JOURNAL OF AQUATIC FOOD PRODUCT TECHNOLOGY
2017, VOL. 26, NO. 9, 1042–1056
https://doi.org/10.1080/10498850.2017.1375588

Taylor & Francis
Taylor & Francis Group

Enrichment of C_{20-22} Polyunsaturated Fatty Acids from Refined Liver Oil of Leafscale Gulper Shark, *Centrophorus squamosus*

Dexy Joseph[a,b‡] and Kajal Chakraborty[a‡]

[a]Marine Bioprospecting Section of Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin, India; [b]Department of Biosciences, Mangalore University, Mangalagangothri, Karnataka State, India

ABSTRACT

The C_{20-22} polyunsaturated fatty acids (C_{20-22} PUFAs), mainly eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), were concentrated from the refined liver oil of deep sea leafscale gulper shark, *Centrophorus squamosus*, by sequential processes of winterization, urea complexation, and argentation chromatography. Winterization at 4°C using acetonitrile as solvent showed significant reduction of the total saturated fatty acid content (12%) with a concomitant increase of polyunsaturated fatty acids (36.3%). The urea complexation process significantly enriched the total polyunsaturated fatty acids (49.5%), with a reduction in saturated fatty acids (83.9%). Silica gel based argentation chromatography further concentrated the esters of C_{20-22} PUFA (>99 percent purity). Nuclear Magnetic Resonance spectroscopy was used as a chemical fingerprinting tool to study the enrichment of C_{20-22} PUFAs at various stages of the purification process. An increase in the proton signal intensity at the olefinic region (at δ 4–6) and a decrease in the aliphatic signals (at δ 0.5–2) showed that the process successfully enriched the C_{20-22} PUFAs.

Abbreviations: PUFA, polyunsaturated fatty acid; **EPA**, eicosapentaenoic acid; **DHA**, docosahexaenoic acid; **FAME**, fatty acid methyl esters; **SFA**, saturated fatty acid; **MUFA**, monounsaturated fatty acid; **NMR**, nuclear magnetic resonance; **GCMS**, gas chromatography mass spectrometry; **FFA**, free fatty acid.

KEYWORDS

C_{20-22} polyunsaturated fatty acids; deep sea leafscale gulper shark; *Centrophorus squamosus*; winterization; urea complexation; argentation chromatography

Introduction

The health benefits of C_{20-22} polyunsaturated fatty acids (PUFA), particularly EPA (eicosapentaenoic acid, 20:5) and DHA (docosahexaenoic acid, 22:6), has increased fish consumption and increased the demand for the PUFA concentrates on a large scale. These essential fatty acids regulate the major biological functions in humans, and their absence can lead to a wide range of diseases, including cardiovascular and auto immune disorders, inflammatory dysfunctions, viral infections, cancer, and other deadly diseases (WHO/FAO, 1980). Deep sea shark liver oil is known for its long-chain C_{20-22} PUFAs, along with other components like alkylglycerols (Bordier et al., 1996) and triterpenes (Davenport and Deprez, 1989). Crude fish oil has been reported to contain unwanted substances, such as saturated fats, cholesterol, pigments, and protenacious matter, which renders them unsuitable for direct use (Ackman et al., 1988). The C_{20-22} PUFAs should be purified from these undesirable raw materials for further utilization.

Several methods were previously reported to acquire fractions rich in PUFAs from crude fish oils. Differences based on polarity or spatial configurations of the fatty acids are the commonly

CONTACT Kajal Chakraborty kajal_cmfri@yahoo.com Marine Biotechnology Division, Central Marine Fisheries Research Institute, Ernakulam North, P.B. No. 1603, Cochin, India.

‡These authors contributed equally to this work.

used methods. These variations are usually related to the presence of double bonds, and therefore, the C_{20-22} PUFAs can be fractionated according to their unsaturation level (Kates, 1972). Physical and chemical methods, such as winterization (Yokochi et al., 1990), urea complexation (Guil-Guerrero and Belarbi, 2001), and enzymatic purification (Shimada et al., 2001; He et al., 2016, 2017) have been reported as commonly available methods to concentrate the long-chain PUFAs. The winterization of oils removes higher melting glycerides, and thus, the crystallized glycerides can be removed by filtration (Gooding, 1962). Urea complexation efficiently concentrates fatty acids with different unsaturation levels (mainly saturated and monounsaturated) by the conversion of tetragonal structured urea to hexagonal crystals with a spiral shaped channel that entraps linear molecules (Hongyan et al., 2016). Argentation chromatography is an easy method to procure EPA and DHA with maximum purity (Guil-Guerrero et al., 2000, 2003). The double bonds in the fatty acids interact with the silver ion (Ag^+) to form fatty acid–silver complexes. The strength of fatty acid–silver complexes was found to be directly proportional with the increasing number of double bonds. Using different solvents and solvent ratios, these complex fatty acids can be eluted based on the number, position, and geometric configuration of double bonds (Li et al., 2009). Argentated silica gel column chromatography has been used to obtain high-purity EPA (Belarbi et al., 2000), DHA (Guil-Guerrero et al., 2001), α-linolenic acid, and stearidonic acid (Ryu et al., 1997) from a variety of fatty acid esters.

Research efforts are continuing to focus on the techniques needed to improve the yield and purity of the long-chain C_{20-22} PUFAs from various marine sources. The leafscale gulper shark belongs to elasmobranch fish species and is known for its abundance of bioactive lipids, such as alkyl glycerol (1-*O*-alkylglycerols), C_{20-22} group of polyunsaturated fatty acids, and squalene content, which contribute to antitumor, immunostimulating, for and hematopoietic properties (Mitre et al., 2005; Szostak and Szostak-Wegierek, 2005). The liver oil from leafscale gulper shark could be a potential source of the C_{20-22} group of polyunsaturated fatty acids. Depending on the shark species, the oil could be rich in 1-*O*-alkylglycerols, the C_{20-22} group of polyunsaturated fatty acids, or squalene. While the chemistry of 1-*O*-alkylglycerols and squalene of the shark and its liver oil have been extensively studied by many researchers (Bordier et al., 1996; Bakes and Nichols, 1996; Deprez et al., 1990; Shimma and Shimma, 1966), there is a lack of information regarding the liver oil composition and C_{20-22} group of polyunsaturated fatty acid profile of many of the shark species, particularly the leafscale gulper shark. Moreover, there have been no comprehensive studies describing the process to concentrate the C_{20-22} group of polyunsaturated fatty acids from shark liver oil. The present study reports the enrichment of C_{20-22} PUFAs from refined liver oil of deep sea leafscale gulper shark, *Centrophorus squamosus* Bonnaterre, 1788 (family Centrophoridae), by the sequential processes of winterization at different temperatures and solvent conditions followed by urea complexation methods. The concentrated C_{20-22} PUFAs were further fractionated by silica gel based argentation chromatography. Nuclear Magnetic Resonance (NMR) spectroscopy based chemical fingerprinting analysis was used to study the enrichment of C_{20-22} PUFAs at various stages of the purification process.

Materials and methods

Materials

Fresh samples of *C. squamosus* were collected from the southwest coast of India (9.97°N, 76.28°E), and their livers were hygienically collected after washing with sterile water. The reagents, solvents, and chemicals used in the study were of analytical grade and procured from E-Merck (Darmstadt, Germany) and/or Sigma-Aldrich (St. Louis, MO, USA).

Extraction and refining of leafscale gulper shark liver oil

Crude oil from *C. squamosus* was prepared by homogenizing the tissue (1 kg); then, the oil was extracted from the homogenate by centrifugation at 8,500 × *g* for 10 min (Thermo Scientific, Biofuge Stratos, Germany). Crude liver oil was subjected to a step-wise refining process of degumming, decolorization, and deodorization (Bimbo, 1998) to produce the refined oil. Briefly, about 100 g of refined oil was saponified by methanolic KOH with constant stirring for 45 min in an inert atmospheric condition using N_2 gas (Haagsma et al., 1982). Water was added to the hydrolyzed mixture, and the unsaponifiable part was separated with a non-polar solvent (*n*-hexane, 3 × 100 ml). The pH of the lower hydromethanolic phase bearing the saponified fatty acids was adjusted to 1.0 with acidified water (HCl–H_2O 1:1 v/v, 150 ml), and the free fatty acids (FAA) were retrieved by extraction with *n*-hexane (3 × 100 ml), washed with water (150 ml) to neutral pH, and dried using anhydrous Na_2SO_4. The residual solids were separated using a Buchner funnel, and the solvents were removed by concentrating in a rotary vacuum evaporator (Heidolph, Germany) to obtain the resultant refined oil in its free fatty acid form (Chakraborty et al., 2016).

Concentration of long-chain C_{20-22} PUFAs

The C_{20-22} PUFAs in the above obtained FFA form of refined oil (100 g) was concentrated by employing winterization (physical) and urea complexation (chemical) techniques. The refined oil in its FFA form was treated with different solvents of variable polarities (acetone, acetonitrile, ethyl acetate, and methanol) at different crystallization temperatures (−20 and 4°C) for a contact time of about 12 h. The liquid phase was separated, filtered through Buchner funnel, dried over anhydrous Na_2SO_4, before being concentrated at 45–50°C to obtain the winterized fatty acids. The optimum winterization method was selected by analyzing the fatty acid composition of the concentrated liquid phase. The fatty acid profile of winterized oil was analyzed by converting the triglycerides to their respective methyl esters. Briefly, 0.5 g oil was subjected to saponification by refluxing with 0.5 N methanoic KOH (5 ml, 20 min, 60°C), and the saponifiable matter was further reacted with methylation reagent (14% BF_3/CH_3OH, 2 ml) for 5 min. The resulting methyl esters of fatty acids (FAMEs) were extracted in *n*-hexane/H_2O (1:2, v/v), and the upper *n*-hexane layer was dried under anhydrous sodium sulfate (Na_2SO_4). The dried hexane layer was injected on a gas chromatograph (PerkinElmer, Shelton, CT, USA) connected with a flame ignition detector and capillary column (SP-2560, 100 m × 0.25 mm i.d., 0.50 μm film) as described earlier (Chakraborty et al., 2016). The individual fatty acid methyl ester was identified by comparing the retention time of the samples with FAME standards (Supelco 37 Component FAME Mix, catalog no. 47885-U, Bellefonte, PA, USA), and the results were expressed as percent of the total fatty acids (%). The optimum winterization method was further corroborated by the results obtained by UV spectrometric analysis.

The winterized fatty acids (WFA) were further concentrated to a greater purity of long-chain C_{20-22} PUFAs using urea complexation. Urea solution was made by adding 150 g of urea to 1 L of 95% MeOH. The WFAs (50 g) were added to the urea solution and heated to 65 ± 5°C with continuous stirring until a clear and homogeneous solution was obtained. The urea solution was cooled to room temperature (at a cooling rate of 0.5°C/min) and subjected to crystallization at two different temperatures (10°C and −20°C). The urea crystals (UC) thus formed were removed from the mother liquor by vacuum filtration. The filtrate was concentrated using a rotary vacuum evaporator to obtain concentrated fatty acids enriched with C_{20-22} PUFAs (CFA). The residual oil obtained was added with deionized water (150 ml) and acidified with dilute HCl (6N, 30 ml) to pH 1–2 under continuous stirring so that traces of urea and MeOH could be eliminated. The fatty acids were extracted with *n*-hexane (3 × 100 ml) in a separating funnel by the process of phase separation. The PUFAs found in the upper *n*-hexane layer were collected and dried over anhydrous Na_2SO_4. The resultant concentrated fatty acids were redissolved in MeOH, flushed with N_2, and stored at −20°C for further fractions.

The fatty compositions of UC and CFA were analyzed by converting to their methyl ester as described in the previous section.

Trans-esterification of concentrated fatty acids

About 10 g of concentrated fatty acids were *trans*-esterified to prepare the concentrated fatty acid methyl esters (CFAMEs) of the triglycerides by refluxing with a Lewis acid catalyst (14% BF_3) in MeOH for 30 min. The reaction was carried out in a heating mantle (45–50°C) in an inert atmospheric condition using N_2. After refluxing, the CFAMEs were cooled under tap water and added with distilled water (20 ml). The fatty acids were extracted with a nonpolar solvent (*n*-hexane), and the upper hexane layer was separated before being washed with distilled water (3 × 100 ml) and concentrated. The resultant CFAMEs (with a recovery yield of 9.4 g) were flushed with N_2 and stored at −20°C for further processing.

Purification of CFAMEs by silica-silver nitrate chromatography

The CFAMEs were separated by normal pressure vacuum liquid chromatography with $AgNO_3$-impregnated neutral silica gel as the stationary phase (Guil-Guerrero and Belarbi, 2001; Hongyan et al., 2016). In brief, silver nitrate solution was prepared by dissolving 10 g of $AgNO_3$ powder in 60 ml ethanol (80% v/v) and stirred for about 10 min. About 50 g of silica gel (0.06–0.2 mm, 70–230 mesh size) was added with 95% ethanol (100 ml) to prepare the silica slurry. This slurry was added with the silver nitrate ($AgNO_3$) solution and subjected to continuous stirring for 2 h to make a homogenous mixture. Ethanol was evaporated under vacuum at 60°C, and the silver impregnated silica was allowed an overnight activation (110°C) in the hot air oven. The silver nitrate-silica powder thus obtained was cooled and kept in the dark in a desiccator until further use. A slurry of silver-impregnated silica (5 g) prepared in *n*-hexane (5 ml) was added into a water-jacketed column (15 cm × 50 mm) previously half-filled with *n*-hexane. The packed height of glass chromatography column (diameter 0.5 cm) was maintained at 9 cm. The concentrated methyl ester (5 g) was diluted in *n*-hexane (5 ml) and added on the column. The column was eluted with sequence of solvents *n*-hexane and acetone with increasing polarity of acetone {100% hexane (F1), 99% hexane: 1% acetone (F2), 97% hexane: 3% acetone (F3), 95% hexane: 5% acetone (F4), 92% hexane: 8% acetone (F5), 90% hexane: 10% acetone (F6), 70% hexane: 30% acetone (F7), 50% hexane: 50% acetone (F8), and 100% acetone (F9)}. The column fractions were dried over anhydrous Na_2SO_4 and concentrated to obtain $C_{20\text{-}22}$ PUFA enriched fatty acids.

Nuclear magnetic resonance (^{1}H-NMR) spectral analysis

The ^{1}H-NMR spectral data of the crude triglyceride extracted from leafscale gulper shark liver and those obtained at various stages of refining process were recorded on a NMR spectrometer (BrukerAvance III, 400 MHz, Bruker, Ettlingen, Germany) equipped with suitable probes (5 mm) using $CDCl_3$ as aprotic NMR solvent, with tetramethylsilane (TMS, Cortec, Paris, France) as an internal standard (δ_{TMS} 0 ppm). The ^{1}H-NMR coupling constants (*J*, expressed as Hz) were recorded in order to understand the resonance splitting characteristics and geometrical isomerism of non-conjugated olefinic bonds. The ^{1}H-NMR data were acquired and processed with BrukerTopSpin™ and MestReNova (Mestrelab Research S.L., ver. 7.1.1-9649) software, respectively.

Statistical methodologies

The statistical evaluations were performed with Statistical Program for Social Sciences 13.0 (SPSS Inc., Chicago, IL, USA; ver. 13.0). Analyses were carried out in triplicate, and the means of all

parameters were examined for significance by analysis of variance (ANOVA). The level of significance for all analyses was $p \leq 0.05$.

Results and discussion

Fatty acid profile of CFA after winterization and urea complexation

The refined oil extracted from leafscale gulper shark liver after low temperature crystallization with acetonitrile at 4°C (with a recovery yield of 67.5 g from 100 g of saponified free fatty acids) showed a greater reduction in the total SFA (∑SFA) (12.49%) compared to the control (33.08%) and those added with other solvents at different temperatures ($p < 0.05$) (Table 1). The ∑MUFA content showed no significant change after the winterization process. The aggregate polyunsaturated fatty acid (∑PUFA) content increased by 36.3% (in relation to control) when solvent acetonitrile was used at 4°C and was found to be higher when compared to other winterization conditions. However, the percent recovery of ∑PUFA content was found to be significantly less (in relation to control) when acetonitrile at −20°C was used (29.7%). Acetone at 4°C produced a result similar to acetonitrile at −20°C (29.7% increment of ∑PUFA). Increases in the contents of $C_{20\text{-}22}$ *n*-3 PUFAs were apparent after winterization in all the combinations, with a maximum increase (29.5%) when solvent acetonitrile was used (Table 1). Notably, the abundance of double bonds in $C_{20\text{-}22}$ fatty acids makes them more polar, and therefore, they exhibited greater solubility in polar solvents, such as acetonitrile and acetone (Hongyan et al., 2016). The present study showed that the winterization process using acetonitrile could effectively eliminate the higher melting point of saturated glycerides from the oil by crystallizing (4°C) them out, with the separation of the unsaturated fatty acids in the liquid. Hwang and Liang (2001) purified ethyl esters from the visceral oil of *Illex argentinus* oil and *Ommastrephes bartrami*. Hongyan et al. (2016) enriched *n*-6 docosapentaenoic acid (DPA, 22:5*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3) from *Schizochytrium* sp. oil by low-temperature solvent crystallization using acetonitrile. They also obtained the majority of PUFA contents in the liquid fractions, while SFAs and MUFAs were found to be prominent in the solid fractions.

The fatty acid obtained after winterization was subjected to urea complexation (with a recovery yield of 23g from 50g of winterized fatty acids), which showed a greater reduction in the content of the total SFAs (83.9%) (Table 2). The ∑PUFA content was significantly increased (49.5%) after urea complexation; notably, the total content of $C_{20\text{-}22}$ *n*-3 PUFAs exhibited a greater increase (49%). No significant increase in the *trans* fatty acid content in the fatty acid concentrate was apparent after winterization followed by urea complexation, which signified that the process did not convert the natural *cis* (*Z*) fatty acids to their *trans* (*E*) geometric isomeric forms during the concentration process. Previous studies demonstrated the use of urea complexation technique to concentrate methyl esters of PUFAs from fatty acid mixtures containing SFAs and MUFAs (Christie, 1982). Urea is crystallized in the presence of long-chain aliphatic compounds and incorporates these compounds within its hexagonal crystals to form a "urea complex." Thus, it allows these long-chain compounds to be easily removed from the solution. The methyl esters of the saturated fatty acids form urea complexes more readily than the methyl esters of unsaturated fatty acids. The present study demonstrated that the urea complexation process using urea: fatty acid at the ratio of 3:1 (with a recovery of 58%) was found to be ideal to attain the maximum enrichment of $C_{20\text{-}22}$ *n*-3 PUFAs from the refined liver oil of deep sea leafscale gulper shark.

Spectral analysis of CFA after winterization and urea complexation

Oil refining may cause changes in the position of double bonds present in the unsaturated fatty acids forming conjugated double bonds. These conjugated double bond functionalities show a typical absorption at 260–280 nm in UV spectrum, whereas PUFAs with methylene interrupted (non-conjugated) double bonds do not show absorption at this region (Chakraborty et al., 2016).

Table 1. Fatty acid profile of refined liver oil of deep sea leafscale gulper shark *C. squamosus* in different conditions of winterization.

Fatty acids	VDO2	Acetone 4°C	Acetone -20°C	Ethyl acetate 4°C	Ethyl acetate -20°C	Methanol 4°C	Methanol -20°C	Acetonitrile 4°C	Acetonitrile -20°C
					Saturated				
14:0	6.01 ± 0.02	4.96 ± 0.02^{a}	4.64 ± 0.03^{b}	4.38 ± 0.03^{c}	5.02 ± 0.03^{a}	4.65 ± 0.02^{b}	4.6 ± 0.03^{b}	4.8 ± 0.02^{d}	4.64 ± 0.03^{b}
16:0	20.64 ± 0.01	11.5 ± 0.02^{b}	18.32 ± 0.03^{c}	15.41 ± 0.15^{d}	18.5 ± 0.02^{c}	18.08 ± 0.02^{c}	20.03 ± 0.02^{e}	5.49 ± 0.03^{f}	6.88 ± 0.03^{g}
18:0	6.41 ± 0.01	6.78 ± 0.02^{a}	7.77 ± 0.15^{b}	6.48 ± 0.03^{c}	8.08 ± 0.03^{d}	7.35 ± 0.04^{e}	8.45 ± 0.02^{f}	1.56 ± 0.02^{g}	8.47 ± 0.04^{f}
∑SFA	**33.08 ± 0.01**	**25.56 ± 0.05^{a}**	**33.2 ± 0.02^{b}**	**29.13 ± 0.02^{c}**	**33.71 ± 0.03^{d}**	**32.73 ± 0.03^{e}**	**35.81 ± 0.02^{f}**	12.49 ± 0.03^{g}	**21.49 ± 0.03^{h}**
					Monounsaturated				
16:1*n*-7 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:1*n*-7 *cis*	8.38 ± 0.01	7.96 ± 0.02^{a}	6.56 ± 0.09^{b}	7.35 ± 0.03^{c}	6.88 ± 0.16^{d}	7.75 ± 0.04^{a}	6.83 ± 0.02^{d}	5.93 ± 0.02^{e}	8.53 ± 0.02^{f}
18:1*n*-9 *trans*	ND	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	ND	0.02 ± 0.00^{a}
18:1*n*-9 *cis*	22.06 ± 0.05	23.65 ± 1.73^{a}	21.62 ± 0.01a^{b}	22.32 ± 0.03^{ab}	20.31 ± 0.03^{b}	22.85 ± 0.04^{ac}	21.36 ± 0.03^{bc}	20.49 ± 0.04^{d}	24.83 ± 0.02^{ae}
22:1*n*-9	0.02 ± 0.00	3.75 ± 0.05^{a}	3.24 ± 0.04b^{d}	3.4 ± 0.01^{c}	3.18 ± 0.04^{d}	3.34 ± 0.05b^{c}	3.18 ± 0.04^{d}	3.42 ± 0.02^{e}	3.63 ± 0.02^{a}
∑ MUFA	**30.5 ± 0.02**	**37.6 ± 0.09^{a}**	**32.01 ± 0.01^{b}**	**33.55 ± 0.07^{c}**	**30.93 ± 0.04^{d}**	**34.4 ± 0.02^{e}**	**31.89 ± 0.04^{f}**	30.22 ± 0.03^{g}	**37.6 ± 0.03^{h}**
					Polyunsaturated				
18:2*n*-6 *trans*	ND	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.02 ± 0.01^{b}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.01 ± 0.01^{b}
18:2*n*-6 *cis*	0.44 ± 0.01	1.27 ± 0.01^{a}	1.05 ± 0.05^{b}	1.2 ± 0.01^{c}	1.11 ± 0.01^{d}	1.12 ± 0.00^{d}	1.09 ± 0.14^{b}	2.42 ± 0.03^{e}	0.94 ± 0.01^{f}
20:4*n*-6	0.19 ± 0.01	0.47 ± 0.00^{a}	0.38 ± 0.00^{b}	0.43 ± 0.00^{c}	0.41 ± 0.01^{d}	0.08 ± 0.00^{e}	0.37 ± 0.00^{b}	0.54 ± 0.00^{f}	0.42 ± 0.00^{d}
20:5*n*-3	0.01 ± 0.00	5.32 ± 0.01^{a}	4.65 ± 0.01^{b}	4.86 ± 0.00^{c}	4.71 ± 0.01^{d}	4.81 ± 0.01^{e}	4.63 ± 0.00^{b}	7.98 ± 0.01^{f}	7.21 ± 0.02^{g}
22:5*n*-3	8.36 ± 0.06	1.84 ± 0.01^{a}	0.09 ± 0.00^{b}	0.21 ± 0.00^{c}	0.22 ± 0.00^{c}	0.09 ± 0.00^{b}	1.75 ± 0.00^{d}	0.19 ± 0.00^{c}	1.77 ± 0.02^{d}
22:6*n*-3	15.51 ± 0.01	23.51 ± 0.02^{a}	23.06 ± 0.03^{b}	24.29 ± 0.03^{c}	21.74 ± 0.01^{d}	20.98 ± 0.02^{e}	19.77 ± 0.03^{f}	25.96 ± 0.05^{g}	23.09 ± 0.03^{b}
∑ PUFA	**25.6 ± 0.01**	**35.09 ± 0.04^{a}**	**31.92 ± 0.03^{b}**	**33.73 ± 0.02^{c}**	**30.62 ± 0.03^{d}**	**29.71 ± 0.04^{e}**	**29.95 ± 0.04^{f}**	**40.2 ± 0.02^{g}**	**36.42 ± 0.01^{h}**
					Fatty acid indices				
∑Trans	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{b}	0.04 ± 0.00^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}
∑*n*-3	23.94 ± 0.02	30.79 ± 0.03^{a}	28.00 ± 0.00^{b}	29.69 ± 0.18^{c}	26.89 ± 0.03^{d}	26.19 ± 0.07^{e}	26.27 ± 0.03^{e}	34.44 ± 0.02^{f}	32.26 ± 0.05^{g}
∑*n*-6	1.66 ± 0.01	4.31 ± 0.00^{a}	3.91 ± 0.04^{b}	4.15 ± 0.02^{a}	3.87 ± 0.12^{b}	3.59 ± 0.04^{c}	3.75 ± 0.03^{b}	5.74 ± 0.02^{d}	4.15 ± 0.01^{a}
∑*n*-3/*n*6	14.51 ± 0.01	7.19 ± 0.04^{a}	7.22 ± 0.01^{b}	7.14 ± 0.01^{a}	7.21 ± 0.03^{ab}	7.4 ± 0.02^{c}	7.05 ± 0.01^{d}	6.01 ± 0.01^{e}	7.8 ± 0.02^{f}
EPA+DHA	23.9 ± 0.01	28.83 ± 0.03^{a}	27.72 ± 0.03^{b}	29.11 ± 0.01^{c}	26.43 ± 0.03^{d}	25.82 ± 0.03^{e}	24.41 ± 0.02^{f}	33.91 ± 0.01^{g}	30.22 ± 0.03^{h}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA – Total saturated fatty acids; ∑MUFA – Total monounsaturated fatty acids ∑PUFA – Total polyunsaturated fatty acids; ∑*Trans* – Total *trans* fatty acids; VDO2– Refined oil after deodorization; Means with different superscripts (a–h) in the same row indicates a statistical difference ($p < 0.05$). ND - not detected.

Table 2. Fatty acid profile of refined liver oil of deep sea leafscale gulper shark *C. squamosus* in different fractions obtained after argentation chromatography.

Fatty acids	CFA	100% Hexane (F1)	99% Hexane (F2)	97% Hexane (F3)	95% Hexane (F4)	92% Hexane (F5)	90% Hexane (F6)	70% Hexane (F7)	50% Hexane (F8)	100% Acetone (F9)
Recovery (%)	-	7.2	7.6	11.6	22.8	6.1	11.5	16.3	9.5	7.2
					Saturated					
14:0	0.43 ± 0.00^{a}	0.34 ± 0.01^{b}	ND	ND	0.01^{c}	ND	ND	ND	ND	ND
16:0	0.08 ± 0.00^{a}	0.11 ± 0.01^{b}	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}	ND	ND	0.01 ± 0.00^{c}	ND	ND	ND
18:0	0.2 ± 0.01^{a}	0.03 ± 0.00^{b}	0.07 ± 0.00^{c}	0.2 ± 0.01^{a}	ND	0.13 ± 0.00^{d}	0.02 ± 0.00^{b}	ND	ND	ND
∑SFA	2.01 ± 0.04^{a}	1.3 ± 0.01^{b}	0.36 ± 0.01^{c}	0.30 ± 0.01^{c}	0.01 ± 0.00^{d}	0.15 ± 0.00^{e}	0.04 ± 0.00^{d}	ND	ND	ND
					Monounsurated					
16:1*n*-7 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:1*n*-7 *cis*	3.31 ± 0.00^{a}	3.15 ± 0.00^{b}	3.62 ± 0.03^{c}	2.32 ± 0.01^{d}	0.07 ± 0.00^{e}	0.4 ± 0.01^{f}	0.21 ± 0.00^{g}	0.2 ± 0.01^{g}	ND	ND
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9 *cis*	3.54 ± 0.02^{a}	3.22 ± 0.00^{b}	4.84 ± 0.01^{c}	3.61 ± 0.00^{d}	0.31 ± 0.01^{e}	0.41 ± 0.00^{f}	0.18 ± 0.00^{g}	0.12 ± 0.00^{h}	ND	ND
22:1*n*-9	8.71 ± 0.00^{a}	3.76 ± 0.02^{b}	9.97 ± 0.06^{c}	6.15 ± 0.00^{d}	0.13 ± 0.00^{e}	0.12 ± 0.00^{e}	0.09 ± 0.00^{e}	ND	ND	ND
∑ MUFA	15.83 ± 0.03^{a}	10.44 ± 0.04^{b}	18.7 ± 0.01^{c}	12.16 ± 0.01^{d}	0.60 ± 0.01^{e}	0.97 ± 0.02^{f}	0.53 ± 0.00^{g}	0.31 ± 0.00^{h}	0.21 ± 0.01^{i}	ND
					Polyunsaturated					
18:2*n*-6 *trans*	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
18:2*n*-6 *cis*	1.84 ± 0.01^{a}	2.00 ± 0.02^{b}	0.83 ± 0.01^{c}	0.98 ± 0.01^{d}	0.57 ± 0.00^{e}	0.03 ± 0.00^{f}	0.93 ± 0.01^{g}	0.09 ± 0.00^{h}	0.05 ± 0.00^{f}	0.08 ± 0.00^{h}
20:4*n*-6	1.23 ± 0.02^{a}	1.25 ± 0.01a^{b}	1.27 ± 0.01^{b}	0.14 ± 0.00^{c}	0.41 ± 0.00^{d}	0.41 ± 0.00^{d}	0.5 ± 0.01^{e}	ND	ND	ND
20:5*n*-3	17.52 ± 0.14^{a}	9.97 ± 0.04^{b}	11.18 ± 0.02^{c}	16.03 ± 0.03^{d}	28.5 ± 0.07^{e}	14.51 ± 0.05^{f}	6.15 ± 0.00^{g}	2.46 ± 0.02^{h}	2.04 ± 0.04^{h}	1.63 ± 0.02^{i}
22:5*n*-3	1.20 ± 0.01^{a}	1.13 ± 0.00^{b}	0.23 ± 0.00^{c}	0.08 ± 0.00^{d}	0.36 ± 0.00^{e}	0.91 ± 0.00^{f}	2.65 ± 0.03^{g}	0.26 ± 0.01^{c}	0.62 ± 0.00^{h}	0.82 ± 0.00^{i}
22:6*n*-3	50.18 ± 0.00^{a}	45.45 ± 0.02^{b}	50.32 ± 0.28^{a}	56.56 ± 0.49^{c}	62.62 ± 0.54^{d}	75.6 ± 0.53^{e}	79.45 ± 0.41^{f}	95.14 ± 0.24^{g}	95.98 ± 0.03^{gh}	97.23 ± 0.21^{h}
∑ PUFA	74.64 ± 0.05^{a}	62.03 ± 0.03^{b}	64.75 ± 0.06^{b}	76.46 ± 0.04^{c}	93.12 ± 0.06^{d}	92.72 ± 0.03^{e}	92.74 ± 0.03^{f}	98.34 ± 0.02^{g}	98.47 ± 0.41^{g}	99.92 ± 0.03^{h}
					Fatty acid indices					
∑*Trans*	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
∑*n*-3	69.23 ± 0.25^{a}	56.57 ± 0.03^{b}	61.79 ± 0.02^{c}	72.72 ± 0.01d^{c}	91.5 ± 0.04^{e}	91.05 ± 0.04^{e}	88.55 ± 0.02^{f}	98.14 ± 0.01^{g}	98.59 ± 0.08^{g}	99.73 ± 0.02^{h}
∑*n*-6	5.48 ± 0.03^{a}	5.51 ± 0.01^{a}	2.97 ± 0.03^{b}	3.53 ± 0.02^{c}	1.63 ± 0.02^{d}	1.51 ± 0.02^{e}	4.12 ± 0.01^{f}	0.18 ± 0.00^{g}	0.06 ± 0.00^{h}	0.20 ± 0.01^{g}
∑*n*-3/*n*6	12.63 ± 0.03^{a}	10.28 ± 0.01^{b}	20.8 ± 0.29^{c}	20.65 ± 0.05^{c}	56.48 ± 0.18^{d}	61.10 ± 0.02^{e}	21.54 ± 0.02^{c}	545.07 ± 1.01^{f}	1644 ± 1.00^{g}	524.63 ± 0.55^{h}
EPA+DHA	67.7 ± 0.09^{a}	55.42 ± 0.21^{b}	61.5 ± 0.22^{c}	72.59 ± 0.13^{d}	91.12 ± 0.03^{e}	90.11 ± 0.09^{e}	85.6 ± 0.05^{f}	97.85 ± 0.02^{g}	98.01 ± 0.01^{g}	98.90 ± 0.01^{g}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA – Total saturated fatty acids; ∑MUFA – Total monounsaturated fatty acids ∑PUFA – Total polyunsaturated fatty acids; ∑*Trans* – Total *trans* fatty acids; FAC – fatty acid concentrate after urea complexation; means with different superscripts (a–j) in the same row indicates a statistical difference ($p < 0.05$). FUC represents fatty acid after urea complexation. ND – not detected.

UV spectroscopic analysis of the oil after winterization (Figure 1B) and urea complexation (Figure 1C) did not show intense absorption intensities at 260–280 nm, which signified that the concentration process did not cause any change in structural configuration in the olefinic systems of fatty acids. Significant reduction in the absorption intensities in the region 200–240 nm was noticed after winterization and urea complexation compared to the control (Figure 1A), which can be interpreted as the reduction in the amount of long-chain saturated carbons after the sequential concentration process.

The quality of the fatty acids after urea complexation was studied by ^{1}H-NMR spectroscopy conjugated with ^{13}C-NMR and DEPT$_{135}$. The NMR peaks were determined with the help of literature data (Guillen and Ruiz, 2003; Knothe and Kenar, 2004; Tyl et al., 2008; Guillen and Ruiz, 2004). Figure 2 represents the stacked spectrum of refined liver oil of deep sea leafscale gulper shark before urea complexation (Figure 2B) and after urea complexation (Figure 2A), which explains the changes in various signal intensities during the concentration process. The methyl protons of saturated acyl group signals were apparent at δ 0.83–0.93. The signal intensities at this region decreased (reduction in proton integrals) after the process of urea complexation. The proton integral at δ 1.2–1.5 showed significant reductions (82.8%), which possibly indicated lower concentration of saturated hydrocarbon groups of the SFAs. A significant increase in proton integral values was observed at δ 2.74–2.92, signifying the CH_2 *bis*-allylic protons (–CH=CH–C**H$_2$**–CH=CH–), which were found to be increased (55%) after urea complexation. The characteristic signals of non-conjugated olefinic protons (–C**H**=C**H**–) in the ^{1}H-NMR spectra appeared at δ 5.2–5.5. A significant increase ($p < 0.05$) in the proton integral was observed (41%) at this region after urea complexation

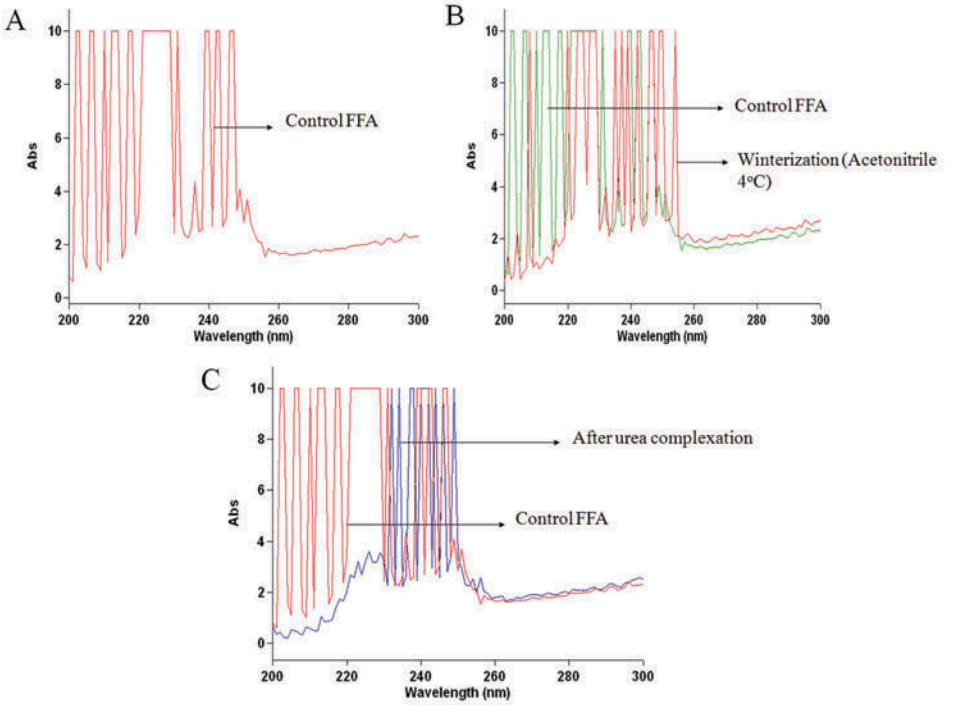

Figure 1. (**A**) UV spectra of the control free fatty acids, (**B**) fatty acids after winterization with solvent acetonitrile at 4°C and (**C**) after urea complexation. Significant reduction in the absorption intensities at the area of 200–240 nm was noticed after winterization and urea complexation compared to the control, which implied that there was a decrease in the amount of hydrocarbon groups or saturated fatty acids after the sequential concentration processes. Furthermore, the urea fractionated non-urea complexed fraction of the fatty acids did not show intense absorption intensities at 260–280 nm, which signified that the concentration process did not cause any change in structural configuration in the olefinic systems of fatty acids.

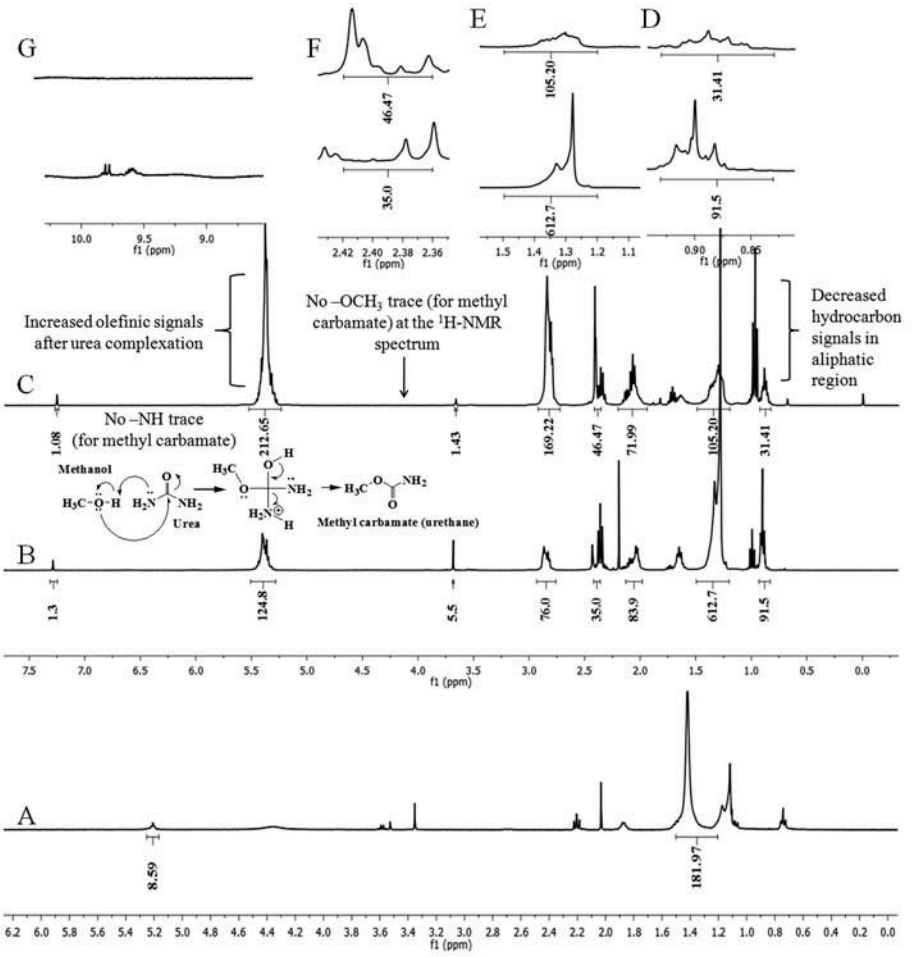

Figure 2. ^{1}H-NMR spectrum of the urea crystals (**A**), FFA before urea complexation (**B**), and after urea complexation (**C**) showing the changes in the intensity of olefinic signals. (**D–F**) shows the enlarged portion of specific regions of the spectrum, while (**G**) shows the aldehydic region in the spectrum **B** and **C**. No residual proton signal traces of methyl urethane were apparent at δ 3.8 and δ 6.0 (broad band) in ^{1}H-NMR due to the presence of $-OCH_3$ and $-NH_2$ groups, respectively (inset: formation of urethane during urea crystallization).

process, which implies that the process successfully increased the olefinic signal intensities. Meanwhile, the olefinic proton signals were found to be insignificant in the urea crystals (Figure 2C); whereas, the aliphatic region (δ 1.2–1.5) was found to be increased. Thus, the urea complexation method could adequately remove the SFAs in the fatty acids, along with the crystals, without losing the unsaturated contents. The presence of aldehydic signals was noted at δ 9.5–9.8 before urea complexation process, and these proton signals were absent after the amide fractionation process (Figure 2G). The double bond configuration was further determined by the *J* values, which were found to be lower and no significant difference was observed after the process. The lower *J* value further ascertained the lower concentration of *trans* fatty acid content. Furthermore, the lack of significant difference in the *J* value after the process of urea complexation inferred that there was no *cis-trans* conversion of the olefinic bonds during the concentration process. The ^{13}C-NMR spectrum before (Figure 3B) and after (Figure 3D) urea complexation and DEPT resonances before (Figure 3A) and after (Figure 3C) the process of urea complexation were analyzed. The olefinic peaks were enveloped at δ 125–135 in the ^{13}C-NMR, and DEPT spectra were found to be increased after

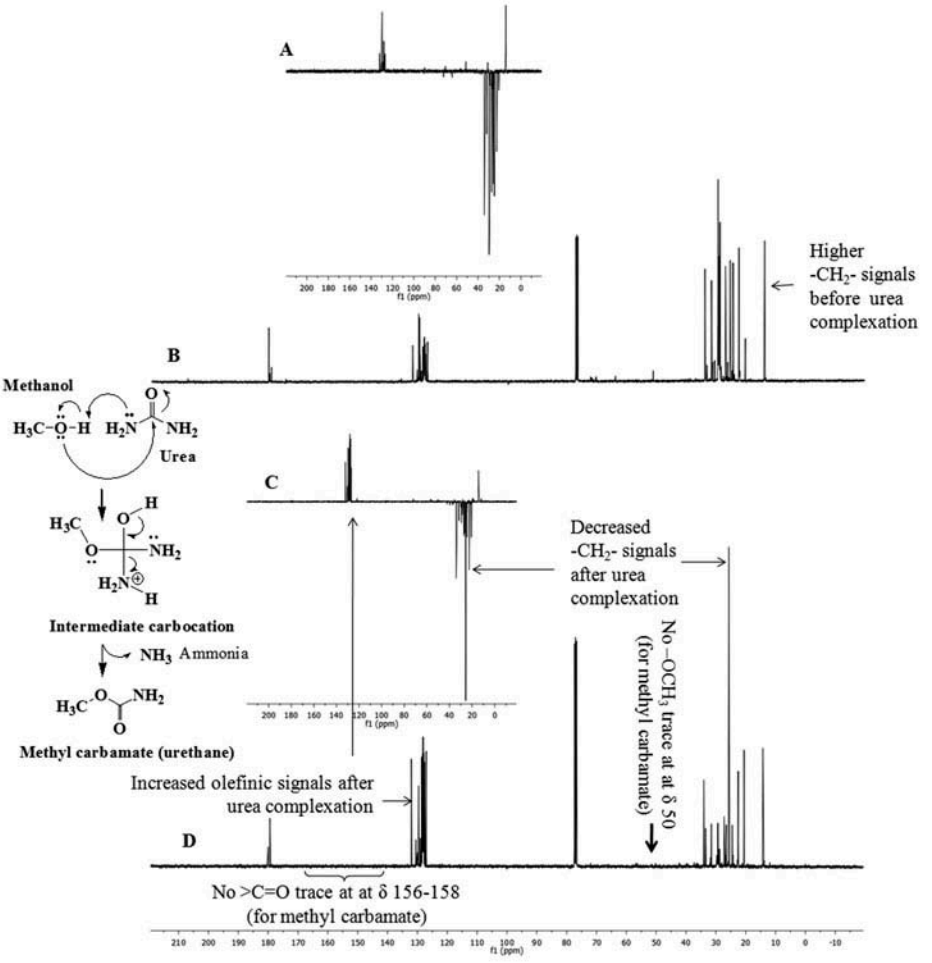

Figure 3. (**A**) $DEPT_{135}$ and (**B**) ^{13}C-NMR spectra of FFA before urea complexation. A greater number of methylene signals at δ 10–30 is indicated by an arrow. (**C**) $DEPT_{135}$ and (**D**) ^{13}C-NMR spectra of FFA after urea complexation reaction showing increased olefinic signals at δ 130 and reduced CH_2 signal intensities at δ 20–35 region. No residual carbon traces at δ 50 and 156–158, respectively, in the ^{13}C-NMR spectrum were attributed to the absence of $-OCH_3$ and –C=O groups, respectively.

the process. The resonances of allylic and *bis*-allylic methylenes were recorded at δ 30 as an envelope and were found to be decreased after urea complexation. Thus, it can be concluded that the urea complexation technique could remove SFAs with methylene envelope $-(CH_2)_n$ in their structure.

Alkyl carbamates are formed by the reaction of urea and methanol/ethanol and were first reported by Liebig and Wohler (1845). Ethyl/methyl carbamate, commonly known as urethane, is a potent carcinogen and has been found in distilled spirits, wines, and in an array of fermented foods like bread (Haddon et al., 1994), soy sauce (Matsudo et al., 1993), and alcoholic beverages (at ppb levels). Previous studies (Ough et al., 1988) have demonstrated that the reaction of ethanol and urea is the main contributor to carbamates in wine. Urethanes can be formed by the reaction between urea and methanol during the concentration of oil using the urea complexation method. Methyl derivatives of urethanes display chemical shift at δ 5.93 and δ 6.0 (broad band) in ^1H-NMR due to the presence of $-OCH_3$ and $-NH_2$ groups, respectively, whereas the corresponding carbon signals appear at δ 50 and 156–158, respectively, in the ^{13}C-NMR spectrum (Wang et al., 2001). It is apparent from the ^1H-NMR (Figure 2) and ^{13}C NMR (Figure 3) that no urethane group signals were

present in the spectra after the process of urea complexation. Hongyan et al. (2016) explained that repeated water washes can remove carbamates formed in the oil during urea complexation. Any trace of methyl carbamate formed might thus have been removed by the water wash in the *trans* esterification process. Furthermore, no trace of urethane was apparent at *m/e* 75 in the gas chromatography mass spectrometry (GCMS) of the product (non-urea crystallized fraction) obtained after urea fractionation process, which demonstrates the absence of this carcinogenic impurity. Thus, the process did not induce the formation of carcinogenic urethanes, and therefore, the method for the concentration of polyunsaturated fatty acids was proved to be safe.

Fatty acid profile of CFAMEs after argentation chromatography

The non-polar solvent *n*-hexane with increasing concentrations of acetone (1, 3, 5, 8, 10, 30, 50, and 100%, v/v) were used to fractionate the FAMEs with differential composition of olefinic bonds. The percent recovery of each fraction during the chromatographic purification is shown in Table 2. After the argentation column chromatography, more than 99% of the samples injected were recovered, which proved that samples were not lost during the process. The fatty acid compositions of the different column eluted fractions (100% *n*-hexane to 100% acetone) were analyzed and are shown in Table 2. The saturated fatty acid esters (14:0, 16:0, and 18:0) with no double bonds were primarily eluted with 100% *n*-hexane as they did not complex with the silver ion. As the concentration of acetone increased, the mobile phase polarity also increased, which eluted greater proportions of unsaturated fatty acids. The MUFAs complexed comparatively less with the silver ions and were eluted at 1–3% of acetone in *n*-hexane as eluent. The ∑*n*-3 fatty acids were found to increase, while the ∑*n*-6 fatty acids were found to decrease as the acetone concentration increased (Figure 4A). The presence of ∑PUFA increased with the increase in the concentration of more polar solvent (acetone), whereas EPA was found to occur at greater concentration at fractions F2–F5, with maximum at F4 (28.03%), and decreased after fraction F5. Notably, the DHA concentration was found to increase substantially when acetone concentration increased to 10%, and this fatty acid was eluted with the maximum purity (>97%) when eluted with 100% acetone (Figure 4B). The % of ∑*trans* fatty acids among the different fractions did not appear to change significantly, which implies that the process did not cause any conversion of the *cis* olefinic bonds to their corresponding *trans* geometrical isomers.

Argentation chromatography has been proved to effectively eliminate the unwanted saturated fatty acids in our study. Saturated fatty acid esters are eluted faster than the unsaturated fatty acid esters during silver-silica fractionation of fatty acids. The elution order of the fatty acid esters was determined by the number, position, and geometric configuration of double bonds. A reversible charge-transfer complexation of Ag^+ with carbon–carbon double bonds helps in the resolving power of Ag–silica gel. Thus, the mobility of the solute in the column is controlled by the intensity and the strength of this complexation (Guil-Guerrero et al., 2000).

NMR spectral analyses of CFAME fractions obtained during argentation chromatography

The purity of the different fractions of CFAME eluted with different solvent systems during the argentated chromatography was studied by extensive spectroscopic techniques, like proton NMR, ^{13}C NMR, and DEPT sequences. An increase in the proton integral at the olefinic region δ 5–5.5 was apparent in the ^{1}H-NMR spectrum, showing a significant enrichment of PUFAs in the fractions eluted with 100% acetone (Figure 5A) and 90% *n*-hexane: acetone (Figure 5B), when compared with the fraction eluted with 100% *n*-hexane (Figure 5C). The lesser *J* value in this region further represented the absence of *trans* olefinic bonds, which signified that the process did not convert the native *cis* (Z) bonds to their *trans* (*E*) forms. The proton integral at δ 1.2–1.5 (zone of hydrocarbon functionality) was found to decrease as the concentration of acetone increased, which indicated the reduction of saturated hydrocarbon moiety of the SFAs after argentation chromatography.

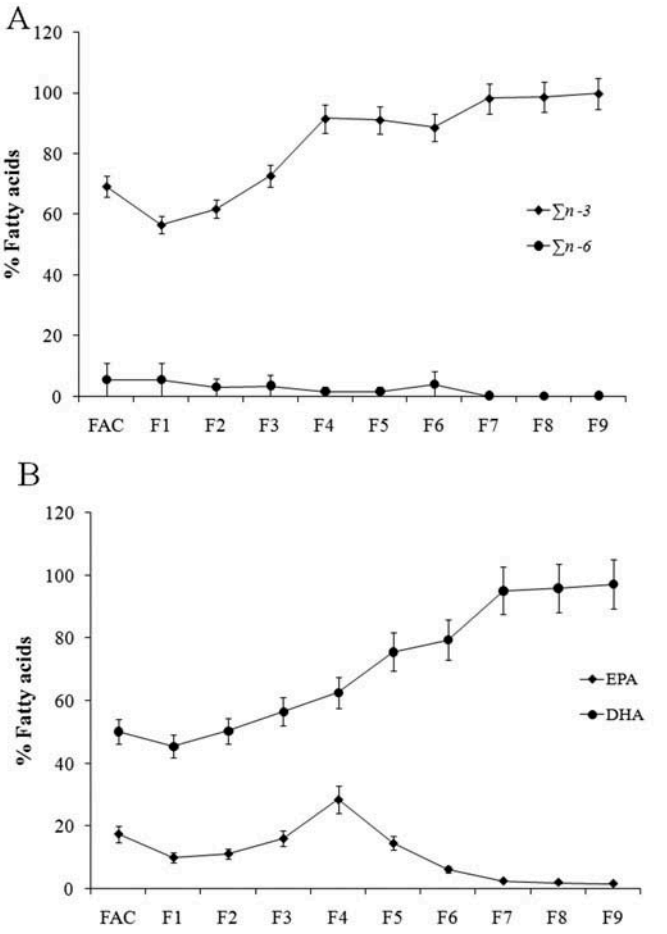

Figure 4. (**A**) Aggregate ∑*n*-3 and ∑*n*-6 fatty acids and (**B**) EPA and DHA (% total fatty acids TFA) at various fractions obtained in the argentation column chromatography.

In the ^{13}C-NMR (Figure 6A) and DEPT spectra (Figure 6B), the characteristic olefinic proton signals were displayed at about δ 125 – 135, which increased in the fractions from 100% *n*-hexane to 100% acetone (Figures 6A and B (a–c). The –CH_2– and –CH_3 carbons appeared at *δ* 15–35, whereas the -CH_2- group hindering the two double bonds occurred downfield at *δ* 38, probably due to the electron withdrawing effects of the olefinic system. The presence of downfield carbons at δ 40 also determined that the carbon is situated in close proximity of a >C=O group (–COOMe) and an olefinic double bond, probably from the long-chain $C_{20\text{-}22}$ *n*-3 PUFAs, such as DHA and EPA. After argentation chromatography, the proton integral values at the olefinic region (δ 5.3) were found to increase, which indicated the enrichment of PUFAs. The integral values of the *bis*-allylic peaks at *δ* 2.5–3 also showed the prevalence of long-chain PUFAs. The presence of these multiples of unsaturated protons explained the preponderance of the fatty acids with greater than three double bonds in the methyl esters. The proton integral values at *δ* 0.5–2.25 were significantly reduced at 100% acetone, which indicated the lesser amount of saturated hydrocarbon groups of SFAs. The ^{13}C-NMR and DEPT spectra were used to corroborate the results obtained by ^{1}H-NMR.

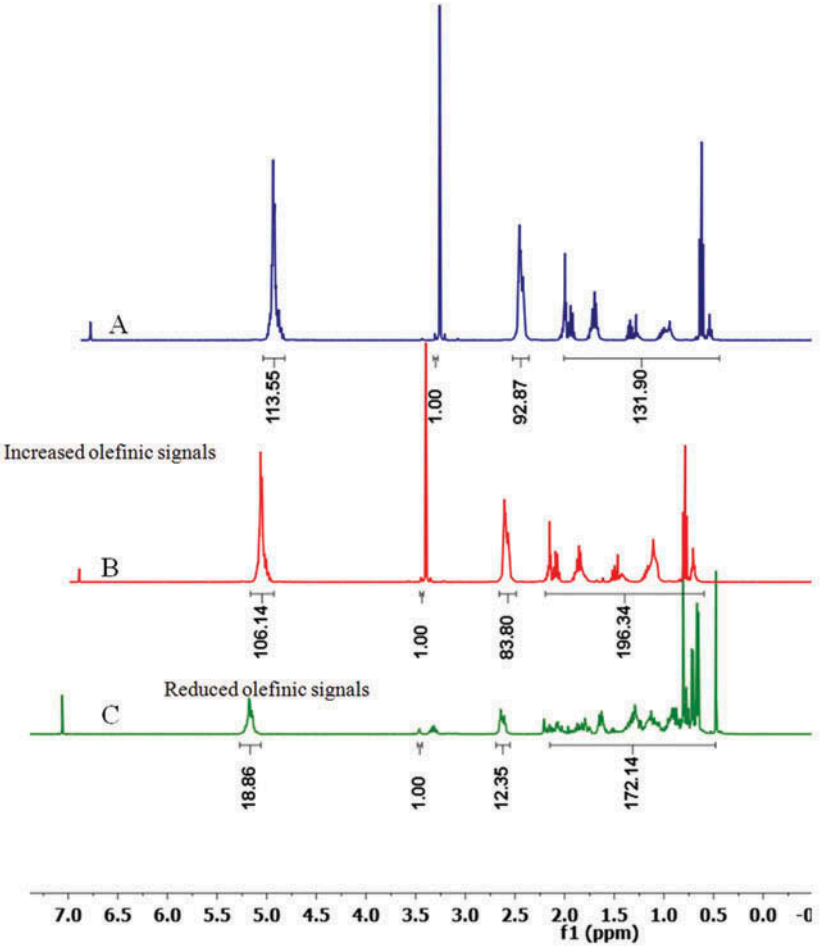

Figure 5. ^{1}H-NMR spectrum of different fractions obtained after argentation chromatography: (**A**) 100% acetone, (**B**) 97:3 (v/v) *n*-hexane: acetone, and (**C**) 100% *n*-hexane showing the changes in the signal intensities.

Conclusions

The refined liver oil of deep sea leafscale gulper shark, *C. squamosus*, was treated by stepwise processes of winterization, urea complexation, and argentation chromatography to obtain concentrated $C_{20\text{-}22}$ PUFAs, mainly 20:5*n*-3 and 22:6*n*-3. The solvent acetonitrile was found to significantly reduce the greater quantities of SFAs and increase the PUFA contents during the process of winterization, without any degradation to the sample material or formation of undesirable impurities, such as *trans* fatty acids. The urea complexation method has proved to effectively eliminate the saturated hydrocarbon moieties from the fatty acids. The characteristic ^{1}H-NMR signals with decreased proton integrals at δ 0.83–0.93 and δ 1.2–1.5 and an increase of proton integrals at δ 5.2–5.5 further demonstrated the efficacy of urea complexation to concentrate long-chain $C_{20\text{-}22}$ PUFAs. Argentated silica chromatography method was used to concentrate the $C_{20\text{-}22}$ *n*-3 PUFAs to greater than 99 percent purity. The methods developed in the present study will help to concentrate $C_{20\text{-}22}$ *n*-3 PUFAs from the refined liver oil of deep sea leafscale gulper shark without affecting their chemical properties.

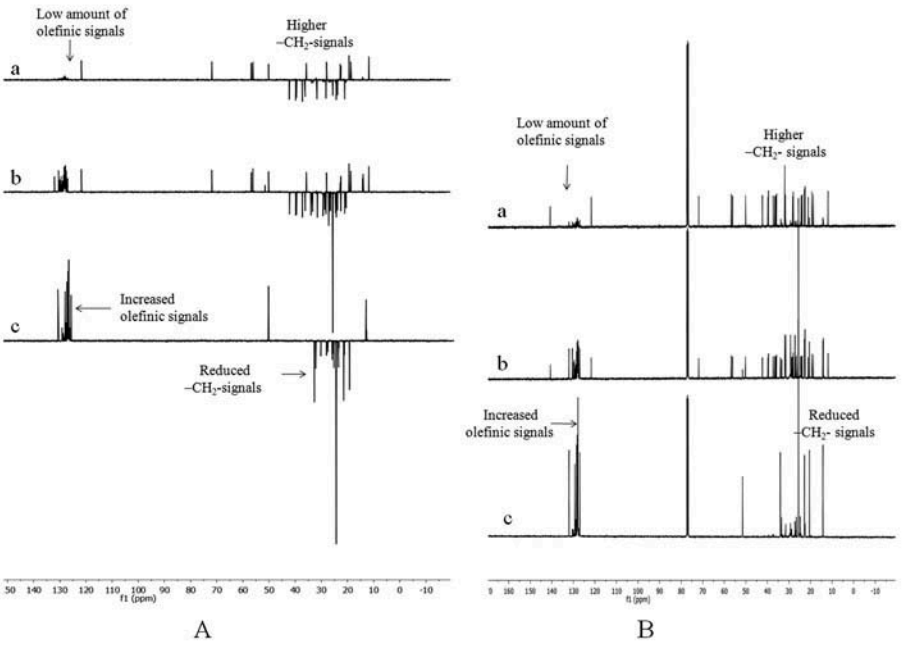

Figure 6. (**A**) ^{13}C-NMR spectrum of fractions obtained after argentation chromatography showing the increase in olefinic and decrease in SFA signals: (a) 100% *n*-hexane, (b) 90:10 *n*-hexane: acetone (v/v), and (c) 100% acetone. (**B**) DEPT spectrum of fatty acid methyl ester fractions obtained after argentation chromatography showing the increase in olefinic and decrease in SFA signals: (a) 100% *n*-hexane, (b) 90:10 (*n*-hexane:acetone), and (c) 100% acetone.

Acknowledgments

The authors thank the Director, Central Marine Fisheries Research Institute for his guidance and support. Thanks are due to the Head, Marine Biotechnology Division, Central Marine Fisheries Research Institute, for facilitating the research activity.

Funding

The authors are thankful to the Science and Engineering Research Board (SERB) of Department of Science and Technology (grant number SR/S0/HS-0124/2012), New Delhi, India, for financial supports. The authors declare no competing financial interest.

References

Ackman, R. G., Ratnayake, W. M. N., and Olsson, B. 1988. The "basic" fatty acid composition of Atlantic fish oils: potential similarities useful for enrichment of polyunsaturated fatty acids by urea complexation. J. Am. Oil Chem. Soc. 65(1): 136–138.

Bakes, M. J., and Nichols, P. D. 1995. Lipid, fatty acid and squalene composition of liver oil from six species of deep-sea sharks collected in southern Australia waters. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 110(1): 267–275.

Belarbi, E. H., Molina, E., and Chisti, Y. 2000. A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. Enzyme Microb. Technol. 26(7): 516–529.

Bimbo, A. P. 1998. Guidelines for characterizing food-grade fish oil. INFORM. 9(5): 473–483.

Bordier, C. G., Sellier, N., Foucault, A. P., and Le Goffic, F. 1996. Purification and characterization of deep sea shark *Centrophorus squamosus* liver oil 1-*O*-aklylglycerol ether lipids. Lipids. 31(5): 521–528.

Chakraborty, K., Joseph, D., and Joseph, D. 2016. Concentration of $C_{20\text{-}22}$ n-3 polyunsaturated fatty acids from *Sardinella longiceps* and fatty acid stabilization. Eur. J. Lipid Sci. Technol. 118(2):208–223.

Christie, W.W. 1982. Lipid Analysis. 2nd edition. Headington Hill Hall, Oxford, UK: Pergamon Press Ltd. Pp. 147–149.
Davenport, S., and Deprez, P. 1989. Market opportunities for shark liver oil. Aust. Fish. 48(11): 8–10.
Deprez, P. P., Volkman, J. K., and Davenport, S. R. 1990. Squalene content and neutral lipis composition of livers from deep-sea sharks caught in Tasmanian waters. Mar. Fresh. Res. 41(3): 375–387.
Gooding, C. M. 1962. Winterization process. U.S. Patent US3, 048,491A, original assignee corn products co.
Guil-Guerrero, J. L., and Belarbi, E. H. 2001. Purification process for cod liver oil polyunsaturated fatty acids. J. Am. Oil Chem. Soc. 78(5): 477–484.
Guil-Guerrero, J. L., Campra-Madrid, P., and Belarbi, E. H. 2000. γ-Linolenic acid purification from seed oil sources by argentated silica gel chromatography column. Process Biochem. 36(4): 341–354.
Guil-Guerrero, J. L., Campra-Madrid, P., and Navarro-Juárez, R. 2003. Isolation of some PUFA from edible oils by argentated silica gel chromatography. Grasas Aceites. 54(2): 116–121.
Guillén, M. D., and Ruiz, A. 2003. Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups in vegetable oils. Eur. J. Lipid Sci. Technol. 105(11): 688–696.
Guillén, M. D., and Ruiz, A. 2004. Study of the oxidative stability of salted and unsalted salmon fillets by ^{1}H nuclear magnetic resonance. Food Chem. 86(2): 297–304.
Haagsma, N., Van Gent, C. M., Luten, J. B., De Jong, R. W., and Van Doorn, E. 1982. Preparation of an ω3 fatty acid concentrate from cod liver oil. J. Am. Oil Chem. Soc. 59(3): 117–118.
Haddon, W. F., Mancini, M. L., Mclaren, M., Effio, A., Harden, L. A., Degre, R. L., and Bradford, J. L. 1994. Occurrence of ethyl carbamate (urethane) in US and Canadian breads: measurements by gas chromatography-mas spectrometry. Cereal Chem. 71(2): 207–215.
He, Y., Li, J., Kodali, S., Chen, B., and Guo, Z. 2016. The near-ideal catalytic property of *Candida antarctica* lipase A to highly concentrate *n*-3 polyunsaturated fatty acids in monoacylglycerols via one-step ethanolysis of triacylglycerols. Biores. Technol. 219: 466–478.
He, Y., Li, J., Kodali, S., Balle, T., Chen, B., and Guo, Z. 2017. Liquid lipases for enzymatic concentration of *n*-3 polyunsaturated fatty acids in monoacylglycerols via ethanolysis: catalytic specificity and parameterization. Biores. Technol. 224: 445–456.
Hwang, L. S., and Liang, J. H. 2001. Fractionation of urea-pretreated squid visceral oil ethyl esters. J. Am. Oil Chem. Soc. 78(5): 473–476.
Kates, M. 1972. Isolation, analysis and identification of lipids. In: Techniques in Lipidology. Work, T. S., and Work, E. (Eds.). Amsterdam: Elsevier. Pp. 268–618.
Knothe, G., and Kenar, J. A. 2004. Determination of the fatty acid profile by ^{1}H-NMR spectroscopy. Eur. J. Lipid Sci. Technol. 106(2): 88–96.
Li, M., Pham, P. J., Wang, T., Pittman, C. U., and Li, T. 2009. Solid phase extraction and enrichment of essential fatty acid methyl esters from soy-derived biodiesel by novel π-complexing sorbents. Bioresour. Technol. 100(24): 6385–6390.
Liebig, J., and Wöhler, F. 1845. Cyansaures Aethyl-und Methyloxyd. Justus Liebigs Ann. Chem. 54(3): 370–371.
Matsudo, T., Aoki, T., Abe, K., Fukuta, N., Higuchi, T., Sasaki, M., and Uchida, K. 1993. Determination of ethyl carbamate in soy sauce and its possible precursor. J. Agric. Food. Chem. 41(3): 352–356.
Mitre, R., Etienne, M., Martnais, S., Salmon, H., Allaume, P., Legrand, P., and Legrand, A. B. 2005. Humoral defence improvement and haematopoiesis stimulation in sows and offspring by oral supply of shark-liver oil to mothers during gestation and lactation. Brit. J. Nutr. 94(5): 753–762.
Mu, H., Jin, J., Xie, D., Zou, X., Wang, X., Wang, X., and Jin, Q. 2016. Combined urea complexation and argentated silica gel column chromatography for concentration and separation of PUFAs from tuna oil: based on improved DPA level. J. Am. Oil Chem. Soc. 93(8): 1157–1167.
Ough, C. S., Crowell, E. A., and Gutlove, B. R. 1988. Carbamyl compound reactions with ethanol. Am. J. Enol. Viticult. 39(3): 239–242.
Ryu, S. N., Lee, J. I., Jeong, B. Y. and Hur, H. S. 1997. U.S. Patent No. 5,672,726. Washington, DC: U.S. Patent and Trademark Office.
Shimada, Y., Sugihara, A., and Tominaga, Y. 2001. Enzymatic purification of polyunsaturated fatty acids. J. Biosci. Bioeng. 91(6): 529–538.
Shimma, Y., and Shimma, H. 1966. On liver oil of deep-sea sharks of Suruga Bay. Bull. Tokai. Reg. Fish. Res. Lab. 48: 53–61.
Szostak, W. B., and Szostak-Wegierek, D. 2005. Health properties of shark oil. Prz. Lek. 63(4): 223–226.
Tyl, C. E., Brecker, L., and Wagner, K. H. 2008. ^{1}H NMR spectroscopy as tool to follow changes in the fatty acids of fish oils. Eur. J. Lipid Sci. Technol. 110(2): 141–148.
Wang, H. L., Kao, H. M., Digar, M., and Wen, T. C. 2001. FTIR and solid state ^{13}C-NMR studies on the interaction of lithium cations with polyether poly (urethane urea). Macromolecules. 34(3): 529–537.
World Health Organization. 1980. Dietary fats and oils in human nutrition: report of an expert consultation held in Rome, 21–30 September 1977.
Yokochi, T., Usita, M. T., Kamisaka, Y., Nakahara, T., and Suzuki, O. 1990. Increase in the γ-linolenic acid content by solvent winterization of fungal oil extracted from *Mortierella* genus. J. Am. Oil Chem. Soc. 67(11): 846–851.