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Eicosapentaenoic Acid Enrichment from Sardine Oil by Argentation Chromatography

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Eicosapentaenoic acid (EPA) derived from chemically hydrolyzed sardine oil was concentrated by urea fractionation using methanol at different temperatures (2, 4, and 6 °C) and urea/fatty acid ratios (2:1, 3:1, and 4:1 w/w) and purified by argentation neutral alumina column chromatography. The individual fatty acids were determined as fatty acid methyl esters (FAME) by gas−liquid chromatography and gas chromatography−mass spectroscopy as FAME and *N*-acyl pyrrolidides. In the mass fragmentation pattern of FAME, the base peak was assigned to be the 1-methoxyethenol moiety ($m/z = 74$) obtained by McLafferty rearrangement. Formation of the cyclic tropylium ion ($m/z = 91$) in fatty acids with four or more double bonds was apparent in FAME−PUFAs. The base peak of *N*-acyl pyrrolidides was the McLafferty rearrangement ion, 1-(pyrrolidin-1-yl)ethenol ($m/z = 113$). The highest concentration of EPA (47.78%) was obtained at the crystallization temperature of 4 °C with a urea/fatty acid ratio of 4:1 (w/w) with 93.74% yield. After complexation of saturated and less unsaturated fatty acids by urea complexation, argentation chromatography resulted in an EPA of high purity (99.6%) with an overall recovery of 54.09% using 50% diethyl ether/*n*-hexane as eluting solvent. The peroxide (POV) and thiobarbituric acid (TBS) values were found to be highest (4.0 mequiv of O_2/kg and 5.2 mg of malondialdehyde/kg, respectively) during urea fractionation at the higher crystallization temperature (6 °C) and higher urea/fatty acid ratio (4:1).

KEYWORDS: Sardine oil; eicosapentaenoic acid (EPA); fatty acid methyl esters (FAME); urea fractionation; argentation column chromatography

INTRODUCTION

The n3 and n6 long-chain polyunsaturated fatty acids (LC-PUFAs), viz., eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3), and arachidonic acid (AA, 20:4n6), are essential fatty acids in the diet of a majority of marine finfish and crustaceans, especially for the larvae and broodstock because they cannot synthesize it de novo from precursor molecules (*1*, *2*). Diets deficient in these PUFAs particularly EPA have been found to have a negative effect on ovarian development, fecundity, and egg quality (*3*). The important natural sources of n3 LC-PUFAs are marine fish oils such as sardine, mackerel, cod, shark, and menhaden, which contain PUFA levels of about 30%. For this reason, marine fish oils are preferentially used as raw material to prepare n3 PUFA concentrates (*4*). However, they contain relatively high levels of triacylglycerols and sterols, which are too heterogeneous, rendering it difficult to isolate individual fatty acids efficiently with a single separation method. Additionally, it was reported that n3 PUFAs were moderately absorbed by the intestine as triglycerides and most promptly absorbed when free fatty acids (FFA) were given orally (*5*, *6*). Therefore, it is convenient to prepare n3 concentrates as FFA after chemical hydrolysis of marine oils. The technologies available for purifying individual PUFAs and PUFA concentrates from fish oil are based on differences in physicochemical properties associated with the number of double bonds in the molecule or the chain length like urea complexation method (*7*). Recently, argentation silica gel column chromatography has been employed to obtain high-purity EPA methyl ester from hydrolysates of fish oil that contained other polyunsaturated fatty acid esters including linolenic acid (18:3n3) and oleic acid (18:1n9) (*8*, *9*). Partial success in purification of linolenic and oleic acid has been achieved by using a mixture of acetonitrile and propionitrile (1:2) at −60 °C (*10*). Argentation silica gel chromatography of urea inclusion adducts from cod liver oil yielded highly pure DHA in the process (100% purity, 64% yield), while for EPA, the recovery in the combined process was 29.6%, and the final purity was 90.6%. 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 (*11*). EPA and DHA have been concentrated from shark liver (*Isurus oxyrinchus*) in one single step, in which fish liver oil was simultaneously extracted, saponified, and concentrated. Additionally, the PUFA concentrate was winterized to crystallize

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the remaining saturated fatty acids, resulting in a further increase in the concentration of DHA and EPA (*12*). The polyunsaturated fatty acids EPA and AA have been purified from the red microalga *Porphyridium cruentum* by the urea inclusion method followed by silica gel column chromatography of the urea concentrate. Total AA and EPA recoveries reached 39.5% and 50.8%, respectively, for purity and approximately 97% for both fatty acids (*13*). Solvent winterization of seed oil and free fatty acids (FFAs) was employed to obtain γ-linolenic acid (GLA) concentrates from seed oils of two Boraginaceae species, *Echium fastuosum* and *Borago officinalis*. Different solutions of seed oils and FFA from these two oils at 10%, 20%, and 40% (w/w) were crystallized at 4, −24, and −70 °C, respectively, using *n*-hexane, acetone, diethyl ether, isobutyl alcohol, and ethanol as solvents. Best results were obtained for *B. officinalis* FFAs in *n*-hexane, reaching a maximum GLA concentration of 58.8% (*14*). Also, there are reports for the production of EPA or DHA concentrates by a combination of techniques such as molecular distillation, fractional distillation, liquid chromatography, and supercritical fluid extraction (*8*).

PUFAs are widely available in a large variety of marine organisms such as microalgae, polychaetes (*15, 16*), finfish, and shellfish, but sardine oil is easily available and inexpensive and contains a considerable amount of PUFAs (33.26%), particularly EPA (>15%), an essential fatty acid for larval and broodstock nutrition in marine aquaculture. The present paper highlights a method for purification of sardine fatty acids with the goal to get a PUFA concentrate with high EPA. The objective of this study is to purify EPA from sardine oil in three main steps: (1) saponification of fish oil to derive free fatty acids, (2) enrichment of PUFA content from the mixed fatty acid concentrate by urea fractionation at three different temperatures (2, 4, and 6 °C) and urea fatty acid ratios (2:1, 3:1, and 4:1 w/w), and (3) separation of the individual n3 fatty acids, particularly high-purity EPA, by normal pressure argentation alumina column chromatography. The oxidative stability of fatty acid concentrates at different stages of purification was also studied.

MATERIALS AND METHODS

Chemicals and Reagents. All solvents used for sample preparation were of analytical grade, and the solvents used for MS analyses were of LC grade (E-Merck, Darmstadt, Germany). Analytical grade solvents were redistilled in an all-glass system. The solvents were nitrogen degassed prior to use. Doubly distilled water was used throughout this work, while all reagents used were of analytical grade and purchased from E-Merck. Standards of fatty acid methyl ester (Supelco TM 37 component FAME mix, catalog no. 47885-U) and boron trifluoride/methanol (14% BF_3 in methanol, w/v) were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Crude sardine oil (*Sardinella longipes*) was obtained from a local fishmeal plant located in Cochin, India, bleached with 4% activated charcoal, and stabilized with butylated hydroxyquinone (TBHQ, 0.01% w/v). The oil was stored under nitrogen at −20 °C, in a 2 L sealed dark amber glass container, until used. All glassware was rinsed with $CHCl_3$−CH_3OH (2: l v/v) and dried under N_2.

Saponification and Extraction of Fatty Acid from Sardine Oil. Sardine oil (50 g) was saponified by refluxing with 100 mL of alkaline solution [prepared by dissolving 250 g of NaOH and 2.5 g of Na_2-EDTA in a 1.5 L solution of water/methanol (1:1) at 60 °C] under continuous stirring for 30−45 min under an inert atmosphere of N_2 following the procedure adopted by Haagsma with modification (*9*). The hydrolyzed mixture was diluted with water (50 mL), and the unsaponifiable matter was extracted successively with diethyl ether (50 mL × 3) and *n*-hexane (5 × 75 mL) and discarded. The pH value of the lower hydroalcoholic phase containing the saponified fatty acids was adjusted to 1.0 with HCl−H_2O (1:1 v/v, 15 mL), and the resultant free fatty acids were recovered by extraction with *n*-hexane (6 × 75 mL), washed with water to neutral pH, and dried over anhydrous $MgSO_4$. The residual solids were removed with a Buchner funnel under suction, and the solvent was evaporated to recover the free fatty acids, dried by passing N_2, weighed (34 g), and converted to methyl esters for further fractionation and gas−liquid chromatographic (GLC) and gas chromatographic−mass spectroscopic (GC/MS) analysis.

Formation of the Fatty Acid−Urea Inclusion Complex. The urea−fatty acid complexation was accomplished following the procedures (*7, 9*) with modification. In the first step of crystallization, urea was added to methanol in the ratio of 1:3 (w/v). The mixture was heated at elevated temperature (65−75 °C) with constant stirring until a clear, homogeneous solution was formed, and the fatty acids derived from saponification were applied in incremental portions into the resulting urea solution, the urea/fatty acid ratios being 2:1, 3:1, and 4:1 (w/w). The solution was cooled to room temperature at a cooling rate of 0.5 °C/min and subsequently crystallized at three different sets of temperatures (2, 4, and 6 °C) for 24 h. The crystals were thereafter kept at −20 °C for 24 h. After thawing, the urea crystals (urea complex fraction) were separated from the mother liquor (non-urea complex fraction) by vacuum filtration. The filtrate containing the unsaturated fatty acids was evaporated using a rotary vacuum evaporator to remove the residual methanol. The solid residue thus obtained was diluted with water (200 mL) and acidified with dilute HCl (6 M, 30 mL) to pH 4−5 under stirring to remove traces of residual urea and methanol. The liberated fatty acids after acidification with dilute HCl were extracted with different solvents (*n*-hexane, chloroform, dichloromethane), and hexane was found as the best with higher recovery percentage of fatty acids. The fatty acids were extracted with *n*-hexane three times (3 × 100 mL) in a separatory funnel to cause the phase separation of urea and concentrated PUFAs. The upper layer of *n*-hexane containing the PUFAs was separated and dried over anhydrous $MgSO_4$. The resulting PUFA concentrate was dissolved in methanol, added with TBHQ (0.01% w/v) as an antioxidant to increase the stability of the fatty acids, and kept under a blanket of N_2 at −20 °C for 48 h.

Acid-Catalyzed Transesterification of Fatty Acid to Methyl Esters (FAMEs). Lipid from the crude sardine oil was extracted by using $CHCl_3$−CH_3OH−H_2O (2:4:1 v/v/v) (*17*). The lipid extract thus obtained was saponified with 0.5 N KOH in CH_3OH. After removal of the nonsaponifiable material with *n*-hexane and acidification with 1 N HCl, the saponifiable materials were extracted with petroleum ether−diethyl ether (1:1 v/v) and transesterified to furnish fatty acid methyl esters (FAME) by reaction (30 min under reflux) with a methylating mixture (14% BF_3/MeOH, 5 mL) in a boiling water bath under an inert atmosphere of N_2 (*18*). The FAME thus obtained was cooled to ambient temperature, and distilled water (20 mL) was added. The solution was extracted with *n*-hexane (10 mL × 6), and the upper *n*-hexane layer was removed and concentrated under an inert atmosphere of N_2. The resulting FAME concentrate was reconstituted in petroleum ether, flushed with N_2 in glass vials, and stored in deep freeze (−20 °C) until required for GC/GC-MS analyses. Analysis was performed in triplicate. Similarly, the fatty acids obtained by urea-aided crystallization at various combinations of temperature (2−6 °C) and urea/fatty acid ratios (2:1, 3:1, and 4:1) were transesterified for analysis by GC/GC-MS. The PUFA concentrates obtained during urea fractionation were directly methylated to furnish the FAMEs, which were extracted with *n*-hexane, and concentrated under inert atmosphere of N_2 to yield a residue. The residue was immediately dissolved in *n*-hexane (30 mL) to be applied on a chromatography column.

Argentation Column Chromatographic Fractionation of FAMEs. The methyl ester of EPA was fractionated from other PUFAs by means of normal pressure liquid column chromatography with $AgNO_3$−impregnated neutral alumina as the stationary phase. Silver nitrate powder ($AgNO_3$, 5 g) was added to ethanol (80% v/v, 30 mL) and dissolved by stirring for 10 min. About 50 g of neutral alumina (0.06−0.2 mm, MW 101.96), column chromatography grade, was slurried in ethanol (96% v/v, 100 mL) and $AgNO_3$ solution (10 mL) under stirring for 2 h. Ethanol was evaporated under vacuum at 60 °C, and the silver-impregnated alumina was activated by overnight heating (110−120 °C)

in the oven to prepare Ag−alumina powder. This material was cooled and kept in the dark in a desiccator until use. The slurry of Ag−alumina gel (500 mg) in *n*-hexane (5 mL) was poured into a water-jacketed column (45 cm × 50 mm i.d.) previously half-filled with *n*-hexane. The packed height of 0.5 cm diameter glass chromatography columns was maintained at 9 cm. The methylated extract (24 mg) obtained in the transesterification step (by using a U/FA ratio of 4:1 at a temperature of 4 °C) was dissolved in *n*-hexane (5 mL) and applied on the chromatography column. The column was eluted with a sequence of solvents (5−50% diethyl ether/*n*-hexane) passed successively through the column with a flow rate of 1.5 mL/min. The eluates were collected as fractions corresponding to the applied solvents. The EPA was purified by passing through the column along with 50% diethyl ether/*n*-hexane. *n*-Hexane was removed from the fractionated liquid layer to obtain highly purified EPA (∼2.11 g).

Silver Ion Thin-Layer Chromatography ($AgNO_3$ TLC). The recovered fatty acid methyl esters from urea fractionation and column chromatography were resolved by TLC (5 cm × 20 cm), precoated with silica gel, and impregnated with $AgNO_3$. Silica gel G (15.0 g) was mixed with a 10% (w/v) solution of $AgNO_3$ (40 mL) in methanol/water (9:1 v/v) and spread in a uniform thickness (0.25 mm). Plates were drained, air-dried, activated at 110−120 °C in dim light for 30 min, and stored in a light-tight desiccator container. The methyl esters were applied to the plate as a narrow band. The plates were developed twice in *n*-hexane/diethyl ether/acetic acid (94:5:1 v/v/v) to separate individual bands. The bands were stained with 2,7-dichlorofluorescein in alcohol (0.1% w/v) and examined under UV light.

Gas−Liquid Chromatography and Gas Chromatography−Mass Spectrometry (GC/MS) Analysis of FAMEs. FAMEs were analyzed by an Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer) equipped with an Elite-5 (cross-bond 5% diphenyl polsiloxane−95% dimethyl polsiloxane) capillary column (30 m × 0.53 mm i.d., 0.50 μm film thickness; Supelco, Bellfonte, PA) using a flame ionization detector (FID). The oven temperature ramp was 110 °C for 1.0 min, followed by an increase of 45 °C/min to 250 °C, where it was held for 1.0 min, followed by an increase of 30 °C/min to 250 °C, where it was held for 1.0 min, followed by an increase of 25 °C/min to 285 °C, where it was held for 2.0 min, until all peaks had appeared. Ultrahigh purity He (>99% purity) was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 285 °C. The injection volume was 1 μL. The detector temperature was 290 °C. FAMEs were identified by comparison of retention times with the known standards (37-component FAME mix; Supelco).

The GC-MS analyses were performed using the electronic impact (EI) ionization mode in a Varian GC (CP-3800) interfaced with a Varian instrument 1200L single quadruple mass spectrometer for confirmation of fatty acid identification. FAMEs were derivatized to *N*-acyl pyrrolidides by condensation of fatty acid methyl ester with a mixture of pyrrolidine (1 mL) and acetic acid (0.1 mL) at 100 °C under reflux (2 h) for GC-MS analyses (*19*). The GC apparatus was equipped with WCOT fused silica capillary column of high polarity (DB-5; 30 m × 0.25 mm i.d., 0.39 mm o.d., and 0.25 μm film thickness; Varian). The polymeric stationary phase was nonpolar (VF-5MS, 5% phenyl-substituted methylsiloxane). The carrier gas was ultrahigh purity He (99.99% purity) with a constant flow rate of 1 mL/min. The injector and detector temperature were maintained isothermal at 300 °C. The injection volume was 1 μL. Samples were injected in split (1:15) mode at 300 °C into the capillary column similar to that used for the GC analyses, and the oven was identically programmed. The ion source and transfer line were kept at 300 °C. Mass spectra were analyzed using Varian Workstation (version 6.2) software.

Mass Spectroscopic Analyses of FAME Derivatives. The following are the mass spectrometric data of FAME derivatives.

Methyl Palmitate. EI-MS *m*/*z* (relative intensity, %): 270 (M^+, 61.11), 239 (15.74), 227 (31.48), 213 (7.41), 199 (14.81), 185 (12.96), 171 (12.96), 157 (7.41), 143 (31.48), 129 (11.11), 87 (74.07), 74 (*100*), 55 (18.52).

Methyl Oleate. EI-MS *m*/*z* (relative intensity, %): 296 (M^+, 20.00), 111 (76.67), 264 (33.33), 222 (26.67), 180 (18.33), 166 (23.33), 152 (23.33), 123 (23.33), 110 (38.33), 97 (75.00), 83 (70.00), 74 (66.67), 69 (78.33), 55 (*100*).

Methyl Linoleate. EI-MS *m*/*z* (relative intensity, %): 294 (M^+, 52.46), 263 (24.59), 220 (8.20), 178 (13.11), 164 (19.67), 150 (21.31), 136 (18.03), 123 (18.85), 109 (37.70), 95 (70.49), 81 (*100*), 67 (91.80), 55 (50.82).

Methyl Linolenate. EI-MS *m*/*z* (relative intensity, %): 292 (M^+, 16.67), 261 (5.00), 236 (6.67), 173 (6.67), 163 (6.67), 149 (20.00), 135 (20.00), 121 (25.00), 108 (56.67), 95 (58.33), 79 (*100*), 67 (56.67), 55 (35.00).

Methyl Arachidonate. EI-MS *m*/*z* (relative intensity, %): 318 (M^+, 1.82), 290 (1.82), 264 (1.82), 175 (5.45), 150 (7.27), 133 (7.27), 105 (30.91), 91 (70.91), 79 (*100*), 67 (80.00), 55 (49.09).

Methyl Eicosapentaenoate. EI-MS *m*/*z* (relative intensity, %): 315 (M^+, 1.67), 175 (6.67), 161 (8.33), 145 (11.67), 131 (18.33), 119 (31.67), 108 (31.67), 91 (70.00), 79 (*100*), 67 (68.33), 55 (48.33).

Methyl Docosahexaenoate. EI-MS *m*/*z* (relative intensity, %): 342 (M^+, 0.60), 145 (4.20), 131 (6.60), 119 (10.80), 108 (11.40), 91 (28.20), 79 (*100*), 67 (20.40).

Mass Spectroscopic Analyses of *N*-Acyl Pyrrolidide Derivatives. The following are the mass spectrometric data of *N*-acyl pyrrolidide derivatives.

1-(Pyrrolidin-1-yl)hexadecan-1-one/Palmitoylpyrrolidine. EI-MS *m*/*z* (relative intensity, %): 309 (M^+, 16.00), 294 (2.00), 168 (8.00), 140 (10.00), 126 (16.00), 113 (*100*), 98 (8.00), 70 (12.00), 55 (14.00).

1-(Pyrrolidin-1-yl)octadec-9-en-1-one. EI-MS *m*/*z* (relative intensity, %): 335 (M^+, 27.56), 250 (8.62), 236 (10.34), 208 (6.90), 196 (5.17), 182 (12.07), 126 (53.45), 113 (*100*), 98 (18.97), 85 (8.62), 72 (20.69), 55 (27.59).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m*/*z* (relative intensity, %): 333 (M^+, 77.97), 290 (10.17), 236 (15.25), 222 (20.34), 182 (16.95), 168 (15.25), 140 (22.03), 126 (44.07), 113 (*100*), 98 (25.42), 70 (42.37), 55 (49.15).

1-(Pyrrolidin-1-yl)octadeca-9,12,15-trien-1-one. EI-MS *m*/*z* (relative intensity, %): 331 (M^+, 44.00), 182 (22.00), 168 (24.00), 140 (26.00), 126 (60.00), 113 (*100*), 98 (30.00), 72 (64.00), 55 (42.00).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14-tetraen-1-one. EI-MS *m*/*z* (relative intensity, %): 357 (M^+, 18.97), 232 (10.34), 180 (10.34), 126 (13.79), 113 (*100*), 85 (17.24), 70 (22.41), 55 (27.59).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14,17-pentaen-1-one. EI-MS *m*/*z* (relative intensity, %): 355 (M^+, 3.85), 286 (7.69), 232 (7.69), 126 (13.46), 113 (*100*), 85 (17.31), 72 (26.92), 55 (21.15).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m*/*z* (relative intensity, %): 381 (M^+, 3.91), 312 (7.05), 272 (7.29), 232 (16.22), 218 (15.76), 192 (8.24), 166 (23.67), 153 (22.85), 113 (*100*), 98 (46.62), 72 (21.98).

Purification Index. The purification indices of the fatty acids were calculated following an earlier procedure (*8*). The yield obtained is defined as the ratio of the weight of the particular component in the product before purification and that of the component after purification. For a process starting from the substance "A" to the end product "B", the yield of the component "x" in the process A−B may be defined as $Y^x_{(a-b)} = (X_b/X_a) \times 100 = (P^x_b/P^x_a)(W^x_b/W^x_a) \times 100$, where X_a and X_b represent the concentrations of the compound "x" (e.g., EPA) in the products A and B. W^x_a and W^x_b are the weights of the component "x" in the product "b" and reactant "a" before and after purification, respectively.

Peroxide Value (POV), Thiobarbituric Acid Reactive Substances (TBS), and Conjugated Diene (CD) Values. The formation of primary products of lipid oxidation (peroxides) at various stages of purification was evaluated by peroxide value (*20*). POV was calculated as the reactive oxygen content and expressed as millimoles of free iodine per kilogram of lipid. To supplement POV values, the level of lipid peroxidation was measured colorimetrically to indicate the presence of malondialdehyde (MDA, $CHOCH_2CHO$) in thiobarbituric acid reactive substance (TBS) assay (*21*, *22*). TBS was calculated and expressed as milligrams of MDA per kilogram of sample. Conjugated diene (CD), another specific parameter to determine the formation of oxidation products, was used to measure the contents of the conjugated diene and conjugated triene of fatty acid concentrates at 233 and 265 nm, respectively (*23*).

Statistical Analyses. Percentage composition individual fatty acid methyl esters were subjected to a one-way analysis of variance

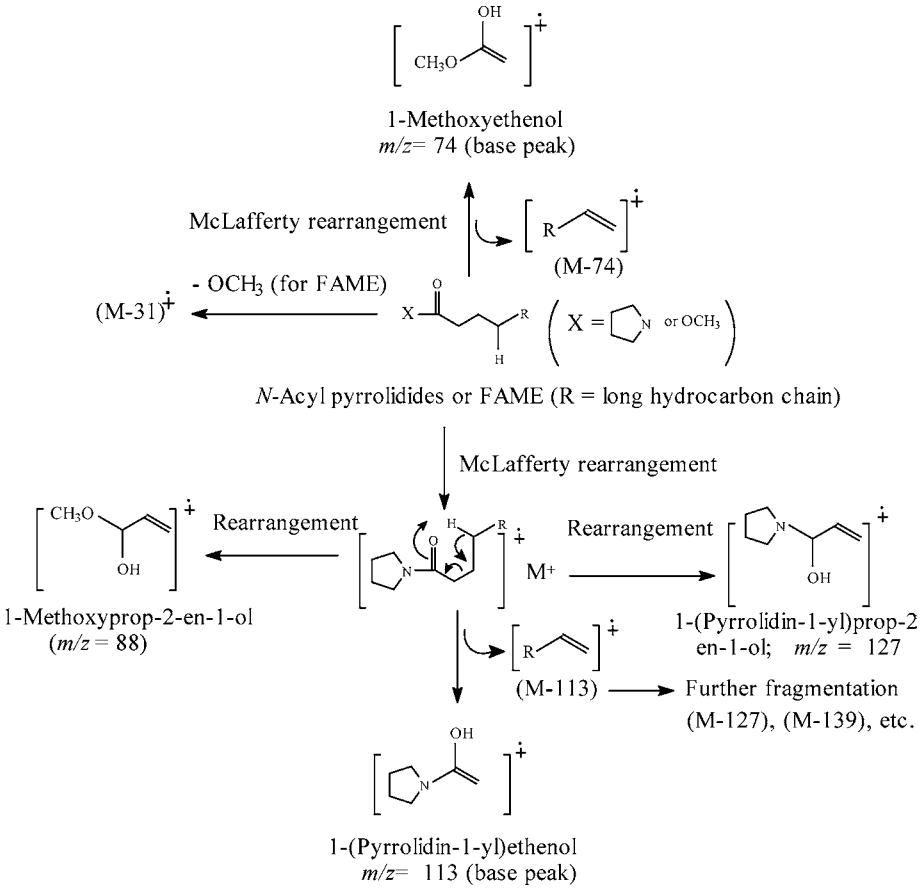

Figure 1. Tentative mass fragmentation pattern of FAME and *N*-acyl pyrrolidides.

(ANOVA) using SPSS (version 10.0) software, and significant means were compared by Tukey's multiple range tests. A significance level of 95% ($p = 0.05$) was used throughout. Arc sin transformation was used prior to statistical analyses of FAME data expressed in percentages. All measurements were performed at least in triplicate ($n = 3$), and values were averaged. On the basis of the significance of treatments LSD at the 5% level of significance ($p = 0.05$) was computed.

RESULTS AND DISCUSSION

Gas Chromatographic−Mass Spectroscopic Analyses of Fatty Acid Methyl Esters and *N*-Acyl Pyrrolidides. GC-MS of FAME was used to determine primarily the chain length and the degree of unsaturation with the molecular ion (M^+). Mass spectrometry of FAME with EI ionization provides meager information only related to the structure of fatty acids, particularly for LC-PUFAs. The molecular ions for SFA and MUFA-FAME were conspicuous as obvious from the mass spectra, and hence the molecular weight is discernible from the EI-MS spectra of FAME. An ion at $(M - 31)^+$ represents the loss of the $-OCH_3$ group, thus confirming the molecular structure as methyl ester. In the mass fragmentation pattern of FAME, the base peak was assigned to be the 1-methoxyethenol moiety ($m/z = 74$) obtained by McLafferty rearrangement per se with the loss of the McLafferty ion ($m/z = 222$) (**Figure 1**). The molecular ion undergoes further rearrangement to furnish 1-methoxyprop-2-en-1-ol ($m/z = 88$). The characteristic ion for SFA is formation of the McLafferty ion ($m/z = 74$) by McLafferty rearrangement, while MUFA methyl esters exhibited the formation of a stable and abundant ion at $M - 74$ after the loss of the McLafferty ion. The spectra further contained lower m/z fragment ions at a difference of $m/z = 14$, definitely supporting the general structure of FAME, viz., $[-CH_3OCO-(CH_2)_nCH_3]^+$ in the homologous series. The McLafferty ion was found to be less conspicuous in EI-MS of LC-PUFAs having more double bonds ($n > 4$), which undergoes complex rearrangement under high-energy EI conditions. The abundance of these fragment ions in SFA and MUFA-FAME was found to be higher than in the EI-MS spectra of LC-PUFAs. Formation of the cyclic tropylium ion ($m/z = 91$) in fatty acids with four or more double bonds was apparent in PUFAs. Cleavage of the tail from the n6 terminal moiety in the methylene-interrupted bond to yield a fragment ion at $m/z = 150$ was found to be characteristic of n6 PUFA methyl esters, while for n3 PUFA methyl esters the characteristic fragment ion was apparent at $m/z = 108$ following cleavage of the tail from the n6 terminal moiety. The EI-MS spectrum of methyl linoleate has an abundant molecular ion ($m/z = 294$) and base peak as the McLafferty ion ($m/z = 74$). In methyl linolenate, a fragment ion at $m/z = 150$ was obvious, which is characteristic for fatty acids with an n6 terminal moiety, while one at $m/z = 108$ defines an n3 terminal group as in methyl eicosapentaenoate and methyl docosahexaenoate. In the mass fragmentation pattern of methyl arachidonate the molecular ion is barely distinguishable, but the characteristic ion for the n3 moiety, at $m/z = 108$, is apparent. In the mass fragmentation pattern of LC-PUFAs with more double bonds ($n \geq 4$), the tropylium ion ($m/z = 91$) does stand out as the diagnostic fragment ion. However, in FAME EI-MS spectra it is difficult to determine the location of the double bond from the spectra. The reason for this is the migration of the double bond along the long aliphatic chain due to destabilization of the positive charge by electron ionization in the mass spectrometer. This drawback is encountered by the EI-MS of fatty acid pyrrolidine derivatives (*N*-acyl pyrrolidides), which minimize the migration, as they are able to stabilize the

charge on the heterocyclic pyrrolidine moiety. In the mass fragmentation pattern of *N*-acyl pyrrolidides, the base peak was assigned to be the McLafferty rearrangement ion, 1-(pyrrolidin-1-yl)ethenol ($m/z = 113$), with the concurrent loss of the M − 113 fragment ion. The molecular ion undergoes further rearrangement to furnish 1-(pyrrolidin-1-yl)prop-2-en-1-ol ($m/z = 127$). A uniform distribution of fragment peaks is apparent at every $m/z = 14$ units, except in the vicinity of the double bond, where the interval is $m/z = 12$ units. For example, in the mass spectrum of 1-(pyrrolidin-1-yl)octadeca-9,12,15-trien-1-one, the double bonds in positions 9 and 12 are located by the gaps of $m/z = 12$ units between ions at $m/z = 196$, 208 and 236, 248, respectively.

Saponification and Extraction of Fatty Acids. Among saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), while 16:1n7 contributed the major share (>31% TFA) among all individual fatty acids in the crude sardine oil (**Table 1**). EPA and DHA were found to be the major n3 PUFAs contributing to 17.8% and 7.67% of TFA, respectively. The n6 fatty acids have a minor share in the total fatty acid content of sardine oil. Solvent extraction resulted in marginal increase of unsaturation (0.85%) in the fatty acid profile, the PUFA exhibited an increase of 6.49%, while MUFA and SFA reduced by 2.96% and 6.91%, respectively (**Table 1**). The n3 fatty acids exhibited an increase of 5.73% in the solvent extract of fatty acids.

LC-PUFA Enrichment by Urea Fractionation. The free fatty acids (34 g) derived from sardine oil were subjected to urea crystallization using methanol as solvent to obtain PUFAs of high purity. Urea complexation of fatty acids is used to concentrate PUFAs from SFAs and MUFAs, where urea occludes straight-chain compounds such as long-chain saturated and monounsaturated fatty acids in a hexagonal crystalline structure, excluding methylene-interrupted polyunsaturated fatty acids (having the −C═C−C−C═C− moiety) due to the irregularities in their molecules caused by the bends at each double bond (*7*). The interfering SFAs and most of the MUFAs (long- and straight-chain molecules) were removed in the form of urea inclusion compound, while the PUFAs remain in solution. Further, as oxidized products do not form urea adducts, the peroxidation of n3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides. The urea inclusion method allows handling of large quantities of material in a more efficient way than fractional crystallization or selective solvent extraction. One of the major parameters quantifying the tendency of fatty acids and esters to combine with urea is the urea adductability values (UAV). The UAV is equal to the carbon number of SFAs and decreases with the unsaturation number. The LC-PUFAs, such as EPA and DHA, have UAV below 4 and, therefore, a very low tendency to form inclusion compounds (*7*).

Different classes of fatty acid composition in the crude sardine oil, solvent-extracted sardine oil, urea concentrate at three different temperatures (2, 4, and 6 °C), and urea/fatty acid ratio (U/FA = 2:1, 3:1, and 4:1 w/w) vis-à-vis sardine oil are depicted in **Table 1**. Among the three different temperatures of crystallization and U/FA ratio, higher U/FA ratio (4:1), irrespective of temperature of crystallization, furnished higher PUFA concentrate with higher recovery. At 6 °C, the total PUFA was found to be 53.59%, with a recovery of 80.81% at the U/FA ratio of 4:1, while at 4 °C the corresponding values were considerably higher ($P^{PUFA}_{c} = 78.35\%$, $Y^{PUFA}_{(b-c)} = 80.28\%$) (**Tables 1** and **2**). The reduction in yield at 2 °C may be due to poor complexation of SFAs and MUFAs, thus furnishing a fatty acid concentrate with comparatively lower PUFA recovery (51.57%). Consequently, the yield of EPA ($Y^{EPA}_{(b-c)}$) was recorded to be higher at 4 °C and U/FA ratio of 4:1 (93.74%) as compared to that at 6 °C (84.37%) (**Table 2**). Linolenic acid (18:3n3) was found to be concentrated at 4 °C and U/FA ratio of 4:1 with a recovery of 53.16%. On the basis of these results, PUFA concentrate obtained from sardine oil at 4 °C temperature of urea crystallization by using a U/FA ratio of 4:1 was selected for subsequent purification by argentation column chromatography.

The purity of 16:1n7, the predominant MUFA, was reduced by 62.4% at the U/FA ratio of 4:1 and a temperature of 4 °C. The yield of total MUFA ($Y^{MUFA}_{(b-c)}$) was found to be 19.04% at the U/FA of 3:1, and at U/FA of 2:1, the recovery was recorded as 29.88%, which were comparatively higher than that obtained at U/FA ratio of 4:1 at 4 °C ($Y^{MUFA}_{(b-c)} = 9.70\%$) (**Table 2**). At higher temperature (6 °C), the urea fractionation could not resolve the SFAs and MUFAs effectively, resulting in lower concentration of PUFAs in the urea concentrate ($Y^{PUFA}_{(b-c)} = 80.81\%$) (**Table 2**). It is likely that, at lower temperature (2 °C), the reaction kinetics to form the urea inclusion complex with SFAs and MUFAs was relatively inefficient, resulting in higher MUFA in the extract. On the contrary, at higher temperature (>2 °C), the urea−fatty acid complex dissociates, resulting in a relatively higher amount of SFA and MUFA in the fatty acid concentrates. The urea complexation reaction raised the EPA content by 2.6-fold, thus allowing further purification by column chromatography.

Separation of LC-PUFAs by Argentation Column Chromatography. Purification of fatty acid methyl esters was performed as these compounds exhibit more stability and better chromatographic behavior than free fatty acids. Normal pressure column chromatography on neutral alumina impregnated with $AgNO_3$ was used to separate different fatty acids according to the differences in their degree of unsaturation. The resolving power of Ag−alumina is largely attributed to a reversible charge-transfer complexation of Ag^+ ion with −C═C− double bonds of unsaturated compounds. The stability of the complex increases with an increasing number of double bonds but decreases with the increasing chain length. The extent and the strength of complexation control the mobility of a solute (fatty acids), as does the polarity of the mobile phase. Under identical conditions, alumina with a greater surface density of silver is better at resolving unsaturated fatty esters than silica gel with a lower Ag^+ loading. The separation achieved by argentation column chromatography was continuously monitored by $AgNO_3$ TLC and GC/GC-MS.

The fatty acid profiles of the various solvent fractions obtained during the course of argentation chromatography are shown in **Table 3**. The fractions containing 5% diethyl ether/*n*-hexane eluted a substantial amount of MUFA esters 16:1n7, 18:1n9, and 17:1 (75.96%), which associates weakly to the stationary phase. The fraction from 25% diethyl ether/*n*-hexane eluted EPA ester of higher purity ($P^{EPA}_{d} = 59.36\%$) and recovery ($Y^{EPA}_{(c-d)} = 30.33\%$). The process was continued by using 50% diethyl ether/*n*-hexane, eluting primarily 20:5n3, and halted the elution of all MUFAs and diunsaturated and triunsaturated methyl esters. Combining the solvent fractions of 5%, 20%, and 50% diethyl ether/*n*-hexane, the final purity of EPA obtained in this step was 99.56%, and the total recovery was calculated as 54.09% (**Table 3**). It is apparent that the preceding urea process removes nearly all SFAs, most of the MUFAs, and also significantly reduces the linoleic (18:2n6) and linolenic (18:3n3) acid levels. The DHA ester having a larger number of double bonds was

Table 1. Percent Fatty Acid Composition of Crude (P^x_a) and Solvent-Extracted Sardine Oil (P^x_b) and PUFA Concentrates Obtained by Urea Fractionation (P^x_c) at Three Different Temperatures (2, 4, and 6 °C) and Urea/Fatty Acid Ratios (U/FA) (2:1, 3:1, and 4:1 w/w)

			% composition of fatty acids in urea concentrate (P^x_c)								
			U/FA at 2 °C			U/FA at 4 °C			U/FA at 6 °C		
fatty acid	CSO[a] (P^x_a)	SE[b] (P^x_b)	2:1	3:1	4:1	2:1	3:1	4:1	2:1	3:1	4:1
						SFAs					
12:0	0.06 ± 0.01	0.05 ± 0.00	0.07 ± 0.02c	0.99 ± 0.04a	0.49 ± 0.15b	ND	ND	ND	ND	ND	ND
14:0	7.04 ± 0.22	6.58 ± 0.39	0.97 ± 0.19c	ND	ND	4.29 ± 0.21b	1.08 ± 0.05c	ND	6.60 ± 0.37a	0.73 ± 0.06c	ND
16:0	0.45 ± 0.09	0.50 ± 0.04	0.55 ± 0.08b	ND	ND	3.68 ± 0.14a	0.41 ± 0.07b	ND	ND	ND	ND
17:0	0.28 ± 0.03	0.15 ± 0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑SFA	7.82	7.28	1.60b	0.99bc	0.49c	7.97a	1.49b	ND	6.60a	0.73c	ND
						MUFAs					
16:1n7	31.56 ± 2.59	30.19 ± 3.17	30.86 ± 1.47a	21.04 ± 2.38ab	12.73 ± 0.63bc	16.91 ± 0.58b	12.80 ± 1.82bc	11.35 ± 1.04c	28.90 ± 1.72a	30.01 ± 2.51a	18.05 ± 1.42b
18:1n9	16.86 ± 1.18	16.79 ± 2.41	17.44 ± 1.15a	16.20 ± 1.36a	8.35 ± 0.49bc	12.22 ± 1.37ab	6.55 ± 0.58b	1.25 ± 0.26c	16.92 ± 0.43a	16.38 ± 1.84a	13.64 ± 2.17a
17:1	0.59 ± 0.08	0.62 ± 0.16	ND	ND	ND	3.95 ± 0.13a	4.11 ± 0.25a	0.17 ± 0.02c	3.00 ± 0.20ab	0.09 ± 0.04c	ND
20:1n11	0.30 ± 0.14	0.24 ± 0.08	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑MUFA	49.30	47.84	48.31a	37.24ab	21.08bc	33.08b	23.45bc	12.78c	48.83a	46.49a	31.69b
						PUFAs					
18:2n6	0.71 ± 0.23	0.96 ± 0.20	2.19 ± 0.32b	1.94 ± 0.17bc	2.09 ± 0.06b	0.86 ± 0.05c	2.22 ± 0.16b	2.23 ± 0.13b	5.75 ± 0.16a	2.28 ± 0.11b	1.17 ± 0.05bc
18:3n3	4.47 ± 0.84	5.11 ± 0.65	1.07 ± 0.25c	4.38 ± 0.52b	5.62 ± 0.18b	4.48 ± 0.33bc	5.34 ± 0.21b	8.33 ± 0.80a	4.86 ± 0.08bc	5.46 ± 0.35b	0.93 ± 0.23c
18:4n3	1.38 ± 0.35	1.40 ± 0.23	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:4n6	0.10 ± 0.02	0.14 ± 0.02	ND	ND	ND	0.22 ± 0.04b	ND	ND	ND	0.55 ± 0.07a	ND
20:5n3	17.80 ± 1.57	18.50 ± 1.54	27.83 ± 2.61bc	29.89 ± 1.48bc	34.96 ± 0.72b	33.63 ± 2.38b	42.96 ± 1.87a	47.78 ± 1.94a	22.37 ± 1.05c	25.80 ± 0.88c	45.71 ± 0.69a
22:5n3	1.14 ± 0.08	1.10 ± 0.22	2.79 ± 0.18ab	3.06 ± 0.09a	4.42 ± 0..16a	1.60 ± 0.16c	3.02 ± 0.08a	2.90 ± 0.61a	1.81 ± 0.19bc	2.36 ± 0.23ab	2.19 ± 0.22b
22:6n3	7.67 ± 1.50	8.21 ± 0.98	15.37 ± 1.03ab	20.34 ± 0.56a	17.03 ± 0.82a	13.33 ± 1.65b	17.73 ± 0.69a	17.11 ± 0.08a	6.28 ± 0.53c	7.31 ± 0.05bc	3.59 ± 0.13c
∑PUFA	33.26	35.42	49.25bc	59.62b	64.11ab	54.11b	71.27a	78.35a	41.09c	43.74bc	53.59b
LSD ($p = 0.05$)	0.95	1.27	0.52	0.67	1.29	2.09	0.83	2.15	1.10	1.48	2.17

[a] CSO implies crude sardine oil. [b] SE implies the solvent extract of sardine oil; individual fatty acid is expressed as a percentage of total identifiable fatty acids. ND = fatty acids identified on the GC trace but not integrated by the instrument. ∑SFA = total saturated fatty acids; ∑MUFA = total monounsaturated fatty acids; ∑PUFA = total polyunsaturated fatty acids. Data presented as mean values of three samples (mean ± standard deviation). Different letters (a–c) within rows indicate significant differences between treatments (ANOVA, Tukey's multiple range test). On the basis of the significance of treatments LSD at the 5% level of significance ($p = 0.05$) was computed. The superscript "x" in P^x_a, P^x_b, and P^x_c represents the individual fatty acids (e.g., EPA) in the products.

Table 2. Percent Yield of Fatty Acids in the Solvent Extract ($Y^x_{(a-b)}$) and Urea Concentrates ($Y^x_{(b-c)}$) at Three Different Urea/Fatty Acid (U/FA) Ratios (2:1, 3:1, and 4:1 w/w) at Three Different Crystallization Temperatures (2, 4, and 6 °C).

		% yield of fatty acids in urea concentrates ($Y^x_{(b-c)}$)								
		CT[a] 2 °C at U/FA			CT 4 °C at U/FA			CT 6 °C at U/FA		
fatty acid	$Y^x_{(a-b)}$	2:1	3:1	4:1	2:1	3:1	4:1	2:1	3:1	4:1
					SFAs					
12:0	56.67 ± 1.94c	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	56.33 ± 1.58b	100 ± 2.87b	437.5 ± 5.40a
14:0	63.56 ± 2.33a	42.16 ± 1.82ab	4.94 ± 0.24c	0.00 ± 0.00	28.17 ± 2.04b	6.38 ± 0.39c	0.00 ± 0.00	5.93 ± 0.42c	0.00 ± 0.00	0.00 ± 0.00
16:0	75.56 ± 2.86ab	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100 ± 4.55a	31.86 ± 2.68c	0.00 ± 0.00	44.26 ± 2.93b	0.00 ± 0.00	0.00 ± 0.00
17:0	36.43 ± 3.19a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
∑SFA	63.30a	38.10b	4.46c	0.00	47.30b	7.95c	0.00	8.84c	5.64c	3.01c
					MUFAs					
16:1n7	65.05 ± 1.55a	40.23 ± 2.03ab	44.23 ± 2.30ab	2.07 ± 0.46c	24.20 ± 0.27b	16.47 ± 1.73c	13.64 ± 0.58c	41.13 ± 1.73ab	28.92 ± 0.37b	18.83 ± 2.33bc
18:1n9	67.72 ± 2.04a	42.35 ± 5.68b	43.41 ± 1.56b	1.56 ± 0.14c	31.45 ± 2.62ab	15.16 ± 2.24c	2.70 ± 0.04c	41.79 ± 0.98b	40.04 ± 2.70b	22.20 ± 1.14bc
17:1	71.46 ± 0.68b	203.4 ± 6.01a	6.46 ± 0.44c	0.00 ± 0.00	100 ± 4.58b	100 ± 2.79b	9.95 ± 0.62c	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1n11	54.40 ± 4.81a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
∑MUFA	65.99a	42.90b	43.24b	3.63c	29.88bc	19.04bc	9.70c	40.63b	32.30bc	19.67c
					PUFAs					
18:2n6	91.94 ± 4.18b	251.7 ± 4.52a	105.7 ± 5.27b	0.13 ± 0.02c	38.71 ± 2.61c	89.85 ± 3.53b	84.31 ± 2.75b	91.79 ± 3.46b	83.86 ± 1.74b	97.20 ± 1.52b
18:3n3	77.74 ± 5.65a	39.97 ± 2.31b	47.55 ± 2.05b	0.11 ± 0.03c	37.88 ± 1.96b	40.60 ± 0.71b	59.16 ± 3.79ab	8.43 ± 0.58c	35.57 ± 2.93bc	49.10 ± 2.35b
18:4n3	68.99 ± 3.11a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4n6	95.20 ± 6.59b	0.00 ± 0.00	174.8 ± 4.88a	0.00 ± 0.00	67.89 ± 3.55c	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5n3	70.67 ± 2.68b	50.82 ± 1.58c	62.06 ± 1.63c	5.24 ± 1.32c	78.54 ± 2.80b	90.22 ± 4.64a	93.74 ± 2.52a	60.53 ± 3.67c	67.05 ± 2.24c	84.37 ± 3.49ab
22:5n3	65.61 ± 3.03	69.16 ± 2.22c	95.47 ± 3.92b	0.25 ± 0.01c	62.84 ± 1.47c	100 ± 5.93b	95.68 ± 3.41b	100 ± 4.72b	100 ± 4.81b	179.4 ± 4.19a
22:6n3	72.79b ± 2.27c	32.15 ± 0.59c	39.62 ± 2.71c	0.41 ± 0.08c	70.15 ± 2.79c	83.91 ± 1.50b	75.64 ± 4.11b	75.32 ± 1.80b	100 ± 3.49a	92.61 ± 2.75a
∑PUFA	72.42a	48.76b	54.95b	6.14c	66.00b	78.18a	80.28a	55.95c	69.85ab	80.81a
others	66.94a	15.46c	42.52b	1.69c	22.11b	15.57bc	33.99b	3.57c	9.43c	67.58a
LSD ($p = 0.05$)	4.54	3.05	3.36	2.76	4.07	3.88	3.97	2.86	1.58	3.76

[a] CT implies crystallization temperature; individual fatty acid is expressed as a percentage of total identifiable fatty acids. $Y^x_{(a-b)}$ = recovery of fatty acids in the solvent extract; $Y^x_{(b-c)}$ = recovery of fatty acids in urea concentrates; all other notations are as indicated under **Table 1**. On the basis of the significance of treatments LSD at the 5% level of significance ($p = 0.05$) was computed. Different letters (a–c) within rows indicate significant differences between treatments (ANOVA, Tukey's multiple range test). Data presented as mean values of three samples (mean ± standard deviation). The superscript "x" in $Y^x_{(a-b)}$ and $Y^x_{(b-c)}$ represents the individual fatty acids (e.g., EPA).

Table 3. Percent Composition (P^x_d) and Recovery ($Y^x_{(c-d)}$) of Fatty Acids in Three Different Eluates Using Three Different Solvent Systems (5%, 25%, and 50% Diethyl Ether in *n*-Hexane) by Argentation Chromatography[a]

	diethyl ether (%)/*n*-hexane (v/v)					
	5%		25%		50%	
fatty acid	P^x_d	$Y^x_{(c-d)}$	P^x_d	$Y^x_{(c-d)}$	P^x_d	$Y^x_{(c-d)}$
			SFAs			
12:0	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
14:0	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
16:0	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
17:0	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
∑SFA	ND	0.00	ND	0.00	ND	0.00
			MUFAs			
16:1n7	43.15 ± 1.29	97.42 ± 0.07	14.74 ± 1.38	31.71 ± 2.38	ND	0.00 ± 0.00
18:1n9	25.84 ± 2.45	100 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
17:1	6.98 ± 0.51	100 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
20:1n11	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
∑MUFA	75.96	98.52	14.74	25.81	ND	0.00
			PUFAs			
18:2n6	1.51 ± 0.26	17.35 ± 1.15	2.13 ± 0.16	23.32 ± 3.49	ND	0.00 ± 0.00
18:3n3	14.12 ± 1.17	43.44 ± 3.04	ND	0.00 ± 0.00	ND	0.00 ± 0.00
18:4n3	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
20:4n6	ND	0.00 ± 0.00	ND	0.00 ± 0.00	ND	0.00 ± 0.00
20:5n3	2.12 ± 0.19	1.14 ± 0.12	59.36 ± 2.24	30.33 ± 2.25	99.56 ± 0.08	54.09 ± 0.15
22:5n3	ND	0.00 ± 0.00	3.47 ± 0.18	29.22 ± 2.09	ND	0.00 ± 0.00
22:6n3	1.43 ± 0.12	2.14 ± 0.26	5.41 ± 0.57	7.72 ± 0.56	0.36 ± 0.03	0.55 ± 0.12
∑PUFA	19.18	15.36	70.37	68.51	99.92	79.72
LSD ($p = 0.05$)	1.32	0.93	1.58	1.20	0.52	0.46

[a] P^x_d = percentage composition of individual fatty acids in the column chromatographic eluates using urea–fatty acid concentrate obtained at 4 °C using U/FA = 4:1; all other notations are as indicated under **Table 1**. On the basis of the significance of treatments LSD at the 5% level of significance ($p = 0.05$) was computed. Data presented as mean values of three samples (mean ± standard deviation). ND = fatty acids identified on the GC trace but not integrated by the instrument.

found to coelute with EPA in this final fraction containing 50% diethyl ether/*n*-hexane.

Silver Ion Thin-Layer Chromatography ($AgNO_3$ TLC). TLC was used as a first-hand tool to know about the progress of purification of PUFAs from other monoenoic and saturated fatty acids during argentation column chromatography. The results were confirmed by using GC/GC-MS to know about the individual fatty acids. The first eluent from column chromatography using 5% diethyl ether/*n*-hexane reveals the separation of FAME up to trienes (18:3n3), the more unsaturated such as 18:2n6 and 18:3n3 were found to be staying at the base of TLC chromatogram (using *n*-hexane/diethyl ether/acetic acid, 94:5:1 v/v/v, as developing solvent). The uppermost band (R_f: 0.86) of saturated fatty acids and the second band of monoenoic fatty acids (R_f: 0.49) were apparent. Tetraene, pentaene, and hexaene methyl esters appeared to be concentrated to >90% in the lower band of the TLC plates (R_f: 0.18−0.33). The second eluent (25% diethyl ether/*n*-hexane) enabled the complete separation of all FAMEs except the saturated and monoenes which comigrate near the solvent front. The eluates from the chromatographic column using 50% diethyl ether/*n*-hexane reveal the separation of EPA from other fatty acids as evident from the TLC chromatogram (R_f: 0.23).

Lipid Oxidation. Lipid oxidation is a complex process in which unsaturated fatty acids react with molecular oxygen, usually via a free radical mechanism, to form hydroperoxides, the primary oxidation products (*24*, *25*). Peroxide, conjugated diene, and TBS values were used as indices to assess the level of lipid oxidation in sardine oil. Changes in peroxide and TBS values of sardine oil during various stages of purification are shown in **Table 4**. The peroxide value measures the formation of peroxide or hydroperoxide groups that are initial products of lipid oxidation. Hydroperoxides decompose to create an assortment of nonvolatile and volatile compounds, such as polymeric triacylglycerides and aldehydes, respectively (*26*).

Table 4. Changes in Various Lipid Oxidation Parameters [Peroxide Value (POV) and Thiobarbituric Acid Reactive Substances (TBS)] during Downstream Processing of EPA

	POV (mequiv of O_2/kg)	TBS (mg of malondialdehyde/kg)
solvent extraction		
solvent extract of fatty acids	4.1 ± 0.28	6.8 ± 0.83
urea crystallization[a]		
crystallization temp 2 °C		
U/FA, 2:1	2.6 ± 0.09	4.1 ± 0.38
U/FA, 3:1	2.8 ± 0.16	3.8 ± 1.07
U/FA, 4:1	4.2 ± 0.37	4.3 ± 0.95
crystallization temp 4 °C		
U/FA, 2:1	2.3 ± 0.04	3.2 ± 0.18
U/FA, 3:1	3.5 ± 0.10	3.6 ± 0.29
U/FA, 4:1	3.9 ± 0.27	3.6 ± 0.62
crystallization temp 6 °C		
U/FA, 2:1	4.0 ± 0.88	5.2 ± 0.09
U/FA, 3:1	5.8 ± 0.53	6.9 ± 1.25
U/FA, 4:1	6.3 ± 1.32	9.4 ± 1.37
column chromatography[b]		
5% diethyl ether/hexane	2.1 ± 0.12	3.0 ± 0.22
20% diethyl ether/hexane	2.9 ± 0.37	2.8 ± 0.17
50% diethyl ether/hexane	2.6 ± 0.08	2.1 ± 0.44
LSD ($p = 0.05$)	0.88	1.03

[a] POV and TBS values as a factor of temperature of crystallization (2, 4, and 6 °C) at three different urea/fatty acid ratios (2:1, 3:1, and 4:1) vis-à-vis sardine oil and solvent extract of fatty acids. [b] POV and TBS values of the eluates from argentation column chromatography (5%, 25%, and 50% diethyl ether in *n*-hexane) as compared to the fatty acid concentrate obtained in urea fractionation at 4 °C using a urea/fatty acid ratio of 4:1. Data presented as mean values of three samples (mean ± standard deviation).

Peroxide values were found to be very marginal in fatty acid concentrates during solvent extraction (4.1 mequiv of O_2/kg). This may be attributed to the antioxidant activity of TBHQ used

in the oil and lower atmospheric exposure. The POV of fatty acid concentrates rose to 6.3 mequiv of O_2/kg at higher crystallization temperature (6 °C) and urea/fatty acid ratio (4:1) and declined during subsequent steps of purification. No significant changes in POV were apparent during urea fractionation at lower temperatures (2 and 4 °C) (**Table 4**). The peroxidation at higher crystallization temperature (6 °C) for urea fractionation was probably due to the release of prooxidants from the oil (*9*). A decrease in POV (2.53 ± 0.19 mequiv of O_2/kg) was noticeable during column chromatographic purification of fatty acids.

Thiobarbituric acid (TBS) values have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes. The TBS value, which measures the malondialdehyde concentration (end product of lipid oxidation) followed a similar pattern. The initial value of TBS was found to be 6.8 mg of malondialdehyde/kg, suggesting that marginal lipid oxidation occurred during solvent extraction (**Table 4**). Higher TBS values were apparent at the higher temperature of crystallization (6 °C) during the course of urea−fatty acid complexation, thus indicating the formation of secondary lipid oxidation products at a higher temperature. Gray (*27*) reported that the TBS value correlate well with the peroxide value only in oils containing fatty acids with ≥3 double bonds such as sardine oil fatty acid concentrate.

No significant difference in conjugated diene values of sardine oil fatty acid concentrates was apparent during the purification process. Diene absorbance at 233 nm and triene absorbance at 265 nm were effected in order to discard EPA degradation. In almost all cases, the absorbance for both wavelengths was negligible, demonstrating the absence of EPA degradation during the downstream processing. Following solvent extraction, a marginal increase in lipid oxidation was obvious during urea fractionation at higher temperature (6 °C) as evidenced by the slight increase in the conjugated diene value. During oxidation of PUFA-containing methylene-interrupted dienes and polyenes, a shift in the double bond positions due to isomerization and conjugate bond formation was apparent due to conversion of the nonconjugated double bonds (−C═C−C−C═C−) in unsaturated fatty acids to conjugated ones (−C═C−C═C−). This was accompanied by increased UV absorption at 233 nm for diene unsaturation and at 265 nm for triene unsaturation. A good correlation has been reported between conjugated diene values and POV, thus suggesting that lower temperature urea crystallization (i.e., 4 °C) enhanced the antioxidant effect of TBHQ as compared to that of higher temperature (6 °C).

In conclusion, the present study enabled the development of a process of two-step purification for achieving concentrated EPA from sardine oil using urea complexation followed by argentation chromatography on neutral alumina. Among three different temperatures of crystallization and U/FA, higher U/FA ratio (4:1 w/w), irrespective of temperature of crystallization, furnished higher recovery of PUFA as well as EPA. The highest concentration of EPA (47.78%) was obtained at the crystallization temperature of 4 °C with a U/FA ratio of 4:1 (w/w) with a recovery of 93.74%. After complexation of saturated and less unsaturated free fatty acids by urea complexation, argentation neutral alumina column chromatography resulted in EPA of high purity (99.6%) with an overall recovery of 54.09% using 50% diethyl ether/*n*-hexane as eluting solvent. The oxidative stability of fatty acid concentrates at different stages of purification revealed that no significant changes in POV, TBS, and CD values were found during urea fractionation at lower temperatures (2 and 4 °C). This established procedure including preparation and purification of EPA would contribute to the industrial application to obtain concentrated EPA.

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