in a boiling water bath (30 min). Then the pressure container was cooled to ambient in a cold water bath. The recovered solution was evaporated under argon (rotary evaporator), and hexane (10 mL) was added. Various amounts of this concentrate were applied to chromatography columns as needed (17).

Fractionation of fatty esters by column chromatography. Argentation silica gel column chromatography was used for fractionation of PUFA esters (Scheme 1). The Ag-silica gel was prepared as follows: 100 g of silica gel (0.06–0.2 mm, 70–230 mesh ASTM; mean pore diameter of 60 nm, specific surface area of 500 m^2 g^{-1}) for column chromatography was slurried in ethanol (200 mL, 10 min). A solution of silver nitrate (10 g) in 70% (vol/vol) ethanol (35 mL) was added. Agitation was continued for 10 min. Ethanol was then evaporated in a rotary evaporator under vacuum at 60°C. The silver-impregnated silica gel was activated by overnight heating

at 120°C. This material was cooled and kept in the dark in a desiccator until needed.

The packed height of glass chromatography columns was varied from 7 to 9 cm in a 0.5-cm diameter column. The exit of the chromatography column was plugged with either glass wool or a sintered glass disc to retain solids. Chromatography columns were packed as follows. A slurry of Ag-silica gel (20 g) in hexane (40 mL) was poured into a column that previously had been half-filled with hexane. A slight flow of hexane was allowed to occur during packing. The hexane level was lowered until it was 1 cm above that of the stationary phase. An aliquot of the methyl-PUFA solution (0.05–0.1 mL) was applied to the chromatography column at specified loadings of extract-to-stationary phase. The maximum acceptable fatty ester loading on the silver-silica gel stationary phase was 4% (w/w) (17). The column was eluted with a sequence of solvents established after several assays. They were consecutively passed through the column, at a flow rate of 1–2 mL/min. The eluates were collected as fractions corresponding to the elution solvent. Unless otherwise noted, a column was eluted with the following sequence of solvents: (i) hexane with 0.5% (vol/vol) acetone; (ii) hexane with 1% (vol/vol) acetone; (iii) hexane with 5% (vol/vol) acetone; (iv) hexane with 10% (vol/vol) acetone; and (v) hexane with 15% (vol/vol) acetone.

GC. An HP 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an autoinjector (model HP 6890) and a flame-ionization detector was used. The separation was done on a Supelco (Bellefonte, PA) Omega wax 250 (30 m × 0.25 mm) fused-silica (0.25 μm) capillary column. The oven time–temperature profile was as follows: 205°C (10 min), 6°C/min to 240°C, 240°C (9 min), giving a total heating time of about 25 min. The internal standard was nonadecanoic methyl ester (19:0 methyl ester) (19).

All essays were done at least in triplicate, and variation on any one sample was routinely less than 10%. Values of a typical experiment are shown in the tables.

RESULTS AND DISCUSSION

The first step of the combined process was CLO saponification (Scheme 1). The results agree with those of Robles-Medina *et al.* (12) for the same oil. The fatty acid profiles of the oil and the extracts are shown in Table 1. It was found that saponification slightly enhanced the percentages of DHA and SA purity. The yield for all fatty acids was 82.4%; yields for SA and DHA were better (99.7 and 98.6%, respectively) than for EPA (83.5%).

The second step was PUFA concentration by the urea inclusion method (Scheme 1), using the FFA that were previously obtained. The purpose of this procedure is to obtain a PUFA concentrate as enriched in EPA as possible and, simultaneously, to maintain the highest yields of this PUFA. Results from other authors (11,12,20,21) indicate that EPA recovery increases quickly from 0 to 30°C for a urea/fatty acid ratio of 4:1 (w/w); thus a screening in this temperature range was done in the present work. Figure 1 shows the temperature influence on the recovery of n-3 PUFA. Although the drop in temperature increases the tendency to form urea compounds, the optimal temperature depends on the particular PUFA (7,11,12,21). In this work, a maximal recovery of PUFA was found at 4°C. EPA percentages in the concentrate were almost constant in the 0–30°C range, with the lower values in the range of 20–30°C. SA percentages were constant for all range of temperatures. However, from 18 to 30°C the percentages of DHA decreased significantly, reaching the lowest value at 22°C (data not shown). On the other hand, total recovery of the three fatty acids (Fig. 1) is maximum for the range 2–6°C, with 10°C being the lowest recovery for the three fatty acids. As shown in this figure, the recovery of the n-3 fatty acids in the range of temperatures between 10–30°C increased notably. Consequently, the range of temperature from 16 to 30°C was chosen to attempt enhancement of EPA recoveries through crystallization.

The crystallization process with urea preferentially selects saturated and monounsaturated fatty acids, and the tendency of fatty acids to combine with urea decreases with increasing unsaturation and decreasing chain length (9). CLO present a very complex fatty acid profile, having 18 fatty acids with a percentage higher than 0.1%, thus the great number of fatty acids that compete to form crystals diminishes the selectivity during the crystallization. Orbital agitation increases the number of encounters between urea and fatty acid molecules during the crystallization and consequently allows the crystal to include the more appropriate fatty acids. Initially, agitation was applied in the range 0–800 rpm, for a range of temperatures from 16 to 30°C. Fatty acid purity percentages of EPA, SA, and DHA were almost constant (data not shown). The highest EPA purity percentages, nearly 30%, were obtained at 20°C, which coincides with other reports (12). EPA yield (Fig. 2A) for all temperatures increased from 60–70% without agitation to 90–97% at 800 rpm. SA yield (Fig. 2B) ranged from 60–75% without agitation to 87–95% at 800 rpm, depending on temperature. DHA yield (Fig. 2C) varied from 53–73% without agitation to 85–99% at 800 rpm.

The recovery process for the three fatty acids was greatly influenced by orbital agitation. A maximal EPA recovery of 75.6% at 28°C through this procedure has been reported; for DHA, 100% at 4°C and 92.5% at 20°C; and for SA 93% at –4°C and 80.5% at 20°C. Note that EPA recovery (Fig. 2A) surpassed 90% for 20°C and 400 rpm, and the recovery for all temperatures increases with increasing agitation until approximately 800 rpm. SA and DHA recoveries (Figs. 2B and 2C) also follow a similar standard.

A multifactorial analysis of variance (ANOVA) was carried out for data on the recoveries of SA, EPA, and DHA. The aim of this procedure was to discover which of the tested variables (temperature, agitation, and fatty acid type) had a significant effect on results. The ANOVA table decomposes the variability of data into contributions due to various factors. Thus, the main effect observed on the data is agitation (F-ratio = 234.5; P = 0.0000), followed by the temperature

TABLE 1
Fatty Acid Composition of Cod Liver Oil and Extract, and Yield Obtained in the Saponification

Fatty acids	Oil[a]	Extract	Yield[b]	Concentration factor[c]
12:0	2.21	2.03	75.7	0.92
14:0	3.83	3.76	80.9	0.98
16:0	10.6	11.1	86.3	1.05
16:1n-7	6.97	6.44	76.1	0.92
16:2n-4	1.02	0.84	67.9	0.82
18:0	2.73	2.69	81.2	0.99
18:1n-9	14.5	17.4	99.0	1.20
18:1n-7	4.90	4.10	68.9	0.84
18:2n-6	1.43	1.74	100	1.22
18:3n-6	0.17	0.20	96.9	1.18
18:3n-3	1.10	1.33	99.6	1.21
18:4n-3	2.29	2.77	99.7	1.21
20:1n-9	9.40	10.2	89.4	1.09
20:2n-6	0.53	0.30	46.6	0.57
20:3n-6	0.47	0.45	78.9	0.96
20:4n-6	1.03	0.78	62.4	0.76
20:5n-3	8.89	8.77	83.5	1.01
22:1n-9	7.57	6.79	73.9	0.90
24:0	1.32	1.11	69.3	0.84
22:3n-3	0.48	0.34	58.4	0.71
22:4n-6	0.50	0.34	56.0	0.68
22:5n-3	1.13	1.07	78.0	0.95
22:6n-3	10.7	12.8	98.6	1.20
Others	6.17	2.66	35.5	0.43
		Rs[d] = 82.4		
Saturates	20.7	20.7	82.4	1.00
Unsaturates	73.1	76.7	86.5	1.05
n-9	31.5	34.4	90.0	1.09
n-7	11.9	10.5	72.7	0.88
n-6	4.13	3.81	76.0	0.92
n-4	1.02	0.84	67.9	0.82
n-3	24.6	27.1	90.8	1.10

[a]Fatty acid percentages obtained by direct transesterification of the oil.
[b]Yield of the saponification for each fatty acid = (% fatty acid in the extract/% fatty acid in the oil) × Rs.
[c]Concentration factor = (% fatty acid in the extract/% fatty acid in the oil).
[d]Rs = yield of the saponification = (amount of fatty acids in the extract/amount of fatty acids in the oil) × 100.

(*F*-ratio = 28.6; *P* = 0.0000) and fatty acid type (*F*-ratio = 19.5%; *P* = 0.0000). The interaction among variables was also significant. The main effect was the interaction between agitation and temperature (*F*-ratio = 4.23; *P* = 0.0000), followed by the interaction between fatty acid type and temperature (*F*-ratio = 3.36; *P* = 0.0004), and finally the interaction between fatty acid type and agitation (*F*-ratio = 2.07; *P* = 0.0386). Therefore, it can be concluded that agitation must be taken into account as well as other variables, such as temperature, when the urea inclusion compound method is applied for PUFA concentration.

In the next step, the PUFA mixture obtained in the previous process was methylated (17,18) and then applied to the chromatography column (Table 2). Our procedure achieved results in agreement with previous reports (22) on purifying α-linolenic acid from perilla oil.

In silver-silica gel fractionation of fatty acid esters, saturated esters elute first, followed by unsaturated ones. The number, position, and geometric configuration of double bonds determine the order of elution of unsaturated fatty esters. Isomers differing in configuration around a single double bond are sometimes easily resolved (15). The resolving power of Ag-silica gel is attributed to reversible charge-transfer complexation of Ag^+ with carbon–carbon double bonds. The extent and the strength of complexation control the mobility of a solute, and so does the polarity of the mobile phase. The loading of silver ion on the gel also affects the retention time of a solute because the loading determines the surface density of Ag^+ ions on the solid matrix, and this in turn influences the frequency of complexation during elution. The areal density also determines whether a molecule of PUFA will simultaneously complex with silver at multiple sites (23). Simultaneous multipoint complexation does not seem plausible during fractionation of cod liver oil fatty esters when the silver loading on the solid phase does not exceed 1.875×10^{-7} kg/m^2 of surface (16). In our case, the estimated areal loading of Ag^+ was 1.27×10^{-7} kg/m^2 of surface; therefore, a molecule of an unsaturated ester apparently interacted with only one silver ion at any given instance.

The fatty acid profiles of the various solvent fractions ob-

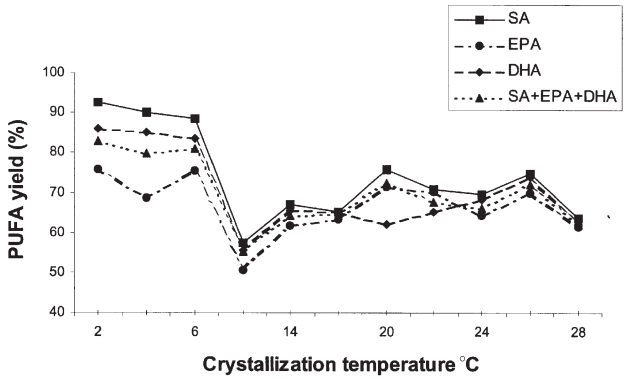


FIG. 1. Yield of n-3 polyunsaturated fatty acids in the nonurea crystalline fraction after urea crystallization at different temperatures without orbital agitation. SA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

tained at the silver chromatography stage are shown in Table 2. This table also shows the fatty acid profile of the concentrate applied to the chromatography column. The CLO PUFA-concentrate was fractionated by means of several volumes of acetone/hexane applied to the chromatography column as single 5-mL volumes. The first solvent fraction, $0.5\%_A$ (i.e., the one containing 0.5% acetone in hexane, 3×5 mL) was found lacking in any type of ester. The second solvent fraction, $0.5\%_B$ (2×5 mL), eluted the saturated methyl esters 12:0, 14:0, 16:0, and 24:0, which are typically eluted until the solvent fraction $0.5\%_D$. Because the saturated esters associate least strongly with the stationary phase, these esters are eluted quantitatively with the more nonpolar eluents. Note that the preceding urea adduct formation involved nearly all the saturated and monounsaturated fatty acids; the 18:2n-6 were also diminished. If the above mentioned process was obviated, to eliminate them it would be necessary to increase the volume of the first solvent fractions, also with other fractions $0.5\%_E$ and successively more apolar solvents.

The elution of the monounsaturated 16:1n-9 and 18:1n-9 esters began with the solvent fraction $0.5\%_c$, however, this pattern is variable and these esters can elute in more polar solvent fractions, such as occurs with the 20:1n-9 ester (begins in the $1\%_A$) and 22:1n-9 (begins in the $1\%_B$). Unexpectedly, low amounts of other more polar fatty esters can elute in these low polarity fractions, as occurs with the polyunsaturated esters 18:4n-3 (0.61% yield) and 20:4n-6 (0.61% yield).

The elution of the polar esters 16:2n-4, 18:3n-3, and 20:5n-3 began with the solvent fraction $1\%_A$, and the subsequent fraction $1\%_B$ continued the elution of the remaining polyunsaturated esters 22:4n-3, 22:5n-3, and 22:6n-3. The polyunsaturated fatty esters 18:4n-3, 20:4n-6, 20:5n-3, and 22:6n-3 can elute in other experiments in the noted solvent fractions or in others with similar polarity. In any case the initial amounts of these fatty esters are low, with significant yields being obtained from the solvent fraction $1\%_B$. Thus, the recovery for the 18:4n-3 ester in this fraction is 36.1%.

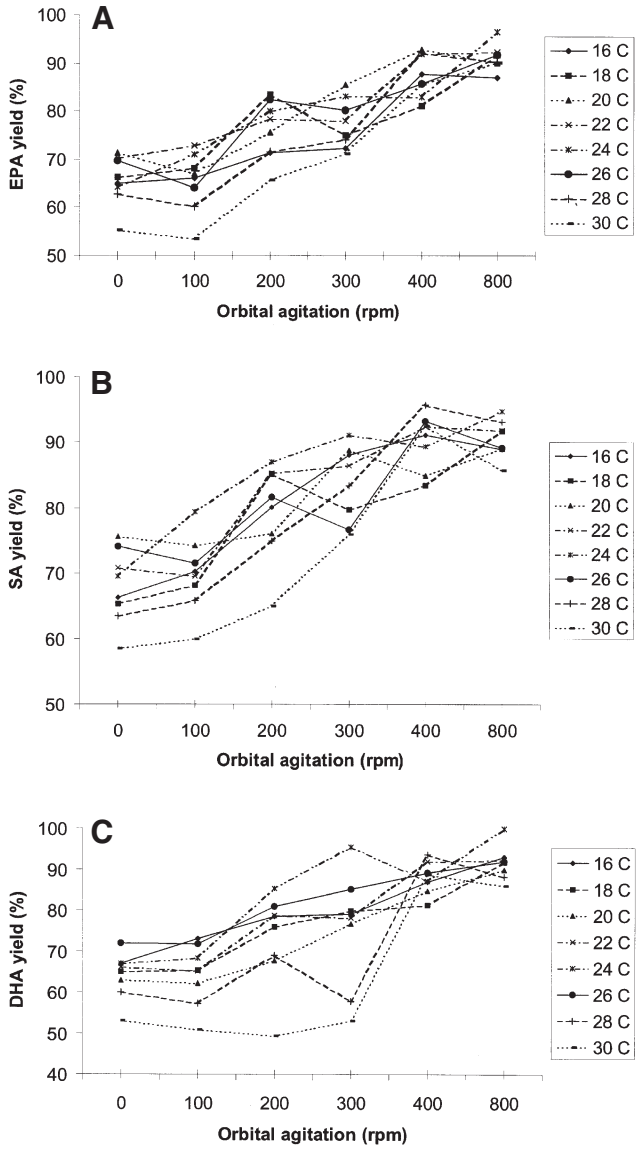


FIG. 2. Results obtained from the nonurea crystalline fraction following urea crystallization at different temperatures and variable speed of orbital agitation: Content of (A) EPA; (B) SA; and (C) DHA. ◆, 16°C; ■, 18°C; ▲, 20°C; ×, 22°C; *, 24°C; ●, 26°C; +, 28°C; –, 30°C. For abbreviations see Figure 1.

However, this fraction had a very complex fatty ester profile, and this ester could not be obtained as a pure product. A solvent fractions series ($5\%_A$–$5\%_E$) was used to purify the 20:5n-3 ester. The best fraction was the $5\%_E$ (90.7% purity; 35.5% yield). All of the preceding 5% fractions (42.5% recovery for the 20:5n-3 ester) could be reused to repurify this ester with lower difficulty considering that these had a simpler fatty acid profile than the mother liquor obtained in the urea-concentrate, although this step has not yet been accomplished. The main contaminants in the fraction $5\%_E$ were the 22:6n-3, 22:6n-3, and 22:1n-9 esters, although the relative amounts among them are very variable in different experiments. From this fraction it was noted that it was not possible

TABLE 2
Purification of CLO PUFA Concentrate Methyl Esters by Silver Nitrate-Silica Gel Chromatography Column

Fatty acid	PUFA	Eluents: % acetone in hexane (vol/vol)															
		0.5%$_A$		0.5%$_B$		0.5%$_C$		0.5%$_D$		1%$_A$		1%$_B$		5%$_A$		5%$_B$	
methyl ester	%	%	Yield[b]	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield
12:0	0.95			21.9	60.6	8.47	22.0										
14:0	0.71			17.9	66.3	4.86	16.9										
16:0	1.45			36.6	66.4	11.4	19.5	2.73	6.08								
16:1n-7	0.77					0.92	2.96	10.2	33.6	8.70	24.5	3.19	26.0	1.00	9.01	0.36	2.78
16:2n-4	1.14									1.23	2.54	2.82	15.5	4.32	21.4	3.82	22.7
18:1n-9	0.37					4.83	34.3	12.0	40.4	1.92	11.3	0.42	7.13				
18:2n-6	0.41							7.00	55.1	5.02	26.6	1.70	26.0				
18:3n-3	0.35									4.45	27.6	1.50	4.29				
18:4n-3	7.16					1.77	0.61	10.8	4.87	39.7	12.1	40.8	36.1	25.7	24.9	11.3	9.39
20:1n-9	0.45									1.93	9.31	2.50	34.9	2.01	31.0		
20:2n-6	0.35					8.27	58.4										
20:3n-6	0.78							4.45	18.4	7.65	21.3	4.40	35.4	1.67	14.9		
20:4n-6	1.95					4.61	5.84	5.49	9.09	13.9	15.5	10.3	33.2	4.60	2.36	1.88	5.74
20:5n-3	22.4									6.95	0.67	20.0	5.61	47.5	2.12	70.3	18.7
22:1n-9	1.64											1.70	6.51	4.39	1.63	5.98	21.7
24:0	0.75			10.4	36.5	8.55	28.2	5.52	23.8								
22:3n-3	0.46											2.09	28.5	1.76	26.6	1.24	16.0
22:5n-3	1.05											2.16	12.9	4.70	31.1	4.06	23.0
22:6n-3	45.8											3.46	0.47				
Total yield[c]					2.63		2.47		3.23		2.17		6.28		6.94		5.95

Fatty acid		Eluents: % acetone in hexane (vol/vol)													
		5%$_C$		5%$_D$		5%$_E$		10%$_A$		10%$_B$		15%$_A$		15%$_{B-E}$	
methyl ester	%	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield	%	Yield
12:0	0.95														
14:0	0.71														
16:0	1.45														
16:1n-7	0.77														
16:2n-4	1.14	2.78	9.24	2.06	4.17	0.59	4.53								
18:1n-9	0.37														
18:2n-6	0.41														
18:3n-3	0.35														
18:4n-3	7.16	6.51	3.45	3.77	4.39	0.79	0.84								
20:1n-9	0.45														
20:2n-6	0.35														
20:3n-6	0.78														
20:4n-6	1.95														
20:5n-3	22.4	78.7	13.3	81.1	8.36	90.7	35.5	51.6	12.4	17.3	2.48	2.49	0.58		
22:1n-9	1.64	6.12	14.1	5.96	8.39	4.27	22.8	3.54	11.6	1.34	2.62	2.41	3.55		
24:0	0.75														
22:3n-3	0.46														
22:5n-3	1.05	3.16	11.4	2.78	6.12	1.01	8.43								
22:6n-3	45.8			1.33	0.07	2.03	0.39	38.5	6.40	78.7	5.51	93.4	10.6	100	64.4
Total yield[c]			3.79		3.21		8.76		5.38		3.21		5.21		29.5

[a]Fatty acid ester percentages of the concentrate applied to the chromatography column.
[b]Yield according to chromatography for each fatty acid ester = (% fatty acid ester in each eluate/% fatty acid ester in the urea concentrate) × yield for the fatty acid esters in each eluate.
[c]Yield of the chromatography for the fatty acid esters in each eluate = (amount of fatty acid esters in each eluate/amount of PUFA concentrate methyl esters) x 100.

to obtain more amounts of the pure 20:5n-3 ester, because the 22:6n-3 ester eluted in the subsequent eluents.

The solvent fractions eluted with 10%$_A$ comprised esters of 20:5n-3 (51.6% purity; 12.4% yield) and 22:6n-3 (38.5% purity; 6.40% yield), and low amounts of the 22:1n-9. The second solvent fraction, 10%$_B$, was richer in the 22:6n-3 ester than previous fractions (78.7% purity; 5.51% yield), although the yield was still low and the previous esters also eluted here. The solvent fraction 15%$_A$ repeated this fatty acid esters profile, but the 22:6n-3 ester had higher purity and yield (93.4 and 10.6%, respectively). From these steps, the successive eluates 15%$_B$–15%$_E$ yielded highly pure 22:6n-3 ester (100% purity; 64.4% yield).

This work shows EPA methyl ester can be obtained at 90.7% purity and 35.5% yield, but other eluates yielding lower purity (5%$_D$, ~81%; 5%$_C$, ~79%; and 5%$_B$, ~70%) can

be reused for successive processes. Because these are free of several contaminant esters, the subsequent purity can be higher than in the previous step, although this aspect requires further confirmatory experiments. DHA recovery was 64.4% with a purity of 100%, although other eluates also have high purity, but lower yields.

The column chromatography could be accomplished directly with fatty acid esters obtained after CLO methylation (17), but the total purity for methyl EPA (82.7%) was lower than the purity shown here. Thus, the urea method increases the overall purity achieved by this procedure because it reduces some PUFA, such as the 16:2n-4, which elute in the medium-polarity solvent fractions. Urea crystallization does not produce a final decrease in the yield if a gentle orbital agitation is applied, as shown in this paper. If saponification yields 83.5% EPA, the overall yield for this ester is ~29.6%. Thus, this procedure seems to be more suitable for DHA purification from CLO than for EPA. The highest purity reported for DHA obtained from fish oil through chromatographic process (HPLC) is 92% (24). Our inexpensive method could be applied easily to obtain high-purity DHA.

In addition, the urea method can be used when the goal is to obtain EPA-DHA concentrates, a product widely used as a dietary supplement in human nutrition. Nevertheless, traces of methyl carbamate (a carcinogen) can be formed (25,26). This problem may be avoided by washing an organic solution containing the PUFA with a mineral acid solution. In any case, the high polarity of the methyl carbamate should prevent its elution through the silica column.

To look for fatty acid peroxidation during the purification process, the fatty esters of EPA and DHA were tested for peroxide value by means of the iodine method (27); results ranged between 6.4 and 11.1 meq O_2/kg, which can be considered as satisfactory. Assays for conjugated diene absorbance at 234 nm and triene absorbance at 268 nm were carried out in order to rule out fatty acid degradation (27). In almost all cases the absorbances at both wavelengths were negligible, which indicates the absence of PUFA degradation during the purification process.

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